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Doctoral Thesis

Genomic and genetic dissection of thyroid cancer

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ABSTRACT/RESUMEN

Thyroid tumors can have two cellular origins and a variety of genetic drivers. Thus, thyroid cancer (TC) is a complex and heterogeneous disease. As much of its etiology remains poorly explored, TC represents an attractive model to study cancer disease process. Herein, we have coupled exhaustive genomic dissection of an exceptional collection of human samples to their genetic characterization, and comprehensive data analysis in order to address several aspects of clinical interest.

In the first part of the study focused on follicular cell-derived cancer, by performing a two-step association study involving 1,820 cases and 2,410 controls, we provide novel insights into the genetic susceptibility of this disease. Apart from underscoring the importance of 9q22.33 locus in disease risk, we identify novel associations at 10q26.12 and 6q14.1 and highlight that genetic heterogeneity between populations could be a part of this disease's hidden heritability. Moreover, we describe the genomic landscape of a total of 165 follicular cell-derived tumors including both papillary and follicular cases. We identify distinct molecular subgroups closely related to oncogenic drivers, and explore the diagnostic and prognostic utility of several genomic features. According to our results, the methylome and miRNome of benign and malignant disease is largely overlapping, which prevents from diagnostic markers' identification. Of note, elevated promoter methylation of *WT1* and *EI24*, and aberrant expression of let-7a and miR-192 could serve as potential novel molecular markers of shorter time to progression.

The second part of this study is focused on the less frequent tumors of the gland arising from C-cells, named medullary thyroid carcinomas (MTC). By characterizing the MTC methylome, we have complemented the genomic dissection of an outstanding collection of 64 frozen and confirmed that this disease comprises of several molecular entities closely related to the underlying mutations. Moreover, taking advantage of a series composed of 103 paraffin embedded tumors we show that even tyrosine kinase inhibitors' (TKI) targets expression differs according to these mutations, and *a priori* genetic screening of MTC patients appears advisable to guide the selection of the most suitable TKI treatment.

To summarize, we have performed genetic and genomic characterization of almost the whole spectrum of thyroid tumors. Our studies have revealed much of molecular mechanisms behind these tumors and shown that they tend to be tightly linked to the causal driver mutations. On the whole, these results are yet another example of the great potential that lies in high-throughput techniques to decipher disease etiology, and discover disease markers.

Los tumores tiroideos pueden tener dos orígenes celulares y estar causados por una gran variedad de mutaciones genéticas, siendo entonces el cáncer de tiroides (CT) una enfermedad compleja y heterogénea. Gran parte de su etiología sigue siendo poco explorada, y el CT representa un modelo atractivo de estudio. En este trabajo, se ha llevado a cabo una disección exhaustiva de aspectos genómicos de una colección sobresaliente de muestras humanas caracterizadas genéticamente, con el fin de abordar varios aspectos de interés clínico.

La primera parte del estudio se centra en el cáncer derivado de célula folicular. Mediante un estudio de asociación en dos etapas que incluía 1,820 casos y 2,410 controles, pudimos confirmar la importancia del locus 9q22.33 en el riesgo de la enfermedad, e identificamos nuevas asociaciones en 10q26.12 y 6q14.1. Nuestros datos sugieren que la heterogeneidad genética entre poblaciones podría en parte explicar la falta de replicación en distintos estudios. Además, describimos las características genómicas de 165 tumores derivados de célula folicular incluyendo tanto casos con patrón de crecimiento papilar como folicular. Identificamos subgrupos moleculares específicamente relacionados con mutaciones concretas, y exploramos la utilidad diagnóstica y pronostica de varias de las características genómicas estudiadas. Así, el metiloma y el miRNoma de enfermedades benignas y malignas se solapan en gran medida, lo cual impide la identificación de marcadores diagnósticos. Sin embargo, la hipermetilación del promotor de *WT1* y *EI24*, y la expresión aberrante de let-7a y miR-192 podrían servir como nuevos marcadores moleculares para predecir el tiempo hasta la progresión.

La segunda parte del estudio se centra en carcinomas tiroideos derivados de las células C, llamados carcinoma medular de tiroides (CMT). Se caracterizó el metiloma de 48 CMT congelados, complementando resultados previos de transcriptoma y miRNoma. Esta enfermedad se compone de varias entidades moleculares estrechamente relacionadas con las mutaciones subyacentes. Utilizando una colección de 103 tumores embebidos en parafina demostramos que la expresión de dianas de inhibidores tirosina quinasas difiere de acuerdo a la mutación. Por tanto, una caracterización genética previa de los pacientes con CMT parece aconsejable para guiar la selección del tratamiento más adecuado.

En resumen, hemos caracterizado genética y genómicamente casi todo el espectro de tumores tiroideos. Nuestros estudios han revelado mecanismos moleculares involucrados en su desarrollo y han demostrado que los perfiles genómicos tienden a estar estrechamente vinculados a mutaciones causales. En conjunto, estos resultados son una muestra del gran potencial de las técnicas de alto rendimiento para descifrar la etiología de la enfermedad, y descubrir marcadores de la enfermedad.

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ABBREVIATIONS

ATC	anaplastic thyroid cancer
AXL	AXL receptor tyrosine kinase
BRAF	B-Raf proto-oncogene
CpG	cytosine-guanine dinucleotide
DTC	well-differentiated thyroid cancer
EGFR	epidermal growth factor receptor
ENCODE	The Encyclopedia of DNA Elements
FA	follicular adenoma
FLT3	fms-related tyrosine kinase 3
FOXE1	forkhead box E1
FTC	follicular thyroid cancer
GWAS	genome-wide association study
HR	hazard ratio
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KLK10	kallikrein-related peptidase 10
LPG	low-penetrance gene
MAF	minor allele frequency
MEN2	Multiple Endocrine Neoplasia type 2
MET	MET proto-oncogene, receptor tyrosine kinase
MTC	medullary thyroid cancer
NGS	next-generation sequencing
NIS	sodium/iodide cotransporter
OMICs	high throughput genomic technologies
PDTC	poorly differentiated thyroid cancer
PTC	papillary thyroid cancer
PTCvf	follicular variant of papillary thyroid carcinoma
RARβ2	retinoic acid receptor β2
RAS	rat sarcoma oncogene
RASSF1	RAS association domain family member 1
RET	rearranged during transfection
SPRY1	Sprouty 1
T3	triiodothyronine
T4	thyroxine
TC	thyroid cancer
TCGA	The Cancer Genome Atlas
Tie2	TEK tyrosine kinase, endothelial
TIMP3	tissue inhibitor of metalloproteinase-3
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
TSHR	thyroid stimulating hormone receptor
UTR	untranslated region
VEGFR	vascular endothelial growth factor receptor
WT	wild type

INTRODUCTION

1. Cancer and OMICs

Although cancer encompasses many different tumor types classified by their cellular origin, they all share abnormal growth and unregulated proliferation. Approximately 14 million new cancer cases are diagnosed yearly worldwide and 8 million of cancer patients die due to the disease. Individual's susceptibility to develop cancer has been for long known to be modulated in a complex manner by genetic predisposition combined with environmental factors (Bartsch and Hietanen, 1996). Ultimately, the disease arises from an accumulation of alterations in critical genes. To date, ~140 such genes have been identified among the common forms of human cancer that, when altered by intragenic mutations, can unleash the unregulated growth and "drive" tumorigenesis (Vogelstein *et al.*, 2013). Importantly, these driver mutations are typically associated with profound changes of other genomic features such as methylation pattern or gene and microRNA expression profiles in neoplastic cells, and it is this complex deregulation that ultimately modulates the malignant potential of each cancer (Balbin *et al.*, 2013). However, for most of the cancer types, the peculiarities of these aberrant molecular changes leading the transformation process remain to be elucidated.

A better understanding of the molecular diversity of cancer may lead to more efficient diagnosis, management of the disease and emergence of novel therapeutic options. In this regard, high throughput genomic technologies (OMICs) have become instrumental in the study of the molecular mechanisms behind cancer in an unbiased manner, as well as an efficient tool for biomarkers discovery. Moreover, recent collaborative efforts in recruiting large sample sets of both non-cancerous and cancerous tissues, their exhaustive genomic characterization and the public availability of the generated data create an ideal environment for genomic dissection of this complex disease (e.g.: The Cancer Genome Atlas [TCGA]: http://cancergenome.nih.gov/, The Encyclopedia of DNA Elements [ENCODE]: http://genome.ucsc.edu/ENCODE/, The Roadmap Epigenomics Mapping Consortium: http://www.roadmapepigenomics.org/).

At this point, is it important to highlight that two main technologies are available for genomic characterization of a given sample. Microarrays have been used for over two decades, and are based on a collection of microscopic DNA molecules attached to a solid surface, which hybridize to the complementary sequence in the sample. A decade ago, next-generation sequencing (NGS) methods based on parallel sample sequencing producing millions of reads concurrently were developed. Nowadays, the cost of the exhaustive NGS methods has become very affordable. Yet, microarrays keep on being used due to the well-established analysis pipelines and less labor-intensive sample preparation.

2. Thyroid gland and thyroid cancer: general concepts

Thyroid is one of the largest human endocrine glands implicated in a wide range of physiological functions via production of thyroid hormones: triiodothyronine (T3), thyroxine (T4) and calcitonin. The former ones regulate the growth and rate of many metabolic processes, while calcitonin is involved in calcium homeostasis.

Macroscopically, the thyroid is a butterfly-shaped organ localized in the neck and composed of two lobes connected via the isthmus. The thyroid gland is covered by a thin fibrous sheath composed of an internal and external layer. From the internal layer, small septa of connective tissue penetrate the gland and divide it into incomplete lobules. Microscopically, these lobules are built up of 20-40 round to oval variable-sized follicles (15-500 μm in diameter) with a central cavity that contains a viscous material called colloid. Colloid is mainly composed of thyroglobulin – the precursor of thyroid hormones. Every colloid follicle is lined with a simple, non-stratified, cuboidal epithelium made up of follicular cells. Adjacent to the thyroid follicles and immersed in the connective tissue, C cells (or parafollicular cells) can be found.

Follicular cells are the main thyroid cell population accounting for >98% of all cellular content of the gland. These endoderm-derived cells are characterized by being polarized and specialized to uptake iodine due to the presence of sodium-iodine symporter (NIS) in their basolateral membrane. Furthermore, they are also known to regulate, produce and secrete triiodothyronine (T3) and thyroxine (T4) hormones (Kopp, 2005). On the other hand, C cells account for only around 1% of thyroid gland cellular content. They are derived from neural crest (ectoderm), lack polarization, are mostly localized in the posterior upper third of the gland and produce calcitonin.

Both of these cell types can be affected by malignant transformation resulting in a disease jointly referred to as thyroid cancer (TC) - the objective of this thesis. Notwithstanding they only represent around 2% of the over-all human cancer burden (accounting for 213,000 new cases yearly worldwide), thyroid tumors represent the most common malignancies of the endocrine system and remain associated with important clinical challenges that shall be discussed further on in the following chapters. TC is a general term encompassing two main cancer entities depending on the cell type affected by transformation. Follicular cell-derived tumors arise from follicular cells of the gland and far outnumber those of C cell origin called medullary thyroid carcinomas (MTC) (DeLellis *et al.*, 2004).

3. Follicular cell-derived thyroid cancer

3.1 Epidemiology, diagnosis and clinical management of follicular cell-derived TC patients

Follicular cell-derived tumors account for >95% of all thyroid neoplasias. They likely arise as a result of interplay between environmental, genetic and hormonal factors. Because of the thyroid's dependence on environmental iodine, the gland is particularly vulnerable to the genotoxic effects of radioactive iodine and to the nongenotoxic effects resulting from iodine deficiency (Dal Maso *et al.*, 2009). As the disease is 2-4 times more frequent in females than in males (Agate *et al.*, 2012) (**Figure 1a**), it has been suggested that a specific susceptibility gene with sex hormone receptor elements may be involved in the pathology of this disease.

The annual incidence of follicular cell-derived thyroid cancer varies among countries ranging from 0.5 to 20 cases per 100 000 individuals each year. This number has been steadily increasing over the past decades (**Figure 1b**). Of note, since follicular cell-derived cancer displays the highest annual percentage change in incidence increase among all cancers (>5%), some authors started to refer to it as to an epidemic disease (Edwards *et al.*, 2014). This increase is largely due to an improvement of the diagnostic techniques, which has caused an "over-diagnosis" of small tumors that would have remained occult in the past (Leenhardt *et al.*, 2004; Li *et al.*, 2013). However, "over-diagnosis" does not fully account for such a dramatic change, suggesting that several unaccounted environmental and genetic factors may be interacting to mediate the disease risk (Pellegriti *et al.*, 2013).

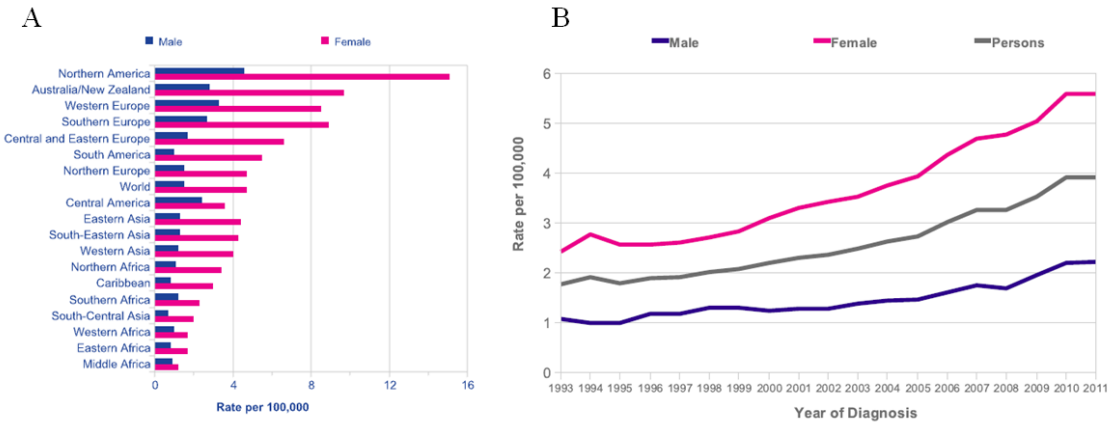

Figure 1. Incidence of follicular cell-derived thyroid cancer. (adapted from Cancer Research UK, www.cancerresearchuk.org) **A)** Follicular cell-derived cancer incidence ranges from 0.5 to 20 cases per 100,000 among countries, and is 2-4 times more frequently diagnosed in women than in men. **B)** The incidence of follicular cell-derived cancer has been steadily increasing over the past years.

The diagnosis of the disease generally starts with finding of a suspicious nodule with signs of intra-nodal vascularization, irregular edges, solid aspect and presence of calcifications by ultrasound imaging technique (Papini *et al.*, 2002). Of note, as little as 5% of thyroid nodules eventually turn out to be malignant lesions, and the accurate diagnosis of the malignant disease remains as unresolved challenge in the clinics (Rossing, 2013). Following ultrasound, potential malignancy is further explored by fine needle aspiration biopsy (Pacini *et al.*, 2012). This technique is based on cytological analysis, which in turn allows for diagnosis of some of the many histological subtypes of TC (**Figure 2**).

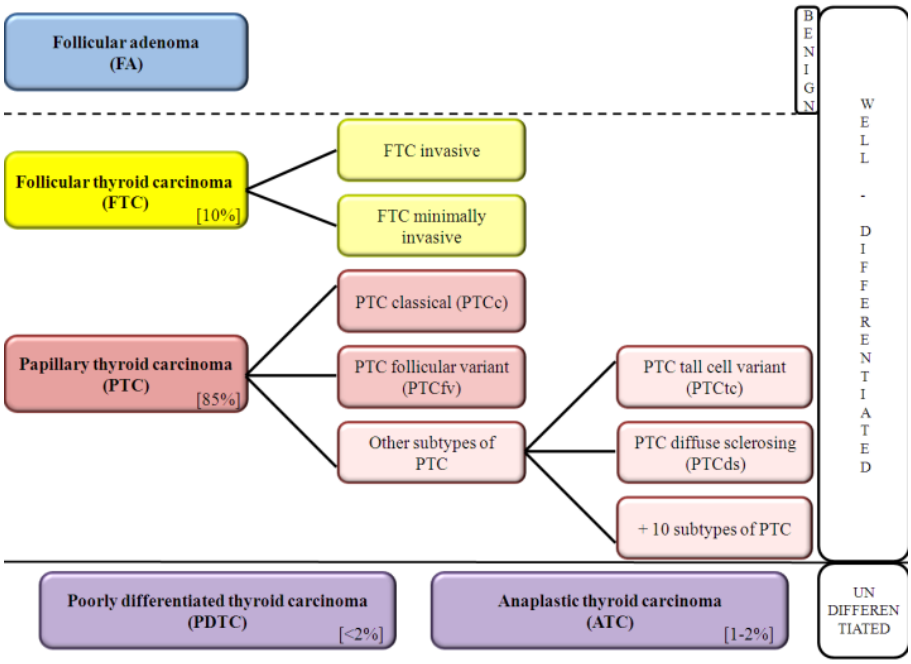

Figure 2. Classification of follicular cell-derived thyroid tumors. This general term represents a highly heterogeneous entity composed of a spectrum of differentiation stages, ranging from benign lesions [follicular adenoma (FA)], to well-differentiated carcinomas such as papillary thyroid carcinoma (PTC) or follicular thyroid carcinoma (FTC), through to undifferentiated, more invasive and lethal human malignancies, such those classified as poorly-differentiated thyroid carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC). Relative prevalence of malignant tumors is detailed in brackets.

In this regard, around 85% of malignant thyroid tumors show characteristic nuclear changes including signs of enlarged and irregular nuclei with dusty to powdery chromatin, small nucleoli, nuclear grooves and often pseudoinclusions (DeLellis *et al.*, 2004). These are features of papillary thyroid carcinomas (PTC). Around 10% of malignant aspirates present dispersed microfollicular arrangement of tumor cells and scant colloid, and often nuclear atypia. These are signs of follicular thyroid carcinomas (FTC). Benign lesions termed follicular adenomas (FA) may also present the cytological features of FTC and nuclear atypia, while some follicular carcinomas may have bland cytological characteristics. Jointly, the tumors described so far (FA,

FTC and PTC) maintain the differentiated state of follicular cells, from which they origin, and are therefore referred to as well-differentiated thyroid cancer (DTC).

Due to cytological similarities between FA and FTC, as much as 25% of the aspirates can result in indeterminate diagnosis. For the accurate diagnosis of the indeterminate cases, there is an urgent need for identification of molecular diagnostic markers of malignancy. In this regard, methods based on mutation screening (Cantara *et al.*, 2010; Nikiforov *et al.*, 2011) or gene expression profiling (Alexander *et al.*, 2012; Alexander *et al.*, 2013) have been developed recently. Nevertheless, the usage of these techniques in the clinics has already been challenged (McIver *et al.*, 2014) and is still limited. Therefore, the diagnosis of malignancy of indeterminate aspirates keeps on depending on the demonstration of capsular or vascular invasion, which is done in histological preparations following surgical sampling of the suspicious nodule (DeLellis *et al.*, 2004). Of note, as little as one out of 5 patients that undergo this "diagnostic" surgery eventually has a malignant tumor.

A small proportion of follicular cell-derived tumors (<5%) lose their differentiation partially (poorly differentiated thyroid carcinoma; PDTC) or completely (anaplastic thyroid carcinoma; ATC). To some extent, these tumors also present characteristic cytological changes, including high cellularity, presence of numerous mitotic figures and necrotic debris. Nevertheless, definite diagnosis may sometimes require histological evaluation (DeLellis *et al.*, 2004). These tumors represent a very different entity as compared to the well-differentiated ones, showing a very adverse clinical evolution with very poor prognosis (<10% 10-year survival (Nikiforov and Nikiforova, 2011)). As they are not the objective of this thesis, they will not be discussed in more detail further on.

Once the malignant diagnosis is confirmed, the standard treatment consists of total or partial thyroidectomy (in case of a localized disease), followed by therapeutic ablation with radioiodine (^{131}I; dose≥100 mCi). The latter approach takes advantage of the capacity of follicular cells to selectively uptake iodine, and is thus applied in order to ablate the remaining thyroid tissue together with microscopic tumor foci (Mazzaferri and Kloos, 2001). In the vast majority of patients with well-differentiated tumors, these procedures lead to full remission (Verburg *et al.*, 2013). However, for patients that develop recurrence, those with persistent disease, and patients with dedifferentiated tumors, the conventional therapy do not provide clinical benefit (Kebebew *et al.*, 2005) and currently, these patients only dispose of palliative forms of treatment based on tyrosine kinase inhibitors (TKIs) (Ho *et al.*, 2013; Brose *et al.*, 2014).

A clinically relevant proportion of patients (up to 30%) develop a recurrence relatively late after diagnosis (>10 years after diagnosis) (Duntas and Grab-Duntas, 2006). Therefore, it is

needed to routinely perform a post-surgical follow-up of all DTC patients based on physical examination, neck ultrasonography, simultaneous determination of serum anti-tiroglobulin antibodies with tiroglobulin and whole-body iodine scanning. In order to identify high risk patients who benefit from the follow-up, it is of utmost importance to uncover prognostic factors, as derived from retrospective studies. There are some clinico-histopathological characteristics already described, which can hint a worse outcome (late age of onset [>45], male gender, size and histology of the tumor, and mutation $BRAF^{V600E}$). However, the predictive value of these features is limited and, to some extent, controversial, and nowadays there are still no molecular markers of recurrence that would be used in the clinical practice.

3.2 Genetic susceptibility to DTC

Follicular cell-derived thyroid tumors arise in their vast majority (>95%) as sporadic entities. The remaining cases emerge in families affected by known hereditary syndromes (Gardner's syndrome, Cowden syndrome, Werner syndrome and Carney complex), or families with a clear familial aggregation of DTC cases, but with no known genetic cause for it. Notwithstanding familial aggregations showing typical Mendelian mode of inheritance are extremely rare, large population studies established that DTC is a highly hereditable disease (Frich *et al.*, 2001; Risch, 2001; Czene *et al.*, 2002). In this regard, first-degree relatives of an individual affected by DTC have an 8.6 – 10.3 times higher risk of developing DTC than general population (Goldgar *et al.*, 1994; Pal *et al.*, 2001; Hemminki *et al.*, 2004). Moreover, the hereditability rate of DTC is the highest among all cancers not showing typical Mendelian inheritance – 53% (Czene *et al.*, 2002). All these observations highlight the important role that genetic elements may be playing in the development of DTC.

Much effort was invested in identification of genetic factors predisposing to DTC. Due to familial manifestation of the disease, involvement of high-penetrance genes was considered and investigated. However, among familial cases, no high-penetrance protein-coding gene predisposing to DTC has been convincingly identified so far. Linkage analyses have only identified putative genomic loci (**Figure 3**), but most of these findings have not been consistently replicated (Malchoff *et al.*, 2000; McKay *et al.*, 2001; Cavaco *et al.*, 2008; He *et al.*, 2009; Suh *et al.*, 2009).

On the other hand, it has become generally accepted that sporadic DTC is a complex disease (Lander, 1996; Chakravarti, 1999). Thus, its genetic predisposition is likely a result of multiple common low-penetrance or rare moderate-penetrance variants (Landa and Robledo, 2011). Moreover, as evidenced by results from our group, gene-gene interactions (Landa *et al.*, 2013) as well as gene-environment interactions (Bufalo *et al.*, 2006; Bufalo *et al.*, 2008) can modulate individual susceptibility. In this scenario, both carefully designed candidate-gene

association studies (Ho *et al.*, 2009; Landa *et al.*, 2009; Landa *et al.*, 2010) and genome-wide association studies (GWAS) (Gudmundsson *et al.*, 2009; Takahashi *et al.*, 2010; Gudmundsson *et al.*, 2012; Kohler *et al.*, 2013; Figlioli *et al.*, 2014) have been used successfully to identify numerous susceptibility variants for DTC (**Figure 3**), of which probably the most important is the variation found at 9q22.33. However, an important part of DTC hereditability remains to be explained.

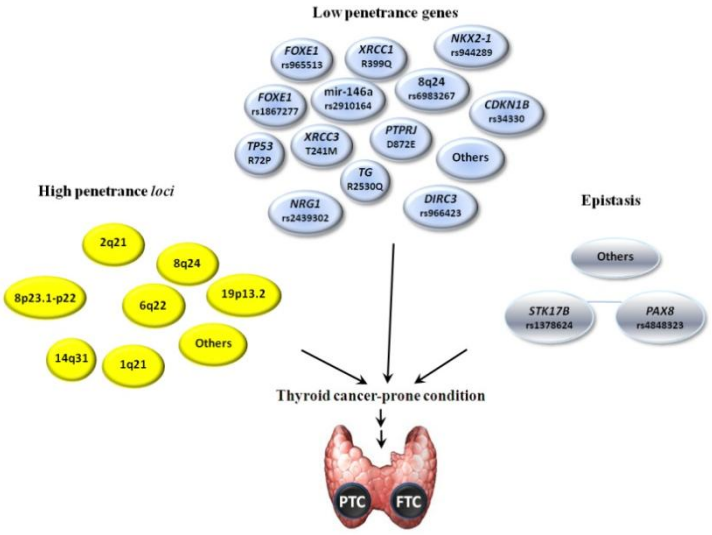

Figure 3. Model of individual genetic predisposition to well-differentiated thyroid cancer. The individual genetic background, either monogenic in familial cases or polygenic in sporadic cases, determines a thyroid cancer-prone condition for a given individual. In addition, gene-gene interactions (epistasis) and gene-environment interactions ultimately determine the development of the disease (adapted from Landa and Robledo, 2011).

Genetic variation at 9q22.33 has been extensively linked to susceptibility to sporadic DTC by both candidate gene (rs1867277; Landa *et al.*, 2009) and genome-wide approaches (rs965513; Gudmundsson *et al.*, 2009, Gudmundsson *et al.*, 2012, Kohler *et al.*, 2013; rs965513 and rs1867277; Takahashi *et al.*, 2010). This locus is exceptionally the only one to be invariably associated with the disease in all GWAS performed until the date and probably represents the best validated genetic factor identified in DTC so far. Interestingly, polymorphisms from this locus were even linked to familial DTC susceptibility (Tomaz *et al.*, 2012). A gene, called forkhead box E1 (*FOXE1*), essential for the development, differentiation, and hormone responsiveness of thyroid gland (Cuesta *et al.*, 2007) is located in this genomic region. Of note, our group was able to functionally demonstrate that variation at 9q22.33 affects transcription of *FOXE1* gene (Landa *et al.*, 2009). Thus far, this is one of the very few known functional mechanisms involving low-penetrance gene action in DTC susceptibility that has been described.

3.2.1 Missing hereditability

Notwithstanding the great effort invested in identification of genetic factors predisposing to DTC, the associations described so far only explain a limited part of the DTC heritability, which will make additional research of particular relevance in the near future. In this regard, better stratification of the patients included in the studies could be a key factor warranting novel findings, since it is not clear neither whether the familial and sporadic disease are distinct entities (Capezzone *et al.*, 2008), nor whether the same SNPs are involved in the etiology of PTC and FTC.

At the time when this thesis was started (2011), first GWA studies addressing the genetic predisposition to DTC had already emerged (Gudmundsson *et al.*, 2009; Takahashi *et al.*, 2010), redundantly uncovering associations with 9q22.33 locus. As mentioned previously, our group reached the same results by a candidate-gene approach, and provided a functional explanation (Landa *et al.*, 2009). In order to address the missing hereditability in an agnostic manner, we aimed to use the largest collection of DTC cases and healthy controls (at that time) composed of 1,820 cases and 2,410 controls in combination with a genome-wide approach.

3.3 Drivers of DTC: the peculiarities of oncogenic signaling

With less than one somatic mutation per megabase, DTC is one of the cancers showing the lowest mutation density (Alexandrov *et al.*, 2013). Importantly, it has been showed that it is a MAPK pathway-driven disease (**Figure 4a**), in which critical genes are frequently mutated via two distinct molecular mechanisms: point mutation or chromosomal rearrangement. It is established that the mutations in MAPK genes are typically mutually exclusive (Soares *et al.*, 2003) and are associated with particular clinical, histopathological and biological characteristics (Adeniran *et al.*, 2006) (**Figure 4b**). Additionally, thyroid cancer progression and possible dedifferentiation often involves a number of secondary mutations that affect PI3K-AKT pathway (reviewed in (Nikiforov and Nikiforova, 2011)) (**Figure 4a**).

3.3.1 RET/PTC rearrangements

The first DTC driver to be discovered was the one concerning the *RET* proto-oncogene in papillary tumors (Fusco *et al.*, 1987). *RET* (REarranged during Transfection) encodes for a receptor tyrosine kinase implicated in nervous system development, kidney morphogenesis and spermatogenesis. It contains 3 functional domains: an extracellular one acting as a ligand-sensitive receptor, a transmembrane one and an intracellular one, which possesses the kinase activity.

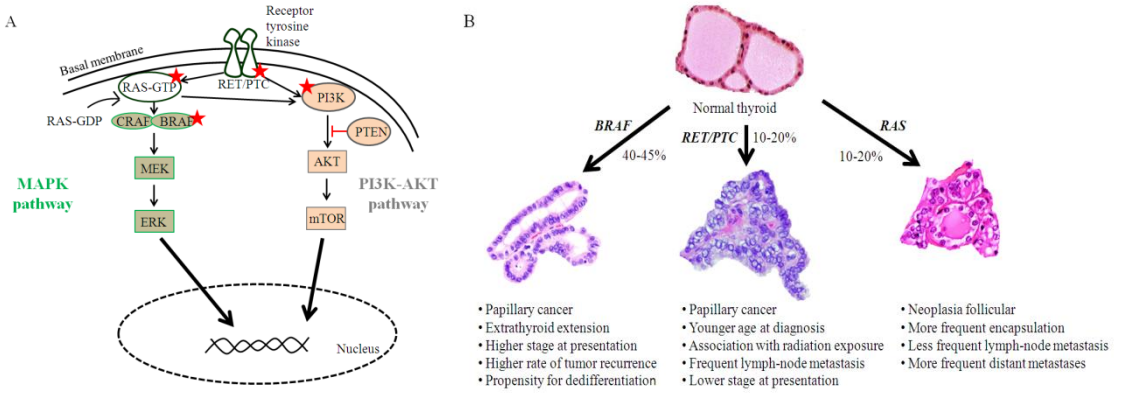

Figure 4. Molecular drivers of DTC. (adapted from (Nikiforov and Nikiforova, 2011)) **A)** The main signaling pathways involved in thyroid carcinogenesis are MAPK and PI3K-AKT pathways. The effector genes frequently affected by mutations are highlighted with red stars. **B)** Most prevalent DTC molecular alterations and their association with clinical and histopathological features of tumors.

The mechanism of oncogenic activation of *RET* in PTC is through a rearrangement with various partners. It results in the juxtaposition of the C-terminal region of the RET protein (containing the kinase domain) with an N-terminal portion of another protein. The generated constitutively active chimeric protein is known as RET/PTC (Santoro *et al.*, 1992; Nikiforov, 2002). To date, there are more than 15 genes known to act as rearrangement partners with *RET*, the most common ones being *CCDC6* ("RET/PTC1") (Grieco *et al.*, 1994), *PRKAR1A* ("RET/PTC2") (Sozzi *et al.*, 1994) and *NCOA4* ("RET/PTC3") (Santoro *et al.*, 1994). These rearrangements account for 10-20% of PTC cases, are especially frequent in pediatric cases and cases associated with radiation exposure (**Figure 4b**), like those related with the nuclear accident in Chernobyl (Bounacer *et al.*, 1997; Rabes *et al.*, 2000).

3.3.2 BRAF alterations

In 2003, mutations in *BRAF* oncogene were associated with the emergence of PTC. *BRAF* is a member of RAF family of serine-threonine kinases, which additionally includes genes *ARAF* and *CRAF*. All three isoforms activate the MAPK pathway, which is involved in regulation of apoptosis, inflammation, cell growth and differentiation (Chang and Karin, 2001).

The transversion of thymine to adenine in the nucleotide 1799 (p.Val600Glu) in the sequence of *BRAF* is the most common genetic alteration in PTC, explaining up to 69% cases in some of the series studied (Kimura *et al.*, 2003). This alteration affects kinase domain of the protein, where the change from nonpolar valine to negatively charged glutamate mimics the phosphorylation of residues Threonine 599 and Serine 602, necessary for BRAF activation (Nikiforova *et al.*, 2003). This results in an increase of protein's kinase activity of 10 to 12 times as compared to the native form of the protein, and as a consequence it can signal to the downstream MAPK effectors as a monomer. Thus, tumors driven by $BRAF^{V600E}$ do not respond

to the negative feedback from ERK to RAF, resulting in high MAPK-signaling (Pratilas *et al.*, 2009) translated in profound phenotypic changes. For instance, expression of genes responsible for iodine uptake and metabolism are greatly reduced in $BRAF^{V600E}$ tumors (Durante *et al.*, 2007). This could be the underlying mechanism accounting for the worse clinical outcome that has been observed among patients harboring *BRAF* mutation (Xing *et al.*, 2005; Lupi *et al.*, 2007) (**Figure 4b**).

Alternatively, mutations in other codons were described (Trovisco *et al.*, 2004), involving a similar transforming mechanism as $BRAF^{V600E}$. Fusions involving *BRAF* have been also identified in PTC (Ciampi *et al.*, 2005; TCGA, 2014). Some fusions supported BRAF signaling with expression and conservation of its kinase domain (*AKAP9/BRAF* in (Ciampi *et al.*, 2005) or *MKRN1/BRAF* in (TCGA, 2014)), while others suggested an alternative, yet unknown, activating mechanism.

3.3.3 RAS mutations

RAS proto-oncogene (RAt Sarcoma) mutations are some of the most frequent genetic alterations found in human cancers in general. *RAS* family of genes (*H-*, *N-* and *K-RAS*) encodes for small GTPases involved in signal transduction within cells. RAS has a high affinity for numerous effectors, including PI3K and RAF proteins (**Figure 4a**).

In DTC scenario, mutations in *RAS* genes are specific of neoplasias with a follicular pattern of growth (FA, FTC and follicular variant of PTC [PTCvf]). The mutations show differences in prevalence among the histological subtypes (20%, 44% and 43% among FA, FTC and PTCfv, respectively (Esapa *et al.*, 1999; Kimura *et al.*, 2003)), and different genes are mutated with different frequency (*N-RAS* mutations most frequent, followed by *K-RAS* (Esapa *et al.*, 1999)). In any case, the alterations tend to affect the GTPase domain (residues 12, 13 and 61) of the protein, conferring insensitivity to inactivation by GTPase activating proteins, and leaving RAS constitutively active. Of note, thyroid tumors driven by oncogenic RAS signal via RAF dimers and are thus responsive to the negative ERK feedback, resulting in a lower MAPK-signaling as compared to $BRAF^{V600E}$-driven tumors (Miller *et al.*, 2009).

3.3.4 Other driver alterations of DTC

RET, *BRAF* and *RAS* alterations explain up to 80% of PTC. Some of the remaining papillary tumors harbor other genetic modifications, such as *NTRK1* rearrangements (Pierotti *et al.*, 1995) or mutations in the novel PTC cancer gene *EIF1AX* (TCGA, 2014). However, these alterations only account for <5% of PTC.

While *RAS* mutations are the most frequent alterations in FTC (40-50%), up to 40% of follicular tumors arise as a consequence of *PAX8/PPARγ* rearrangement (Nikiforova *et al.*, 2002). The resulting fusion protein is composed of the promoter region and DNA binding domain of thyroid-specific transcription factor *PAX8* and almost the entire sequence of the nuclear receptor *PPARγ*. It causes the activation of NFKβ signaling pathway (Kato *et al.*, 2006), resulting in increased proliferation and decreased apoptosis. Other, less frequent alterations among FTCs (<10%) affect PI3K-AKT pathway genes *PIK3CA* and *PTEN* (Nikiforov and Nikiforova, 2011).

3.4 DTC in the age of molecular genomics: missing markers of malignancy and progression

High-throughput technologies have been instrumental in deciphering signal pathways associated with specific clinical features allowing for biomarkers identification in other cancer types. In 2011, little was known about the molecular fingerprints in DTC. Of note, ours was one of the pioneer groups to apply mRNA expression profiling to a large set of thyroid tumors (Montero-Conde *et al.*, 2008). Similarly to an earlier work of Giordano and colleagues (Giordano *et al.*, 2005), we observed that expression profiles tightly relate to histological classes. Moreover, we proposed a prognosis identifier based on a 23-gene expression signature. This study set a foundation for subsequent characterization of other genomic features in our large collection of thyroid tumors in search for biomarkers. Since the observed differences in gene expression could be caused by distinct gene regulatory mechanisms, such as DNA methylation or even action of microRNAs, these were the two genomic features we set off to study in detail.

3.4.1 DNA methylation and its involvement in TC

DNA methylation is the most thoroughly studied and understood epigenetic modification, which is based on addition of a methyl group to cytosine residue in context of cytosine-guanine dinucleotide sequence (CpG). It is typically associated with repressed regulatory regions or active gene transcripts (Cedar and Bergman, 2009; Smith and Meissner, 2013). The global distribution of CpG methylation, termed “DNA methylome”, is variable between different cell types and dynamic during cell differentiation. Importantly, deep deregulation of DNA methylation patterns is a hallmark of cancer, where global loss of methylation often affects heterochromatin repeats leading to genome instability, while local discrepancies in DNA methylation can lead to aberrant expression of oncogenes and tumor suppressors.

At the beginning of this thesis project, only a handful of studies were available addressing the role of aberrant DNA methylation in the etiology of DTC. All of these studies

used a candidate gene-based approach, and found an inverse relationship between DNA methylation and gene expression of genes either involved in thyroid gland function, such as thyroid-stimulating hormone receptor (*TSHR*) (Xing *et al.*, 2003) or *NIS* (Venkataraman *et al.*, 1999), or well-known tumor suppressors, e.g. tissue inhibitor of metalloproteinase-3 (*TIMP3*), retinoic acid receptor β2 (*RARβ2*) (Hu *et al.*, 2006; Brait *et al.*, 2012) and *RAS* association domain family protein 1 (*RASSF1*) (Schagdarsurengin *et al.*, 2002). Later on, the first exploratory genome-wide study involving 8 thyroid tumors uncovered there are differences in methylomes reflecting distinct histological subtype of TC (Rodriguez-Rodero *et al.*, 2013). However, due to obvious reasons, more studies were needed in order to decipher the precise TC methylome. Thus, we aimed to provide a more detailed characterization, focusing not only on the specificities of DTC methylome with respect to histology, but also on the underlying drivers and differential patients' outcome. Moreover, we explored the relationship between DNA methylation and gene expression using data generated for the same tumors.

3.4.2 microRNAs in TC

MicroRNAs (miRNAs) are small non-coding RNAs, which negatively regulate gene expression at the post-transcriptional level. They bind to semi-complementary sites at 3'-UTR of targeted mRNA, which can result in mRNA degradation, translational truncation, or both (Bartel, 2004; Liu *et al.*, 2008). Each single miRNA is promiscuous with its targets, and can regulate several hundred genes, while a single gene can be targeted by several miRNAs (Lewis *et al.*, 2003). In this way, miRNAs can regulate the expression of approximately one third of protein-coding genes (Bartel and Chen, 2004). Thus, they are involved in a wide variety of processes, including cellular proliferation, apoptosis as well as developmental processes and differentiation. Similar to DNA methylomes, miRNA signatures vary between distinct tissues, and diverge in a pathological state.

Due to their potential clinical utility, miRNAs have been extensively studied in DTC. The vast majority of the data on deregulated miRNAs was generated using microarray technologies, which could be a source of bias. Notwithstanding this fact, up-regulation of miR-146b and the miR-221~222 cluster are one of the best validated changes in papillary tumors (He *et al.*, 2005; Pallante *et al.*, 2006; Nikiforova *et al.*, 2008). Only recently, the first studies applying next-generation sequencing technology started to emerge (Swierniak *et al.*, 2013; TCGA, 2014). So far, the only histological subtype, in which the miRNAs were sequenced, is PTC. We used our large collection of DTC tumors and NGS in order to complete the spectrum of deregulated miRNAs involved in the other, less frequent, DTC subtypes, as well as to assess the relationship between miRNA and gene expression, and the possible prognostic role of miRNAs.

3.4.3 DTC in The Cancer Genome Atlas project

It is worthy to note that an important effort was invested through the TCGA project to gather 500 papillary tumors (of various subtypes) and the corresponding normal tissues. These samples were characterized for their genetic alternations, gene and miRNA expression as well as DNA methylation. All but the last genomic feature were assessed using NGS, while DNA methylation was measured by microarray technology (450 K Infinium Methylation Array from Illumina). The generated data is publically available, and the resulting genomic characterization of PTC was recently published (http://cancergenome.nih.gov/cancersselected/thyroid, (TCGA, 2014)). It re-confirmed that different driver alterations lead to different pathologies with distinct signaling and differentiation characteristics, raising the possibility of subdividing this histological class into several molecular subtypes. With respect to the current thesis, this public resource provided a valuable source of data for validation of some of our findings.

4. Medullary thyroid carcinoma

4.1 Epidemiology, diagnosis and clinical management of MTC patients

MTC is a rare disease that accounts for approximately 1-2% of all thyroid malignancies. Conversely to the follicular cell-derived TC, MTC affects both genders with a similar frequency. Around 75% of cases are sporadic in nature, while the remaining 25% arise as a manifestation of the hereditary disease termed Multiple Endocrine Neoplasia type 2 syndrome (MEN 2).

MTC is characterized by a solid mass of polygonal shaped cells with frequently observed amyloid deposition. Aspirates from medullary carcinomas are typically hypercellular with loosely cohesive cells. Hyperchromic nuclei with granular chromatin as well as multinucleate giant cells are commonly found. MTC arises from the calcitonin-producing C cells of the gland, and the increased serum level of this hormone is often used as a diagnostic marker, as well as a marker of disease relapse (DeLellis *et al.*, 2004).

The diagnosis and treatment of MTC patients was revolutionized in 1993, when the implication of *RET* in the emergence of the disease was described (Mulligan *et al.*, 1993). Ever since, the genetic testing is leading the clinical management of familial cases. Prophylactic thyroidectomy, which timing depends on the underlying genetic driver, is carried out ideally before the disease emerges. When it comes to the sporadic MTCs, the only possibly curative treatment option is complete thyroidectomy. Since medullary carcinoma tends to metastasize early and particularly frequently locally to cervical lymph nodes, the surgery is often coupled with lymphadenectomy. In total, up to 50% of patients may present with nodal metastases and up to 15% may have distant metastases (to lungs, bones and liver) (DeLellis *et al.*, 2004). For patients with residual or recurrent disease after primary surgery, or for those with distant metastases, the most appropriate management (surgery, chemotherapy or radiotherapy) is not well established, as the traditional treatment options show little to no benefit for the patient (Orlandi *et al.*, 2001). The 10-year survival rate of the patients with advanced disease is less than 20% (Leboulleux *et al.*, 2004).

4.1.1 Targeted therapies for advanced MTC

As *RET* codes for a tyrosine kinase receptor, MTC patients with advanced disease have recently been treated with TKIs in a palliative manner (Schlumberger *et al.*, 2009; Kurzrock *et al.*, 2011; Almeida and Hoff, 2012; Durante *et al.*, 2013; Fox *et al.*, 2013). The results of clinical trials showed that these drugs may achieve remarkable response in some MTC patients while provoking strong toxicities in others. The molecular basis for the large variability in TKI response is unknown.

Currently, two TKIs are approved for treatment of MTC patients. Vandetanib (ZD6474, Zactima) is a potent inhibitor of RET, EGFR and VEGFR2/3 and was approved in 2011 for treatment of adults with advanced MTC. Some of the most common adverse events associated with this drug such as diarrhea, rash, nausea can be adequately managed, while others such as the corrected QT interval elongation are of particular concern (Grande *et al.*, 2013). In 2012, Cabozantinib (XL184, Exelixis) was approved to treat metastatic MTC by inhibition of RET, VEGFR2, MET, KIT, VEGFR1/3, FLT3, Tie2 and AXL. Opposite to Vandetanib, QT interval elongation was not observed in any patient enrolled in the EXAM trial (Elisei *et al.*, 2013).

There are other TKIs such as Sorafenib, Sunitinib, Motesanib, and Axitinib currently being tested in different stages of clinical trials (Almeida and Hoff, 2012).

4.2 MTC arising within MEN 2: general aspects and implication of genetics

Mutations in *RET* proto-oncogene explain more than 95% of MEN 2 cases. In the remaining families not harboring mutations in *RET*, the driver(s) remain to be discovered (Cerrato *et al.*, 2009). In any case, the MEN 2 families display a dominant autosomal inheritance model, with a variable clinical expression pattern, and almost complete penetrance (according to the specific *RET* mutation). Inherited MTCs often emerge from precursor C-cell hyperplasia, are mostly multifocal and bilateral in character, and manifest at an early age (DeLellis *et al.*, 2004).

More than 90% of MEN 2 affected individuals develop MTC, but there are other endocrine diseases associated with the syndrome and depending on the clinical manifestation, MEN 2 syndrome can be subdivided into two different subtypes: Multiple Endocrine Neoplasia type 2A (MEN 2A) and Multiple Endocrine Neoplasia type 2B (MEN 2B) (Wells *et al.*, 2015). A half of MEN 2A and 2B patients develop pheocromocytoma (PCC), while hyperparathyroidism occurs in approximately 20-30% of MEN 2A individuals (Eng *et al.*, 1996).

Even if the MEN 2B subtype is the less frequent one, it is characterized by the most aggressive clinical manifestation of MTC. If MEN 2B patients do not undergo prophylactic thyroidectomy at an early age (less than 1 year), they are prone to developing metastatic MTC in childhood (Skinner *et al.*, 1996). MEN 2A is the more prevalent subtype (accounting for 95% of all MEN 2 cases) showing less aggressiveness as compared to the former one. However, patients often present cervical lymph node metastases (Cohen and Moley, 2003). These subtypes and the associated phenotypes relate well to the genetic alterations the patients harbor (**Table 1**), and the genetic testing is therefore used to lead the clinical management. In this regard, hereditary MTCs were divided into four different risk categories according to the

germline mutation with associated recommendations for treatment (Kloos *et al.*, 2009). The most severe phenotype is expectable in patients harboring the *RET*^{M918T} mutation, followed by mutations affecting the codon 634.

Mutated *RET* codon	Risk level	Syndrome	Clinical recommendation
883, 918	D	MEN 2B	Thyroidectomy within the first year of life
634	C	MEN 2A	Thyroidectomy before 5 years of age
609, 611, 618, 620, 630	B	MEN 2A	Thyroidectomy at 5-10 years of age
768, 790, 804, 891	A	MEN 2A	Periodic biochemical testing for MTC if thyroidectomy was not performed

Table 1. Genotype-phenotype correlation among MEN 2 patients. MTC risk level correlates with the mutated codon of *RET* (level D is the most severe). Adapted from (Kloos *et al.*, 2009).

4.3 MTC oncogenic drivers

4.3.1 RET signaling in MTC: mechanisms of aberrant activation

RET is the major genetic player in the emergence of MTC. As stated previously, it explains up to 95% of familial cases, and up to 50% of sporadic cases (Dvorakova *et al.*, 2006; Elisei *et al.*, 2008). The mechanism of oncogenic transformation differs from the one described previously in PTC (chapter 3.3.1).

Under normal physiological conditions, binding of calcium and ligand-specific coreceptor to the extracellular domain of RET is needed for RET homodimerization and activation (Airaksinen and Saarma, 2002). Following homodimerization, specific tyrosine residues are phosphorylated in order to serve as docking sites for adaptor proteins. Different activated sites trigger downstream signaling to different pathways. For instance, tyrosine 1015 is a binding site for phospholipase C that activates protein kinase C (PKC), while phosphorylated tyrosine 1062 serves as a docking site for multiple adaptors, including Shc, FRS2, Dok family proteins, insulin receptor substrate 2, and Enigma (Takahashi, 2001). Then, various pathways, such as MAPK, PI3K, JNK and ERK pathway, are activated affecting cell survival, differentiation, proliferation and chemotaxis (de Groot *et al.*, 2006).

In MTC scenario, RET is constitutively active due to distinct gain-of-function mutations. Most of MEN 2B and sporadic cases carry the *RET*^{M918T} mutation, which shows the highest transformation capacity resulting from activation due to disruption of an auto-inhibited head-to-tail RET TK homodimer (Knowles *et al.*, 2006). On the other hand, most of the MEN 2A mutations affect the extracellular cystein-rich domain of RET (codons 609, 611, 618, 620, 630, 634) (Kouvaraki *et al.*, 2005; Marx, 2005; Zbuk and Eng, 2007), allowing for a ligand-

independent homodimerization of the protein (Knowles *et al.*, 2006). The remaining MEN 2A mutations affect the intracellular kinase domain (codons 768, 790, 791, 804, 891) (de Groot *et al.*, 2006). However, little is known regarding the specific *RET* mutation-driven signaling pathways, even though the distinct mutations display differences in phosphorylation of docking sites, triggering specific intracellular signaling cascades (de Groot *et al.*, 2006).

4.3.2 RAS mutations and sporadic MTC

As shown recently, 18-80% of sporadic MTC lacking somatic *RET* mutations harbor somatic mutations of *H-RAS*, *K-RAS* or rarely *N-RAS* (Moura et al., 2011; Boichard et al., 2012; Ciampi et al., 2013). Interestingly, Ras is one of the many signaling effectors that mediate RET intracellular effects. The mutations found in MTC affect the same codons as previously discussed for follicular cell-derived tumors (chapter 3.3.3), triggering the same changes in the protein. The MTC genotype-phenotype correlation seems to encompass *RAS* positive MTCs as well, since Ciampi *et al*. described a higher prevalence of disease-free patients among those *RAS*-mutated when compared to *RET*-related cases (Ciampi *et al.*, 2013), suggesting *RAS* mutations give rise to a milder phenotype.

In total, *RET* and *RAS* mutations explain up to 75% of all sporadic tumors. In the remaining cases, termed "wild type" (WT), where the molecular driver is unknown, the transforming mechanism remains to be uncovered.

4.4 MTC in the molecular genomics era: from disease etiology to therapy

Notwithstanding the great technological possibilities, much of MTC etiology remains unknown likely due to the rareness of the disease and, in consequence, the difficulty to collect an informative sample set. There is an urgent need to identify signaling pathways associated with familial and sporadic forms of the disease in order to identify targetable molecules.

One of the first approximations using microarray technology in order to uncover disease-related signaling cascades was actually carried out in our laboratory. We characterized the transcriptome of the largest series of primary MTCs described so far (Maliszewska *et al.*, 2013). Together with the other works using similar approach (Jain *et al.*, 2004; Ameur *et al.*, 2009), it became clear that distinct *RET* mutations lead to activation of different signaling cascades. Interestingly, the over-expression of genes related to epithelial to mesenchymal transition and tumor invasion and metastases was notable among the poor-prognosis MTCs caused by the RET^{M918T} mutation. Moreover, miRNome of the same tumors was also explored in the laboratory (unpublished data). This is of particular interest, as very little is known about the aberrant expression of miRNAs in MTCs. So far, three microarray-based studies have been published on the topic (Nikiforova *et al.*, 2008; Abraham *et al.*, 2011; Santarpia *et al.*, 2013).

Little conclusions can be drawn from these articles as the authors study a low number of MTC tumors regardless of the genetic drivers they harbor.

Similarly to follicular cell-derived cancer, the previously generated data set the basis for integrative genomic analysis. During this thesis project, we produced genome-wide data on DNA methylation in order to shed more light into the complex regulation leading to aberrant gene expression. DNA methylation is perhaps the least studied genomic feature of MTC, as only two candidate gene-based studies are available, exploring the promoter methylation of tumor suppressors *RASSF1* (Schagdarsurengin et al., 2002) and Sprouty 1 (*SPRY1*) (Macia *et al.*, 2012). Later on, two MTC samples were included in an exploratory genome-wide study showing low levels of DNA methylation is attributable to MTC (regardless of the driver alteration) (Rodriguez-Rodero *et al.*, 2013). Nevertheless, the MTC methylome is still poorly characterized. As no integrative genomic studies of these rare tumors have been carried out so far, the combination of transcriptome, miRNome and DNA methylome data of the same samples could be a powerful tool to retrieve important information about the biology of this disease.

Moreover, given the limited therapeutic options and the great promise targeted therapies hold, we wanted to explore the possible biological reason underlying the diverse response to TKIs observed in the clinics among MTC patients. In order to do so, we firstly took advantage of the genome-wide transcriptomic data, as well as of an exceptional collection of formalin fixed paraffin-embedded MTC tumors, in order to confirm our hypothesis that the expression of key target proteins of these drugs vary in MTC according to the specific mutation.

OBJECTIVES

The final goal of this work was to couple exhaustive genomic dissection of an exceptional collection of human thyroid cancer samples to their genetic characterization in order to address several disease aspects of clinical interest. The specific aims of this thesis were:

1) To uncover novel low-penetrance genes associated with risk of developing well-differentiated thyroid cancer by applying a genome-wide association study.

2) To describe the methylome and miRNome of well-differentiated follicular cell-derived thyroid tumors, and to assess the correlation between DNA methylation, miRNA and mRNA expression.

3) Evaluate the potential utility of DNA methylation and miRNA signatures as diagnostic and/or prognostic markers of well-differentiated follicular cell-derived thyroid tumors.

4) To complement the genomic characterization of medullary thyroid cancer by exploring its methylome. Also, to assess the correlation between DNA methylation, miRNA and mRNA expression in order to uncover regulatory axes important in the disease etiology.

5) Assess potential associations between medullary thyroid cancer genetic drivers and the expression of several targets of tyrosine kinase inhibitors in order to uncover whether the mutations could serve in guiding the choice of the most suitable treatment.

ARTICLES

ARTICLE 1

Article 1: Thyroid cancer GWAS identifies 10q26.12 and 6q14.1 as novel susceptibility loci and reveals genetic heterogeneity among populations.

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Abstract

Thyroid cancer (TC) is the most heritable cancer of all those not displaying typical Mendelian inheritance. Notwithstanding the great effort that has been made to identify the causal genetic factors, most of these remain unknown. Here, by performing a two-step association study in differentiated thyroid cancer involving 1,820 cases and 2,410 controls, we provide novel insights into the genetic susceptibility of this disease.

Our study highlights that the 9q22.33 locus contains the most strongly associated low penetrance common variants in TC. In addition, novel variants at 10q26.12 and 6q14.1 within or close to the *WDR11-AS1* and *HTR1B* genes, respectively, were detected to be associated with risk of the disease in a population-specific manner. The findings herein described suggest that heterogeneity in genetic susceptibility between populations is a key feature to take into account when exploring risk factors related to TC. This heterogeneity may also explain at least part of the lack of replication of findings between studies.

On the whole, this study depicts how genetic heterogeneity between populations influences thyroid cancer susceptibility and uncovers it as a part of the hidden heritability of this disease.

Personal contribution: I participated in the extraction and preparation of the samples. I also contributed to the discussion of the results and drafting of the paper.

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Thyroid cancer GWAS identifies 10q26.12 and 6q14.1 as novel susceptibility loci and reveals genetic heterogeneity among populations

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Thyroid cancer is the most heritable cancer of all those not displaying typical Mendelian inheritance. However, most of the genetic factors that would explain the high heritability remain unknown. Our aim was to identify additional common genetic variants associated with susceptibility to this disease. In order to do so, we performed a genome-wide association study in a series of 398 cases and 502 controls from Spain, followed by a replication in four well-defined Southern European case-control collections contributing a total of 1,422 cases and 1,908 controls. The association between the variation at the 9q22 locus near *FOXE1* and thyroid cancer risk was consistent across all series, with several SNPs identified (rs7028661: OR = 1.64, $p = 1.0 \times 10^{-22}$, rs7037324: OR = 1.54, $p = 1.2 \times 10^{-17}$). Moreover, the rare alleles of three SNPs (rs2997312, rs10788123 and rs1254167) at 10q26.12 showed suggestive evidence of association with higher risk of the disease (OR = 1.35, $p = 1.2 \times 10^{-04}$, OR = 1.26, $p = 5.2 \times 10^{-04}$ and OR = 1.38, $p = 5.9 \times 10^{-05}$, respectively). Finally, the rare allele of rs4075570 at

Key words: thyroid cancer, susceptibility, genetic heterogeneity, FOXE1, HTR1B

Abbreviations: CI: confidence interval; DIRC3: disrupted in renal carcinoma 3; FOXE1: Forkhead box protein E1; FTC: follicular thyroid carcinoma; fvPTC: papillary thyroid carcinoma follicular variant; GWAS: genome-wide association study; HTR1B: 5-hydroxytryptamine (serotonin) receptor 1B; LD: linkage disequilibrium; LPG: low penetrance genes; NKX2-1: thyroid transcription factor 1; OR: odds ratio; PTC: papillary thyroid carcinoma; QC: quality control; SNP: single nucleotide polymorphism; TSHR: thyroid-stimulating hormone receptor; WDR11-AS1: WDR11 antisense RNA 1; WDTC: well-differentiated forms of thyroid cancer

Additional Supporting Information may be found in the online version of this article.

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6q14.1 conferred protection in the series studied (OR = 0.82, $p = 2.0 \times 10^{-04}$). This study suggests that heterogeneity in genetic susceptibility between populations is a key feature to take into account when exploring genetic risk factors related to this disease.

What's new?

Thyroid cancer shows the highest genetic susceptibility among all cancers with non-Mendelian hereditability. The authors performed a two-step association study involving 1820 cases and 2410 controls in Europe and identify the 9q22 locus near the *FOXE1* locus as the most important low-penetrance variation in thyroid cancer. In addition, novel variations at 10q26.12 and 6q14.1 were found associated with risk of the disease in a population-specific manner, underscoring how genetic heterogeneity among populations influences thyroid cancer susceptibility.

Thyroid cancer is the most common endocrine malignancy, accounting for around 1% of all new cancer diagnoses yearly. Around 85% of all thyroid tumors are diagnosed as papillary thyroid carcinomas (PTC), while follicular thyroid carcinomas (FTC) account for up to 10% of cases. As both of these carcinomas tend to preserve the differentiated state of the follicular cell from which they originate, they are also termed well-differentiated thyroid cancer (DTC). Of note, as many as 10% of PTC patients have a family history of the disease in a first- or a second-degree relative, suggesting that this is a highly heritable disease.[1–3] In fact, the genetic proportion of susceptibility is the highest among all cancers not showing typical Mendelian hereditability—53%.[1] Since pedigrees displaying a Mendelian mode of inheritance are extremely rare, it is not surprising that no high-penetrance protein-coding gene predisposing to PTC has been identified so far. Linkage analyses have only identified putative genomic loci, but most of these findings have not been consistently replicated.[4–8]

Therefore, it has become generally accepted that genetic predisposition to DTC is likely due to common low-penetrance or rare moderate-penetrance variants.[9] Moreover, there is evidence that gene-gene[10,11] and gene-environment interactions[12,13] modulate individual susceptibility. In this scenario, both carefully designed candidate-gene association studies[14–16] and genome-wide association studies (GWAS)[17–20] have been used to identify susceptibility variants for DTC.

Even if the genome-wide approach does not offer a straightforward biological explanation for all associations found, it undoubtedly detects association signals on unexpected genes or genomic regions, unlikely to have been included in a candidate-gene approach. Four such studies have been carried out so far in DTC.[17–20] While the first three GWAS were conducted on a relatively small series of patients, they all detected an association with locus 9q22 (rs965513), located close to thyroid-specific transcription factor *FOXE1*. Other weaker associations were detected with rs944289 at 14q13.3 (near *NKX2-1*),[18] rs966423 at 2q35 (in an intron of *DIRC3*) and rs2439302 at 8p12 (in an intron of *NRG1*).[17] The last GWAS to be performed in DTC used a considerably higher number of samples.[19] Apart from robustly replicating the associations with SNPs at 9q22 and 2q35, it became especially apparent from the study by Kohler *et al.* that inter-population heterogeneity can be relevant for some of the SNPs emerging as influencing the risk of DTC.[19] The associations described in these studies only explain a limited part of the heritability of DTC, which will make additional research on specific populations of particular relevance in the near future.

Herein, we performed a GWAS in a collection of 398 cases and 502 controls from Spain, followed by a replication step in four different well-characterized Southern European case-control series, three from different Spanish regions and one from Italy. Importantly, Italy is among the countries with the highest incidence rate of DTC in the world. On the other hand, Spain generally displays low incidence rates, but there are some regional differences in the disease prevalence (Cancer Incidence in Five continents, IX, 2000, http://ci5.iarc.fr/CI5i-ix/ci5i-ix.htm). We once again confirm the implication of the 9q22 locus harboring *FOXE1* in DTC risk, and also detect suggestive novel associations that point to 10q26.12 and 6q14.1 regions as associated with DTC susceptibility.

Material and Methods

Ethics statement

Written informed consent was obtained from all participants in accordance with the protocols approved by the "Comité de bioética y bienestar animal del Instituto de Salud Carlos III" and the Ethics Committee of the COR (Regional Cancer Center), Padova, Italy, which approved this study.

Subjects

All participants in this study were of European ancestry. Five series of thyroid cancer cases were recruited in distinct regions of Spain and Italy from January 1st, 2002 to December 31st, 2011. Tumor histology was assessed by pathologists at each participating institution. The median time from diagnosis to recruitment was 4 years (range 1–19). Personal and clinical data, such as family history of thyroid cancer and

Table 1. Characteristics of the discovery and replication series used in the study

	GWAS	Italy	Galicia	Spanish network 2	Catalonia	Total
Controls	**502**	**532**	**437**	**531**	**408**	**2,410**
Sex (M/F)	257/245	255/277	84/353	309/222	98/310	1,003/1,407
Median age (range)	55 (20–89)	72 (12–101)	49 (22–97)	72 (34–95)	50 (20–86)	
Cases	**398**	**541**	**287**	**240**	**354**	**1,820**
Sex (M/F)	66/332	114/427	57/230	49/191	80/274	366/1,454
Median age (range)	46 (10–85)	50 (5–87)	50 (11–92)	46 (13–85)	45 (13–88)	
Histology						
FTC	45 (11%)	45 (8%)	37 (13%)	27 (11%)	45 (13%)	199
PTC	248 (62%)	475 (88%)	176 (61%)	166 (69%)	262 (74%)	1,327
fvPTC	105 (27%)	21 (4%)	74 (26%)	47 (20%)	47 (13%)	294

Abbreviations: FTC, follicular carcinoma; PTC, papillary carcinoma; fvPTC, papillary carcinoma follicular variant.

tumor subtype, stage, and surgery was collected by questionnaire, completed by clinicians at recruitment. In all five patient series, predominantly sporadic (>97% of cases) and adult cases (>98% of cases) were recruited. Controls free of thyroid cancer and without family history of cancer were recruited from the same geographical regions as the cases.

Discovery series (series I). We recruited 417 patients from diverse geographic locations of Spain through the Spanish hospital network to be used in the GWAS. A series of 505 cancer-free controls was available from the same geographical regions as control group. Twenty-two individuals (19 cases and 3 controls) were excluded because their ages were unknown. The final set of samples consisted of 398 cases and 502 controls.

Replication series. A total of four independent case-control series were recruited. The first comprised 541 cases and 532 controls from three Italian hospitals. The second included 287 cases and 437 controls from hospitals located in Galicia, in Northwestern Spain. A third Spanish series recruited in Madrid was composed of 240 cases and 531 controls. A fourth independent group of Spanish thyroid cancer patients and controls was obtained from hospitals located in Catalonia, and included 354 cases and 408 controls. Overall, the replication study comprised 1,422 thyroid cancer cases, including 1,268 PTC (of which 189 corresponded to fvPTC) and 154 FTC (Table 1) and 1,908 controls. Demographic and histological information is shown in Table 1.

DNA isolation, genotyping and quality control

Blood or saliva samples were obtained from all cases and controls. Genomic DNA was extracted from peripheral blood lymphocytes by automated methods according to the manufacturer's instructions (Magnapure, Roche, Madrid, Spain), or manually, using standard methods. The Oragene DNA Self-Collection Kit (DNA Genotek, Ottawa, Canada) was used for DNA extraction from saliva. DNA concentration was quantified in all samples prior to genotyping using the Quant-iT PicoGreen dsDNA Reagent (Invitrogen, Eugene, OR).

Genotyping for the GWAS was performed using the Affymetrix Axiom Genome-Wide CEU array (Affymetrix, Santa Clara, CA) using solely DNA samples extracted from blood lymphocytes. This genotyping panel is designed to maximize coverage of common alleles (MAF >1%) across the genome in European populations. SNPs were called using the Axiom GT1 algorithm implemented in the Affymetrix Genotyping Console software (v.4.1.2.837) and transformed into PLINK format[21] using the Genotyping Data Filter v.3.3.2.[22] A total of 628,348 markers were successfully called and corresponded uniquely to base-pair substitutions.

SNPs showing the lowest p values for association ($p < 0.0001$) in the initial series and seven additional SNPs previously reported to be associated with DTC[10,23,24] (rs2910164 at 5q33.3 in mir-146a gene, rs6983267 at 8q24.21 in *LOC727677* intron, rs1867277 and rs965513 at 9q22 near *FOXE1*, rs2284734 at 14q31.1 in an intron of *TSHR*, rs664677 at 11q22.3 in *ATM* intron and rs944289 at 14q13.3 near *NKX2-1*) were genotyped in the replication study using MassARRAY SNP genotyping system (Sequenom Inc., San Diego, CA) at the node of the Spanish National Genotyping Center of the University of Santiago de Compostela (CeGen-ISCIII, www.cegen.org). Multiplexed assays were designed using the Sequenom Assay Design v3.1 software for three plexes containing 59 SNPs. This assay design was used to genotype the 3,330 samples included in this replication step according to the manufacturer's instructions for the Sequenom iPLEX GOLD chemistry. Three trio samples provided by "Centre d'Etude du Polymorphisme Humain" (CEPH) were used as positive controls, and non-DNA controls and replicates were included in each plate. Genotyping specificity was assessed by including two DNA duplicates (an intra-assay and an inter-assay duplicate) and a negative control in each 96-well plate genotyped, yielding 100% consistent results. In addition, each plate included case and control samples. The resulting data were analyzed using the MassARRAY Typer 4.0 Analyzer software.

Imputation was performed in the initial series for additional SNPs at 9q22 (rs965513 and rs1867277), and at

14q13.3 (rs944289) based on data from the 1000 Genomes Project using Beagle v3.[25]

Statistical analysis

Statistical tests were carried out using PLINK software, version 1.07,[21] IBM SPSS Statistics version 20 (IBM SPSS Statistics) and R for Windows.

For each SNP, associations were assessed by applying logistic regression to estimate odds ratios (ORs) with 95% confidence intervals (CIs) and p values. All analyses were performed assuming a log-additive effect for each polymorphism (the genotypes were coded as 0, 1 or 2 according to the number of minor alleles). Gender and age were included as covariates in all analyses. Moreover, for locus 9q22, we tested for residual association by adding rs7028661 as covariate.

A fixed-effects model of the Mantel-Haenszel method[26] was applied to pooled data from the series. Between-series heterogeneity was assessed by Cochran's Q test and I^2 statistics. If the heterogeneity was significant (p_{het} value <0.05 for Q statistics), a random effects model was applied.

Gene-base tests (sequence kernel association test, SKAT)[27] were applied to explore the cumulative effect of SNPs in or near a given gene within each population and in the combined data set, as implemented in skatMeta R library (http://cran.r-project.org/).

A power analysis was conducted on our discovery series sample size using the following assumptions: 400 patients with thyroid cancer and 500 controls, $\alpha = 0.0001$ and a minor allele frequency of 0.3. We estimated that we had >30%, >57% and >80% power of detecting a per allele odds ratio of 1.4, 1.5 and 1.6, respectively.

Results

Quality control (QC) procedures included exclusion of SNPs with frequencies <5% ($n = 109{,}205$), missing genotypes for >5% of samples ($n = 41{,}432$) and Hardy-Weinberg Equilibrium test p values $<10^{-6}$ in either the control or the all-samples groups ($n = 3{,}139$). SNPs were also tested for differences in genotyping success rates in cases and controls, and those with p values $<10^{-6}$ were excluded ($n = 2{,}090$). The final set of markers consisted of 474,624 SNPs genotyped in 398 cases and 502 controls.

Fifty-two SNPs showed statistical significance below $p < 0.0001$ in the initial series and are listed in Supporting Information Table S1. These SNPs and seven additional ones, selected based on a literature search, were considered for further replication steps (59 SNPs in total). Table 2 shows results for those SNPs, which displayed the strongest association signals in the fixed-effects meta-analysis of the initial and replication series, highlighted in bold in Table 2. While the results shown in Table 2 were obtained comparing all cases *versus* controls (regardless of the histological diagnosis), similar results were obtained when only PTC cases were considered (data not shown).

9q22 locus

A significant association with SNPs from 9q22 locus, where the *FOXE1* gene is located, was also detected in the replication series, confirming once more the role of this locus in DTC susceptibility. The SNPs previously associated with the disease, rs965513[18] and rs1867277,[16] had to be imputed for the genome-wide step, as they were not included on the platform used (rs965513: OR = 1.73, $p = 2.2 \times 10^{-07}$, rs1867277: OR = 1.39, $p = 1.2 \times 10^{-03}$). As shown in Supporting Information Table S2, the meta-analysis for these two SNPs revealed a strong association (rs965513: OR = 1.65, $P = 2.7 \times 10^{-23}$, rs1867277: OR = 1.41, $P = 3.4 \times 10^{-12}$). Moreover, we detected associations with three other variants in the same chromosomal location (Table 2). In all studied populations, strong LD was observed between rs965513 and rs7028661, and between rs1867277 and rs10122541 (Supporting Information Figure S1). We assessed whether the associations observed remained after adjusting for the most strongly associated genotyped variant by adding in rs7028661 as a covariate in the logistic regression analyses. The association with the disease remained significant in the meta-analysis of all the other 9q22 SNPs: those genotyped (rs7037324: OR = 1.25, $p = 1.3 \times 10^{-04}$, rs10122541: OR = 1.22, $p = 7.4 \times 10^{-04}$) and those imputed (rs1867277: OR = 1.18, $p = 2.1 \times 10^{-03}$, rs965513: OR = 1.34, $p = 0.037$).

Other known SNPs

Apart from two SNPs at 9q22, only one of the seven previously published SNPs included in the replications steps (see Material and Methods) reached significance in the final meta-analysis (Supporting Information Table S2). It was the SNP rs944289 at 14q13.3 localized upstream of *NKX2-1* gene. This polymorphism did not reach the threshold of significance when imputed in the discovery phase ($p = 0.07$), but was found to be nominally significant in the Catalonian ($p = 0.003$) and Italian ($p = 0.01$) replicates, reaching a significant p values of 1.5×10^{-5} in the meta-analysis (Supporting Information Table S2). Additionally, a meta-analysis combining our results with those previously published on this locus[18,20,24] (Supporting Information Table S3) showed a significant association with the disease (OR = 1.30, $p < 0.0001$). Of note, Cochran's Q test and I^2 statistics did not reveal significant heterogeneity among the populations ($I^2 = 0\%$, $p_{het} = 0.60$).

The allele frequency of the four markers not replicated was not different among the populations, in which the SNPs were originally detected, and those studied here.

Novel SNPs

Results for the genotyped SNPs in the replication phase are listed in Supporting Information Table S4. Several SNPs in the GWAS series were located at the same locus. This was the case for three SNPs from 10q26.12 localized in intronic regions of *WDR11-AS1* (rs2997312, rs10788123 and

Table 2. Association results for the top replicated SNPs

SNP	chr	Position	Gene	Origin	*N* (controls/cases)	MAF controls	MAF cases	OR	95%CI	*p*
rs4075570	6	77903809	*HTR1B*	1_GWAS	(502/398)	0.35	0.28	0.64	0.51–0.80	9.6×10^{-05}
				2_Italy	(532/541)	0.36	0.33	0.85	0.70–1.04	0.12
				3_Galicia	(437/286)	0.36	0.29	0.74	0.59–0.92	0.0083
				4_Spanish network 2	(531/240)	0.33	0.30	0.90	0.65–1.26	0.54
				5_Catalonia	(407/354)	0.34	0.35	1.05	0.85–1.29	0.68
				Meta-analysis	**(2,409/1,819)**			**0.82**	**0.74–0.91**	$\mathbf{2.0 \times 10^{-04}}$
								$p_{het} = 0.025$	I^2 = 64.2%	0.029
rs7028661	9	100538470	*FOXE1*	1_GWAS	(502/398)	0.33	0.44	1.71	1.38–2.11	7.8×10^{-07}
				2_Italy	(531/541)	0.34	0.49	1.97	1.61–2.41	5.5×10^{-11}
				3_Galicia	(437/287)	0.34	0.38	1.17	0.95–1.44	0.15
				4_Spanish network 2	(531/240)	0.33	0.47	1.84	1.33–2.54	2.2×10^{-04}
				5_Catalonia	(408/353)	0.32	0.46	1.73	1.41–2.14	2.4×10^{-07}
				Meta-analysis	**(2,409/1,819)**			**1.64**	**1.49–1.81**	$\mathbf{1.0 \times 10^{-22}}$
								$p_{het} = 0.007$	I^2 = 71.5%	2.1×10^{-07}
rs10122541	9	100628268	*FOXE1*	1_GWAS	(502/398)	0.32	0.43	1.66	1.34–2.06	3.6×10^{-06}
				2_Italy	(528/541)	0.40	0.50	1.64	1.34–2.00	1.4×10^{-06}
				3_Galicia	(437/284)	0.36	0.42	1.26	1.02–1.56	0.030
				4_Spanish network 2	(531/238)	0.33	0.45	1.76	1.28–2.44	5.9×10^{-04}
				5_Catalonia	(408/354)	0.33	0.44	1.54	1.26–1.90	3.5×10^{-05}
				Meta-analysis	**(2,406/1,815)**			**1.54**	**1.40–1.70**	$\mathbf{1.1 \times 10^{-17}}$
								$p_{het} = 0.304$	I^2 = 17.31%	
rs7037324	9	100658318	*FOXE1*	1_GWAS	(502/398)	0.32	0.44	1.73	1.39–2.14	6.3×10^{-07}
				2_Italy	(528/540)	0.40	0.50	1.64	1.35–2.01	1.2×10^{-06}
				3_Galicia	(432/286)	0.37	0.42	1.23	0.99–1.51	0.060
				4_Spanish network 2	(531/240)	0.34	0.46	1.69	1.23–2.33	0.0013
				5_Catalonia	(407/351)	0.34	0.45	1.54	1.25–1.89	4.0×10^{-05}
				Meta-analysis	**(2,400/1,815)**			**1.54**	**1.39–1.70**	$\mathbf{1.2 \times 10^{-17}}$
								$p_{het} = 0.177$	I^2 = 36.7%	
rs2997312	10	122567702	*WDR11-AS1*	1_GWAS	(502/398)	0.10	0.14	2.13	1.53–2.96	7.0×10^{-06}
				2_Italy	(530/540)	0.13	0.15	1.27	0.95–1.68	0.10
				3_Galicia	(437/286)	0.10	0.11	1.02	0.71–1.45	0.93
				4_Spanish network 2	(531/240)	0.11	0.14	1.31	0.82–2.08	0.26
				5_Catalonia	(408/353)	0.11	0.13	1.19	0.86–1.65	0.28

Table 2. Association results for the top replicated SNPs (Continued)

SNP	chr	Position	Gene	Origin	*N* (controls/cases)	MAF controls	MAF cases	OR	95%CI	*p*
				Meta-analysis	**(2,408/1,817)**			**1.35**	**1.16–1.57**	**1.2×10^{-04}**
								$p_{het} = 0.032$	$I^2 = 62.0\%$	0.021
rs10788123[1]	10	122568882	*WDR11-AS1*	1_GWAS	(502/398)	0.20	0.27	1.69	1.32–2.16	3.2×10^{-05}
				2_Italy	(530/540)	0.23	0.26	1.26	1.01–1.57	0.043
				3_Galicia	(435/286)	0.23	0.23	0.95	0.74–1.23	0.72
				4_Spanish network 2	(531/240)	0.24	0.26	1.16	0.81–1.66	0.41
				Meta-analysis	**(1,998/1,464)**			**1.26**	**1.11–1.43**	**5.2×10^{-04}**
								$p_{het} = 0.018$	$I^2 = 70.4\%$	0.077
rs1254167	10	122601203	*WDR11-AS1*	1_GWAS	(502/398)	0.08	0.13	2.42	1.70–3.44	9.9×10^{-07}
				2_Italy	(532/540)	0.11	0.14	1.27	0.95–1.71	0.11
				3_Galicia	(437/286)	0.10	0.09	0.90	0.63–1.29	0.57
				4_Spanish network 2	(530/240)	0.09	0.13	1.25	0.76–2.04)	0.39
				5_Catalonia	(408/353)	0.09	0.12	1.40	1.01–1.96	0.047
				Meta-analysis	**(2,409/1,817)**			**1.38**	**1.18–1.62**	**5.9×10^{-05}**
								$p_{het} = 0.004$	$I^2 = 74.1\%$	0.048
rs944289[2,3]	14	36649246	*NKX2-1*	1_GWAS	(502/398)	0.56a	0.61a	1.21a	0.98–1.48	0.072
				2_Italy	(529/540)	0.60	0.66	1.30	1.06–1.60	0.012
				3_Galicia	(437/286)	0.54	0.57	1.16	0.94–1.43	0.17
				4_Spanish network 2	(531/240)	0.56	0.61	1.11	0.81–1.53	0.50
				5_Catalonia	(408/352)	0.51	0.63	1.36	1.11–1.66	0.0033
				Meta-analysis	**(2,407/1,816)**			**1.24**	**1.13–1.37**	**1.5×10^{-05}**
								$p_{het} = 0.762$	$I^2 = 0\%$	

[1]Not successfully genotyped in the Catalonian series.
[2]Not genotyped in the original GWAS, but added in a posterior validation step, thus its genotype was imputed.
[3]ORs and allele frequencies calculated for the common (T) allele, for an easier comparison with results from Ref. 18.
Abbreviations: MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

rs1254167) and three from 6q14.1 in the proximity of the *HTR1B* gene (rs4075570, rs12206214 and rs4463226). Findings from more detailed LD analyses from the genome-wide step of the 10q26.12 and 6q14.1 loci are provided in Supporting Information Figure S2. All but the last two SNPs from 6q14.1 had p values below the cut-off to be considered as significantly associated in the meta-analysis (Table 2). These other two SNPs remained close to statistically significant in the meta-analysis: rs12206214 (meta-analysis p value 0.0025, with nominal significance in Italian replicate) and rs4463226 (meta-analysis p value 0.0006, nominal significance in the Galician replicate). Both genes showed a suggestive p value in the meta-analysis of the SKAT gene-base test: $p = 9.37 \times 10^{-4}$ for the combined effect of the three SNPs of *HTR1B* and $p = 7.23 \times 10^{-5}$ for the three SNPs in *WDR11-AS1* (meta-analysis performed excluding Catalonian population due to the lack of data for rs10788123).

As shown in Table 2, the estimated OR from the Galician replication series was that which most differed from the others for four of the five SNPs, for which significant heterogeneity was observed, possibly due to heterogeneity among the included populations. Therefore, a random effects model was also applied when the p_{het} was smaller than 0.05. Even if the estimated p values (underlined in Table 2) were greater, the associations assessed by this more rigorous method remained statistically significant at $p < 0.05$ for all but one SNP (rs10788123).

Discussion

The genetic contribution to thyroid cancer risk is greater than that of any other cancer, and the effect extends beyond the nuclear family.[1,28] However, most of the hereditable risk to DTC remains unexplained.[9] Here, we performed a GWAS in order to look for common variants predisposing to PTC and FTC. We further confirmed some of the previously reported genetic associations with DTC. Moreover, we detected a suggestive association with DTC for several SNPs within the 10q26.12 and 6q14.1 genomic loci.

All the GWAS in DTC performed until now have consistently found an association between the locus 9q22 and disease risk. This locus was the only one associated with susceptibility to DTC in all of the populations considered in the present study, confirming it harbors the most important low-penetrance variation in DTC. On the other hand, we observed two high-risk LD blocks at 9q22. Importantly, the two variants associated with DTC in other studies (rs965513[18] and rs1867277[16]) lie in distinct LD blocks, which is consistent with previous reports.[24] Further analyses revealed that SNPs of 9q22 remained statistically significantly associated with disease risk after adjusting for rs7028661, localized in the same LD block as rs965513. This suggests that even though this latter SNP is the most validated low-penetrance variation in DTC, additional independent disease-associated genetic variation is found in the locus. Further fine-mapping and functional studies are required to identify the underlying risk-modifying variant(s) at this locus.

A SNP at 14q13.3 adjacent to *NKX2-1* was reported by Gudmundsson *et al.*[18] to be associated with risk of the disease, and replicated by Jones *et al.*[24] but not in other studies.[20] We replicated this association in meta-analysis of our data. Furthermore, as the meta-analysis involving all GWAS studies published so far with data on rs944289[18,20,24] confirmed the significant association of variation at locus 14q13.3 with DTC, it suggests this genomic region plays a relevant role as a DTC risk factor, although probably having a more subtle effect than variation at 9q22.

However, associations previously reported by others and included in the replication steps of this study (rs2910164, rs6983267, rs664677 and rs2284734) were not replicated. Similarly, no association signal was observed in the genome-wide step of this study for other markers identified previously (rs966423 at 2q35 and rs2439302 at 8p12[17] or rs10136427 at 14q24.3 and rs7267944 at 20q12[29]). As all of these genetic variants were described in a single study, without replication by subsequent studies, they could be associated with the disease in a population-specific manner. Indeed, there are important differences observed among specific European populations in thyroid cancer prevalence suggesting that attention should be paid to the inter-population heterogeneity in genetic susceptibility.

In this regard, the associations observed using our data appeared to be different for the Galician population for some SNPs, despite the lower number of cases and controls. Specifically, rs7028661 in *FOXE1* was more weakly associated with DTC in the Galician than in the other series. This type of population effect could be reflecting different population history[30] and possibly gene-environment interactions.[31] Galicia is a large, but quite isolated region in North-western Spain, surrounded by important geographic barriers and characterized by specific cultural characteristics including language, which could have contributed to the emergence of genetic diversity. In fact, founder effects have been commonly observed in Galicia. For instance, the founder mutation *BRCA1* 330A>G (p.R71G) explains more than 50% of all inherited breast cancer cases in this population,[32] while in other Spanish as well as Caucasian populations it is not observed.

The disparity observed among different DTC GWAS is similar to findings reported in other diseases, such as breast cancer.[33] It seems that to identify novel low-penetrance variants conferring very small effects, very large numbers of cases and controls (>10,000 of each) need to be used in order to achieve sufficient statistical power, which generally requires international collaboration.[34] Indeed, the limitation of the current study is the sample size available. Still, we were able to identify novel low-penetrance variants associated with DTC that were heterogeneously validated in different populations. It seems plausible that the differences observed among the studied cohorts are due to genetic heterogeneity

and not to other factor; the genotyping was done in only one centre and the results obtained were consistent after restriction to only one disease subtype.

Regarding the novel associations, we focused our attention on loci exhibiting more than one association signal. Two low correlated SNPs (rs10788123 and rs1254167, $r^2 = 0.32$), located at 10q26.12 in intronic regions of *WDR11-AS1*, were associated with risk of DTC. This gene codes for an antisense RNA to *WDR11*, which is a scaffold protein involved in many cellular processes, including cell cycle progression, signal transduction, apoptosis, and gene regulation. Therefore, the disruption of the *WDR11* regulation axis may represent a potentially transforming molecular mechanism in DTC etiology. Of note, this locus has been found to be disrupted in cancer by translocation events,[35] and proposed to have a tumor suppressor role. The eQTL analysis Genevar tool (http://www.sanger.ac.uk/resources/software/genevar/) found a significant association between rs10788123 and *WDR11* expression in lymphocytes. Although data on thyroid tissue is not available in this browser, it is tempting to suggest that this SNP could potentially interfere with the expression of the *WDR11* gene in the thyroid.

Finally, rs4075570 located at 6q14.1 in an intergenic region with very few nearby genes, is significantly associated with *HTR1B* expression in lymphocytes and adipocytes according to the Genevar browser. This gene codes for a serotonin receptor involved in a wide variety of physiological functions. The role of the *HTR1B* gene has already been studied in lung, breast, ovary and endometrial cancer.[36–39] Methylation-mediated decreased *HTR1B* expression was apparently a common feature among most of these neoplasias, pointing to a potential tumor suppressor role. Notwithstanding this, there is evidence that this receptor can activate ERK,[40] a well-known member of the MAPK pathway, whose deregulation is the causal aberrance of thyroid cancer.[41] Finally, this receptor seems to mediate serotonin-induced angiogenic signaling,[42] which is yet another hallmark of cancer. Therefore, although further confirmation is warranted, the association of several SNPs belonging to a gene involved in the MAPK pathway and angiogenic signaling converts *HTR1B* in an attractive potential low penetrance DTC gene.

Although only variation at 9q22 reached genome-wide statistical significance ($p < 10^{-7}$), the fact that several independent association signals were observed within 10q26.12 and 6q14.1 loci, increase the interest of our findings, suggesting that these two regions could carry new risk factors related to DTC. Taking into account the effect of population heterogeneity demonstrated in this study, the existence of risk markers whose role in thyroid cancer susceptibility is exclusive to a specific population seems to be a feasible possibility. It could explain at least part of the lack of inter-study replication often observed in thyroid carcinoma and the consideration of this phenomenon may give rise to important insights into this complex disease.

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ARTICLE 2

Article 2: DNA methylation profiling of well-differentiated thyroid cancer uncovers markers of recurrence free survival.

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Abstract

Previous transcriptome profiling in the laboratory showed the expression patterns are subtype specific and capable of identifying high-risk patients. In this following work, we aimed to uncover the potential regulators of the differentially expressed genes. Firstly, we set off to study DNA methylation. Although methylation is an emerging hallmark of cancer, little is known about aberrant epigenetic profiles in thyroid cancer. This study aimed to characterize, at the genome-wide level, the DNA methylation patterns of well-differentiated thyroid tumors, previously classified according to the presence of *RET/PTC* rearrangements, or mutations in *BRAF* or *RAS* genes. Moreover, we aimed to uncover the regulatory DNA methylation changes by integration of transcriptomic data from the same tumors in the analysis.

According to the results of this study, follicular and papillary tumors present distinct methylation profiles, closely linked to specific mutations. We were able to identify genes whose expression is controlled by methylation of their promoters, as well as aberrant methylation events with potential diagnostic and prognostic value. In this regard, it is remarkable the diagnostic value of *KLK10* for *BRAF*-positive tumors, and hypermethylation of *EI24* and *WT1*, which confers higher risk of recurrence, as independent prognostic markers. These findings could have an impact on the clinical management of thyroid cancer patients.

Personal contribution: I extracted and prepared the samples for this study. I performed genetic screening of the samples. I was actively involved in all the statistical analysis, and contributed to the formation of the hypothesis. I also contributed to the discussion of the results and drafting of the paper.

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DNA methylation profiling of well-differentiated thyroid cancer uncovers markers of recurrence free survival

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Cancer Genetics

Thyroid cancer is a heterogeneous disease with several subtypes characterized by cytological, histological and genetic alterations, but the involvement of epigenetics is not well understood. Here, we investigated the role of aberrant DNA methylation in the development of well-differentiated thyroid tumors. We performed genome-wide DNA methylation profiling in the largest well-differentiated thyroid tumor series reported to date, comprising 83 primary tumors as well as 8 samples of adjacent normal tissue. The epigenetic profiles were closely related to not only tumor histology but also the underlying driver mutation; we found that follicular tumors had higher levels of methylation, which seemed to accumulate in a progressive manner along the tumorigenic process from adenomas to carcinomas. Furthermore, tumors harboring a *BRAF* or *RAS* mutation had a larger number of hypo- or hypermethylation events, respectively. The aberrant methylation of several candidate genes potentially related to thyroid carcinogenesis was validated in an independent series of 52 samples. Furthermore, through the integration of methylation and transcriptional expression data, we identified genes whose expression is associated with the methylation

Key words: well-differentiated thyroid cancer, methylation, *BRAF*, *RAS*, prognostic markers

Abbreviations: *AKT3*: v-akt murine thymoma viral oncogene homolog 3; *BRAF*: v-raf murine sarcoma viral oncogene homolog B1; CIMP: CpG island methylator phenotype; *COL4A2*: collagen type IV alpha 2; *DLEC1*: deleted in lung and esophageal cancer 1; *EI24*: etoposide induced 2.4 mRNA; FA: follicular adenoma; FDR: false discovery rate; FTC: follicular thyroid carcinoma; fvPTC: papillary thyroid carcinoma follicular variant; *KLK10*: kallikrein-related peptidase 10; *NIS*: sodium-iodine symporter; PcG: PolyComb Group; PTC: papillary thyroid carcinoma; *RARβ2*: retinoic acid receptor β2; *RASSF1*: *RAS* association domain family protein 1; *RET*: "rearranged during transfection" protooncogene; RFS: recurrence-free survival; *TIMP3*: tissue inhibitor of metalloproteinase-3; *TSHR*: thyroid-stimulating hormone receptor; *WT1*: Wilms tumor 1

Additional Supporting Information may be found in the online version of this article.

*V.M. and R.B. contributed equally to this work

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status of their promoters. Finally, by integrating clinical follow-up information with methylation levels we propose etoposide-induced 2.4 and Wilms tumor 1 as novel prognostic markers related to recurrence-free survival. This comprehensive study provides insights into the role of DNA methylation in well-differentiated thyroid cancer development and identifies novel markers associated with recurrence-free survival.

What's new?

Follicular cell-derived carcinomas of the thyroid gland, which are the most common endocrine malignancies, are of special interest for molecular research, given their common cellular origin. However, whether epigenetic modifications contribute to the heterogeneous nature of follicular thyroid malignancies remains unclear. Here, genome-wide characterization of DNA methylation patterns of well-differentiated thyroid tumors shows that tumors with distinct subtypes and mutational status have unique methylation profiles, offering insight into the biology underlying the heterogeneity and differential outcomes of thyroid cancers. Novel markers associated with recurrence-free survival were also identified and could be used for patient classification.

Follicular cell-derived carcinoma arises from the main cell population of the thyroid gland and is the most common endocrine malignancy, accounting for more than 95% cases. This general term represents a highly heterogeneous entity composed of a spectrum of differentiation stages, ranging from benign lesions such as follicular adenoma (FA), to well-differentiated but mostly indolent carcinomas such as papillary thyroid carcinoma (PTC) or follicular thyroid carcinoma (FTC), through to undifferentiated, more invasive and lethal human malignancies, such those classified as poorly differentiated thyroid carcinoma and anaplastic thyroid carcinoma. Most well-differentiated carcinomas can be effectively clinically managed and have an excellent prognosis. However, a subset of these tumors behave aggressively, and there is currently no effective treatment for them.[1] As all these malignancies arise from the same cell type, it is of great interest to understand the molecular alterations giving rise to the observed heterogeneity.

Genetics has been shown to play an important role in the development of this disease. The most recurrent point mutations and rearrangements tend to affect the effector genes of the MAPK pathway, such as v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*), the *RAS* family and the "rearranged during transfection" protooncogene *RET*. These early alterations have been shown to be exclusive, subtype-specific and to a certain extent prognostic. Virtually all tumors bearing *RAS* mutations present a follicular pattern of growth (FA, FTC or PTC follicular variant [fvPTC]), while *BRAF* mutations and rearrangements in the *RET* gene are characteristic of PTC.[2] It is widely accepted that *BRAF*-positive tumors tend to have a worse prognosis.[3,4] Conversely, *RAS* mutations are detected among both follicular carcinomas and adenomas, thus having diagnostic value but not prognostic value, and leaving many clinical questions to be answered.

At present, high-throughput techniques are being used to identify altered pathways related to the development of specific tumor types or clinical features. In thyroid cancer, it has been demonstrated that aberrant expression patterns can predict a patient's prognosis.[5] Moreover, these patterns have been closely linked to the presence of particular mutations and shown to be specific to each.[5–7] Although it might be expected that other genomic features such as methylation are also closely related to the particular mutated gene that leads to carcinogenesis, little is known about aberrant epigenetic profiles specific to individual thyroid cancer subtypes. Studies published to date have followed either a strict candidate gene-based approach or have used a limited number of samples.[8] Therefore, the genes identified so far as repressed by aberrant methylation are either involved in thyroid gland function (*e.g.*, thyroid-stimulating hormone receptor [*TSHR*],[9] sodium-iodine symporter [*NIS*][10] or have a tumor suppressor gene function (*e.g.*, tissue inhibitor of metalloproteinase-3 [*TIMP3*], retinoic acid receptor β2 [*RARβ2*],[11,12] *RAS* association domain family protein 1 [*RASSF1*][13]. A global view of genome-wide aberrant methylation in thyroid cancer is still lacking.

In this study, we quantitatively profiled the genome-wide DNA methylation of 83 primary thyroid tumors (18 FA, 18 FTC and 47 PTC) and 8 samples of adjacent normal thyroid tissue using the 27 K Infinium Methylation Array. We identified disease subtype- and mutation-specific DNA methylation patterns and propose novel markers related to recurrence-free survival (RFS). Moreover, by integrating methylation data with mRNA expression, we were able to identify genes whose expression is associated with the methylation status of their promoter regions, thereby adding new insights into thyroid carcinogenesis.

Material and Methods

Sample collection and patient follow-up

One hundred and thirty-two thyroid tumors were snap frozen following surgery at Hospital Sant Pau and Hospital Sabadell in Barcelona (Spain) and at Hospital Arnau de Vilanova in Lleida (Spain), and stored at −80°C. Of these, 83 primary thyroid tumors (42 PTC, 5 fvPTC, 18 FA and 18 FTC) not previously profiled at the genome-wide DNA methylation level[8] were used in the discovery phase of the study, and the remaining 49 tumor samples (24 PTC, 9 fvPTC, 12

FA and 4 FTC) in the replication phase. Sections of each sample were evaluated by a pathologist and, when necessary, non-tumoral tissue was dissected. We studied eight adjacent normal thyroid tissues in the discovery and three in the replication series. At least 80% of the cells were cancerous in all tumor samples, while non-tumor samples had no observable tumor epithelium. Tumors were classified as PTC, fvPTC, FA and FTC according to the criteria proposed by WHO classification of tumors of the endocrine system, by three pathologists with experience on thyroid pathology (XM, MRB and EL). All cases in the PTC group exhibited the typical cytological and architectural features of the classical variant. Strict criteria were used for fvPTC; tumors showed unquestionable cytological features together with a follicular pattern of growth. For FA, presence of capsule and absence of hyperplasic changes in the adjacent thyroid tissue was required. For FTC, obvious evidence of vascular and capsular invasion was also required. Genomic DNA was extracted from all samples using the DNeasy Blood and Tissue kit (QIAGEN) according to the manufacturer's protocol.

The clinical follow-up of the patients was performed by physical examination, neck ultrasonography, simultaneous determination of serum anti-tiroglobulin antibodies with tiroglobulin (basal, or after thyrotropin stimulation by thyroid hormone withdrawal or the administration of recombinant human thyrotropin) and whole-body iodine scanning. If there was a suspicion of local or distant disease, other imaging techniques such as CT, MRI, PET-CT or scintigraphy were used. The frequency and the type of technique used depended on the postoperative staging, which was also used to assess prognosis and to guide adjunctive therapy.

Mutation analysis

All PTCs were screened by Sanger sequencing for *BRAF* mutations at codon 600 in exon 15, while FA, FTC and fvPTC samples were screened for mutations in *H-, N-* and *K-RAS* at mutational hotspots on codons 12 and 13 of exon 2 and codon 61 of exons 3. When available, cDNA from PTC samples was also screened for *RET/PTC1* and *RET/PTC3* rearrangements as previously described.[5]

DNA methylation assay, data processing and data analysis

Briefly, genomic DNA was bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) following the manufacturer's recommended procedures. Genome-wide promoter DNA methylation profiling was performed at the Spanish "Centro Nacional de Genotipado (CEGEN-ISCIII)" (www.cegen.org) using the Illumina Infinium HumanMethylation 27K Platform (Illumina, San Diego, CA) as described previously.[14] This assay generates DNA methylation data for 27,578 CpG dinucleotides covering 14,473 unique genes. The raw intensity data were quartile-normalized using the R package, HumMeth27QCReport.[15] For each CpG site, methylation levels were quantified using β-values, which represent the proportion of methylation, calculated as $M/(M + U)$, where M is the methylated probe intensity and U the unmethylated probe intensity. β-Values range from 0 to 1, with 0 being completely unmethylated and 1 being completely methylated. M-Values, defined as $\log_2(M/U)$, were used for statistical analyses; negative values indicate less than 50% methylation and positive values indicate more than 50% methylation.[16] We excluded probes that were detected in less than 95% of the samples (16 probes), probes designed for sequences on either the X or the Y chromosome (1,084 or 7 probes, respectively) as well as probes that potentially hybridized to more than one genomic locus (538 probes). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE51090 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51090).

Unsupervised hierarchical clustering was performed using Cluster 3.0 software with "complete linkage" (Pearson correlation, uncentered metrics). The clusters were subsequently visualized using Treeview (http://rana.stanford.edu/software), and Principal Component Analysis (PCA) was performed using R CRAN version 2.15.3 (R, 2013).

Differences in DNA methylation status between normal thyroid tissue and specific subtypes were tested using POMELLO II, applying either a t-test with 200,000 permutations or linear models (limma).[17] To account for multiple hypotheses testing, p-values were adjusted using Benjamini's false discovery rate (FDR) correction. We defined a probe to be hypomethylated or hypermethylated when it displayed a mean M-value difference (ΔM-value) < -1.4 or >1.4, respectively, between a particular tumor group and normal tissue, and had a FDR < 0.05.

Methylation status validation: selection of candidate genes and bisulfite sequencing

Three of the most differentially methylated probes, all with a high fold-change across the experimental groups, were selected for validation. Biological functions were considered as additional criteria to select candidate promoter regions. Technical validation of microarray results was performed using bisulfite sequencing, first in a subset of the original discovery series (comprising 4 FA, 7 FTC, 13 PTC and 8 adjacent normal thyroid tissue samples). The candidate markers were then validated in 52 independent samples (24 PTC, 9 fvPTC, 12 FA, 4 FTC and 3 adjacent normal thyroid tissue samples).

From the bisulfate-treated DNA, at least two independent nested PCRs (for each sequence to be studied) were performed using two sets of primers specifically designed to contain no CpG sites (Supporting Information Table S1). The pooled PCR products were purified (High Pure PCR product Purification Kit, Roche) and analyzed by Sanger sequencing (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems). Primary tumors were classified as hyper- or hypomethylated when the studied locus showed an increase or a

Table 1. Summary of the main clinical and pathological characteristics of samples used in this study

Clinical characteristics	Discovery series ($n = 83$) Number (%)[1]	Replication series ($n = 49$) Number (%)[1]
Gender		
Male	17 (20.5)	13 (26.5)
Female	66 (79.5)	35 (71.5)
Missing	0 (0)	1 (2.0)
Age		
Median	47	45
Min-max	13–78	20–84
Histology		
PTC	42 (50.6)	24 (49.0)
fvPTC	5 (6.0)	9 (18.4)
FTC	18 (21.7)	4 (8.2)
FA	18 (21.7)	12 (24.4)
NT	8	3
Recurrence[2]		
Yes	14 (21.5)	7 (18.9)
No	47 (72.3)	24 (64.9)
Missing	4 (6.2)	6 (16.2)
Follow-up (months)		
Median	36	46.5
IQR	13.5–84	21–73.25
Mutation		
BRAF^{V600E}	23 (27.7)	13 (26.5)
RAS	13 (15.7)	2 (4.1)
RET/PTC1	3 (3.6)	0 (0)
Negative	44 (53.0)	44 (69.4)

A total of 132 tumor samples and 11 normal adjacent tissues were used in this study, divided in the discovery series (83 tumors and 8 normal thyroid tissues) and replication series (49 tumors and 3 normal samples).
IQR, interquartile range.
[1]The percentage is calculated taking into account only the total number of tumors (normal tissues were not included).
[2]The data on recurrence are only included for the malignant tumors (adenomas were not taken into account).

decrease, respectively, in methylation level of over 20% relative to the average methylation of normal thyroid samples.

Integration of gene expression and DNA methylation

To identify genes whose expression is associated with methylation of their promoters, we assessed correlations between methylation and mRNA expression using two approaches. First, mRNA expression was compared to DNA methylation using the 31 primary thyroid tumors[5] for which data were available. Expression of all the available genes identified as differentially methylated (FDR < 0.05, ΔM-value $> |1.4|$) was examined in this study. For genes with multiple probes included in the methylation array, the probe with the highest variance was selected, as previously described.[18] Correlation was measured using the Spearman coefficient. Second, we used an independent mRNA expression dataset available from GEO (http://www.ncbi.nlm.nih.gov/geo; GEO data base GSE27155).[6] This database contains both histopathological and genetic information on the samples included. We merged the list of genes with differential methylation (FDR < 0.05, ΔM-value $> |1.4|$) with that of those identified as differentially expressed (t-test, FDR < 0.05).

Identification of genes whose methylation is of potential prognostic value

To identify methylation changes that could serve as potential prognostic markers, we integrated available clinical follow-up data of 60 patients with the methylation profiles. First, we performed supervised analysis with the POMELO II tool using methylation data for carcinoma samples from individuals free of the disease for at least 5 years and those with recurrence within 5 years after the appearance of the disease. Next, we chose probes with the most significant changes in methylation (FDR < 0.05, ΔM-value $> |1.4|$), and using SPSS (IBM SPSS Statistics version 19) we conducted an univariate Cox regression analysis to determine the impact of methylation status on RFS. RFS was defined as the time between initial diagnosis and relapse or death by the disease, with observations censored at last follow-up if no event had occurred. p-Values were adjusted using Benjamini's FDR correction.

Results

DNA methylation profiles reflect histology and *RAS*/*BRAF* mutational status in well-differentiated thyroid cancer

The main clinical and pathological characteristics, as well as the somatic tumoral mutation status, of the patients included in the study are summarized in Table 1 (more detailed information is available in Supporting Information Table S2). The prevalence of the mutations found among our samples is similar to that previously described.[2] We excluded probes that appeared to be constitutively unmethylated (M-value < -2.0, corresponding to a β-value <0.2; 8,657 probes) or methylated (M-values >2.0, corresponding to a β-value >0.8; 717 CpGs) in all samples. The vast majority of the unmethylated probes were in CpG islands (97.8%); DAVID functional annotation analysis[19] returned GO terms such as primary metabolic processes (Benjamini–Hochberg-adjusted p-value $= 1.8 \times 10^{-18}$) and cellular metabolic processes (Benjamini-Hochberg-adjusted p-value $= 4.8 \times 10^{-24}$) as best hits (Supporting Information Fig. S1), suggesting an enrichment of housekeeping genes. Most of the methylated probes were located at non-CpG islands (63.6%), and no functional enrichment of the genes involved was observed. Further analyses were performed with the remaining 17,274 probes.

An unsupervised hierarchical cluster analysis of the 912 CpGs with standard deviation >1.2 identified two distinct clusters based on histological subtype and the underlying mutation (Fig. 1*a*). More specifically, "cluster 1" was enriched with FTC (p-value < 0.0001) and showed statistically significantly higher levels of methylation when compared to normal tissue samples grouped in "cluster 2" (p-value $= 2.7 \times 10^{-7}$). "Cluster 2" comprised two sub-clusters. "Cluster 2A" consisted of tumors with substantial histological heterogeneity, including, among others, the majority of FA samples (11 out of 18 FA) and all the normal thyroid tissue samples. Of note, the branch lengths between the normal thyroid samples were shorter than those between the tumor samples, indicating greater heterogeneity in methylation profiles among the latter. The tumors grouped in this sub-cluster also showed higher levels of methylation compared to normal tissue (p-value $= 4.6 \times 10^{-3}$). Finally, "cluster 2B" showed a robust methylation profile and comprised 36 tumors, 35 of which were PTCs; this sub-cluster included all those with the $BRAF^{V600E}$ mutation and *RET/PTC1* rearrangement. The methylation levels of samples in "cluster 2B" showed no statistical difference compared with normal tissue samples, suggesting methylation profiles differences between tumors with follicular and papillary growth patterns.

Although no clustering of samples harboring *RAS* mutations was observed using unsupervised analysis, it is noteworthy that those fvPTC bearing a *RAS* mutation were grouped in clusters 1 and 2A, together with *RAS*-FTC and *RAS*-FA, while the only tumor bearing a *BRAF* mutation clustered with *BRAF*-PTC tumors. Therefore, we applied a principal components analysis (PCA) using the 912 CpGs with highest methylation variability, and confirmed the grouping of samples according to their mutational status (Fig. 1*b*). Using publically available data from The Cancer Genome Atlas (TCGA) project (87% of the 912 probes used for the unsupervised analysis were also included in the 450K platform), we were able to confirm the robust clustering of thyroid tumors according to primary mutation, *BRAF versus RAS* (Fig. 1*c*).

Identification of differentially methylated regions in well-differentiated thyroid cancer

We identified 9 hypomethylated probes (9 genes) in FA, 83 (77 genes) in FTC and 53 (51 genes) in PTC. We also observed 89 hypermethylated CpGs (83 genes) in FA, 460 (416 genes) in FTC and 39 (31 genes) in PTC. A Venn diagram analysis revealed that a substantial proportion of differentially methylated regions identified in FA was also altered in FTC. Sixty-nine (83%) hypermethylated and six (67%) hypomethylated probes in FA were also hyper- and hypomethylated, respectively, in FTC (Figs. 2*a* and 2*b* and Supporting Information Fig. S2). Table 2 summarizes the 20 most significant subtype specific probes identified as well as their corresponding Δβ-values. An extended list of all differentially methylated probes is listed in Supporting Information Table S3.

After dividing FA and FTC samples according to their mutational status, we assessed associations of individual probes with each of the genetic subgroups. For *RAS*-positive tumors, we identified 72 probes (70 genes) and 203 probes (181 genes) to be hypomethylated in FA and FTC, respectively. Hypermethylated were 263 probes (258 genes) and 454 probes (426 genes) in FA and FTC, respectively. In tumors with no mutations, we identified on one hand 11 hypomethylated probes (11 genes) in FA and 77 (71 genes) in FTC, and on the other hand 105 hypermethylated probes (97 genes) in FA and 587 (528 genes) in FTC (Figs. 2*c* and 2*d* upper and Supporting Information Fig. S2).

After dividing PTC samples according to the genetic alterations they harbored, we identified 126 hypomethylated probes (121 genes) in *BRAF*-positive tumors, 74 (72 genes) in *RAS*-positive tumors and 7 (7 genes) in tumors with no detectable mutations (16 tumors); we found 78 hypermethylated probes (70 genes) in *BRAF*-related tumors, 141 (132 genes) *RAS*-mutated tumors and 84 (78 genes) in tumors with no mutations. No probes were found to be specific to PTC tumors harboring the *RET/PTC1* rearrangement (Figs. 2*c* and 2*d* lower and Supporting Information Fig. S2). All hypo- and hypermethylated genes for each tumor subtype are listed in Supporting Information Table S3.

Furthermore, we aimed to identify the differentially methylated probes specifically associated with mutational status, independently of histology, by separately comparing with normal tissue all *RAS*-tumors, all *BRAF*-tumors and all non-mutated tumors. We obtained 450 probes from these analyses (Supporting Information Table S3) that, when used to perform PCA, resulted in a robust separation of mutated samples into two main groups: *BRAF*-positive samples together with *RET/PTC1* samples and samples harboring *RAS* mutations (Fig. 3).

For all differentially methylated probes identified (both mutation- and subtype-related), we also investigated the genomic context of their location, as it is well-known that hypo- and hypermethylation target different genomic regions in cancer (Supporting Information Fig. S3). We observed that hypermethylation in thyroid tumors occurred preferentially within a CpG island, whereas hypomethylation tended to affect probes outside of CpG islands (p-value < 0.0001). In addition and as previously reported,[20] hypermethylation occurred preferentially at stem cell PolyComb Group (PcG) target genes (p-value < 0.0001), while hypomethylated probes were highly enriched with CpGs that are heavily methylated in Embryonic Stem Cells (p-value < 0.0001).

Validation and replication of differentially methylated loci

We chose the promoter regions of three genes for validation using bisulfite sequencing; hypermethylation of two of them (*COL4A2* and *DLEC1*) was more common in thyroid neoplasias in general than in normal tissues, while hypomethylation

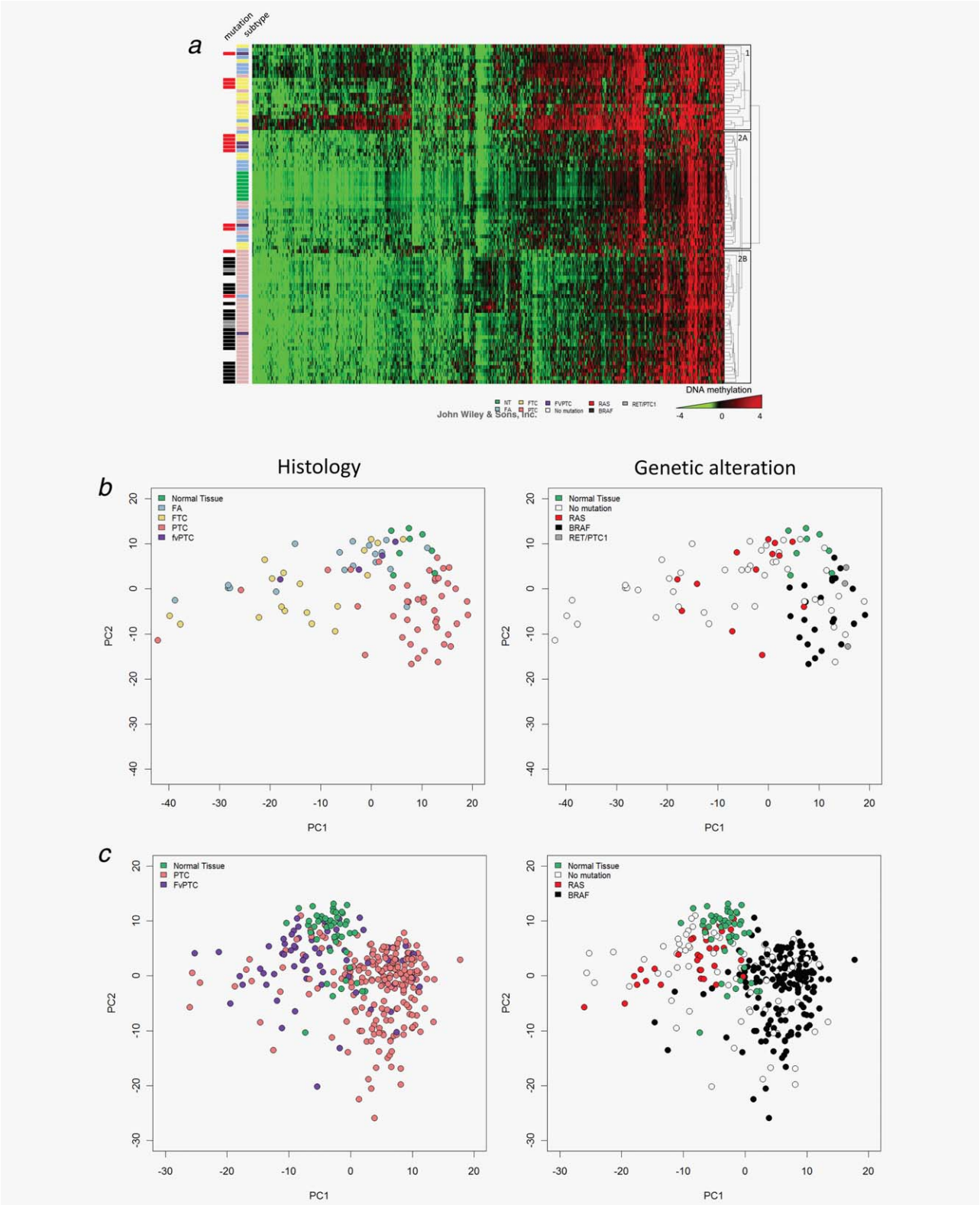

Figure 1. (*a*) Unsupervised hierarchical cluster analysis. Hierarchical cluster analysis of 83 primary thyroid tumors and 8 adjacent normal tissue samples based on the 912 CpGs with the greatest variability (SD > 1.2). The analysis divided the sample set into two main clusters. "Cluster 1" was statistically significantly enriched with FTC samples. In "cluster 2B" the majority of PTC was gathered, while in "cluster 2A" normal tissues, showing a very homogeneous profile, were localized together with the majority of FA. (*b*) Principal component analysis. PCA analysis of 83 primary thyroid tumors and 8 adjacent normal tissue samples based on the 912 CpGs with the greatest variability (SD > 1.2). PCA analysis showed a clear relationship between DNA methylation and histology as well as genetic alterations. The three tumors containing a *RET/PTC* rearrangement group with *BRAF*-positive samples. (*c*) Principal component analysis. PCA analysis using DNA methylation data from the TCGA project (including 304 primary thyroid tumors and 50 adjacent normal tissue samples) based on the same CpGs with the greatest variability (SD > 1.2) identified in our study.

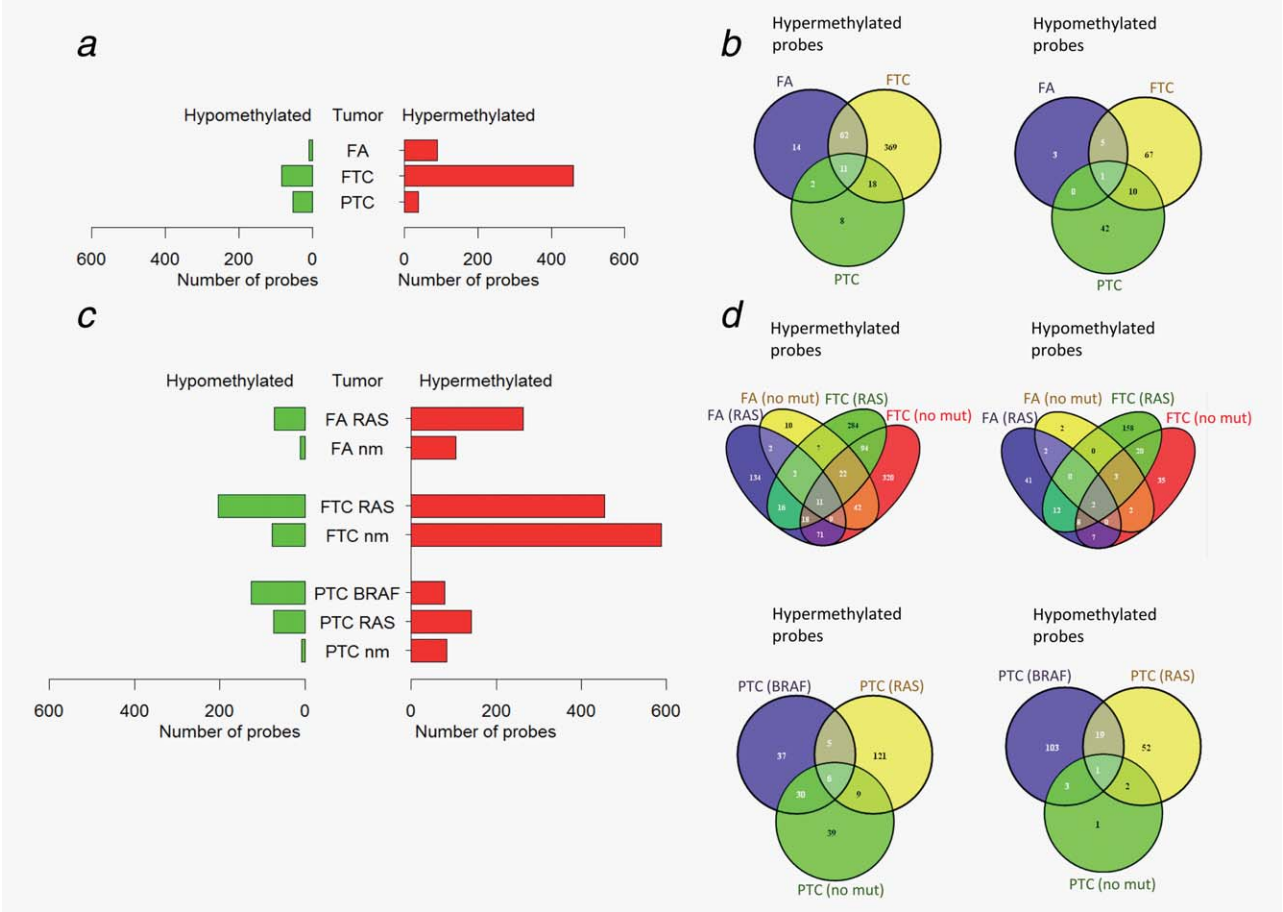

Figure 2. Differentially methylated probes. (*a*) Subtype-specific probes identified using POMELO II based on FDR < 0.05 and ΔM-value $> |1.4|$. (*b*) Venn diagram showing the overlap between the identified subtype-specific hyper- and hypomethylated probes. (*c*) Mutation-specific probes identified using POMELO II and the criteria listed above. (*d*) Venn diagram analysis showing overlap between the identified mutation-specific hypermethylated and hypomethylated probes, respectively.

of *KLK10* was specific to PTC tumors harboring the *BRAF*^{V600E} mutation. Comparison of quantitative methylation values at these three CpG sites from the HumanMethylation 27K Platform and bisulfite sequencing in 32 samples confirmed the accuracy of the array-based measurement (Supporting Information Fig. S4). The analysis by bisulfite sequencing assessed not only the methylation state of the CpG within the probe but also the flanking CpGs, revealing that the differential methylation affected a larger region (Supporting Information Fig. S4). These results validate the use of the single CpG sites interrogated by Illumina Infinium HumanMethylation 27K Platform as surrogate reporters of regional methylation. In addition, we replicated the findings for the three candidate genes in an independent series of 24 PTC, 9 fvPTC, 12 FA, 4 FTC and 3 normal thyroid tissues (Supporting Information Table S2).

In the discovery series, as measured by the array, *COL4A2* was hypermethylated in 66% of PTC, 21% of FA and in 56% of FTC samples; in the independent replication series hypermethylation was observed in 41% of PTC and 33% of FA samples. The lack of *COL4A2* hypermethylation among FTC samples could be due to the fact that only four samples were available. Conversely, *DLEC1* showed hypermethylation in the discovery series in 23% of PTC, 42% of FA and 56% of FTC samples, compared to 23% of PTC, 8% of FA and 75% of FTC samples in the replication series. In the replication series, we confirmed the *KLK10* hypomethylation in all *BRAF*-positive samples (Supporting Information Fig. S4).

Correlation between DNA methylation and mRNA expression

The integration of methylation and expression data available[5] from 31 thyroid tumors allowed us to examine gene expression levels of a limited number of genes (4,029 genes on both platforms that represent 27.8% of the genes included on the methylation array). We examined the correlation between DNA methylation and mRNA expression in all histological groups where at least five samples were available for the analysis. In PTCs with the *BRAF*^{V600E} mutation, we observed an inverse correlation with expression for 13.3% (6 out of 45

Table 2. TOP 20 subtype-specific probes for FA, FTC and PTC

Probe ID	Gene ID	FDR	Δ*M*-value	Δβ-value	Chr.	CpG Island
TOP 20 FA-specific probes						
cg06367117	*ALDOC*	2.00E-07	2.71093869	0.3926528	17	TRUE
cg08047457	*RASSF1*	9.00E-07	2.29675986	0.40019958	3	TRUE
cg21554552	*RASSF1*	2.40E-06	2.44631867	0.36554431	3	TRUE
cg17568996	*NFAM1*	2.42E-05	2.25126535	0.33678735	22	FALSE
cg06821120	*RASSF1*	0.0001938	1.7311015	0.33777293	3	TRUE
cg27219973	*GNRHR*	0.0008154	1.54777294	0.2221765	4	FALSE
cg13926569	*PAPSS2*	0.0013162	1.72337985	0.27635483	10	FALSE
cg09606564	*MFAP4*	0.0013598	1.4726875	0.25294503	17	FALSE
cg16393207	*GDPD5*	0.0013598	1.54616899	0.22478436	11	TRUE
cg14129786	*MGMT*	0.0014039	1.66225486	0.22401578	10	TRUE
cg14973995	*TETRAN*	0.0014039	2.07575889	0.30959818	4	TRUE
cg18055007	*DDAH2*	0.0014133	1.88659804	0.25590677	6	TRUE
cg17582777	*EFNA3*	0.0017439	−1.53694597	−0.23233439	1	FALSE
cg05656364	*VAMP8*	0.0021883	−1.55332458	−0.16488351	2	FALSE
cg22995106	*COG4*	0.0022767	1.40257423	0.1805763	16	TRUE
cg15692239	*ALDOC*	0.0022767	2.53820971	0.20161756	17	TRUE
cg21402071	*CHRNB4*	0.0028915	1.82059373	0.20535253	15	FALSE
cg26365553	*MADD*	0.0031854	1.42934142	0.24722019	11	FALSE
cg12783776	*SERPING1*	0.0033165	1.9289042	0.27738869	11	TRUE
TOP 20 FTC-specific probes						
cg21554552	*RASSF1*	7.00E-07	2.98852759	0.45417166	3	TRUE
cg08047457	*RASSF1*	9.00E-07	2.72818463	0.47218605	3	TRUE
cg14679230	*LIPE*	3.80E-06	1.45037569	0.1459732	19	FALSE
cg04972979	*C20orf54*	5.50E-06	1.61451484	0.30256229	20	FALSE
cg16517394	*TNFSF4*	9.60E-06	1.8536169	0.23950413	1	FALSE
cg00804392	*RHOH*	1.11E-05	1.85216994	0.29420054	4	TRUE
cg05467458	*SLC7A9*	1.52E-05	1.70161824	0.28134819	19	FALSE
cg20802392	*CTSK*	1.52E-05	1.98033411	0.27687162	1	TRUE
cg26218269	*MAB21L2*	1.58E-05	1.72450003	0.21028063	4	TRUE
cg06367117	*ALDOC*	1.58E-05	3.13695217	0.45480542	17	TRUE
cg16779976	*BLNK*	1.58E-05	−1.5932182	−0.17493057	10	FALSE
cg04629204	*EXTL1*	1.68E-05	1.4815062	0.24451418	1	FALSE
cg14120436	*GNB5*	2.62E-05	1.59384173	0.20025587	15	FALSE
cg20592700	*WIPI2*	2.62E-05	−1.50386259	−0.13059051	7	TRUE
cg00777121	*RASSF1*	4.72E-05	1.4952662	0.24566678	3	TRUE
cg10861599	*TNFSF4*	4.72E-05	1.57521415	0.19950722	1	FALSE
cg20356482	*FBP2*	5.00E-05	1.40957532	0.20331262	9	TRUE
cg09538582	*KRTHA5*	5.45E-05	1.47233264	0.09209393	17	FALSE
cg20394284	*JAK2*	6.48E-05	1.69088777	0.22587538	9	TRUE
TOP 20 PTC-specific probes						
cg13019092	*PDZK1*	<0.0000001	1.4330228	0.15003065	1	FALSE
cg07763768	*C9orf45*	<0.0000001	1.85389182	0.22681097	9	TRUE
cg02423618	*SPATA8*	<0.0000001	−1.56480549	−0.20065839	15	FALSE

Table 2. TOP 20 subtype-specific probes for FA, FTC and PTC (Continued)

Probe ID	Gene ID	FDR	Δ*M*-value	Δβ-value	Chr.	CpG Island
cg18302652	*IL8*	2.58E-05	−1.69117713	−0.22868075	4	FALSE
cg17568996	*NFAM1*	7.01E-05	1.87197435	0.24967876	22	FALSE
cg24497819	*SELPLG*	0.0001279	−2.22238036	−0.3194521	12	FALSE
cg19385139	*COL4A2*	0.0002352	1.95997347	0.2680565	13	FALSE
cg03001305	*STAT5A*	0.000285	−2.54273891	−0.31643091	17	FALSE
cg15262516	*COL4A2*	0.0004566	1.40539859	0.16244608	13	FALSE
cg04057858	*UNQ9391*	0.0008376	−1.40846284	−0.18092863	8	FALSE
cg02523400	*SERPIND1*	0.0009559	2.57803439	0.31279995	22	FALSE
cg27105123	*EPS8L1*	0.0009877	−1.60910587	−0.23177057	19	FALSE
cg03733371	*LIPH*	0.0012087	−2.5252893	−0.35653317	3	FALSE
cg18752880	*C1QTNF3*	0.0014062	1.47593113	0.26132674	5	FALSE
cg04756629	*LOC400696*	0.0015227	−1.8391597	−0.21467597	19	FALSE
cg12385643	*UGT1A6*	0.0016378	−1.65626606	−0.18292765	2	FALSE
cg12530080	*PMCHL1*	0.0016882	−1.81689239	−0.26447097	5	FALSE
cg18343292	*MS4A7*	0.0016882	−1.49789853	−0.18361614	11	FALSE
cg27009703	*HOXA9*	0.0017272	1.75344159	0.18988395	7	TRUE
cg10236239	*SULT1C2*	0.001969	1.48508253	0.23764054	2	FALSE

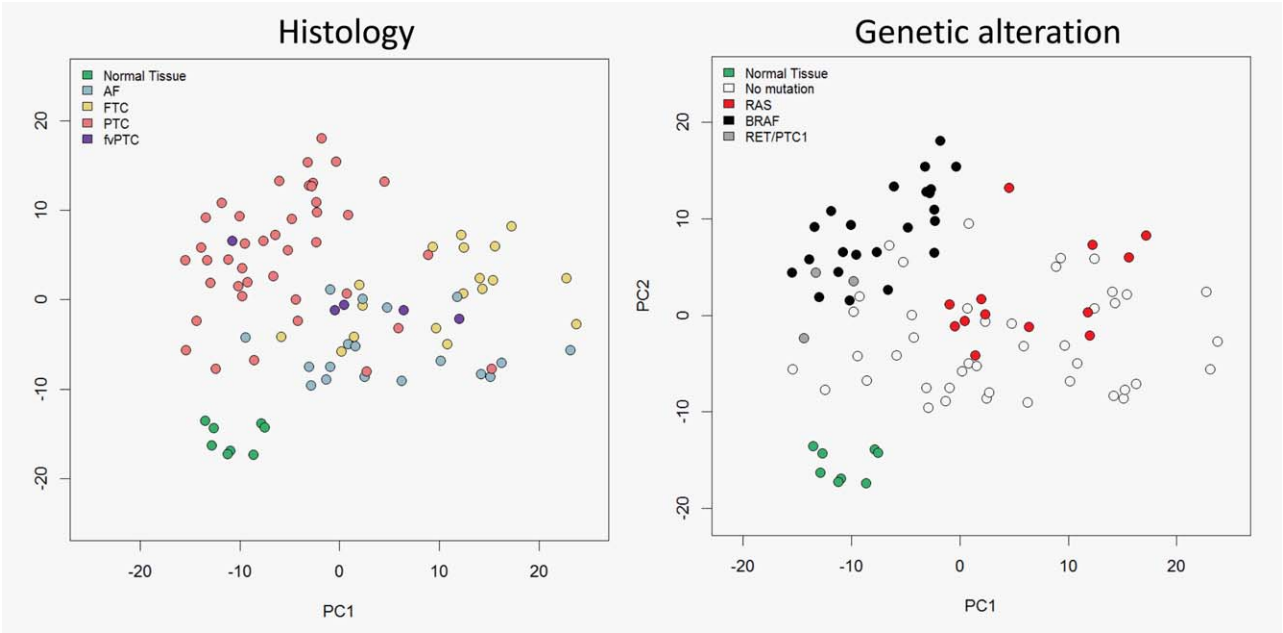

Figure 3. PCA analysis using 450 mutation-specific probes. PCA analysis using 450 probes identified to be differentially methylated in supervised cluster analysis. The probes were identified to be specifically associated with the mutational status and independent of the histology and their usage resulted in a robust separation of samples into two main groups: *BRAF*- and *RET/PTC*-positive tumors and *RAS*-positive tumors.

genes available for integration) of genes with significant changes in methylation. Among FTC, 10.4% (16/153) of genes showed a similar trend, while in the case of FA it was 11.6% (5/43).

Additionally, we identified differentially expressed genes using an mRNA expression data from an independent case series.[6] After comparing these genes with the lists of differentially methylated genes we found that 10% (7 out of 70 differentially methylated genes) and 2.9% (12/416) of the genes hypermethylated in PTC (*BRAF*-related) and FTC, respectively, were down-regulated. Moreover, we found that 20.7% (25/121) of the hypomethylated genes in PTC (*BRAF*-related)

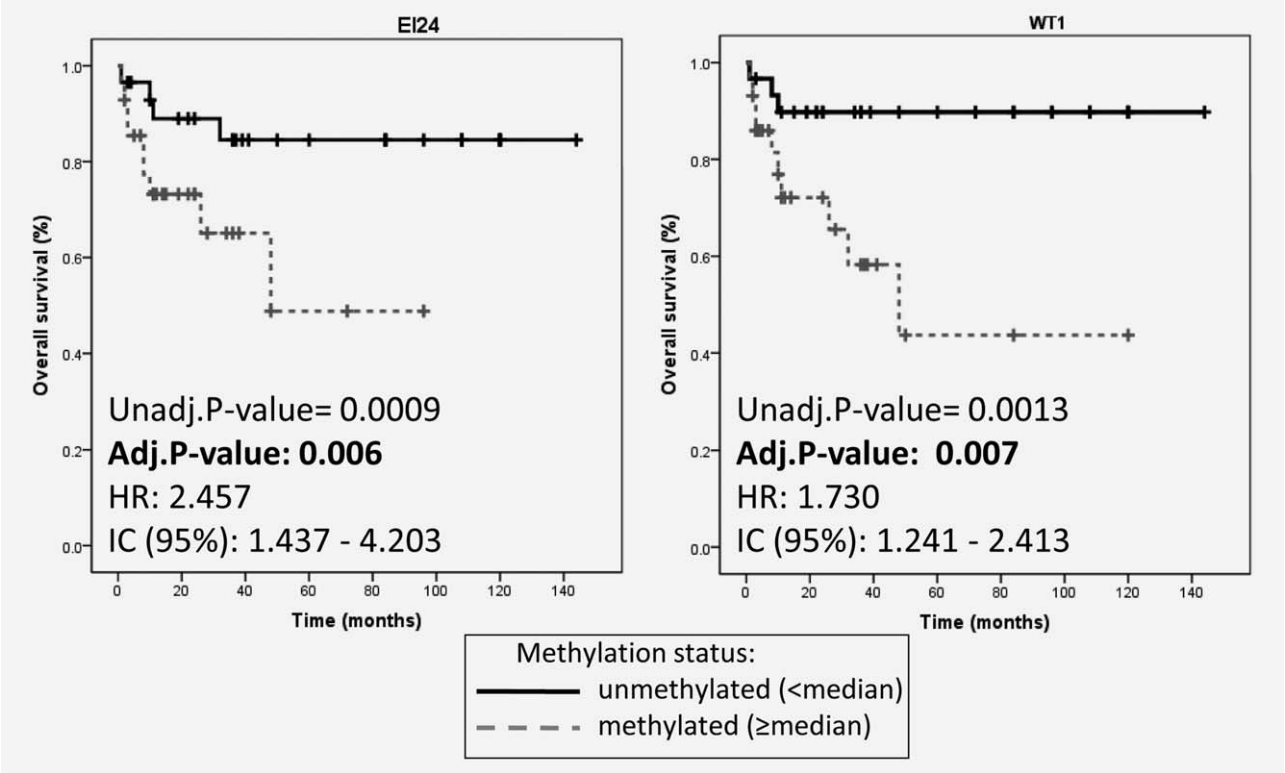

Figure 4. Prognostic value of the methylation status of *EI24* and *WT1* genes. RFS of 60 thyroid cancer patients based on the methylation levels, considered as a continuous variable, of each of the proposed prognostic factors. RFS was defined as the time between initial diagnosis and recurrence or death due to the disease, with follow-up censored at last contact if no event had occurred. The unadjusted *p*-values (Unadj. *p*-value) were obtained from Cox regression analysis, and were corrected for multiple testing (adj. *p*-value, shown in bold).

and 1.9% (1/77) in FTC were up-regulated. We did not, however, find any association between changes in methylation and gene expression in FA.

A substantial portion of the genes whose expression we observed to be inversely correlated with the methylation level of their corresponding promoters has been already described to play a role in the tumorigenic process (*CTSF, KLK10, PHLDA2, RUNX1, TACSTD2, BAALC, CTSF, HMGA1, RASSF2, IMPDH1* and *TNFRSF10C*). Moreover, for *BRAF*-related PTC they also included genes from the MAPK pathway (*MAPK13, DUSP5* and *RAP1GA1*) and genes involved in apoptosis (*LCN2, RIPK1* and *LGALS1*), whereas for FTC we observed a down-regulation by hypermethylation of several genes involved in innate immune response (*C7, SERPING1, TRAF3, PYCARD* and *CFH*). All genes identified in this analysis are listed in Supporting Information Table S4.

Identification of methylation-related prognostic markers

The analysis of 34 samples (PTC and FTC) with available follow-up information identified 32 probes differentially methylated among patients with and without recurrence (Supporting Information Table S3). We performed survival analysis of 60 patients to evaluate the impact of methylation levels of these genes on RFS, obtaining significant associations with risk of recurrence for etoposide induced 2.4 (*EI24*) and Wilms tumor 1 (*WT1*) (Fig. 4). Among all known risk factors associated with poor prognosis (sex, age of onset, mutation in *BRAF*, FTC and tumor size), only the latter showed statistical significance in our study ($p = 0.016$). After including tumor size as a covariable, the association of *EI24* and *WT1* methylation levels with prognosis remained significant ($p = 0.004$; HR = 2.08; CI: 1.262–3.445 and $p = 0.006$; HR = 1.64; IC = 1.149–2.335, respectively); results from the univariate analysis are shown in Figure 4. In addition, the association of *EI24* and *WT1* methylation level with recurrence remained significant in multivariable analysis including separately each of the remaining clinical variables known to be related to poor prognosis.

Discussion

Alterations in DNA methylation have been shown to play a role in tumorigenesis and disease progression in many malignancies, including thyroid cancer. Until recently, technical limitations have restricted methylation studies to the characterization of a handful of candidate loci[9–11,13] and one genome-wide exploratory study, mainly focused on identification of subtype specific methylation patterns.[8] Here, we describe quantitative DNA methylation levels at more than 26,000 loci across 14,000 gene promoters. By assaying the largest collection of thyroid tumors described so far, we were able to not only confirm methylation changes seen in previously published candidate loci studied but also identify novel

recurrent ones. Our results suggested that in thyroid cancer, aberrant methylation targets specific genomic regions, particularly PcG-associated stem cell genes and sequences that are highly methylated in stem cells, which are also commonly epigenetically deregulated in other cancer types.[20] Moreover, according to our results, the methylation patterns in thyroid cancer are specific to the follicular and papillary patterns of growth as well as to the underlying mutational event. Furthermore, by comparing DNA methylation with mRNA expression data, we further confirmed that the relationship between methylation and expression is complex and context-dependent.[21] Finally, by integrating methylation data with clinical information we were able to propose novel prognostic markers in well-differentiated thyroid cancer.

Alterations in DNA methylation have been observed in early cancers and precursor lesions, suggesting that they play an important role in malignant initiation,[20,22,23] and our observations are largely consistent with this hypothesis. It has been proposed that follicular adenoma is a precursor lesion for follicular thyroid carcinoma, as evidenced by both the simultaneous presence of carcinomas in benign lesions and the similarity in the molecular alterations observed in FA and FTC.[24] The few differences in methylation between FA and normal thyroid tissue samples that were also observed in FTC could represent the initiating changes, providing an additional piece of evidence that FA may give rise to FTC, and new insights about the critical steps in follicular cell neoplastic transformation. We also observed a progressive gain of promoter CpG-island hypermethylation between benign- (83 probes) and malignant-stage tumors (460 probes), which confirms previous findings.[20,23]

Although in other cancer types distinct methylation patterns have been found to be associated with the presence of specific mutations,[25–29] to our knowledge, ours is the first study to assess this in thyroid cancer. We observed a robust separation of mutated samples, especially evident for fvPTC, where the mutation apparently was tightly associated with their methylation pattern and subsequent clustering. These findings are consistent with those from a recent genome-wide methylation study in two thyroid cancer cell lines showed they undergo hypermethylation in an important proportion of genes upon the knockdown of *BRAF*.[30] Given that TCGA project's methylation data validated the pattern according to the mutation, it seems reasonable to conclude that methylation pattern is specific to the particular mutation involved in thyroid cancer. However, the biological mechanism explaining this remains unknown, and further experiments are needed.

We found it particularly striking that PTCs had a higher proportion of hypomethylated probes. In cancer, hypomethylation is more prominent in large inter-genic satellite regions and has been related to genomic instability,[31,32] whereas PTC has been described as the thyroid cancer subtype with least structural rearrangements.[33] As the platform used in the study was biased towards gene promoters, it is likely that the observed hypomethylation events on unique sequences could cause increased expression of cancer-promoting genes, rather than genomic instability. An integrative study applying various OMICs approaches to a common series of PTC tumors is required to shed light on the relationship between hypomethylation and genomic instability.

The results for PTC tumors harboring the $BRAF^{V600E}$ mutation specifically caught our attention. The presence of $BRAF^{V600E}$ has been strongly associated with the "CpG island methylator phenotype" (CIMP) in colorectal cancer,[25,28] but it has been suggested that this mutation is not sufficient to induce CIMP in a colorectal cell line.[26] Rather, to promote its oncogenic effects, it requires additional cooperative events, often of an epigenetic nature,[26,34] which bypass the senescence and apoptosis that this mutation induces in cells.[27,35] Importantly, this tumor suppressor mechanism has been recently described in thyroid carcinogenesis[36] even though it remains to be established which events are associated with its impairment. Concomitant activation of v-akt murine thymoma viral oncogene homolog 3 (*AKT3*) was reported to overcome *BRAF*-induced senescence in melanoma cells.[37] Indeed, in our experimental setting, we observed strong *AKT3* promoter hypomethylation (FDR $= 6.6 \times 10^{-6}$, ΔM-value $= -2.26$). However, we did not observe a correlation between *AKT3* methylation and expression, probably due to the fact that the corresponding CpG dinucleotide arrayed did not lie within a CpG island. Nevertheless, a tendency towards elevated expression of *AKT3* specifically in PTC has been reported by others.[38] In addition, the over-activation of the mTOR pathway, which is classically regulated through the phosphatidylinositol-3-kinase (PI3K)/AKT pathway, has been recently reported to be strongly associated with *BRAF* mutation-positive PTC.[39] Further studies are necessary to decipher the precise role of *AKT3* in the development of *BRAF*-related thyroid tumors.

In both case series considered, the hypermethylation of the promoter regions of *COL4A2* and *DLEC1* was confirmed in follicular cell-derived tumors, while the hypomethylation of *KLK10* was strongly associated with *BRAF* mutation-positive PTC. *KLK10* is a member of the kallikrein family of genes, which are secreted serine proteases that have been extensively studied in cancer due to their involvement in extracellular matrix degradation as well as their promising role as disease biomarkers.[40] Hypomethylation of *KLK10* has been recently associated with biochemical recurrence in prostate cancer.[41] Conversely, *COL4A2* encodes one of the six subunits of type IV collagen, the major structural component of basement membranes. The C-terminal portion of the protein, known as canstatin, is an inhibitor of angiogenesis and tumor growth.[42] Finally, *DLEC1* is a candidate tumor-suppressor gene, which is commonly deleted in various carcinomas; more importantly, it has been reported to be epigenetically repressed in many tumor types.[43,44]

To gain insights into the functional implications of epigenetic changes, we integrated the DNA methylation data with gene expression profiling data. The integration with an independent series of samples identified a relatively lower

proportion of correlated genes in FTC, and we observed no correlations for FA, which was probably due to the small number of samples included in the original study.[6] Nevertheless, in general, we observed similar proportions of genes showing correlation to those reported in previous studies.[18,25] The products of some of the correlated genes in PTC samples harboring the $BRAF^{V600E}$ mutation were clustered in the MAP kinase cascade, which further confirms the importance of impairment of this pathway in the development of this tumor subtype. In FTC samples, we observed an enrichment of genes involved in innate immunity response mechanisms, known for a long time to promote carcinogenesis.[45]

We found that elevated levels of methylation of at least two genes known to participate in carcinogenesis were associated with increased risk of recurrence of thyroid cancer. Interestingly, both genes, *EI24* and *WT1*, exhibited a significant association with poor prognosis even after adjustment for relevant clinical variables. Although preliminary, the associations of these novel markers with disease recurrence could potentially serve to better stratify patients. Specifically, *EI24* is a putative tumor-suppressor gene, the expression of which is impaired in several types of cancer by either aberrant methylation or deletion.[46] More importantly, this impairment has been found to be associated with tumor invasiveness[46] and poor response to treatment.[47] *WT1* encodes a transcription factor, mutated in a small subset of patients with Wilm's tumors, and whose expression has been suggested to be indicative of minimal residual disease in leukemias.[48,49] Furthermore, its methylation status has been recently proposed to be correlated with time to recurrence in prostate cancer.[50]

To summarize, the assessment of genome-wide DNA methylation profiles in the largest series of well-differentiated thyroid tumors described so far allowed us to identify and replicate distinct epigenetic signatures that reflect the underlying tumor histology as well as the mutation status. Specific aberrant methylation associated with the early development of this disease was found, and DNA methylation events associated with changes in gene expression were identified. We proposed novel prognostic markers, which according to our data are independent of the already established ones.

Acknowledgments

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ARTICLE 3

Article 3: MicroRNA deep-sequencing reveals master regulators of follicular and papillary thyroid tumors.

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Abstract

In this study, the action of microRNAs as gene expression regulators was considered. We have characterized the complete spectrum of deregulated microRNAs using our series of 127 genetically classified thyroid tumors, including 49 follicular cases, as well as 17 normal thyroid tissues. The use of deep-sequencing allowed us to accurately quantify miRNAs across the genome. These data were also assessed along with the transcriptomic data for the same tumors and clinical follow-up of the corresponding patients.

We found that follicular and papillary tumors present distinct microRNA profiles that are closely linked to specific driver mutations. It was especially interesting to uncover few miRNAs consistently up-regulated across all tumor classes, suggesting their role as "master regulators" of thyroid transformation. Furthermore, we detected deregulation of several novel miRNAs associated with specific tumor subgroups. For example, down-regulation of the miR-1247 was observed in follicular tumors in particular. By integrating these miRNA data with gene expression data, we were able to identify target genes for these key miRNAs, which is a novel finding for thyroid pathologies.

Further, based on an analysis of clinical follow-up information, we propose a prediction model for disease relapse based on the expression of 2 miRNAs (miR-192 and let-7a) and clinicopathological features.

Personal contribution: I extracted and prepared the samples for this study. I performed the genetic screening of known genetic drivers. I also contributed to the discussion of the results, formation of the hypothesis and drafting of the paper.

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MicroRNA deep-sequencing reveals master regulators of follicular and papillary thyroid tumors

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MicroRNA deregulation could be a crucial event in thyroid carcinogenesis. However, current knowledge is based on studies that have used inherently biased methods. Thus, we aimed to define in an unbiased way a list of deregulated microRNAs in well-differentiated thyroid cancer in order to identify diagnostic and prognostic markers. We performed a microRNA deep-sequencing study using the largest well-differentiated thyroid tumor collection reported to date, comprising 127 molecularly characterized tumors with follicular or papillary patterns of growth and available clinical follow-up data, and 17 normal tissue samples. Furthermore, we integrated microRNA and gene expression data for the same tumors to propose targets for the novel molecules identified. Two main microRNA expression profiles were identified: one common for follicular-pattern tumors, and a second for papillary tumors. Follicular tumors showed a notable overexpression of several members of miR-515 family, and downregulation of the novel microRNA miR-1247. Among papillary tumors, top upregulated microRNAs were miR-146b and the miR-221~222 cluster, while miR-1179 was downregulated. *BRAF*-positive samples displayed extreme downregulation of miR-7 and -204. The identification of the predicted targets for the novel molecules gave insights into the proliferative potential of the transformed follicular cell. Finally, by integrating clinical follow-up information with microRNA expression, we propose a prediction model for disease relapse based on expression of two miRNAs (miR-192 and let-7a) and several other clinicopathological features. This comprehensive study complements the existing knowledge about deregulated microRNAs in the development of well-differentiated thyroid cancer and identifies novel markers associated with recurrence-free survival.
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MicroRNAs (miRNAs) are potent regulators of gene expression in a tissue-specific manner. Their deregulation has been demonstrated to be a hallmark of cancer. It has been suggested that miRNA profiles can reliably identify the cell origin of tumors and that they are specific to differentiation stage and driver alterations, and associated with progression and response to treatment.[1–3] Thus, their clinical

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utility has been extensively studied for many cancer types.

It is especially appealing to explore the diagnostic and prognostic value of miRNA profiles in thyroid cancer, since this is a very complex and heterogeneous disease showing various stages of differentiation. Further, the accurate diagnosis of some patients remains an unresolved challenge in the clinical setting.[4] The vast majority of thyroid tumors develop from follicular epithelial cells of the gland, often after the emergence of a driver mutation altering the MAPK pathway (affecting key genes such as *BRAF*, *RAS*, or *RET*). They include benign (follicular adenoma) and malignant forms (papillary thyroid carcinoma; follicular carcinoma). Both papillary and follicular thyroid carcinomas may progress to poorly differentiated thyroid carcinoma or lose their differentiation completely (anaplastic thyroid cancer), or the dedifferentiated disease can emerge spontaneously.[5] There is an urgent need to understand the biology underlying both progressive and dedifferentiated disease, as there is still no effective treatment available for patients diagnosed with these subtypes.[6] Although the remaining thyroid cancers, termed well-differentiated thyroid carcinomas, are generally indolent malignancies with well-established clinical management and excellent prognosis,[7] a clinically relevant proportion presents recurrent disease and it is equally important to pinpoint predictive markers of disease relapse.

MiRNA profiling has been already extensively applied to well-differentiated thyroid cancer. Similarly to other genomic features,[8–10] the miRNA expression fingerprints have been shown to be subtype- and driver alteration-specific (reviewed in Pallante *et al*[11]). Moreover, the role of polymorphisms in the complementary sites of target mRNAs was initially proposed in the context of papillary carcinomas,[12] and polymorphisms in the miRNA sequence itself were shown to be predisposing to thyroid cancer,[13] highlighting the role of miRNAs in thyroid tumorigenesis. Upregulation of miR-146b and the miR-221 ~ 222 cluster are the most commonly documented miRNA changes related to papillary tumors.[12,14–17] However, little data are available on downregulated miRNAs, or on the changes present in less frequent histological subtypes such as follicular tumors. It is also important to highlight that apart from recent miRNA deep-sequencing studies using papillary tumors,[16,18,19] the remaining data on deregulated miRNAs in thyroid cancer were generated at a time when only ~300 miRNAs had been identified, compared with the ~1300 miRNAs that are known today.[11]

We have performed a miRNA deep-sequencing study using the largest collection of thyroid samples published to date, comprising of 127 thyroid tumors (including 26 follicular adenomas, 23 follicular carcinomas and 78 papillary thyroid carcinomas) and 17 normal thyroid tissues. We not only confirm some of the miRNA expression changes previously described as implicated in thyroid cancer, but also identify novel ones, such as the downregulation of miR-1247 in tumors with a follicular pattern of growth. By integrating miRNA and mRNA expression data from the same tumors, we identify several possible targets for the top novel deregulated candidates. Finally, we propose a relapse prediction model based on expression of two miRNAs and several other clinicopathological features.

Materials and methods

Thyroid Sample Collection and Patient Follow-Up

One hundred and twenty-seven thyroid tumors were snap frozen following surgery at *Hospital Sant Pau* and *Hospital Sabadell* in Barcelona (Spain) and at *Hospital Arnau de Vilanova* in Lleida (Spain), and stored at − 80 °C. An informed consent was obtained from all the study participants, and the study was approved by the Institutional Review Board (*comité de bioética y bienestar animal*) of the *Instituto de Salud Carlos III*. Of the samples collected upon surgery, 26 were follicular adenomas, 23 follicular carcinomas, and 78 papillary carcinomas. Sections of each sample were evaluated by a pathologist and, when necessary, non-tumoral tissue was dissected. We studied 17 normal thyroid tissues in total, which were obtained from patients with localized disease that underwent hemithyroidectomy and gave consent to take a sample from the unaffected contralateral thyroid lobule. Nine corresponded to tumors included in the study, while for the eight remaining normal thyroid tissues, the matched tumors were not available. At least 80% of the cells were cancerous in all tumor samples, while non-tumor samples had no observable tumor epithelia. Tumor samples were grouped into two sets, the first composed of tumors from *Sant Pau* and *Sabadell* hospitals (16 follicular adenomas, 17 follicular carcinomas, 35 papillary carcinomas, and 8 normal thyroid tissues), and the second composed of tumors from *Arnau de Vilanova* (10 follicular adenomas, 6 follicular carcinomas, 43 papillary tumors, and 9 normal thyroids). The histological classification criteria applied have been previously described.[9]

The clinical follow-up of the patients was carried out by physical examination, neck ultrasonography, simultaneous determination of serum anti-tiroglobulin antibodies with tiroglobulin (basal, or after thyrotropin stimulation by thyroid hormone withdrawal, or the administration of recombinant human thyrotropin), and whole-body iodine scanning. If there was a suspicion of local or distant disease, other imaging techniques such as CT, MRI, PET-CT, or scintigraphy were used. Both structural and biochemical recurrence were considered events in the analysis of recurrence-free survival.

Genotyping

Using Sanger sequencing, all papillary samples were screened for *BRAF* mutations at codon 600 in exon

15, while follicular adenomas, follicular carcinomas, and follicular variants of papillary tumors were screened for mutations in *H-*, *N-*, and *K-RAS* at mutational hotspots on codons 12 and 13 of exon 2, and codon 61 of exons 3. When available, cDNA from papillary samples was also screened for *RET/PTC1* and *RET/PTC3* rearrangements.

RNA Extraction and Next-Generation Sequencing

Whole RNA from tumor set 1 was extracted using TRIzol (Life Technologies, MD, USA) according to the manufacturer's instructions. RNAs from tumor set 2 were further purified using RNeasy MinElute Cleanup columns (Qiagen, Valencia, CA, USA). The integrity of RNA was assessed using Agilent BioAnalyzer 2100 (Agilent Technologies).

RNA samples were processed as described in the 'TruSeq Small RNA Sample Preparation Guide' (Illumina part # 15004197 Rev. A of November 2010). Briefly, 1 μg of purified total RNA containing the small fraction of RNA was sequentially ligated to 3' and 5' adapters using the truncated form of T4 RNA ligase 2 and the T4 RNA ligase, respectively. Reverse transcription with SuperScript II reverse transcriptase was then used to yield cDNA adapter-ligated libraries that were amplified by PCR with Phusion DNA polymerase and Illumina RNA PCR primers. cDNA-amplified libraries were pooled and separated by polyacrylamide gel electrophoresis, and a fraction of 145–160 bases was extracted. The purified fraction constituted the multiplexed, purified libraries that were applied to an Illumina flow cell to generate clusters, and sequenced on the Genome Analyzer IIx with SBS TruSeq v5 reagents following manufacturer's protocols.

Statistical Analyses

Detailed statistical methods are presented in Supplementary Materials and Methods. Briefly, after quality assessment,[20] adapter removal,[21] and sequence mapping,[22] differential miRNA expression assessment was carried out using the Bioconductor package edgeR.[23] Benjamini and Hochberg's[24] correction was applied to ensure a false discovery rate (FDR) below 1% and those miRNAs meeting this criterion and with a fold change >2 or <0.5 were considered for further analysis.

For 38 samples of tumor set 1 (see Supplementary Table S1), mRNA expression data were available from previous studies.[10] Taking advantage of the available data from mRNA and miRNA expression from the same tumors, MirRGate, a specific tool for miRNA targets' identification, was used for target prediction (http://mirgate.bioinfo.cnio.es/API/). The miRNA was considered regulatory if its expression was negatively correlated with that of the target mRNA as assessed by Pearson's correlation test.

For recurrence-free survival analysis, a shrinking LASSO regression applied via the R package 'glmnet' (v.1.9–8)[25] was used for variable selection. Recurrence-free survival was defined as the time between initial diagnosis and relapse or death due to the disease, with observations censored at the last follow-up if no event had occurred. MiRNA covariates for which the estimated corresponding regression coefficient (β) did not shrink to zero ($\beta \neq 0$) were considered active coefficients, and evaluated in further steps. An exploratory univariate Cox regression was carried out for these miRNA covariates using the 'survival' package in R (v.2.37–7). Those with FDR < 0.05 were considered statistically significant, and evaluated in the multivariate Cox regression model. A signature score (SScore) representative of the signature expression for each sample was calculated as the sum of each miRNA expression level multiplied by the value of the coefficient obtained in the LASSO regression (β). The median value of the SScore was used to stratify tumors into two groups: high risk and low risk. The final multivariate Cox proportional hazards model was established with a miRNA signature expression based on the goodness of the fit by the Akaike Information Criteria (AIC) along with all remaining covariates, adjusting for the following confounding variables: histological subtype, tumor stage, gender, and age at diagnosis.

Results

MiRNA Sequencing Reveals Differential miRNAs Expression Patterns Among Thyroid Tumors

Altogether, data from 127 thyroid tumors including 26 follicular adenomas, 23 follicular carcinomas and 78 papillary carcinomas and 17 normal tissues were generated using next-generation miRNA sequencing. However, since slightly different extraction methods were used for the two tumor sets (see Materials and Methods section), we analyzed them separately, in order to avoid the introduction of technical bias in the results. Tumor set 1 was chosen as discovery series in part because expression profiling data were also available. Tumor set 2 was used to validate results from tumor set 1. The histopathological and mutational characteristics of both tumor sets are summarized in Table 1 (for further information, see Supplementary Table S1).

Unsupervised hierarchical clustering of data from the discovery set revealed the existence of two main clusters (Figure 1a), which resembled those described in a recent DNA methylation study.[9] Follicular tumors (both carcinomas and adenomas) were localized in the same branch, together with normal tissues, while papillary tumors were in a separated branch. Moreover, a separation of samples according to the mutational status was evident. This observation led us to perform a supervised analysis subdividing samples according to both histology and

Table 1 Summary of the main clinical and pathological characteristics of samples

Clinical characteristics	*Tumor set 1 (n = 68) Number (%)*	*Tumor set 2 (n = 59) Number (%)*
Gender[a]		
Male	18 (27)	9 (15)
Female	50 (73)	50 (85)
Age[a]		
Median	43	49
Min-max	13–77	22–78
Histology[a]		
Conventional variant of papillary thyroid carcinoma	26 (38)	31 (53)
Follicular variant of papillary thyroid carcinoma	6 (9)	7 (12)
Other variants of papillary thyroid carcinoma[b]	3 (4)	5 (8)
Follicular thyroid carcinoma	17 (25)	6 (10)
Follicular adenoma	16 (24)	10 (17)
Mutation[a]		
BRAF^{V600E}	19 (28)	17 (29)
RAS	12 (18)	1 (2)
RET/PTC1	3 (4)	0 (0)
Negative	34 (50)	41 (69)
Recurrence[c]		
Yes	16 (31)	14 (29)
No	36 (69)	34 (69)
Missing	0 (0)	1 (2)
Follow-up (months)[d]		
Median (interquartile range)	72 (41–96)	24 (10–36)
Normal thyroid tissue	8	9

A total of 127 tumor samples and 17 normal tissues were used in this study, divided in the discovery series (tumor set 1 = 68 tumors and 8 normal thyroid tissues) and replication series (tumor set 2 = 59 tumors and 9 normal samples).
[a]The percentage was calculated taking into account only the total number of tumors (normal tissues were not included).
[b]Other variants of papillary thyroid carcinoma include tall-cell variant, diffuse sclerosing, and oncocytic variant (for more information see Supplementary Table S1).
[c]The data on recurrence are only included for the malignant tumors (neither normal tissues nor adenomas were taken into account).
[d]Median follow-up time and interquartile range were calculated only for disease-free patients with a malignant disease.

genetics. Of the 808 significantly differentially expressed miRNAs, 170 and 100 were significantly up- and downregulated, respectively. It is worth noting that 7 and 13 miRNAs were consistently up- and downregulated, respectively, in all thyroid tumors (regardless of their malignancy, histological or mutational status; Figure 1b).

The comparison of each histological subtype with normal thyroid samples allowed us to identify a long list of subtype-specifically deregulated miRNAs (Figure 1c). Ninety miRNAs were overexpressed in follicular adenomas by at least twofold compared to normal tissue. There were 114 and 26 overexpressed in follicular and papillary carcinomas, respectively. The miRNAs with maximum changes exhibited extreme overexpression in follicular adenomas and follicular carcinomas (3452- and 1866-fold, respectively), and 101-fold for papillary samples. Conversely, there were 32 miRNAs downregulated at least twofold in adenomas (maximum 9), 53 in follicular carcinomas (maximum 17), and 42 in papillary tumors (maximum 13). According to a Venn diagram analysis, it seemed papillary samples had a distinct miRNA expression spectrum to that of tumors with a follicular pattern of growth (Figure 1c). Even having relatively few tumors classified as follicular variant of papillary carcinoma, a Venn diagram analysis showed they share more features with follicular tumors than papillary ones (Supplementary Figure S1). Moreover, there was substantial overlap between the deregulated miRNAs identified in follicular adenomas and follicular carcinomas (Figure 1b and c); 89% of upregulated and 94% of downregulated miRNAs identified in the former were also up- and downregulated, respectively, in the latter. A large number of the highly upregulated miRNAs in both of these histogroups belonged to the gene family miR-515 (miR--517a/b, -518a/b/c/e/f, and -516a/b). Other commonly highly upregulated molecules were miR-182, miR--183, and miR-96. The most downregulated miRNA among tumors with a follicular pattern of growth was miR-1247, which has not previously been implicated in thyroid cancer, followed by several members of the miR-199 family (miR-199a/b). Downregulation of miR-150 seemed to be follicular carcinoma specific as it was not detected among adenomas. For samples with a papillary pattern of growth, miR-146b and the miR-221 ~ 222 cluster were the most upregulated miRNAs in tumor set 1, followed by miR-21 and -31. MiR-1179 was one of the most downregulated molecules in these tumors. All deregulated miRNAs are listed in Supplementary Table S2.

As the specific driver mutation genotype was available, it was possible to group tumors according to this information, and to compare miRNA expression with that of normal thyroid tissues. Because of low prevalence, *RET/PTC1*-related tumors were not further considered. Figure 1b clearly shows that *RAS*-mutated tumors shared deregulated miRNAs with tumors with a follicular pattern of growth (both adenomas and carcinomas).

This rationale allowed us to identify extended lists of deregulated miRNAs associated with the presence of specific driver alterations. There were 61 upregulated miRNAs in *BRAF*-mutated and 56 in *RAS*-related tumors (showing a maximum 134-fold and 16-fold expression, respectively). However, 53 and 51 miRNAs were downregulated in BRAF- and *RAS*-positive samples, respectively (maximum 25- and 17-fold, respectively). In both cases, the most significantly overexpressed miRNAs were those previously described for papillary tumors (miR-146b and the miR-221 ~ 222 cluster). Conversely, miR-7 and miR-204 were the most downregulated molecules in

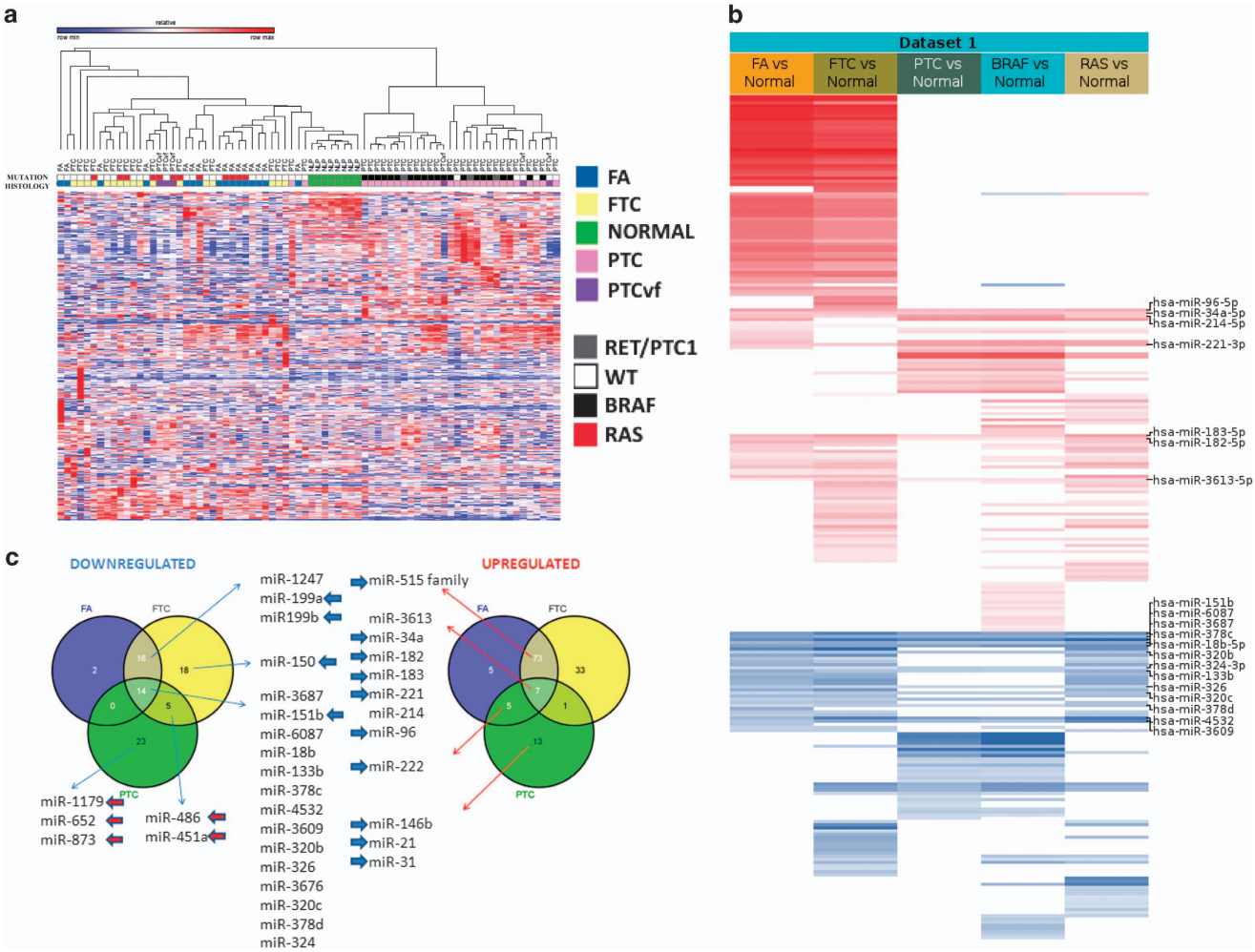


Figure 1 miRNoma from the discovery series. (**a**) Unsupervised hierarchical cluster analysis of 68 primary thyroid tumors and 8 normal tissue samples divided the sample set into two main clusters. 'Branch 1' was composed of all follicular carcinomas (FTC) and follicular adenomas (FA) as well as all normal tissues. 'Branch 2' included the majority of papillary samples (PTC). Separation of samples according to the driver mutation was also apparent. (**b**) Heatmap representation of differentially expressed miRNAs. Tumors were compared to normal thyroid tissues based on histological as well as driver mutation grouping, which was apparent from the unsupervised analysis. Color legend: red, upregulated; blue, downregulated. The 13 down- and 7 up- commonly deregulated miRNAs among all tumor classes (regardless of the malignancy and mutational status) are listed. (**c**) Venn diagram analysis of subtype-specific differentially expressed miRNAs revealed substantial overlap between follicular adenomas (FA) and follicular carcinomas (FTC), while papillary samples (PTC) showed a distinct miRNA expression signature. Moreover, 14 miRNAs were commonly upregulated while 7 were downregulated among all three subtypes. Only miR-3676 was not present when the mutation was considered (**b**). Blue arrows denote miRNAs previously described elsewhere to be implicated in thyroid cancer. Red arrows denote molecules described in Swierniak *et al*.[16]

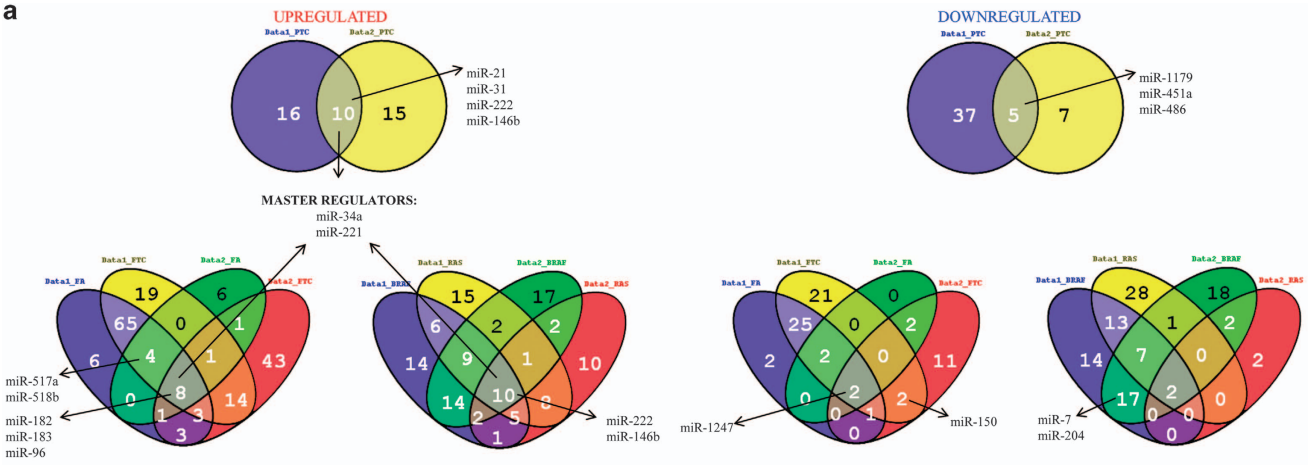
BRAF tumors; the latter was statistically significant when *BRAF* tumors were compared with wild-type papillary samples (data not shown). All deregulated miRNAs are listed in Supplementary Table S2.

Validation of Deregulated miRNAs

A smaller number of deregulated miRNAs was observed in tumor set 2, possibly due to the slightly different RNA extraction procedure followed. Nevertheless, two out of the three commonly upregulated miRNAs had been already detected in tumor set 1 (miR-221 and miR-34a), termed from now on 'master regulators'.

When it comes to the subtype-specifically deregulated miRNAs, a considerable proportion of these had been already detected in the discovery phase (62, 34, and 43% among follicular adenoma-, follicular thyroid carcinoma-, and papillary thyroid carcinoma-specific probes; Figure 2a and Supplementary Table S3). It seemed that the grouping of samples according to the mutational status was more robust, as a higher proportion of miRNAs was validating the results of tumor set 1 (60 and 58% in BRAF and *RAS*, respectively; Figure 2a and Supplementary Table S3). Commonly deregulated miRNAs in both tumor sets are summarized in Supplementary Table S4.

As detailed in the Figure 2, upregulation of miR-96, -182, and -183 was confirmed for tumors with a follicular pattern of growth, while a considerable increase in some members of the miR-515 family was only detected among follicular adenomas



b

miR	subtype	Tumor set 1(%)	Tumor set 2(%)
↑miR-96	Follicular Adenomas	100	90
	Follicular Carcinomas	94	50
↑miR-182	Follicular Adenomas	100	90
	Follicular Carcinomas	94	67
↑miR-183	Follicular Adenomas	97	100
	Follicular Carcinomas	100	92
↑miR-518b	Follicular Adenomas	69	60
↑miR-517a	Follicular Adenomas	63	40
↑miR-21	Papillary Carcinomas	100	93
↑miR-31	Papillary Carcinomas	95	81
↑miR-222	Papillary Carcinomas	93	89
↑miR-146b	Papillary Carcinomas	100	82

miR	subtype	Tumor set 1(%)	Tumor set 2(%)
↓miR-1247	Follicular Adenomas	100	90
	Follicular Carcinomas	100	100
↓miR-150	Follicular Carcinomas	100	100
↓miR-451a	Papillary Carcinomas	97	97
↓miR-486	Papillary Carcinomas	97	97
↓miR-1179	Papillary Carcinomas	97	94
↓miR-7	*BRAF*+ Papillary Carcinomas	100	100
↓miR-204	*BRAF*+ Papillary Carcinomas	100	100

Figure 2 Validation step. (**a**) Venn diagram analysis identified those molecules commonly detected in both tumor sets and therefore considered validated. (**b**) Proportion of tumors of both sets showing deregulation of some of the commonly deregulated miRNAs. Arrow next to the miRNA name indicates whether the molecule was detected as downregulated (↓) or upregulated (↑).

of tumor set 2. Significant downregulation of miR-1247 was observed for tumors with follicular pattern of growth in both tumor sets, while, miR-150 downregulation was validated to be follicular carcinoma specific. In papillary tumors, several upregulated (miR-21, -31, -222, and -146b) as well as downregulated (miR-451a, -486, and -1179) molecules were validated in the tumor set 2. The downregulation of miR-7 and -204 was again strongly associated with the presence of *BRAF* mutation in the validation set. The proportions of tumors showing these deregulations are detailed in Figure 2b.

Integrated Gene Expression Reveals Possible Targets for Novel miRNAs

For those novel deregulated miRNAs common to both tumor sets, we integrated the miRNAseq data with gene expression data available for the tumors from tumor set 1 using miRGate software (Figure 3).

MiR-1247 was less expressed in all tumors with a follicular pattern of growth (follicular adenomas, follicular carcinomas, and follicular variant of papillary tumors) than in normal tissue (fold change 3–46). According to validated and predicted interaction data available, the negative regulation of relevant targets could be impaired. These include molecules involved in cell proliferation (*FGFR4*), migration (*BAIAP2L1* and *PTK2*), apoptosis (*FAM129B*), as well as a thyroid-specific transcription factor (*PAX8*). Mir-150 was downregulated in follicular thyroid carcinomas (8- to 10-fold change) and its expression correlated negatively with genes involved in proliferation (*FGF12*, *PRKCA*, and *TGFBR1*) and invasive growth (*TGFBR1*). The compromised expression of miR-1179 seen in papillary tumors (three- to ninefold change) could lead to the deregulation of molecules involved in cell cycle progression (*ESP8* and *ANXA4*) or invasiveness (*HPN* and *MMP13*). Finally, miR-7 and miR-204 were severely downregulated in *BRAF*-positive samples (25- to 29- and 11- to 23-fold change, respectively), potentially affecting a long list of possible targets, including genes involved in extracellular matrix remodeling (*KLK10*, *MMP9*, *MMP15*, and *MMP16*), angiogenesis (*AMOT*, *CLIP1*, *CTSS*, and *GAB2*), and epithelial-mesenchymal transition (*CDH11* and *DUSP6*).

A 2-Gene miRNA Signature Associated with Recurrence

Using the linear regression selection method LASSO, 16 miRNAs were selected for inclusion in the recurrence-free survival analysis. Univariant analysis and correction for multiple testing pointed to the relevance of five of them (let-7a-3p, miR-30e-5p, miR-192-3p, let-7d-3p, and miR-493-5p). Applying Akaike Information Criteria the best model included two molecules (miR-192 and let-7a) whose expression individually (Figure 4a), but even more

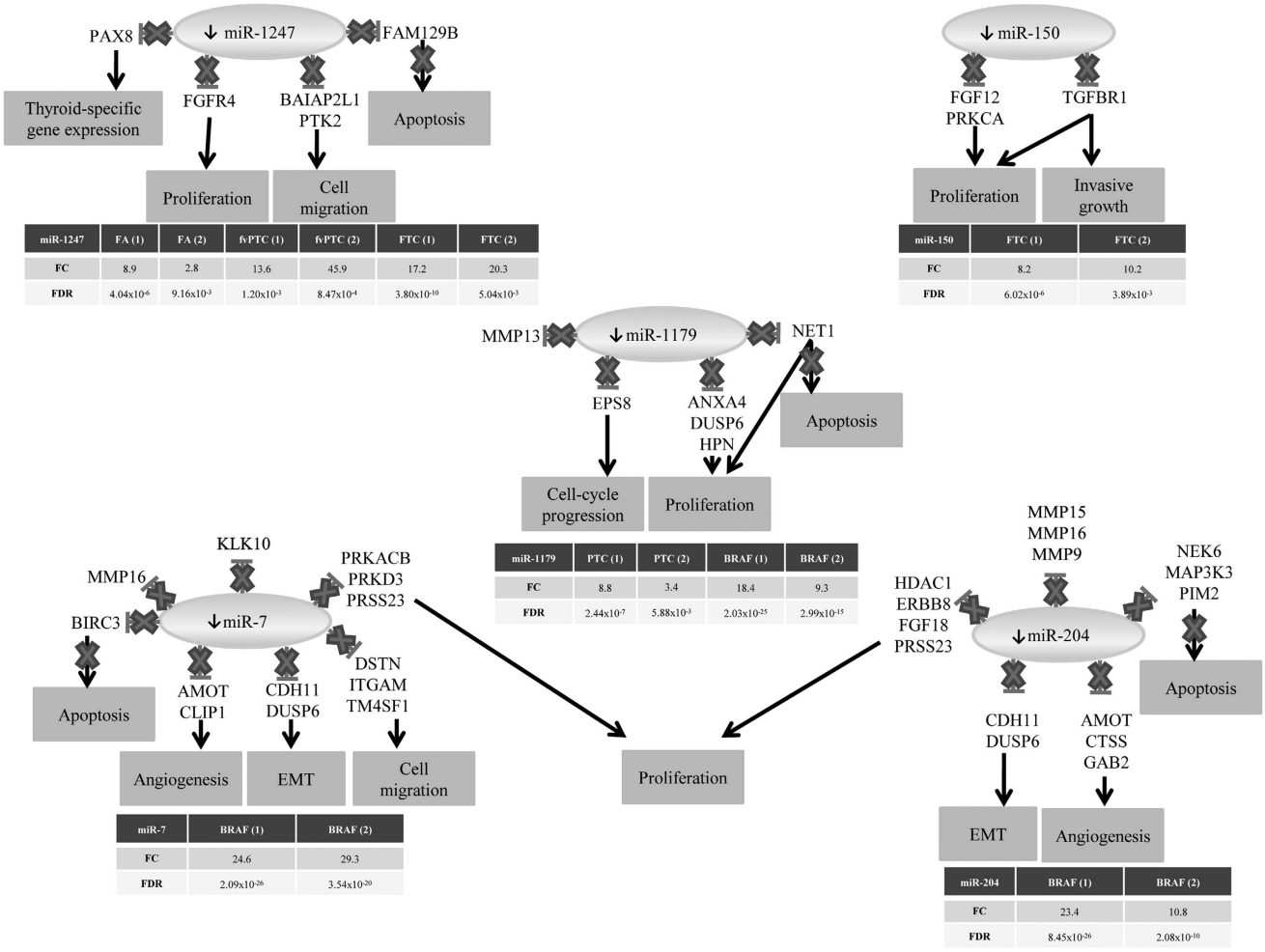


Figure 3 Predicted and validated targets of novel miRNAs. MiRGate software was applied to identify possible targets of novel molecules identified in this study. A small downward arrow to the left of specific microRNAs refers to downregulation. Larger arrows denote where downregulation of the miRNA gives rise to a lack of inhibition of the target gene pointed to. (1) refers to results from tumor set 1, while (2) refers to those from tumor set 2. EMT, epithelial-mesenchymal transition; FA, follicular adenoma; FC, fold change; FDR, false discovery rate; FTC, follicular thyroid carcinoma; fvPTC, follicular variant of papillary thyroid carcinoma; PTC, papillary thyroid carcinoma.

significantly in combination (Figure 4b), discriminated patients with a higher probability of recurrence. In this model, a combination of increased expression of let-7a, together with decreased miR-192 expression, was associated with an increased risk of recurrence. Even after correcting for other important clinical features, such as subtype, stage, gender, and age, the miRNAs remained significant predictors of disease relapse in the final prediction model (Supplementary Figure S2).

Discussion

The currently available evidence suggests that miRNA deregulation could be a crucial event in thyroid carcinogenesis. Most of what is known about deregulated miRNAs has been based on microarray profiling or real-time PCR, both of which have inherent biases. Approaches based on next-generation sequencing permit the detection and simultaneous quantification of the miRNAs present in a specimen,[26] thus affording an in-depth, unbiased characterization of full miRNomes. In the present work, using two independent series of well-differentiated thyroid tumors and next-generation sequencing, we were able to identify deregulated miRNAs related to the specific histological subtypes and driver mutations. These included not only miRNAs previously reported to be related to thyroid cancer, but also novel recurrent markers. We propose possible targets for these novel molecules based on *in silico* prediction using miRNA and mRNA expression data from the same tumors. Finally, we describe a model based on a two-miRNA signature that could be used in relapse prediction.

Two miRNA molecules, miR-221 and miR-34a, were found to be consistently deregulated among all the tumor groups studied, suggesting that they play a key role, both in the first steps of the malignant

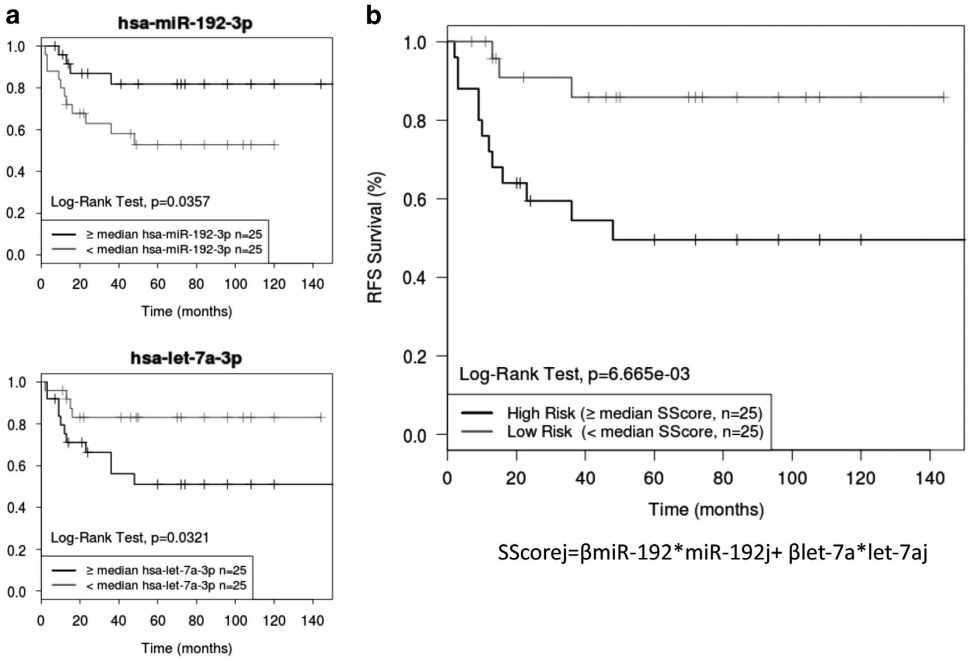


Figure 4 Markers of recurrence-free survival. (**a**) Individual Kaplan–Meier curves for each signature member (miR-192 and let-7a) with expression dichotomized at the median value. (**b**) Kaplan–Meier survival analysis. A signature score (SScore) representing the signature expression for each sample was calculated as $SScore^j = \beta_{miR\text{-}192} \times miR\text{-}192^j + \beta_{let\text{-}7a} \times let\text{-}7a^j$, where β_x is the coefficient for x obtained from the LASSO regression. The score was dichotomized at the median to create two groups for comparison.

transformation process in thyroid carcinogenesis, and in more advanced forms of the disease. That is, they may act as master regulators in thyroid cancer. The involvement of miR-221 in carcinogenesis has already been studied, as its expression is increased in various neoplasias. Our results confirm those of a previous microarray study, where the authors observed upregulation of miR-221 in various histological subtypes of thyroid cancer (follicular adenomas, follicular carcinomas, and papillary carcinomas).[14] In thyroid cells, it was shown that miR-221 has oncogenic properties, as it induces cell cycle progression by targeting $p27^{Kip1}$.[27] To date, the overexpression of miR-34a has been described in both papillary tumors[12,17] and papillary thyroid carcinoma cell lines.[28] Recently, it has been suggested that its oncogenic effect in PTC involves activation of the PI3K/Akt/Bad pathway,[29] which is consistent with it having a master regulator role. Moreover, both miR-34a and -221 have been very recently identified in the TCGA data set as crucial regulators of the immune response activities among all papillary carcinoma variants studied by this consortium,[19] further confirming our results.

As observed with other genomic features,[4,9] the miRNomes of tumors with a follicular pattern of growth were largely similar, pointing to a possible progressive evolution of carcinomas from follicular adenomas.[30] The observed upregulation of miR-96, miR-182, and miR-183 in both follicular adenomas and follicular carcinomas has been reported previously.[14,31,32] Of the other molecules that had been previously reported to be deregulated in thyroid cancer,[32] it is noteworthy that several members of the miR-515 family were highly overexpressed in both follicular adenomas (showing a maximum fold change of 3452) and follicular carcinomas (maximum fold change of 1867) in tumor set 1. However, for few of those was that overexpression replicated in tumor set 2. Similarly, downregulation of the miR-199 family was detected in the discovery phase of our study, confirming previously published findings, but not in the validation phase.[31,32] It is likely that the use of a purification column introduces bias into miRNA profiles. Indeed, the effect of extraction technique on miRNA expression detection has been previously reported by others.[33] The identification of deregulated miRNAs common to both tumor sets, despite the different methodologies employed, adds weight to the evidence that they play a role in thyroid cancer.

One interesting novel marker was miR-1247, which was found to be downregulated in all tumors with a follicular pattern of growth (including even adenomas), indicating it could be an early event in the development of these neoplasias. The lack of negative regulation of its predicted targets could give proliferative potential to the follicular cell. Indeed, it has recently been confirmed that this molecule plays a tumor-suppressive role in pancreatic cancer by inhibiting proliferation and tumorigenesis and triggering G_0/G_1 cell cycle arrest.[34] Further, miR-150 was downregulated specifically in follicular carcinomas. In line with its recently described downregulation in poorly differentiated thyroid tumors,[35] the candidate targets identified here (*FGF12*, *PRKCA*,

and *TGFBR1*) could lead to a more aggressive neoplasm.

As reported in tumors with follicular growth pattern, we observed upregulation in papillary tumors of previously described markers, such as miR-146b, the miR-221 ~ 222 cluster, miR-21 and miR-31.[12,14,15,17] Several other miRNAs (miR-486, -873, -1179, -451a, and -652), firstly described in the first next-generation sequencing study performed in papillary thyroid carcinoma,[16] were also detected. This consistency with findings from earlier studies served as an external validation of our results. Particularly noteworthy was the consistent miR-204 downregulation observed in both tumor sets, specifically associated with the presence of the *BRAF* mutation. The targets of this miRNA were predicted to impair several cellular processes, such as those maintaining epithelial physiology,[36] which could explain the relatively poor prognosis described for these patients.[37] Another interesting marker was miR-7, highly significantly associated with *BRAF* mutation when compared to normal thyroid tissues in tumor set 1, and with wild-type papillary carcinomas in the validation set. Moreover, one of the predicted targets of miR-7 was *KLK10*, which our group recently identified as specifically hypomethylated in *BRAF*-mutated tumors and correlated with gene expression.[9] Thus, overexpression of miR-7 represents a second mechanism by which this molecule may be specifically upregulated in *BRAF*-positive papillary thyroid tumors, thereby highlighting its importance.

Though most well-differentiated tumors can be effectively clinically managed, there is a subset of patients who develop recurrences and often respond poorly to the current therapeutic options. Here, we propose a relapse prediction model based on a two-miRNA signature composed of a combination of let-7a increase together with a miR-192 decrease. The let-7 family of miRNAs are well-established tumor suppressors;[38] their increased expression has been detected in the serum from papillary thyroid cancer patients (compared to healthy individuals or benign cases) as associated with the presence of multifocal lesions ($P < 0.001$).[39] Thus, further studies are required to elucidate the role of this miRNA family in thyroid tumorigenesis. However, the tumor suppressive role of miR-192 has been robustly functionally demonstrated,[40–42] and is consistent with our current observation. In this regard, the value of the tumor set 2 was limited for validation, as the time of follow-up was shorter. Thus, while the predictive value of our signature requires validation, it seems that these miRNAs could be considered as markers of recurrence, acting independently of other known prognostic factors, and therefore potentially contributing to an improved stratification of thyroid cancer patients.

To summarize, this is the first next-generation sequencing study to be applied to all major histological subtypes of well-differentiated thyroid cancer in the largest tumor collection reported so far. We update the current knowledge about deregulated miRNAs by confirming some of those that have been previously reported as well as by describing novel ones. According to our results, there are at least two miRNA molecules (miR-34a and miR-221) acting as master regulators of thyroid carcinogenesis. Moreover, by integrating miRNA and mRNA expression data for the same tumors, we were able to explore the possible targets of the novel molecules detected, and thus shed further light on the biological mechanisms involved in thyroid carcinogenesis. By integrating clinicopathological data with the miRNA expression, we identified a two-miRNA signature associated with disease recurrence.

Acknowledgments

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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ARTICLE 4

Article 4: Methylation profiling reveals markers specific to distinct *RET* mutations in Medullary Thyroid Carcinoma.

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In preparation to be sent for peer-review

Abstract

Medullary thyroid carcinoma (MTC) is one of the orphan rare cancers, whose etiology is so far little explored. Ours is one of the pioneer groups, where OMICs techniques (mRNA and miRNA profiling) were applied to dissect the disease. In this work, we have complemented the acquired genomic data by studying the methylome in the largest MTC cohort published until today. We show that the DNA methylation profiles differ according to different *RET* mutations, with the most distinctive profile found among RET^{M918T}-related MTCs characterized by a large number of hypomethylation events. Moreover, through the integration of methylation with mRNA and miRNA expression data available from the same tumors, we identified genes whose expression is negatively correlated with the methylation status of their promoters. For *PLCB2*, *DKK4* and *MMP20* genes as well as miR-10a, -30a and -200c, we also assessed the impact of promoter methylation levels on expression of the genes in MZ-CRC-1 and TT cell lines. Finally, we validated three DNA methylation markers specific of distinct *RET* mutations in an independent set of 25 MTCs by bisulfite pyrosequencing.

On the whole, this integrative genomic study uncovers some interesting regulatory axes that could play a role in MTC etiology, and underscores the importance of DNA methylation regulation in the disease process.

Personal contribution: I participated in the conception and design of the study. I extracted and prepared the samples for this study. I performed the genetic screening of known genetic drivers. I performed the statistical analyses. I technically validated the array results and performed the *in vitro* experiments with the MTC cell lines. Finally, I contributed to the discussion of the results and the drafting of the paper.

Title: Methylation profiling reveals markers specific to distinct *RET* mutations in Medullary Thyroid Carcinoma

Running title: MTC methylation markers

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ABSTRACT

Medullary thyroid carcinoma (MTC) accounts for 1-2% of thyroid malignancies. Around 75% of them are sporadic, and the remaining 25% are hereditary and related to Multiple Endocrine Neoplasia type 2 syndrome. Although a genotype-phenotype correlation related to specific germline *RET* mutations is well established, the disease etiology specifically associated with each mutation still remains largely unknown. Here, we investigated the role of aberrant DNA methylation in the development of MTC. We performed DNA methylation profiling assessing >27,000 CpGs across the genome in the largest MTC series reported to date, comprising 48 molecularly characterized tumors. We observed significant differences between the methylation patterns among the samples bearing the RET^{M918T}, RET^{C634X} mutation and "wild-type" (WT) tumors; those RET^{M918T} –related had a larger number of hypomethylation events when compared to RET^{C634X} – positive and WT tumors. Moreover, through the integration of methylation with mRNA and miRNA expression data of the same tumors, we identified genes whose expression is negatively correlated with the methylation status of their promoters. For *PLCB2*, *DKK4* and *MMP20* genes as well as miR-10a, -30a and -200c, we also assessed the impact of promoter methylation levels on expression of these genes in MZ-CRC-1 and TT cell lines. Finally, we validated the aberrant methylation events of three of the genes in an independent set of 25 MTCs by bisulfite pyrosequencing.

INTRODUCTION

Medullary thyroid carcinoma is a malignant tumor of the thyroid gland showing C-cell differentiation accounting for up to 2% of all thyroid cancers (DeLellis *et al.*, 2004). Around 25% of MTC cases are inherited attributable to germline mutations in the "rearranged during transfection" (*RET*) proto-oncogene, while the remaining forms are sporadic. The majority of the latter arise due to somatic mutations either in *RET* (Leboulleux *et al.*, 2004) or, as described recently, in *RAS* family of genes (Moura *et al.*, 2011; Ciampi *et al.*, 2013). Of note, there is a distinct genotype-phenotype correlation described for these alterations (Romei *et al.*, 1996; Elisei *et al.*, 2008; Ciampi *et al.*, 2013). However, the underlying molecular mechanisms specifically altered according to each of the driver mutations require further study.

Microarray profiling has been already used to define genomic signatures linked to particular driver mutations in cell lines and other thyroid cancer subtypes (Giordano *et al.*, 2005; Montero-Conde *et al.*, 2008; Hou *et al.*, 2011; Rodriguez-Rodero *et al.*, 2013; Mancikova *et al.*, 2014; Mancikova *et al.*, 2015); uncovering molecular events associated with progression, recurrence etc. However, data on MTC are scarce due to the disease's low prevalence and, in consequence, the difficulty to collect an informative sample set. So far, only a handful of studies using mRNA expression arrays have been published reporting the MTC expression profiles are mutation-specific (Jain *et al.*, 2004; Ameur *et al.*, 2009; Maliszewska *et al.*, 2013). Interestingly, the over-expression of genes related to epithelial to mesenchymal transition (Jain *et al.*, 2004) and tumor invasion and metastases (Ameur *et al.*, 2009; Maliszewska *et al.*, 2013) was notable among MTCs caused by the RET^{M918T} mutation, widely accepted as associated with poor prognosis (Elisei *et al.*, 2008). On the other hand, the miRNA profiling studies so far available focused rather on the patients' outcome than on the genetics, and successfully uncovered some molecular events related to metastasis and worse outcome (Nikiforova *et al.*, 2008; Abraham *et al.*, 2011; Santarpia *et al.*, 2013).

Undoubtedly, some of the differentially expressed genes identified in the previous profiling studies could be regulated by aberrant methylation. However, this epigenetic mechanism, whose de-regulation is a well-known hallmark of cancer, has so far been explored in a rather limited manner in MTC, either focused on specific candidate genes, such as *RAS* association domain family protein 1 (*RASSF1*) (Schagdarsurengin *et al.*, 2002) and Sprouty 1 (*SPRY1*) (Macia *et al.*, 2012), or exploring the whole methylome in a very few samples (Rodriguez-Rodero *et al.*, 2013). Thus, it remains poorly characterized.

Herein, we quantitatively profiled the largest cohort of medullary tumors published until today composed of 48 samples for the DNA methylation levels of >27,000 CpGs across the genome. We observed significant differences between the methylation patterns among the

samples carrying the RET^{M918T}, RET^{C634X} mutation and "wild-type" tumors. By means of integrating methylation data with mRNA and microRNA expression data of the same tumors, we were able to identify genes whose expression was controlled by the methylation of their promoters, thereby adding new insights into MTC carcinogenesis. The negative effect of promoter methylation on gene expression of *DKK4*, *PLCB2*, miR-10a, -30a and miR-200c was also validated in two MTC cell lines (MZ-CRC-1 and TT). Finally, validation of methylation levels of selected protein-coding genes was performed in a subset of arrayed samples by bisulfite sequencing, and subsequently in an independent cohort of 25 formalin-fixed paraffin-embedded (FFPE) MTC samples by pyrosequencing.

MATERIAL AND METHODS

Human MTC tissue samples and cell lines

Forty-eight fresh frozen MTC tumor samples were collected at the Spanish National Cancer Research Center (CNIO) in collaboration with the CNIO Tumor Bank. Written informed consent was obtained from all study participants and the study was approved by institutional review board (*Comité de bioética y bienestar animal* of the *Instituto de Salud Carlos III*). Sections of each sample were evaluated by a pathologist. Only samples in which at least 80% of the cells were cancerous were used in this study, and cases with high amyloid content were excluded. Moreover, twenty-five unrelated FFPE MTC samples and 2 MTC cell lines were used in the validation steps. MZ-CRC-1 cell line is derived from a metastatic MTC and harbors the *RET*^{M918T} mutation, while TT cell line has a mutation in the 634th codon of *RET*. Genomic DNA from all the samples was extracted using the DNeasy Blood and Tissue kit (QIAGEN) according to the manufacturer's protocol. Total RNA was extracted from 5x10^6 of MZ-CRC-1 and TT cells and 1 ml of TRIzol® (Life Technologies) using the standard conditions.

Mutation analysis

All samples were screened for hotspot mutations of *RET* gene in exons 10, 11, 15 and 16 by Sanger sequencing. If negative, the hotspot codons 12, 13 and 61 of exons 2 and 3, respectively, of all *RAS* genes were screened by the same technique. Tumors were classified as "wild-type" (WT) if no mutation was found in neither of the genes screened.

DNA methylation assay, data processing and data analysis

Briefly, genomic DNA was bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) following the manufacturer's recommended procedures. Genome-wide promoter DNA methylation profiling was performed using the Illumina Infinium HumanMethylation 27K Platform (Illumina, San Diego, CA, USA) as described previously (Bibikova *et al.*, 2009). This assay generates DNA methylation data for 27,578 CpG dinucleotides covering 14,473 unique genes. For each CpG site, methylation levels were quantified using β-values, which represent the proportion of methylation, calculated as M/(M+U), where M is the methylated probe intensity and U the unmethylated probe intensity. β-values range from 0 to 1, with 0 being completely unmethylated and 1 being completely methylated. We excluded probes that were detected in less than 95% of the samples (24 probes), probes designed for sequences on either the X or the Y chromosome (1,085 or 7 probes, respectively), as well as probes with missing value in at least one of the samples (22 probes).

Unsupervised hierarchical clustering was carried out using Cluster 3.0 software with "average linkage" (Pearson correlation, uncentered metrics). The clusters were subsequently visualized using Treeview (http://rana.stanford.edu/software). Principal Component Analysis (PCA) was performed using R CRAN version 2.15.3 (R, 2013). Differences in DNA methylation status among tumor groups (based on the driver mutations present in the samples) were tested using POMELLO II, applying linear models (limma) (Morrissey and Diaz-Uriarte, 2009). Tumors with less frequent *RET* mutations were not considered in the analysis. To account for multiple hypotheses testing, *p*-values were adjusted using Benjamini's False Discovery Rate (FDR) correction. We defined a probe to be hypomethylated or hypermethylated when it displayed a mean β-value difference (Δβ-value) < -0.2 or > 0.2, respectively, among particular tumor groups, and had a FDR<0.05.

Integration of gene expression and DNA methylation

Since we disposed of both mRNA (Maliszewska *et al.*, 2013) and miRNA expression data for 33 and 31 of the tumors used in this study, respectively, we aimed to identify genes whose expression is correlated with the methylation status of their corresponding promoter regions. In total, 8,622 protein-coding genes were included on both platforms and thus available for integration. In case of miRNA integration, there were 254 probes included on the methylation platform that mapped to promoters of 110 microRNA genes already annotated by the manufacturer (Bibikova *et al.*, 2009). To this number, we added 423 additional probes belonging to putative promoter regions of 151 miRNAs identified using PROmiRNA method (Marsico *et al.*, 2013). These additional probes and their corresponding miRNA genes are listed in Supplementary Table S1. Correlation was measured by the Spearman coefficient using R CRAN version 2.15.3 (R, 2013).

Confirmation of negative correlation between methylation and gene expression using MZ-CRC-1 and TT cell lines

Cell culture and 5-Aza-2-'Deoxycytidine treatment

MZ-CRC-1 and TT were cultured in Dulbecco's modified Eagle medium Gluta MAX (DMEM, Invitrogen), supplemented with 10% (v/v) foetal bovine serum (FBS, PAA laboratories), 1% (v/v) penicillin/streptomycis and 0.6% (v/v) Fungizone (Gibco). MZ-CRC-1 cells at 60% of confluence were treated with 2.5 μg/ml 5-Aza-2-'Deoxycytidine (Sigma). After 48 hours of incubation, the treatment was renewed and on the following day, the cells were collected for subsequent analyses.

DNA methylation level assessment

MTC cell lines and bisulfite sequencing using primers listed in Supplementary Table S2 were used to assess methylation levels of the selected promoters (belonging to 3 protein-coding genes and 3 miRNA genes) according to protocols described elsewhere (Mancikova *et al.*, 2014).

qRT-PCR

For the assessment of expression of the selected protein-coding genes, one microgram of total RNA was reverse transcribed using Superscript II (Invitrogen) and an oligo dT14 primer following manufacturer's instructions. The amounts of *DKK4*, *MMP20* and *PLCB2* mRNA were quantified by real-time PCR with the Sequence Detection System 7900HT (Applied Biosystems, Foster City, CA), using primers designed to be specific for the three genes (Supplementary Table S2) and probes from the Universal ProbeLibrary Set, Human (Roche). Normalization was carried out with the internal standard β-actin (*ACTB*).

For miRNA gene expression quantification, 10 nanograms of total RNA were used for first-strand cDNA synthesis using miRCURY LNA Universal RT miR PCR system (Exiqon) and LNA miR-PCR primer/SYBR Green mix (Exiqon) was used for subsequent quantification of miR-10a, -30a and -200c according to the manufacturer's recommendations. MiR-16 was selected as reference gene for normalization.

Negative controls were included in all PCR series and assays were carried out in triplicates. The ΔΔ*C*t method was used for the calculation of mRNA content (Livak and Schmittgen, 2001).

Methylation status validation

Three of the most differentially methylated probes, all showing negative correlation with expression of the corresponding protein-coding gene, were selected for validation. Biological functions were considered as additional criteria to select candidate promoter regions. The technical validation of microarray results in a subset of the original discovery series (comprising 6 RET^{M918T}, 6 RET^{C634X}, 3 *RAS*, 2 "wild-type" tumors and 6 tumors bearing other *RET* mutations) was performed using bisulfite sequencing as described elsewhere (Mancikova *et al.*, 2014). The candidate markers were then validated by pyrosequencing in 25 independent FFPE MTC samples (8 RET^{M918T}, 4 RET^{C634X}, 3 *RAS* and 10 "wild-type" tumors). The region of interest was firstly amplified from the bisulfite-treated DNA by a set of primers designed with PyroMark assay design software (version 2.0.01.15). After the PCR amplification, pyrosequencing was performed using PyroMark Q24 reagents, vacuum prep workstation, equipment and software (Qiagen). Primers used in these steps are listed in Supplementary Table S2.

RESULTS

Molecular characterization of the MTC discovery series

Among the tumors whose genome-wide DNA methylation levels were measured, 13 samples harbored the *RET*^{M918T} mutation, 14 *RET*^{6C34X}, 6 tumors harbored a *RAS* mutation, and 9 tumors carried different, less frequent, *RET* mutations. The remaining 6 tumors did not carry any alteration in the studied genes and were considered "wild-type" (WT). The results of the mutational screening together with the main clinico-histopathological characteristics of the samples are summarized in the Supplementary Table S3.

MTC methylation profiles relate closely to the *RET* mutational status

After excluding all the possible sources of bias (see Material and Methods), we identified probes that appeared to be constitutively unmethylated in all samples (β-value <0.2; 11,915 probes). Of these, the majority (97.5%) was located within CpG islands adjacent to house-keeping genes (Benjamini-Hochberg adjusted *p*-value $7.1x10^{-10}$). On the other hand, 179 probes were constitutively methylated (β-values among all the samples>0.8) in our data. Both constitutively unmethylated and methylated probes were excluded from the further analysis. A PCA of the remaining 14,346 probes (belonging to 9,216 consensus coding sequences) did not reveal any apparent batch effect affecting the data (Supplementary Figure S1).

Unsupervised hierarchical analysis using the probes with the highest variance among the data set (SD>0.2; 851 probes) pointed towards the existence of differences among the two principal clusters based on underlying genetics (Figure 1). Only two samples did not fall into either of them. Interestingly, cluster B showed significantly higher levels of methylation when compared to cluster A (*p*-value $3.0x10^{-9}$), and was enriched with WT cases and those harboring *RET*^{C634X} mutation, while cluster A was composed mostly of *RET*^{M918T}-positive tumors (p-value<0.02). We did not observe any clear clustering of the *RAS*-mutated samples.

Supervised analysis allowed us to identify a list of differentially methylated probes associated with a specific genetic condition, especially long for *RET*^{M918T}-related tumors. In this regard, the results confirmed those of unsupervised clustering, as in this group there were more hypomethylated probes (Figure 2A, Supplementary Table S4). Interestingly, when exploring the genes affected by hypomethylation, DAVID functional analysis (Huang da *et al.*, 2009) returned KEGG pathways such as cytokine-cytokine receptor interaction (p-value $1.5x10^{-7}$) or JAK/Stat signaling pathway (p-value $2.3x10^{-4}$). Moreover, *DKK4*, previously described to be up-regulated in *RET*^{M918T}-related MTCs (Maliszewska *et al.*, 2013), was affected by hypomethylation. As could have been expected from the unsupervised clustering result, the lists of differentially methylated probes characteristic of the other genetic conditions (*RET*^{C634X}- and *RAS*-related

tumors) were considerably shorter and did not allow identification of any pathway enrichment. Nevertheless, hypomethylation of *GAL* was detected among RET^{C634X}-positive tumors when compared to WT, and this event could cause the increased expression of this molecule reported elsewhere (Maliszewska *et al.*, 2013).

It was noteworthy that hypermethylation affected more frequently probes located within CpG islands (p-value 0.0014) as well as those located near to stem cell PolyComb Group target genes (p-value<0.0001). Hypomethylated probes, on the other hand, were enriched with CpGs that are heavily methylated in Embryonic Stem Cells (p-value<0.0001) (Figure 2B).

Genome-wide integration reveals those DNA methylation changes potentially functional in MTC

The fact of finding differential methylation in genes previously reported to be deregulated in MTC indicated these epigenetic changes might be functional. Therefore, we systematically explored genes regulated by promoter methylation. When considering protein-coding genes, mRNA expression data was available for 93.55% genes from the methylation array. Of these, almost 12% was showing significant negative correlation between expression and methylation (Table 1). Moreover, expression data of 31 matching samples for 78 microRNA genes was available for the integration. In this case, 5 genes (6.9%) were showing negative correlation (Table 1).

Importantly, some of the genes showing inverse correlation between expression and methylation were also found as differentially methylated in the previous analyses (Table 1). Of these, *PLCB2*, *DKK4*, *MMP20* and miR-10a were selected for further studies. Moreover, two additional miRNA genes (miR-30a and -200c) showing negative correlation between expression and methylation, but not differential methylation, were also studied further due to their biological function (Cheng *et al.*, 2012; Kumarswamy *et al.*, 2012; Santarpia *et al.*, 2013). As assessed by bisulfite sequencing, the levels of methylation of these 6 genes differed among the two available MTC cell lines (Figure 3A). Apart from two genes that were not expressed by none of the cell lines (*MMP20* and miR-30a), the expression of the remaining confirmed the results of *in silico* predictions of inverse correlation between DNA methylation and gene expression (Figure 3B). Moreover, it was possible to achieve re-expression of all genes but *MMP20* in MZ-CRC-1 cell line by treatment with 5-Aza-2-'Deoxycytidine (Figure 3C), further confirming the functionality of these epigenetic changes.

Validation of aberrant methylation of candidate oncogenes

CpGs from promoters of 3 differentially methylated genes (*DKK4*, *MMP20*, *PLCB2*) were selected for validation steps. These genes also showed a negative correlation between

DNA methylation levels and gene expression, and have been previously described to have tumor-growth promoting role (Bertagnolo *et al.*, 2007; Liu *et al.*, 2011; Maliszewska *et al.*, 2013; Takeuchi *et al.*, 2013). As depicted in Figure 4, the results of bisulfite sequencing in a subset of the original sample set revealed a high concordance with those array-based (R^2 ranging from 0.6597 to 0.8008). Moreover, we were able to replicate the findings in an independent FFPE series of 25 MTCs by bisulfite pyrosequencing (Figure 4), even if the differences between the DNA methylation levels of the compared groups were smaller, probably since the DNA source was a paraffin tissue.

DISCUSSION

In the current genomic era, an increasing number of cancers are being studied in a collaborative manner via international consortia (such as the TCGA project, http://cancergenome.nih.gov/). The results of such studies generate a comprehensive integrative view of the disease that responds to many clinically relevant questions. However, there are still some orphan cancer types that are being neglected by these efforts, generally due to their low prevalence, and thus small overall public health impact. In this regard, medullary thyroid carcinoma is one of the least prevalent subtypes of thyroid cancer, but responsible for a large proportion of thyroid cancer-related deaths (Roman *et al.*, 2006). Herein, in the largest cohort of MTC samples reported to date, we apply an integrative approach focused on the effects of DNA methylation in the etiology of this tumor. Not only do we find that underlying genetics relate closely with the genome-wide DNA methylation fingerprints, but also that the aberrant methylation events affect specific genomic loci pointing towards the existence of epigenetic progenitor cell signature in MTC (Zhuang *et al.*, 2012). Furthermore, we identify both protein-coding and microRNA genes, whose expression is negatively correlated with the methylation status of their promoters and confirm some of these findings in MTC cell lines. Finally, we also validate some of the DNA methylation markers in an independent collection of samples.

So far, the knowledge generated by mRNA profiling studies in MTC indicates that this genomic feature is driven by the driver alteration (Jain *et al.*, 2004; Ameur *et al.*, 2009; Maliszewska *et al.*, 2013). The current work is the first one reporting the same for another genomic attribute -DNA methylation- since the only genome-wide study on MTC did not address this aspect (Rodriguez-Rodero *et al.*, 2013). Actually, we observed larger proportion of hypomethylation events in *RET*^{M918T}-related MTCs as compared to the other genetic classes. Interestingly, global hypomethylation using the same platform was correlated with poorer prognosis in different gynecological cancers (Zhuang *et al.*, 2012), which would be in concordance with the clinically worst behavior of *RET*^{M918T}-positive MTC (Elisei *et al.*, 2008). Moreover, this deregulation affected genes enriched within pathways such as cytokine-cytokine interaction and JAK/Stat, previously reported as activated in this genomic class (Maliszewska *et al.*, 2013), and related to the malignant tumor behavior. Thus, it seems that at least some of the deregulated pathways in this tumor subgroup are indeed affected at this epigenetic regulatory level.

DNA methylation is traditionally believed to be a regulator of gene expression. In case of protein-coding genes, the classical view on the inverse effect of methylation on expression is being challenged by recent technical advances (Suzuki and Bird, 2008). Many cancer-orientated studies report a surprisingly low percentage of genes fulfilling this rule (Hinoue *et al.*, 2012;

Kulis *et al.*, 2012; Selamat *et al.*, 2012) that we confirm in the current one. The situation with microRNAs is even more complex given the difficulties with predicting their promoter regions (Krol *et al.*, 2010). Recently, a methodology was developed that recognizes putative promoter regions of miRNAs using both sequence- and histone-based techniques (Marsico *et al.*, 2013). By integrating this strategy in our analysis, we were able to identify CpGs belonging to additional putative miRNA promoters included on the 27K array. However, the overall portion of miRNA genes showing negative correlation between methylation and expression was still lower than in case of protein-coding genes. This suggests that even with the new promoter-recognizing algorithms, our understanding of the complex relationship between DNA methylation and gene expression is still limited.

Nevertheless, in the majority of the genes selected for validation of the inverse correlation between expression and methylation in MTC cell lines, we could confirm the *in silico* results. Of these genes, *DKK4* has been already connected with the MTC pathogenesis in a previous study (Maliszewska *et al.*, 2013), and here we propose the aberrant methylation as the regulatory mechanism responsible for its over-expression in *RET*^{M918T}-related tumors. On the other hand, *PLCB2* plays a role in multiple transmembrane signal transduction pathways involving inositol lipids' metabolism. In cancer, its over-expression has been associated with mitosis promotion, migration and poor outcome (Bertagnolo *et al.*, 2006; Bertagnolo *et al.*, 2007). Our findings are in agreement given we found hypomethylation in the *RET*^{M918T}-postive tumor samples, which was negatively correlated with the gene expression. Even though the prognostic utility of *MMP20* gene from the family of metalloproteinases, has been already demonstrated in cancer (Liu *et al.*, 2011), we did not detect its expression in the studied cell lines.

In case of miRNA genes, the expression of miR-200c has been recently described as causative of the metastatic potential of human MTCs (Santarpia *et al.*, 2013). Interestingly, according to our results in the MTC cell lines, the aberrant methylation of miR-200c putative promoter could underlie its differential expression among patients with distinct outcome. MiR-10a is mostly exerting oncogenic effect (Weiss *et al.*, 2009; Bryant *et al.*, 2012; Long *et al.*, 2012) and we have found little DNA methylation of its promoter in both MTC cell lines. Interestingly, there is evidence from hepatocellular carcinoma that miR-10a is negatively regulated by DNA methylation (Shen *et al.*, 2012), which got further validated by our findings. Finally, our miRNA array results pointed to the relevance of down-regulation of miR-30a in *RET*^{M918T}-related MTCs (unpublished data). We found corresponding hypermethylation of its promoter in MZ-CRC-1 cells. This molecule deserves further studies due to its tumor-suppressive features (Cheng *et al.*, 2012; Kumarswamy *et al.*, 2012). Moreover, PROM1, a

cancer stem cell marker found over-expressed in *RET*^{M918T}-positive tumors (Maliszewska *et al.*, 2013), is one of its predicted targets according to targetscan tool (http://www.targetscan.org/).

To sum, this comprehensive genome-wide DNA methylation study performed using the largest cohort of MTC samples reported to date provides insights into the involvement of this regulatory epigenetic mechanism in the etiology of this disease. According to our results, hypomethylation may induce activation of key pathways related to the malignant tumor behavior of *RET*^{M918T}-related MTCs. Moreover, we were able to confirm the regulatory role of DNA methylation for of *DKK4, PLCB2*, miR-10a, -30a and miR-200c using MZ-CRC-1 and TT cell lines. The validation of aberrant methylation markers in an independent cohort of samples warranted the accuracy of our results.

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	Protein-coding genes	microRNA genes
Total # genes available for integration	8,622	78
r<0	5,569 (64.6%)	52 (66.7%)
***P*<0.05**	1,033 (11.9%)	5 (6.9%)
Differentially methylated	54	1

Table 1. Genome-wide integration of DNA methylation, mRNA and miRNA expression data. Correlation between CpG methylation and adjacent gene expression was measured by the Spearman coefficient.

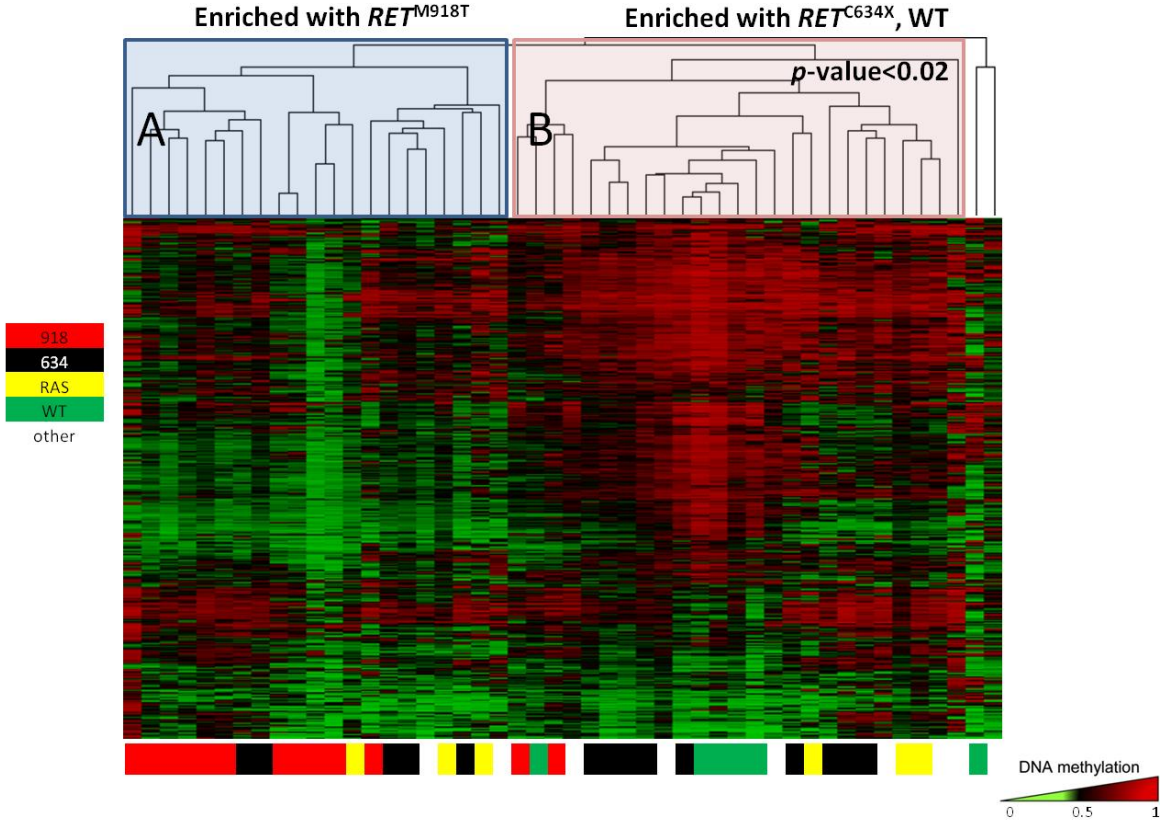
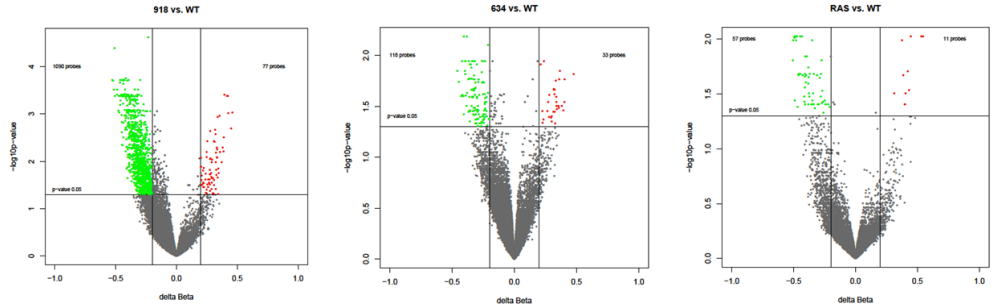


Figure 1. Methylome of the discovery series. Unsupervised hierarchical cluster analysis of 48 medullary tumors divided the sample set into 2 main clusters. "Cluster A" was composed mostly of *RET*^{M918T}-related samples. "Cluster B" included the majority of *RET*^{C634X}- and wild-type cases.

2A



2B

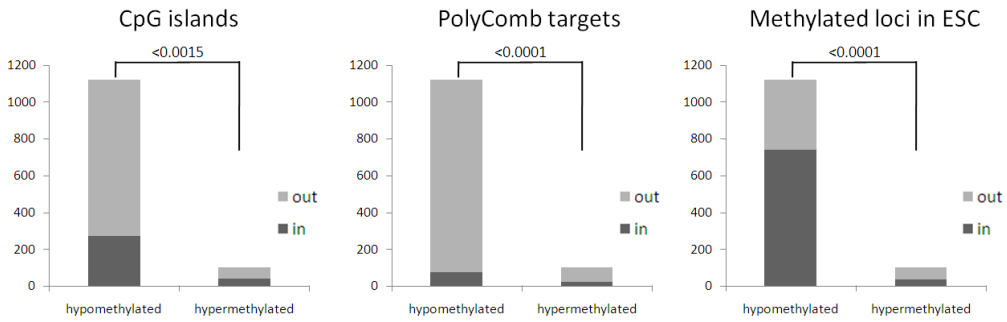


Figure 2. Identification of differentially methylated probes. A) Volcano plots, from each of the supervised analysis carried out, identifying differentially hypomethylated (green) and hypermethylated (red) probes, defined based on FDR<0.05 and Δβ-value ≥ |0.2|. **B)** Number of probes hypermethylated and hypomethylated, by location with respect to: CpG islands; gene targeted by PolyComb Repressive Complex; loci that are heavily methylated in Embryonic Stem Cells.

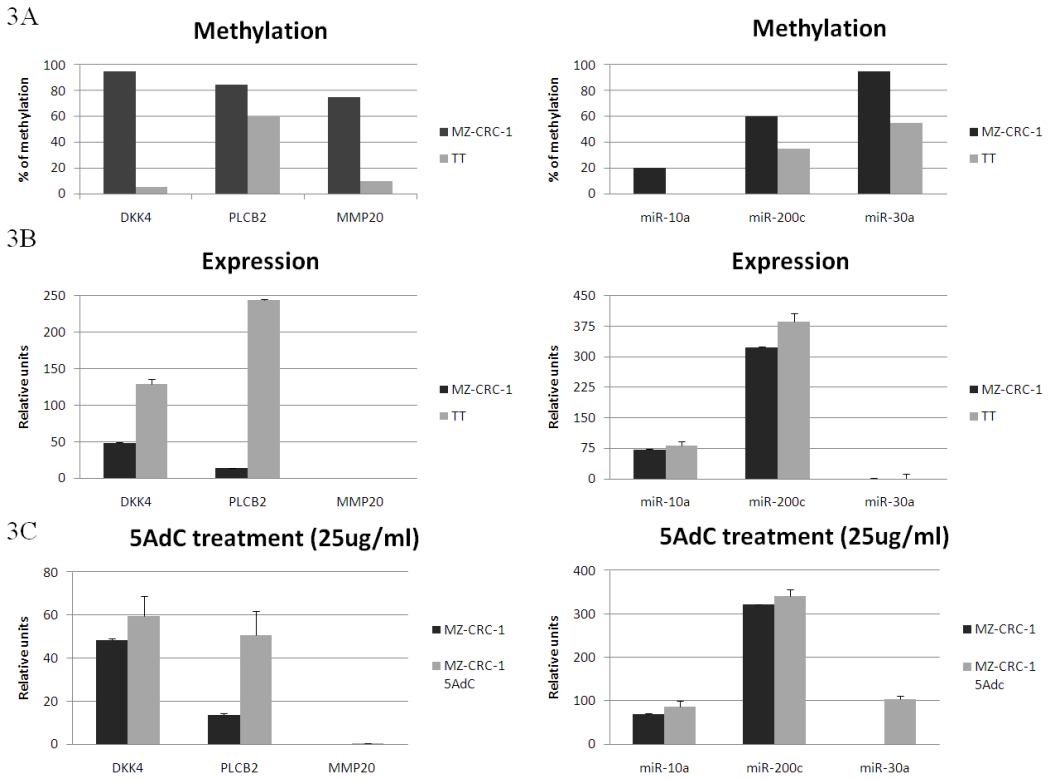


Figure 3. Confirmation of negative correlation between methylation and gene expression in MZ-CRC-1 and TT cell lines. A) DNA methylation levels of promoter CpGs of *DKK4*, *PLCB2*, *MMP20*, miR-10a, -200c and -30a as measured by bisulfite sequencing. **B)** Expression of *DKK4*, *PLCB2*, *MMP20*, miR-10a, -200c and -30a shows an opposite trend as compared to the DNA methylation levels of the genes, confirming *in silico* results. **C)** 5-aza-2′-deoxycytidine (5AdC) treatment causes the reactivation of *DKK4*, *PLCB2*, miR-10a, -200c and -30a expression.

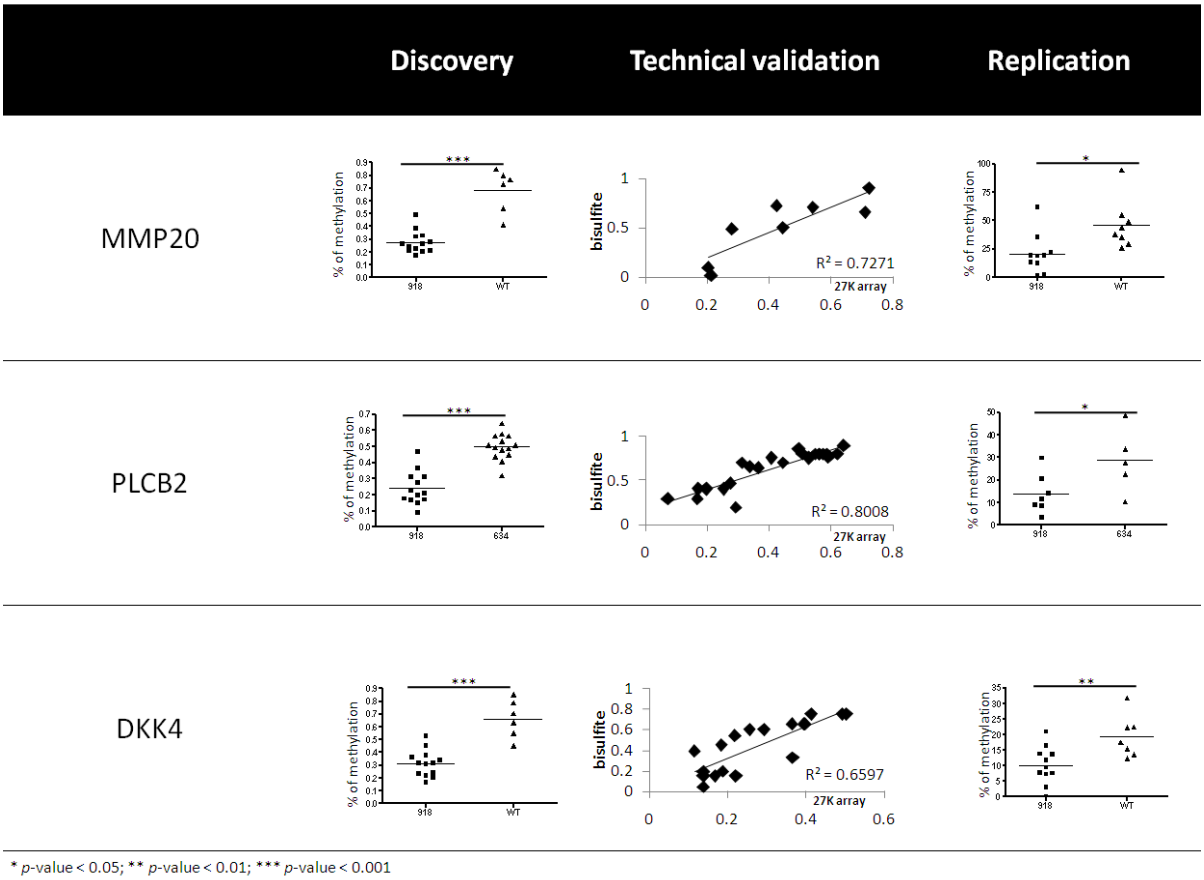


Figure 4. Validation of selected loci. Three CpGs from promoter regions of *MMP20*, *PLCB2* and *DKK4* were selected for validation. In the left panel, results from the discovery series are represented. In the middle panel, correlation between the results from Illumina Infinium HumanMethylation 27K Platform and bisulfite sequencing for selected loci is shown. Bisulfite sequencing was performed in a subset of samples included in the discovery series (6 RET^{M918T}, 6 RET^{C634X}, 3 *RAS*, 2 "wild-type" tumors and 6 tumors bearing other *RET* mutations). In the right panel, results of bisulfite pyrosequencing of 25 independent MTC samples (8 RET^{M918T}, 4 RET^{C634X}, 3 *RAS* and 10 "wild-type" tumors) are depicted.

ARTICLE 5

Article 5: Influence of *RET* mutations on the expression of tyrosine kinases in medullary thyroid carcinoma.

Authors: Cristina Rodríguez-Antona, Iván Muñoz-Repeto, Lucia Inglada-Pérez, Aguirre A. de Cubas, **Veronika Mancikova**, Marta Cañamero, Agnieszka Maliszewska, Álvaro Gómez, Rocío Letón, Luis J. Leandro-García, Iñaki Comino-Méndez, Lara Sanchez, Cristina Álvarez-Escolá, Javier Aller, Alberto Cascón and Mercedes Robledo

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Abstract

Management of sporadic medullary thyroid carcinoma (MTC) has substantially changed recently due to the development of targeted cancer drugs. MTC patients with metastatic or locally advanced disease are treated with small-molecule tyrosine kinase inhibitors (TKIs) with promising results. Yet, some of MTC patients need to discontinue the treatment due to severe toxicities, and the molecular basis for the large variability in TKI response is unknown.

According to our results (Maliszewska *et al.*, 2013) and those from other laboratories (Jain *et al.*, 2004; Ameur *et al.*, 2009), there is emerging evidence that gene expression profiles in MTC are driven by the underlying genetics. Therefore, we decided to investigate if the key TKIs targets are expressed in MTCs in a mutation-dependent manner by means of immunohistochemistry. Apart from showing that multiple TKI targets are highly expressed in a subset of MTCs, we found that MTC samples with the RET^{C634X} mutation exhibited a higher expression of VEGFR3 and KIT than the RET^{M918T}-mutated and non-mutated *RET* tumor samples (P=0.005 and P=0.007, respectively) and a lower expression of VEGFR1 (P=0.04).

These results seem crucial for MTC patients' enrollment into TKI trials and the choice of the most appropriate treatment.

Personal contribution: I helped with genetic screening of the samples.

Influence of *RET* mutations on the expression of tyrosine kinases in medullary thyroid carcinoma

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Abstract

The therapeutic options for patients with metastatic medullary thyroid carcinoma (MTC) have recently increased due to the development of tyrosine kinase inhibitors (TKIs), some of which have achieved remarkable clinical responses in MTC patients. However, the molecular basis for the large variability in TKI responses is unknown. In this exploratory study, we investigated the expression of eight key TKI target proteins (EGFR, KIT, MET, PDGFRB, VEGF (VEGFA), VEGFR1 (FLT1), VEGFR2 (KDR), and VEGFR3 (FLT4)) by immunohistochemistry in 103 molecularly characterized MTC samples and identified the associated clinical and molecular features. A number of MTC samples exhibited a high expression of VEGFR2 and VEGFR3, which were overexpressed in 57 and 43% of the MTC samples respectively. VEGFR1, PDGFRB, VEGF, KIT, and MET were present in 34–20% of the cases, while EGFR was highly expressed in only 10% of the MTC samples. Some proteins exhibited large differences in expression between sporadic and familial cases, suggesting that different *RET* mutations may be associated with the immunohistochemical profiles. MTC samples with the C634 *RET* mutation exhibited a higher expression of VEGFR3 and KIT than the M918T *RET*-mutated and non-mutated *RET* tumor samples ($P=0.005$ and $P=0.007$ respectively) and a lower expression of VEGFR1 ($P=0.04$). Non-mutated *RET* MTC cases exhibited a lower expression of PDGFRB ($P=0.04$). Overall, this is the first study, to our knowledge, to show that multiple TKI targets are highly expressed in a subset of MTCs, suggesting that molecular stratification of patients may have the potential to improve TKI therapies for MTC.

Key Words
- medullary thyroid carcinoma
- *RET* mutation
- tyrosine kinase inhibitors
- EGFR
- KIT
- MET
- PDGFRB
- VEGF
- VEGFR1
- VEGFR2
- VEGFR3

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Introduction

Medullary thyroid carcinoma (MTC) arises from the parafollicular cells of the thyroid gland. Sporadic MTC accounts for 75% of the cases, and the remaining 25% is inherited in nature as part of multiple endocrine neoplasia type 2 syndrome. The *RET* proto-oncogene plays a major role in MTC development, with 30–50% of the sporadic MTCs carrying somatic *RET* mutations (Uchino *et al*. 1999). *RET* mutations involved in MTC are gain-of-function alterations that increase RET kinase activity, resulting in a constant activation of downstream signaling pathways that ultimately lead to tumor growth (Schuffenecker *et al*. 1998, Randolph & Maniar 2000, Nagy *et al*. 2004). The presence of specific *RET* mutations determines the age of presentation and aggressiveness of the tumor, allowing for genetic screening and recommendations for preventive surgical management in familial cases (Brandi *et al*. 2001, Cote & Gagel 2003).

Patients with MTC undergo total thyroidectomy and lymph node dissection. However, because MTC is derived from neuroendocrine cells, it is unresponsive to radioiodine and TSH suppression, and it is unclear as to which is the most appropriate treatment for patients with residual or recurrent disease after primary surgery and for those with distant metastasis. Until recently, management of metastatic disease has primarily been oriented toward the relief of symptoms (Wells *et al*. 1982, Giraudet *et al*. 2007), but in the last few years, much effort has been devoted to developing clinical trials using targeted therapies.

In addition to *RET* mutations, vascular endothelial growth factor (VEGF)-mediated angiogenesis, leading to increased tumor growth and invasiveness, has been recognized as an important feature in MTC. Thus, targeted molecular therapies that inhibit oncogenic kinases such as RET and tyrosine kinase receptors involved in angiogenesis could be important for the treatment of metastatic or locally advanced MTC. In this regard, a number of small-molecule inhibitors that selectively inhibit tyrosine kinase receptors, such as vandetanib, sorafenib, sunitinib, axitinib, motesanib, and cabozantinib (XL184), have shown remarkable clinical responses in MTC patients, inducing partial responses and stabilization of the disease in a substantial number of patients (Cohen *et al*. 2008, Schlumberger *et al*. 2009, Carr *et al*. 2010, Lam *et al*. 2010, Robinson *et al*. 2010, Wells *et al*. 2010, 2012, Ahmed *et al*. 2011, Hong *et al*. 2011, Kurzrock *et al*. 2011). Although comprehensive clinical trials for MTC should ideally incorporate patient and tumor characteristics, most of these studies include only a small number of MTC patients who are not characterized or are heterogeneous with respect to the specific *RET* mutation. Some kinase receptors, such as EGFR and VEGFR2, have been shown to be overexpressed in subsets of primary MTC tumors and metastases (Rodriguez-Antona *et al*. 2010). However, currently such information is lacking for most of the tyrosine kinase inhibitor (TKI) targets, and it is unknown whether they are expressed in MTC and whether this expression is associated with specific clinical and molecular features. Therefore, we set out to investigate the expression of eight key TKI target proteins in an outstanding series of 103 molecularly characterized MTC cases. This is the first study, to our knowledge, to demonstrate that multiple TKI targets are highly expressed in a subset of MTCs in a *RET* mutation-dependent manner. This information might be critical for the inclusion of MTC patients in future clinical trials and, ultimately, for improving treatment response.

Subjects and methods

Human MTC samples

A total of 103 paraffin-embedded MTC samples from 101 patients were obtained from the Spanish National Cancer Centre in collaboration with the CNIO tumor Bank. Institutional Review Board approval was obtained for the study, and informed consent was obtained from all the patients. The patients were aged from 11 to 80 years (median age 50 years), and 56% were females. Of the 103 MTC samples, 92 corresponded to primary tumors and 8 to metastases (see Table 1). The mutational status of the *RET* proto-oncogene in exons 10, 11, 13, 14, 15, and 16 was assessed from genomic DNA using standard PCR conditions, primers, and automated sequencing as described previously (Ceccherini *et al*. 1993). The tumor samples corresponded in most cases to patients diagnosed as sporadic or familial, based on the analysis of the *RET* proto-oncogene in peripheral blood samples. Familial cases carried germline mutations in exon 10, 11, 14, or 15, while sporadic cases carried somatic *RET* mutations in exon 10, 11, 15, or 16, or were classified as 'no mutation in *RET*' when no somatic mutations were found in exon 10, 11, 13, 14, 15, or 16 (Table 1). Among the apparently sporadic cases (without *RET* mutations in blood samples), four tumors were classified as 'undetermined *RET* mutation' due to the failure of the PCR analysis caused by low tumor DNA quality. Among the familial cases, one

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Table 1 Characteristics of the 101 MTC patients included in the study and mutational status of the corresponding samples

Characteristics	*n*	Percentage (%)
Age (years)		
Median	50	
Range (min–max)	38–59 (11–80)	
Gender		
Female	57	56
Male	40	40
Unknown	4	4
Type of tumor		
Primary	92	89
Metastatic	8	8
Unknown	3	3
Genetic features[a]		
Sporadic	**69**	**68**
RET exon 10 (C618, F619, and C620)	3	4
RET exon 11 (C634, c.1894_1900 del GAGCTGT ins A)	5	7
RET exon 15 (A883)	1	1
RET exon 16 (M918T)	17	25
No mutation in *RET*[b]	39	57
Undetermined *RET* mutation[c]	4	6
Familial	**27**	**27**
RET exon 10 (C618)	4	15
RET exon 11 (C634)	20	74
RET exon 14 (V804)	1	4
RET exon 15 (S891)	1	4
Undetermined *RET* mutation[c]	1	4
Unknown	**5**	**5**
Undetermined *RET* mutation[c]	5	100

[a]Percentages in bold type refer to all the patients, whereas percentages in regular type refer to the specific subgroup (sporadic, familial, or unknown).
[b]No mutations found in *RET* exons 10, 11, 13, 14, 15, and 16.
[c]Tumors in which *RET* exon 10, 11, 13, 14, 15, or 16 could not be assessed.

tumor derived from a patient with a family history of multiple neoplasia type 2 (second-degree relative diagnosed with a pheochromocytoma and mother with a MTC) was classified as 'undetermined *RET* mutation' because no blood sample was available and the tumor DNA was of low quality. Five cases could not be classified as sporadic or familial (referred to as 'unknown') because blood samples were not available.

Immunohistochemical study

Hematoxylin and eosin-stained sections of each tumor sample were examined by two pathologists to confirm the diagnosis and to select MTC areas representative of each tumor to construct tissue microarrays (TMAs). Three TMAs containing all the tumor samples (103) were constructed as described previously with two cores of each tumor placed at different positions in the TMAs (Cascon *et al.* 2005).

The three paraffin-embedded TMAs were used for the detection of EGFR, KIT, MET, PDGFRB, VEGF (VEGFA), VEGFR1 (FLT1), VEGFR2 (KDR), and VEGFR3 (FLT4) proteins by immunohistochemistry (IHC) using specific antibodies. The suppliers, dilutions, visualization systems, and immunostainers used for the antibodies are given in Supplementary Table 1, see section on supplementary data at the end of this article. Two independent experienced pathologists (I Muñoz-Repeto and M Cañamero) evaluated the intensity and extension of staining for all the antibodies by visual examination under a microscope. Not only the tumoral cells but also the stroma (fibroblasts, inflammatory cells, and blood vessels) were evaluated, taking into account the fact that these tumors are highly cellular with low stromal component, mostly consisting of blood vessels. Since each TMA included two different tumor cylinders from each case, immunohistochemical scoring was done after examining both samples.

The IHC scoring used was as follows: for EGFR, VEGFR2, and PDGFRB, tumor samples with moderate/strong staining were considered positive (Maderna *et al.* 2007, Rodriguez-Antona *et al.* 2010); for KIT, both the intensity and extent of staining were evaluated (Miliaras *et al.* 2004), and the intensity of staining was graded as 0 (absent), 1 (weak), 2 (moderate), or 3 (strong), and the extent of staining was evaluated semiquantitatively and categorized as 0 (0% of cells), 1 (<10%), 2 (between 10 and 50%), 3 (between 50 and 80%), and 4 (more than 80%), and aggregate scores were obtained for each case (range 0–7) and cases with scores >3 were regarded as KIT-positive; MET protein was considered positive when its expression was positive for 30% of the tumor cells with moderate/strong staining (Lee *et al.* 2010); for VEGF, the intensity of staining was estimated on a four-tiered scale encoded as 0 (absent), 1 (weak), 2 (moderate), and 3 (strong), and immunopositivity was defined by strong staining (Duncan *et al.* 2008); for VEGFR1, a tumor was considered positive if cytoplasmic expression was detected; for VEGFR3, the percentage of cells with positive staining was evaluated for each case and the median was calculated, and tumor samples with a percentage of positive cells higher than the median (50% of the positive tumor cells) were considered to have a high expression of VEGFR3. The staining intensity for each protein marker analyzed and the number of tumor samples included in each category are given in Supplementary Table 2, see section on supplementary data at the end of this article.

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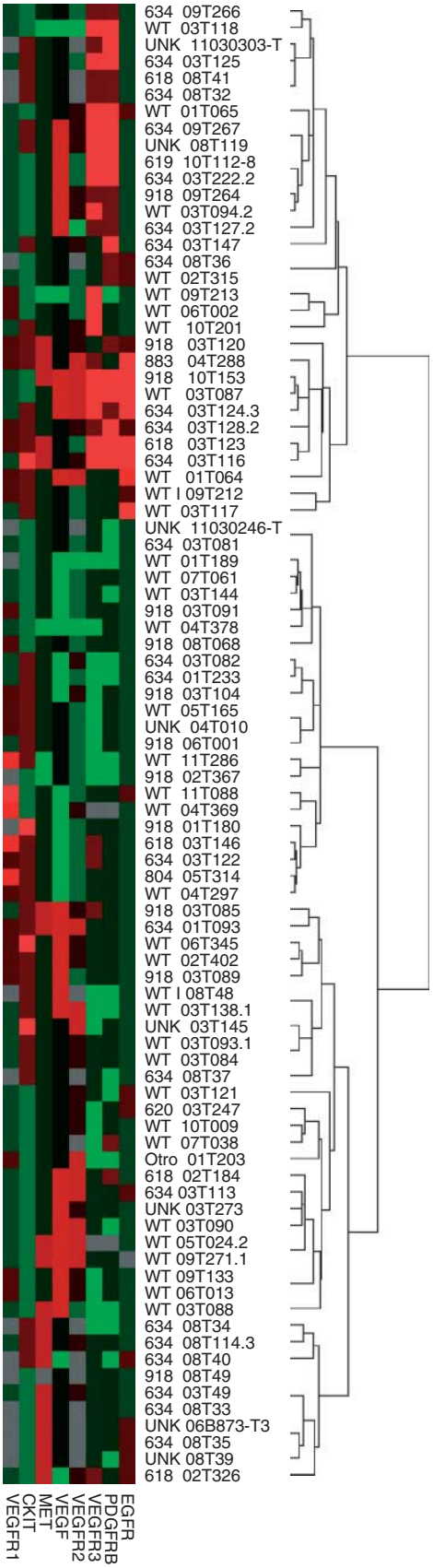

Clustering

Hierarchical unsupervised cluster analysis of the 92 primary tumor cases was carried out by the average linkage clustering method using GeneCluster 3.0 (mean centered; de Hoon *et al.* 2004) and viewed in a visualizer (TreeView) that displays cluster profiles and relevant cluster member information. Immunohistochemical results were represented by a range of colors from green to red, with the brightest green representing the lowest staining intensity for each marker and the brightest red the highest one (Fig. 1). Three samples in which more than three antibodies failed to yield results were excluded from the analysis.

Statistical analyses

All statistical analyses were carried out using SPSS version 17.0 statistical software. The χ^2 test or Fisher's exact test was used to compare variables representing patient characteristics (male/female) and tumor types (primary tumors/metastases and sporadic/familial) with IHC protein expression (Tables 2 and 3). In addition, logistic regression analyses were carried out to obtain odds ratios (ORs) and 95% CI, which are given in Supplementary Table 4, see section on supplementary data given at the end of this article. For *RET* mutation analysis, we considered independently each of the major *RET*-mutated groups: C634, M918T, and no *RET* mutation. The C634 *RET*-mutated group included both familial and sporadic forms with germline and somatic mutations respectively; the M918T *RET*-mutated group corresponded exclusively to sporadic forms with M918T somatic mutation; and the 'no mutation in *RET*' group corresponded exclusively to sporadic forms in which germline mutations were discarded and no somatic *RET* mutation was detected (see Table 1). Other less frequent *RET* mutations were not considered for further analysis as they represented a heterogeneous group. To calculate the correlation between the expression of the different proteins evaluated, we used the Spearman's test. Since this is an exploratory study, no correction of *P* values due to multiple testing was performed, and bilateral *P* values <0.05 were considered significant.

Figure 1
Hierarchical clustering of 89 primary MTC samples. The staining intensity for each immunohistochemical marker is represented as a range of colors between the brightest green (lowest expression) and the brightest red (highest expression). Gray squares indicate that no data was available.

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Table 2 Protein expression of EGFR, KIT, MET, PDGFRB, VEGF, VEGFR1, VEGFR2, and VEGFR3 according to the type of MTC

Protein	Primary *n* (%)	Metastatic *n* (%)	*P*	Sporadic[a] *n* (%)	Familial[a] *n* (%)	*P*
EGFR−[b]	83 (91)	5 (71)	NS	58 (92)	24 (89)	NS
EGFR+[c]	8 (9)	2 (29)		5 (8)	3 (11)	
KIT−	65 (75)	8 (100)	NS	50 (83)	14 (54)	0.0040
KIT+	22 (25)	0 (0)		10 (17)	12 (46)	
MET−	63 (83)	4 (50)	0.049	48 (84)	14 (78)	NS
MET+	13 (17)	4 (50)		9 (16)	4 (22)	
PDGFRB−	60 (70)	3 (38)	NS	46 (78)	13 (50)	0.010
PDGFRB+	26 (30)	5 (63)		13 (22)	13 (50)	
VEGF−	62 (69)	4 (50)	NS	41 (65)	20 (77)	NS
VEGF+	28 (31)	4 (50)		22 (35)	6 (23)	
VEGFR1−	45 (62)	8 (100)	0.046	31 (58)	14 (78)	NS
VEGFR1+	27 (38)	0 (0)		22 (41)	4 (22)	
VEGFR2−	32 (43)	2 (29)	NS	23 (42)	8 (44)	NS
VEGFR2+	42 (57)	5 (71)		32 (58)	10 (56)	
VEGFR3−	50 (57)	4 (57)	NS	39 (65)	10 (38)	0.022
VEGFR3+	37 (43)	3 (43)		21 (35)	16 (61)	

[a]Only primary tumor cases included in the analysis.
[b]A negative expression is indicated by '−'.
[c]A positive expression is indicated by '+'.

Results

Immunohistochemical study in MTC cases

The different TKI targets tested in the 103 MTC samples available for the study presented a variable expression (Supplementary Figure 1, see section on supplementary data given at the end of this article). Many MTCs showed positive staining with 57, 43, 34, 33, 32, 23, 20 and 10% positive cases for VEGFR2, VEGFR3, VEGFR1, PDGFRB, VEGF, KIT, MET and EGFR, respectively. The IHC intensity observed for each marker is summarized in detail in Supplementary Table 2.

The expression of several TKI targets was correlated. The strongest correlations corresponded to PDGFRB, which exhibited a positive correlation with VEGFR3 and a negative correlation with VEGFR1 (correlation coefficients of 0.51 and −0.34, $P=9\times10^{-8}$ and 0.002 respectively). A positive correlation was also detected between VEGF and VEGFR2 (correlation coefficient of 0.37, $P=0.0006$). The expression of EGFR correlated with that of MET and VEGFR3, and the expression of MET correlated with that of VEGFR2 and VEGF (see Supplementary Table 3, see section on supplementary data given at the end of this article). Similar results were observed when only primary tumor samples were analyzed.

Unsupervised clustering analysis of the primary MTCs based on the eight immunohistochemical markers evaluated clustered tumor samples into two main groups (Fig. 1). The left branch, which grouped 35% of the MTC cases, was characterized by positive PDGFRB and VEGFR3 staining and included most cases with EGFR overexpression. The right branch of the cluster included some of the VEGFR2-, VEGF-, MET-, KIT-, and VEGFR1-positive cases and cases displaying a negative expression for all the tested proteins. The left branch tended to be enriched in cases carrying the C634 *RET* mutation, either germline or somatic (35 vs 21%, in the left and right branches respectively), while the right branch was apparently enriched in tumors with the M918T *RET* somatic mutation (14 vs 6%).

Protein expression of TKI targets in different MTC types

Gender was associated with the expression of VEGFR1 (45% of female MTC cases exhibited positive staining vs 16% of the male cases, $P=0.007$) and VEGF (23 and 46% positive cases for females and males respectively, $P=0.017$), but age at diagnosis was not associated with IHC staining. We next determined whether the expression of the markers could be influenced by the type of tumor: primary tumors vs metastases and sporadic vs familial. MET displayed a higher expression in the metastatic cases than in the primary tumor cases ($P=0.049$; Table 2), similar to EGFR, PDGFRB, and VEGF, although differences for the latter proteins did not reach statistical significance (see Table 2). On the other hand, VEGFR1 displayed a higher expression in the primary tumor samples ($P=0.046$), similar to KIT, although this protein did not show any statistically significant difference. Some of the

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Table 3 Protein expression of EGFR, KIT, MET, PDGFRB, VEGF, VEGFR1, VEGFR2, and VEGFR3 according to the *RET* mutation in primary MTCs

Protein	C634 mut[a] *n* (%)	Rest[b] *n* (%)	*P*	M918T mut[c] *n* (%)	Rest[b] *n* (%)	*P*	Wt *RET*[d] *n* (%)	Rest[b] *n* (%)	*P*
EGFR−[e]	22 (92)	56 (90)	NS	14 (93)	64 (90)	NS	33 (92)	45 (90)	NS
EGFR+[f]	2 (8)	6 (10)		1 (7)	7 (10)		3 (8)	5 (10)	
KIT−	13 (54)	48 (83)	0.0069	12 (92)	49 (71)	NS	29 (83)	32 (68)	NS
KIT+	11 (46)	10 (17)		1 (8)	20 (29)		6 (17)	15 (32)	
MET−	13 (77)	46 (84)	NS	9 (75)	50 (83)	NS	31 (91)	28 (74)	0.054
MET+	4 (22)	9 (16)		3 (25)	10 (17)		3 (9)	10 (26)	
PDGFRB−	13 (54)	43 (75)	0.058	10 (71)	46 (69)	NS	27 (82)	29 (60)	0.040
PDGFRB+	11 (46)	14 (25)		4 (29)	21 (31)		6 (18)	19 (40)	
VEGF−	18 (75)	41 (67)	NS	10 (70)	49 (67)	NS	23 (64)	36 (73)	NS
VEGF+	6 (25)	20 (33)		5 (30)	21 (33)		13 (36)	13 (27)	
VEGFR1−	14 (82)	28 (55)	0.044	4 (44)	38 (64)	NS	20 (61)	22 (63)	NS
VEGFR1+	3 (18)	23 (45)		5 (56)	21 (36)		13 (39)	13 (37)	
VEGFR2−	7 (41)	25 (47)	NS	6 (54)	26 (44)	NS	15 (45)	17 (46)	NS
VEGFR2+	10 (59)	28 (53)		5 (44)	33 (56)		18 (55)	20 (54)	
VEGFR3−	8 (33)	39 (67)	0.0047	9 (64)	38 (56)	NS	24 (71)	23 (48)	0.041
VEGFR3+	16 (67)	19 (33)		5 (36)	30 (44)		10 (29)	25 (52)	

[a]Tumors with an activating mutation in *RET* residue C634, either germline or somatic.
[b]The rest of the tumors that do not have the analyzed genetic characteristic.
[c]Tumors with the activating somatic *RET* mutation M918T.
[d]Sporadic tumors with no mutations in *RET* exons 10, 11, 13, 14, 15, and 16.
[e]A negative expression is indicated by '−'.
[f]A positive expression is indicated by '+'.

markers exhibited large differences in expression in relation to the inherited character of the disease. We found that positive KIT, PDGFRB, and VEGFR3 staining was more frequently associated with familial forms than with sporadic forms (*P* values of 0.0040, 0.010, and 0.022 respectively; Table 2; see also Supplementary Table 4). On the other hand, EGFR, MET, VEGF, VEGFR1, and VEGFR2 exhibited similar positive staining in familial and sporadic forms. Because familial cases are associated with specific mutations, these data suggest that different *RET* mutations can predict differences in the expression of some of these proteins.

Expression of TKI targets according to *RET* mutations

Because the metastatic samples exhibited altered expression of some of the proteins studied (see Table 2), we examined only primary tumor cases to determine the effect of specific *RET* mutations on protein levels. Cases with the C634 *RET* mutation included both germline and sporadic tumors, while cases with the M918T *RET* mutation and cases with no mutation in *RET* were all sporadic forms. We found that 67% of the MTC cases with the C634 *RET* mutation expressed VEGFR3, compared with 36 and 29% respectively of the MTC cases with the M918T *RET* mutation and the MTC cases without the *RET* mutation ($P=0.0047$; see Table 3; OR=4.1, 95% CI=1.5–11; Supplementary Table 4). The expression of KIT was also more frequently found in the C634 *RET* mutation cases than in the M918T *RET* mutation cases and non-mutated *RET* cases ($P=0.0069$; Table 3; OR=4.1, 95% CI=1.4–11; Supplementary Table 4). By contrast, VEGFR1 exhibited a lower expression in the C634 *RET* mutation cases than in the M918T or non-mutated *RET* cases (18, 56, and 39% respectively, $P=0.044$; Table 3). The expression of PDGFRB was higher in the C634 and M918T *RET* mutation cases than in the non-mutated *RET* cases ($P=0.040$; Table 3). Other proteins such as EGFR, VEGF, and VEGFR2 did not display differences in expression among the different types of *RET*-mutated tumors, while MET had a tendency toward a lower expression in MTC cases without *RET* mutations (Table 3).

The expression of KIT, PDGFRB, VEGFR1, and VEGFR3 was also examined in stroma: blood vessels, inflammatory cells, and fibroblasts (data not shown). KIT was only present in the inflammatory population in scattered cells; PDGFRB was expressed in blood vessels and fibroblasts, with an expression level ranging from moderate to high; and VEGFR1 and VEGFR3 were expressed in most blood vessels and occasionally in inflammatory cells. In addition, the expression of KIT was significantly lower in MTC cases with the C634 mutation ($P=0.0018$); no statistically significant differences for the expression of PDGFRB, VEGFR1, and VEGFR3 were found.

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Discussion

The lack of effective therapies for MTC may be changed drastically by the use of already available TKIs and the development of novel targeted drugs. Some of the TKIs have demonstrated remarkable clinical responses in MTC patients, and several new trials with these molecules are currently being conducted (Cohen *et al*. 2008, Schlumberger *et al*. 2009, Carr *et al*. 2010, Lam *et al*. 2010, Robinson *et al*. 2010, Wells *et al*. 2010, 2012, Ahmed *et al*. 2011, Hong *et al*. 2011, Kurzrock *et al*. 2011). However, there is a large inter-patient variability in TKI responses. The molecular basis for this variability is unknown, and the identification of biomarkers that could identify MTC patients who will probably benefit from these different drugs will improve clinical trial outcomes and ultimately progression-free survival and overall survival of patients with a disease that not long ago was untreatable. In this study, we used a large retrospective series of well-characterized MTC patients to define the expression of TKI target proteins in relation to specific clinical and tumor characteristics to provide data that could be used for rational selection of patients for TKI treatment.

So far, vandetanib and cabozantinib, which have markedly improved the progression-free survival of MTC patients (Schoffski *et al*. 2013, Thornton *et al*. 2012), are the only agents that have been approved for the treatment of this disease, but a wide variety of additional multi-targeted kinase inhibitors have entered clinical trials and several have shown clinical benefit in MTC patients. Promising results have been obtained with agents that primarily target angiogenesis and inhibit VEGF receptors at nanomolar concentrations. Because of the structural similarity between kinases, many of these molecules also have an effect on RET and other kinases, such as PDGFR, MET, and KIT, which could be all-important for the clinical responses observed. However, axitinib does not have an anti-RET activity and has displayed objective responses in MTC patients, suggesting that VEGFR might be as important as RET for targeted therapy (Cohen *et al*. 2008). Comparison of outcomes among the various phase II trials carried out so far is limited by variations in patient eligibility and response assessment. In addition, in most cases, genetic characteristics of the different MTC patients included (e.g. sporadic or familial and type of *RET* mutation) have not been reported.

In this study, we examined the expression of eight key proteins for TKI action (EGFR, KIT, MET, PDGFRB, VEGF, VEGFR1, VEGFR2, and VEGFR3) in 103 paraffin-embedded MTC samples characterized for *RET* mutations. We observed that these proteins were highly expressed in a subset of MTCs in a coordinated manner (Fig. 1, Supplementary Table 3). A previous publication that examined VEGF, VEGFR1, and VEGFR2 in 38 MTC samples has reported 95, 96, and 91% positive expression for these proteins respectively (Capp *et al*. 2010). In the present study, we adopted more strict criteria to define immunopositivity and, thus, the proportions of MTC samples exhibiting overexpression of these proteins were lower (32, 34, and 57% respectively). Applying criteria the same as those used by Capp *et al*., we obtained similar results for VEGF and VEGFR2, but observed a lower expression of VEGFR1 (see Supplementary Table 2). This difference could be due to the different VEGFR1 antibody used. The expression of some proteins was significantly different between primary tumor and metastatic samples (Table 2), but the number of metastatic samples included in this study was small, and we cannot rule out the possibility that additional changes would be detected in a larger series. In fact, a previous study by our group, including more metastatic cases and paired primary tumor/metastatic samples, had found a significant overexpression of VEGFR2 in the metastatic samples (Rodriguez-Antona *et al*. 2010). In the present study, we also found a higher expression of VEGFR2 in the metastatic samples than in the primary tumor samples (71 vs 55%), but it did not reach statistical significance. Additional histopathological information and clinical outcome data were not available to conduct additional analyses. Differences in the expression of the proteins were evident when comparing sporadic and familial MTC cases (Table 2), with a higher expression of KIT, PDGFRB, and VEGFR3 being observed in the familial cases. Most hereditary MTCs are caused by *RET* mutations affecting residue C634 and most sporadic cases have the M918T *RET* mutation (see Table 1), suggesting that the expression of TKI targets and *RET* mutations may be associated. In agreement with this, we found that MTC cases exhibiting VEGFR3 and KIT overexpression were mainly C634 *RET*-mutated cases; VEGFR1-positive cases were mainly M918T *RET*-mutated cases and cases without *RET* mutations; and MTC cases exhibiting PDGFRB overexpression were mainly tumors without *RET* mutations. VEGFR2, which is a target of several TKIs, exhibited similar staining among the different *RET*-mutated tumor cases, with the number of positive cases exceeding 40% in all cases (Table 3). When we examined the expression of KIT, PDGFRB, VEGFR1, and VEGFR3 in the tumor stroma, only KIT displayed a statistically significant difference in the C634 *RET*-mutated tumor cases, suggesting that for the

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expression of the receptors, *RET* mutation is only relevant in the tumor cells. In general, and although a validation with an independent series is required to confirm these data, these results indicate that different TKI treatments could be more effective according to the specific *RET* mutation present in the MTC. It could be suggested that C634 *RET*-mutated cases (mainly hereditary), with a higher expression of VEGFR3, PDGFRB, and KIT, might benefit from drugs with a high affinity for these targets, while for the M918T *RET*-mutated cases (mostly sporadic), TKIs targeting RET and VEGFR2 might be more appropriate. In fact, a phase III clinical trial comparing vandetanib vs placebo has found that the response rate to this drug is greater in patients with sporadic tumors who had a M918T *RET* mutation (Wells *et al*. 2012). However, it is also important to note that intratumoral heterogeneity and changes in tumor molecular profile through the acquisition of new somatic mutations suggest that combinations of more than one TKI may be more effective than single-agent treatments and that changing to different TKIs over time might also be needed.

In conclusion, this study shows for the first time to our knowledge that a substantial number of MTCs exhibit high expression levels of kinases targeted by TKIs for which promising results have been obtained in recent clinical trials. Furthermore, the expression of these targets is associated with clinical and molecular characteristics of the MTCs, supporting the notion that these data could be used for the identification of patients most likely to benefit from specific TKIs, thus helping to design rational clinical trials and perform a molecular selection of treatments to ultimately improve the clinical response of MTC patients.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-12-0316.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

C Rodríguez-Antona was responsible for study design, data analysis, data interpretation, manuscript writing, and final approval of the manuscript. I Muñoz-Repeto, L Inglada-Pérez, A A de Cubas, and M Cañamero were involved in data analysis, data interpretation, and final approval of the manuscript. V Mancikova and A Cascón were responsible for data collection, data interpretation, and final approval of the manuscript. A Maliszewska, L Sanchez, A Gómez, R León, C Álvarez-Escolá, and J Aller were responsible for data collection and final approval of the manuscript. L J Leandro-García and I Comino-Méndez were responsible for data interpretation and final approval of the manuscript. M Robledo was responsible for study design, data interpretation, manuscript writing, and final approval of the manuscript.

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ARTICLE 6

Article 6: VEGF, VEGFR3 and PDGFRB protein expression is influenced by *RAS* mutations in Medullary Thyroid Carcinoma.

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Abstract

The work from article 5 underscored the effect that distinct *RET* mutations exert on the expression of key TKI targets. While this project was being carried out, the involvement of *RAS* somatic alterations emerged as an important driver of sporadic MTCs (Moura *et al.*, 2011; Boichard *et al.*, 2012; Ciampi *et al.*, 2013). This prompted us to complete the mutational screening of the stained MTCs by additionally testing for *RAS* mutations and carrying out the corresponding statistical analyses. We found out that *RAS*-mutated MTCs in general express lower levels of several targets when compared to *RET*-related tumors, and in particular none of them expressed neither PDGFRB nor MET. This could be an important finding given that *RAS* alterations have been related to TKI treatment resistance in other cancer types and should be taken into consideration when choosing the most adequate treatment for MTC patients.

Personal contribution: I performed the genetic screening of the samples. I also participated in discussion of the results and drafting of the paper.

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VEGF, VEGFR3, and PDGFRB Protein Expression Is Influenced by *RAS* Mutations in Medullary Thyroid Carcinoma

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Background: Tyrosine kinase inhibitors (TKIs) have achieved remarkable clinical results in medullary thyroid carcinoma (MTC) patients. However, the considerable variability in patient response to treatment with TKIs remains largely unexplained. There is evidence that it could be due, at least in part, to alterations in genes associated with the disease via their effect on the expression of TKI targets. The objective of this study was to evaluate the influence of *RAS* mutations on the expression levels in MTC tumors of eight key TKI target proteins.

Methods: We assessed by immunohistochemistry the expression of EGFR, KIT, MET, PDGFRB, VEGF, VEGFR1, VEGFR2, and VEGFR3 in a series of 84 primary MTC tumors that had previously been molecularly characterized, including 14 *RAS*-positive, 18 RET^{M918T}-positive, and 24 RET^{C634}-positive tumors, as well as 15 wild-type tumors with no mutations in the *RET* or *RAS* genes.

Results: In contrast to *RET*-positive tumors, *RAS*-positive tumors expressed neither PDGFRB nor MET ($p = 0.0060$ and 0.047, respectively). Similarly, fewer *RAS*-positive than *RET*-related tumors expressed VEGFR3 ($p = 0.00062$). Finally, wild-type tumors expressed VEGF more often than both *RAS*- and *RET*-positive tumors ($p = 0.0082$ and 0.011, respectively).

Conclusions: This is the first study identifying that the expression of TKI targets differs according to the presence of *RAS* mutations in MTC. This information could potentially be used to select the most beneficial TKI treatment for these patients.

Introduction

Upon neoplastic transformation, parafollicular cells of the thyroid gland give rise to medullary thyroid carcinoma (MTC), a rare malignancy that accounts for approximately 2–5% of all thyroid neoplasias (1). Around 75% of MTCs are sporadic, while the remaining cases arise as a manifestation of the hereditary multiple endocrine neoplasia type 2 syndrome (MEN2). Interestingly, in both scenarios, different activating point mutations in the ''rearranged during transfection'' (*RET*) proto-oncogene have been shown to lead to carcinogenesis (2,3). It has recently been described that a substantial proportion of non-*RET*-mutated sporadic MTCs are caused by mutations in *RAS* genes (4,5). Both types of molecular alterations have been shown to have an impact on the evolution of MTC: while *RAS* gene mutations have been associated with better outcomes, *RET* mutations give rise to a more aggressive phenotype with a worse prognosis (4).

The clinical outcome of MTC depends greatly on when in the disease process the patient is diagnosed. The 10-year survival rate of patients with MTC diagnosed at an advanced stage is less than 20% (6), mainly because treatment with cytotoxic drugs and/or standard radiotherapy has been proven to be ineffective (7). In this regard, because *RET* is a tyrosine

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kinase receptor, MTC patients with metastatic or locally advanced disease have more recently been treated with small-molecule tyrosine kinase inhibitors (TKIs), with promising results (8–13). Remarkably, it has already been shown that the expression of the key target proteins of these drugs varies in MTC according to the specific *RET* mutation present (14), a finding that could undoubtedly have an important impact on clinical practice. However, little is known about how *RAS*-positive MTC patients respond to TKI drugs.

On the basis of the evidence that expression of TKI target proteins is associated with the presence of particular genetic mutations and, more importantly, since *RAS* mutations have also been associated with resistance to TKI therapy for other tumor types (15), we assessed the relationship between the expression of key TKI targets in a series of 84 molecularly characterized primary MTC tumors. We observed differences in the frequency of expression of VEGFR3, MET, and PDGFRB between *RAS*- and *RET*-mutated tumors. Wild-type (WT) tumors, those with no mutations in these two genes, more frequently expressed VEGF. These results could be of clinical importance when enrolling these patients in clinical trials of TKI treatments.

Materials and Methods

Human MTC samples

Eighty-four formalin-fixed paraffin-embedded MTC primary tumor samples were collected at the Spanish National Cancer Research Center (CNIO) in collaboration with the CNIO Tumor Bank. Written informed consent was obtained from all study participants, and the study was approved by the institutional review board (Comité de Bioética y Bienestar Animal) of the Instituto de Salud Carlos III. The tumor samples corresponded in most cases to patients diagnosed as sporadic or familial, based on the analysis of the *RET* proto-oncogene in peripheral blood samples. The mutational status of exons 10, 11, 13, 14, 15, and 16 of *RET* was assessed in genomic germline DNA using standard PCR conditions, primers, and automated sequencing, as previously described (16). When no *RET* mutation was found in peripheral blood, the same *RET* screening was performed in the corresponding tumor. Twenty-nine *RET*-negative samples were subsequently screened for somatic alterations in *H*-, *N*-, and *K-RAS* mutation hotspots: codons 12 and 13 in exon 2, and codon 61 in exon 3, as previously described (5) (see Table 1 for details).

Of the 84 formalin-fixed paraffin-embedded samples, 79 were distributed across 3 tissue microarrays as previously described (14), and 5 were evaluated as complete sections. The tissue microarrays were constructed with 2 cores of 1 mm from each tumor. Sections of each tissue microarray and individual tumors were immunostained using antibodies specific for the EGFR, KIT, MET, PDGFRB, VEGF, VEGFR1, VEGFR2, and VEGFR3 proteins. The immunohistochemistry (IHC) protocols used, immunostaining, and the scoring applied are detailed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/thy) and have been previously described (14). Briefly, for EGFR, VEGFR2, and PDGFRB, tumors with moderate/strong staining were considered positive (17,18); for KIT, both the intensity and the extent of the staining were evaluated, and cases with aggregate scores >3 were regarded as positive (19). The MET protein was considered positive when the expression was positive for 30% of tumor cells with moderate/strong staining (20); for VEGF, the intensity of the staining was estimated on the 4-tiered scale (0, absent; 1, weak; 2, moderate; 3, strong), and immunopositivity was defined by strong staining (21); for VEGFR1, a tumor was considered positive if cytoplasmic expression was detected; for VEGFR3, tumors with a percentage of cells with positive staining greater than the observed median (50%) were considered positive.

Statistical analysis

All statistical analyses were performed using SPSS version 17.0. The χ^2-test or Fisher's exact test was used to assess associations between mutation status and IHC expression of each protein. *RET*-mutated tumors were classified into three groups: *RET*-mutated group as a whole (including all *RET*-related tumors, regardless the mutation), RET^{C634} tumors, and RET^{M918T} tumors. The RET^{C634}-mutated group included tumors from familial and sporadic cases with germline and somatic mutations, respectively; the RET^{M918T}-mutated group comprised exclusively tumors from sporadic cases. Tumors were classified as *RAS*-mutated regardless of the particular *RAS* gene involved, although we also carried out an analysis stratified by gene. The

Table 1. Molecular Characteristics of the 84 Medullary Thyroid Carcinoma Primary Tumors Included in the Study

Tumor group	*Total = 84,* N *(%)*	*Specific mutation*	*Other features*
RAS gene mutation	14 (17%)	*H-RAS* exon 2 ($n=3$; 4%)	Sporadic ($n=3$)
		H-RAS exon 3 ($n=7$; 8%)	Sporadic ($n=7$)
		K-RAS exon 2 ($n=2$; 2%)	Sporadic ($n=2$)
		K-RAS exon 3 ($n=2$; 2%)	Sporadic ($n=2$)
RET gene mutation	55 (65%)	*RET* M918T ($n=18$; 22%)	Sporadic ($n=18$)
		RET C634 ($n=24$; 29%)	Sporadic ($n=4$)
			Familial ($n=20$)
		RET 618 ($n=6$; 7%)	Sporadic ($n=1$)
			Familial ($n=5$)
		RET 611, 619, 620, 804, 883, or 891 ($n=6$; 7%)	Sporadic ($n=4$)
			Familial ($n=2$)
		Other ($n=1$; 1%)	Sporadic ($n=1$)
WT	15 (18%)		

H-RAS accession number: ENSG0000017477; *K-RAS* accession number: ENSG00000133703.

WT group consisted exclusively of tumors from sporadic cases in which both germline and somatic *RET* mutations and *RAS* somatic mutations were not found. Two-sided *p*-values <0.05 were considered statistically significant.

Results

The results of mutational screening are summarized in Table 1. In our series, 55 (65%) tumors harbored a mutation in *RET* proto-oncogene (27 familial and 28 sporadic), with codons 634 and 918 being the most frequently affected (24 and 18 cases, respectively). Other, less frequent *RET* mutations were present in 13 tumors. A *RAS* gene mutation was found in 14 of the 29 *RET*-negative primary tumors (48%), which represented 17% of the entire collection of primary tumors. The majority of the mutations were located in *H-RAS*, while four were in *K-RAS*.

There were clear differences in the immunohistochemical expression of the TKI receptors (Fig. 1). Table 2 summarizes significant results of the analysis of IHC status with the underlying mutated gene. Briefly, we observed differences in the frequency of expression of four key TKI target genes (*PDGFRB*, *VEGFR3*, *MET*, and *VEGFR1*) between *RAS* gene-mutated samples and *RET*-mutated tumors, and one, VEGF, when comparing *RAS* with WT.

Notably, all *RAS*-related samples lacked PDGFRB expression, while *RET*-related MTC frequently stained positive (0% vs. 40%, $p=0.0060$). In order to assess whether this association was because of one *RET* mutation in particular, the analysis was repeated considering only the two more prevalent *RET* mutations. PDGFRB expression was more strongly associated with RET^{C634}-mutated (46%) than RET^{M918T}-mutated cases (35%, $p=0.0032$ and 0.024, respectively). *RAS*-related tumors less often expressed VEGFR3 than *RET*-related tumors ($p=0.00062$), seemingly more associated with harboring a RET^{C634} mutation ($p=3.7\times10^{-4}$) than the RET^{M918T} change ($p=0.0067$). Although VEGFR1 expression in *RAS*-mutated tumors was not significantly different from that in *RET*-mutated tumors as a whole, it was compared with the RET^{C634}-mutated group (60% vs. 18%, $p=0.039$). Finally, *RAS*-related tumors less often expressed MET than *RET* tumors, mainly because of RET^{M918T} samples (0% vs. 36%, $p=0.042$).

In order to identify a characteristic staining profile for WT cases, we compared this group to *RET* and *RAS* samples separately. In both comparisons, the only significant difference observed was in the frequency of VEGF expression, which was more common in the WT group ($p=0.011$ and 0.0082, respectively).

Discussion

The appropriate clinical management of familial MTC is already well established, including prophylactic thyroidectomy at an early age, determined according to the particular

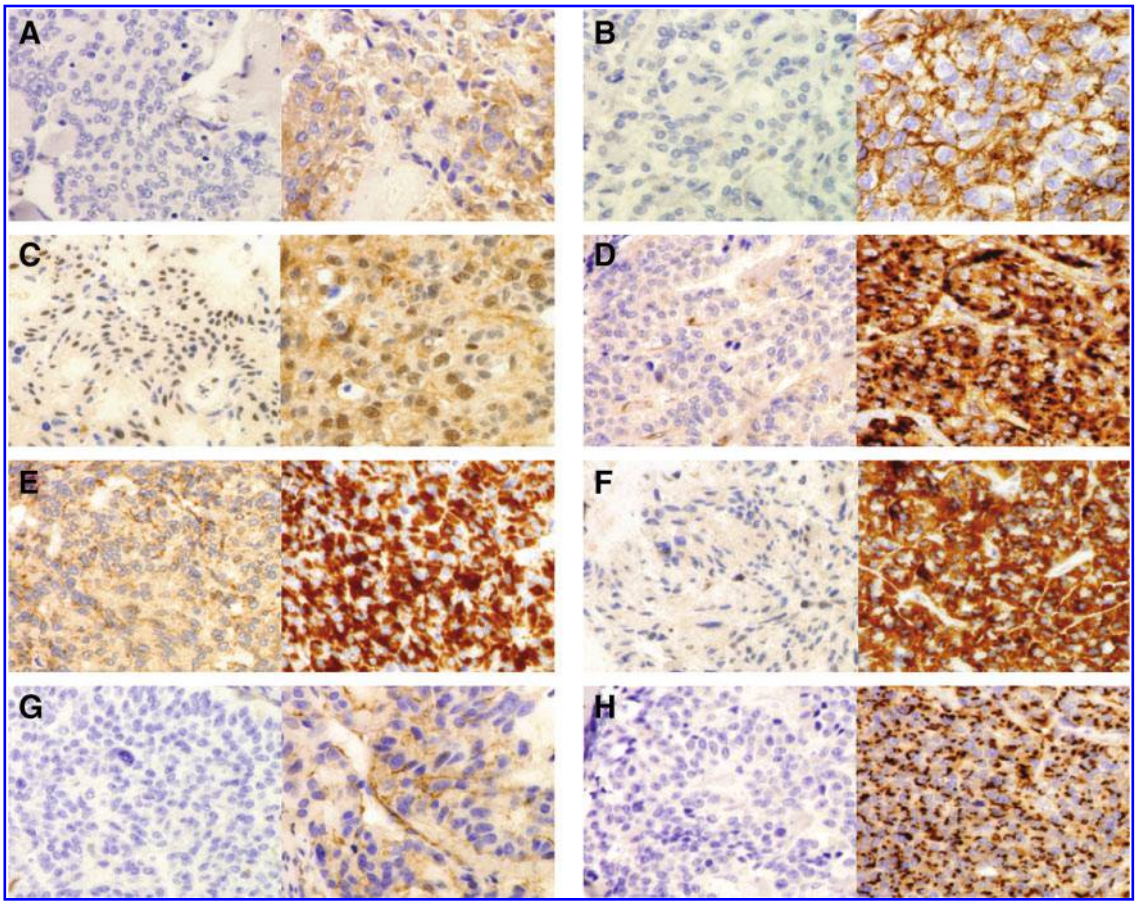

FIG. 1. Examples of immunohistochemical staining of the selected proteins. Representative cases are shown with low and high protein expression of KIT **(A)**, EGFR **(B)**, MET **(C)**, PDGFRB **(D)**, VEGF **(E)**, VEGFR2 **(F)**, VEGFR1 **(G)**, and VEGFR3 **(H)**.

Table 2. Proportion of Tumors with Proteins Differentially Expressed

	RAS *vs.* RET			*WT vs.* RET			*WT vs.* RAS		
Proteins[a]	RAS	RET	p	*WT*	RET	p	*WT*	RAS	p
VEGF	2/12 (17%)	15/52 (29%)	0.49	9/13 (69%)	15/52 (29%)	**0.011**	9/13 (69%)	2/12 (17%)	**0.0082**[b]
PDGFRB	0/13 (0%)	21/53 (40%)	**0.0060**[b]	2/12 (17%)	21/53 (40%)	0.19	2/12 (17%)	0/13 (0%)	0.22
VEGFR1	6/10 (60%)	13/37 (35%)	0.28	6/13 (46%)	13/37 (35%)	0.52	6/13 (46%)	6/10 (60%)	0.68
VEGFR2	4/10 (40%)	22/39 (56%)	0.48	10/13 (77%)	22/39 (56%)	0.19	10/13 (77%)	4/10 (40%)	0.10
VEGFR3	1/14 (7%)	31/53 (59%)	**0.00062**[b,c]	5/12 (42%)	31/53 (59%)	0.35	5/12 (42%)	1/14 (7%)	0.065
MET	0/12 (0%)	12/40 (30%)	**0.047**	4/14 (29%)	12/40 (30%)	0.99	4/14 (29%)	0/12 (0%)	0.10

	RAS *vs.* RET^{C634}			RAS *vs.* RET^{M918T}		
Proteins[a]	RAS	RET^{C634}	p	RAS	RET^{M918T}	p
VEGF	2/12 (17%)	6/24 (25%)	0.69	2/12 (17%)	6/16 (38%)	0.40
PDGFRB	0/13 (0%)	11/24 (46%)	**0.0032**[b]	0/13 (0%)	6/17 (35%)	**0.024**[b]
VEGFR1	6/10 (60%)	3/17 (18%)	**0.039**[c]	6/10 (60%)	5/10 (50%)	0.99
VEGFR2	4/10 (40%)	10/17 (59%)	0.44	4/10 (40%)	6/12 (50%)	0.69
VEGFR3	1/14 (7%)	16/24 (67%)	**0.00037**[b,c]	1/14 (7%)	10/17 (59%)	**0.0067**[b,c]
MET	0/12 (0%)	4/17 (24%)	0.12	0/12 (0%)	5/14 (36%)	**0.042**

[a]Only proteins with a *p*-value ≤ 0.10 in at least one of the comparisons are shown.
[b]Remained statistically significant when considering only tumors with mutations in *H-RAS*. *H-RAS* accession number: ENSG0000017477.
[c]Remained statistically significant when considering only tumors with mutations in *K-RAS*. *K-RAS* accession number: ENSG00000133703.
p-Values < 0.05 highlighted in boldface.

germline mutation detected (22). It is the management of sporadic and *de novo* patients with germline *RET* mutations that presents a clinical challenge. These patients are often diagnosed at advanced stage with local or distant metastases (23), for which the standard therapeutic options are not effective (7).

Since the transforming event in 30–50% of MTC sporadic cases is the activation of *RET* by point mutations, one promising approach to extend the progression-free survival of patients with advanced disease is targeted therapy to inhibit tyrosine kinase receptors. However, the molecular basis underlying the great variability in the response of MTC patients to TKI treatment remains unknown (8). There is emerging evidence that expression profiles in MTC are driven by the underlying genetics (24) and, in consequence, that the expression of TKI target proteins could be dictated by particular mutations (14). In our series, the proportion of non-*RET* but *RAS*-positive MTCs was 48% (17% of the entire collection), with *H-* and then *K-RAS* being the most often mutated genes, and *N-RAS* mutations being totally absent. The reported prevalence of *RAS* gene mutations in non-*RET* MTC varies considerably, ranging from 17.6% to 81.0% (4,5,25), explaining nevertheless a substantial proportion of sporadic patients. Therefore, there is an urgent need to distinguish this subset of patients and to find out if they share a specific targetable expression pattern.

Currently, two main TKIs are available in the treatment of advanced MTC. Vandetanib (ZD6474), which targets VEGFR2, VEGFR3, RET, and EGFR, was the first TKI approved for the treatment of adults with symptomatic or progressive MTC (9). Recently, this drug has been used for treatment of pediatric patients with MTC, who harbor almost exclusively the RET^{M918T} mutation, with encouraging results (10). In addition, Cabozantinib (XL184), which inhibits VEGFR2, MET, RET, KIT, VEGFR1/3, FLT3, Tie2, and AXL (11), was approved by the FDA for metastatic MTC in 2012.

Recent results from phase III clinical trials showed shorter progression-free survival in RET^{M918T}-negative patients treated with Vandetanib when compared with M918T-mutated patients (26). Even though *RAS* gene mutation status was not assessed in this study, according to the known *RAS* gene mutations' prevalence, it seems reasonable to assume that a proportion of the RET^{M918T}-negative patients with worse response to Vandetanib carried *RAS* alterations, which according to our results express less frequently some of its targets. Ciampi *et al.* (4) reported a higher but not significant prevalence of disease-free patients among the patients with *RAS*-mutated MTC. Thus, it could be expected that less *RAS*-positive patients were included in TKI trials, which usually require patients to have advanced metastatic disease. However, it should be noted that even *RET* mutations with lower transforming capacity eventually trigger advanced disease. In addition, our findings could also explain in part the tendency for longer progression-free survival observed for *RET*-mutated patients treated with Cabozantinib when compared with *RAS*-mutated patients (60 vs. 47 weeks) (13), as the latter group expresses much less frequently important targets of this drug. Additionally, differential expression of other Cabozantinib targets (e.g., Flt-3, Tie2, or AXL) could also contribute to differences in drug response.

There are other TKIs such as sorafenib, sunitinib, motesanib, and axitinib, currently being tested in clinical trials to treat aggressive MTC. Some of these drugs target PDGRFB (12), which according to our observations was not expressed by *RAS*-related tumors at all. On the other hand, it was particularly interesting that a major part of WT tumors expressed VEGF, suggesting that antiangiogenic therapy could be an option for these patients. In this regard, bevacizumab, a humanized monoclonal antibody that produces inhibition of angiogenesis by inhibiting vascular endothelial growth factor, has shown promising results in various cancers.

This is an exploratory study, which requires further confirmation in an independent series of samples. Moreover, we

did not have access to information about the treatment that patients received or their response to treatment. Thus, it was not possible to assess the impact of the differential expression of TKI targets on the treatment outcomes of these patients.

To conclude, this is the first report evaluating the expression of key TKI target proteins in *RAS*-related MTC tumors. *RAS*-related MTCs do not express MET and PDGFRB, and stain less frequently for VEGFR3. VEGF was notably more frequently expressed in WT MTCs. These findings could have an important impact on treatment decisions for MTC patients based on the likelihood of benefiting from a particular therapy, and therefore constitute a first step toward personalized medicine for these patients.

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DISCUSSION

In 2012 in the USA, thyroid cancer accounted for approximately 5% of all female malignancies (being the fifth most common female cancer), and 1.5% men cancer cases (Siegel *et al.*, 2012). Even if the increasing incidence is converting TC into an epidemic, much of the disease etiology remains poorly explored. The main objective of this thesis project was to provide a better understanding of various aspects of the disease by applying an exhaustive genetic and genomic characterization to an exceptional collection of human samples (blood & saliva in Article 1, tumor samples in Articles 2-6) from individuals affected by TC.

1. Follicular cell-derived thyroid cancer (Articles 1-3)

1.1 Missing heritability (Article 1)

The genetic contribution to thyroid cancer risk is greater than that of any other cancer, and the first-degree relatives of an individual affected by DTC have more than 8 times higher risk of developing the disease than general population (Goldgar *et al.*, 1994; Pal *et al.*, 2001; Hemminki *et al.*, 2004). Thus, it might come as surprising that the majority of genetic factors related to DTC remains undetermined. For now, involvement of mutations in high-penetrance genes seems little plausible as no such alterations have been convincingly replicated (Malchoff *et al.*, 2000; McKay *et al.*, 2001; Cavaco *et al.*, 2008; He *et al.*, 2009; Suh *et al.*, 2009). These observations imply that the follicular cell-derived thyroid cancer susceptibility is polygenic in nature, meaning that a relatively large number of low-penetrance genes (LPGs) could be involved. Variation in these LPGs is associated with a small increase in the disease risk, ranging from 1.1 to 1.6 (Fletcher and Houlston, 2010), creating a requirement for a rather large sample sets in order to detect these associations. In a disease such as TC, which prevalence keeps on being relatively low, this represents an important limiting factor obstructing the identification of genetic risk factors.

In the last years, both carefully designed candidate gene-based and genome-wide association studies have been applied to discover novel TC LPG. As a result, variants in many potential LPGs were identified (such as in *RET* (Lesueur *et al.*, 2002; Ho *et al.*, 2005), *ATM* (Akulevich *et al.*, 2009), *CHEK2* (Cybulski *et al.*, 2004), *RAD52* (Siraj *et al.*, 2008), *VEGFA* (Hsiao *et al.*, 2007), *CYP2D6* (Lemos *et al.*, 2007), *NAT2* (Hernandez *et al.*, 2008), *FOXE1* (Gudmundsson *et al.*, 2009; Landa *et al.*, 2010) and *NKX2*-1 (Gudmundsson *et al.*, 2009; Matsuse *et al.*, 2011; Gudmundsson *et al.*, 2012)). Some of these LPGs are involved in DNA repair pathways (such as *ATM*, *RAD52* or *CHEK2*), and could thus be linked to irradiation - a well-known risk factor of TC. Others are closely related to the function and development of thyroid gland (such as *FOXE1* and *NKX2-1*).

However, the mentioned LPGs are in their great majority described by a single study and lack subsequent validation. This could be potentially explained by limited detection power, a recurrent pitfall of many of these studies, and a consequence of a moderate sample size and the minor allele frequency of the assessed variants. Another possible limitation affecting many of the above-mentioned LPGs is the fact that it is not accounted for the disease heterogeneity in the study design and patients with tumors of different histological subtypes are grouped together in the final analyses. This could be affecting the results, as it is not known whether the same variants are involved in the etiology of different histological subtypes of TC. In fact, previous results from our laboratory strongly point towards involvement of distinct SNPs in PTC, FTC and PTCvf susceptibility. Finally, it is worthy to consider that some associations could be related to the disease in a population-specific manner. This could well be due to distinct genetic background of different populations and possibly gene-environment interactions, and could ultimately underlie the observed world-wide differences in the TC prevalence.

In this scenario, we performed a two-step association study in differentiated thyroid cancer involving 1,820 cases and 2,410 controls. At the time the study was designed, it was involving the largest collection of TC patients in the genome-wide discovery step, warranting novel insights into the genetic susceptibility of this disease. During the course of the thesis, a work involving more numerous sample collection emerged (Kohler *et al.*, 2013). Both ours and the work by Kohler and colleagues show that there are not too many TC LPGs that are universally associated with the disease in all populations. As we determined in the meta-analysis of all TC GWAS, for now SNPs at two genomic loci (9q22.33 and 14q13.3 close to *FOXE1* and *NKX2-1* genes, respectively) were invariably detected in all studies and could therefore be considered *bona fide* thyroid cancer SNPs. Moreover, variation at 9q22.33 serves as an exceptional example of a universally validated genetic factor that acts above known environmental factors, as depicted in the work of Takahashi and colleagues (Takahashi *et al.*, 2010).

Apart from re-confirming the associations at 9q22.33 and 14q13.3, we detected novel ones at 10q26.12 and 6q14.1. SNPs from these loci were clearly heterogeneously associated with the disease in the studied populations. Nevertheless, we were able to find a correlation between the genotype of rs10788123 from 10q26.12 and rs4075570 from 6q14.1 and the expression of *WDR11* and *HTR1B* genes, respectively, showing they may be expression quantitative trait loci (eQTL). *WDR11* plays a role in many processes including cell signaling, apoptosis and gene regulation, while *HTR1B* is involved in MAPK signaling and angiogenesis (Leone *et al.*, 2000; Zamani and Qu, 2012). Thus, even if the novel associations did not reach

genome-wide statistical significance, it is very tempting to suggest their involvement in TC susceptibility.

In order to work with homogeneous patients' sets, we conducted the analyses sub-stratifying the affected individuals according to their histopathological diagnosis. We were able to confirm the associations from 9q22.33, 14 q13.3, 10q26.12 and 6q14.1 among PTC patients, who represented the vast majority of all the cases. When considering exclusively FTC patients, we obtained significant results for 9q22.33 locus ($p<0.05$), and borderline significant ones for 14q13.3 and 10q26.12 loci ($p<0.1$). However tempting, we consider we did not have a sufficient number of FTC cases that would allow us to unequivocally conclude if the same SNPs are involved in the etiology of FTC or not.

Taking into consideration the Hazard Ratios (HR) corresponding to the variants that are being detected, it is clear that on its own, each low-penetrance locus has a relatively small effect on thyroid cancer risk and would not produce dramatic familial aggregation. However, in combination with other genetic loci and/or environmental factors, particularly given how common these can be, variants of this kind might significantly alter disease risk. Yet, studies that would consider cooperative influence of several SNPs on the TC risk are extremely rare (Landa *et al.*, 2013). Nevertheless, these studies of epistasia hold a great promise of providing novel insights into the genetic mechanisms underlying TC inheritance. Moreover, it is expectable that in order to identify novel low-penetrance variants with small associated HR, very large numbers of cases and controls (>10 000 of each) need to be used in order to achieve sufficient statistical power. Hopefully, this need will culminate in international collaboration in the future, which would add missing pieces to the TC heritability puzzle.

To sum up, the findings herein described suggest that heterogeneity in genetic susceptibility between populations is a key feature to take into account when exploring risk factors related to TC. This phenomenon may also explain at least part of the disparity observed between TC association studies. On the whole, this study depicts how genetic heterogeneity between populations influences TC susceptibility and uncovers it as a part of the hidden heritability of this disease.

1.2 In search of markers of malignancy and progression (Articles 2&3)

Follicular cell-derived thyroid cancer represents an attractive and intriguing model to study cancer initiation and progression, since it shows all relevant stages of cancer evolution as benign precursor lesions, indolent carcinomas and aggressive and highly invasive forms. Much of the processes occurring at the stage-switching points remain unknown, which is associated with important unresolved issues in the clinics.

In this thesis, we had the opportunity to add to the already existing data on gene transcriptome (Montero-Conde *et al.*, 2008) the information about miRNome and DNA methylome of a large sample set of thyroid tumors. As shown in **Figure 5**, there were 30 samples characterized at all three genomic levels. Moreover, all samples were characterized for their somatic alterations, and these were taken into account when the data analyses were performed. Unlike in the TCGA project, which comprehensively characterized exclusively papillary tumors, we were able to generate data about an important number of follicular neoplasias (both adenomas and carcinomas). This provided us with a generous data platform to address the two main clinical issues that persistently complicate TC patients' management: the lack of markers of malignancy leading to an excess of diagnostic thyroidectomies, and those related to disease progression. By doing so, we aimed to decipher the missing pieces in the DTC cancer etiology.

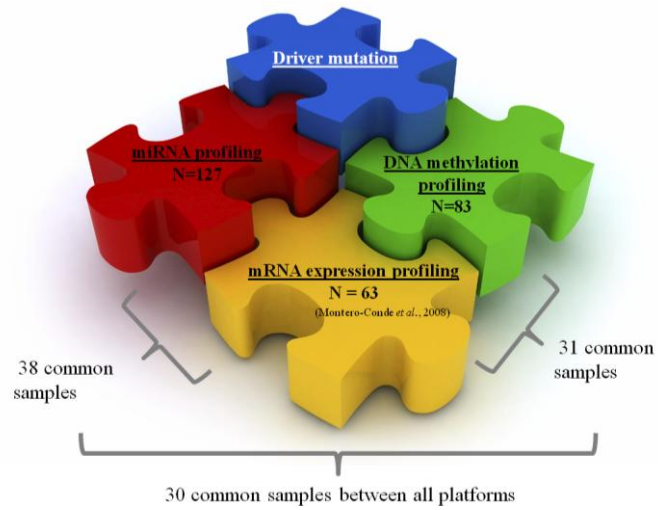

Figure 5. OMICs strategy used in order to decipher DTC etiology and identify novel biomarkers. Several genomic features were characterized at a genome-wide level, and the data was integrated, bearing in mind not only distinct histology, but also the underlying driver mutation and patients' outcome. Data on gene expression were available from previous studies in the laboratory (Montero-Conde *et al.*, 2008).

As revealed by both miRNA and DNA methylation profiles, there are essentially two molecular subtypes in DTC, which correlate greatly with the histological subdivision into tumors with papillary and follicular pattern of growth. Nevertheless, as evidenced from the behavior of PTCvf, it is the underlying driver mutation that ultimately leads the genomic profiles. In this way, we could observe that *BRAF*-mutated PTCvf tumors always cluster together with papillary tumors (in DNA methylation and miRNA profiling, as well as in the previously available mRNA transcriptional profiling), while *RAS*-mutated ones invariably gather with follicular tumors. This observation is in agreement with previous studies (Giordano *et al.*, 2005; Nikiforova *et al.*, 2008; Ellis *et al.*, 2014), and can even be extrapolated to the

findings from the TCGA consortium (TCGA, 2014). With respect to the latter study, where authors genomically dissected exclusively papillary tumors, they could observe a strong association between *RAS* mutations and follicular variant histology. Moreover, just as in our unsupervised analyses, in the TCGA data set, the *RET/PTC*-driven tumors share genomic properties with *BRAF*^{V600E}-mutated samples.

When it comes to the specificities of each molecular subtype, papillary tumors show similar levels of DNA methylation when compared to normal thyroid tissues, and characteristically over-express miR-146b and the miR-221 ~ 222 cluster, confirming previous results (He *et al.*, 2005; Pallante *et al.*, 2006; Tetzlaff *et al.*, 2007; Jazdzewski *et al.*, 2008; Nikiforova *et al.*, 2008; Swierniak *et al.*, 2013). Of note, *BRAF*-mutated PTCs show aberrant hypomethylation of a number of important genes (such as *AKT3* or *KLK10*) accompanied by a remarkable down-regulation of miR-7 and miR-204. Conversely, follicular tumors on the whole show higher genome-wide levels of DNA methylation, which is again observable in the TCGA data (TCGA, 2014). MicroRNA sequencing additionally revealed an extreme over-expression of several members of miR-515 family, accompanied by already described up-regulation of miR-182, -183 and -96 (Nikiforova *et al.*, 2008; Rossing *et al.*, 2012; Dettmer *et al.*, 2013; TCGA, 2014). Importantly, we were able to pinpoint several miRNAs with a possible master regulator function. According to our results, miR-34a and miR-221 are invariable up-regulated and thus play a role in all TC histological subtypes, while down-regulation of novel miR-1247 is specific to all tumors with follicular pattern of growth. Given the increased role of miRNAs in determining cancer phenotype, these candidates deserve further investigation.

Moreover, mRNA transcriptomic data from matching tumors allowed us to identify molecules that are potentially regulated by DNA methylation and/or action of miRNAs. An attractive candidate for further studies, which according to our analysis is regulated in a complex manner, is *KLK10*. It is a member of the kallikrein family of genes, which are secreted serine proteases that have been extensively studied in cancer due to their involvement in extracellular matrix degradation as well as their promising role as disease biomarkers (Borgono and Diamandis, 2004; Olkhov-Mitsel *et al.*, 2012). According to our data, this molecule is specifically involved in the pathology of *BRAF*-positive tumors. A negative regulator of *KLK10* – miR-7 – was found extremely down-regulated in *BRAF*-related tumors, and the promoter region of this gene was found highly hypomethylated. Further functional studies should uncover what advantage could the up-regulation of KLK10 give to the PTC cancer cells.

Such a broad classification into only two molecular classes already hints that it might be difficult to pinpoint diagnostic markers of malignancy differentiating between benign follicular adenomas and their malignant counterparts in this particular data set. The results of

unsupervised analyses of both DNA methylation and miRNA data showed that even though a FTC-specific profile could be intuited, it was always disrupted by some FA samples. These samples could well be having a molecular profile closer to carcinogenic transformation. It is expectable that the transformation is a multi-step process (Rhodes *et al.*, 2004), and we did observe a cumulative increase in the DNA methylation levels between benign and malignant tumors. Nevertheless, on the whole, there was a large overlap (>82%) among the deregulated events identified in adenomas as compared to those from follicular carcinomas (**Figure 6**). These results further confirm that FA could be a precursor lesion of FTC (Arora *et al.*, 2008), and suggest that several steps take place in the conversion between these two TC stages. At this point, it is worthy to note that even though our miRNome characterization was exhaustive using deep-sequencing techniques, we were only able to array roughly 0.01% of all genomic CpGs. Thus, a deeper DNA methylome characterization could uncover additional information, potentially even markers of malignancy.

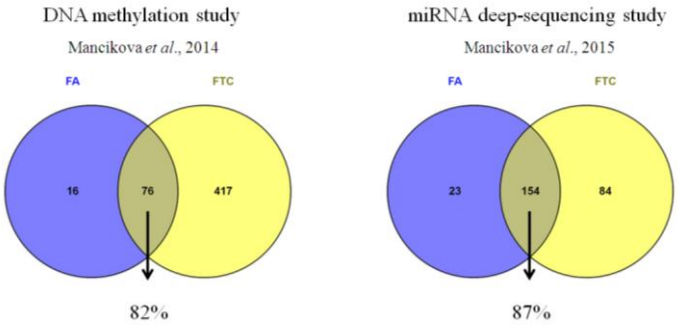

Figure 6. A large overlap between alterations identified in FA and FTC. Both the DNA methylation and miRNA transcription study revealed a large overlap between the deregulated events identified in adenomas and those from follicular carcinomas. This implies that malignancy markers remain elusive.

Another issue that complicates the clinical management of the TC patients is the lack of prognostic markers that would characterize those of them at high risk of eventually developing a disease recurrence. Thyroid cancer recurrences are prone to appear after long periods of time following the initial diagnosis (even more than 10 years). This makes it especially challenging to investigate markers of recurrence-free survival, as retrospective studies need to count with long term follow-up periods. In this thesis, we fortunately worked with samples with a median follow-up of 6 years, which gave us an excellent opportunity to evaluate the prognostic value of DNA methylation and miRNA signatures. We were able to identify several alterations that have a prognostic value in our tumor set. Elevated levels of DNA methylation of *WT1* and *EI24* were associated with worse prognosis. Expression or methylation levels of *WT1* have been proposed as markers of disease recurrence in many cancers (Trka *et al.*, 2002; Weisser *et al.*, 2005; Kobayashi *et al.*, 2011), while *EI24* expression has been found to be associated with tumor invasiveness and poor prognosis to treatment (Mork *et al.*, 2007; Mazumder Indra *et al.*, 2011).

In the case of miRNA markers, it was the combination of high levels of let-7a with lower levels of miR-192 that discriminated the best between the low-risk and high-risk patients. The increased expression of let-7 has been detected in serum from papillary thyroid cancer patients as associated with the presence of multifocal lesions (Yu *et al.*, 2012), while miR-192 display a robust tumor suppressive role (Song *et al.*, 2008; Feng *et al.*, 2011; Kim *et al.*, 2011). In all analyses, other important clinical features, such as subtype, stage, gender, mutational status and age were corrected for, and the associations of the novel prognostic markers with recurrence remained significant. However, it is important to state that these markers require further validation. In this regard, the TCGA data set was of little use, as the tumors involved have a rather short follow-up and thus the series is in this regard little informative yet.

On the whole, according to our results at the resolution used in this thesis, the profiles of benign and malignant follicular tumors are highly overlapping, preventing from diagnostic markers identification. Nevertheless, we were able to identify several promising novel markers of recurrence-free survival, whose potential utility deserves further study.

2. Medullary thyroid cancer (Articles 4-6)

2.1 Genomic dissection of Medullary Thyroid Carcinoma (Article 4)

In the current genomic era, the precise molecular etiology of MTC remains elusive. MTC is so far a disease of few genetic drivers (*RET* and *RAS*), but a variety of mutations whose emergence has important clinical consequences (Ciampi *et al.*, 2013). Yet, there are some limitations that have been obstructing a true genomic dissection of the disease, such as the challenge to collect an informative sample set, and perhaps partially also the fact it is not possible to compare the disease state to a normal one due to low cell fraction of C-cells in the normal thyroid (about 1%). In this scenario, we planned to finish the comprehensive characterization of several genomic aspects of the largest collection of MTC samples published so far, and provide an integrative overview of the disease etiology.

It was previously demonstrated in MTC that mRNA transcriptomic profiles are altered according to the driver mutation (Jain *et al.*, 2004; Ameur *et al.*, 2009; Maliszewska *et al.*, 2013). Similarly, explorative studies involving a limited number of tumors showed that miRNA signatures could potentially serve as prognostic markers in this disease (Abraham *et al.*, 2011; Santarpia *et al.*, 2013). These findings encouraged us to continue with the MTC methylome characterization, paying special attention to the underlying genetics of the tumors (thus following a similar strategy as in case of follicular cell-derived thyroid cancer). According to the results of the unsupervised analysis, there are two main methylome profiles in MTC, which to a certain extend relate to the genetic alterations. In detail, RET^{M918T}-related tumors show higher frequency of hypomethylation events, while RET^{C634X}-related tumors together with those tumors not harboring mutations neither in *RET* nor *RAS* share a more methylated profile. These distinct profiles once more indicate that medullary thyroid tumors comprise of several molecular entities.

Integrative analysis using matching mRNA, miRNA and DNA methylation data available for 31 samples uncovered those DNA methylation changes, which have a negative regulatory effect on gene expression. It caught our attention that promoters of several genes previously described by our group as up-regulated in distinct MTC genetic classes (*DKK4* in RET^{M918T}-, and *GAL* among RET^{C634X}-positive tumors, respectively (Maliszewska *et al.*, 2013)), displayed corresponding promoter hypomethylation. In agreement with our findings, these genes showed oncogenic properties in other cancers (Berger *et al.*, 2005; Hawes *et al.*, 2006; Matsui *et al.*, 2009; Sugimoto *et al.*, 2009). Moreover, we found enrichment for cytokine-cytokine interaction and JAK/Stat pathway among the genes affected by hypomethylation in RET^{M918T}-related MTCs. These pathways were already found aberrantly activated in this genetic

class in our previous work (Maliszewska *et al.*, 2013). Altogether, these findings show that some of the deregulated genes and pathways involved in distinct MTC molecular subclasses are affected at this epigenetic regulatory level.

Interestingly, we could also find several miRNA genes that seemed to be regulated by DNA methylation. miRNAs have gained importance in cancer, serving as markers of cell of origin, differentiation stage or response to treatment (Calin *et al.*, 2005; Lu *et al.*, 2005; Calin and Croce, 2006). Even if they fine-tune the expression of 1/3 of human genes, their own regulation remains elusive (Krol *et al.*, 2010). Here, by identifying putative promoters (Marsico *et al.*, 2013), we could demonstrate that at least the miRNAs tested (miR-10a, -30a and -200c) show that promoter DNA methylation negatively control their expression. Surely further research and algorithm development to define miRNA promoters will lead to unveiling more information on miRNA regulation.

When considering results from this thesis in light of those previously obtained in the laboratory, an interesting regulatory axis was uncovered. PROM1 is over-expressed in *RET*^{M918T}-positive MTCs (Maliszewska *et al.*, 2013), and due to its cancer stem cell marker properties it could be linked to resistance to treatment. Our unpublished data highlighted a sharp down-regulation of miR-30a in the same tumor group. Using MAGIA tool, a negative correlation between PROM1 and miR-30a expression was found in matching tumors (R=-0.74, FDR=0.037). Finally, using *in silico* target prediction softwares, we confirmed PROM1 as a possible miR-30a target. According to the current integration, this post-transcriptional regulation could be further regulated by epigenetics, as we found that the miR-30a down-regulation might be caused by DNA methylation in *RET*^{M918T}-related MTCs.

By our focused analyses taking into account the underlying genetics of MTC, we were able to unveil several interesting regulatory axes in *RET*^{M918T}-positive MTCs that deserve further studies. Moreover, according to our results, WT tumors share a similar methylome with *RET*^{C634X}-positive tumors and thus perhaps similar pathways might be implicated in the emergence of these two MTC subclasses. Probably due to sample number limitations, we could not unmask any methylation markers of *RAS*-positive tumors. Thus, a continued interest in MTC genomics warrants further insights into its etiology. However, it is important to gather a diverse sample collection, where the less frequent genetic classes (WT, *RAS*-mutated, less frequent *RET* mutations) would be represented by a reasonable number of tumors.

2.2 Implication of genetics in treatment of MTC patients with targeted therapies (Articles 5-6)

Medullary thyroid carcinoma is one of the least prevalent subtypes of thyroid cancer, but responsible for a large proportion of thyroid cancer-related deaths (Roman *et al.*, 2006). Genetic screening has been leading the clinical management of familial MTC cases for over 20 years. Among the sporadic patients, it became equally important to uncover the genetic driver of the disease, and either rule out germinal *RET* mutations, or, if a potential index case is uncovered, proceed with screening of the family members. Understanding the genetic foundation of the disease recently led to applying TKIs as an alternative to the conventional therapies. Importantly, some of the TKIs targets have been shown to be over-expressed in subsets of primary MTCs and metastases (Rodriguez-Antona *et al.*, 2010). This might be the underlying reason for which this alternative palliative treatment has achieved encouraging results in many patients, inducing partial responses and stabilization of the disease. Yet, the therapy has to be discontinued due to important toxicities in others. The molecular basis for the large variability in TKI response remains largely unknown.

As detailed above, there is piling evidence that several genomic attributes, including expression profiles, are driven by the underlying genetics. Therefore, we wondered if the expression of TKI target proteins could also be traced back to particular mutations, as this could potentially affect the response. Using an exceptional collection of 103 paraffin-embedded MTCs, we only considered *RET* mutations in the first approximation. By means of unsupervised analysis, we observed that TKI target proteins' expression differs according to the *RET* status in primary tumors. In detail, MTC samples with the RET^{C634X} mutation exhibited a higher expression of VEGFR3 and KIT than the RET^{M918T}-mutated and non-mutated *RET* tumor samples and a lower expression of VEGFR1. These results could be underlying the greater response rate to vandetanib of sporadic patients who harbored RET^{M918T} mutation (Wells *et al.*, 2012) and highlight that molecular stratification of patients may have the potential to improve TKI therapies for MTC.

Subsequently, we set off to establish if *RAS*-mutated MTCs share a TKIs targetable profile, as these alterations have been related to treatment resistance in other cancer types (Linardou *et al.*, 2008). We found that *RAS*-positive MTCs indeed express lower levels of several targets when compared to *RET*-related tumors. This could be explaining the tendency for shorter progression-free survival observed for *RAS*-mutated patients treated with cabozantinib when compared with *RET*-mutated patients (47 *vs*. 60 weeks) (Sherman *et al.*, 2013). Importantly, even though the etiology of the tumors not harboring neither *RET* nor *RAS* mutations remains unknown, it seems these patients could benefit from anti-angiogenic therapy, as they more often express VEGF.

Taking all this into consideration, *a priori* genetic screening of MTC patients appears advisable to guide their enrollment in TKIs clinical trials. One interesting aspect to address in the future similar studies is to assess the relationship between TKI targets' expression profile and the ultimate patients' treatment outcome. Moreover, some other genomic factors could be potentially affecting the treatment response. It is encouraging to see that genomics is slowly starting to be taken into account in the current MTC clinical trials' designs, such as in the case of microRNAs (https://clinicaltrials.gov/ct2/show/NCT02268734). Surely, similar efforts will lead to important advances in the treatment options of MTC patients in the near future.

CONCLUSIONS

1. We demonstrate that there are two *bona fide* thyroid cancer susceptibility loci: 9q22.33 and 14q13.3. The novel detected variants from 10q26.12 and 6q14.1 are associated with the disease risk in a population-specific manner. The genetic heterogeneity between populations could explain the lack of replication of already identified LPGs and needs to be taken into account as a part of the hidden heritability of this disease.

2. As revealed by both miRNA and DNA methylation profiles, there are essentially two molecular subtypes in DTC, which correlate greatly with the histological subdivision into tumors with papillary and follicular pattern of growth and are ultimately determined by the driver mutation.

3. The methylome and miRNome of benign tumors highly overlaps with those of their malignant counterparts, which complicates the identification of differential markers that could serve for precise diagnosis of malignant disease.

4. *WT1* and *EI24* hypermethylation, and the combination of aberrant expression of let-7a and miR-192 could serve as potential novel molecular markers of time to progression of patients with well-differentiated follicular cell-derived tumors, and may contribute to identification of those at high risk of disease recurrence.

5. DNA methylome profiles are closely related to distinct *RET* mutations in MTCs and verify that there are several molecular subgroups in this disease closely linked to the driver mutations. Hypomethylation of effector genes of cytokine-cytokine interaction and JAK/Stat pathway could be underlying the aberrant activation of these pathways described among RET^{M918T}-related MTCs.

6. *A priori* genetic screening of MTC patients should be considered to guide the choice of the most suitable TKI treatment, as the TKI targets' expression differs according to the distinct MTC genetic drivers.

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OTHER PUBLICATIONS

1. Clin Cancer Res. 2015 Mar 30. pii: clincanres.2804.2014. [Epub ahead of print]

Title: DNA methylation profiling in pheochromocytoma and paraganglioma reveals diagnostic and prognostic markers.

Authors: de Cubas AA, Korpershoek E, Inglada-Perez L, Letouze E, Curras-Freixes M, Agustin FF, Comino-Mendez I, Schiavi F**, Mancikova V**, Eisenhofer G, Mannelli M, Opocher G, Timmers H, Beuschlein F, de Krijger RR, Cascon A, Rodriguez-Antona C, Fraga MF, Favier J, Gimenez-Roqueplo AP, Robledo M

ABSTRACT

PURPOSE: Pheochromocytoma and paraganglioma (PPGL) are rare neuroendocrine tumors, associated with highly variable post-operative evolution. The scarcity of reliable PPGL prognostic markers continues to complicate patient management. In this study, we explored genome-wide DNA methylation patterns in the context of PPGL malignancy to identify novel prognostic markers.

EXPERIMENTAL DESIGN: We retrospectively investigated DNA methylation patterns in PPGL with and without metastases utilizing high-throughput DNA methylation profiling data (Illumina 27K) from two large, well-characterized discovery (n=123; 24 metastatic) and primary validation (n=154; 24 metastatic) series. Additional validation of candidate CpGs was performed by bisulfite pyrosequencing in a second independent set of 33 paraffin-embedded PPGLs (19 metastatic).

RESULTS: Of the initial 86 candidate CpGs, we successfully replicated fifty-two (47 genes), associated with metastatic PPGL. Of these, 48 CpGs showed significant associations with time to progression even after correcting for *SDHB* genotype, suggesting their value as prognostic markers independent of genetic background. Hypermethylation of *RDBP* (negative elongation factor complex member E) in metastatic tumors was further validated by bisulfite pyrosequencing (Δβmetastatic-benign=0.29, p=0.003; HR: 1.4 (CI95%: 1.1-2.0), p=0.018), and may alter transcriptional networks involving (RERG, GPX3, and PDZK1) apoptosis, invasion, and maintenance of DNA integrity.

CONCLUSION: This is the first large-scale study of DNA methylation in metastatic PPGL that identifies and validates prognostic markers, which could be used for stratifying patients according to risk of developing metastasis. Of the three CpGs selected for further validation, one (*RDBP*) was clearly confirmed, and could be used for stratifying patients according to the risk of developing metastases.

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Title: Whole-exome sequencing identifies MDH2 as a new familial paraganglioma gene.

Authors: Cascón A, Comino-Méndez I, Currás-Freixes M, de Cubas AA, Contreras L, Richter S, Peitzsch M, **Mancikova V**, Inglada-Pérez L, Pérez-Barrios A, Calatayud M, Azriel S, Villar-Vicente R, Aller J, Setién F, Moran S, Garcia JF, Río-Machín A, Letón R, Gómez-Graña Á, Apellániz-Ruiz M, Roncador G, Esteller M, Rodríguez-Antona C, Satrústegui J, Eisenhofer G, Urioste M, Robledo M

ABSTRACT

Disruption of the Krebs cycle is a hallmark of cancer. *IDH1* and *IDH2* mutations are found in many neoplasms, and germline alterations in *SDH* genes and *FH* predispose to pheochromocytoma/paraganglioma and other cancers. We describe a paraganglioma family carrying a germline mutation in *MDH2*, which encodes a Krebs cycle enzyme. Whole-exome sequencing was applied to tumor DNA obtained from a man age 55 years diagnosed with multiple malignant paragangliomas. Data were analyzed with the two-sided Student's t and Mann-Whitney U tests with Bonferroni correction for multiple comparisons. Between six- and 14-fold lower levels of *MDH2* expression were observed in *MDH2*-mutated tumors compared with control patients. Knockdown (KD) of *MDH2* in HeLa cells by shRNA triggered the accumulation of both malate (mean ± SD: wild-type [WT] = 1±0.18; KD = 2.24±0.17, P = .043) and fumarate (WT = 1±0.06; KD = 2.6±0.25, P = .033), which was reversed by transient introduction of WT MDH2 cDNA. Segregation of the mutation with disease and absence of MDH2 in mutated tumors revealed *MDH2* as a novel pheochromocytoma/paraganglioma susceptibility gene.

3. Pharmacogenomics J. 2014 Nov 4. doi: 10.1038/tpj.2014.67. [Epub ahead of print]

Title: High frequency and founder effect of the *CYP3A420 loss-of-function allele in the Spanish population classifies *CYP3A4* as a polymorphic enzyme.**

Authors: Apellániz-Ruiz M, Inglada-Pérez L, Naranjo ME, Sánchez L, **Mancikova V**, Currás-Freixes M, de Cubas AA, Comino-Méndez I, Triki S, Rebai A, Rasool M, Moya G, Grazina M, Opocher G, Cascón A, Taboada-Echalar P, Ingelman-Sundberg M, Carracedo A, Robledo M, Llerena A, Rodríguez-Antona C

ABSTRACT

Cytochrome P450 3A4 (*CYP3A4*) is a key drug-metabolizing enzyme. Loss-of-function variants have been reported as rare events, and the first demonstration of aCYP3A4 protein lacking functional activity is caused by *CYP3A4**20 allele. Here we characterized the world distribution and origin of *CYP3A4**20 mutation. *CYP3A4**20 was determined in more than 4000 individuals representing different populations, and haplotype analysis was performed using *CYP3A* polymorphisms and microsatellite markers. *CYP3A4**20 allele was present in 1.2% of the Spanish population (up to 3.8% in specific regions), and all *CYP3A4**20 carriers had a common haplotype. This is compatible with a Spanish founder effect and classifies *CYP3A4* as a polymorphic enzyme. This constitutes the first description of a *CYP3A4* loss-of-function variant with high frequency in a population. *CYP3A4**20 results together with the key role of *CYP3A4* in drug metabolism support screening for rare *CYP3A4* functional alleles among subjects with adverse drug events in certain populations.

4. J Clin Endocrinol Metab. 2014 Jul;99(7):E1376-80. doi: 10.1210/jc.2013-3879. Epub 2014 Mar 31.

Title: *H-RAS* mutations are restricted to sporadic pheochromocytomas lacking specific clinical or pathological features: data from a multi-institutional series.

Authors: Oudijk L, de Krijger RR, Rapa I, Beuschlein F, de Cubas AA, Dei Tos AP, Dinjens WN, Korpershoek E, **Mancikova V**, Mannelli M, Papotti M, Vatrano S, Robledo M, Volante M.

ABSTRACT

CONTEXT: Somatic or germline mutations in up to 15 disease-causative genes are detectable in up to 50% of patients with pheochromocytoma (PCC) and paraganglioma (PGL). Very recently, somatic *H-RAS* mutations were identified by exome sequencing in approximately 7% in sporadic PCCs and PGLs, in association with male sex and benign behavior.

OBJECTIVE: To explore the prevalence of *RAS* mutations in a cohort of 271 PCC and PGL from a European registry and to compare the genotype with clinical and pathological characteristics of potential clinical interest.

SETTING AND DESIGN: Genetic screening for hotspot mutations in *H-*, *N-*, and *K-RAS* genes was performed by means of Sanger sequencing or pyrosequencing methods on tumor DNA in a series of patients with (n = 107) or without (n = 164) germline or somatic PCC/PGL-related gene mutations.

RESULTS: Overall, *H-RAS* mutations were detected in 5.2% of cases (14/271), which were confined to sporadic PCCs resulting in a prevalence of 10% (14/140) in this cohort. In contrast, no mutations were found in PCC with PCC/PGL-related gene mutations (0/76) or in PGL (0/55) harboring or not mutations in PCC/PGL susceptibility genes. In this large series, *H-RAS* mutations in PCCs lacked any significant correlation with pathological or basic clinical endpoints.

CONCLUSIONS: Somatic *H-RAS* mutations are restricted to a relevant proportion of sporadic PCC. These findings provide the basis to study potential *H-RAS*-dependent correlations with long-term outcome data.

5. PLoS One. 2013 Sep 23;8(9):e74765. doi: 10.1371/journal.pone.0074765. eCollection 2013.

Title: An epistatic interaction between the *PAX8* and *STK17B* genes in papillary thyroid cancer susceptibility.

Authors: Landa I, Boullosa C, Inglada-Pérez L, Sastre-Perona A, Pastor S, Velázquez A, **Mancikova V**, Ruiz-Llorente S, Schiavi F, Marcos R, Malats N, Opocher G, Diaz-Uriarte R, Santisteban P, Valencia A, Robledo M

ABSTRACT

Papillary Thyroid Cancer (PTC) is a heterogeneous and complex disease; susceptibility to PTC is influenced by the joint effects of multiple common, low-penetrance genes, although relatively few have been identified to date. Here we applied a rigorous combined approach to assess both the individual and epistatic contributions of genetic factors to PTC susceptibility, based on one of the largest series of thyroid cancer cases described to date. In addition to identifying the involvement of *TSHR* variation in classic PTC, our pioneer study of epistasis revealed a significant interaction between variants in *STK17B* and *PAX8*. The interaction was detected by MD-MBR ($p = 0.00010$) and confirmed by other methods, and then replicated in a second independent series of patients (MD-MBR $p = 0.017$). Furthermore, we demonstrated an inverse correlation between expression of PAX8 and STK17B in a set of cell lines derived from human thyroid carcinomas. Overall, our work sheds additional light on the genetic basis of thyroid cancer susceptibility, and suggests a new direction for the exploration of the inherited genetic contribution to disease using association studies.

6. J Clin Endocrinol Metab. 2013 Jul;98(7):2811-21. doi: 10.1210/jc.2012-3566. Epub 2013 May 10.

Title: DNA methylation signatures identify biologically distinct thyroid cancer subtypes.

Authors: Rodríguez-Rodero S, Fernández AF, Fernández-Morera JL, Castro-Santos P, Bayon GF, Ferrero C, Urdinguio RG, Gonzalez-Marquez R, Suarez C, Fernández-Vega I, Fresno Forcelledo MF, Martínez-Camblor P, **Mancikova V**, Castelblanco E, Perez M, Marrón PI, Mendiola M, Hardisson D, Santisteban P, Riesco-Eizaguirre G, Matías-Guiu X, Carnero A, Robledo M, Delgado-Álvarez E, Menéndez-Torre E, Fraga MF.

ABSTRACT

OBJECTIVE: The purpose of this study was to determine the global patterns of aberrant DNA methylation in thyroid cancer.

RESEARCH DESIGN AND METHODS: We have used DNA methylation arrays to determine, for the first time, the genome-wide promoter methylation status of papillary, follicular, medullary, and anaplastic thyroid tumors.

RESULTS: We identified 262 and 352 hypermethylated and 13 and 21 hypomethylated genes in differentiated papillary and follicular tumors, respectively. Interestingly, the other tumor types analyzed displayed more hypomethylated genes (280 in anaplastic and 393 in medullary tumors) than aberrantly hypermethylated genes (86 in anaplastic and 131 in medullary tumors). Among the genes indentified, we show that 4 potential tumor suppressor genes (*ADAMTS8*, *HOXB4*, *ZIC1*, and *KISS1R*) and 4 potential oncogenes (*INSL4*, *DPPA2*, *TCL1B*, and *NOTCH4*) are frequently regulated by aberrant methylation in primary thyroid tumors. In addition, we show that aberrant promoter hypomethylation-associated overexpression of MAP17 might promote tumor growth in thyroid cancer.

CONCLUSIONS: Thyroid cancer subtypes present differential promoter methylation signatures, and nondifferentiated subtypes are characterized by aberrant promoter hypomethylation rather than hypermethylation. Additional studies are needed to determine the potential clinical interest of the tumor subtype-specific DNA methylation signatures described herein and the role of aberrant promoter hypomethylation in nondifferentiated thyroid tumors.

7. Endocr Relat Cancer. 2013 Jun 24;20(4):477-93. doi: 10.1530/ERC-12-0183. Print 2013 Aug.

Title: Integrative analysis of miRNA and mRNA expression profiles in pheochromocytoma and paraganglioma identifies genotype-specific markers and potentially regulated pathways.

Authors: de Cubas AA, Leandro-García LJ, Schiavi F, **Mancikova V**, Comino-Méndez I, Inglada-Pérez L, Perez-Martinez M, Ibarz N, Ximénez-Embún P, López-Jiménez E, Maliszewska A, Letón R, Gómez Graña A, Bernal C, Alvarez-Escolá C, Rodríguez-Antona C, Opocher G, Muñoz J, Megias D, Cascón A, Robledo M

ABSTRACT

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are rare neuroendocrine neoplasias of neural crest origin that can be part of several inherited syndromes. Although their mRNA profiles are known to depend on genetic background, a number of questions related to tumor biology and clinical behavior remain unanswered. As microRNAs (miRNAs) are key players in the modulation of gene expression, their comprehensive analysis could resolve some of these issues. Through characterization of miRNA profiles in 69 frozen tumors with germline mutations in the genes *SDHD*, *SDHB*, *VHL*, *RET*, NF1, *TMEM127*, and *MAX*, we identified miRNA signatures specific to, as well as common among, the genetic groups of PCCs/PGLs. miRNA expression profiles were validated in an independent series of 30 composed of *VHL*-, *SDHB*-, *SDHD*-, and *RET*-related formalin-fixed paraffin-embedded PCC/PGL samples using quantitative real-time PCR. Upregulation of miR-210 in *VHL*- and *SDHB*-related PCCs/PGLs was verified, while miR-137 and miR-382 were confirmed as generally upregulated in PCCs/PGLs (except in *MAX*-related tumors). Also, we confirmed overexpression of miR-133b as *VHL*-specific miRNAs, miR-488 and miR-885-5p as *RET*-specific miRNAs, and miR-183 and miR-96 as *SDHB*-specific miRNAs. To determine the potential roles miRNAs play in PCC/PGL pathogenesis, we performed bioinformatic integration and pathway analysis using matched mRNA profiling data that indicated a common enrichment of pathways associated with neuronal and neuroendocrine-like differentiation. We demonstrated that miR-183 and/or miR-96 impede NGF-induced differentiation in PC12 cells. Finally, global proteomic analysis in *SDHB* and *MAX* tumors allowed us to determine that miRNA regulation occurs primarily through mRNA degradation inPCCs/PGLs, which partially confirmed our miRNA-mRNA integration results.

8. Hum Mol Genet. 2013 Jun 1;22(11):2169-76. doi: 10.1093/hmg/ddt069. Epub 2013 Feb 14.

Title: Tumoral *EPAS1* (*HIF2A*) mutations explain sporadic pheochromocytoma and paraganglioma in the absence of erythrocytosis.

Authors: Comino-Méndez I, de Cubas AA, Bernal C, Álvarez-Escolá C, Sánchez-Malo C, Ramírez-Tortosa CL, Pedrinaci S, Rapizzi E, Ercolino T, Bernini G, Bacca A, Letón R, Pita G, Alonso MR, Leandro-García LJ, Gómez-Graña A, Inglada-Pérez L**, Mancikova V**, Rodríguez-Antona C, Mannelli M, Robledo M, Cascón A

ABSTRACT

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are chromaffin-cell tumors that arise from the adrenal medulla and extra-adrenal paraganglia, respectively. The dysfunction of genes involved in the cellular response to hypoxia, such as *VHL*, EGL nine homolog 1, and the succinate dehydrogenase (*SDH*) genes, leads to a direct abrogation of hypoxia inducible factor (*HIF*) degradation, resulting in a pseudo-hypoxic state implicated in PCC/PGL development. Recently, somatic post-zygotic mutations in *EPAS1* (*HIF2A*) have been found in patients with multiple PGLs and congenital erythrocytosis. We assessed 41 PCCs/PGLs for mutations in *EPAS1* and herein describe the clinical, molecular and genetic characteristics of the 7 patients found to carry somatic *EPAS1* mutations; 4 presented with multiple PGLs (3 of them also had congenital erythrocytosis), whereas 3 were single sporadic PCC/PGL cases. Gene expression analysis of *EPAS1*-mutated tumors revealed similar mRNA *EPAS1* levels to those found in *SDH*-gene- and *VHL*-mutated cases and a significant up-regulation of two hypoxia-induced genes (*PCSK6* and *GNA14*). Interestingly, single nucleotide polymorphism array analysis revealed an exclusive gain of chromosome 2p in three *EPAS1*-mutated tumors. Furthermore, multiplex-PCR screening for small rearrangements detected a specific EPAS1 gain in another *EPAS1*-mutated tumor and in three non-*EPAS1*-mutated cases. The finding that *EPAS1* is involved in the sporadic presentation of the disease not only increases the percentage of PCCs/PGLs with known driver mutations, but also highlights the relevance of studying other hypoxia-related genes in apparently sporadic tumors. Finally, the detection of a specific copy number alteration affecting chromosome 2p in *EPAS1*-mutated tumors may guide the genetic diagnosis of patients with this disease.

9. Clin Cancer Res. 2012 Aug 15;18(16):4441-8. Epub 2012 Jun 20.

Title: Regulatory polymorphisms in β-tubulin IIa are associated with paclitaxel-induced peripheral neuropathy.

Authors: Leandro-García LJ, Leskelä S, Jara C, Gréen H, Avall-Lundqvist E, Wheeler HE, Dolan ME, Inglada-Perez L, Maliszewska A, de Cubas AA, Comino-Méndez I, **Mancikova V**, Cascón A, Robledo M, Rodríguez-Antona C

ABSTRACT

PURPOSE: Peripheral neuropathy is the dose-limiting toxicity of paclitaxel, a chemotherapeutic drug widely used to treat several solid tumors such as breast, lung, and ovary. The cytotoxic effect of paclitaxel is mediated through β-tubulin binding in the cellular microtubules. In this study, we investigated the association between paclitaxel neurotoxicity risk and regulatory genetic variants in β-tubulin genes.

EXPERIMENTAL DESIGN: We measured variation in gene expression of three β-tubulin isotypes (I, IVb, and IIa) in lymphocytes from 100 healthy volunteers, sequenced the promoter region to identify polymorphisms putatively influencing gene expression and assessed the transcription rate of the identified variants using luciferase assays. To determine whether the identified regulatory polymorphisms were associated with paclitaxel neurotoxicity, we genotyped them in 214 patients treated with paclitaxel. In addition, paclitaxel-induced cytotoxicity in lymphoblastoid cell lines was compared with β-tubulin expression as measured by Affymetrix exon array.

RESULTS: We found a 63-fold variation in β-tubulin IIa gene (*TUBB2A*) mRNA content and three polymorphisms located at -101, -112, and -157 in TUBB2A promoter correlated with increased mRNA levels. The -101 and -112 variants, in total linkage disequilibrium, conferred *TUBB2A* increased transcription rate. Furthermore, these variants protected from paclitaxel-induced peripheral neuropathy [HR, 0.62; 95% confidence interval (CI), 0.42-0.93; P = 0.021, multivariable analysis]. In addition, an inverse correlation between *TUBB2A* and paclitaxel-induced apoptosis (P = 0.001) in lymphoblastoid cell lines further supported that higher TUBB2A gene expression conferred lower paclitaxel sensitivity.

CONCLUSIONS: This is the first study showing that paclitaxel neuropathy risk is influenced by polymorphisms regulating the expression of a β-tubulin gene.