UNIVERSIDAD AUTONOMA DE MADRID

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BIOCHEMISTRY DEPARTMENT

Genomic Alterations in Familial and Sporadic Epithelial Ovarian Carcinomas and their Clinical Relevance

Doctoral thesis

Marta Magdalena Kamieniak

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Doctoral thesis submitted to the Autonoma University of Madrid
for a degree of Doctor of Philosophy by
M.Sc. in Biotechnology,

Marta Magdalena Kamieniak

Thesis directors

Dr. Javier Benítez Ortiz

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HUMAN GENETICS GROUP
HUMAN CANCER GENETICS PROGRAMME
SPANISH NATIONAL CANCER RESEARCH CENTRE

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CERTIFICA:

Que Doña **Marta Magdalena Kamieniak**, Máster en Biotecnología por la Universidad de Ciencias de la Vida de Varsovia, ha realizado la presente Tesis Doctoral **"Genomic alterations in familial and sporadic epithelial ovarian carcinomas (EOC) and their clinical relevance"** y que a su juicio reúne plenamente todos los requisitos necesarios para optar al **Grado de Doctor en Biociencias Moleculares**, a cuyos efectos será presentado en la Universidad Autónoma de Madrid. El trabajo ha sido realizado bajo mi dirección, autorizando su presentación ante el Tribunal Calificador.

Y para que así conste se extiende el presente certificado,

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V^{O}B^{O} del Director de la Tesis:

V^{O}B^{O} del Tutor de la Tesis:

Dr. **María José García,** Investigadora de grupo de Genética del Cáncer Humano del Centro Nacional de Investigaciones Oncológicas (CNIO)

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Que Doña **Marta Magdalena Kamieniak**, Máster en Biotecnología por la Universidad de Ciencias de la Vida de Varsovia, ha realizado la presente Tesis Doctoral **"Genomic alterations in familial and sporadic epithelial ovarian carcinomas (EOC) and their clinical relevance"** y que a su juicio reúne plenamente todos los requisitos necesarios para optar al **Grado de Doctor en Biociencias Moleculares**, a cuyos efectos será presentado en la Universidad Autónoma de Madrid. El trabajo ha sido realizado bajo mi dirección, autorizando su presentación ante el Tribunal Calificador.

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This thesis, submitted for the degree of Doctor of Philosophy
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ABBREVIATIONS

aCGH	array-based Comparative Genomic Hybridization
ASR	Age-Standardized incidence Rate
BAC	Bacterial Artificial Chromosome
BMI	Body Mass Index
BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2
CCC	clear cell carcinoma
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A (P16)
CNV	Copy Number Variants
COGS	Collaborative Oncological Gene-Enviromental Study
CR	complete remission
DAPI	4',6-diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery database
DDR	DNA damage repair
DHPLC	Denaturing High Performance Liquid Chromatography
DNA	Deoxyribonucleic acid
EC	endometrioid carcinoma
EDTA	ethylenediaminetetraacetic acid
EOC	Epithelial Ovarian Cancer
ER	estrogen receptor
FDR	False Discovery Rate
FET	Fisher Exact Test
FFPE	Formalin Fixed Paraffin Embedded
FIGO	International Federation of Gynecology and Obstetrics
FISH	Fluorescent in situ Hybridization
GEO	Gene Expression Omnibus
HBOC	Hereditary Breast and Ovarian Cancer Syndrome
HD	homozygous deletion
HE	hematoxylin and eosin staining
HGSOC	High Grade Serous Ovarian Carcinoma
HNPCC	Hereditary Non Polyposis Colorectal Cancer (Lynch syndrome)
HR	homologous recombination
HRT	hormonal replacement therapy
HUGO	Human Genome Organisation
IHC	immunohistochemistry
IPA	Ingenuity Pathway Knowledge Base
LGSOC	Low Grade Serous Carcinoma
LOH	loss of heterozygosity
MC	mucinous carcinoma
MCR	Minimal Common Region
MDACC	M. D. Anderson Cancer Center
MMR	mismatch repair
NCBI	National Center for Biotechnology Information

NP	nonpolymorphic
OC	oral contraceptives
OS	Overall Survival
PARP	Poly ADP (Adenosine Diphosphate) Ribose Polymerase
PFS	Progression Free Survival
PJS	Peutz Jeghers syndrome
PR	progesterone receptor
RPKM	reads per kilobase per million reads
RT	room temperature
SNP	single nucleotide polymorphism
STK11	Serine Threonine Kinase 11
TCGA	The Cancer Genome Atlas
TMA	tissue microarray
TNM	tumor-node-metastasis classification system
TP53	tumor protein p53
TSG	tumor suppressor gene
UPD	uniparental disomy
WECCA	Weighted Clustering of Called aCGH Data
YAC	Yeast Artificial Chromosome

ABSTRACT

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy mainly due to lack of specific symptoms and effective screening methods that allow an early detection. It is also a very heterogeneous disease with various histological subtypes and classification criteria. Epithelial ovarian carcinomas from carriers of *BRCA1* and *BRCA2* mutations present distinct clinical and histopathological features when compared to sporadic tumors. In particular, it has been shown that *BRCA1/2* carriers respond better to standard therapy, show improved survival and are likely to respond to PARP inhibition. However, few studies have attempted to characterize genomic changes occurring in hereditary EOC and inconsistent results have been obtained. In addition, due to just a short-term survival benefits achieved by current treatment of advanced cases another priority in the field is to develop better prognostic and/or predictive markers that help to improve patients' outcome and to improve quality of life.

With these antecedents, in this study we aimed to characterize the profile of genomic alterations in hereditary and sporadic ovarian tumors and to assess the usefulness of DNA copy number changes, as potential prognostic and predictive biomarkers.

To address this we conducted a high-resolution array-based Comparative Genomic Hybridization (aCGH) profiling of 53 familial and 15 sporadic paraffin-embedded EOCs. We also integrated this data with immunohistopathological and clinical features in order to define potential common and subtype-specific features and to identify DNA copy number changes associated with survival or other biologically relevant characteristics. Three additional datasets consisting of 103 EOCs characterized by FISH, 411 high-grade serous ovarian carcinomas (HGSOCs) from TCGA and 1436 EOCs from the KM-plotter, *in-silico* tool, were used for validation of potential biomarkers.

Our results indicate that a high level of genomic instability and a greater contribution of losses versus gains are a common feature in EOC. We also found that sporadic and familiar EOC exhibit a similar global pattern of DNA copy number changes and that groups of EOC defined based on their DNA copy number profiles

show an association with histotype, FIGO stage and proliferation-related markers rather than with their familiar or sporadic condition. We identified common, recurrently altered regions in hereditary and sporadic tumors that would encompass genes potentially fundamental for ovarian carcinogenesis, independently from *BRCA1/2* mutations. Importantly, despite global similarity between sporadic and hereditary EOC we found that extensive genomic loss was significantly higher in tumors from *BRCA1* and *BRCA2* mutation carriers making this feature potentially clinically relevant in guiding the selection of BRCA-related patients, who are likely to respond to PARP inhibitors. Finally, integration of immunohistopathological and clinical data led us to demonstrate at the DNA copy-number level in 563 tumors and at the gene expression level in 1436 EOC that the 6q24-26 deletion is an independent marker of favorable outcome in ovarian cancer. In particular, our results indicate prognostic utility in HGSOCs, the most common and aggressive EOC subtype. This finding has a potential clinical value as the deletion can be analyzed by FISH and guide patients´ selection towards more conservative therapeutic strategies to reduce side-effects and improve quality of life.

RESUMEN

El cáncer epitelial de ovario es la neoplasia ginecológica más letal principalmente debido a la ausencia de síntomas específicos y a la falta de métodos de cribado efectivos que permitan una detección precoz. Se trata de una enfermedad muy heterogénea con numerosos subtipos histológicos y diferentes criterios de clasificación. Los carcinomas epiteliales de ovario (CEO) de pacientes portadoras de mutaciones en los genes *BRCA1* y *BRCA2* presentan unas características clínicas e histopatológicas distintivas. En concreto se ha demostrado que las portadoras de dichas mutaciones responden mejor a la quimioterapia convencional, presentan mejores supervivencias y tienden a responder al tratamiento con inhibidores de PARP. Sin embargo en muy pocos estudios se ha abordado la caracterización de los cambios genómicos que tienen lugar en los CEO hereditarios y estos han producido resultados contradictorios. Por otra parte los tratamientos estándar actuales ofrecen pobres perspectivas de supervivencia a las pacientes con tumores diseminados. Por ello, otra prioridad en este campo es el desarrollo de mejores marcadores pronósticos y predictivos que permitan mejorar la expectativa y calidad de vida de las pacientes.

Con estos antecedentes en el presente estudio hemos abordado la caracterización del patrón de cambios genómicos de los tumores de ovario familiares y esporádicos. Asimismo hemos evaluado los cambios en el número de copias de ADN como potenciales biomarcadores pronósticos y predictivos.

Para ello hemos analizado mediante Hibridación Genómica Comparativa en soporte de array (aCGH) 53 tumores de ovario familiares y 15 tumores de ovario esporádicos, todos ellos embebidos en parafina. Hemos integrado los resultados derivados de dicho análisis con datos inmunohistopatológicos y clínicos para definir alteraciones potencialmente comunes y específicas de los distintos grupos tumorales y para identificar cambios en el número de copias de ADN asociados con supervivencia u otras características biológicamente relevantes. Con el objeto de validar posibles biomarcadores candidatos se han usado tres series adicionales de tumores consistentes en 103 CEO caracterizados mediante FISH, 411 carcinomas serosos de alto grado del estudio del TCGA y 1436 CEO recogidos en la herramienta *in-silico* KM-plotter.

Nuestros resultados señalan que la alta inestabilidad genómica y la mayor contribución de las pérdidas en relación con las ganancias serían rasgos característicos de los CEO. También hemos constatado que los CEO familiares y esporádicos exhibirían un patrón similar de cambios en el número de copias de ADN y que grupos de tumores definidos en función de dichas alteraciones se asociarían con el subtipo histológico, el estadio FIGO y marcadores de proliferación y no con su origen esporádico o hereditario. Hemos identificado alteraciones recurrentes comunes a ambos tipos de tumores que contendrían genes potencialmente importantes para la patogénesis de cáncer de ovario independientemente de la existencia de mutaciones en los genes *BRCA1* y *BRCA2*. Cabe resaltar que a pesar de la similitud global entre los CEO hereditarios y esporádicos hemos observado que las pérdidas genómicas serían particularmente acusadas en los tumores de portadoras de mutaciones en *BRCA1* y *BRCA2.* Esta característica podría tener relevancia clínica en la selección de pacientes "*BRCA-like"* para las cuales se han descrito buenas respuestas a tratamientos con inhibidores de PARP. Por último, la integración de datos immunohistopatológicos y clínicos nos ha permitido demostrar en 563 CEO a nivel de cambios en el número de copias de ADN y en 1436 CEO a nivel de expresión génica que la deleción en 6q24-26 sería un marcador independiente de pronóstico favorable en cáncer de ovario. En concreto nuestros resultados señalan valor pronóstico en los tumores serosos de alto grado que constituyen el subtipo más común y agresivo. Este hallazgo presentaría una potencial aplicación clínica ya que mediante la determinación de la deleción con FISH se podrían seleccionar pacientes susceptibles de beneficiarse de estrategias terapéuticas más conservadoras que presentan menores efectos secundarios y repercusión en la calidad de vida.

INTRODUCTION

1 OVARIAN CANCER

Ovaries are female reproductive organs located on either side of the uterus below the fallopian tubes and responsible for hormone secretion and more importantly for housing and releasing oocytes or eggs (germ cells), which are essential for reproduction (Rosai, 2004) (*Figure 1A*).

The ovary is covered by a single layer called the ovarian surface epithelium (SE). The ovarian stroma is divided into an outer-cortical and inner medullar sections, but the bounders between them are indistinct. The cortex is where the follicles and oocytes are found at various stages of development and degeneration and is made of tightly packed connective tissue. The medulla is where the ovarian vasculature is found and is primarily loose stromal tissue (Rosai, 2004) (*Figure 1B*).

Different ovarian tumors are classified on the basis of their cell or tissue of origin. Approximately 90% of all ovarian cancers are epithelial and the other 10% are made up by gonadal-stromal (6% occurrence) and germ cell (4% occurrence) tumors (Holschneider & Berek, 2000). Ovarian cancer of epithelial cell origin is the most common type and it will be the focus of this study.

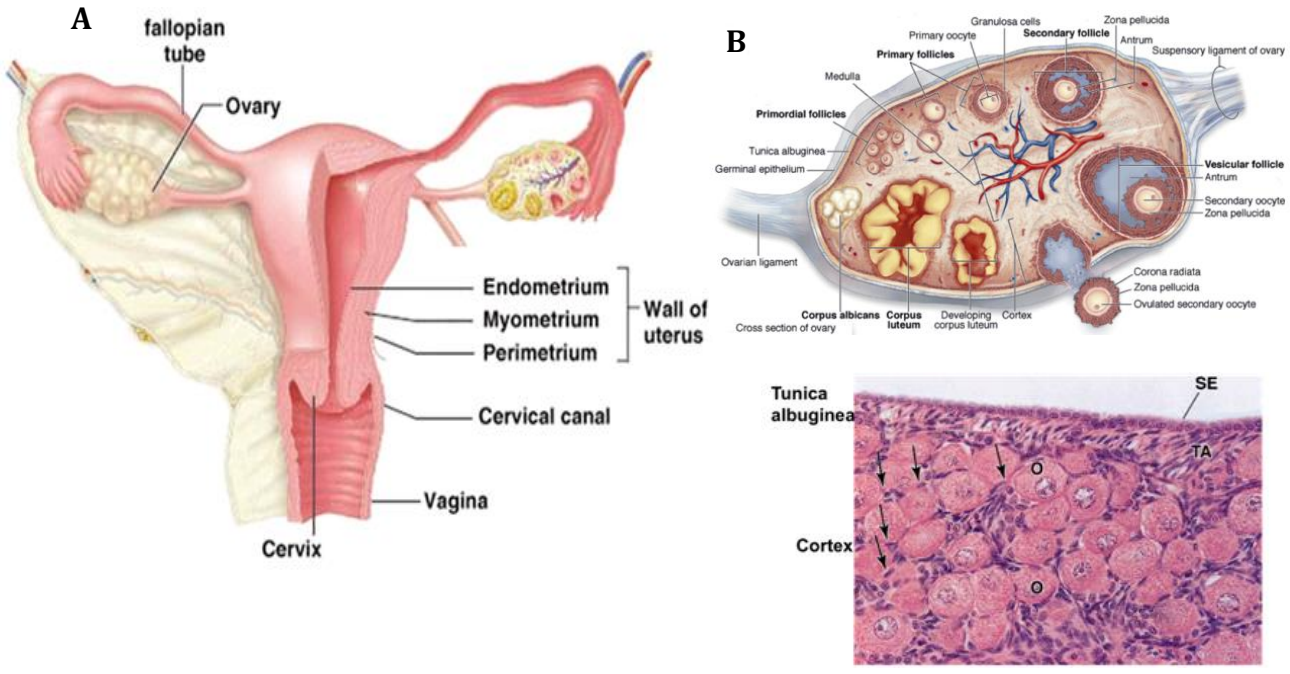

Figure 1. Structure of the gynecological track **(A)** and the cross section of ovary **(B)** SE, surface epithelium; O, oocyt *(www.accessmedicine.com)*

2 EPITHELIAL OVARIAN CANCER (EOC)

2.1 EPIDEMIOLOGY

Ovarian cancer is the seventh most common and most deadly cancer worldwide. Among gynecological malignancies it is the second most common (after cervical cancer) and first cause of cancer-related deaths (Ferlay J *et al*, 2013).

The lifetime risk of ovarian cancer is 0.67%, meaning that one in 149 women will develop the cancer in the general population by the age of 74. However the risk varies significantly worldwide with higher rates observed in more developed countries (1.01%) reaching the highest in Central and Eastern Europe (1.25%; 1 in 80 women) and lowest in the third world countries (0.43%) (Ferlay J *et al*, 2013).

The age-standarized worldwide incidence rate (ASR) is 6 per 100,000 women per year, and it is higher for Caucasian, than for African and Asian women (Ferlay J *et al*, 2013) *(Figure 2).*

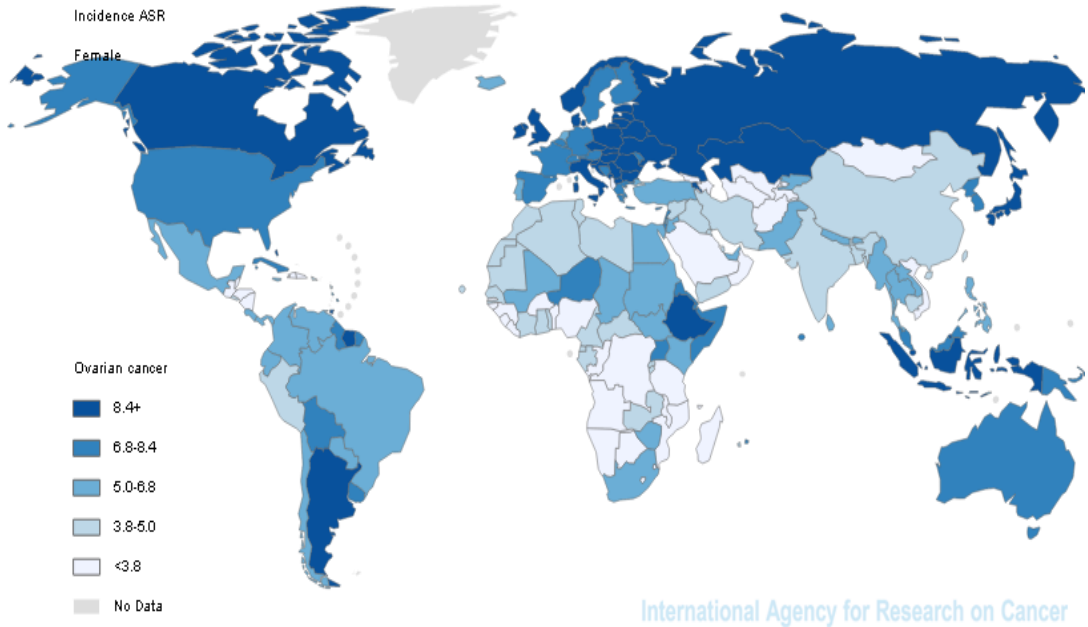

Figure 2. Estimated age-standarized incidence rate of ovarian cancer per 100,000 women (Globocan 2012)

Spain occupies an intermediate position worldwide, with ASR of 8 cases and mortality rate of 4 cases per 100.000 women per year, placing Spain on fifth position with the lowest mortality rate of this cancer in Europe (Ferlay J *et al*, 2013).

In ovarian cancer incidence is strongly related to age, with the highest rates being in older women. The largest number of patients with epithelial ovarian cancer is found in the 60–64 years old women (Berek *et al*, 2012).

2.2 CLASSIFICATION OF EOC

2.2.1 Histological subtypes

Epithelial ovarian canner is subdivided into benign, borderline (intermediate) and malignant (carcinomas) depending on the degree of cell proliferation, nuclear atypia, and the presence or absence of stromal invasion. About 10% of epithelial ovarian neoplasms are borderline tumors that show higher (than in benign counterparts) degree of cellular proliferation and variable nuclear atypia in the absence of stromal invasion. Despite the lack of stromal invasion they can progress to low grade serous carcinomas (LGSOCs) and invade underlying tissues (Prat, 2012b).

The malignant epithelial tumors (carcinomas) account for the majority of EOC (90% of cases) (Prat, 2012a). Initially they were considered as a single entity, however currently on the basis of their immunohistopathological and molecular features they are classified into five main subtypes: high-grade serous carcinomas (HGSCs) (70%), endometrioid carcinomas (ECs) (10%), clear cell carcinomas (CCCs) (10%), mucinous carcinomas (MCs) (3 %), and low grade serous carcinomas LGSOCs (<5%) (Prat, 2012b). Other less common types are Brenner, small cell, mixed and undifferentiated carcinomas.

2.2.2 Type I and Type II tumors

Besides the standard stratification of the EOC based on histopathological and immunohistochemical characteristics a more recent dualistic model categorizes various types of ovarian cancer into two designated types: type I and type II (Shih Ie & Kurman, 2004). Type I tumors are considered as clinically indolent, diagnosed at early stages and are

represented by low-grade serous, low-grade endometrioid, clear cell, and mucinous subtype. They grow slowly and develop in a stepwise manner from well-recognized precursors, namely borderline tumors. In contrast, type II tumors are usually highly aggressive, present at higher stage and are diagnosed as high-grade serous, high-grade endometrioid or undifferentiated carcinomas. They are rarely associated with morphologically recognizable precursor lesions and evolve rapidly, metastasizing early in their course. They account for approximately 75% of all epithelial ovarian carcinomas, show greater genomic instability, molecular homogeneity and have a uniformly poor outcome (Kurman & Shih Ie, 2010).

The morphologic differences between both tumor types are reflected substantially by distinct molecular features (Cho & Shih Ie, 2009). Type I tumors are more genetically stable and display wide range of mutations specific for each histological type (Kuo *et al*, 2009). Thus, *KRAS, BRAF, and ERBB2* mutations are characteristic for majority (75%) of low-grade serous carcinomas, while aberrations in the Wnt signaling pathway involving somatic mutations of *CTNNB1* (encoding β-catenin), *PTEN* and *PIK3CA* are specific for low-grade endometrioid carcinomas. Mucinous tumors present *KRAS* mutations in more than half of the cases, while clear cell carcinomas show a high percentage of *PIK3CA* activating mutations (Kurman & Shih Ie, 2010). The prototypic type II tumors, high-grade serous carcinomas, are characterized by very frequent *TP53* mutations (>80% of cases) and *CCNE1* amplification, but rarely present the mutations found in type I tumors (Cho & Shih Ie, 2009).

2.3 CLINICAL MANAGEMENT OF EOC

Cancer staging and grading are used to predict the clinical behavior of malignancies, establish appropriate therapies, and facilitate exchange of precise information between clinicians. During the staging/grading process, patients are placed in standardized categories according to the anatomical location of dissemination and the pathologic characteristics of their tumors.

2.3.1 Ovarian cancer staging and grading

The extent of tumoral spread, also known as *stage of disease,* at diagnosis is typically established by radiological evaluation and surgical excision and it is essential for determining the correct treatment strategy for an individual patient.

Currently used staging system published in Twenty-sixth Volume of the FIGO (International Federation of Gynecology and Obstetrics) Annual Report unifies tumor-node-metastasis (TNM) and FIGO classification (Heintz *et al*, 2006). TNM system determinates the extent of the tumor spread, assigning "T" if it is only confined to the ovary and nearby organs, "N" if involves lymph nodes, or "M" if already affects distant organs (Fleming *et al*, 1997). Staging according to FIGO guidelines defines 4 stages: Stage I reflecting the tumor that is strictly confined to the organ of origin; Stage II - tumor extended beyond the site of origin involving local adjacent organs or structures; Stage III - more extensive tumor involvement, i.e., wide infiltration reaching neighboring organs; and Stage IV - clearly distant metastasis (Heintz *et al*, 2001). Those basic stages are then classified into substages, as a reflection of specific clinical, pathological, or biological prognostic factors within a given stage.

In addition, several *grading systems* are used for ovarian carcinoma, however most of them are based on analyzes of histologic type, architectural pattern, nuclear/cytologic atypia, mitotic index, or a combination of these features.

Currently there are two commonly used grading systems: the universal grading system proposed by Shimizu-Silverberg (Silverberg, 2000) and the two-tier for serous carcinomas defined by M. D. Anderson Cancer Center (MDACC) (Malpica et al, 2004).

Table1. Proposed universal grading system for invasive ovarian carcinomas *(Silverberg, 2000)*

Score	Predominant architectural pattern	Cytologic atypia[a]	MFs/10 HPFs[b]
1	Glandular	Slight	0–9
2	Papillary	Moderate	10–24
3	Solid	Marked	>/–25

[a] As defined in text and in references 23 and 26.
[b] Counted in the most active region at 10× 40× on a Nikon Optiphot microscope (field diameter 0.663 mm, field area 0.345 mm^2).
Total score 3 to 5 = grade 1; 6 or 7 = grade 2; 8 or 9 = grade 3.

The 3-tier Shimizu-Silverberg classification is based on the Nottingham system for breast carcinomas and uses three parameters: architectural pattern

(glandular, papillary, solid), nuclear pleomorphism, and mitotic activity. Each parameter is given a score of 1–3 and a final grade is defined based on the summation of the scores, as shown in *Table 1* (Silverberg, 2000).

More recently, a 2-tier system, where tumors are stratified into low and high-grade, has been proposed by MDACC for serous carcinomas. This grading is primarily based on the assessment of nuclear atypia with the mitotic rate used as a secondary feature. According to this two-tier system, tumors with mild to moderate cytologic atypia are designated as low-grade, whereas tumors with marked cytologic atypia as high-grade (Malpica *et al*, 2004).

Besides the use of one of those universal grading systems there is an increasing tendency to employ different grading system for each histopathological subtype, as recommended by the Royal College of Pathologists Ovarian Cancer Datasets in the United Kingdom.

2.3.2 Diagnosis: Symptoms and screening methods

Epithelial Ovarian Cancer is called a ‘‘silent killer” due to lack of specific symptoms until it spreads into the pelvis and upper abdomen. In these advanced stages it manifests with pelvic or abdominal pain or pressure, abdominal swelling, nauseas, dyspepsia, and early satiety (Behtash *et al*, 2008). Ovarian cancer screening is preformed through pelvic examination, ultrasonography, computer tomography and measurements of the levels of CA125 serum marker, methods which are currently regarded as not specific and sensitive enough.

Therefore, due to lack of specific symptoms and effective screening methods, ovarian cancer is often diagnosed at advanced stages (Heintz et al, 2006) and the estimated 5-year survival rate of these cases is around 27% (Siegel et al, 2012).

2.3.3 Treatment

Specific ovarian carcinoma treatment recommendations are dependent on the stage of the disease, however according to the consensus statement of the 4th Ovarian Cancer Consensus Conference of the Gynecologic Cancer InterGroup (GCIG) in 2011, the treatment cornerstones are the maximal cytoreductive surgery followed by platinum-taxane adjuvant chemotherapy (Thigpen *et al*, 2011). In case of moderately and well differentiated low stage tumors (I and II) surgery alone may be an adequate treatment option, but should include hysterectomy, bilateral salpingo-oophorectomy, and omentectomy. In case of higher grade low stage tumors the surgery might be followed either by radiation therapy or by chemotherapy based on platins alone or in combination with paclitaxel.

Patients with high stage tumors (III and IV) are subjected for maximal debulking surgery followed by a systemic standard chemotherapy based on carboplatin and paclitaxel. In the inoperable cases, neo-adjuvant chemotherapy is given to reduce the tumor load before a second attempt of surgical cytoreduction. Maximal removal of the tumor is an essential prognostic factor (Ramirez *et al*, 2011).

Growing understanding of ovarian cancer landmarks in the recent years has led to the development of molecular-driven targeted therapies that have being more widely used in the combination to the standard chemotherapy. Currently the main strategies involve targeting angiogenesis by inhibiting VEGF pathway with bevacizumab or targeting cells with defective homologous recombination (e.g. *BRCA1/2* mutation) with PARP inhibitors, based on the concept of "synthetic lethality" (Banerjee & Kaye, 2013) that assums that targeting the second gene from the synthetic lethal pair will selectively kill defective (tumoral) cells.

2.3.4 Prognostic and predictive factors

In addition to maximal cytoreduction, stage of the tumor determined according to FIGO system and age of initial diagnosis represent well established prognostic factors (Bristow *et al*, 2002; Heintz *et al*, 2003; Thigpen *et al*, 1993). Also growing number of evidence points to the association of *BRCA1/2* germline mutations carriers with significantly better prognosis (Alsop *et al*, 2012; Bolton *et al*, 2012; Vencken *et al*, 2011; Yang *et al*, 2011). Specific histologic subtype and grade have been shown to have some significant relevance

too, however, the independent contribution of each of them after adjustment for tumor stage, has not been well established.

In addition, several possible molecular markers have been suggested to have prognostic or predictive role, but few have been proven in the subsequent studies and still none of them is used in the clinical practice. The most recent meta-analysis evaluating few of them (*Bcl-2, EGFR, GST, LRP, p16, p21 and TNF- α*) reported lack of association with patients prognosis or response (Xu *et al*, 2013).

2.4 RISK FACTORS

The etiology of ovarian cancer is not completely revealed yet. There are many different factors such as genetic, but also environmental, reproductive, medical and lifestyle-related that can modify the risk of ovarian cancer.

2.4.1 Non-genetic risk factors

Among non-genetic risk factors increasing age is far the strongest one (Cancer Research UK, 2013). Unlike some other cancer types, risk rates for ovarian carcinomas keep rising constantly until woman's eighties.

Reproductive factors

Epithelial Ovarian Cancer risk is strongly modulated by reproductive and menstrual factors. In general all events which interrupt ovulation and decrease the total number of ovulatory cycles such as pregnancy, breastfeeding, and use of oral contraceptives reduce the risk of ovarian cancer, while those that prolong exposure to ovulation such as nulliparity and infertility increase that risk.

Reproductive factors that decrease the risk of ovarian cancer include short menstrual history (late age of menarche and early age of menopause), early age at first birth as well as last pregnancy at a later age, a greater number of pregnancies and longer periods of breastfeeding (Siskind *et al*, 1997; Titus-Ernstoff *et al*, 2001; Whittemore *et al*, 1992). Oral contraceptives (OCs) are an established protective factor, effect of which increases with the

duration of OC intake (Hunn & Rodriguez, 2012; King, 2011). Whereas hormonal replacement therapy (HRT) increases the risk with a duration of treatment, becoming significant after seven years. Higher increase of risk is observed in case for oestrogen-based HRT, in contrast to oestrogen-progestin HRT (Pearce *et al*, 2009) (Cancer Research UK, 2013).

Habits and environmental factors

Higher body mass index (BMI>30) (Protani *et al*, 2012) and height over 1.7m (Schouten *et al*, 2008) were reported to increase the risk. Other factors such as use of talk (Huncharek *et al*, 2003; Wu *et al*, 2009), medical radiation exposure or night-shift work have been reported to modify the ovarian cancer risk.

2.4.2 Genetic risk factors

The single greatest ovarian cancer risk factor is a family history of the disease. Having a single first degree relative affected with ovarian cancer increases the lifetime risk to 5% and to at least 7% with two or more first-degree relatives (Stratton *et al*, 1998).

Although familial aggregation of cancer may be caused by both genetic and non-genetic factors shared within families twin studies indicated a greater role of genetic factors (Lichtenstein *et al*, 2000).

2.5 HEREDITARY EPITHELIAL OVARIAN CANCER

2.5.1 Hereditary predisposition to ovarian cancer

Hereditary nature of the ovarian carcinomas can be recognized based on the assessment of several features like many individuals in the affected family, earlier age of onset, multiple primary cancers in the same individual, bilaterallity or existence of multifocal cancers (Berliner & Fay, 2007).

Hereditary carcinomas occur more often in the context of several hereditary autosomal dominant syndromes, among which Hereditary Breast and Ovarian Cancer Syndrome (HBOCS) confers the greatest risk. Other syndromes such as Hereditary Non-

Polyposis Colorectal Cancer (HNPCC), Peutz-Jeghers syndrome or Cowden's disease also confer high risk for developing hereditary ovarian cancer, but explain only smaller percentage of such cases.

Initially, it was believed that only around 10% of ovarian cancer cases could be explained by an underlying genetic syndrome, however more recent data indicate that just 2 of them, Hereditary Breast and Ovarian Cancer Syndrome and Hereditary Nonpolyposis Colorectal Cancer, account for at least 20% of ovarian cases (Weissman *et al*, 2012b).

Currently at least 25% of newly diagnosed ovarian tumors are explained by germline mutations in a single gene suggesting that much higher proportion of ovarian cases is hereditary in nature (Pal *et al*, 2005; Pennington & Swisher, 2012; Walsh *et al*, 2011). Besides the high-risk genes, several intermediate-risk genes have been recently identified as well as a number of low penetrance variants *(Figure 3).* Common low penetrance variants confer little risk individually, but when inherited in a combination may contribute to explain part of the familial risk.

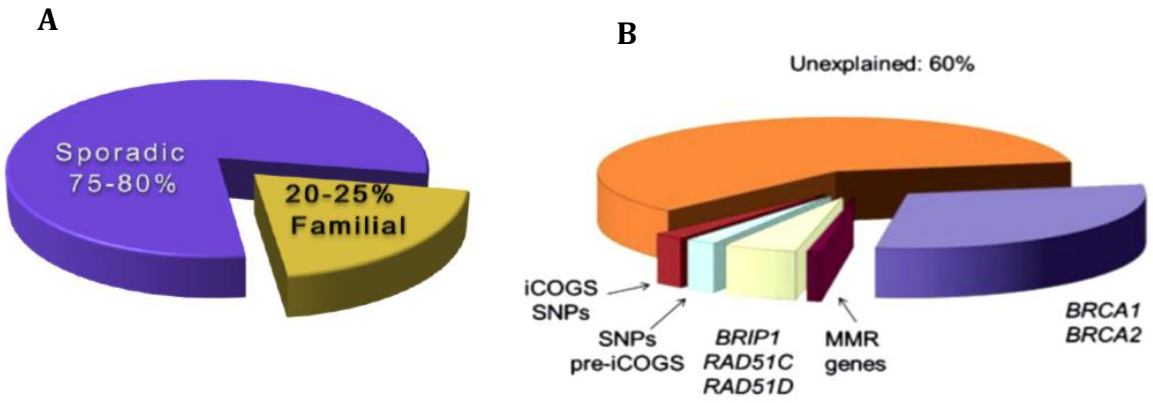

Figure 3. Percentage of ovarian cancer cases having familial antecedents (**A**) and proportion of different types of genetic risk explaining those cases (**B**) (adapted from http://www.nature.com/icogs/) MMR-mismatch repair; SNP-single nucleotide polymorphim; COGS-Collaborative Oncological Gene-Environment Study; iCOGS: custom Illumina iSelect genotyping array desined by the COGS consortia

2.5.2 BRCA1 and BRCA2: Hereditary Breast and Ovarian Cancer Syndrome (HBOCS)

The strongest risk for epithelial ovarian cancer is conferred by germline mutations in one of two high-susceptibility genes, *BRCA1* and *BRCA2,* which cause Hereditary Breast and Ovarian Cancer Syndrome (HBOCS). The HBOCS is an autosomal dominantly inherited disease characterized by an increased risk for breast and ovarian cancer as well as for other

neoplasms, such as prostate or pancreatic cancer. The percentage of HBOCS families explained by mutations in *BRCA1* or two or more ovarian cancer cases can be attributed to germline mutations in one of these two genes (Ramus *et al*, 2007).

The *BRCA1* gene is located at 17q21 and contains 24 exons spread over 80 kb that encode for a 1,863 aa protein (Miki *et al*, 1994). The BRCA1 protein consists of four major protein domains: the zinc-finger RING domain located at the N-terminus, the BRCA1 serine domain and two BRCT domains, composed of repetitive sequences for interactions with key proteins involved in DNA repair or metabolism, located at the C-terminus. The *BRCA2* gene, located at 13q12.3, contains 27 exons, 26 of which encode for a 3,418 aa protein. The BRCA2 protein contains two functional domains known as BRC repeats essential for the DNA repair and interaction with RAD51.

It is estimated that in general population, approximately 0.125% to 0.20% carry a mutation in either of these genes (Pal *et al*, 2005; Walsh *et al*, 2011; Zhang *et al*, 2011). However, the mutations' frequencies vary between the populations. In geographically or culturally isolated ethnic groups like Ashkenazi Jewish (Oddoux *et al*, 1996), Finnish, Islandic (Tulinius *et al*, 2002), but also in Polish (Gorski *et al*, 2004) and Spanish (Diez *et al*, 2003) exist population-specific mutations, called founder mutations, which have much higher prevalence. For instance, the prevalence of *BRCA1/2* mutations in Ashkenazi Jewish ovarian cancer cases is 35-40%, as 2.3% of this population harbors one of three founder mutations (Moslehi *et al*, 2000; Struewing *et al*, 1997).

The lifetime risk of developing ovarian cancer differs between the two genes and there have been a number of studies aiming to estimate that risk. According to two recent, large meta-analysis the lifetime risk for ovarian cancer was estimated to be approximately 40% for BRCA1 and 18% for BRCA2 (Chen et al, 2006; Chen & Parmigiani, 2007), and 22% and 18%, respectively, in the Spanish population (Milne et al, 2008). The variation in the risk estimates is accounted by many factors and depends not only on the population studied, but also on factors like study design, patient ascertainment method, mutation type and location and additional genetic and environmental factors that may modify the risk.

BRCA2 depends on different factors, but it has been reported that up to 50% of families with

Regarding the role of *BRCA1* and *BRCA2* many cellular and biochemical functions of both genes have been discovered. Importantly they have been defined as tumor suppressors,

and 'caretakers' sensing the DNA damage and participating in the repair process. Specifically, both BRCA1 and BRCA2 are necessary for double-stranded DNA breaks repair by homologous recombination (HR). Their inactivation allows other genetic defects to accumulate leading to genomic instability. Loss of BRCA1 function results in defects in DNA repair, transcription, centrosome duplication, G2/M cell cycle checkpoint regulation, spindle formation and also in chromosomal instability (Brodie & Deng, 2001; Deng, 2006; Rosen *et al*, 2006b). Cells lacking BRCA2 are deficient in the repair of double-strand DNA breaks, as reflected in a hypersensitivity to ionizing radiation (Venkitaraman, 2001; Venkitaraman, 2002).

2.5.3 Other high-risk ovarian cancer susceptibility genes

In addition to *BRCA1* and *BRCA2* there are several other genes that confer high susceptibility to develop ovarian cancer in the context of other autosomal dominant tumor predisposition syndromes.

The Hereditary Non-Polyposis Colorectal Cancer Syndrome (HNPCC) also known as Lynch syndrome explains 2-4% of ovarian carcinomas. Germline mutations in one of four mismatch-repair (MMR) genes (*MLH1, MSH2, MSH6*, and *PMS2*) account for approximately 36%, 38%, 14%, and 15% of this syndrome, respectively (Palomaki *et al*, 2009). In addition, smaller proportion of affected individuals may have germline deletions in *EPCAM* which inactivate *MSH2* through epigenetic silencing (Weissman *et al*, 2011). The cumulative risk of ovarian cancer associated with this syndrome is estimated to be between 4 and 11% (Weissman *et al*, 2012a; Weissman *et al*, 2012b), with an average age of onset of 42.7 years (Watson *et al*, 2001) and the tumors are usually moderately or well differentiated, of serous (32%), endometrioid (29%), mixed (24%), mucinous (19%) or clear cell (18%) histology (Crijnen *et al*, 2005).

A small percentage of hereditary ovarian cancer cases is also explained by another rare (reported incidence: 1:8300 and 1:200,000 births) autosomal dominant syndrome known as Peutz-Jeghers syndrome (PJS) (Allen & Terdiman, 2003; Lindor *et al*, 2008). Between 50 to 90% of the patients with this disorder are explained by germline mutations in serine threonine kinase 11 gene (*STK11*) (Amos *et al*, 2004; Salloch *et al*, 2010; Volikos *et al*, 2006). Individuals with PJS show an increased by 10-18 fold risk for a wide variety of epithelial malignancies (Giardiello *et al*, 2000; van Lier *et al*), among them, ovarian carcinomas.

2.5.4 Recently described high- to moderate- susceptibility genes

In 2010 *RAD51C,* a gene essential in homologous recombination (HR), was found to present germline mutations in patients from high-risk breast and ovarian families negative for *BRCA1* and *BRCA2* mutations (BRCAX families) (Meindl *et al*, 2010; Vaz *et al*, 2010; Vuorela *et al*, 2011). A relative risk for carriers of the *RAD51C* muataions was estaimated to be 5.9, that equals to more than 9% of cumulative risk of developing ovarian cancer by the age 80 (Loveday *et al*, 2012). However, currently ongoing international study will help to fully clarify the frequency and risk conferred by those mutations.

Identification of *RAD51C* mutations promped investigators to study the role of *RAD51D,* other RAD51 paralog, in cancer susceptibility (Loveday *et al*, 2011). Mutations were identified in 0.9% of BRCAX families and were more prevalent in families with more than just one ovarian cancer case and increased with each additional ovarian tumor. The relative risk of ovarian cancer for this gene is estimated to be 6.3, which confers 10% cumulative risk by 80 years old (Loveday *et al*, 2011). As for *RAC51C*, further analysis of large series of ovarian cancer cases from the general population will be necessary to accurately estimate the frequency and penetrance of *RAD51D* mutations and to eventually facilitate the clinical implementation of these genes.

Table 2 Genes associated with hereditary EOC (Pennington & Swisher, 2012)

Genes implicated in hereditary ovarian carcinoma
FA–BRCA pathway genes
• *BRCA1*
• *BRCA2*
• *RAD51C*
• *RAD51D*
• *BRIP1*
• *BARD1*
• *CHEK2*
• *MRE11A*
• *NBN*
• *PALB2*
• *RAD50*
Mismatch repair genes
• *MLH1*
• *MSH2*
• *MSH6*
• *PMS2*
Other genes
• *TP53*

Recently two rare frameshift mutations in *BRIP1* were found to be associated with markedly increased risk of ovarian cancer (Rafnar *et al*, 2011). The mutation identified in the Icelandic population was associated with 8.1 times higher risk of developing ovarian cancer, while the one found in Spanish individuals was estimated to increase the risk 25 times.

Recent massively parallel sequencing of 21 tumor suppressor genes in ovarian cancer patients identified deleterious germline mutations in 6 of them, which had not been previously associated with hereditary ovarian cancer: *BARD1, BRIP1, CHEK2, MRE11A, NBN* and *RAD50* (Walsh *et al*, 2012). However the overall proportion of ovarian carcinomas explained by mutations in these genes and their penetrance, remain to be established and validated.

Current data, indicate existence of at least 16 genes (*Table 2*) (some of those still awaiting further evidence) that would confer high to moderate risk to ovarian cancer (Pennington & Swisher, 2012).

2.5.5 Common low-penetrance susceptibility variants

All high-susceptibility and moderate susceptibility genes described so far account for about 36% of familial EOC cases (Bahcall, 2013) .

Several genetic models are proposed to explain residual familial inheritance; one of those predicts an existence of other highly penetrant, but very rare genes.

Alternatively, several more moderate risk genes could account for a remaining part of familial risk and multiple case families. Finally, there may be many low risk (low penetrance) genes that individually would confer small relative risks, but whose combined inheritance would contribute to risk increments.

Results from the Collaborative Oncological Gene-Enviroment Study (COGS) using a large-scale genome-wide association meta-analysis have confirmed previously reported ovarian cancer risk variants and described new ones. In total 12 ovarian cancer susceptibility loci located at 2q31, 3q25, 8q21, 8q24, 9p22.2, 10p12, 17p12, 17q12, 17q21 and 19p13 and 5p15 (two variants in this locus) were identified (Bojesen *et al*, 2013; Bolton *et al*, 2010; Goode *et al*, 2010; Permuth-Wey *et al*, 2013; Pharoah *et al*, 2013; Song *et al*, 2009; White *et al*, 2010). Very recently, two additional variants at the NF-Kb pathway genes: *IL1A* and *TNFSF10* have also been reported (Charbonneau *et al*, 2014). However, altogether these alleles explain only 4% of excess familial aggregation (*Figure 3B*) and their effects on risk are modest, varying from 0.63 and 1.48 (Pharoah *et al*, 2013). On the basis of what is known about the architecture of genetic susceptibility for other cancers, it is probable that many more common susceptibility alleles exist, accounting for the most of 60% of unexplained inheritance (McClellan & King, 2010; Pharoah *et al*, 2013).

2.5.6 Clinical features of hereditary EOC

Hereditary ovarian tumors are usually diagnosed in younger patients, especially those in *BRCA1* mutation carriers (52-55 yrs) and only slightly earlier in *BRCA2* carriers (59-60yrs) than in general population (60-62yrs), which is consistent with different penetrance rate of those genes (Alsop *et al*, 2012; Bolton *et al*, 2012; Walsh *et al*, 2011; Yang *et al*, 2011).

Despite the fact that most of the *BRCA1/2* carriers are diagnosed at advanced stages (Pennington & Swisher, 2012; Walsh *et al*, 2012) and their tumors seem to show more aggressive characteristics, they have been reported to be associated with favorable survival outcomes, some studies referring it only to *BRCA2*-mutation carriers (Bolton *et al*, 2012; Hyman *et al*, 2011; TCGA, 2011; Yang *et al*, 2011). This more favorable outcome for BRCA mutation carriers is likely to be associated with increased sensitivity of BRCA-deficient cells, that have impaired homologous recombination repair (HR), to cytotoxic drugs such as platinum-based agents (Alsop *et al*, 2012; Bolton *et al*, 2012; Vencken *et al*, 2011; Yang *et al*, 2011). Greater survival advantage observed specifically in *BRCA2*-carriers has been associated with different role of both BRCA genes in DNA double strand repair, with *BRCA2* being more directly involved in the homologous recombination itself (Hyman *et al*, 2011; Yang *et al*, 2011).

2.5.7 Immunohistopathological features of hereditary EOC

Ovarian carcinomas associated with *BRCA1/2* mutations present a distinct histopathological phenotype, with the majority being high-grade serous of solid type (Bolton *et al*, 2012; Boyd *et al*, 2000; Evans *et al*, 2008; Lakhani *et al*, 2004; Mavaddat *et al*, 2012). Besides serous histology *BRCA1/2* mutation carriers are also represented (to lower extent) by other high grade subtypes, including endometrioid, clear cell and undifferentiated carcinomas. In contrast mucinous and borderline neoplasms account only for about 2% of those cases (Evans *et al*, 2008). *BRCA1*-associated high grade serous carcinomas would also show specific cell morphology, presenting solid, pseudoendometroid and transitional cellular type, higher mitotic index and greater number of tumor infiltrating lymphocytes (Soslow *et al*, 2012).

Immunohistochemical characteristic of familial ovarian tumors is poorly established due to few existing studies, usually small sample series and lack of conclusive results.

However, p53 overexpression is the most consistently reported feature of *BRCA1/BRCA2* mutation cases (Lakhani *et al*, 2004; Munoz-Repeto *et al*, 2013). In addition a study that included 40 immunohistochemical markers found an association of *BRCA1*-related ovarian carcinomas with higher expression of progesterone receptor and nuclear EGFR, the later being more frequently overexpressed also in BRCAX (65%) tumors (in contrast to *BRCA2*-related carcinomas (19%) (Munoz-Repeto *et al*, 2013).

2.5.8 Gene expression pattern of hereditary EOC

So far there have been few studies aiming to characterize gene expression profiles in hereditary ovarian carcinomas. One such study described a gene expression signature that would discriminate between *BRCA1*- and *BRCA2*-mutant tumors allowing classification of sporadic cancers as either BRCA1- or BRCA2-like (Jazaeri *et al*, 2002). However, a recent study did not replicate these findings, reporting *BRCA1*-mutated tumors as the outlier group in gene expression, with *BRCA2* and wild-type tumors being more closely related (George *et al*, 2013b). Other study found similar gene expression profiles in *BRCA1* mutated and *BRCA1* wild-type tumors (just two genes being defined as differentially expressed) and also in the group with epigenetically silenced *BRCA1* (no genes identified as differentially expressed) (Pradhan *et al*, 2010).

2.6 DNA COPY NUMBER CHANGES IN EOC

2.6.1 Genomic instability in cancer

Somatic chromosomal copy number alterations and rearrangements are a cardinal feature inherent to almost all solid tumors and lead to altered expression and function of genes residing within the affected region of the genome (Albertson *et al*, 2003; Mitelman, 2000). Such structural changes are consequences of underlying genomic instability leading to chromosomal missegregation and repetitive cycles of DNA strand breaks and rejoining. Since such alterations commonly harbor either oncogenes or tumor suppressor genes depending on whether they are present in increased or decreased copy number, respectively, their proper identification is crucial. Defining DNA copy number altered regions and especially the genes involved, offers a basis for better understanding of a cancer development and more

importantly, provides improved tools for clinical cancer management, such as new diagnostics and therapeutic targets.

2.6.2 DNA copy number variation detection methods

Historically, cytogenetic analysis of Giemsa-stained metaphase chromosomes was applied to ascertain chromosomal abnormalities. This technique was used to identify balanced and unbalanced structural and numerical chromosomal changes, however it was not sensitive enough to detect subtle rearrangements (less than 4 Mb). Further implementation of fluorescent *in situ* hybridization (FISH) improved the diagnostic resolution and, until recently, had been considered the method of choice for detecting chromosomal imbalances and rearrangements. However, this technique is time-consuming and targeted, meaning that requires prior knowledge of the chromosomal region(s) of interest and therefore interrogates one or more candidate chromosomal loci at a time.

Only the development of comparative genomic hybridization (CGH) in 1992 (Kallioniemi *et al*, 1992), which was initially invented as a molecular tool in cytogenetics, opened the possibility for genome-wide copy number screening. CGH effectively reveals any DNA copy number changes (i.e., gains, amplifications, or losses) that are present in at least 30–50% of the specimen cells (Kallioniemi *et al*, 1994). However, it does not detect balanced translocations, inversions, and other aberrations that do not change copy number. The theoretical detection limit of CGH has been estimated to be about 2 Mb (Piper *et al*, 1995).

Substitution of the metaphase chromosomes with array-based CGH (aCGH) established by Solinas-Toldo *et al* (Solinas-Toldo *et al*, 1997) and further refined by Pinkel *et al* (Pinkel *et al*, 1998) has simplified the analysis procedure and solved many technical issues connected with cytogenetic chromosome preparations. However, the main advantage of this new aCGH technique was the ability to perform copy number analyses with much higher resolution, than was ever possible using chromosomal CGH (Davies *et al*, 2005; Lockwood *et al*, 2006; Pinkel & Albertson, 2005).

In aCGH, equal amounts of labeled genomic DNA from a test and a reference sample are co-hybridized to an array containing the DNA targets. Those interrogating probes (targets) used for the microarrays' construction are pieces of human genomic DNA, initially in

the form of yeast artificial chromosome (YAC; 0.2–2 Mb in size), bacterial artificial chromosomes (BACs) or P1 (PAC) clones (size of 75–200 kb), smaller insert clones such as cosmids (size of 30–40 kb) and fosmids (size of 40–50 kb), or more recently oligonucleotides (25–85 mers) that are automatically spotted and immobilized onto glass slides using split metal pins or glass capillaries.

In aCGH, either a pool male/female DNA, or, more reliable, matched normal DNA from the same person are used as a reference. Genomic DNA from the patient and reference are labeled with fluorescent dyes and hybridized onto the sildes containing the arrayed probes from the genome. Slides are scanned and the spots' intensities are measured and quantified using feature extraction software. The resulting ratio of the fluorescence intensities is proportional to the ratio of the copy numbers of DNA sequences in the test and reference genomes. If the intensities of the dyes are equal the region is interpreted as having equal quantity of DNA in the test and reference samples; if the ratio is altered indicates either a loss or a gain of the patient DNA at that specific genomic region.

In addition to aCGH, high-density SNP arrays, principally developed to detect single nucleotide variation, have become more popular for copy number profiling. The advantage of SNP arrays is, that in contrast to standard aCGH they are able to detect loss of heterozygosity events (LOH) and copy number-neutral LOH in form of acquired uniparental disomy (UPD). These type of regions are particularly interesting in cancer research, because of their probability of containing either a mutated tumor suppressor gene (TSG) or oncogene with loss of their normal allele (Nowak *et al*, 2009). However, they have also some limitations, because SNPs in these arrays are not uniformly distributed across the genome and are sparse in regions with segmental duplications or complex aberrations (Carter, 2007). To overcome these limitations, the new generations of SNP genotyping arrays have now incorporated additional nonpolymorphic (NP) markers to provide more comprehensive coverage of the human genome.

Recently emerging next-generation sequencing technologies provide increasingly high-resolution analyses of copy-number alterations in cancer genomes. They enable to achieve extremely high resolution, more than 3 times higher than the current generation microarrays. Therefore they overpower traditional microarrays in the precision of mapping chromosomal breakpoints and detection of extremely small intragenic events. In addition, they are also able to detect structural rearrangements and minimize the effect of the contamination of the tumor sample with normal cells by performing deeper sequencing (Campbell *et al*, 2008; Chiang *et al*, 2009). However the cost and to some extent the analysis

and interpretation of the obtained data is still a bottle neck for their wide implementation in the copy number analysis (Chiang *et al*, 2009; Shendure, 2008).

2.6.3 DNA copy number changes in familial and sporadic EOC

As for other tumor types DNA copy number profiling has been widely applied in ovarian cancer research in order to define the genomic aberrations in the tumors, search for genes involved in the carcinogenesis of particular cancer subtypes, get more insight in diagnostic classification and assessment of tumor progression or patients' prognosis.

Since late 1960s, when the first karyotypes of ovarian cancer were published (Yamada *et al*, 1966) it was clear that epithelial ovarian cancer is characterized by highly aneuploid cells with high heterogeneity both within and between individual cases. Improvements in cytogenetic techniques lead to the publication of more than 150 ovarian cancer karyotypes by 1990 (Mitelman, 2008) but only the development of the comparative genomic hybridization (CGH) in 1992 (Kallioniemi *et al*, 1992) enabled identification of the most common DNA copy number changes. By 2000, the most frequent gains such as: 8q, 3q, 1q and 20q and losses at 4q, 5q, 8p, 22q, 18q and 17p had been well annotated, however identification of specific target genes remained problematic due to high cytogenetic complexity and heterogeneity of ovarian cancer (Gorringe & Campbell, 2009). Currently HGSOCs, as the most common and aggressive subtype, are getting a lot of researchers' attention. The most recent and comprehensive study of HGSOC carried out by The Cancer Genome Atlas (TCGA) revealed that these tumors are characterized by surprisingly high level of genomic instability with complex chromosomal alterations, which is in contrast to many other solid tumors (Bowtell, 2010; TCGA, 2011). That study identified 8 recurrent gains and 22 losses, all of which had been previously reported. Five of the gains and 18 of the losses occurred in more than 50% of the tumors.

Although sporadic EOC tumors have been quite extensively studied, much less is known about DNA copy number changes in hereditary ovarian tumors.

The few existing studies have rendered contradictory results, either reporting no significant differences in the number of genetic alterations between hereditary and sporadic cases (Ramus *et al*, 2003; TCGA, 2011; Zweemer *et al*, 2001), or on the contrary observing higher genomic instability in hereditary cases (Israeli *et al*, 2003; Patael-Karasik *et al*, 2000). Also divergent data have been produced regarding global degree of genomic instability or

type of dominating alterations, indicating higher contribution of gains (Israeli *et al*, 2003) or losses (Leunen *et al*, 2009) in the overall genomic instability of hereditary cases. Such contradictory results, might be due to the limited number of tumors included (Israeli *et al*, 2003; Leunen *et al*, 2009; Patael-Karasik *et al*, 2000), the use of low resolution techniques (Patael-Karasik *et al*, 2000; Ramus *et al*, 2003; Zweemer *et al*, 2001) or application of different algorithms (Leunen *et al*, 2009; TCGA, 2011) *(Table 3).*

Table 3. Summary of a few existing CGH studies on familial EOC

Study	Method resolution	Samples	Copy number alterations characteristics for given group of tumors			Main results	
			group	gains	losses		
TCGA,2011	aCGH oligo 1M Agilent	489 HGSOC: 27 BRCA1 20 BRCA2 442 sporadic	BRCA1/2 impared			none of the regions were significantly more frequently altered in BRCA1/2 impaired cases (germline mt +somatic mt + BRCA1 methylaion) vs. BRCA1/2 wild-type	
Domanska et al, 2010	aCGH BAC 32k	12 BRCA1 8 HNPCC 24 sporadic	BRCA1	7q36 11q13-q24 (11q13) 17p13	4q34-35 8p23-21 8p21-p12 12q21-q23 12q14 13q12-q32 17q22-23 (17q21) 18p11-p21 19p13	Gains were more common in sporadic tumors while losses in BRCA1 cases	
Leunen et al, 2009	aCGH BAC 1M	13 EOC: 5 BRCA1 8 sporadic	BRCA1	2q24 2q31 10q (10q11) 11q22 13q22	5q 7 8q22-ter12q24 15q15 15q2516 17 18 19 22q13	Copy number gains-the most frequent in sporadic tumors, while copy number losses in BRCA1 tumors, no significant differences in the length of the alterations between two groups, longer CNL in BRCA1 vs. sporadic	
Caserta et al, 2008	aCGH BAC 1M	10 EOC with familail history	Familial	8q 9q 12p	6q 9p 10q 17p 21q 22q		
Ramus et al,2003	CGH	141 EOC: 46 BRCA1 18 BRCA2 44 BRCAX 28 sporadic	BRCA1	9pter-p21, 11q14-q22 (11q13)	12q21-23 13cen-q14 17p 17cen-q21	No significant differences in number of genetic alterations between BRCA1, BRCA2 BRCAX or sporadic	41 CNA regions were identified to differentiate between 4 groups
			BRCA2& BRCAX	no regions significantly enriched			
Isreali et al, 2003	CGH	46 EOC: 11 BRCA1 8 BRCA2 27 sporadic	BRCA1	1q21-23, 2q, 3q26, 8q24-ter	9q,19	The average nr of genomic alterations was significantly higher in carriers vs. non-carriers group, the pattern of the chromosome	The mean nr of gains was around 2 times higher than losses in each group
			BRCA2	1p, 1q, 2q,3q26, 8q24-ter	16,22		
Zweemer et al, 2001	CGH	36 EOC: 13 BRCA1 2 BRCA2 21BRCAX	Familial	2q31-32 3q26 6cen-p23 8q23-qter 11q22 13q22 17q24-25	8p21-pter 9q31-33 11p15 12q24 13q14 15q11-15 15q24-25 16q22-qter 17p12-13 18q21 20q13 22q13 Xp21-22	The mean nr of copy number losses more than two times higher than gains	Losses at 8p21-pter, 12q24, 15q11-15, 15q24-25, 22q13 considered as much more frequent in familial than in sporadic cases
Patel-Karasik et al, 2000	CGH	12 EOC: 3 BRCA1 1 BRCA2	BRCA1		9q 19q	More genomic alterations in carriers than in sporadic cases	
Tapper et al,1998	CGH	36 EOC: 16 BRCA1 4 BRCA2 20 sporadic	BRCA1	2q24-q32 amplification		The ratio of copy nr gains to losses: 3 vs1	Extensive genomic similarity between familial and sporadic cases

BRCA impared: tumors with *BRCA1*/2 germline or somatic mutations or *BRCA1* methylation; all other BRCA1/2 in Table refer to tumors from germline mutation; HGSOC-High Grade Serous Ovarian Carcinomas; HNPCC- Hereditary Non-Polyposis Colorectal Cancer

2.6.4 DNA copy number changes as clininaly relevant markers in EOC

Few of the studies focused on describing DNA copy number changes in sporadic or familial epithelial ovarian aimed to define specific aberrations that may have clinical relevance in predicting outcome in ovarian cancer (Baumbusch *et al*, 2013; Bruchim *et al,*

2009; Engler *et al*, 2012; Hu *et al*, 2003; Nanjundan *et al*, 2007; Suzuki *et al*, 2000; Wang *et al*, 2012b; Yamamoto *et al*, 2009).

Some markers that may be applied in predicting tumor progression and recurrence have been described (Bruchim *et al*, 2009; Hu *et al*, 2003; Wang *et al*, 2012a). For example higher risk of recurrence has been proposed to be associated with 5p gain, while 1p gain and 5q loss with its decrease (Bruchim *et al*, 2009). Also, amplification at 5q31–q35 has been linked to poor prognosis, while 4p16 loss to better outcome (Birrer *et al*, 2007). Nevertheless, most studies that focused on assessment of specific alterations were limited by an absence of independent copy number validation datasets (Bruchim *et al*, 2009; Hu *et al*, 2003; Nanjundan *et al*, 2007; Yamamoto *et al*, 2009). Other studies including independent validation series were mainly focused on description of general features (ie. genomic instability or LOH profiles) more difficult to implement in the clinic than distinct individual changes (Baumbusch *et al*, 2013; Wang *et al*, 2012a).

In order to define novel prognostic and predictive markers in EOC some limitations have to be addressed. Among them, the use of large discovery series and high-resolution platforms; availability of clinical data that allow to adjust the results for already established prognostic factors and, very important, robust validation.

OBJECTIVES

It has been reported that hereditary and sporadic EOC present distinct oncogenic pathways. However, little is still known about their resemblance or divergence at the genomic level. Given the recently confirmed relevance of DNA copy number changes as drivers of ovarian oncogenesis and the growing clinical implications of the *BRCA1/2* mutation status, we proposed the following objectives:

1. To characterize the genomic alteration profiles of familial (BRCA1, BRCA2 and BRCAX) and sporadic EOC at high resolution level in order to:
    - Describe the general rate and pattern of genomic instability in the different tumor groups
    - Define altered regions, genes and pathways common to all tumor groups and specific to each of them
2. To find associations between DNA copy number-based groups of EOC and immuno-histopathological and clinical features
3. To define DNA copy number changes with prognostic and/or predictive value

OBJETIVOS

Se ha descrito que los CEO hereditarios y esporádicos presentan diferentes vías oncogénicas. Sin embargo todavía se conoce poco acerca de sus similitudes o diferencias a nivel genómico. Recientemente se ha confirmado el importante papel que tendrían las alteraciones en el número de copias de ADN en la oncogénesis de los tumores de ovario. Teniendo esto en cuenta y también la creciente implicación clínica de las mutaciones en los genes *BRCA1* y *BRCA2*, hemos propuesto los siguientes objetivos:

1. Caracterizar los perfiles de alteración genómica de los CEO familiares (BRCA1, BRCA2 y BRCAX) y esporádicos con herramientas de alta resolución para:

    - Describir los niveles y patrones generales de inestabilidad genómica en los distintos grupos de tumores

    - Definir alteraciones en regiones, genes y rutas celulares comunes entre los distintos grupos de tumores y específicos de cada uno de ellos

2. Determinar si grupos de CEO definidos de acuerdo con su patrón de alteraciones genómicas presentan asociación con características immunohistopatológicas y clínicas

3. Definir alteraciones en el número de copias de ADN con valor pronóstico y/o predictivo

MATERIALS AND METHODS

I PATIENTS AND TUMORS

I.1 DISCOVERY SERIES

Discovery series consisted of 75 formalin-fixed paraffin-embedded (FFPE) epithelial ovarian tumors. Fifty-seven corresponded to patients from high-risk breast and ovarian cancer families (21 with mutation in *BRCA1*, 6 with mutation in *BRCA2* and 30 without either mutations) and 18 to sporadic cases.

Families selected for this study fulfilled one of the following criteria: (a) at least two cases of ovarian cancer in the same family line; (b) at least one case of ovarian cancer and at least one case of breast cancer in the same family line; (c) at least one woman with both breast and ovarian cancer; (d) at least one woman with bilateral ovarian cancer. All families were ascertained at the Spanish National Cancer Research Center (CNIO) familial cancer consultancy and at different Spanish hospitals: Hospital Fundación Alcorcón**,** Hospital Doce de Octubre, Fundación Jiménez Díaz and Hospital Ramón y Cajal (Madrid); Hospital Sant Pau (Barcelona); Hospital Donostia (San Sebastian); Hospital General de Albacete (Albacete). Index cases were analyzed for germline mutations throughout the coding regions and splice site boundaries of the *BRCA1* and *BRCA2* genes by a combination of different methods including denaturing high performance liquid chromatography (DHPLC) and direct sequencing (Beristain *et al*, 2007; de la Hoya *et al*, 2001; Diez *et al*, 2003; Llort *et al*, 2002; Milne *et al*, 2008; Osorio *et al*, 2000). Individuals with no mutations in *BRCA1* or *BRCA2* genes were designated as BRCAX cases. Ovarian tumors from index cases, confirmed carriers or non-tested obligate carriers were collected from *BRCA1* and *BRCA2* positive families. Tumors from index cases and first-degree relatives of index cases were collected from *BRCA1/2* negative families (BRCAX families).

Sporadic cases (with no reported first or second degree relative with breast or ovarian cancer) were obtained from one single institution (Hospital Virgen del Rocio, Sevilla) and were selected to match the histopathological distribution of the familial series.

The study was approved by The Ethical Committees of the participating centers and written informed consent was given by each individual involved in the study.

1.1.1 Histopathological classification of tumors

All tumors were blindly reviewed by two pathologists (Ivan Muñoz-Repeto and Jose Palacios) and classified histopathologically. Immunohistochemical expression of markers such as Wilms Tumor protein (WT1), tumor protein p53 (TP53), estrogen receptor (ER), progesterone receptor (PR) and cyclin-dependent kinase inhibitor 2A (p16) (CDKN2A) was performed in order to assist in the differential diagnosis (Kalloger *et al*, 2011; Kobel *et al*, 2009). The antibodies, dilutions, suppliers, visualization systems, immunostainers and scoring of the staining are shown in Supplementary Table 1. Grading of serous tumors was performed according to two-tier M. D. Anderson Cancer Center (MDACC) system (Gilks *et al*, 2008; Malpica *et al*, 2004) while the rest of the histological types were graded according to World Health Organization criteria (Organization, 2004; Silverberg, 2000).

A subgroup of tumors within the type II carcinomas was defined in order to allow for comparisons between more homogenous groups of high-grade neoplasms. This subgroup consisted of high-grade serous tumors of solid growth pattern and undifferentiated carcinomas (hereafter referred as to "subgroup of type II tumors"). Detailed information is shown in *Table 4*.

1.1.2 Patients´ clinical data

Comprehensive clinical data (e.g. FIGO stage, response to the therapy and survival data) was retrieved with an approved clinical form from all the patients and summarized in *Table 4*.

Included patients were diagnosed and underwent surgical intervention between 1990 and 2008. Surgical resections were classified as optimal (less than or equal

1cm) or suboptimal (greater than 1cm diameter of residual tumor). Progression Free Survival (PFS) was calculated from the date of primary surgery to the date of disease progression as specified by a rise in CA125 or radiological or surgical evidence of relapse. The length of Overall Survival (OS) was defined from the date of primary laparotomy to the date of patient death. For both analyses, time was censored at the date of the last follow-up.

Overall Survival (OS) data was available for 76% (n=52) of the patients, while Progression Free Survival (PFS) for 57% (n=39) of the patients. Median follow up time for was 67 months (95% CI: 59-75): 72 months (95% CI: 62-82) for familial and 36 months (95% CI: 12-60) for sporadic cases. Platinum sensitivity was defined as progression free survival of more than 6 months after the last dose of platinum-based adjuvant therapy and was observed in 26 out of 31 patients (83%) for whom sufficient clinical information was available. Response to chemotherapy was evaluated retrospectively according to the World Health Organization evaluation criteria (Miller *et al*, 1981). This evaluation was based on data from medical records describing patients´ clinical condition and CA125 levels at 3–4 week intervals. Complete remission (CR) was defined as disappearance of all clinical and biochemical symptoms of ovarian cancer evaluated after completion of first-line chemotherapy and confirmed at 4 weeks.

1.2 INDEPENDENT VALIDATION SERIES

Two independent series were used to validate the associations of 6q24-26 deletion with patient´s outcome.

The **first series** was composed of 103 EOCs randomly distributed on 4 tissue microarrays (TMAs). It contained 77 sporadic and 26 familial tumors (7 BRCA1, 9 BRCA2, 10 BRCAX) with available clinical data. Forty-nine per cent of tumors in this series were classified as serous (35% HGSOCs and 14% LGSOCs), 24% as clear cell carcinomas, 14% as endometrioid and 13% as others (including 1% with missing information). More than half of them (59%) were of high FIGO stage (III and IV). Patients were diagnosed and underwent surgical intervention between 1991 and

2010. Definition and evaluation of clinical data variables was conducted as specified for the discovery series.

The **second series** consisted of 411 high grade ovarian serous ovarian carcinomas (HGSOC) from The Cancer Genome Atlas (TCGA) with publically available DNA copy number and clinical data (TCGA, 2011). This series included mainly sporadic (91%), high grade (87%), and high (III and IV) FIGO stage (95%) serous adenocarcinomas.

In addition, 1436 EOCs with gene expression and clinical data available from the online survival analysis tool - Kaplan Meier plotter (Gyorffy *et al*, 2012) were used for validation at the gene expression level. This large dataset of EOCs is a unification of 10 different gene expression studies using Affymetrix platform. Available tumors were mainly serous (74%), high FIGO (72%), and high grade (61%) EOCs.

2 METHODS

2.1 TISSUE MICROARRAYS (TMAs) CONSTRUCTION

Ovarian tumors from the discovery and the first validation series were placed on 4 TMAs that were constructed as previously described (Munoz-Repeto *et al*, 2013; Palacios *et al*, 2003). Briefly, representative areas of the tumors selected based on hematoxylin and eosin-staining (HE) were marked on individual paraffin blocks. Then two tissue cores (1-mm diameter) for each specimen were obtained from the selected area as shown in the *Figure 4*. These tissue cores were arrayed in a predetermined order into a receptor paraffin block using a tissue microarray workstation (Beecher Instruments, Silver Spring, MD), in the Immunohistochemistry Unit at the CNIO. In addition, at least 2 non-neoplastic control tissues (amygdala and/or ovarian) were included in each TMA.

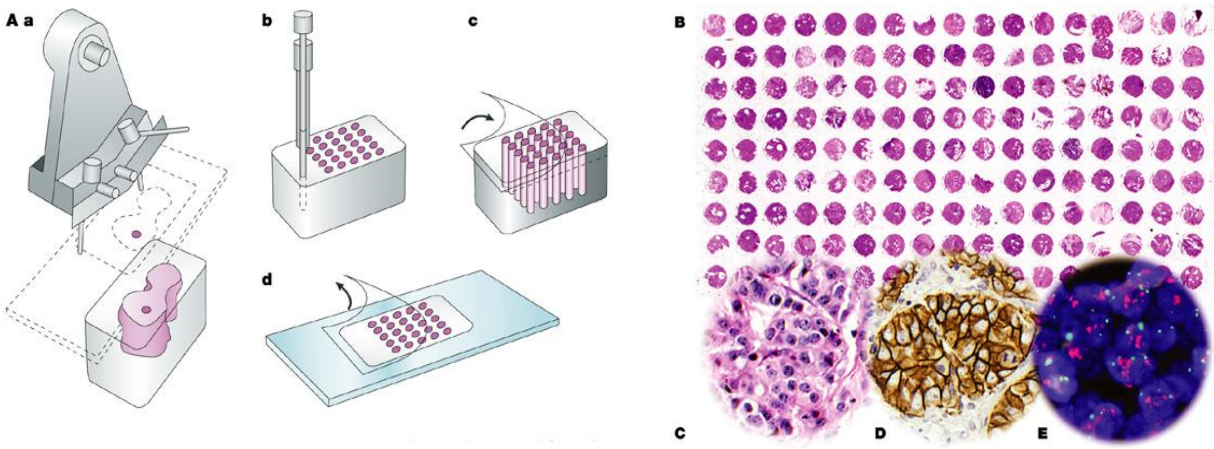

Figure 4. Construction of tissue microarrays: (**A**) Cylindric tissue cores are removed from a conventional ('donor') paraffin block using a tissue microarrayer; these are released into premade holes of an empty ('recipient') paraffin block. Regular microtomes can be used to cut tissue microarray sections. (**B**) Overview of a haematoxylin-eosin (H&E) stained TMA section. Each tissue spot measures 0.6 mm in diameter. (**C–E**) Examples of magnifications of sectors from tissue spots from different experiments. (**C**) H&E staining (**D**) Immunohistochemistry (**E**) FISH analysis (adapted from (Sauter *et al*, 2003)

2.2 IMMUNOHISTOCHEMICAL ANALYSIS

2.2.1 Immunohistopathological characterization of the tumors

The immunohistochemical staining was performed on the 4 previously built TMAs using 30 IHC markers and the EnVision method with a heat-induced antigen retrieval step at the Immunohistochemistry Unit at CNIO. The expression of the 30 markers was assessed independently by two pathologists Ivan Muñoz-Repeto and Jose Palacios. Evaluated proteins were involved in a variety of different cellular processes such as hormone signaling (ER, PR and AR), proliferation (topoisomerase IIα, Ki-67), cell cycle (CCND1, CCNE1, CDKN2A, p21, p27, p53, RB1, β-tubulin III), apoptosis (BCL-XL, survivin), cell adhesion (E-cadherin), tumor progression (KLK7, MMP7, PIK3CA), angiogenesis (CD105, VEGF), signaling (C-KIT, EGFR, β-catenin) or DNA repair (ERCC1, XPG, XPF, RAD50, RAD51 and CHEK2). The antibodies, dilutions, suppliers, visualization systems, immunostainers and scoring used are shown in *Supplementary Table 1*. Between 100 and 150 cells per core were scored to determine the percentage of positive nuclei, cytoplasm, or membranes, depending on the marker. Nuclear staining was evaluated for ER, PR, AR, p53, Ki-67, CCND1 and CCNE1, p27, p21, RB1 topoisomerase IIα, survivin, RAD50, RAD51, XPF, XPG, CHEK2, ERCC1, EGFR, MMP7, KLK7, PIK3CA, E-cadherin and β-catenin. Cytoplasmic staining was assessed for CDKN2A, BCL-XL, survivin, VEGF, C-Kit, EGFR, E-cadherin and β-catenin. Membrane staining was evaluated for EGFR, E-cadherin and β-catenin. The thresholds to determine over-expression of each marker were established based on literature (*Supplementary Table 1*) as described elsewhere (Bali et al, 2004; Brun et al, 2008; Duncan et al, 2008; Honrado et al, 2005; Lin et al, 2001; Ni et al, 2004; Raspollini et al, 2004; Rosen et al, 2006a; Schindlbeck et al, 2007; Schmandt et al, 2003; Steffensen et al, 2009; Tangjitgamol et al, 2009; Xia et al, 2009). The percentage of stained nuclei, independent of the intensity, was scored for ER, PR, AR, Ki-67, p53, CCND1, CCNE1, p27, p21, RAD51, XPF, XPG, CHEK2, ERCC1, EGFR, MMP7 and KLK7. For EGFR, E-cadherin and β-catenin expression, the percentage of cells with membrane staining and staining intensity was determined.

2.2.2 Immunohistochemical validation of aCGH results

Three of the evaluated antibodies targeting genes altered by high-amplitude events (homozygously deleted *CDKN2A* and *RB1* and amplified *CCNE1*) were used to validate the aCGH hybridizations and analytical approaches. Expression levels in the amplified and deleted samples were compared to the mean expression levels in tumors with normal DNA copy number status at the corresponding locus (according to the aCGH profiles).

2.3 aCGH: HYBRIDIZATIONS AND DATA PRE-PROCESSING

2.3.1 DNA isolation and labeling

Total genomic DNA was extracted from three 10-µm-thick FFPE tissue sections per tumor. After deparaffination and rehydration, sections were hematoxylin and eosin (HE) stained and tumor areas were delimited by a pathologist and macrodissected with a surgical blade to ensure at least 80% tumor content. Tumor and conserved normal tissue (when available) were separately dissected and placed in independent tubes. DNA extraction was carried out according to standard protocols including overnight proteinase K digestion and a column-based commercially available kit following the manufacturer´s instructions (QIAamp DNA mini kit; Qiagen, Westburg, Leusden, The Netherlands). DNA quantity and quality was assessed using the NanoDrop ND-1000 UV-VIS Spectrophotometer version 3.2.1. (Nanodrop Technologies, Wilmington, DE, USA).

Labeling of test and reference DNA was performed with the Enzo Agilent aCGH labeling kit according to the manufacturer´s instructions (Enzo Life Sciences, Farmingdale, NY, USA). Briefly, 500 ng genomic DNA was combined with a mixture of random primers and reaction buffer to a final volume of 39µl. The DNA was denatured at 99^0C for 10 min, and placed on ice. While on ice, 10µl cyanine 3-dUTP and cyanine 5-dUTP nucleotide mixture was added to the test and reference DNA, respectively. At the end 1µl of Klenow DNA polymerase was added to each sample.

After 4 hr incubation at 37^{0}C, 5µl stop buffer was added to stop the reaction. Labelled DNA was purified using the QIAquick PCR Purification Kit (Qiagen, Westburg, Leusden, NL). DNA yield and specific dye incorporation was measured with NanoDrop.

$$SpecificActivity = \frac{(pmol\ per\ \mu L\ dye)}{(\mu g\ per\ \mu L\ genomic\ DNA)}$$

Only samples with specific activity >15 were used for hybridizations.

In 45 out of 75 hybridizations (60%) patient-matched normal DNA was used as a reference (in 26 from conserved normal tissue within the paraffin blocks and in 19 from the patient´s peripheral blood). In the remaining 30 hybridizations (40%) a pool of normal DNA from 20 healthy females was used as a reference (http://www.kreatech.com/products/megapool-reference-dna.html).

2.3.2 Hybridizations

Hybridizations and preprocessing of the data were carried out in Department of Pathology (Microarray Core Facility) and Department of Epidemiology and Biostatistics at the VU University Medical Center Univeristy. The slides used for hybridizations contained four arrays, each with 180,880 *in situ* synthesized 60-mer oligonucleotides (4x 180K, Agilent Technologies, Palo Alto, CA) representing 169,793 unique chromosomal locations evenly distributed across the genome (space ~ 17kb), and 4,548 additional unique oligonucleotides, located at 238 of the Cancer Census genes (http://www.sanger.ac.uk/genetics/CGP/Census/). Cy5-Labeled tumor DNA was combined with an equivalent amount of Cy3-labeled reference DNA in a total volume of 39 µl and mixed with 11µl of 10x blocking agent (Agilent Technologies) and 55µl of 2x hybridization buffer (Agilent Technologies). In addition 6.5 µg of human Cot-1 DNA (Invitrogen, Breda,NL) was used to block repetitive sequences. The hybridization mixture was heated at 95^{0}C for 3 min and immediately incubated at 37^{0}C for 30 min. After centrifugation for 60 sec at 14,000rpm, the hybridization mixture was applied to the slide and placed in an assembly chamber for 24 hr at 65^{0}C and 20 rpm (Agilent Technologies). Next, slides were washed in the following steps: 1

min in the wash buffer 1 at room temperature (RT), 5 min with wash buffer 1 at RT with rotation speed (~750 rpm), 1 min with wash buffer 2 at 37^0C with rotation speed (~750 rpm), and finally 1 min in acetonitrile at RT.

2.3.3 Scanning and image acquisition

Hybridized slides were scanned with Agilent Array scanner MAF0013 (Agilent Technologies), with the laser set to 635nm, Power at 80 and a scan resolution of 3µm. To avoid ozone bleaching, microarrays were scanned in an ozone-free environment (less than 2 ppb ozone). Agilent Feature Extraction software (version 9.5.3) (Agilent Technologies) was used for quantification of the fluorescence intensities of scanned images. Local background was subtracted from the median intensities of both Cy3 and Cy5 channels. The tumor to normal ratio was calculated for each probe, then log2 transformed (log_2 ratio) and normalized against the median of the ratios of all autosomes. Reproducibility and reliability of each single microarray was assessed using Quality Control metrics, which included computation of the average green and red signal intensity at all the probes and using non-hybridizing control probes and quantification of background signal (noise) and signal-to-noise ratio. Average signal intensity >150 with signal-to-noise ratio >20 were regarded as satisfactory. Schematic representation of the aCGH method is shown in *Figure 5.*

The oligonucleotides were mapped according to the human genome build NCBI36/hg18 assembly (March 2006).

2.3.4 Deposition of the aCGH raw data in a public database

The aCGH raw data generated in this study have been deposited at NCBI's Gene Expression Omnibus (Edgar *et al*, 2002) and are accessible through GEO accession number GSE41253 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41253).

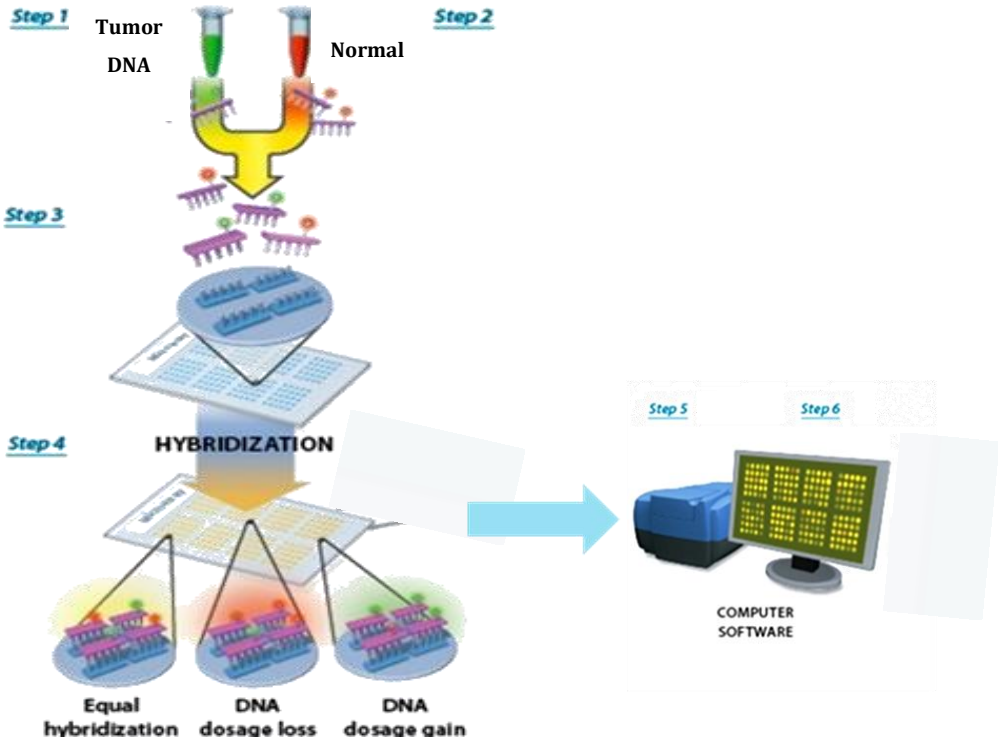

Figure 5. Scheme of two-color aCGH **Step 1-3** Normal (control) and tumor DNA are labeled and applied to the slides containing the arrayed probes from the genome **Step 4** Tumor and control DNA compete with each other to hybridize to the oligonucleotides on the array; **Step 5-6** Scanner measures the spots' intensities and quantify them using feature extraction software

2.3.5 Data normalization, segmentation and calling

Data were processed in the R programming environment v.2.13 (http://www.r-project.org). The $\log_2$ tumor-to-normal ratio was calculated for each spot and normalized to the median values of autosomes. Sex chromosomes were excluded from the analysis.

Possible wave bias was removed with the Wave Smoothing method (van de Wiel *et al*, 2009). Segmentation and calling was done with DNAcopy algorithm implemented in CGHcall v.2.5 with cellularity set to 0.7 and median normalization. Segments with a probability score higher than 0.5 were considered amplified, gained or lost and corresponding ordinal values were assigned (2,1,-1, respectively and 0 in no copy number change).

Visualization and further analysis of data was performed in Nexus Copy Number v5.1 (BioDiscovery, Inc; El Segundo, CA). For defining the exact location of amplifications, gains and losses called data was used. For determination of homozygous deletions raw log2 data were applied to Nexus Copy Number v5.1 and Rank Segmentation algortim was used with the following settings: a significance threshold: 1.0E-5 , maximum contiguous probe spacing of: 1,000 kb and minimum number of probes per segment: 3, was used.

Although the use of patient-matched normal and tumor samples in most hybridizations allowed elimination of germline polymorphic copy number variants (CNV), genomic regions covered entirely by CNVs previously described in the human genome (from The Center for Applied Genomics' Database of Genomic Variants, http://projects.tcag.ca/variation/tableview.asp?table=DGV_Content_Summary.txt) were removed from the analysis. Cancer Census genes located at the defined regions were obtained from http://www.sanger.ac.uk/genetics/CGP/Census/.

2.4 aCGH: DOWNSTREAM DATA ANALYSIS

2.4.1 Overall genomic instability rate: number and length of alterations

To determine the degree of genomic instability in each subgroup of tumors (BRCA1/2/X and sporadic), total number of alterations and number of alterations of a particular type (homozygous deletions/losses/gains/amplifications) per sample were calculated. Also, total size of altered genome and size accounted by gains and losses were calculated by adding up the lengths of individual segments. Next, average number of changes and average size of altered genome were calculated for sporadic tumors and for each group of familial tumors. Also, in order to determine the relative contribution of each type of change (losses or gains) we computed the ratio of the average number of losses to the average number of gains within each tumor subtype.

Similarly, we calculated the ratio of the average lost genome length to the average gained genome length.

2.4.2 Common and potentially specific altered regions

In order to visualize the general pattern of chromosomal changes, frequency plots were generated and a list of recurrent Minimal Common Regions (MCRs) of alterations for each tumor subtype (BRCA1/2/X and sporadic) were defined using Nexus software with "Aggregate cut off" (minimum frequency) of 25% and the "Peaks Only" option to refine the size of the altered region. High-amplitude DNA copy number changes (amplifications and homozygous deletions) were considered recurrent when present in at least two cases from the group. MCRs for each group has been defined with minimum frequency of 25% for gains and 35% for losses. For the region to be considered commonly altered across tumor groups it had to be present among the top 60 most frequent MCRs of alterations in at least three of the groups.

To define potential group-specific alterations, significant differences in frequency of alterations between tumor subtypes were detected by Fisher Exact Test (FET) implemented in Nexus (with minimum recurrence differences between groups of 25% for gains and 35% for losses, and a p-value < 0.05). We also ran CGHtest (R package and www.few.vu.nl/~mavdwiel) to define regions exhibiting significant differences in frequency between tumor subtypes, using the chi-square test and false discovery rate (FDR) correction with 10,000 permutations, data simplification at the level of 0.01 and the "stepup" option. Regions defined to be significant (with FET and chi-square test, P<0.05) and an FDR < 0.2 (as defined by CGHtest) were listed as potentially group-specific. To further refine this list, our regions were compared with the regions defined in the TCGA ovarian study that characterized a series of 489 mainly sporadic high-grade ovarian carcinomas (TCGA, 2011). Frequencies of regions of amplification, gain, loss and homozygous deletion in our list were compared to frequencies of "High Gains", "Low Gains", "Shallow deletions" and "Deep Deletions", respectively, reported for the same chromosomal locations in the TCGA study (using

"wide peaks" ranges). Only candidate familial-specific regions from our list that were not defined as significant focal alterations in the TCGA series were considered to have some specificity for one of the familial groups. Similarly, regions significantly associated with sporadic tumors in our series that were also defined as significantly frequent in the TCGA study were considered to show some specificity for sporadic cases.

2.4.3 Biological pathways altered by copy number changes

In order to know about the specific pathways and processes that might be targeted by copy number alterations in each tumor group we performed a pathway enrichment analysis. The analysis was performed using the lists of the genes encompassed within the minimal common regions of gains, losses, amplifications and homozygous deletions generated per each group, in the previous steps. Those lists were used to identify functions and processes enriched in copy number altered genes over the whole genome, in each tumor group. A comprehensive pathway enrichment analysis was performed integrating results from three publicly available databases: FatiGO (Babelomics v4), DAVID v6.7 (Database for Annotation, Visualization and Integrated Discovery) and Ingenuity Pathway Knowledge Base v9 (IPA). In case of IPA the direction of the alterations: either copy number increase (gains and amplifications) or copy number loss (deletions and homozygous deletions) was associated with each HUGO gene identifier. Only pathways defined to be significantly altered ($P < 0.05$) (enriched in gained and lost genes) by at least two different tools with at least one significant after correction for multiple testing (FDR<0.05) were considered.

2.4.4 Unsupervised analysis

WECCA (Weighted Clustering of Called aCGH Data) R package (Van Wieringen *et al*, 2008) was used for unsupervised hierarchical clustering (total linkage and overall similarity algorithms) and generation of the heatmaps for called aCGH data. The maximum pairwise symmetrized Kullback Leibler divergence score (Tumminello *et al*, 2007) was used to define regions that best distinguished rendered clusters. Regions with the highest scores (≥1.5 for carcinomas; ≥3 for type II tumors) were defined as the ones differentiating clusters the best. Their exact location was defined based on GRCh37/hg19 assembly. The CGHtest (R package and www.few.vu.nl/~mavdwiel) was used to determine whether the selected regions were significantly differently represented between clusters after the Benjamini–Hochberg FDR correction (with 10,000 permutations and the step-up procedure) that accounted for multiple testing. An FDR less than 0.01 was considered statistically significant.

2.5 ASSOCIATION OF DEFINED REGIONS WITH SURVIVAL

Defined regions were evaluated for their associated with overall and progression free survival using multivariate Cox regression model with forward conditional method in which all the tested regions were included together with all the confounding factors significant in the series (P<0.05): FIGO stage, and residual tumor. Final estimation of Hazard Ratios and P-values of the significant regions was calculated in the Cox regression model adjusted for two significant cofactors (FIGO stage, and residual tumor).

2.6 VALIDATION OF THE PROGNOSTIC VALUE OF 6q24-q26 DELETION

2.6.1 Validation in an independent series of tumors by Fluorescence in situ Hybridization (FISH)

Fluorescence *in situ* hybridization was carried out in the Molecular Cytogentics Group (CNIO) using an independent series of 103 EOCs included in TMAs described in the Patients and Tumors section. Thirty-five additional tumors from the discovery series were analyzed to technically confirm aCGH data.

FISH analysis was performed according to Vysis' protocol (Vysis, Downers Grove, IL, USA) with slight modifications (Moreno-Bueno *et al*, 2003). In brief, the test probe, composed of three BAC clones: RP11-608N7, RP11-68I24, RP11-100N9 mapping to the 6q25.1 region (157099063-157530401) was labeled by nick translation with dUTP-SpectrumOrange (Abbott Molecular, IL, USA). Similarly two BACs: RP11-410B13 and RP11-107P14 targeting 6p21 (43490072-43543812) were labeled with dUTP-SpectrumGreen (Abbott Molecular, IL, USA) and used as reference for 6q deletion.

The BACs were obtained from BACPAC Resource Center (BPRC) at the Children's Hospital Oakland Research Institute (Oakland, CA). The probes were blocked with Cot-1 Human DNA (Roche Diagnostics GmbH, Mannheim, Germany) to suppress repetitive sequences. Probes specificity was confirmed on normal peripheral blood metaphase cells.

Paraffinated tissue slides were deparaffinated and boiled in a pressure cooker with 0.5 mM EDTA (pH 8.0) for 10 minutes and incubated with pepsin at 37°C for 21 minutes and dehydrated. The probe was first denatured at 96°C for 5 minutes and hybridized overnight at 37°C in a humid chamber. After posthybridization washes, the tissue samples were counterstained with DAPI VECTASHIELD solution (Vector Laboratories Inc., Burlingame, CA, USA) for chromatin counterstaining. Cell images were captured using an Olympus BX61 microscope with a 100×/1.30 NA oil objective and a cooled charge-coupled device camera (CoolSNAP ES; Photometrics)

connected to a computer running the CytoVision Image Analysis System (Applied Imaging, Newcastle, UK).

The FISH analysis was performed by two investigators who had no prior knowledge of the genetic, clinical, or immunohistochemical features of the tumors. On average 5 (3-7) high-power fields with well defined-nuclei were analyzed per each sample (always in duplicates). Deletion was considered as positive, when at least 100 cells/per tissue core showed one red signal less than green in the same nuclei. The deletion status in the tumors was then analyzed for association with patient´s outcome.

2.6.2 Validation using data from TCGA ovarian study

To validate the association of the 6q24.2-q26 deletion with patients outcome data for 411 HGSOCs from the TCGA ovarian cancer study (TCGA, 2011) was used. Normalized $\log_2$ ratios from 1M Agilent Sure Print Human Microarray platform were downloaded from TCGA website (https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm) and subjected to the segmentation and calling (in the Structural Computational Biology Group) using the algorithms applied in the discovery series. Tumors were considered deletion-positive if at least 90% of the defined region (6q24.2-q26, 145,593,087-162,867,181) was lost. Deletion status was subsequently analyzed for associations with patients' survival data (provided by TCGA).

2.6.3 Validation at the global gene expression level

The association of the 6q24-q26 deletion with survival was validated at the gene expression level using KM-plotter (Gyorffy *et al*, 2012) with the JetSet probset (Li *et al*, 2011). The KM-plotter is an online tool that allows the assessment of the prognostic value of the expression levels of microarray-quantified genes in ovarian cancer patients. The current database is set up using gene expression data and survival information of 1436 ovarian cancer patients downloaded from Gene

Expression Omnibus and The Cancer Genome Atlas (10 different datasets). The mean expression of the genes from the 6q24-q26 region was used and the data was dichotomized at the automatically selected best fitted cut-off into higher and lower expressing groups. Kaplan–Meier and log-rank test were used to characterize the distribution and estimate the outcomes. In addition Cox proportional hazard model was used to estimate the hazard ratios and 95% Confidence Intervals. The association with overall survival was assessed in all 1436 EOC with available OS data and was further confirmed in 799 HGSOCs (grade3) and in 675 high FIGO stage (III and IV) HGSOCs tumors alone.

2.6.4 Definition of candidate genes at the 6q24-q26

In order to propose individual candidate genes that might explain the observed association with patient survival, we first identified those genes in the 6q24-26 region whose loss had an impact on expression. To address that we used 232 tumors from TCGA ovarian study for whose copy number and expression data were available and assessed a total of 81 genes localized at 6q24.2-q26 for whom RNAseq data (RPKM) was available. The Wilcoxon rank test was used to compare the expression values of each gene between tumors with normal copy number status and tumors with genomic loss at each locus. Significant genes were then evaluated for potential association between their expression levels and patient survival. Before running survival analysis, RPKM values were normalized, by subtracting the mean of all samples and dividing by the standard deviation, in order to allow a direct comparison of hazard ratios between genes.

Next, the rescaled gene expression values for each of 296 HGSOCs from TCGA study (for whom clinical and RPKM data were available) were included as an explanatory variable in Cox regression models, together with cofactors significantly associated with survival in the series (FIGO stage, age of diagnosis and BRCA mutation status).

2.7 STATISTICAL ANALYSES

Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL). Comparison of continuous variables was assessed using a two-tailed Student's t-test for variables with approximate normal distribution (as determined by Kolmogorov-Smirnov test), or by Mann-Whitney test otherwise. For categorical data (FIGO stage, tumor histology, grade, *BRCA1/2* mutation status, and some IHC markers) Pearson´s Chi-squared Test or Fisher Exact Test was applied (in case of expected values less than 5). All tests were two-sided and *P*-value<0.05 was considered statistically significant. Overall survival (OS) and progression-free survival (PFS) were defined as specified in the "Patients´ clinical data" section (1.1.2).

2.7.1 Survival analysis

Estimation of survival time distribution was performed using Kaplan-Meier method and differences between survival curves were assessed for statistical significance with log-rank test, if the proportional hazard assumption was valid or Gehan–Breslow–Wilcoxon test otherwise. To adjust for other prognostic factors, potentially acting as confounding variables for each tumor series we created a multivariate Cox proportional hazards regression model including all possibly confounding variables: FIGO stage, residual tumor, age of diagnosis, BRCA mutation status, grade, histological type. In the final model, with tested variable, all covariables with *P*-values<0.05 were included. The prognostic value of 6q24-26 deletion was tested (in discovery and validation sets) by comparing patients positive for the deletion (having at least 90% of the region lost) versus all the others.

All statistical tests were two-sided and nominal *P*-values less than 0.05 were considered statistically significant.

RESULTS

I CLINICO-HISTOPATHOLOGICAL CHARACTERISTICS OF THE FAMILIAL OVARIAN TUMOR SERIES

Most of our familial series was represented by epithelial ovarian carcinomas, however in the series were also 4 borderline entitles, all of which belonged to BRCAX group. The rest of the carcinomas were mainly represented by high-grade serous tumors, however the histopathological features, like histotype and FIGO stage differed between the *BRCA1/2*- mutation carriers and BRCAX group. Most of the mutation carriers were of serous type (BRCA1, 90%; BRCA2, 100%) and of high FIGO stage (FIGO≥III; BRCA1, 57%; BRCA2, 67%) *(Table 4).* Two BRCA1 cases were classified as low grade-endometrioid and one as clear cell but none of the carriers were classified as mucinous or undifferentiated. In contrast, BRCAX group was more heterogeneous and presented a wider range of histological subtypes. Also a higher percentage of BRCAX tumors were diagnosed at early stage compared to carriers (FIGO=I; BRCAX, 31%; BRCA1, 5%; BRCA2, none) *(Table 4).* As expected, hereditary patients were diagnosed at a significantly younger age than sporadic ones (51 *v* 62 yrs, p=0.001) with BRCA2 having the highest age of onset among all hereditary cases, and that was only marginally different than of that from sporadic cases (55 vs 62 yrs, P=0.06).

As specified in Material and Methods a subgroup of tumors within the type II carcinomas was defined with high grade serous carcinomas, to allow for comparisons between more homogenous groups. This subgroup of familial carcinomas was enriched (although not significantly) in *BRCA1/2* mutation carriers, compared to the whole series of carcinomas (type II, 14/22, 64%; whole series 27/53, 51%). Also within this more homogeneous set of tumors the differences regarding age of diagnosis are more distinguishable. More details about patients' and tumors' characteristics are summarized in *Table 4.*

Table 4. Clinicopathological features of familial and sporadic series

			Familial subtypes		
	Sporadic	**Familial all**	***BRCA1***	***BRCA2***	***BRCAX***
Total series	**n=18**	**n=57**	**n=21**	**n=6**	**n=30**
BORDERLINE TUMOURS	***3***	***4***	***-***	***-***	***4***
CARCINOMAS	***15***	***53***	***21***	***6***	***26***
Age at diagnosis					
Median	**62**	**51**	**52**	**55**	**48**
Range	51-78	34-72	36-70	45-72	34-72
Stage					
I	3 ***20%***	9 ***17%***	1 ***5%***	-	8 ***31%***
II	3 ***20%***	7 ***13%***	5 ***24%***	-	2 ***8%***
≥III	9 ***60%***	24 ***45%***	12 ***57%***	4 ***67%***	8 ***31%***
NA	-	13 ***25%***	3 ***14%***	2 ***33%***	8 ***31%***
Grade (Serous)[a]					
Low	-	8 ***19%***	4 ***21%***	1 ***17%***	3 ***18%***
High	12 ***100%***	30 ***71%***	12 ***63%***	5 ***83%***	13 ***76%***
NA	-	4 ***10%***	3 ***16%***	-	1 ***6%***
Grade (Non-Serous)[b]					
1	2 ***67%***	3 ***27%***	1 ***50%***	-	2 ***22%***
2	-	5 ***46%***	1 ***50%***	-	4 ***44%***
3	1 ***33%***	3 ***27%***	-	-	3 ***33%***
Histologic subtype					
Serous	12 **80%**	42 ***79%***	19 ***90%***	6 ***100%***	17 ***65%***
Endometrioid	1 **7%**	3 ***6%***	2 ***10%***	-	1 ***4%***
Mucinous	-	1 ***2%***	-	-	1 ***4%***
Clear cell	2 **13%**	3 ***6%***	1 ***5%***	-	3 ***12%***
Undifferenciated	-	3 ***6%***	-	-	3 ***12%***
Mixed	-	1 ***2%***	-	-	1 ***4%***
Complete response					
Yes	9 **60%**	24 ***45%***	11 ***52%***	1 ***17%***	12 ***46%***
No	4 **27%**	10 ***19%***	4 ***19%***	1 ***17%***	5 ***19%***
NA	2 **13%**	19 ***36%***	6 ***29%***	4 ***67%***	9 ***35%***
Progression-free survival					
mean (months)	**42.3**	**23.7**	**33.9**	**13.5**	**15.6**
95%CI	*(16-69)*	*(12-35)*	*(12-56)*	*(8-19)*	*(6-26)*
Overall Survival					
mean (months)	**98.1**	**106.5**	**84.7**	**61.2**	**113.4**
95%CI	*(77-119)*	*(79-134)*	*(55-114)*	*(35-87)*	*(73-154)*
TYPE II CARCINOMAS [c]	***9***	***22***	***10***	***4***	***8***
Age at diagnosis					
Median	**60**	**48.5**	**48**	**54**	**49**
Range	*51-71*	*36-71*	*36-71*	*50-60*	*40-64*
Complete response					
Yes	6 ***67%***	11 ***50%***	6 ***60%***	-	5 ***63%***
No	3 ***33%***	2 ***9%***	1 ***10%***	1 ***25%***	0 ***0%***
NA	0 ***0%***	9 ***41%***	3 ***30%***	3 ***75%***	3 ***38%***
Progression-free survival					
mean months	**29.5**	**23.5**	**32.8**	**14.0**	**11.0**
95%CI	*(3-56)*	*(1-46)*	*(9-18)*	*(20-22)*	*(15-54)*
Overall Survival					
mean months	**89.6**	**127.0**	**104.0**	**77.0**	**165.0**
95%CI	*(74-104)*	*(110-144)*	*(93-115)*	*(62-102)*	*(148-182)*

[a] Grade of serous tumors according to the M. D. Anderson Cancer Center (MDACC) criteria (Malpica et al, 2004); [b]Grade of non-serous tumors according to the World Health Organization criteria (Organization, 2004; Silverberg, 2000); [c]Subgroup of type II carcinomas as described in Materials and Methods; *NA*, Not Available. Percentages shown are relative to the total number of carcinomas or type II carcinomas in each group except for grade where percentages are relative to total number or serous or non-serous tumors in each group

2 GENOMIC INSTABILITY IN FAMILIAL AND SPORADIC EOC

2.1 GLOBAL PATERN OF GENOMIC INSTABILITY IN EOC

To reveal potential general differences in the rate and pattern of genomic instability between familial and sporadic EOCs, genomic alterations were first visualized by generating frequency plots. These plots display DNA copy number gains and losses in each chromosomal location across the genome *(Figure 6).*

Overall the genomic instability level was high in all the EOC groups and the general pattern of alterations was not substantially different between familial (all subtypes) and sporadic tumors. In general most frequently lost and gained regions were common to both tumor groups *(Figure 6-II and 6-III).*

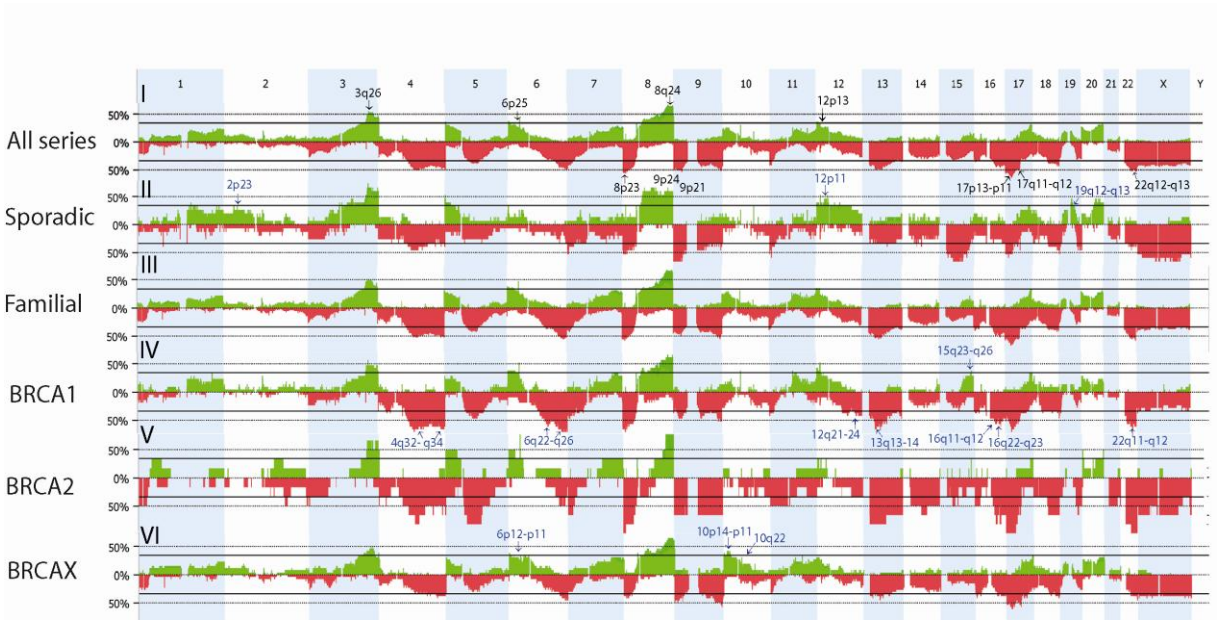

Figure 6. Frequency plots of copy number gains (in green) and losses (in red) defined in all carcinomas and subgroups. The proportion of tumors with gained/lost regions is plotted on the y-axis versus genomic location on the x-axis. Common recurrently altered regions across all four subgroups or present in >55% of the whole series are marked with black arrows on the general plot (for all series), while group-specific regions identified for sporadic, *BRCA1* and BRCAX tumors are identified on the corresponding plots with blue arrows. Simplified chromosomal locations are given next to the arrows (using the same color code).

Likewise, there were no significant differences between familial and sporadic tumors regarding the average total number of alterations and the average total length

of genome altered per tumor considering all carcinomas and the subgroup of type II neoplasms *(Table 5).* Both, familial and sporadic carcinomas showed very unstable genomic profiles with an average fraction of haploid genome altered of 27.9% (893Mb) and 28.75% (921Mb), respectively *(Table 5).* Group of type II carcinomas showed an average of more than 60 aberrations per tumor that involved more than 1Gb of the altered genome *(Table 5).*

Despite this general similarity, a separate analysis of gains and losses and stratification of familial tumors according to their *BRCA1/2* mutation status revealed some differences.

Table 5. Across tumor subtype and intra tumor subtype comparisons of average number and length of copy number alterations

						Across tumor-type comparisons					
Number of alterations	**Familial**	**BRCA1**	**BRCA2**	**BRCAX**	**Sporadic**	**FvsS**	**B1vsB2**	**B1vsBX**	**B1vsS**	**B2vsBX**	**B2vsS**
All carcinomas	*n=53*	*n=21*	*n=6*	*n=26*	*n=15*			*p-value*			
Gains	19.2	21.7	14.5	18.4	25.7	0.11	0.36	0.34	0.29	0.35	*0.10*
Losses	26.1	29.6	32.7	21.9	25.6	0.85	0.57	*0.08*	0.43	*0.06*	0.24
Amp.	3.4	4.4	1.5	3.0	7.3	0.17	**0.02**	**0.03**	0.87	0.26	**0.04**
HD	4.5	7.4	4.2	2.3	3.8	0.44	0.60	**0.01**	*0.09*	0.14	0.38
Total	53.1	63.0	52.8	45.7	62.4	0.46	0.88	0.10	0.93	0.38	0.97
Av. nr of losses/av. nr of gains	1.4	1.4	2.3	1.2	1.0	_	_	_	_	_	_
Gains v. Losses (*p value*)[a]	***0.05***	***0.02***	***0.01***	0.31	0.57	_	_	_	_	_	_
Type II carcinomas [b]	*n=21*	*n=9*	*n=4*	*n=8*	*n=8*			*p-value*			
Gains	22.7	25.9	14.3	23.4	30.1	0.16	0.16	0.78	0.38	0.39	*0.06*
Losses	32.2	36.1	30.8	28.5	29.4	0.96	0.70	0.28	0.45	0.61	0.82
Amp	4.1	3.8	1.5	5.9	10.0	0.29	*0.09*	0.70	0.48	0.10	*0.07*
HD	6.5	9.7	4.3	4.1	3.3	0.58	0.35	0.16	0.38	0.93	0.94
Total	65.6	75.4	50.8	61.9	72.8	0.73	0.60	0.54	0.92	0.61	0.22
Av. nr of losses/av. nr of gains	1.4	1.4	2.2	1.2	1.0	_	_	_	_	_	_
Gains v. Losses (*p value*)[a]	***0.01***	0.09	***0.04***	0.26	0.46	_	_	_	_	_	_
Length of alterations [Mb]	**Familial**	**BRCA1**	**BRCA2**	**BRCAX**	**Sporadic**	**FvsS**	**B1vsB2**	**B1vsBX**	**B1vsS**	**B2vsBX**	**B2vsS**
All carcinomas	*n=53*	*n=21*	*n=6*	*n=26*	*n=15*			*p-value*			
Gains+Amp	298	340	241	279	358	0.38	0.28	0.43	0.83	0.66	0.21
Losses+HD	595	701	907	442	562	0.77	**0.02**	**0.01**	0.26	**< 0.001**	**0.05**
Total	893	1042	1148	721	921	0.84	0.43	**0.02**	0.41	**<0.01**	0.24
Av. length of losses/av. length of gains	2.0	2.1	3.8	1.6	1.6	_	_	_	_	_	_
Gains v. Losses (*p value*)[a]	***<0.001***	***<0.001***	***<0.001***	***0.04***	0.10	_	_	_	_	_	_
Type II carcinomass [b]	*n=21*	*n=9*	*n=4*	*n=8*	*n=8*			*p-value*			
Gains+Amp	321	337	225	351	432	0.26	0.31	0.90	0.33	0.37	0.13
Losses+HD	692	745	910	525	656	0.79	0.18	*0.09*	0.54	**0.01**	0.10
Total	1013	1082	1134	876	1088	0.75	0.48	0.54	0.98	0.19	0.46
Av. length of losses/av. length of gains	2.2	2.2	4.0	1.5	1.5	_	_	_	_	_	_
Gains v. Losses (*p value*)[a]	***<0.001***	***<0.001***	***<0.001***	0.22	0.14	_	_	_	_	_	_

P-value ≤ 0.05 considered significant and highligthed in bold, borderline significant in italics. [a]*P-values* corresponding to intra tumour comparisons (gains versus losses within a particular group).[b]Subgroup of type II carcinomas as defined in Materials and Methods;
F: Familial; S: Sporadic; B1: *BRCA1* mutated; B2: *BRCA2* mutated; BX: BRCAX mutated; Amp.:Amplifications; HD: Homozygous Deletions.

2.2 NUMBER OF DNA COPY NUMBER CHANGES

BRCA1 and BRCA2 tumors showed greater average number of losses and homozygous deletions (HD) than sporadic or BRCAX tumors. In contrast, sporadic cases presented the highest average number of gains and amplifications of all tumor subtypes *(Figure 7A) (Table 5)*. A similar pattern was observed when only group of type II tumors was considered *(Figure 7B* and *Table 5).*

Comparisons between number of gains versus number of losses performed within each tumor subtype revealed a similar average number of both events in sporadic tumors (25.7 *v.* 25.6, respectively). In familial cases, however, the average number of losses was 1.4 times greater than the average number of gains, with differences mostly attributed to BRCA1 (29.6 losses v. 21.7 gains, *P*=0.02) and BRCA2 tumors (32.7 losses v. 14.5 gains, *P* =0.009) *(Figure 7C* and *Table 5).* This pattern was also observed in the subgroup of type II tumors, with significant and borderline significant differences between numbers of gains and losses in BRCA2 and BRCA1 tumors, respectively *(Figure 7D and Table 5).*

2.3 LENGTH OF GENOME ALTERED BY DNA COPY NUMBER CHANGES

In agreement with the analysis of the number of alterations, we found that BRCA1 and BRCA2 tumors presented a significantly higher average length of genome altered by DNA losses when compared to sporadic or BRCAX cases *(Figure 7E and Table 5).* BRCAX tumors showed the lowest average length of genome altered by losses. This pattern was partially maintained in the subgroup of type II carcinomas, with significant differences between BRCAX and BRCA2 tumors *(Figure 7F* and *Table 5)*. Sporadic cases displayed more genome affected by copy number gains than familial tumors (358 *v.* 298 Mb) showing even larger difference in a subgroup of type II carcinomas although differences did not reach statistical significance.

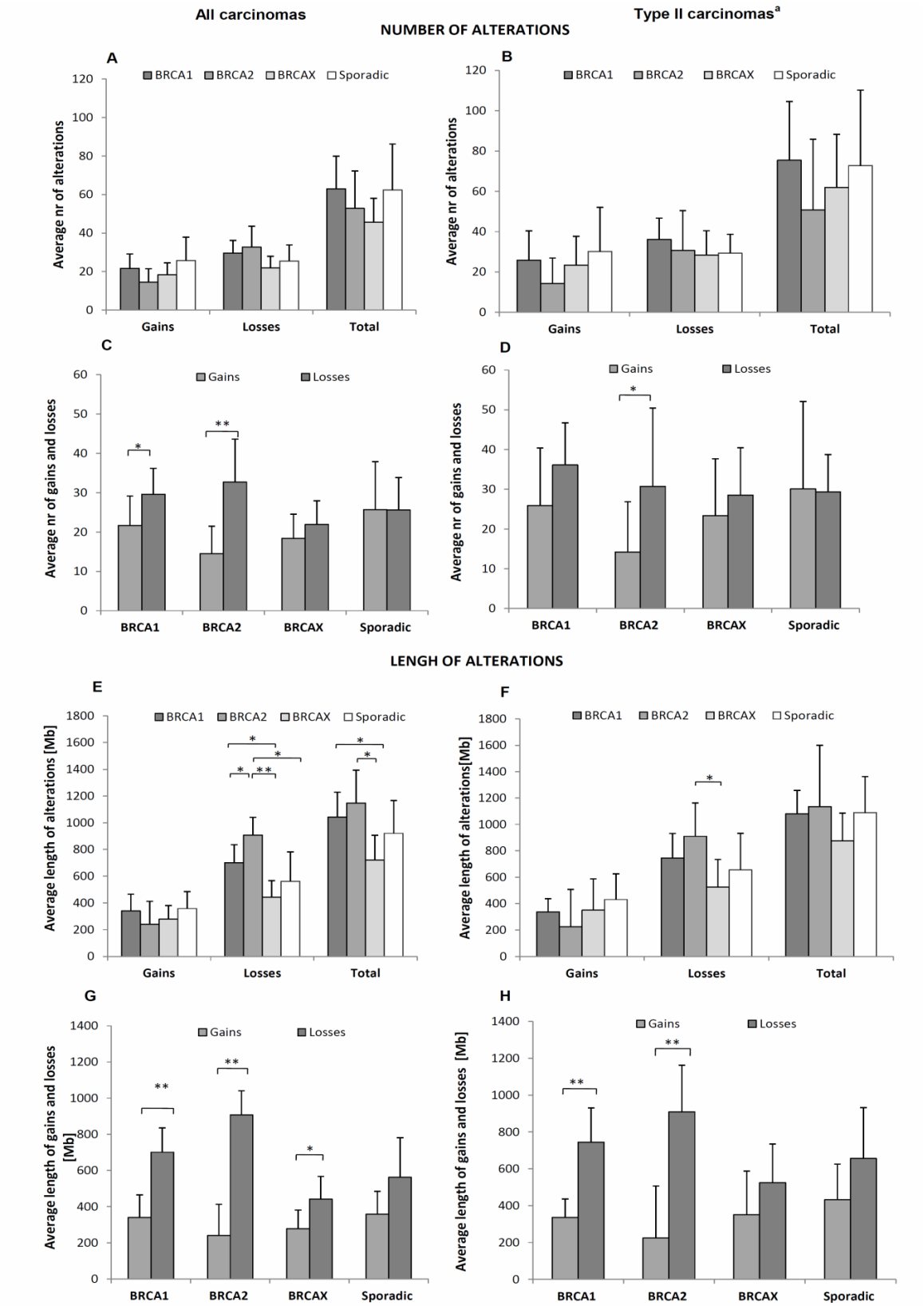

Figure 7. Average number (**A-D**) and length (**E-H**) of copy number alterations in different groups of ovarian carcinomas (**A, C, E, G**) and type II carcinomas (**B, D, F, H**). Significant differences in number and length of alterations between (**A-B, E, F**) and within (**C, D, G, H**) tumor groups are indicated with *(p<0.05) or ** (p<0.01). Error bars represent 95% Confidence Intervals.

Interestingly, in all tumor subtypes, including sporadic carcinomas, which showed a similar average number of both types of alterations, more genetic material was lost than gained *(Figure 7G-H* and *Table 5).* In BRCA1 and BRCA2 tumors the average length of lost genome was 2.1 and 3.8-fold greater than the length of gained material, respectively. In sporadic and BRCAX tumors differences were less marked (1.6-fold in both) *(Figure 7G* and *Table 5).* Differences between length of genome gained and lost per tumor were statistically significant in all familial tumors (BRCA1, BRCA2 and BRCAX), while only a trend was observed in sporadic cases (P=0.09). In the subgroup of type II tumors only differences in *BRCA1/2* carriers remained significant (P<0.001) *(Figure 7H* and *Table 5).*

2.4 MCRS OF DNA COPY NUMBER CHANGES DEFINED IN EACH TUMOR GROUP

In order to define the most commonly altered copy number changes in each tumor group we defined minimal common regions (MCRs) gained in more than 25% or lost in more 35% of the group as shown in the *Supplementary Table 2* and *3*,respectively. These regions served to identify the alterations shared between different tumor groups and also to define those potentially specific to each group.

2.5 DNA COPY NUMBER CHANGES SHARED ACROSS TUMOR GROUPS

To determine DNA copy number alterations that might be fundamental for the development and progression of ovarian carcinomas regardless their *BRCA1/2* mutation status, we sought to define frequently altered regions in all tumor groups.

A summary of common gains and losses identified as recurrent in at least three of the analyzed tumors subtypes (BRCA1, BRCA2, BRCAX and sporadic) is shown in *Table 6*. Regions recurrently gained in the all four tumors subtypes were 6p25.3,

8q24.2-q24.3, and 12p13.33-p13.32, and regions exhibiting the highest frequencies were 3q26.2 and 8q24.2-q24.3 (*Figure 6-I* and *Table 6*). These regions and others defined as recurrently gained included from 1 up to 45 genes and spanned well known or potential oncogenes such as *MECOM, PIK3CA*, *FOXQ1, MYC, CCND2* and *CANT1.*

Regions recurrently lost in all four subgroups were defined at 9p24.3, 9p21.3, 17q11.2-q12, 22q12.3, 22q13.1 and 22q13.31-q13.33. Alterations with the highest incidence were found at 8p23.3-p23.1 and 17p13.3-p11.2 (*Figure 6-I* and *Table 6*). Many of these deleted regions and the others qualifying as recurrent across tumor subtypes encompassed tumor suppressors previously linked to ovarian carcinogenesis (e.g. *MCPH1, CDKN2A, CDKN2B* and *NF1*). However, other regions pointed to less well characterized suppressors not previously associated with ovarian cancer (e.g. *FANCC, TSC1, CREBBP, CDH11* and *EDA2R*).

Table 6. Recurrent Minimal Common Regions (MCRs) of gains and losses shared across tumor subtypes

Cytoband	Region	Size [bp]	Frequency[a] (%)	Nr of genes	Genes of interest[b]	Other genes in the region[c]
Gains						
3q26.1	161,858,336-163,987,311	2,128,975	37	6		*ARL14 PPM1L B3GALNT1 NMD3 C3orf57 OTOL1*
3q26.2	170,336,781-170,346,939	10,158	**56**	1	***MECOM***	
3q26.32	180,277,629-180,352,072	74,443	54	1	***PIK3CA***	
5p14.3 - p14.2	19,297,228-23,809,228	4,512,000	28	5	*CDH18 CDH12 PRDM9*	*CDH18 GUSBP1 PMCHL1 CDH12 PRDM9*
6p25.3	1,189,126-1,285,230	96,104	38	1	*FOXQ1*	
6p25.1 - p24.3	5,776,997-8,041,186	2,264,189	29	16	*NRN1 TXNDC5*	
6p24.1	13,408,897-13,486,913	78,016	32	2	*TBC1D7*	*GFOD1*
7q32.2 - q32.3	129,620,363-130,651,373	1,031,010	28	18		
7q33 - q34	134,477,470-142,682,704	8,205,234	30	80	*CREB3L2 KIAA1549* ***BRAF***	
7q35 - q36.1	144,378,243-147,685,879	3,307,636	28	3		*CNTNAP2 MIR548F4 MIR548T*
8q24.21	128,807,376-129,291,694	484,318	**68**	2	***MYC***	*PVT1*
8q24.3	141,522,457-143,860,659	2,338,202	**65**	21	***TRAPPC9*** *PSCA*	
10q11.23	51,473,718-51,664,989	191,271	31	3		*FAM21A FAM21B ASAH2*
12p13.33 - p13.32	54,933-5,102,331	5,047,398	37	45	***KDM5A CCND2***	
17q25.1 - q25.3	70,225,044-72,286,125	2,061,081	30	26	*FOXJ1*	
17q25.3	74,387,898-78,774,742	4,386,844	26	93	***CANT1 ASPSCR1***	
20p13	0-3,018,638	3,018,638	41	61	*ANGPT4*	
Losses						
4q24	103,855,981-104,152,060	296,079	51	4		*MANBA UBE2D3 CISD2 NHEDC1*
4q28.3 - q31.21	136,561,996-142,584,570	6,022,574	46	19		
4q34.3 - q35.1	182,303,764-182,934,067	630,303	49	1		*NCRNA00290*
6q26	161,740,093-161,828,041	87,948	44	1	*PARK2*	
8p23.3 - p23.1	1,190,650-6,260,759	5,070,109	**56**	9	*MCPH1*	
8p21.2 - p21.1	26,260,243-28,304,348	2,044,105	47	21		
9p24.3	0-1,631,746	1,631,746	51	11		
9p24.1	8,056,737-8,434,111	377,374	50	1	*PTPRD*	
9p23 - p22.3	12,386,932-14,108,018	1,721,086	51	5	***NFIB***	*TYRP1 C9orf150 MPDZ FLJ41200*
9p22.3	14,622,555-16,229,616	1,607,061	35	8		
9p22.3 - p21.3	16,479,078-20,933,035	4,453,957	51	17	***MLLT3***	
9p21.3	21,969,065-22,051,061	81,996	54	3	***CDKN2A CDKN2B***	*CDKN2BAS*
9q21.33	88,865,092-89,129,765	264,673	44	2		*LOC494127 C9orf170*
9q22.32	96,898,992-97,116,235	217,243	43	1	***FANCC***	
9q22.33 - q31.1	100,644,626-101,678,237	1,033,611	43	3	***NR4A3***	*GALNT12 COL15A1 TGFBR1 ALG2 SEC61B*
9q33.1	117,953,363-118,381,663	428,300	46	3		*PAPPA LOC100128505 ASTN2*
9q33.2	123,060,025-123,208,621	148,596	46	2		*GSN STOM*
9q33.3	128,975,869-129,006,542	30,673	35	1		*RALGPS1*
9q34.13-q34.2	133,483,430-135,001,392	1,517,962	51	17	***TSC1***	
9q34.2	135,339,532-135,424,778	85,246	53	2		*TMEM8C ADAMTSL2*
11p15.5 - p15.4	826,091-2,876,898	2,050,807	41	44		
13q12.13	25,719,218-25,817,850	98,632	44	1		*CDK8*
16p13.3	3,759,661-3,782,779	23,118	35	1	***CREBBP***	
16q21	62,541,693-64,974,691	2,432,998	40	3	***CDH11***	*LOC283867 CDH5*
16q22.3 - q23.1	73,046,913-74,862,172	1,815,259	40	20		
17p13.3	1,431,769-1,639,791	208,022	**57**	11		
17p11.2	19,082,703-20,046,930	964,227	**63**	15		
17q11.2 - q12	26,420,214-29,077,825	2,657,611	54	4	***NF1***	*TMEM98 SPACA3 ACCN1*
18q21.2	48,644,312-51,715,504	3,071,192	40	9		
18q21.32 - q21.33	55,721,386-57,214,344	1,492,958	40	2		*PMAIP1 MC4R*
18q23	75,912,794-76,117,153	204,359	44	3		*ADNP2 LOC100130522 PARD6G*
22q12.3	30,817,999-32,856,500	2,038,501	43	12		
22q12.3	32,856,500-34,737,030	1,880,530	53	10		
22q12.3	34,737,030-35,049,039	312,009	43	6	***MYH9***	*RBFOX2 APOL3 APOL4 APOL2 APOL1*
22q13.1	37,668,125-37,724,138	56,013	53	2		*APOBEC3A APOBEC3B*
22q13.1	43,530,191-46,673,931	3,143,740	53	32		
22q13.31 - q13.33	46,673,931-49,472,215	2,798,284	54	40		
Xq11.1 - q12	62,673,834-65,512,219	2,838,385	35	14		
Xq12	65,512,219-66,014,702	502,483	46	1	*EDA2R*	
Xq12-q13.1	66,014,702-68,066,344	2,051,642	35	5	*AR*	*OPHN1 YIPF6 STARD8 EFNB1*
Xq27.3	144,141,243-146,657,619	2,516,376	41	21		

Gained and lost regions listed as shared across tumor subtypes if present among the top 60 most frequent ly altered regions (minimum frequency = 25%) in at least three tumour subtypes (B1, BRCA1; BRCA2, B2; BRCAX, BX; S, sporadic). In bold regions found to be recurrent in all 4 tumour groups. [a]Global frequency of the alteration in whole tumour set frequencies greater or equal to 55% highlighted in bold. [b]Genes of interest selected from Cancer Census (in bold) or based on their function and previously published data. [c]Rest the genes in the defined region listed if less than seven.

2.6 GROUP-SPECIFIC DNA COPY NUMBER CHANGES

Besides many similarities observed in the pattern of genomic instability between familial and sporadic tumors, some of the alterations were shown to be more associated with particular tumor type. The group-specific alterations were defined based on significant differences in the frequency of copy number alterations between two tumor groups using Fisher's Exact Test (FET). Preselected group-specific alterations (P<0.05 and FDR<0.02) were further refined by comparison to the regions defined in 489 HG-SOC from TCGA ovarian study (TCGA, 2011) (*Table 7*).

Since *BRCA1/2* mutation carriers presented the largest amount of genome lost, alterations of this type were also the most frequent among the ones defined as specific for this group. Among the lost regions significantly the most frequently found in BRCA1 than in sporadic tumors were: 4q32.3-q34.1, 6q22.3-q26 or 12q21.2-q23.2 spanning genes of tumor suppressive or potential suppressive function such as *TNFAIP3, PERP or PLAGL1 (Table 7).*

Due to high variability of BRCAX tumors, regarding their grade and histological subtype and also lower overall genomic instability rate, specific regions associated with these tumors were difficult to distinguish. However, gains at 6p12.3-p11.2 and gains spanning chromosome 10 (10p14-p13, 10p11.23-p11.21, 10q22.1-q22.2) were significantly more frequent in this tumor group when compared to BRCA1 tumors *(Figure 6-IV, Table 7).* In particular, copy number gain at 10q22.1-q22.2 was identified exclusively in the BRCAX tumors. Further indication, that these alterations might be associated specifically with these tumors was revealed when more homogenous subgroup of high-grade type II BRCAX cases was considered. Then, exactly the same alterations at 10p and 10q were found to be significantly more frequent (by more than 45%) in BRCAX cases (in comparison to BRCA1 cases, p= 0.017 for gains at p arm and p=0.026 for gains at q arm at chromosome 10).

Among the alterations more significantly represented in sporadic cases we only identified changes that involved gain of genomic material. Gains at 2p23.3, 12p11.22 and 19q12-q13.11 were significantly over-represented in sporadic tumors *(Table 7),* with the latter containing the *CCNE1* gene that was also amplified in these tumors.

Table 7. List of regions potentially associated to particular tumor types

Regions most frequently altered in familial tumors

Cytoband	Alteration type	Start [bp]	End [bp]	Size [bp]	Nr of genes	Frequency (%)[a]			Pair-wise comparisons[b]		Genes of interest[d]
						BRCA1	BRCAX	Sporadic	BRCA1 vs BRCAX	BRCA1 vs Sporadic	
4q32.3 - q34.1	*Loss*	165,057,469	174,917,900	9,860,471	34	**61**	35	21		**0.008**-0.048	
6p12.3 - p11.2	*Gain*	50,179,546	57,296,041	7,116,495	41	5	**32**	12	0.01-0.03		*RAB23 BAG2*
6q22.33 - q23.2	*Loss*	127,627,130	137,934,880	10,307,750	65	**55**	30	14		**0.006**-0.043	***MYB LAMA2***
6q23.3 - q24.2	*Loss*	137,934,880	145,417,355	7,482,475	31	**61**	32	21	0.021-0.043	**0.008**-0.049	***TNFAIP3*** *PERP PLAGL1*
6q24.2 - q26	*Loss*	145,417,355	162,400,039	16,982,684	90	**68**	37	18		**0.0002**-0.025	***ESR1 LATS1 ARID1B PARK2***
8q22.1	*Amp*	98,814,783	98,930,489	115,706	1	**29**	4	0	0.035	0.03	*LAPTM4B*
10p14 - p13	*Gain*	9,434,405	15,042,168	5,607,763	29	14	**42**	14	0.035		
10p11.23 - p11.21	*Gain*	30,565,751	37,925,305	7,359,554	21	10	**31**	7	0.039		
10q22.1 - q22.2	*Gain*	55,246,043	75,909,014	20,662,971	116	0	**19**	0	0.023		***PRF1***
12q21.2 - q23.2	*Loss*	75,082,367	100,468,698	25,386,331	103	**39**	19	2		**0.002**-0.049	***BTG1***
12q23.3 - q24.11	*Loss*	106,833,069	109,514,249	2,681,180	38	**33**	4	13	0.015		
12q24.12 - q24.31	*Loss*	110,401,635	123,147,556	12,745,921	140	**40**	11	11	0.02-0.043	0.04	***PTPN11 BCL7A***
12q24.31 - q24.33	*Loss*	123,989,527	130,437,809	6,448,282	18	**43**	23	11		0.04	
13q13.3	*Loss*	35,225,910	35,520,955	295,045	2	**57**	31	22		0.05	
13q13.3	*Loss*	36,092,590	39,288,740	3,196,150	16	**67**	35	28	0.041	0.02	*LHFP SMAD9*
13q13.3 - q14.11	*Loss*	39,288,740	41,013,566	1,724,826	16	**67**	35	25		0.01-0.025	
13q14.3	*Loss*	50,990,104	51,674,267	684,163	10	**67**	35	28		0.02	
15q23 - q24.1	*Gain*	68,342,150	70,408,845	2,066,695	13	**29**	8	0	0.022		
15q25.1	*Gain*	76,307,772	76,370,118	62,346	3	**29**	4	0	0.035	0.02	
15q25.1-q26.1	*Gain*	76,370,118	88,100,329	11,730,211	123	**30**	8	0	**0.009**-0.022		***NTRK3***
16q11.2 - q12.1	*Loss*	45,018,886	45,660,495	641,609	8	**48**	15	27	0.025		
16q22.3 - q23.1	*Loss*	69,917,525	74,862,172	4,944,647	44	**57**	33	22		0.05	
22q11.22 - q12.1	*Loss*	21,378,067	26,356,535	4,978,468	67	**57**	27	33	0.043		***BCR SMARCB1***

Regions most frequently altered in sporadic tumours

Cytoband	Alteration type	Start [bp]	End [bp]	Size [Kb]	Nr of genes	Frequency (%)[a]			Pair-wise comparisons[b]	Genes of interest[d]
						Familial	Sporadic	TCGA[c]	Familial vs Sporadic	
2p23.3	*Gain*	28,250,360	28,933,896	683,536	5	8	**33**	40	0.020	
12p11.22 - p11.1	*Gain*	30,129,837	33,668,057	3,538,220	14	18	**48**	45	**0.01-0.04**	
19q12 - q13.11	*Gain*	33,763,985	39,417,419	5,653,434	33	10	**39**	31	**0.005-0.029**	***CCNE1 CEBPA***
19q13.11 - q13.12	*Gain*	39,863,018	40,801,847	938,829	31	6	**27**	34	0.038	
19q13.12 - q13.2	*Gain*	41,343,753	44,808,705	3,464,952	88	4	**28**	27	0.004-0.037	
19p13.12 - p13.11	*Amp*	15,460,257	16,706,329	1,246,072	31	0	**13**	12	0.046	***TPM4***
19q12	*Amp*	33,105,133	35,503,735	2,398,602	8	0	**13**	23	0.046	***CCNE1***

Regions that exibited significant differences in frequency across tumor subtypes after performing pair-wise comparisons (significant, $p < 0.05$, using Fisher's Exact Test and Chi-square test and with FDR < 0.2 as defined by CGHtest). [a]Frequency of alteration in the different tumour subtypes with the highest value across groups highlighted in bold. [b] Fisher's Exact Test p -value obtained for the specified comparison is provided; in order to simplify the table, small contiguous regions (< 1Mb apart) were combined, listed as a single region and range of p-values obtained for individual segments quoted. Among regions significantly more frequent in familial subtypes compared with sporadic cases only those not reported in the TCGA study were included. [c]Frequency of alteration reported in the TCGA study at same chromosomal location. [d]Genes of interest belong to The Cancer Census (in bold)-downloaded from http://www.sanger.ac.uk/genetics/CGP/Census/ (ver.Dec 2010) or are potentially cancer-related genes based on their function or previous reports.

2.7 AMPLIFICATIONS AND HOMOZYGOUS DELETIONS

2.7.1 High-level copy number gains

The high resolution of our platform allowed us to identify focal high-amplitude copy number changes. The most frequent regions of amplification with their distributions across the groups of tumors are shown in *Table 8 part A.* Fifty-nine narrow amplifications (median length of 739Kb spanning on average 11 genes) were identified in at least 2 cases. In general amplifications tended to occur in the sites of frequent gains delineating potential driver oncogenes.

The most frequent site of amplification is 8q22-ter with the highest peak at 8q24.21-q24.3 containing *MYC* oncogene. Although this high copy number event was the most prevalent in familial tumors reaching 30% in *BRCA1/2* mutation carriers, it was frequently gained in sporadic cases (in more than 60% of the cases). However, common region of amplification present in sporadic and at least two familial groups spanned more proximal parts of this chromosome, with 8q22.3 being the most frequent. This high copy number change encompassed only one gene – *YWHAZ,* that was previously implicated in, among others, breast and ovarian cancer (Bergamaschi *et al*, 2011; Li *et al*, 2010) *(Table 8 part A* and *Table 9).*

Other prevalent (12%) site of common amplification was found at 11q13.1, spanning just 2 genes. One of whose was the Metastasis Associated Lung Adenocarcinoma Transcript 1, *MALAT1, (Table 8 part A, Table 9)* reported to be involved in cell growth, cell cycle progression and invasion of cervical (Guo *et al*, 2010) and endometrial cancer (Yamada *et al*, 2006).

Among amplifications, identified to be significantly more prevalent in one of the tumor groups, was 8q22.1 - found to be amplified exclusively in BRCA1 cases, in 29% of this group. This high copy number gain consisted of only one gene - *LAPTM4B*, whose overexpression has been previously implicated to poor prognosis and metastasis of many gynecological carcinomas (Meng *et al*, 2010; Yin *et al*, 2011) *(Table 8 part A* and *Table 9).*

Table 8, partA. List of regions amplified in at least 2 tumors

	<3Mb			>10%						
	<1Mb			>15%						
Regions of Amplification (AMP)	**Size [bp]**	**Cytoband Location**	**Nr of genes**	**% of CNV Overlap**	**Frequency of AMP in the whole series %**	**Nr of samples with AMP per group**				**Genes of interest[a]**
						ALL SAMPLES n=68	***BRCA1 n=21***	***BRCA2 n=6***	***BRCAX n=26***	***Sporadic n=15***	
chr1:32,503,266-32,521,648	18,382	p35.1	1	0	2.94	2	2				***LCK***
chr1:39,041,303-41,074,169	2,032,866	p34.3 - p34.2	38	32	4.41	3	1		2		***MYCL1***
chr1:44,435,677-46,030,327	1,594,650	p34.1	35	20	2.94	2	1		1		***MUTYH***
chr1:49,695,021-49,890,370	195,349	p33	1	11	2.94	2	1		1		
chr1:70,947,563-76,146,686	5,199,123	p31.1	18	21	2.94	2			1	1	
chr1:78,525,513-81,820,296	3,294,783	p31.1	3	60	4.41	3	**1**		**1**	**1**	*PTGFR*
chr1:117,469,284-120,219,596	2,750,312	p13.1 - p12	18	41	2.94	2	1		1		
chr2:113,742,362-113,760,303	17,941	q13	1	0	2.94	2			1	1	***PAX8***
chr3:170,112,026-170,346,939	234,913	q26.2	1	0	5.88	4	1			3	***MECOM***
chr3:173,712,240-173,805,956	93,716	q26.31	1	1	4.41	3	1			2	
chr3:176,050,626-176,152,887	102,261	q26.31	1	0	4.41	3			1	2	
chr3:179,526,769-181,046,005	1,519,236	q26.32 - q26.33	12	5	5.88	4			1	3	***PIK3CA***
chr4:54,639-2,564,364	2,509,725	p16.3	49	52	2.94	2	1		1		***FGFR3 WHSC1***
chr5:0-230,625	230,625	p15.33	1	70	2.94	2				2	
chr7:155,939,892-158,642,803	2,702,911	q36.3	16	48	5.88	4	2		2		
chr8:47,740,011-49,156,916	1,416,905	q11.1 - q11.21	6	43	2.94	2	1		1		
chr8:55,294,302-56,780,890	1,486,588	q11.23-q12.1	4	15	2.94	2	1			1	
chr8:59,887,163-61,522,646	1,635,483	q12.1	2	11	2.94	2	1			1	
chr8:67,839,021-70,431,804	2,592,783	q13.1 - q13.2	13	31	2.94	2			2		
chr8:77,272,553-77,956,021	683,468	q21.11	2	11	2.94	2			1	1	
chr8:81,539,648-81,601,407	61,759	q21.13	1	0	2.94	2			1	1	
chr8:85,047,239-87,355,110	2,307,871	q21.2 - q21.3	15	76	5.88	4	**2**		**1**	**1**	
chr8:92,323,386-92,443,508	120,122	q21.3	1	16	7.35	5	**3**		**1**	**1**	
chr8:94,918,806-95,029,680	110,874	q22.1	1	0	5.88	4	3		1		
chr8:96,742,090-97,658,770	916,680	q22.1	6	12	7.35	5	**3**		**1**	**1**	
chr8:98,814,783-98,930,489	115,706	q22.1	1	20	8.82	6	6				*LAPTM4B*
chr8:101,926,982-102,101,553	174,571	q22.3	1	42	11.76	8	**5**		**1**	**2**	*YWHAZ*
chr8:107,121,208-108,087,190	965,982	q23.1	2	6	10.29	7	4		3		
chr8:110,438,342-111,177,707	739,365	q23.1 - q23.2	4	1	11.76	8	4		4		
chr8:115,228,831-116,155,561	926,730	q23.3	2	56	11.76	8	3	1	4		
chr8:118,543,986-118,626,272	82,286	q24.11	1	0	14.71	10	3	1	6		
chr8:122,701,184-123,202,812	501,628	q24.13	2	25	13.24	9	3	1	5		
chr8:128,537,094-128,997,964	460,870	q24.21	2	47	19.12	13	5	2	6		***MYC***
chr8:139,929,002-142,315,091	2,386,089	q24.23 - q24.3	8	16	22.06	15	7	2	6		*TRAPPC9*
chr11:64,958,966-65,036,226	77,260	q13.1	2	76	11.76	8	**3**		**2**	**3**	*MALAT1*
chr11:70,950,388-71,160,423	210,035	q13.4	2	96	2.94	2			2		
chr11:77,593,734-78,131,024	537,290	q14.1	4	19	2.94	2			1	1	
chr11:107,562,790-107,606,652	43,862	q22.3	2	0	2.94	2	1		1		***ATM***
chr12:109,681-215,162	105,481	p13.33	4	73	2.94	2	1		1		
chr12:20,338,662-20,713,421	374,759	p12.2	1	1	2.94	2			1	1	
chr12:23,013,927-25,315,520	2,301,593	p12.1	10	49	2.94	2			1	1	***KRAS***
chr12:31,378,017-32,257,281	879,264	p11.21	6	53	2.94	2			2		
chr13:49,453,707-51,187,789	1,734,082	q14.3	14	10	2.94	2			1	1	
chr13:99,498,597-99,556,452	57,855	q32.3	1	46	4.41	3	1		1	1	
chr13:110,288,867-114,142,980	3,854,113	q34	31	43	2.94	2			1	1	
chr17:35,083,091-35,415,740	332,649	q12 - q21.1	11	10	5.88	4			2	2	***ERBB2***
chr17:73,546,263-78,774,742	5,228,479	q25.3	108	80	2.94	2	1		1		***CANT1 ASPSCR1***
chr19:8,939,055-10,745,326	1,806,271	p13.2	33	27	5.88	4			2	2	*MUC16*
chr19:11,997,917-12,450,517	452,600	p13.2	13	26	2.94	2			1	1	
chr19:12,608,550-12,678,645	70,095	p13.13	7	1	2.94	2			2		
chr19:11,997,917-12,678,645	680,728	p13.2-p13.13	20	13	4.41	3			2	1	
chr19:15,460,257-16,706,329	1,246,072	p13.12 - p13.11	31	20	2.94	2				2	***TPM4***
chr19:34,558,302-35,503,735	945,433	q12	6	15	4.41	3			1	2	***CCNE1***
chr19:37,341,279-37,864,742	523,463	q13.11	5	2	4.41	3			1	2	
chr19:41,650,275-41,889,373	239,098	q13.12	7	27	2.94	2				2	
chr19:55,895,448-57,132,339	1,236,891	q13.33	58	47	2.94	2	1			1	***KLK2***
chr20:48,184,070-49,413,927	1,229,857	q13.13 - q13.2	13	76	4.41	3			2	1	
chr20:51,530,308-52,731,992	1,201,684	q13.2	7	45	5.88	4			2	2	
chr20:54,436,225-55,409,396	973,171	q13.31	12	3	5.88	4	**1**		**2**	**1**	
av.	1,127,536		11								
median	739,365		5								

Amplifications shorter than <3Mb and <1Mb or present in more than 5% or more than 10% of the samples are shown against grey and dark grey bakgrounds (respectively). Regions depicted in bold were defined in sporadic and at least 2 familial subtypes.[a]Genes of interest belong to The Cancer Census (in bold)-downloaded from http://www.sanger.ac.uk/genetics/CGP/Census/ (ver.Dec 2010) or are potentially cancer-related based on their function or previous reports. Regions which were less than 1Mb apart and were presented by the same set of samples were joined together and listed under the same cytoband with corresponding list of genes. AMP:amplification

In addition, two amplified regions at chromosome 19 were found to be significantly more frequently altered in sporadic cases. In particular amplification of 19q12 with *CCNE1* indentified in 13% of those cases and gained in 47% of them. *CCNE1* was also amplified in one BRCAX tumor, but it was not altered in any of BRCA1/2 mutation carriers (Table 8 part A). Similarly other amplifications spanning known oncogenes as: *ERBB2* (17q21.32) or *PIK3CA* (3q26) were found exclusively in non-BRCA1/2 mutation carriers *(Table 8 part A* and *Table 9).* As previously mentioned sporadic cases showed more scattered pattern of high copy number alterations, while for familial cases, chromosome 8q was a main site of this type of aberrations.

2.7.2 Homozygous deletions

In addition to high copy number changes, 57 focal homozygous deletions (HD) (median length of 465 Kbp spanning 6 genes on average) were identified in at least 2 samples *(Table 8, part B).*

The most frequent (9%) and present in each tumor group HD was found at 17q11.2 and contained only one gene, the known Ras pathway inhibitor *NF1*. Also with the same frequency, 13q14.2 was homozygously deleted in sporadic and familial tumours and spanned only one gene, the crucial cell cycle regulator *RB1.* Other frequent HD common for sporadic and familial tumors was defined at 8p23.2-p23.1. One of the two genes located in this region was the early DNA-damage-response gene *MCPH1*, indicated to be involved in double strand DNA repair and recruitment of many important DDR proteins to the DNA damage sites, thus involved in maintenance of genomic stability (Lin *et al*, 2010). Another common HD found across familial and sporadic tumors was identified at the fragile site on chromosome 3 (3p14.2, FRA3B) that contained the known tumor suppressor- FHIT (Table 8 part B and Table 9).

Deletions of 17q21.31 (with *BRCA1* gene) were more frequent in *BRCA1/2* mutation carriers than in BRCAX and sporadic tumors *(Supplementary Table 3).* The MCR of loss at this region (17q21-q23) in BRCAX tumors did not include *BRCA1* but

spanned the double-strand break repair protein *BRIP1.* The deletion of 13q13.1 (with *BRCA2*) was the most prevalent in tumors with *BRCA2* mutation.

Table 8, part B . List of homozygous deletions (HD) present in at least 2 tumors

<3Mb <1Mb >4% >5%

Region of Homozygous deletion (HD)	Size [bp]	Cytoband Location	Nr of genes	% of CNV Overlap	Frequency of HD in whole series %	Nr of samples with HD per group					Genes of interest[a]
						ALL SAMPLES n=68	*BRCA1 n=21*	*BRCA2 n=6*	*BRCAX n=26*	*Sporadic n=15*	
chr1:2,540,513-3,280,448	739,935	p36.32	5	53.77	2.94	2	2				***PRDM16***
chr1:4,132,552-4,616,506	483,954	p36.32	2	4.29	4.41	3	3				*AJAP1*
chr3:0-1,784,961	1,784,961	p26.3	2	82.15	2.94	2			2		
chr3:60,530,653-60,943,377	412,724	p14.2	1	54.71	4.41	3		1	1	1	***FHIT***
chr4:106,820,243-107,041,888	221,645	q24	3	4.83	2.94	2	1			1	
chr4:113,838,849-114,526,629	687,780	q25 - q26	2	2.22	2.94	2				2	
chr4:171,227,282-174,806,729	3,579,447	q33 - q34.1	9	20.39	2.94	2		1	1		
chr4:182,480,402-183,363,793	883,391	q34.3 - q35.1	2	3.22	2.94	2	1	1			
chr4:184,634,599-184,832,402	197,803	q35.1	4	0.37	2.94	2		1		1	
chr5:52,244,809-52,290,900	46,091	q11.2	1	4.20	2.94	2		1		1	
chr5:58,539,730-58,712,374	172,644	q11.2	1	0.00	2.94	2	1			1	
chr5:78,450,689-78,629,730	179,041	q14.1	2	33.03	2.94	2	1			1	
chr5:90,191,978-93,011,123	2,819,145	q14.3 - q15	7	13.51	2.94	2	1			1	
chr5:95,249,110-95,428,565	179,455	q15	1	95.35	4.41	3	1		1	1	
chr6:101,029,808-102,134,923	1,105,115	q16.3	2	4.53	2.94	2			1	1	
chr6:107,387,034-107,631,596	244,562	q21	3	81.73	2.94	2			2		
chr6:108,774,892-109,080,592	305,700	q21	2	5.24	2.94	2	1		1		
chr6:161,879,964-162,306,746	426,782	q26	1	78.96	2.94	2	2				*PARK2*
chr6:169,254,237-169,719,190	464,953	q27	2	26.09	2.94	2	2				*THBS2*
chr7:76,979,716-77,300,067	320,351	q11.23	4	0.45	2.94	2	2				
chr7:84,158,437-85,080,631	922,194	q21.11	1	39.86	2.94	2	1			1	
chr8:0-206,224	206,224	p23.3	3	99.96	7.35	5	4	1			
chr8:6,177,456-6,455,595	278,139	p23.2 - p23.1	2	60.71	7.35	5	3	1		1	*MCPH1*
chr8:17,000,420-19,725,139	2,724,719	p22 - p21.3	17	30.82	5.88	4	3			1	***PCM1*** *MTUS1*
chr8:22,096,663-26,106,924	4,010,261	p21.3 - p21.2	37	30.39	7.35	5	4			1	*DOCK5 CDCA2 EBF2*
chr8:28,636,935-33,247,321	4,610,386	p21.1-p12	21	2.21	4.41	3	2			1	***WRN*** *DUSP4 HMBOX1 NRG1*
chr9:1,963,556-2,857,740	894,184	p24.3 - p24.2	5	9.59	2.94	2	1			1	
chr9:9,696,731-10,144,546	447,815	p23	1	61.04	2.94	2	1		1		*PTPRD*
chr9:22,105,292-22,397,667	292,375	p21.3	1	17.35	2.94	2			1	1	***CDKN2A CDKN2B***
chr9:94,133,038-94,416,949	283,911	q22.31	6	0.00	2.94	2			1	1	***OMD***
chr9:125,691,025-125,787,766	96,741	q33.2	1	48.68	2.94	2	2				
chr10:28,293,954-28,890,162	596,208	p12.1 - p11.23	4	7.38	2.94	2	1	1			
chr11:4,745,401-4,860,114	114,713	p15.4	5	16.84	2.94	2			1	1	
chr13:47,841,552-47,847,231	5,679	q14.2	1	0.00	8.82	6	2		3	1	***RB1***
chr16:65,629,269-65,702,942	73,673	q22.1	2	42.89	2.94	2			1	1	***CBFB***
chr16:80,791,319-82,209,820	1,418,501	q23.3	2	15.01	4.41	3	3				*CDH13*
chr17:24,273,465-25,846,831	1,573,366	q11.2	23	3.07	2.94	2	1		1		
chr17:26,516,613-26,548,438	31,825	q11.2	1	0.00	8.82	6	1	1	2	2	***NF1***
chr17:28,829,318-30,302,330	1,473,012	q12	10	11.33	2.94	2	2				
chr19:0-357,847	357,847	p13.3	9	95.38	2.94	2	2				
chr19:5,023,641-5,531,524	507,883	p13.3	4	1.39	2.94	2	1			1	
chr19:61,705,416-61,805,010	99,594	q13.43	4	0.81	2.94	2	1		1		
chr19:61,855,409-62,293,274	437,865	q13.43	6	10.75	4.41	3	1		1	1	***ZIM2 PEG3***
chr22:25,360,415-26,504,223	1,143,808	q12.1	2	41.37	2.94	2	2				***MN1***
chr22:31,132,973-31,848,817	715,844	q12.3	5	0.69	2.94	2	1			1	
chr22:46,034,061-48,551,789	2,517,728	q13.31 - q13.33	4	23.97	4.41	3	2		1		
chrX:10,897,824-12,431,990	1,534,166	p22.2	5	7.42	2.94	2			1	1	
chrX:13,120,045-19,564,783	6,444,738	p22.2 - p22.12	45	16.95	2.94	2	1		1		
chrX:24,744,413-25,039,949	295,536	p22.11 - p21.3	2	9.22	4.41	3	1		1	1	*POLA1*
chrX:31,763,941-33,579,700	1,815,759	p21.1	1	25.62	5.88	4	1		1	2	
chrX:38,519,008-39,470,641	951,633	p11.4	1	5.06	4.41	3	1		1	1	
chrX:43,295,963-43,705,583	409,620	p11.3	3	4.98	5.88	4	1		2	1	
chrX:44,198,148-44,786,777	588,629	p11.3	3	38.41	4.41	3	1		2		***KDM6A***
chrX:46,905,791-48,631,317	1,725,526	p11.3 - p11.23	45	57.76	4.41	3	2		1		***SSX4, WAS, GATA1***
chrX:49,634,473-49,949,828	315,355	p11.23 - p11.22	10	4.16	4.41	3	1		1	1	
chrX:64,513,775-66,111,261	1,597,486	q11.1 - q12	8	23.40	2.94	2	1		1		*EDA2R*
chrX:99,548,530-99,782,384	233,854	q22.1	3	53.65	2.94	2				2	
av.	1,000,040		6	26	4						
median	464,953		3	15	3						

Homozygous deletions: shorter than <3Mb and <1Mb or present in more than 4% or more than 5% of the samples are shown agains grey and dark grey bakgrounds (respectively). Regions depicted in bold were defined in sporadic and at least 2 familial subtypes.[a]Genes of interest belong to The Cancer Census (in bold)-downloaded from http://www.sanger.ac.uk/genetics/CGP/Census/ (ver.Dec 2010) or are potentially cancer-related based on their function or previous reports. Regions which were less than 1MB apart and were presented by the same set of samples were joined together and listed under the same cytoband with corresponding list of genes.HD-homozygous deletion.

Table 9. Distribution of most frequent amplifications and homozygous deletions across tumor subtypes

Cytoband	Region	Size [bp]	Frequency[a] %	Nr of samples with alteration					Nr of genes	Genes of interest[b]	Other genes in the region[c]
				All	B1	B2	BX	S			
Amplifications											
3q26.2	170,112,026-170,346,939	234,913	5.9	4	1			3	1	***MECOM***	*MECOM*
3q26.32 - q26.33	179,526,769-181,046,005	1,519,236	5.9	4			1	3	12	***PIK3CA***	
7q36.3	155,939,892-158,642,803	2,702,911	5.9	4	2		2		16		
8q21.2 - q21.3	85,047,239-87,355,110	2,307,871	5.9	4	2		1	1	15		
8q21.3	92,323,386-92,443,508	120,122	7.4	5	3		1	1	1		*SLC26A7*
8q22.1	94,918,806-95,029,680	110,874	5.9	4	3		1		1		*PDP1*
8q22.1	96,742,090-97,658,770	916,68	7.4	5	3		1	1	6		*LOC100506773 GDF6 UQCRB MTERFD1 PTDSS1 SDC2*
8q22.1	98,814,783-98,930,489	115,706	**8.8**	6	6				1	*LAPTM4B*	*LAPTM4B*
8q22.3	101,926,982-102,101,553	174,571	**11.8**	8	5		1	2	1	*YWHAZ*	*YWHAZ*
8q23.1	107,121,208-108,087,190	965,982	**10.3**	7	4		3		2		*OXR1 ABRA*
8q23.1 - q23.2	110,438,342-111,177,707	739,365	**11.8**	8	4		4		4		*PKHD1L1 EBAG9 SYBU KCNV1*
8q24.11	118,543,986-118,626,272	82,286	**14.7**	10	3	1	6		1		*MED30*
8q24.13	122,701,184-123,202,812	501,628	**13.2**	9	3	1	5		2		*HAS2 HAS2AS*
8q24.21	128,537,094-128,997,964	460,870	**19.1**	13	5	2	6		2	***MYC***	*MYC PVT1*
8q24.23 - q24.3	139,929,002-142,315,091	2,386,089	**22.1**	15	7	2	6		8	*TRAPPC9*	*COL22A1 KCNK9 TRAPPC9 CHRAC1 EIF2C2 PTK2 DENND3 SLC45A4*
11q13.1	64,958,966-65,036,226	77,260	**11.8**	8	3		2	3	2	*MALAT1*	*MIR612 MALAT1*
17q12 - q21.1	35,083,091-35,415,740	332,649	5.9	4			2	2	11	***ERBB2***	
19p13.2	8,939,055-10,745,326	1,806,271	5.9	4			2	2	33	*MUC16*	
20q13.2	51,530,308-52,731,992	1,201,684	5.9	4			2	2	7		*TSHZ2 ZNF217 SUMO1P1 BCAS1 CYP24A1 PFDN4 DOK5*
20q13.31	54,436,225-55,409,396	973,171	5.9	4	1		2	1	12		
Homozygous deletions											
1p36.32	4,132,552-4,616,506	483,954	4.4	3	3				2	*AJAP1*	*LOC284661 AJAP1*
3p14.2	60,530,653-60,943,377	412,724	4.4	3		1	1	1	1	***FHIT***	*FHIT*
5q15	95,249,110-95,428,565	179,455	4.4	3	1		1	1	1		*ELL2*
8p23.3	0-206,224	206,224	**7.4**	5	4	1			3		*OR4F21 RPL23AP53 ZNF596*
8p23.2 - p23.1	6,177,456-6,455,595	278,139	**7.4**	5	3	1		1	2	*MCPH1*	*MCPH1 ANGPT2*
8p22 - p21.3	17,000,420-19,725,139	2,724,719	**5.9**	4	3			1	17	***PCM1*** *MTUS1*	
8p21.3 - p21.2	22,096,663-26,106,924	4,010,261	**7.4**	5	4			1	37	*DOCK5 CDCA2 EBF2*	
8p21.1-p12	28,636,935-33,247,321	4,610,386	4.4	3	2			1	21	*WRN DUSP4 HMBOX1 NRG1*	
13q14.2	47,841,552-47,847,231	5,679	**8.8**	6	2		3	1	1	***RB1***	*RB1*
16q23.3	80,791,319-82,209,820	1,418,501	4.4	3	3				2	*CDH13*	*CDH13 MIR3182*
17q11.2	26,516,613-26,548,438	31,825	**8.8**	6	1	1	2	2	1	***NF1***	*NF1*
19q13.43	61,855,409-62,293,274	437,865	4.4	3	1		1	1	6	*PEG3 ZIM2*	*LOC147670 ZNF835 ZIM2 PEG3AS PEG3 MIMT1*
22q13.31 - q13.33	46,034,061-48,551,789	2,517,728	4.4	3	2		1		4		*FLJ46257 MIR3201 FAM19A5 C22orf34*
Xp22.11 - p21.3	24,744,413-25,039,949	295,536	4.4	3	1		1	1	2		*POLA1 ARX*
Xp21.1	31,763,941-33,579,700	1,815,759	**5.9**	4	1		1	2	1		*DMD*
Xp11.4	38,519,008-39,470,641	951,633	4.4	3	1		1	1	1		*MID1IP1*
Xp11.3	43,295,963-43,705,583	409,620	**5.9**	4	1		2	1	3		*MAOA MAOB NDP*
Xp11.3	44,198,148-44,786,777	588,629	4.4	3	1		2		3	***KDM6A***	*FUNDC1 DUSP21 KDM6A*
Xp11.3 - p11.23	46,905,791-48,631,317	1,725,526	4.4	3	2		1		45	***SSX4 WAS GATA1***	
Xp11.23 - p11.22	49,634,473-49,949,828	315,355	4.4	3	1		1	1	10		

Amplifications and homozygous deletions with frequencies greater than 5% (≥4 samples) and 4% (≥3 samples), respectively. Cytobands in bold indicate regions found in sporadic and at least two of the familial subtypes. B1, BRCA1; BRCA2, B2; BRCAX, BX; S, sporadic. [a]Global frequency of the alteration in whole tumor set; frequencies greater than 5% are highlighted in bold. [b]Genes of interest selected from Cancer Census (in bold) or based on their function and previously published data. [c]Rest of genes in the defined region (a maximum of 6 genes per region is shown).

2.8 IMMUNOHISTOCHEMICAL VALIDATION OF aCGH RESULTS

In order to validate our aCGH results we assessed the correlation between the assigned DNA copy number and the immunohistochemical expression of three genes targeted by high-amplitude events: *CDKN2A* and *RB1* located at homozygously deleted regions, and *CCNE1* that was found amplified. Immunohistochemical analysis showed complete lack or much lower expression of CDKN2A and RB1 in tumors with HD at these loci compared to the mean value of samples with a flat profile at 9p21.3 and 13q14.2, respectively *(Figure 8 A,B).* Tumors exhibiting *CCNE1* amplification presented much higher expression compared to the mean value of tumors with normal DNA copy number at this *locus (Figure 8 C).*

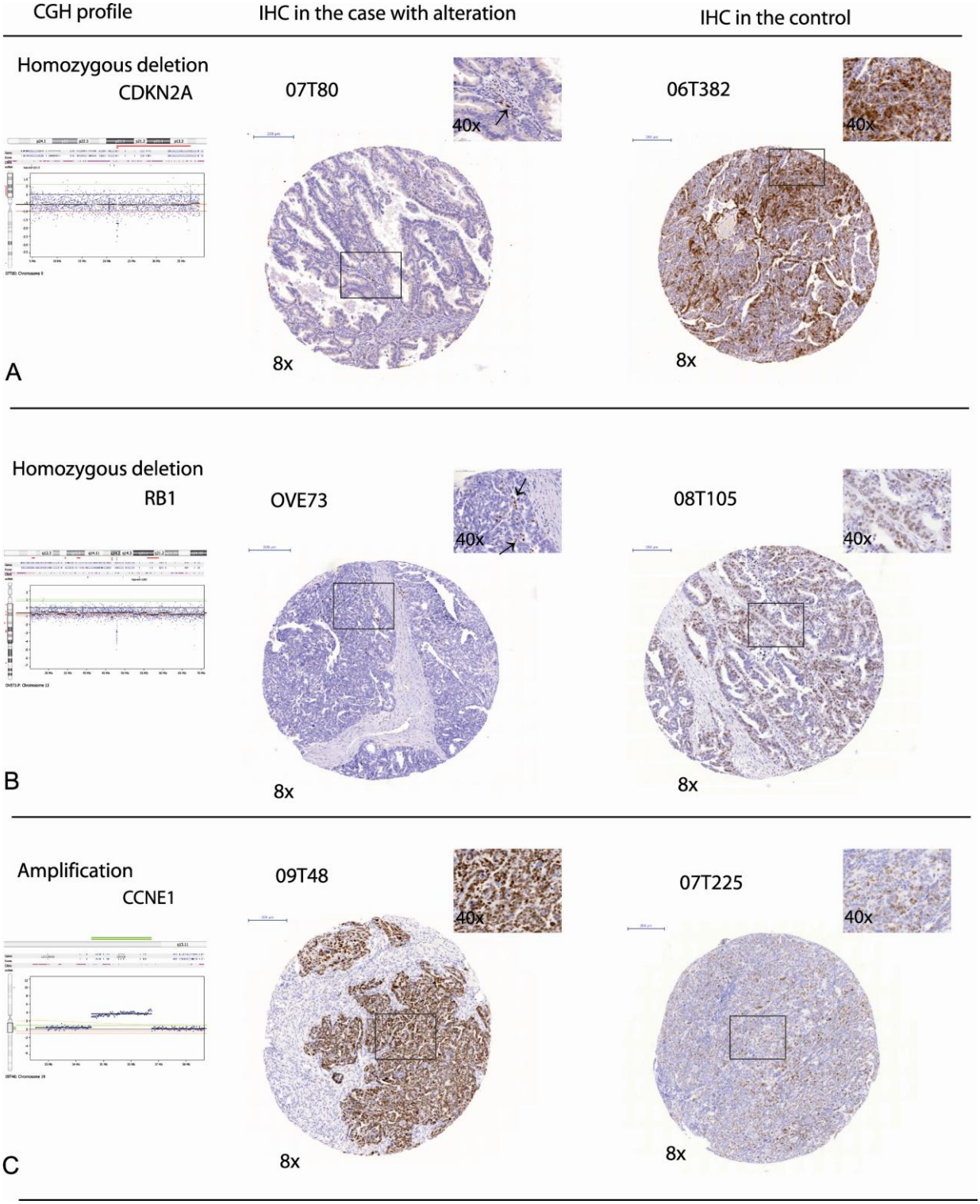

Figure 8. Immunohistochemical results: expression of three markers evaluated in the tumors with high copy number changes identified by aCGH: homozygous deletion of *CDKN2A* (**A**); *RB1* (**B**) and amplification of *CCNE1* (**C**). From the left to the right: CGH profile at the loci affected by copy number changes with the chromosomic location on x-axis and corresponding log2ratios on the y-axis; expression of the IHC antibody in the same tumoral sample (8 x magnification) with corresponding 40 x magnification of the part of the tumor (arrows pointing to internal controls for homozygous deletions); the most right panel shows expression of given antibody in the control sample (at 8x and 40x magnification) representing mean expression of the samples with normal DNA copy number status at this loci. The corresponding expression levels of the three antibodies, are shown in the right down corner next to each sample. The integral control is either macrophage within tumoral mass (*CDKN2A*) or stained tumoral cell (*RB1*).

2.9 PATHWAYS OF BIOLOGICAL RELEVANCE

To determine biological processes, which might be affected by copy number changes in each tumor group we performed pathway enrichment analysis using publicly available databases and the lists of genes identified within MCRs of gains, losses, amplifications and homozygous deletions per tumor group.

As shown in Table 10 there were no striking differences in the pathways significantly enriched in the different tumor groups. Cell cycle regulation and checkpoint pathways were found significantly associated to all tumor groups. In addition immune response pathways typically altered in cancer like Jak-STAT and Toll-like receptor signaling were enriched in BRCA1 and sporadic tumors, as well as was PIK3/AKT-signaling that is commonly found activated in ovarian tumors.

However, besides these similarities some pathways, like DNA repair through homologous recombination and Ras pathway were more frequently altered in BRCA1 tumors. As expected, loss of *BRCA1* function in DNA damage response was defined to be specifically associated with this tumor group *(Table 10).*

In sporadic series in addition to the pathways involved in immune response other activated (enriched in gained and amplified molecules) functions were related to oncogenic pathways (*MAPK-, EGFR-, PDGF-, VEGF- and IGF1*-signaling) suggesting their relevant role in the oncogenesis of sporadic tumors *(Table 10).*

High heterogeneity of BRCAX tumors together with their lower overall genomic instability lessened the chances of identifying many significantly enriched pathways. However cycle regulation and DNA replication-related pathways were identified as significantly enriched in this group *(Table 10).*

Table 10. The most significantly altered pathways determined by at least 2 different tools:

Term	Significance determined by diffrent tools: FATIGO	DAVID	IPA	Specific term defined by IPA
BRCA1 TUMORS				
Jak-STAT signaling pathway	**	*	*	
Cell cycle	**	*	**	***Cyclins and Cell Cycle Regulation***
				Cell Cycle: G1/S Checkpoint Regulation
IL 2 signaling pathway	**		*	
Homologous recombination	*		*	*Role of BRCA1 in DNA Damage Response*
Regulation of cell cycle progression by Plk3	*		*	*PI3K/AKT Signaling*
Toll-like receptor signaling pathway	*	*		
Ras Pathway	*	*		
SPORADIC TUMORS				
Natural killer cell mediated cytotoxicity	**	**	**	***Natural Killer Cell Signaling***
Jak-STAT signaling pathway	**	**	*	***Role of JAK1 and JAK3 in Cytokine Signaling***
MAPK signaling pathway	**	**	*	***ERK/MAPK Signaling***
PI3 kinase pathway	*	**	**	***Role of PI3K/AKT Signaling***
Signaling by PDGF/VEGF	**	**		
Signaling by EGFR	**	**		
Cell Cycle: G1/S Check Point		**		
Cell cycle	**	*		
Cyclins and Cell Cycle Regulation		*		
Toll-like receptor signaling pathway	*	*		
mTOR signaling pathway	*	*		
IGF-1 Signaling Pathway	*	*		
p53 pathway		**	*	
BRCAX TUMORS				
Cell Cycle, Mitotic	*	*	*	***DNA Double-Strand Break Repair by Homologous Recombination***
Cell Cycle Checkpoints	*		*	*Cell Cycle: G1/S Checkpoint Regulation*
DNA Replication	*		*	

**p <0.05; **p <0.01*; * - significant after correction for multiple testing FDR<0.05; in bold- pathways determined by all three tools

3 DNA COPY NUMBER-BASED UNSUPERVISED CLUSTERING OF EOCs

3.1 FAMILIAL STATUS OF THE EOCs STRATIFIED BY DNA COPY NUMBER PROFILING

In addition to systematic comparison of copy number alterations across different tumor subtypes, we also carried out unsupervised analysis of the aCGH data to unveil possible association between particular patterns of genomic changes and the sporadic or familial status of tumors (or specific familial subytpe, BRCA1/2/X).

Unsupervised hierarchical clustering stratified our series of ovarian carcinomas into two main clusters (A and B) *(Figure 9A).* There were no significant differences found between tumors from both clusters (or from smaller subgroups) either according to their general familial or sporadic condition or according to their specific BRCA mutation status *(Figure 9A).* Similarly, unsupervised hierarchical clustering of the subgroup of type II tumors also rendered two clusters (IIA and IIB) without significant enrichment in tumors from particular BRCA subgroups *(Figure 9B).*

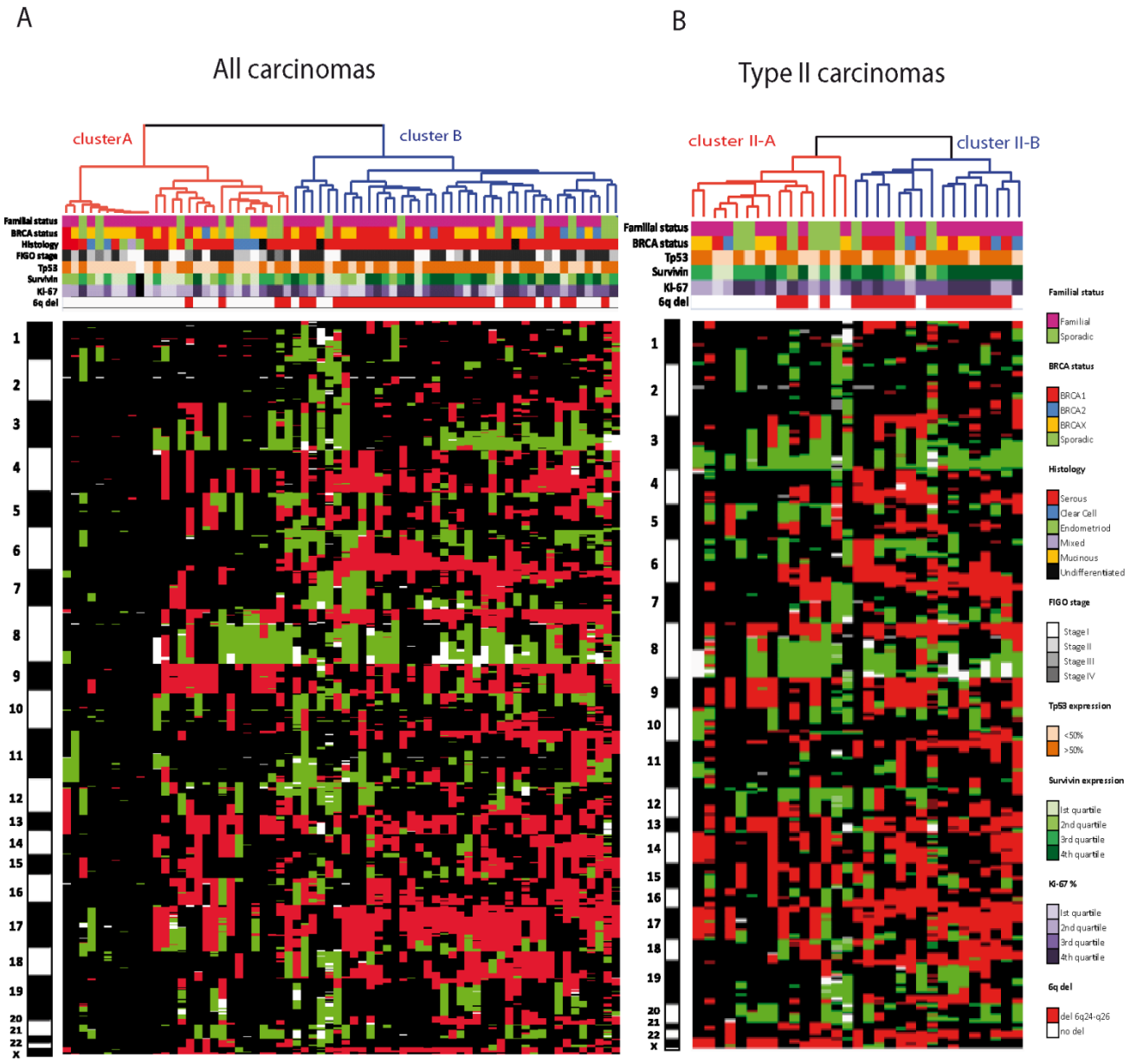

Figure 9. DNA copy number-based unsupervised hierarchical clustering of 68 primary ovarian carcinomas (**A**) and 31 type II carcinomas (n=31) (**B**). Each column represents a tumor sample and each row corresponds to DNA copy number changes mapped according to chromosomal location. Colors correspond to different copy number categories: red, loss; green, gain; white, amplification; black, lack of copy number changes. Dendrogram highlights the division of the samples into two main clusters. Hereditary or sporadic condition of tumors, immunohistopathological features and the 6q deletion status are represented by color labels shown below the dendrogram.

3.2 IMMUNOHISTOPATHOLOGICAL CHARACTERIZATION OF EOC CLUSTERS DEFINED BY THEIR DNA COPY NUMBER PROFILES

Since having or not *BRCA1/2* mutation did not seem to stratify the tumors into groups of specific pattern of DNA copy number alterations, we sought to determine whether tumors that shared a similar genomic instability profile may share a distinctive and biologically meaningful pattern of immunohistopathological features.

Cluster B, which comprised the most genomically unstable tumors, with higher number and length of alterations, was significantly enriched in high FIGO stage (P=0.03) and serous tumor type (versus all other subtypes, P=0.001). It was also characterized by higher proliferative rate (as measured by Ki-67 immunostaining) and increased expression of p53 and the antiapoptotic marker survivin *(Figure 9A , Table 11).*

No more significant associations were found with any other of 27 immunohistochemical markers (all the evaluated markers are listed in the *Supplementary Table 4* and *Supplementary Table 5*).

Table 11. Correlation of the clusters defined by unsupervised hierarchical clustering of 68 EOC carcinomas with immunohistopathological and genomic instability features:

Carcinomas		Cluster A n=28		Cluster B n=40		P-value
BRCA status, n *(%)*						
Familial		**20**	*(71.4)*	**33**	*(82.5)*	
BRCA1		***6***		***15***		
BRCA2		***1***		***5***		0.2[+]
BRCAX		***13***		***13***		
Sporadic		**8**	*(28.6)*	**7**	*(17.5)*	
Histology, n *(%)*						
Serous		**16**	*(57.1)*	**38**	*(95)*	
Endometrioid		**4**	*(14.3)*	**0**	-	
Mucinous		**1**	*(3.6)*	**0**	-	*0.001*[+]
Clear cell		**5**	*(17.8)*	**0**	-	
Undifferenciated		**1**	*(3.6)*	**2**	*(5)*	
Mixed		**1**	*(3.6)*	**0**	-	
FIGO stage, n *(%)*						
Low (I,II)		**10**	*(35.7)*	**4**	*(10)*	
High (III,IV)		**12**	*(42.9)*	**27**	*(67.5)*	*0.03*[+]
NA		**6**	*(21.4)*	**9**	*(22.5)*	
Grade (only serous)						
Low		**3**	*(18,7)*	**5**	*(13.2)*	0.82[+]
High		**12**	*(75)*	**31**	*(81.6)*	
NA		**1**	*(6.3)*	**2**	*(5.2)*	
Genomic instability						
Median number per tumor (interquartile range)						
Amplifications		**2**	*(1-4.3)*	**3**	*(1-5.8)*	0.371
Gains		**11.5**	*(6.8-21.5)*	**21.5**	*(13.3-31)*	*0.005*
Losses		**14**	*(6.8-20.3)*	**32**	*(28-41.8)*	*<0.0001*
HD		**1**	*(0-2.3)*	**4**	*(2-7.8)*	*<0.0001*
Median length per tumor[‡] *(interquartile range)*						
Gains		**155.2**	*´52.7-329.7)*	**338.8**	*(204.7-458.4)*	*0.004*
Losses		**309.9**	*´16.9-671.3)*	**752.8**	*(597.8-927.8)*	*<0.0001*
Immunohistochemical markers						
Median expression[#] *(interquartile range)*						
Nuclear Survivin		**12.0**	*(3-18)*	**21.5**	*(11.5-25.9)*	*0.001*
Ki-67		**34.5**	*(23.5-50)*	**51**	*(36.8-67.8)*	*0.024*
p53, n *(%)*	**Neg**	17	*(60.7)*	**Neg**	13	*(32.5)*	*0.024*[+]
	Pos	10	*(35.7)*	**Pos**	27	*(67.5)*	
	NA	1	*(3.6)*	**NA**	-	-	

[+]For categorical variables (*BRCA* status, Histology, FIGO stage, Grade, and p53 expression) Fisher Exact Test was used; All the other variables, expressed by continous values, were compared with Mann-Whitney test. *P*-values <0.05 were considered significant and shown in *italics*; HD, Homozygous Deletions; [‡]Expressed in Megabases; [#]The percentage of stained nuclei, independent of the intensity, Neg, negative; Pos, positive

When more homogenous group of type II carcinomas was considered, Cluster II-B, of more pronounced genomic loss, showed some evidence of higher expression of Progesterone Receptor (PR) (P=0.05) and survivin (P=0.06) *(Table12).*

Table 12. Correlation of the clusters defined by unsupervised hierarchical clustering of 31 Type II EOC with immunohistopathological and genomic instability features:

Type II EOC[$]	Cluster A-II n=15		Cluster B-II n=16		P-value
BRCA status, n *(%)*					
Familial	**9**	*(60)*	**13**	*(19.4)*	
BRCA1	***3***		***7***		
BRCA2	***1***		***3***		0.29[†]
BRCAX	***5***		***3***		
Sporadic	**6**	*(40)*	**3**	*(4.5)*	
Genomic instability					
Median number per tumor (interquartile range)					
Amplifications	**4**	*(2.8-5)*	**3**	*(1-6.8)*	0.42
Gains	**20**	*(10.8-31.3)*	**22**	*(14.5-27.5)*	0.79
Losses	**21**	*(15-28.3)*	**36**	*(31.3-50.8)*	*<0.0001*
Homozygous dele	**3**	*(1-5)*	**6**	*(2.5-10.8)*	*0.017*
Median length per tumor [‡] (interquartile range)					
Gains	**380.8**	*(195-571.4)*	**257.8**	*(204.7-423.3)*	0.19
Losses	**548.4**	*(360.3-801.9)*	**879.2**	*(597.8-996)*	*0.01*
Immunohistochemical markers					
Median expression[#] (interquartile range)					
Nuclear Survivin	***18***	*(6-22)*	***25***	*(17.6-35.5)*	0.06
PR	***4***	*(0-16.5)*	***23***	*(4-47)*	0.05

[$]Subgroup of type II carcinomas as described in Materials and Methods [†]For categorical variables (*BRCA* status) Fisher Exact Test was used; All the other variables, expressed by continous values, were compared with Mann-Whitney test. *P*-values <0.05 were considered significant;and shown it *italics* [‡]Expressed in Megabases; [#]The percentage of stained nuclei, independent of the intensity, Neg, negative; Pos, positive

3.3 DNA COPY NUMBER DEFINED GROUPS OF EOCs AND THEIR ASSOCIATION WITH PATIENTS' SURVIVAL

We further aimed to determine whether groups of ovarian tumors defined according to their copy number features differed in terms of patients' prognosis.

Univariate Cox regression analysis of clinicopathological variables confirmed the association of known prognostic factors, such as FIGO stage and status of debulking surgery with both overall survival (OS) and progression-free survival (PFS) *(Table 13).* Age, grade, histotype or BRCA mutation status did not show an association with survival in our series. Similarly, the DNA copy number-based defined clusters (A and B) were not significantly associated with survival in the univariate analysis. However, adjustment for significant cofactors (FIGO stage and debulking status) revealed an association of cluster B with better OS (HR=0.28, 95% CI:0.08-0.93; *P*=0.04) *(Table 14).*

Table 13. Univariate survival analysis

	OS			PFS		
Variable	***HR (95% CI)***		***P-value***	***HR (95% CI)***		***P-value***
Univariate analysis, all carcinomas						
Age of diagnosis	1.06	*(0.99-1.12)*	0.07	1.01	*(0.96-1.05)*	0.80
FIGO stage	7.77	*(1.03-58.64)*	*<0.001*	1.70	*(0.84-3.47)*	*0.02*
Debulking status *(optimal vs. suboptimal)*	3.18	*(1.07-9.47)*	*0.03*	1.48	*(0.67-3.3)*	*0.05*
Histological type	1.09	*(0.77-1.54)*	0.62	1.58	*(0.93-2.7)*	0.37
Clusters *(B vs.A)*	0.83	*(0.33-2.12)*	0.35	0.90	*(0.42-1.96)*	0.62
BRCA status: carriers *vs. non-carriers*	1.73	*(0.7-4.27)*	0.23	1.02	*(0.51-2.03)*	0.98
BRCA1 vs. non-carriers	1.60	*(0.59-4.31)*	0.35	0.90	*(0.42-1.93)*	0.79
BRCA2 vs. non-carriers	2.48	*(0.64-9.66)*	0.19	1.46	*(0.49-4.37)*	0.50

Univariate Cox regression analysis in the whole series of carcinomas with Overall Survival (OS) and Progression Free Survival (PFS) as endpoints. FIGO stage (I, II, III, IV), debulking status (optimal, residual tumour <1cm; suboptimal , residual tumour>1cm); histological type (serous versus all other). HR, Hazard Ratio. CI, Confidence Interval.*P- values* were calculated with log-rank test and the significant ones (<0.05) shwon in *italics*

To further corroborate this result, the analysis was repeated within high FIGO stage carcinomas only, as these tumors represent the majority of the series and the association of cluster (B) with improved survival was found already in the univariate analysis (HR=0.29, 95%CI:0.09-0.93, *P*=0.028) *(Figure 10A).*

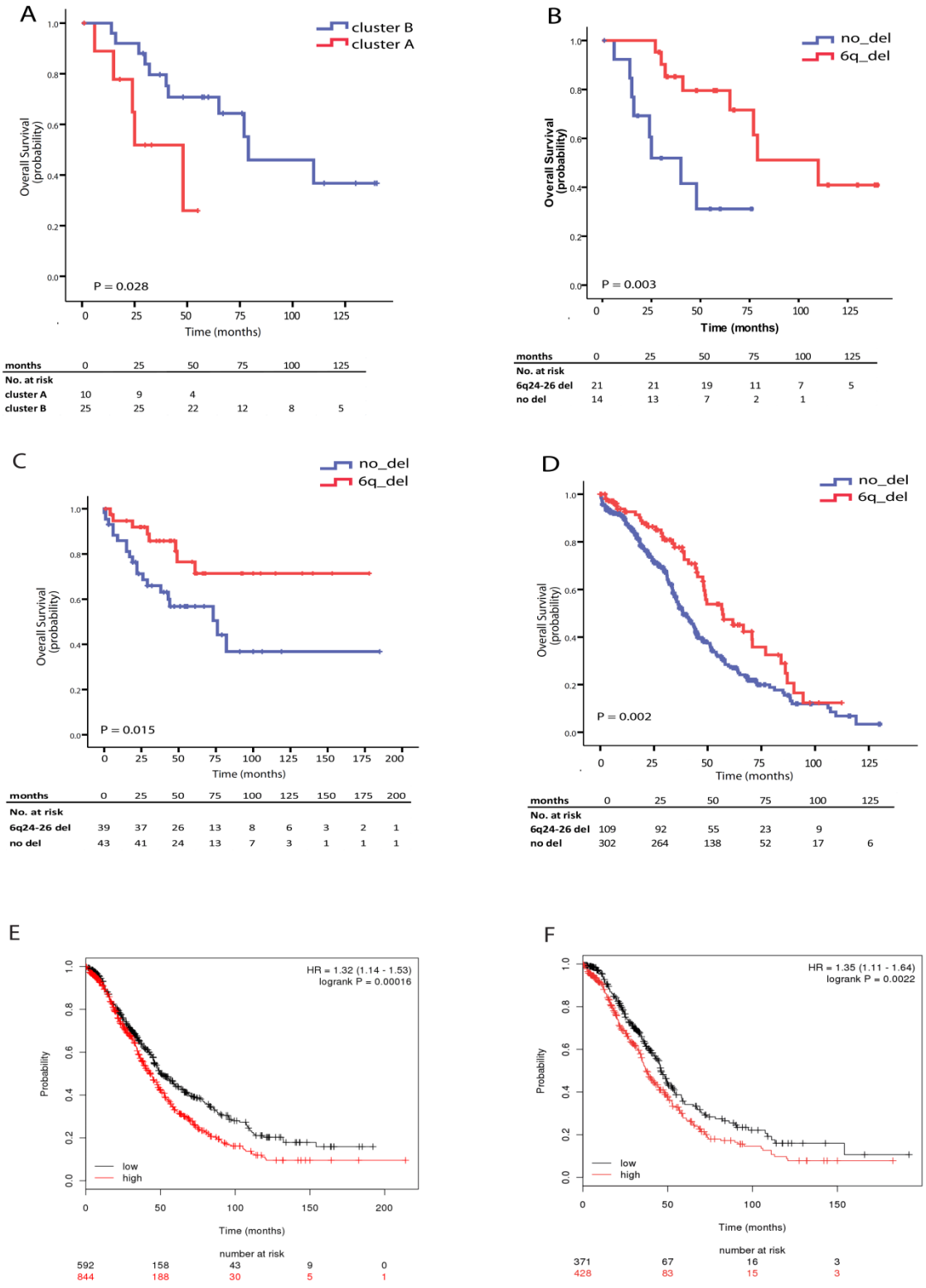

Figure 10. Kaplan-Meier survival curves. Overall Survival of high FIGO stage EOCs patients from the discovery series according to (**A**) clusters defined by DNA copy number-based unsupervised analysis (log-rank *P*=0.028) and (**B**) the presence of the 6q24.2-26 deletion (log-rank *P*=0.003). Validation of the association observed between the presence of the 6q24.2-26 deletion and improved overall survival in two independent series from (**C**) EOCs where the deletion was evaluated by Fluorescence *in situ* Hybridization (log-rank *P*=0.015) and (**D**) HGSOC patients from the TCGA study (log-rank test *P*=0.002). Lower expression of the genes within 6q24.2-26 region predicts longer Overall Survival in (**E**) all 1436 EOCs (log-rank *P*=0.0002) and in (**F**) 799 HGSOC carcinomas (log-rank *P*=0.002), as shown using an online tool KM-plotter from publicly-available microarray data.(Gyorffy *et al*, 2012)

More importantly it remained significant on adjustment for debulking status (HR=0.27, 95%CI=0.08-0.94, P_{adj}=0.04) *(Table 14).* To minimize the effect of death not due to ovarian cancer we confirmed the association after censoring all follow-up five years after diagnosis, both overall and for high FIGO stage carcinomas only *(Table 14).*

Table 14. Multivariate Cox regression model of prognostic factors for overall and 5-yrs survival

	Tumours[T]	Comparison	P-value	HR	HR (95% CI) Lower	Upper	P(adj)
Clusters							
Overall Survival (OS)							
	All carcinomas	***cluster B vs A***	0.345‡	0.28	0.08	0.93	*0.04*
		FIGO stage		11.77	1.21	114.19	*0.03*
		debulking status		1.87	0.59	5.92	0.28
	High FIGO	***cluster B vs A***	*0.028*	0.27	0.08	0.94	*0.04*
		debulking status		1.52	0.48	4.76	0.48
	Type II carcinomas	***cluster IIB vs IIA***	0.07‡	0.09	0.01	1.127	0.06
		debulking status		2.92	0.36	23.89	0.32
5-yrs survival							
	All carcinomas	***cluster B vs A***	0.28	0.26	0.08	0.91	*0.03*
		FIGO stage		8.45	0.89	80.44	0.06
		debulking status		1.31	0.40	4.36	0.66
	High FIGO	***cluster B vs A***	*0.028*	0.27	0.08	0.95	*0.04*
		debulking status		0.99	0.30	3.35	0.99
	Type II carcinomas	***cluster IIB vs IIA***	0.10	0.10	0.01	1.33	0.08
		debulking status		2.74	0.32	23.30	0.36
6q24.2-6q25 region							
Overall Survival (OS)							
	All carcinomas	***6q24.2-6q25***	*0.028*‡	0.14	0.04	0.49	*0.002*
		FIGO stage		19.02	1.94	186.85	*0.012*
		debulking status		1.48	0.45	4.87	0.52
	High FIGO	***6q24.2-6q25***	*0.003*	0.13	0.04	0.48	*0.002*
		debulking status		1.17	0.35	3.85	0.80
	HGSOC	***6q24.2-6q25***	*0.004*	0.17	0.04	0.72	*0.016*
		debulking status		2.06	0.56	7.58	0.28
	Type II carcinomas	***6q24.2-6q25***	0.10	0.09	0.01	1.13	0.06
		debulking status		2.92	0.36	23.89	0.32
5-yrs survival							
	All carcinomas	***6q24.2-6q25***	*0.026*	0.12	0.03	0.44	*0.001*
		FIGO stage		13.66	1.45	128.79	*0.02*
		debulking status		0.96	0.27	3.39	0.95
	High FIGO	***6q24.2-6q25***	*0.002*	0.12	0.03	0.44	*0.002*
		debulking status		0.70	0.20	2.51	0.59
	HGSOC	***6q24.2-6q25***	*0.005*	0.19	0.05	0.81	*0.025*
		debulking status		1.63	0.41	6.45	0.49
	Type II carcinomas	***6q24.2-6q25***	0.06	0.10	0.01	1.33	0.08
		debulking status		2.74	0.32	23.30	0.36

P-values in the univariate analysis calculated with log-rank test or ‡Gehan–Breslow–Wilcoxon test, when applicable; *P(adj)* as calcualted in multivariate analysis by using Cox regression model in: the whole series of carcinomas, high-FIGO stage carcinomas and high-grade serous carcinomas (HGSOC) with Overall Survival (OS) and 5-year survival as endpoints. FIGO stage (high, ≥III; low, I&II), debulking status (optimal, residual tumour <1cm; suboptimal , residual tumour>1cm). HR, Hazard Ratio. CI, Confidence Interval, significant *P- values* (<0.05) are shown in *italics*

Considering type II tumors only, the association of the more genomically unstable cluster II-B with better prognosis showed some, but weak evidence, in the univariate analysis (*P*=0.07) and after adjustment for debulking status in both OS and 5-years survival (HR=0.09, 95%CI=0.01-1.13, P_{adj}=0.06 and HR=0.10, 95%CI=0.01-1.33, P_{adj}=0.08, respectively) (*Table 14).*

3.4 EXPLAINING DIFFERENCES IN SURVIVAL OF PATIENTS FROM DIFFERENT CLUSTERS

3.4.1 BRCA1/2 mutation status

Due to the fact that ovarian cancer patients with germline *BRCA1/2* mutations show improved survival rate (Alsop *et al*, 2012; Bolton *et al*, 2012; Pennington & Swisher, 2012) and considering that, although not significant, cluster B showed some enrichment in mutation carriers *(Table 11),* the association of the clusters with survival was adjusted also for this factor (in addition to FIGO and debulking status) even if BRCA condition was not significant in the univariate analysis performed in our series *(Table 12),* Nevertheless, the association remained statistically significant (HR=0.28, 95%CI:0.08-0.96, P_{adj} =0.043) indicating that *BRCA1/2* mutation status did not explain the better survival of patients from the higher genomic instability cluster.

3.4.2 Genomic instability level

In ovarian tumors and other neoplasms it has been shown that higher genomic instability is associated with worse prognosis (Carter *et al*, 2006; Choi *et al*, 2009; Cope *et al*, 2013; Walther *et al*, 2008). However, extreme genomic instability provides beyond particular level, no growth advantage for cancer cell viability and is deleterious for cell survival (Baumbusch *et al*, 2013; Birkbak *et al*, 2011; Roylance *et al*, 2011). Therefore, we hypothesized that this effect might explain the better outcome of the patients with tumors in the more genomically unstable clusters.

In order to address this question we assessed the association with survival of genomic instability (GI), measured as the total length of altered genome, and included

as an explanatory variable dichotomised at the median, or categorized into quartiles. Patients with tumors with GI above the median had worse prognosis (HR=6.51, 95%CI: 1.2 - 35.07, P_{adj}=0.029) *(Figure 11)* and gradually increasing GI was also associated with worse outcome (HR=2.36 per quartile of GI, 95%CI: 1.17- 4.77, P_{adj}=0.016),(Cope *et al*, 2013) suggesting that in our series extreme levels of GI do not hinder tumor development (Carter *et al*, 2006; Kronenwett *et al*, 2004; Walther *et al*, 2008). Moreover, the association of cluster with survival was stronger after adjustment for this variable (HR=0.20, 95%CI: 0.056 – 0.75, P_{adj}= 0.018).

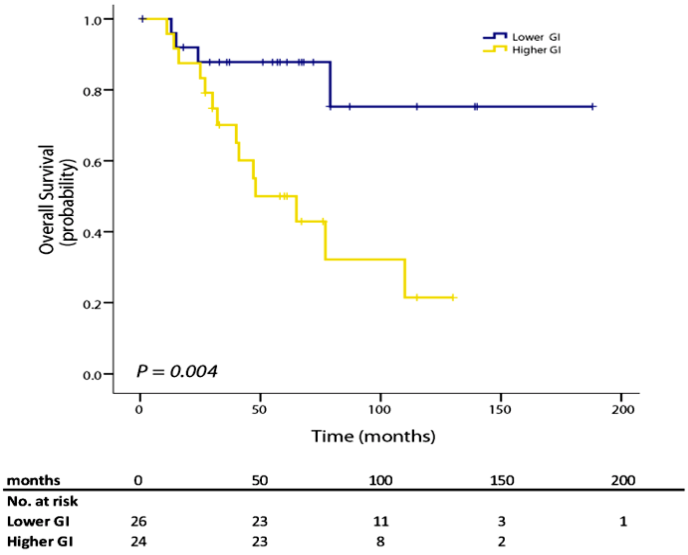

Figure 11. Kaplan-Meier survival plot for overall survival according to genomic instability (GI) level defined using median total length of the genome altered (log-rank *P*=0.004)

3.4.3 Specific DNA copy number alterations

After ruling out the level of genomic instability as a factor that may explain the differences in the survival between clusters, we hypothesized that specific copy number changes might explain the observed association.

For this purpose we determined altered regions that significantly (FDR<0.01) differentiated clusters A and B (and clusters IIA and IIB) by calculating the maximum pairwise symmetrized Kullback Leibler divergence score for each chromosomal

region rendered in the clustering analysis (as described in Materials and Methods). Top scored regions, significantly differentiating clusters after FDR correction are shown in *Table 15*. All top regions were then tested for their association with overall survival using Kaplan-Meier estimates.

Only one deletion at 6q24.2-6q26 (145,593,087-162,867,181), spanning 152 genes, more predominant in cluster B *(Table 15)* was found to be significantly related to better outcome (HR=0.51, 95%CI: 0.2-1.29, *P*=0.028) *(Figure 10B).*

This association remained significant after adjustment for FIGO stage and debulking status (HR=0.14, 95%CI=0.04-0.49, P_{adj} =0.002) *(Table 14)* and on limiting the analysis to high FIGO stage tumors only (HR=0.13, 95%CI=0.04-0.48, P_{adj}=0.002) *(Table 14)* and to HGOCs only (HR=0.17, 95%CI=0.04-0.72, P_{adj}=0.016) *(Table 14).* The association was also maintained on additional adjustment for age, grade, and *BRCA1/2* mutation status. Among the regions differentiating clusters IIA and IIB in the subgroup of type II tumors the same deleted region (6q25.1) characteristic for cluster II-B *(Table 15),* showed a tendency towards better prognosis (P_{adj}=0.062).

Among regions differentiating clusters II-A and II-B (type II tumors only) a deleted region in the same chromosomal location (6q25.1), characteristic of cluster II-B *(Table 15)* showed weak evidence of association with better prognosis in the univariate analysis (*P*=0.1) and after adjustment for debulking status (HR=0.09, 95%CI=0.01-1.13, P_{adj} =0.062).

Table 15 List of regions differentiating clusters in the whole set of carcinomas and in type II tumors

Cytoband	Start [bp]	End [bp]	Size [bp]	Nr of genes	Kullback Leibler score	Enriched cluster	Genes of interest‡
All carcinomas							
Gains							
7q32.3-q36.2	131,691,734	155,928,338	24,236,604	206	1.80	***B***	***CREB3L2 KIAA1549 BRAF*** *EZH2* ***EPHA1***
Losses							
6q21-q22	107,800,817	114,592,693	6,791,876	46	1.54	***B***	***FOXO3*** *TRAF3IP2 LAMA4*
6q24.2-q26	145,593,087	163,760,007	18,166,920	152	1.98	***B***	*ESR1 ARID1B PARK2*
7p22.1-p21.3	5,337,272	16,586,072	11,248,800	53	1.74	***B***	***PMS2 ETV1***
7p21.1	16,606,047	19,615,529	3,009,482	11	1.50	***B***	
7p15.3	19,627,792	20,373,135	745,343	5	1.47	***B***	
11p15.4	4,350,150	6,370,707	2,020,557	76	1.63	***B***	
17p11.2	19,083,766	26,409,508	7,325,742	108	1.83	***B***	
17q12	33,728,116	35,544,912	1,816,796	46	1.67	***B***	***MLLT6 LASP1 ERBB2***
17q21.31	38,511,969	38,540,145	28,176	1	1.45	***B***	***BRCA1***
18q23	75,803,559	76,116,026	312,467	6	1.52	***B***	
22q13.1	39,132,840	39,146,045	13,205	1	1.83	***B***	***MKL1***
Type II carcinomas †							
Gains							
5p13.2-p13.1	35,965,963	42,072,451	6,106,488	37	3.48	***II-A***	***LIFR*** *RICTOR*
5p12	44,072,044	46,150,784	2,078,740	3	3.11	***II-A***	***FGF10***
19p13.2	8,200,910	10,716,707	2,515,797	80	4.05	***II-A***	***MUC16***
19p13.13	12,615,927	12,627,741	11,814	1	3.34	***II-A***	
Losses							
1p36.33	784,458	1,500,487	716,029	41	5.82	***II-B***	
4p16.3-p16.1	13,036	11,781,764	11,768,728	117	3.82	***II-B***	***FGFR3 WHSC1***
4p15.33-p15.31	13,722,505	23,836,873	10,114,368	30	3.75	***II-B***	
4p15.2-q13.2	25,620,436	68,863,978	43,243,542	132	3.55	***II-B***	***PHOX2B FIP1L1 CHIC2 PDGFRA KIT KDR***
6q14.1	78,073,601	81,332,468	3,258,867	10	3.01	***II-B***	
6q25.1	151,948,867	152,073,215	124,348	2	3.12	***II-B***	*ESR1*
8p11.23	39,356,595	39,378,051	21,456	1	3.04	***II-B***	
11p13-11p12	31,789,321	43,759,278	11,969,957	47	3.38	***II-B***	***WT1 LMO2***
19p13.3	210,395	566,610	356,215	12	3.12	***II-B***	
19q13.12-q13.32	41,497,831	50,990,870	9,493,039	272	4.93	***II-B***	***AKT2 CD79A CIC BCL3 CBLC ERCC2***

†Subgroup of type II carcinomas as described in Materials and Methods; Regions differentiating clusters in whole set of carcinomas (Clusters A and B) and in Type II tumours (Clusters IIA and IIB) were defined by using the pairwise symmetrized Kullback Leibler (KL) divergence score implemented in WECCA and chi-square test corrected for multiple testing (significance, *FDR*<0.01.) .‡Genes of interest were selected from Cancer Census (in bold) or based on their function and previously published data. Regions which were less than 1MB apart and showed similar KL score were joined together and listed under the same cytoband. Highlighted regions were found to be associated with better prognosis.

4 PROGNOSTIC VALUE OF THE 6q24.2-q26 DELETION IN OVARIAN CANCER

4.1 VALIDATION IN AN INDEPENDENT OVARIAN CANCER SERIES BY FISH ANALYSIS

In order to validate our findings we performed FISH using a 6q25.1 probe *(Figure 12)* in an independent series of 103 EOCs of different histology that also included a subset of familial cases. Eighty-four tumors were successfully hybridized. The deletion was detected in 48% of successfully hybridized cases and was associated with significantly better overall survival (P=0.015) *(Figure 10C).* After adjusting for the only significant covariable (FIGO stage) in this series, the 6q deletion was confirmed to be an independent prognostic marker for overall survival (HR=0.38, 95%CI=0.15-0.96, P_{adj}=0.042). In addition, to acknowledge the atypical histological composition of this tumor series and different status of the residual disease the association was further proved on adjustment for histological type, tumor grade and debulking status (HR=0.25, 95%CI=0.065-0.96, P_{adj}=0.045). The results were also consistent on adjusting for age, *BRCA1/2* mutation status.

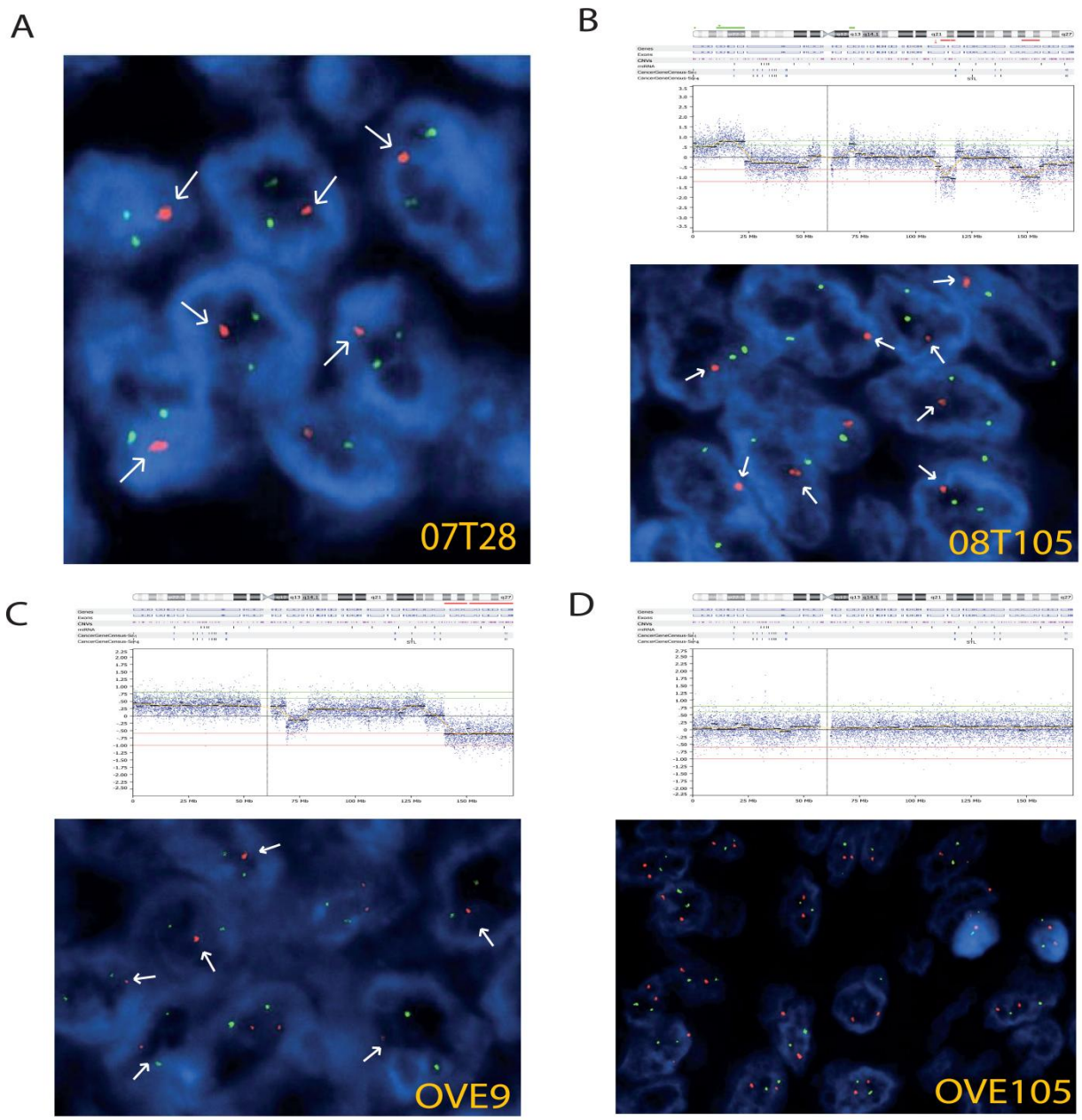

Figure 12. Evaluation of the 6q24.2-26 deletion by Fluorescence *in situ* Hybridization (FISH) on paraffin-embedded tissue sections. Test (red) and reference (green) probes mapped to 6q25.1 and 6p21, respectively. Presence of one single red signal is indicated with an arrow. (**A**) Tumor from validation series showing deletion at 6q25.1. (**B, C, D**) Chromosome 6 array-CGH profiles (top panels) of tumors from the discovery series and corresponding FISH analysis (lower panels) confirming the presence of the deletion (**B, C)** and normal DNA copy number at this locus (**D**). Magnification: 100x

4.2 VALIDATION IN THE TCGA OVARIAN CANCER SERIES

Since HGSOCs are the most common and lethal EOCs we aimed to further validate the association of the 6q24.2-q26 deletion with disease outcome in this specific histotype by using 411 HGSOCs from the TCGA ovarian cancer study (TCGA, 2011). Normalized $\log_2$ ratios from 1M Agilent Sure Print Human Microarray platform were downloaded from TCGA website (https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm) and subjected to the segmentation and calling algorithms used in our discovery series (Materials and Methods). Tumors were considered deletion-positive if at least 90% of the defined region (6q24.2-q26, 145,593,087-162,867,181) was lost. The deletion was associated with survival advantage in the univariate analysis (P=0.002) *(Figure 10D)* and, once adjusted for significant confounders (FIGO stage, *BRCA1/2* mutation status, and age at diagnosis) (HR=0.67, 95%CI=0.48-0.93, P_{adj}=0.019). Further adjustment for debulking status produced consistent results. The prognostic value of the marker was also confirmed for 5yr survival (HR=0.61, 95%CI=0.41-0.89, P_{adj}=0.010).

4.3 VALIDATION BY USING GLOBAL GENE EXPRESSION AT 6q24.2-q26 AND A META ANALYSIS APPROACH

Assuming that copy number status has an impact on the mRNA level we evaluated the prognostic value of the deletion at the gene expression level using KM-plotter (Gyorffy *et al*, 2012), which integrates gene expression and clinical data from 10 different data sets for 1436 EOCs patients (Materials and Methods). We found that low mean expression of the genes within 6q24.2-26 region was associated with longer OS in all 1436 EOCs patients (HR=1.32, 95%CI=1.14-1.53, log-rank P=0.0002) and on limiting the analysis to 799 HGSOCs (HR=1.35, 95%CI=1.11-1.64, log-rank P=0.002) *(Figure 10E* and *Figure 10F*, respectively) and to 675 high FIGO stage (III&IV) HGSOCs only (HR=1.31, 95%CI=1.04-1.64, log-rank P=0.02). The results were consistent once 5yrs follow up period was considered. In addition, to acknowledge the effect of confounding factors, the association was evaluated separately in the

stratified groups (optimally and suboptimally debulked tumors; high and low FIGO stage tumors) producing consistent results.

4.4 DEFINITION OF CANDIDATE GENES EXPLAINING SURVIVAL ASSOCIATION

Next, in order to propose individual candidate genes that might explain the observed association, we selected only those genes whose copy number status actually had an impact on the expression level. By using 232 tumors with accessible copy number and expression data from TCGA study we found that 76% of the examined genes in the region (62 out of 81 with available RNAseq data) were significantly down-regulated when lost (FDR<0.05) (Materials and Methods and *Supplementary Figure 1*). Of these, multivariate Cox regression analysis identified four genes whose downregulation was significantly associated with better survival independently of known prognostic factors (FIGO stage, age at diagnosis, and *BRCA1/2* mutation status) (P_{adj}<0.05) and eight additional genes that showed borderline associations (P_{adj}<0.1) (Materials and Methods and *Table 16*).

Table 16 . List of the genes from the region whose expession was associated with overall survival

Gene symbol	Gene name	HR	HR (95% CI)		
			Lower	Upper	$P_{(adj)}$
SLC22A2	*solute carrier family 22,member 2*	1.34	1.17	1.54	*<0.0001*
ARID1B	*AT rich interactive domain 1B (SWI1-like)*	1.18	1.02	1.36	*0.029*
SLC22A3	*solute carrier family 22,member 3*	1.17	1.01	1.36	*0.040*
SAMD5	*sterile alpha motif domain containing 5*	1.17	1.01	1.37	*0.043*
GRM1	*glutamate receptor, metabotropic 1*	1.16	0.99	1.37	0.07
TAB2	*TGF-beta activated kinase 1/MAP3K7 binding protein 2*	1.13	0.98	1.30	0.08
PPIL4	*peptidylprolyl isomerase (cyclophilin)-like 4*	1.15	0.98	1.35	0.08
TIAM2	*T-cell lymphoma invasion and metastasis 2*	1.13	0.98	1.32	0.09
AKAP12	*A kinase (PRKA) anchor protein 12*	1.13	0.98	1.31	0.09
GTF2H5	*general transcription factor IIH, polypeptide 5*	1.15	0.98	1.36	0.10
ULBP1	*UL16 binding protein 1*	1.12	0.98	1.29	0.10
SASH1	*SAM and SH3 domain containing 1*	1.11	0.98	1.26	0.10

HR, Hazard Ratio, CI, Confidence Interval; p-values were calculated with cox proportional hazard model for each gene individually adjusting for cofactors (FIGO stage, age of diagnosis and *BRCA1/2* mutation status), significant *P*-values (<0.05) are highlighted in *italics*

DISCUSSION

Epithelial ovarian cancer is a very heterogeneous disease with many histological subtypes and classification criteria. It represents more a range of different diseases sharing an anatomical location (Prat, 2012b). Therefore, current efforts in the field pursue the stratification of EOCs into biologically meaningful groups that actually reflect different clinical behavior. As for other neoplasms the emphasis is now made on gaining knowledge about the molecular alterations that characterize the different groups of tumors, which ultimately might determine response to treatment and patients´ outcome.

So far few studies have specifically analyzed the DNA copy number changes that characterize the different groups of hereditary ovarian tumors (*BRCA1, BRCA2* and BRCAX) or have compared these changes with those observed in sporadic cases (Israeli *et al*, 2003; Leunen *et al*, 2009; Patael-Karasik *et al*, 2000; Ramus *et al*, 2003; Zweemer *et al*, 2001). Moreover, the few studies conducted have yielded contradictory results, which might be due to limited number of tumors included (Israeli *et al*, 2003; Leunen *et al*, 2009; Patael-Karasik *et al*, 2000), the use of low resolution platforms (Patael-Karasik *et al*, 2000; Ramus *et al*, 2003), the application of different algorithms or the dissimilar characteristics of the comparisons made (TCGA, 2011).

With these antecedents and given the recently confirmed relevance of copy number changes as drivers of ovarian oncogenesis (TCGA, 2011) and the growing clinical implications of the *BRCA1/2* mutation status, we aimed to determine how hereditary and sporadic ovarian tumors relate to genomic instability and to define common and/or distinct events occurring in the genesis and evolution of these neoplasms. In our study we tried to address some of the limitations of prior studies by using a high-resolution aCGH platform and separately considering copy number gains and losses. Also, in contrast to the majority of previous studies, we not only analyzed tumors from carriers of *BRCA1/2* mutations, but also from non-*BRCA1/2* hereditary patients (BRCAX tumors) as these have been particularly poorly characterized.

I. HIGH SIMILARITY BETWEEN DNA COPY NUMBER PROFILES OF SPORADIC AND FAMILIAR EOC

Our findings indicate lack of substantial differences in the global pattern of DNA copy number changes between sporadic and familial EOCs. The general profile of genomic instability between those tumors was comparable as reflected by similar frequency plot overviews and similar total number and length of copy number alterations. Also this resemblance was illustrated by the existence of several shared regions found to be recurrently altered in each group of tumors. These common events point to the involvement of genes fundamental for ovarian carcinogenesis, selected throughout the evolution of the tumors and providing advantage to any cancer cell, independently of the existence of germinal mutations in the *BRCA1/2* genes.

Some of the possible candidates have been previously implicated in ovarian carcinogenesis such as *PIK3CA, MECOM* and *MYC* oncogenes, found within recurrently amplified and gained regions or *NF1* and *RB1* tumor suppressors, defined within commonly deleted genomic regions. In addition we also defined other less characterized genes, whose gain of function (*CDH12, FOXQ1, TXNDC5, CCND2, FOXJ1*) and/or abrogation (*FANCC, TSC1, CREBBP, CDH11, EDA2R*) might be crucial for ovarian cancer development and/or progression. Exploration of the therapeutic opportunities provided by these targets, to which a majority of tumors are likely to be addicted, is an attractive possibility. For instance, it has been suggested that modulation of cellular activities of the forkhead transcription factor FOXQ1 may have an application in cancer therapy, since its inhibition blocks epithelial to mesenchymal transition and results in cancer cell sensitization to a variety of chemotherapeutic agents (Qiao et al, 2011). Therapeutic approaches targeting cyclin D gene have also been explored (Dong et al, 2010; Tiedemann et al, 2008) and might be applicable to EOCs presenting aberrant *CCND2* expression due to DNA-copy number gains. Likewise, m-TORC1-directed therapies may be more effective in cancer patients, whose tumors present TSC1 (tuberous sclerosis complex 1) genomic losses as it has been proposed for patients whose tumor harbor *TSC1* somatic mutations (Iyer et al, 2012).

Also exemplifying the absence of marked differences in the profile of genomic changes of carriers and non-carriers of *BRCA1/2* mutations, unsupervised hierarchical clustering did not stratify tumors according to their familial or sporadic condition, nor did it according to their *BRCA1/2* mutation status. These findings are in contrast to what has been observed in familial BRCA1 and BRCA2 breast tumors, which show an association with particular molecular subtypes (defined with expression arrays) and specific patterns of copy number changes (Bergamaschi et al, 2006; Jonsson et al, 2005; Melchor et al, 2008; Stefansson et al, 2009). Lack of segregation of ovarian tumors from carriers and non-carriers of *BRCA1/2* germline mutations, based on their genomic instability pattern, would support a model according to which homologous recombination (HR) deficiency, arising through distinct mechanisms including germline, but also somatic inactivation of the *BRCA1/2* genes, methylation of *BRCA1* or other members of the pathway, and *EMSY* amplification, is not only a frequent event explaining about 50% of high-grade serous ovarian carcinomas (Bowtell, 2010; TCGA, 2011), but also an event occurring in the initial phases of tumor growth. Such events mimicking biologic behavior of *BRCA1/2* deficient tumors and their phenotypic characteristics has been termed "BRCAness". This notion was started in 1996 following the few studies on inactivation of *BRCA1/2* genes in sporadic ovarian tumors, pointing to their resemblance of *BRCA*-related tumors (Esteller et al, 2000; Foster et al, 1996; Geisler et al, 2002).

Although our results support the hypothesis only at the genomic level, other evidence from gene expression profiling of sporadic and familial tumors also indicates lack of consistent separation of high grade ovarian carcinomas according to *BRCA1/2* status (George *et al*, 2013a; Pradhan *et al*, 2010)

2. EXTENSIVE GENOMIC LOSS IN BRCA1/2 EOC

Interestingly, despite this similarity between sporadic and hereditary tumors, some differences in the overall degree of genomic instability were revealed when gains and losses were analyzed separately. Greater contribution of losses than gains was observed in all tumor subtypes, however the extent of this phenomenon was

more prominent in carriers of *BRCA1* and *BRCA2* mutations, both in global terms (comparison of losses made across tumor subtypes) and relative to the number of gains (comparison within each tumor subtype).

Some prior studies, including the most comprehensive one conducted by the TCGA Research network in high-grade serous ovarian carcinomas reported no differences in the global degree of instability between tumors with *BRCA1/2* inactivating events and those with functional *BRCA1/2* genes (Ramus *et al*, 2003; TCGA, 2011). However, no distinction was made between gains and losses, and only comparison of total changes was conducted. Earlier studies already suggested the relevance of loss of heterozygosity (LOH) in ovarian tumors from *BRCA1* and *BRCA2* mutation carriers (Leunen *et al*, 2009; Walsh *et al*, 2008; Wang *et al*, 2012a), but included very few familial cases (Leunen et al, 2009; Walsh et al, 2008) or used low-resolution platforms (Leunen et al, 2009). Our results derived from analysis made across tumor types, within each tumor subgroup and particularly when taking into account only a subgroup of high-grade type II tumors highlight the relevance of genomic loss in *BRCA1* and *BRCA2* tumors, a phenomenon that would not merely reflect differences related to the higher grade or more prevalent serous histotype of those tumors.

Our findings suggest that in the oncogenesis of ovarian tumors, and in particular of hereditary BRCA1 and BRCA2 carcinomas, loss of function of tumor suppressors might be under greater selection pressure than gain of function of proto-oncogenes at least through DNA copy number-related mechanisms. In fact, a gain of function of proto-oncogenes (*MAPK*, *EGFR*, *PDGF*, *VEGF*, and *IGF1*) in sporadic cases rather than in *BRCA1* tumors was supported by the pathway enrichment analysis. However, despite a potential selection pressure for loss events in tumors from carriers we did not found enrichment of particular suppressor pathways in the BRCA1 associated tumors. This fact, and the lack of clear segregation of BRCA1 and BRCA2 tumors in the unsupervised analysis would suggest that most of the genomic loss in carriers would not involve a consistent set of specific critical regions (or specific suppressor genes) recurrently selected during evolution of these particular tumors. Alternatively, greater involvement of loss events in ovarian tumors might be related to impairment of HR function, with grosser effects in BRCA1 and BRCA2

tumors due to their central role in the pathway. It should be noted that the results derived from the pathway enrichment assessment would be limited by the fact that the analysis was performed entirely based on genes identified to be altered only at the DNA copy number level without further integration with gene expression data.

3. DNA COPY NUMBER PROFILES OF BRCAX TUMORS RESEMBLE SPORADIC MORE THAN OTHER FAMILIAL CASES

Interestingly, in our study, that included a representative group of familial BRCAX cases, we found that this group shares more similarities with sporadic cases than with BRCA1 and BRCA2 tumors. While BRCA1 and BRCA2 were characterized by extensive genomic loss, BRCAX tumors presented the lowest total number of alterations overall and in particular of losses. Also, the greater involvement of losses compared to gains in tumor from carriers was less marked in BRCAX tumors and similar to that observed in sporadic cases.

This would be consistent with the fact that the predominant role of genomic losses in EOCs might be to a great extent determined by HR defects and in particular with this feature being more prominent due to specific HR impairment by *BRCA1* and *BRCA2* dysfunctions. Up to 50% of sporadic cases are expected to show HR impairment through different mechanisms that include *BRCA1/2* germline mutations (despite lack of familial history), somatic mutations and epigenetic silencing and also through alterations in other genes of the pathway (Bowtell, 2010; TCGA, 2011). In BRCAX tumors the presence of *BRCA1* and *BRCA2* germline mutations has been ruled out through genetic testing and this, may at least partly account for the lower rate of losses observed in this group of tumors. It is possible that these tumors may have germline HR disruption by loss of function of other genes involved in the pathway (such as *RAD51D*, *RAD51C, BRIP1, CHEK2* or *BARD1*), loss of which may have less prominent impact on the genomic instability level. However alterations in these genes may explain only a low percentage of familiar cases (about 6% altogether) (Pennington *et al*, 2013). Also, the lower rate of losses in BRCAX cases might suggest

that majority of unknown susceptibility genes responsible for the ovarian cancer risk in the BRCAX families might belong to pathways different than HR.

4. GROUP-SPECIFIC COPY NUMBER ALTERATIONS AS POTENTIAL BIOMARKERS OF BRCAness

Although lack of clear segregation of hereditary and sporadic tumors in the unsupervised analysis indicates that there is not a clear pattern of critical regions consistently related to each subgroup of tumors, we were able to define some alterations potentially associated with BRCA1 and sporadic tumors. The individual regions with significantly different frequency in the different groups of tumors might reflect an accumulation of few selected genomic events acquired during development of tumors of a particular genetic background.

BRCA1 associated regions are of particular interest, as might be pointing to genes whose loss or gain is selectively required to permit cell growth of the highly genomically unstable-*BRCA1* defective cells. Importantly they may serve as biomarkers to identify tumors with BRCAness (Rigakos & Razis, 2012; Wysham *et al*, 2012), which has important implications in the clinical setting given the enhanced response to PARP inhibitors shown by EOCs from *BRCA1* and *BRCA2* mutations carriers (Ratner *et al*, 2012). In our series we reported that losses at 4q32.1-q35.2, 13q13.3-q14.3, 17q11.1-q11.2, 17q12, 17q21.32-q21.33, 17q24.3-q25.1 and 22q13.31 are more specifically related to BRCA1 cases and these alterations were also previously shown to be associated with this tumor group (Domanska et al, 2010; Ramus et al, 2003; Zweemer et al, 2001).

In contrast much fewer regions were reported to be specifically associated with sporadic cases, most of them occurred at chromosome 19 and consisted of copy number gains or amplifications. Reassuringly, the largest so far ovarian cancer study of TCGA (TCGA, 2011) reported that amplifications of two of these regions, 19p13.13 and 19q12, the latest encompassing *CCNE1,* were the only ones significantly enriched in high-grade serous sporadic EOCs. This finding reinforces the proposed role for *CCNE1* and of other proteins implicated in cell cycle progression as important

contributors to ovarian carcinogenesis in tumors with intact *BRCA1/2* function (Berns & Bowtell, 2012; Bowtell, 2010; TCGA, 2011).

Given the fact that patients with *BRCA1/2*-mutated ovarian tumors present relatively uniform behavior with high overall response rates to first-line platinum-based therapy (Boyd *et al*, 2000; Vencken *et al*, 2011), long disease-free intervals, and improved overall survival rates (Alsop *et al*, 2012; Bolton *et al*, 2012; Yang *et al*, 2011), it is of a great importance to identify markers that help to identify BRCA-related patients, who present better response to standard treatment regimes and are more likely to benefit from the treatment with PARP inhibitors. Screening for somatic and/or germline mutations to identify those patients is impractical for large populations and also non informative for other kinds of defects in the HR pathway that can lead to *BRCA*ness.

Although the early attempts to identify *BRCA*ness features, mainly based on gene expression profiling in *BRCA1/2* deficient and proficient tumors, (Jazaeri *et al*, 2002; Konstantinopoulos *et al*, 2010) led to define a gene signature that was successfully predicting *BRCA*-like phenotype, it did not present further clinical utility. In this study, we propose several DNA copy number regions, specifically associated with *BRCA1* mutation carriers that may be used to guide the selection of BRCA-related patients. In addition, in the light of recent findings reporting mutual exclusivity between *BRCA1/2* impairment and *CCNE1* amplification (Bowtell, 2010; TCGA, 2011), the predictive value of each of the identified regions, together with the absence of mutually exclusive ones, may help to develop a scoring system, that would more accurately predict a *BRCA*ness phenotype. The big advantage of such DNA-based markers is the fact that they can be easily analyzed on paraffin sections by FISH, therefore being particularly suitable for routine clinical applications. In addition, the greater rate of copy number losses that has been found specifically associated with *BRCA1* and *BRCA2* mutation carriers may also serve as a marker itself.

The usefulness and robustness of these particular DNA copy number changes and of the rate of genomic loss in defining the *BRCA1/2* phenotype need to be validated in larger cohorts and prospective studies. However, in addition to other approaches of feasible implementation in the clinical setting, such as the sequencing

of a reduced panel of informative genes (ie. DNA repair, chromatin remodeling and DNA cohesion related genes) (Bajrami *et al*, 2013) copy-number changes might demonstrate utility in predicting the BRCA-like phenotype in EOCs.

5. DNA COPY NUMBER PROFILES DIFFERENTIATES EOCs INTO GROUPS OF DIFFERENT IMMUNOHISTOPATHOLIGICAL AND CLINICAL FEATURES

In addition to gain insight into the differences and similarities of familial and sporadic EOCs, we were interested in defining novel prognostic and predictive biomarkers in epithelial ovarian cancer. Since this neoplasm is the most lethal gynecological malignancy (Ferlay J et al, 2013) the identification of novel molecular markers that may explain the different clinical behavior of EOC patients is of critical importance. In this context we have investigated whether stratification of EOCs on the basis of DNA copy number may delineate novel categories of tumors with different underlying biology, as defined by a distinct immunostaining pattern and/or, more importantly, by a different clinical outcome.

Since our data derived from supervised and unsupervised analysis of DNA copy number profiles showed lack of clear separation of familial and sporadic EOCs we sought to determine what other features might characterize the tumors that shared a similar genomic instability pattern.

We found that the cluster of EOCs exhibiting greater genomic instability (cluster B) was associated with high FIGO stage and serous histological subtype. Almost the entire high genomic instability group was composed of serous tumors, of mainly high grade, indicating that their copy number profile was substantially different from all the other histological types. Noteworthy, all the other histological types fell in the cluster of lower genomic instability. This separation mostly coincided with the distinction between type I and type II tumors, with the latter ones falling into more genomically instable cluster, in agreement with the characteristics of type II

tumors that present higher rate of genomic instability,than type I (Kurman & Shih Ie, 2011b).

Although 30 different immunohistochmical markers were used only three showed to significantly differentiate the groups of tumors defined based on their genomic instability profiles. The three markers pointed to greater aggressiveness and cellular turnover of the more gnomically unstable tumors.

Positive staining of TP53 is an indicator of mutated TP53, as the mutations of this tumor suppression gene result in a conformational change of the protein, stabilizing it and allowing for immunohistochemical detection. More frequent positive p53 staining in the tumors from the more genomically unstable cluster is likely to be explained by greater enrichment of type II tumors in this cluster, as p53 mutation is a specific feature of these tumors (present in more than 80% of the cases) (Kurman & Shih Ie, 2011b). Loss of p53 function allows uncontrolled replication of genetically damaged cells, that otherwise would be halted in the p53 proficient cells. This would explain the fact that those highly genomically unstable tumors (type II) have dysfunctional p53, that allows them to proliferate despite their genomic aberrations (Kar *et al*, 2007; O'Neill *et al*, 2005).

Significantly higher Ki-67 expression indicates higher cellular proliferation of those more advanced and genomically unstable tumors present in cluster B. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2,and mitosis), but absent from resting cells (G0), make it an excellent marker for determining the growth fraction within tissues (Scholzen & Gerdes, 2000). The other significantly associated marker-survivin may be another indicator of high proliferative activity of those tumors (Fields *et al*, 2004) although its main function is an inhibition of apoptosis through downstream caspase binding (Altieri, 2003). Expression of this marker has not been proved to have an independent predictive or prognostic value, however it has been reported to be correlated with other markers of unfavorable prognosis like advanced tumor stage, high histological grade, p53 mutation (Cohen *et al*, 2003) supporting the finding that more aggressive tumors show higher level of genomic instability and a specific pattern of copy number changes that can be distinguished from more indolent tumors.

6. DELETION AT 6q24-q26 AS AN INDEPENDENT PROGNOSTIC MARKER IN OVARIAN CANCER

The tumor groups defined based on their copy number changes did not only show some specific immunohistopathological features, but also a significantly different outcome. Surprisingly, the cluster of higher genomic instability, a feature related to more aggressive tumors (Kurman & Shih Ie, 2011a), showed significant association with better overall survival. However, this association could only be revealed when factors, known to have an impact on the survival of ovarian cancer patients and also found to be significant in our tumor series (such as FIGO stage and debulking status) were considered in the model. Since the better survival group was enriched in high FIGO stage tumors, this factor was masking the association of the tumor group with patients' survival. That presumption was further confirmed by analyzing more homogenous groups composed of high FIGO stage carcinomas alone, where the association was observed already at the univariate level, and was further shown to be independent of other cofactors. Importantly, since prior reports have shown that extreme levels of genomic instability and the presence of mutations in the BRCA genes are associated with improved prognosis (Alsop *et al*, 2012; Baumbusch *et al*, 2013; Birkbak *et al*, 2011; Bolton *et al*, 2012) we also considered both variables as possible confounders in our analysis. Finally, the examination of distinctive DNA copy number changes characterizing the cluster of tumors with better survival led us to propose a specific copy number loss at 6q24.2-26 as an independent marker of favorable prognosis in ovarian cancer. To define molecular determinants of EOC patients' outcome, previous studies have focused either on single genes (Bacic *et al*, 2012; Fujiwara *et al*, 2012; Kim *et al*; Madhuri *et al*, 2012; Quinn *et al*, 2013) or gene expression signatures (Berchuck *et al*, 2005; Crijns *et al*, 2009; Konstantinopoulos *et al*, 2010; Sabatier *et al*, 2009; Spentzos *et al*, 2004; Verhaak *et al*, 2013). However, the robustness and reproducibility of these new markers/signatures is still questionable as they seem difficult to be translated into clinical practice and to overpower well established markers such as FIGO stage and debulking status (Yoshida *et al*, 2009). So far very few studies have aimed to identify copy number regions that may predict ovarian cancer patients' outcome (Bruchim *et al*, 2009; Engler *et al*, 2012). In

addition, none of them led to a definition of a prognostic marker that can be implemented into clinics. This could be explained by limited sample sizes, insufficient clinical information, use of low resolution platforms or lack of a robust validation.

In this regards we could demonstrate the prognostic value of the 6q24-26 deletion at the DNA copy number level in a total of 563 tumors and at the gene expression level in 1436 EOCs. Furthermore, the deletion proved to be prognostic in HGSOCs, the most common and aggressive EOC subtype. Importantly, since its prognostic utility was confirmed after further adjustment for *BRCA1/2* mutation status the association between the 6q24.2-26 loss and an outcome appears to be driven by mechanisms other than those proposed to mediate the survival advantage of *BRCA1/2* mutation carriers (Alsop *et al*, 2012; Bolton *et al*, 2012).

The long arm of chromosome 6 is frequently altered in many human malignances including leukemias (Burkhardt *et al*, 2006; Mancini *et al*, 2005), lymphomas (Nelson *et al*, 2008; Rinaldi *et al*, 2006; Schwaenen *et al*, 2009; Tagawa *et al*, 2005), thymomas (Penzel *et al*, 2003; Rieker *et al*, 2005), central nervous system neoplasms (Ichimura *et al*, 2006; Li *et al*, 2013; Monoranu *et al*, 2008; Rousseau *et al*, 2010; Yin *et al*, 2009) breast cancer (Chappell *et al*, 1997; Devilee *et al*, 1991; Saito *et al*, 2009; Theile *et al*, 1996), ovarian cancer (Caserta *et al*, 2008; Foulkes *et al*, 1993; Hansen *et al*, 2002; Orphanos *et al*, 1995; Saito *et al*, 1992; Tibiletti *et al*, 1996) and many others such as melanomas and colon, stomach and liver carcinomas (Carvalho *et al*, 2001; Cui *et al*, 2011; Guo *et al*, 2011; Knosel *et al*, 2003; Vajdic *et al*, 2003; van Gils *et al*, 2008). The most common alteration of this chromosome is a loss at 6q24-26, which has been widely studied as a potential location for genes with a tumor suppressive role (Hayashi *et al*, 2012; Stilgenbauer *et al*, 1999; Sun *et al*, 2003). However, only a few reports have associated this loss with clinical outcome, reaching contradictory results, depending on the cancer type. Some of them associated the deletion with poor prognosis and tumor recurrence (Cui *et al*, 2011; Fischer *et al*, 2004; Letessier *et al*, 2007; Schwaenen *et al*, 2009), while others indicated a favorable outcome (Dalsass *et al*, 2013; Monoranu *et al*, 2008; Pfister *et al*, 2009).

These discrepancies might be explained by distinct tumor biology and treatment strategies. In so far the largest study on EOC by TCGA (TCGA, 2011) the only 6q loss defined among the 50 significant focal losses was 6q27, but not 6q24.2-26. This might be due to different methodology and composition of the tumor series. More likely, it may be attributable to the fact that we did not define the 6q24-26 deletion based on recurrence in HGSOCs, but through comparison of genomic aberrations between tumor clusters found to be associated with survival.

7. GENES POTENTIALLY EXPLAINING THE PROGNOSTIC VALUE OF 6q24-q26 DELETION

In order to identify a plausible mechanism explaining the association of the 6q24-26 deletion with improved outcome and to explore its potential clinical implications we attempted to define some candidate genes within the region.

We showed that 6q24.2-26 loss has an impact on a gene expression, as evidenced by downregulation of 76% genes from the lost region, and a significant association of lower mean expression level of all those genes with longer survival. Then by multivariate Cox regression analysis we identified several candidate genes whose downregulation was significantly associated with better survival. It is noteworthy that despite the fact that 6q24.2-26 loss was associated with survival, and the expression of most loci within the region was associated with loss, the expression of only few individual genes was associated with an outcome. This might be explained by the presence of "passenger" genes at the lost region, whose association with survival at the copy number level would result from co-deletion with the "driver/s" neighboring gene/s. The analysis of gene expression and survival would account for additional mechanisms of down-regulation other than loss (i.e. epigenetic changes, mutations) decreasing the confounding effect of physical position and helping to pinpoint gene/s likely to drive the survival association.

One of the candidate genes whose expression was significantly associated with improved survival was the DNA-binding subunit of SWI/SNF chromatin remodeling complex *ARID1B*. The presence of this gene in the complex is mutually exclusive with

ARID1A, which was found to be mutated in clear cell and endometrioid ovarian carcinomas (Ayhan *et al*, 2012; Jones *et al*, 2010; Wiegand *et al*, 2010; Xiao *et al*, 2012). Although, *ARID1A*-containing SWI/SNF complexes have been associated with tumor suppression (Blais & Dynlacht, 2007; Nagl *et al*, 2007) the role of *ARID1B*-complexes is not entirely clear. They have been shown to present both a tumor suppressive (Khursheed *et al*, 2013) and pro-proliferative function (Nagl *et al*, 2007), depending on the context. In addition high expression levels of *c-Myc*, detected in various types of cancers, among them ovarian, are particularly dependent on *ARID1B* (Nagl *et al*, 2007). All these findings are consistent with improved survival being associated with loss of function of *ARID1B*, as well as for other genes in the region with oncogenic properties, such as the TGF-Beta Activated Kinase1/MAP3K7 (*TAB2*), the T-cell lymphoma invasion and metastasis 2 gene (*TIAM2*) or the Glutamate Receptor (*GRM1*) (Martino *et al*, 2012; Speyer *et al*, 2012; Wangari-Talbot *et al*, 2012).

Among the genes in the region with oncogenic properties an interesting candidate seemed to be *ESR1,* due its mutagenic role in the response to estrogen stimuli and implication of steroid hormones in ovarian cancerogenesis (Ahmad & Kumar, 2011). However, we did not find a significant association between its expression and survival. Based on the previous evidence on breast cancer, very inconsistent and even opposing roles of estrogen have been proposed. Similarly in ovarian cancer the evaluation of its prognostic significance has led to conflictive results. Some studies reported that *ESR1* expression predicts favorable outcome (Bizzi *et al*, 1988; Burges *et al*, 2010; Halon *et al*, 2011) while others showed an association with worse prognosis (Alonso *et al*, 2009; Geisler *et al*, 1996; Schlumbrecht *et al*, 2010). The latest metanalysis including 23 studies focused on the role of *ESR1* in EOC showed lack of association of *ESR1* with patients' outcome (Zhao *et al*, 2013).

Other genes in the regions could be affecting tumor progression in the context of treatment. Among them the General Transcription factor IIH Polypeptide 5 (*GTF2H5)* that plays an essential role in the nucleotide-excision repair (NER) (Giglia-Mari *et al*, 2006; Theil *et al*, 2013). Given the fact that up to 50% of high-grade EOC have a defective HR (TCGA, 2011) compromised expression of genes involved in another DNA repair pathway in HR deficient cells might lead to synthetically lethal interaction thus enhancing cancer cells' sensitivity to cytotoxic drugs used to treat ovarian cancer (Chernikova *et al*, 2012). If this effect could be demostrated in *in vitro* cytotoxicity assays, *GTF2H5* might be used as predictive marker for platinum sensitivity in ovarian cancer.

Also in the context of therapy, a recent genome-wide synthetic lethal screen for sensitivity to the PARP inhibitor olaparib, revealed that genes that control chromatin remodeling and sister chromatic cohesion seem to modulate the response to olaparib maybe through DNA-damage repair involvement (Bajrami *et al*, 2013). Based on this findings and given its chromatin remodeling function, impairment of the already mentioned *ARID1B*, might have an alternative implication as predictor of response to PARP inhibition and/or to cytotoxic treatment.

Among other candidate genes from the region we have also identified *ULPB1*, one of the NKG2D ligands involved in the immune response. These proteins (ULBP1/2/3 and family of RAET1F-M genes) are rarely expressed in normal healthy tissues, but are present at high levels in different cancer types and cancer-derived cell lines (Coudert & Held, 2006). NKG2D pathway leads to activation of cytotoxic lymphocytes and subsequent recruitment of antitumor immune response (Raulet, 2003), however its involvement varies markedly between different tumors. In ovarian cancer high expression of NKG2D ligands- in particular ULBP2 (Li *et al*, 2009), RAET1G, RAET1E (McGilvray *et al*, 2010) have been associated with poor prognosis. Although it is not really clear how those immune response molecules influence disease progression, it is suggested the high levels of those proteins can hinder the infiltration of cytotoxic T lymphocytes and lead to unfavorable outcome by allowing tumor cells to escape from immune surveillance.

Individual loss of the above mentioned, or of other genes in the deleted region, might indeed have an impact on patients' survival; however, it is also plausible that it is not just a single gene that needs to be deleted to influence tumor progression, but rather a combination of them. Moreover we should not overlook the role of non-coding DNA fragments e.g. regulatory elements, miRNAs or specific sequences, as some of them have been shown to be essential for maintaining chromosomal structure, centromere function or homologous recognition (Subirana & Messeguer, 2010).

8. CLINICAL RELEVANCE OF 6Q24-Q26 DELETION IN EOC

While the specific genes involved in the association are further delineated and the mechanistic determinants are unveiled, the present study has shown that the 6q24.2-26 deletion is an independent marker of favorable outcome in EOCs. In particular our data indicate prognostic utility in HGSOCs, the most prevalent and aggressive EOC histotype. These findings have potentially relevant clinical value as this marker could help to guide the selection of patients, whose favorable prognosis would support the use of new treatment regimens focused on improving tolerability without jeopardizing efficacy. Also, the deletion, together with other emerging prognostic and predictive biomarkers in ovarian cancer, could be used to better balance patients between treatment arms in clinical trials to reduce the risk of confounding. Importantly, DNA-based markers that can be analyzed by FISH, such as this deletion, are particularly suitable for routine clinical applications due to their robustness and suitability for use with paraffin sections. In addition, the deletion, together with other emerging prognostic and predictive biomarkers in ovarian cancer, could be used to better balance patients between treatment arms in clinical trials to reduce the risk of confounding. Future research in this line should be dedicated to the prospective validation of this marker and further characterization of tumors that carry the deletion, as well as of the genes in the region. This may not only offer insights into tumor biology, but may eventually lead to the development of effective targeted therapies.

CONCLUSIONS

1. Our results indicate that sporadic and familiar EOCs exhibit a similar global pattern of DNA copy number changes as reflected by comparable frequency plots and lack of stratification by unsupervised hierarchical clustering. Overall, high levels of genomic instability and greater contribution of losses versus gains was a common feature in EOCs.

2. We defined a set of recurrent DNA copy number changes shared by sporadic and familial EOCs that encompassed known and putative cancer-related genes. These commonly altered regions and genes would point to key events in ovarian carcinogenesis in general, regardless the existence of germinal mutations in the BRCA1 or BRCA2 genes.

3. Despite general similarity between sporadic and hereditary EOCs, we found that extensive genomic loss was significantly higher in tumors from BRCA1 and BRCA2 mutation carriers. In addition we could also define a few, but potentially specific, BRCA1-associated alterations. These hallmark features might be clinically relevant as it could help to identify BRCA-related patients, who present better prognosis when treated with standard regimes and are likely to respond to PARP inhibitors.

4. DNA copy number profiles of BRCAX cases presented the lowest total number of alterations overall and in particular of losses resembling more sporadic than BRCA1/2 tumors. Also, the greater involvement of losses compared to gains was less marked in this tumor group and similar to that observed in sporadic cases, supporting that prominent genomic loss is particularly related to BRCA1 and BRCA2 dysfunctions.

5. Groups of EOCs defined based on their DNA copy number profiles showed an association with histotype, FIGO stage and proliferation-related markers. In particular we found that EOCs of greater genomic instability are more likely to be of higher FIGO stage, serous subtype and show increased expression of TP53, Ki-67 and survivin.

6. Deletion at 6q24-q26 was found to be an independent prognostic marker for overall and 5yrs survival in patients with EOC. In particular, our results indicate prognostic utility in high grade serous ovarian carcinomas, the most common and aggressive subtype. This marker has a potential clinical value, as it can be analyzed by FISH on tumor sections and guide selection of patients towards more conservative therapeutic strategies in order to reduce side-effects and to improve quality of life.

CONCLUSIONES

1. Nuestros resultados indican que los carcinomas epiteliales de ovario (CEO) esporádicos y familiares presentan un patrón similar de cambios en el número de copias de ADN tal como señalan sus gráficas de frecuencias comparables y la ausencia de segregación mediante clasificación jerárquica no supervisada. Un rasgo general a todos los CEO fue el alto nivel de inestabilidad genómica y la mayor contribución de pérdidas con respecto a ganancias.

2. Hemos definido un conjunto de alteraciones en el número de copias de ADN comunes entre los CEO esporádicos y familiares que contienen genes ya vinculados con la oncogénesis y otros potencialmente relacionados con dicho proceso. Estas regiones y genes compartidos apuntarían a cambios cruciales para desarrollo del cáncer de ovario independientemente de la presencia de mutaciones germinales en los genes *BRCA1* o *BRCA2.*

3. A pesar de la similitud global entre los CEO esporádicos y familiares, la pérdida de material genético fue especialmente prominente en los tumores BRCA1 y BRCA2. Además se definió un pequeño número de regiones potencialmente asociadas a los tumores BRCA1. Estas características distintivas podrían contribuir a la identificación de pacientes "*BRCA-like*", con mejor pronóstico y mejor respuesta tanto a la quimioterapia convencional como a los inhibidores de PARP.

4. Los tumores BRCAX presentaron el menor número de alteraciones tanto globales como de pérdidas, pareciéndose más en ese sentido a los tumores esporádicos que a los tumores BRCA1 y BRCA2. Asimismo la contribución relativa de pérdidas en comparación a las ganancias fue menos acusada tal como se observó en los tumores esporádicos. Todo ello apoyaría que la abundante pérdida de material genético estaría especialmente relacionada con alteraciones en los genes *BRCA1* y *BRCA2*.

5. Grupos de CEO basados en su patrón de alteraciones en el número de copias de ADN mostraron asociación con el subtipo histológico, el estadio FIGO y marcadores de proliferación. En concreto observamos que los CEO con mayor inestabilidad genómica tendrían más probabilidades de presentar a estadios FIGO superiores, ser de subtipo seroso y presentar mayor expresión de TP53, Ki-67 y survivina.

6. La deleción 6q24-q26 constituiría un marcador pronóstico independiente de supervivencia global y a los cinco años en pacientes con CEO. En particular, nuestros resultados señalan valor pronóstico en los tumores serosos de alto grado, el subtipo más común y agresivo. Este marcador tendría un potencial valor clínico ya que puede analizarse mediante FISH en secciones tumorales y guiar la selección de pacientes candidatos a recibir tratamientos más conservadores para minimizar los efectos secundarios y mejorar la calidad de vida.

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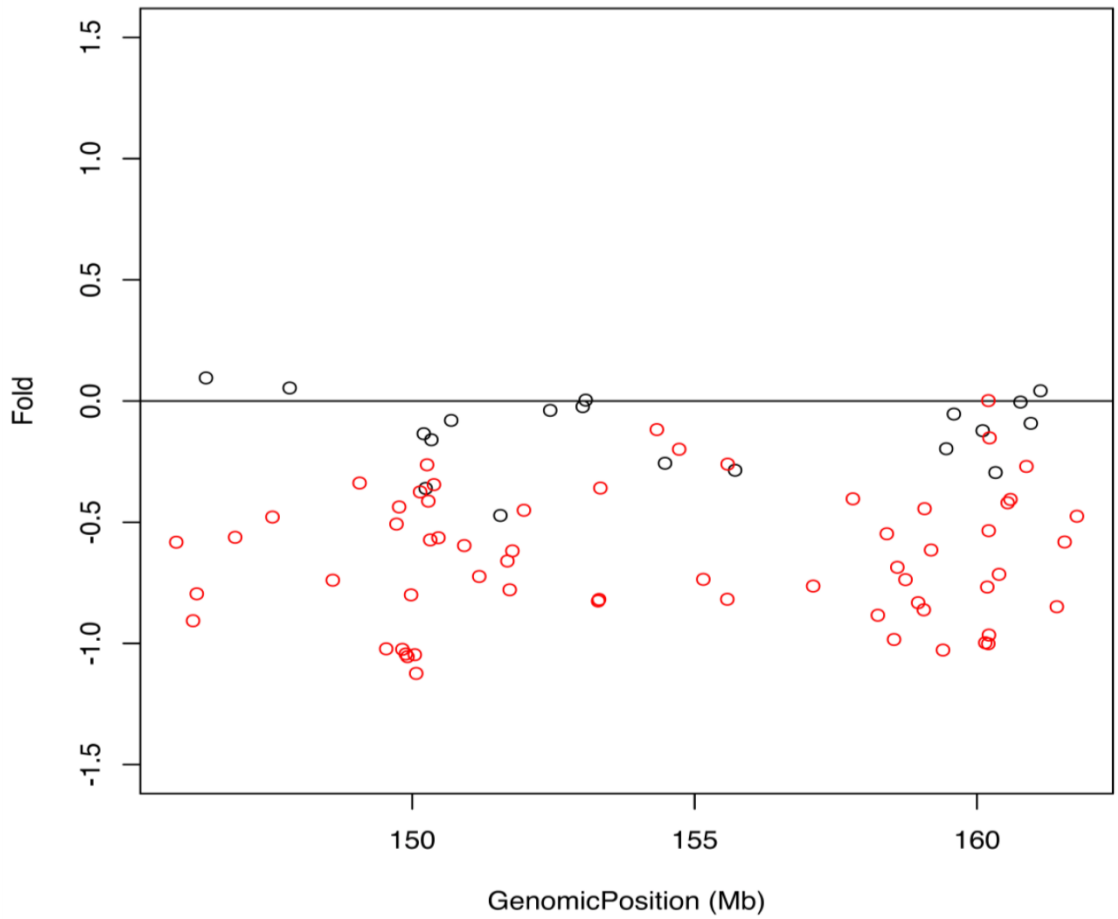
APPENDIX

SUPPLEMENTARY MATERIALS



Supplementary Figure1. Association between copy number status of 81 genes from the 6q24-26 region and their expression level (based on RPKM values) from TCGA studies. Fold change (log-ratio) in gene expression between cases with loss *v* normal copy number status at this locus on the y-axis; genomic location on the 6q chromosome on the x-axis; significant changes (*FDR* < 0.05) are marked in red.

Supplementary Table 1. List of antibodies evalauted in this study

Antibody	Clone	Dilution	Supplier	Visualization System & Immunostainer	Threshold
WT1 FLEX†	6F-H2	1:1	DAKO	En Vision FLEX/ DAKO Autostainer	>10%
AR	AR441	1:100	DAKO	En Vision FLEX/ DAKO Autostainer	>10%
β-Catenin	14	1:100	BD Transduction Lab	En Vision FLEX/ DAKO Autostainer	> 5% with strong staining
BCL-XL	2H12	1:10	Zymed	En Vision FLEX/ DAKO Autostainer	IRS 6
CD105	4G11	1:50	Novocastra	Vision Bio System/ Leica BOND MAX	Any positive cell
Chek2	DCS 270.1	1:25	Novocastra	Vision Bio System/ Leica BOND MAX	76.5% (mean)
C-kit	Polyclonal rabbit	1:200	DAKO	En Vision FLEX/ DAKO Autostainer	10% with moderate/strong staining
Cyclin D1 FLEX	SP4 Rabbit	1:1	DAKO	En Vision FLEX/ DAKO Autostainer	>10%
Cyclin E	13AE	1:10	Novocastra	En Vision FLEX/ DAKO Autostainer	>10%
E-Cadherin FLEX	NCH-38	1:1	DAKO	En Vision FLEX/ DAKO Autostainer	> 5% with strong staining
EGFR	EGFR.113	1:10	Novocastra	En Vision FLEX/ DAKO Autostainer	Any positive cell
ER-ALPHA FLEX	SP1	1:1	DAKO	En Vision FLEX/ DAKO Autostainer	>10%
ERCC1	D-10	1:50	Santa Cruz	Vision Bio System/ Leica BOND MAX	10-49% with strong staining or ≥ 50% with moderate/strong staining
Ki-67 FLEX	MIB-1	1:1	DAKO	En Vision FLEX/ DAKO Autostainer	≥ 36% (median)
KLK7	Polyclonal goat	1:25	R&D Systems	Vision Bio System/ Leica BOND MAX	Score mean ± standard deviation*
MMP7	SMP294	1:100	Abcam	Vision Bio System/ Leica BOND MAX	Any positive cell (nuclear) and Score mean ± standard deviation*
P16	E6H4	1:1	MTM	En Vision FLEX/ DAKO Autostainer	IRS 6
P21 (WAF1)	EA10	1:10	Calbiochem	En Vision FLEX/ DAKO Autostainer	>10%
P27	57	1:1000	BD Transduction Lab	En Vision FLEX/ DAKO Autostainer	>50%
P53 FLEX	DO-7	1:1	DAKO	En Vision FLEX/ DAKO Autostainer	>50%
PIK3CA	C73F8	1:100	Cell Signaling	En Vision FLEX /DAKO Autostainer	Any positive cell (nuclear) and ≥ 50% (cytoplasmic)
PR FLEX	636 Mouse	1:1	DAKO	En Vision FLEX/ DAKO Autostainer	>10%
RAD50	13B3/2C6	1:300	Abcam	Vision Bio System/ Leica BOND MAX	80% with strong staining
RAD51	51RAD01	1:25	Neomarkers	En Vision FLEX/ DAKO Autostainer	5.5% (nuclear) and strong staining (cytoplasmic)
RB1	63-245	1:100	BD Pharmigen	En Vision FLEX/ DAKO Autostainer	>10%
Survivin	Polyclonal rabbit	1:1000	R&D Systems	En Vision FLEX/ DAKO Autostainer	>10%
Topoisomerase IIα	Ki-S1	1:250	DAKO	En Vision FLEX/ DAKO Autostainer	IRS 4 (median)
TUBB3	TUJ1	1:500	Santa Cruz	En Vision FLEX/ DAKO Autostainer	10% with moderate/strong staining
VEGF	SP28	1:2	Abcam	Vision Bio System/ Leica BOND MAX	Strong staining
XPF	SPM228	1:2	Abcam	Vision Bio System/ Leica BOND MAX	>80%
XPG	8H7	1:100	Neomarkers	Vision Bio System/ Leica BOND MAX	13% (median)

WT1:Wilms tumor 1; AR: androgen receptor; EGFR: epidermal growth factor receptor; ER: estrogen receptor; ERCC1: excision repair cross-complementing rodent repair deficiency, complementation group 1; KLK7: kallikrein 7;MMP7: matrix metalloproteinase 7; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; PR: progesterone receptor; RB1: retinoblastoma 1; TUBB3: tubulin, beta 3 class III; VEGF: vascular endothelial growth factor; XPF: xeroderma pigmentosum complementation group F; XPG: xeroderma pigmentosum complementation group G;
IRS: immunoreactive score (intensity of the staining - 0: no reaction; 1: weak; 2: moderate and 3: strong - and percentage of positive cells - 0: no positive cells; 1: <10% positive cells; 2: 10-50% positive cells; 3: 51-80% positive cells and 4: >80% positive cells - was scored. The final score was derived by multiplying the percentage of positive cells with staining intensity and ranged between 0 and 12). *T-test;
† WT1 was evaluated in order to perform histopathological classification
Further information about scoring methods and thresholds are described elsewhere (Munoz-Repeto et al, 2013)

Supplementary Table 2 Minimal common regions of gains in at least 25% of the tumour group

BRCA1 n=21				BRCA2 n=6				BRCAX n=26				Sporadic n=15			
Cytoband	Freq of gain	Freq of HCG	Genes of interest#	Cytoband	Freq of gain	Freq of HCG	Genes of interest#	Cytoband	Freq of gain	Freq of HCG	Genes of interest#	Cytoband	Freq of gain	Freq of HCG	Genes of interest#
				1p34 - p31	33	0	***MYCL1 MPL MUTYH TAL1 EPS15***								
1q21 - q22	29	0	***MUC1***									1q21	40	0	
1q23 - q24	29	0	***PBX2***									1q23 - q25	33	0	***SDHC FCGR2B PBX1***
												1q31	27	7	
1q41	33	0													
												1q42	33	7	
												2p23	33	0	
												2p22	33	7	
												2p16	27	0	***BCL11A***
												2p14	27	0	
												2p13 - p12	27	7	
				2q22 - q24	33	0									
												3p14 - p13	27	0	***MITF FOXP1***
												3p11	40	0	*EPHA3*
												3q11	40	0	
												3q13 - q21	46	0	
3q23 -q25	28	0	***GMPS MLF1***									3q22 - q23	40	0	***FOXL2***
3q26	58	5	***MECOM***	3q26	67	0	***MECOM PIK3CA***	3q25 - q26	44	0	***GMPS MLF1 PIK3CA***	**3q26** - q27	63	17	***PIK3CA MECOM***
3q29	48	0		3q28 - q29	67	0	***LPP TFRC***								
5p15.33	29	0		5p15.33 - p13.3	50	0						**5p15.2-p15.1**	40	13	
5p15.1 - p14	29	0										5p14- **p13**	33	7	***LIFR***
6p25- **p22**	38	5	***IRF4 DEK FOXQ1***	6p25 - p22	50	0	***IRF4 DEK FOXQ1***	6p25 - p24	36	0	*FOXQ1*	6p25.3	33	0	*FOXQ1*
								6p21	31	0	***HMGA1***	**6p21 - p12**	27	7	***TFEB CCND3***
								6p12 - p11	32	0					
												7p14	27	0	
7q32	33	0													
7q33-34	33	0	***CREB3L2 KIAA1549 BRAF***												
7q35-**36**	33	9						7q35-36	31	4	***EZH2***				
								8p11	27	0		8p11	27	0	***HOOK3***
								8q11	34	4					
8q12.1	43	0	***PLAG1 CHCHD7***									**8q12.1**-q12.2	64	7	***PLAG1***
								8q13	46	8					
8q13.3 - **q21.3**	40	14						8q21.11	42	0					
												8q21.13-q21.3	67	7	
8q22.1	50	28	*LAPTM4B*					8q22.1-q22.3	46	0	***CCNE2 LAPTM4B YWHAZ***	**8q22.1-q22.3**	64	7	***COX6C CCNE2 YWHAZ***
8q22.1-q22.2	50	22													
								8q23	54	15		8q23	60	0	
8q24	65	29	***MYC RECQL4 TRAPPC9 PSCA***	**8q24**	**83**	33	***MYC RECQL4 TRAPPC9 PSCA***	**8q24**	65	22	***MYC TRAPPC9 PSCA***	8q24	64	0	***RECQL4 MYC PSCA***
								10p15	38	0					
								10p14 - p13	42	0					
								10p12	42	0					
10q11	38	0						10q11	29	0	***RET***	10q11	27	0	

11q13	35	14	*MALAT1*					**11q13**	28	8	*MALAT1*			
11q14	33	5												
								11q22	**27**	4	***ATM***	11q22	27	0	***ATM***
								11q25	31	4	***FLI1***				
12p13	45	5	***KDM5A CCND2***	**12p13**	33	16	***KDM5A CCND2***	**12p13**	31	4	***KDM5A CCND2***	**12p13**	48	7	***KDM5A CCND2 ZNF384***
12p12	33	0										**12p12**- p11	50	7	***KRAS***
												12q13	40	0	
												12q14	40	7	***HMGA2***
15q23 - q24	29	0													
15q26	35	0		15q26	33	0						**17q12 - q21**	27	13	***ERBB2***
								17q22	27	0					
								17q23	27	0	***CLTC***				
								17q24	27	0					
17q25	33	5	***CANT1 ASPSCR1 FOXJ1***	17q25	33	0	***CANT1 ASPSCR1 FOXJ1***	**17q25**	35	8	***CANT1 ASPSCR1***	17q24 - q25	33	0	*FOXJ1*
												18p11	27	0	
												19p13	27	13	***JAK3 TPM4***
												19q12	47	13	***CCNE1***
												19q13	37	13	***CEBPA***
20p13	29	0		20p13	50	0		**20p13 - p12**	27	4					
								20p11	27	0					
				20q11	33	0									
												20q12 - **q13.2**	47	8	
				20q13.33	**50**	16	***SS18L1***	20q13.33	33	0	***SS18L1***				

Regions gained in more than 25% of the tumor group with corresponding frequency of alteration (per group); whenever amplification was found within MCR of gain, the peak frequency of amplification was mentioned and region (with corresponding gene) was shown in **bold and underlined;** alterations shared by sporadic and at least 2 familial groups were highlighted with darker background (only exact overlap of locations was considered). Regions with blue backrogund were found to be significantly more frequent in particular group; whenever more than 1 region of gain was found within the same cytoband the average frequency of all the alterations was calculated; #genes of interest belong to The Cancer Census (**in bold)** downloaded from http://www.sanger.ac.uk/genetics/CGP/Census/ or are potentially cancer-related because of their function or previous reports;HCG-high copy number gain

Supplementary Table 3 Minimal common regions of losses >35% and homozygous deletions for tumor group

BRCA1 n=21				BRCA2 n=6				BRCAX n=26				Sporadic n=15			
Cytoband	Freq of loss	Freq of HD	Genes of interest#	Cytoband	Freq of loss	Freq of HD	Genes of interest#	Cytoband	Freq of loss	Freq of HD	Genes of interest#	Cytoband	Freq of loss	Freq of HD	Genes of interest#
				1p36	50	0	***PRDM16 RPL22 SDHB PAX7 MDS2***								
				2q31	50	0	***CHN1 HOXD13 HOXD11***								
				2q37	50	0									
				3p26	50	0									
4p15	38	5		4p16-p15	50	0									
4q21	50	0		4q13-q22	59	0									
4q24	71	5													
				4q24-q26	83	0	***TET2***					4q22-q24	47	5	***RAP1GDS1 TET2***
												4q25-q26	47	11	
4q27-31	65	0	***IL2***					4q28	38.5	0		4q28 - q31	45	6	***IL2***
4q32-q34	62	0		4q32	67	16.67		4q31-q32	39	0					
4q34-q35	67	5		4q34-q35	67	16.67						4q34-q35	40	0	***DUX4***
5q13	43	0		5q12-q21	83	0	***PIK3R1***								
5q14-15	48	10													
6q21	52	5													
6q22-q23	59														
6q23-**q25**	64	10	***TNFAIP3***					6q25	42	3					
6q26-27	71	10	*ARID1B* ***FGFR1OP MLLT4***	6q24-q27	50	0	*ESR1 ARID1B*	6q26 6q27	46 46	3 0	***FGFR1OP MLLT4***				
7p22	48	5	***PMS2***									7p22	53	0	
7p21 - p13	40	0	***HOXA13 JAZF1***	7p22-p21	67	0	***CARD11 PMS2 ETV1***								
7q11	5	10													
				8p23	100	16.67	*MCPH1*	8p23	38	0	*MCPH1*				
8p22 - **p12** (**8p21**)	60	14	***PCM1 WRN***					8p22	38.5	0		8p23-p21	50	6	***PCM1 MCPH1***
												8p12	47	6	***WRN***
												8p11	40	0	
9p24	38	5	*FOXD4*					9p24-p23	50	0	*(FOXD4 PTPRD)*				***JAK2 NFIB MLLT3***
9p22-p21	48	5	***MLLT3 CDKN2A CDKN2B***	9p24-p13	67	0	***JAK2 NFIB MLLT3 CDKN2A CDKN2B FANCG PAX5*** *FOXD4 PTPRD*	9p23-22 9p21	46 50	3 3	***NFIB*** ***CDKN2A CDKN2B***	9p24-p21	67	6	*FOXD4 PTPRD* ***CDKN2A CDKN2B***
9p13	38	0										9p13	53	0	
9q21	48	5						9q21	38	3	***GNAQ***	9q13-q21	47	6	***GNAQ***
9q33	52	10		9q12-q34 *(9q21)*	67	16.67	***GNAQ SYK OMD FANCC XPA NR4A3 TAL2 SET FNBP1 ABL1 NUP214 TSC1 BRD3 NOTCH1***	9q22 - q31 9q32-33	42 50	3 0	***SYK OMD FANCC XPA NR4A3***	9q22-q31 9q33	45 40	6 0	***OMD FANCC NR4A3***
9q34	52	0	***TSC1 BRD3 NOTCH1***					9q34	56	3	***ABL1 NUP214***	9q34	47	0	
11p15	40	0	***HRAS CARS***	11p15-p11	50	0	***HRAS CARS NUP98 LMO1 FANCF WT1 LMO2 EXT2 DDB2***					11p15	47	6	
12q21 - q22	43	5	***BTG1***												
12q24	43	5	***BCL7A***												

13q12	48	0	***CDX2 FLT3***	13q12-q21 (*13q14*)	83	16.67	***CDX2 FLT3 BRCA2 LHFP LCP1 RB1*** *(SMAD9)*	13q12	40	0						
13q13-**14**	67	10	***LHFP*** ***RB1*** *(SMAD9)*					13q13	38	0	***BRCA2***					
								13q14	44	10	***RB1***					
13q21.31-	57	0						13q21.1-q22.	40	0		13q21	40	0		
13q31	48	0														
13q34	48	5														
				14q22-q32	58.5	0	***KTN1 GPHN RAD51L1***					14q22	40	0		
												15q12	60	0		
				15q14-q21	50	0	***BUB1B C15orf21***					15q14-q23	66	6	***C15orf21 TCF12***	
												15q25	51	0		
16p13	38	5	***CREBBP***	16p13	50	0	***TSC2 CREBBP SOCS1 TNFRSF17 ERCC4***	16p13	40	0	***CREBBP ERCC4***					
16q11-**q21**	47		***CYLD HERPUD1***	16q21	83	0	***CDH11***									
16q21-**q23**	57	14	***CDH11 CBFB***	16q22-q23	83	0		16q21 - q24	38	3	***CDH11 CBFB CDH1 MAF***					
17p13	52	5		17p13-q12 (*17q11*)	100	16.67	***USP6 TP53 PER1 GAS7 MAP2K4 NF1 SUZ12***	17p13	54	0	***USP6***	17p13	60	0	***PER1***	
								17p12	62	3						
17p11	71	5										17p11	53	0		
17q11	65	5	***NF1***					**17q11**	50	7	***NF1***	**17q11**	40	11	***NF1***	
17q12	65	10						17q12	50	0	***MLLT6 LASP1***	17q12	40	0		
17q21	57	0	***BRCA1***	17q21	58.5	0	***BRCA1***	17q21 - q23	41	0	***COL1A1 MSI2 BRIP1***					
17q23- q24	38	0	***CD79B DDX5***													
18q12	38	0														
18q21	38	5		18q21-q23	67	0	***MALT1 BCL2***	18q21	38.5	3		18q21	40	0	***MALT1***	
18q22 - q23	38	0						18q23	42.3	3		18q23	53	6		
19p13	43	10										19p13	47	6	***FSTL3 STK11 TCF3***	
				19q13	67	0						19q13	47	6	***ZNF331***	
				21q11-q22	50	0	***OLIG2 RUNX1***									
22q11-**12**	54	10	***BCR SMARCB1 MN1 CHEK2 EWSR1 NF2 MYH9***	22q12-q13	100	0	***MYH9 PDGFB PDGFB MKL1 EP300***	22q12-13	41	3	***EWSR1 NF2 MYH9 PDGFB***	22q12	42	6		
22q13	65	10										22q13	40	0	***PDGFB MKL1 EP300***	
				Xp22-p21	67	0	*POLA1 ARX*	Xp22	42	3		Xp22	60	6		
												Xp21	67	11		
				Xp11	67	0		**Xp11**	42	10	***KDM6A***	Xp11	67	6	***SSX2 SSX2B KDM5C***	
				Xq11-q23 (Xq21)	67	16.67	***MSN NONO*** *EDA2R*	Xq11 - q13	38	3	***MSN*** *EDA2R*	Xq11-q13	67	6	***MSN*** *EDA2R*	
												Xq21-q22	67	11		
												Xq23-q25	67	0		
								Xq26	42	0						
								Xq27	42	0		Xq27	53	6		
				Xq28	67	0	***MTCP1***					Xq28	60	6		

Regions lost in more than 35% of the tumor group with corresponding frequency of alteration (per group);whenever homozygous deletion (HD) was found within MCR of loss, the peak frequency of HD was mentioned and ovarlapped region (with corresponding gene) was underlined (or **bold and underlined** in case of 2 or more HD);whenever more than 1 region of loss was found within the same cytoband the average frequency of all the alterations was calculated; alterations shared by sporadic and at least 2 familial groups were highlighted with redish background (only exact overlap of locations was considered); regions with blue backrogund were found to be significantly more frequent in particular group; #genes of interest belong to The Cancer Census (in bold) were downloaded from http://www.sanger.ac.uk/genetics/CGP/Census/ or were potentially cancer-related because of their function or previous reports; genes only homozygously lost within MCR of loss highligthed in blue;HD-homozygous deletion

Supplementary Table 4. Immunohistological characterization of tumors in clusters A and B defined by unsupervised hierarchical clustering by IHC markers-expression defined as postive or negative

Carcinomas		Cluster A n=28			Cluster B n=40		P-value
Immunohistochemical markers							
number of positivly and negativly stained tumors							
B-CATANIN cytoplasmtic, n (%)	**Pos**	11	*(39.3)*	**Pos**	24	*(60)*	0.14
	Neg	16	*(57.1)*	**Neg**	16	*(40)*	
	NA	1	*(3.6)*	**NA**	-	-	
B-CATANIN membrane, n (%)	**Pos**	3	*(10.7)*	**Pos**	5	*(12.5)*	1.00
	Neg	24	*(85.7)*	**Neg**	35	*(87.5)*	
	NA	1	*(3.6)*	**NA**	-	-	
B-CATANIN nuclear, n (%)	**Pos**	2	*(7.1)*	**Pos**	*4*	*(10)*	1.00
	Neg	25	*(89.3)*	**Neg**	*36*	*(90)*	
	NA	1	*(3.6)*	**NA**	-	-	
BCL-XL, n (%)	**Pos**	15	*(53.6)*	**Pos**	23	*(57.5)*	1.00
	Neg	12	*(42.9)*	**Neg**	17	*(42.5)*	
	NA	1	*(3.6)*	**NA**	-	-	
C-KIT, n (%)	**Pos**	0	*(0)*	**Pos**	1	*(2.5)*	1.00
	Neg	27	*(96.4)*	**Neg**	39	*(97.5)*	
	NA	1	*(3.6)*	**NA**	-	-	
CD105, n (%)	**Pos**	2	*(7.1)*	**Pos**	9	*(22.5)*	0.18
	Neg	25	*(89.3)*	**Neg**	30	*(75)*	
CYCLIN D1, n (%)	**Pos**	21	*(75)*	**Pos**	32	*(80)*	1.00
	Neg	5	*(17.9)*	**Neg**	8	*(20)*	
	NA	2	*(7.1)*	**NA**	-	-	
CYCLIN E, n (%)	**Pos**	25	*(89.3)*	**Pos**	39	*(97.5)*	0.56
	Neg	2	*(7.1)*	**Neg**	1	*(2.5)*	
	NA	1	*(3.6)*	**NA**	-	-	
E-CADHERIN cytoplasmatic, n (%)	**Pos**	12	*(42.9)*	**Pos**	19	*(47.5)*	1.00
	Neg	15	*(53.6)*	**Neg**	21	*(52.5)*	
	NA	1	*(3.6)*	**NA**	-	-	
E-CADHERIN membrane, n (%)	**Pos**	4	*(14.3)*	**Pos**	9	*(22.5)*	0.54
	Neg	23	*(82.1)*	**Neg**	31	*(77.5)*	
	NA	1	*(3.6)*	**NA**	-	-	
EGFR cytoplasmatic, n (%)	**Pos**	7	*(25)*	**Pos**	13	*(32.5)*	0.79
	Neg	19	*(67.9)*	**Neg**	27	*(67.5)*	
	NA	2	*(7.1)*	**NA**	-	-	
EGFR membrane, n (%)	**Pos**	2	*(7.1)*	**Pos**	3	*(7.5)*	1.00
	Neg	24	*(85.7)*	**Neg**	37	*(92.5)*	
	NA	2	*(7.1)*	**NA**	-	-	
EGFR nuclear, n (%)	**Pos**	15	*(53.6)*	**Pos**	23	*(57.5)*	1.00
	Neg	11	*(39.3)*	**Neg**	17	*(42.5)*	
	NA	2	*(7.1)*	**NA**	-	-	
ER, n (%)	**Pos**	17	*(60.7)*	**Pos**	29	*(72.5)*	0.43
	Neg	10	*(35.7)*	**Neg**	11	*(27.5)*	
	NA	1	*(3.6)*	**NA**	-	-	

ERCC1, n (%)	**Pos**	15	*(53.6)*	**Pos**	20	*(50)*	0.81
	Neg	12	*(42.9)*	**Neg**	19	*(47.5)*	
	NA	1	*(3.6)*	**NA**	1	*(2.5)*	
KALLIKREIN 7, n (%)	**Pos**	11	*(39.3)*	**Pos**	14	*(35)*	0.80
	Neg	16	*(57.1)*	**Neg**	26	*(65)*	
	NA	1	*(3.6)*	**NA**	-	-	
P21, n (%)	**Pos**	21	*(75)*	**Pos**	23	*(57.5)*	0.12
	Neg	6	*(21.4)*	**Neg**	17	*(42.5)*	
	NA	1	*(3.6)*	**NA**	-	-	
P27, n (%)	**Pos**	13	*(46.4)*	**Pos**	13	*(32.5)*	0.21
	Neg	14	*(50)*	**Neg**	27	*(67.5)*	
	NA	1	*(3.6)*	**NA**	-	-	
P53, n (%)	**Pos**	10	*(35.7)*	**Pos**	27	*(67.5)*	*0.02*
	Neg	17	*(60.7)*	**Neg**	13	*(32.5)*	
	NA	1	*(3.6)*	**NA**	-	-	
PIK3CA, n (%)	**Pos**	13	*(46.4)*	**Pos**	*22*	*(55)*	0.62
	Neg	14	*(50)*	**Neg**	*17*	*(42.5)*	
	NA	1	*(3.6)*	**NA**	*1*	*(2.5)*	
PR, n (%)	**Pos**	12	*(42.9)*	**Pos**	21	*(52.5)*	0.80
	Neg	14	*(50)*	**Neg**	19	*(47.5)*	
	NA	2	*(7.1)*	**NA**	-	-	
RAD50, n (%)	**Pos**	20	*(71.4)*	**Pos**	30	*(75)*	1.00
	Neg	7	*(25)*	**Neg**	10	*(25)*	
	NA	1	*(3.6)*	**NA**	-	-	
RAD51, n (%)	**Pos**	11	*(39.3)*	**Pos**	21	*(52.5)*	0.46
	Neg	16	*(57.1)*	**Neg**	19	*(47.5)*	
	NA	1	*(3.6)*	**NA**	-	-	
RB1, n (%)	**Pos**	24	*(85.7)*	**Pos**	34	*(85)*	1.00
	Neg	3	*(10.7)*	**Neg**	5	*(12.5)*	
	NA	1	*(3.6)*	**NA**	1	*(2.5)*	
TOPOISOMERASE II ALFA, n (%)	**Pos**	10	*(35.7)*	**Pos**	13	*(32.5)*	0.80
	Neg	17	*(60.7)*	**Neg**	27	*(67.5)*	
	NA	1	*(3.6)*	**NA**	-	-	
TUBULIN 3, n (%)	**Pos**	12	*(42.9)*	**Pos**	18	*(45)*	1.00
	Neg	15	*(53.6)*	**Neg**	22	*(55)*	
	NA	1	*(3.6)*	**NA**	-	-	
VEGF, n (%)	**Pos**	5	*(17.9)*	**Pos**	7	*(17.5)*	1.00
	Neg	22	*(78.6)*	**Neg**	33	*(82.5)*	
	NA	1	*(3.6)*	**NA**	-	-	
XPF, n (%)	**Pos**	23	*(82.1)*	**Pos**	35	*(87.5)*	1.00
	Neg	4	*(14.3)*	**Neg**	5	*(12.5)*	
	NA	1	*(3.6)*	**NA**	-	-	

Staining defined as positive or negative according to the threshold defined separately for each marker (Supplementary Table 1), Fisher Exact Test was used for comparisons; All the variables, expressed by continuous values, were compared with Mann-Whitney test. *P*-values <0.05 were considered significant and shown in *italics*;

Supplementary Table 5. Immunohistological characterization of tumors in clusters A and B defined by unsupervised hierarchical clustering by IHC markers-evaluated as %

	Cluster A		*Cluster B*		
Carcinomas	**n=28**		**n=40**		***P-value***
Immunohistochemical markers					
Median expression # (interquartile range)					
AR	**10.0**	*(1-56.5)*	**29.0**	*(4-63)*	0.28
CHEK2	**84.5**	*(79-91)*	**84.5**	*(68-91.5)*	0.97
CYCLIN E	**67.0**	*(45-79)*	**55.5**	*(44-70.2)*	0.20
CYCLIN D1	**24.0**	*(11-51)*	**26.0**	*(11.2-41.5)*	0.76
CDKN2A	**90.0**	*(30-100)*	**99.5**	*(40-100)*	0.34
ER	**33.5**	*(0-75.5)*	**43.0**	*(4.8-78)*	0.53
KI-67	**34.5**	*(23.5-50)*	**51**	*(36.8-67.8)*	*0.02*
MMP7	**42.5**	*(10-100)*	**42.0**	*(10-65)*	0.49
P21	**17.0**	*(10.5-43.5)*	**14.5**	*(6.6-28.6)*	0.13
P27	**47.5**	*(31.5-69.5)*	**40.0**	*(21.2-55.6)*	0.24
PR	**5.5**	*(0-35)*	**10.5**	*(2.1-44.2)*	0.13
RAD50	**97.0**	*(94.5-99)*	**97.0**	*(95-98)*	0.90
RAD51	**4.0**	*(1.5-10)*	**5.5**	*(2.25-9.5)*	0.42
RB1	**46.0**	*(25-70.5)*	**26.5**	*(12-59)*	0.14
SURVIVIN Cytoplasmatic	**40.0**	*(0-80)*	**50.0**	*(15.2-73.7)*	0.79
SURVIVIN Nuclear	**12.0**	*(3-18)*	**21.5**	*(11.5-25.9)*	*0.001*
TOPOISOMERASE II ALFA	**29.5**	*(12-43.5)*	**33.0**	*(15.3-48.7)*	0.51
XPF	**96.0**	*(90-98.5)*	**95.0**	*(92.25-98)*	0.98
XPG	**3.0**	*(1-15)*	**3.5**	*(0.5-11.3)*	0.99

The expression evaluated as a percentage of stained nuclei, membrane or cytoplasm as detailed in Materials and Methods; comparison between clusters with Mann-Whitney test. *P*-values <0.05 were considered significant and shown in *italics*;