



RAPID COMMUNICATION

Regulatory effects of miR-19a on MAD2 expression and tumorigenesis in gastric cancer



Gastric cancer (GC) is worldwide the sixth most diagnosed and third leading cause of cancer deaths, with poor and late prognosis, probably due to post-surgery adjuvant treatment resistance and lack of a thorough panel of prognostic markers. We have previously shown that mitotic arrest deficient 2 (MAD2, encoded by *MAD2L1*), a key protein of the spindle assembly checkpoint, is relevant in GC cells; its interference impairs migration and growth, while its overexpression correlates with tumorigenesis.¹ Here we show a similar correlation with overall survival (OS) in a pilot patient series. We hypothesized that MAD2 overexpression might relate to micro-RNA (miRNA) deregulation. Bioinformatic analysis identified miRNAs specifically targeting *MAD2L1*-3'UTR. Expression of miR-19a and miR-203 inversely correlated with *MAD2L1* expression in GC cell lines and patients' samples. A broader analysis using Cancer Genome Atlas data showed that only high miR-19 levels correlated with a better OS, especially in patients overexpressing *MAD2L1*. In GC cells, miR-19a expression reduced cell migration and invasion capability and increased apoptosis, in combination with classical and new antitumoral drugs. We propose that miR-19a is a critical regulator of MAD2 protein in GC, with potential clinical use as a prognostic biomarker, and as a model for MAD2 interfering agent design with therapeutic potential.

To strengthen the evidence that MAD2 over-expression correlates with GC cells tumorigenicity, we studied a cohort of 44 GC patients divided into two groups according to high ($n = 16$) and low/normal *MAD2L1* expression ($n = 28$). General features are summarized in Table S1. No association between *MAD2L1* expression and age, sex, stage, Lauren's classification, and Her2⁺ expression, or progression-free survival was observed in GC patients ($P < 0.05$). However, significant differences were detected in mortality, as illustrated in the Kaplan–Meier (K-M) curve, which showed early differences in OS between both populations (Fig. 1A and Table S2); survival median of patients with low and high *MAD2L1* expression reached 54 vs. 20 months, respectively. This trend was also detected in a wider panel

of patients ($n = 592$) available in the K-M plot (29.5 vs. 19.5 months in low vs. high *MAD2L1* expression) (Fig. S1).

miRNAs efficiently regulate the levels of tumorigenic proteins and, in GC, many are directed to a plethora of signaling proteins.² We undertook an *in silico* analysis to search for miRNAs that could specifically regulate MAD2 levels in GC. We used the public dataset GSE30070, from Gene Expression Omnibus (GEO) database that describes the miRNA expression profile in 90 GC samples collected prior to chemotherapy treatment and 34 samples from healthy volunteers. After curation of the resulting data (Fig. S2), miR-19a, miR-203, and miR-224 turned up as possible *MAD2L1* regulators in GC.

In cellulo analysis pointed at a functional role of at least two of the identified miRNAs (Fig. S3). Expression levels of *MAD2L1*, miR-19a, miR-203, and miR-224 were evaluated by RT-qPCR in a panel of GC cell lines (Fig. S3A). An inverse correlation was found between *MAD2L1* (indicated in each graph with a bold line) and miR-19a or miR-203 expression, but not with that of miR-224 (discarded from then on). Luciferase assays in 293-T cells co-transfected with psi-CHECK-3'UTR *MAD2L1* and pEGP-miR-19a or pEGP-miR-203 confirmed they both target *MAD2L1*-3'UTR (Fig. S3B). Enforced expression of both miRNAs resulted in significantly lowering both RNA and protein levels (Fig. S3C).

We studied the correlation between the levels of *MAD2L1* and miR-19a or miR-203 in a subset of the 44 GC patients studied above and found that, as in the GC cell lines, high *MAD2L1* expression correlated with lower levels of miR-19a and, to a lesser extent, of miR-203 (Fig. 1B). We thus aimed at assessing the potential use of miR-19a as a new GC biomarker. We tested the association of miR-19a with survival performance. GC samples (The Cancer Genome Atlas (TCGA): $n = 372$) were divided into two different groups according to miR-19a expression. The mean value was used as a cut-off value ($P < 0.05$; high expression group ($n = 122$) and low expression group ($n = 250$)). K-M analysis (Fig. 1C; Table S3) showed that the OS rate of the low group was markedly worse than that of the high group, with a P -value equal to 0.021 (Log Rank–Mantel-Cox), suggesting that down-regulation of miR-

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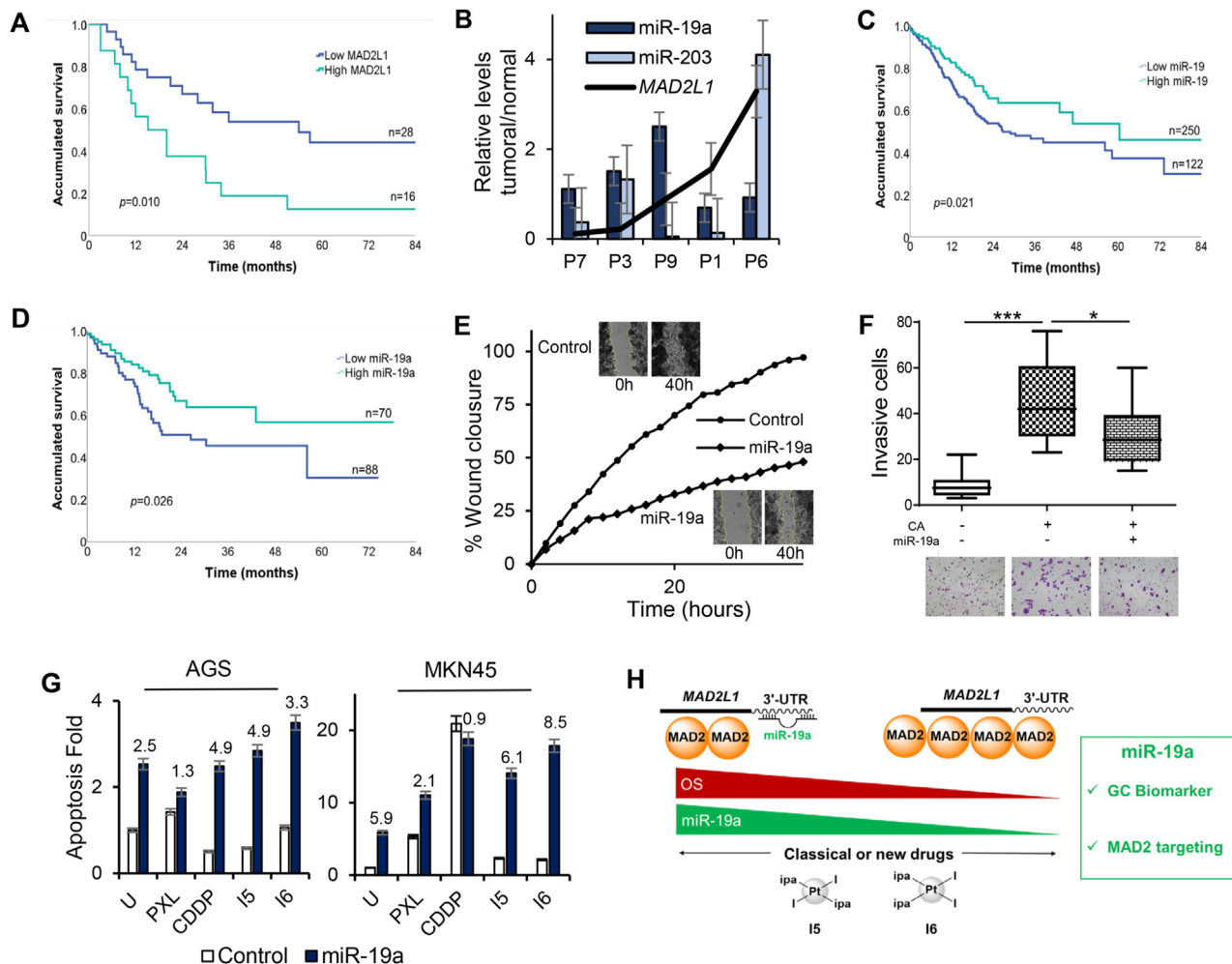


Figure 1 miR19a serves as a new potential GC biomarker and *MAD2* interfering agent. **(A)** *MAD2L1* expression correlates with GC patients' OS. K-M analysis of 84-month accumulative survival curves of a panel of 44 GC patients divided in high *MAD2L1* ($n = 16$) versus low *MAD2L1* expression ($n = 28$). The statistical significance was evaluated with the Mantel–Cox test. **(B)** miR-19a/miR-203 vs. *MAD2L1* expression in a subset of GC patients. RT-qPCR of *MAD2L1*, miR-19a, and miR-203 in RNA isolated from tumoral and adjacent normal tissue samples of five of the GC patients panel analyzed in panel A. **(C)** High miR-19a predicts better prognosis, independently on *MAD2* expression. K-M analysis with the log-rank test indicated that low miR-19a expression ($n = 250$, blue lines), had a significant impact on OS ($p = 0.021$). **(D)** miR-19a expression correlates with good prognosis even in patients overexpressing *MAD2*. K-M analysis of 5-year OS curves of patients with high *MAD2L1* expression and high or low miR-19a. Database TCGA for stomach adenocarcinoma was used. The statistical significance was evaluated with the Mantel–Cox test. **(E)** miR-19a reduces GC cell migration. AGS cells were transfected with pEGP-null (control), or pEGP-miR-19a (miR-19a). The graph shows the percentage of wound closure over the study time using the ImageJ program. Control (●), miR-19a (◆). Inset: Representative images of the first (0 h) and last picture (40 h) of control and miR-19a transfected cells taken during the wound healing experiment. Images were taken at $10\times$ magnification, every 2 h for 40 h. The yellow line represents the wound border. **(F)** miR-19a reduces GC cell invasion. The graph shows the quantification of stained invasive cells from MKN45 and miR-19a-expressing MKN45 cells using Transwell membrane assay, without (–) and with (+) 20% fetal bovine serum (FBS) as a chemoattractant (CA) for 48 h. Representative photographs of the experiment are also shown. Scale bar = $50\ \mu\text{m}$. Statistical differences were assessed by one-way ANOVA ($*P < 0.05$; $***P < 0.001$). **(G)** miR-19a induces apoptosis in GC cells, in synergy with classical and new antitumoral metallodrugs. AGS (left) and MKN45 (right) cells were transfected with pEGP-null (control) or pEGP-miR-19a (miR-19a). 24 h after transfection, cells were treated for a further 24 h with PXL, CDDP, I5, and I6 at IC₅₀ concentration (as determined for each cell line, see supplementary methods and materials for details). The graph shows the fold induction of apoptosis referred to as control, untreated cells (U). Numbers on top of miR-19a bars indicate fold-induction in miR-19a-transfected over untransfected cells in each of conditions. **(H)** Graphical Summary. Bad prognosis in patients overexpressing *MAD2* may be due to down-regulation of miR-19a, as OS parallels its expression. We propose miR-19a as a GC prognostic biomarker. Additionally, because miR-19a enforced expression targets *MAD2L1*, miR-19a could serve as a model to design *MAD2* interfering agents which, together with appropriate (and maybe synergistic) classical or new antitumoral compounds (such as platinum iodide derivatives I5 and I6) could help to achieve more successful GC therapy strategies.

19a may contribute to the malignant progression of GC. In contrast, no difference could be detected between high or low miR-203 expression (Fig. S4A). We then assessed the predictive prognosis capacity of miRNA-19a in the high *MAD2L1* subgroup, according to the receiver operating characteristic curve. We found that the OS prediction for 1, 2, and 5 years in the group with high miR-19a expression was 84.2%, 64.0%, and 56.9%, significantly more favorable than that found in the low expression group (73.9%, 48.6%, and 30.5%), respectively (Fig. 1D and Table S4). The results suggest that the ratio of *MAD2L1*/miRNA19-a in GC patients correlates with poor clinical outcomes, and miR-19a could be considered a prognostic biomarker.

To estimate if interference with *MAD2* expression by enforced miR-19a might affect tumorigenic phenotype, we quantitatively assessed the migration and invasion capacity of GC cells by performing wound healing and Transwell membrane assays. miR-19a expression significantly inhibited migration (40% vs. > 95% wound closure by 40 h; Fig. 1E), unlike miR-203 (Fig. S4B). Invasion capability was also significantly reduced in miR-19a-expressing MKN45 cells, compared to untransfected cells (Fig. 1F).

Some miRNAs may affect the response to cancer treatments,³ as is the case for miR-19a in arsenic trioxide treatment of bladder cancer.⁴ We asked if miR-19a expression would affect GC cells apoptosis as a response to classical (Paclitaxel-PXL, Cisplatin-CDDP) or new antitumoral metal-lodrugs, I5 and I6, two of our new cisplatin iodide derivatives, reported to provoke a G2/M arrest.⁵ Mere expression of miR-19a increased 2.5 times and 5.9 times respectively the rate of apoptosis in untreated AGS and MKN45 cells, to similar levels elicited by PXL only (Fig. 1G). Moreover, apoptosis was also enhanced by miR-19a in GC cells concomitantly treated with the different drugs, although to different degrees depending on the compound and the cell line. Thus, miR-19a synergized modestly with PXL in both cell lines (1.3 times and 2.1 times), efficiently with CDDP, but only in one of them (4.9 times in AGS since in MKN45 the effect of the drug is highly apoptotic on its own), and rather efficiently with both I5 and I6, in both AGS, 4.9 times and 3.3 times and MKN45, 4.9 times and 6.1 times, respectively. These platinum derivatives also affected *MAD2* RNA and protein levels (Fig. S5).

In summary, our data indicate that a better GC prognosis parallels high levels of miR-19a and low of *MAD2* (Fig. 1H). This inverse correlation allows us to propose miR-19a as a predictive biomarker in GC. Additionally, its enforced expression appears to provoke apoptosis and even synergize with anti-tumoral conventional and new drugs in GC cells. This could provide an encouraging scenario to test GC patient- and tumor-oriented new therapeutic strategies by designing agents that would interfere with *MAD2* over-expression and synergize with *ad hoc* chemotherapeutics.

Conflict of interests

The authors have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gendis.2023.02.025>.

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