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Cancer Research

An Illegitimate microRNA Target Site within the 3′ UTR of *MDM4* Affects Ovarian Cancer Progression and Chemosensitivity

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Abstract

Overexpression of *MDM4* (also known as MDMX or HDMX) is thought to promote tumorigenesis by decreasing *p53* tumor suppressor function. Even modest decrease in Mdm4 levels affects tumorigenesis in mice, suggesting that genetic variants of *MDM4* might have similar effects in humans. We sequenced the *MDM4* gene in a series of ovarian cancer cell lines and carcinomas to identify mutations and/or single nucleotide polymorphisms (SNPs). We identified an SNP (SNP34091) in the 3′-UTR of *MDM4* that creates a putative target site for hsa-miR-191, a microRNA that is highly expressed in normal and tumor tissues. Biochemical evidence supports specific miR-191–dependent regulation of the *MDM4-C*, but not *MDM4-A*, variant. Consistently, the A-allele was associated with statistically significant increased expression of MDM4 mRNA and protein levels in ovarian carcinomas. Importantly, the wild-type genotype (A/A) is more frequent (57.8% vs. 42.2% for A/C and C/C, respectively) in patients with high-grade carcinomas than in patients with low-grade carcinomas (47.2% vs. 52.5% for A/A and A/C + C/C, respectively). Moreover, A/A patients who do not express the estrogen receptor had a 4.2-fold [95% confidence interval (CI) = 1.2–13.5; $P = 0.02$] increased risk of recurrence and 5.5-fold (95% CI = 1.5–20.5; $P = 0.01$) increased risk of tumor-related death. Unexpectedly, the frequency of *p53* mutations was not significantly lower in A/A patients. We conclude that acquisition of an illegitimate miR-191 target site causes downregulation of *MDM4* expression, thereby significantly delaying ovarian carcinoma progression and tumor-related death. Importantly, these effects appear to be, at least partly, independent of *p53*. *Cancer Res; 70(23); 1–9. ©2010 AACR.*

Introduction

Epithelial cancer of the ovary is the leading cause of death among the malignancies of the female reproductive system in the Western world despite being a relatively uncommon gynecologic cancer (1). Because the symptoms of ovarian cancer are nonspecific, most women present with advanced stages of disease at the time of diagnosis (2). The 5-year overall survival rates are only 30% to 40% for advanced stages (FIGO stages III and IV) of ovarian cancer (1, 3). Tumor debulking is the standard component of initial surgery for patients with advanced disease (4). Cytoreductive surgery of the tumor is followed by chemotherapy with carboplatin and paclitaxel. Unfortunately, the disease recurs in nearly all patients owing to acquisition of chemoresistance. Therefore, factors that predict and/or modulate chemoresistance are of key importance in the treatment of patients with ovarian carcinomas.

An intact *p53* pathway is an important determinant of the response to platinum-based chemotherapeutic treatments (5, 6). The importance of *p53* in tumor suppression is highlighted by the frequency with which inactivating, somatic mutations are found in human cancers (7). Even modest change in the levels of *p53* and/or of key regulators of its activity can significantly affect *p53* tumor suppressive function and therefore cancer development and/or progression (8). Naturally occurring sequence variations within *p53* (SNP, codon72, rs1042522, C/G) in human populations cause measurable perturbations of *p53* function (9). Importantly, polymorphisms

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and somatic genetic alterations of genes encoding *p53* and its regulators have been shown to affect ovarian cancer (10, 11). For instance, a single nucleotide polymorphism (SNP) in the P2 promoter of MDM2, one of the key regulators of *p53* (12), was recently associated with an earlier age of tumor onset in estrogen receptor (ER) overexpressing ovarian carcinomas (6).

Mdm4 is another critical negative regulator of *p53* and is thought to contribute to cancer development via its ability to inhibit *p53* function (13). In keeping with this hypothesis, it is frequently overexpressed in human cancers that retain wild-type *p53* (14–18). Recent data suggest that even modest variation in Mdm4 levels in mice cause measurable perturbations of *p53* tumor suppressive function (19).

Thus, *MDM4* could harbor genetic variant(s) that affect carcinogenesis in humans. Here we report that SNP34091 (A/C) in the 3′-UTR of *MDM4* creates a new functional illegitimate target site for hsa-miR-191, a microRNA (miRNA) that is highly expressed in both normal and tumor tissues. miR-191 binds to the 3′-UTR of the *MDM4-C* allele but not that of the *MDM4-A* allele. Consequently, MDM4 protein expression is reduced in ovarian cancer patients with the A/C or C/C genotype. Importantly, the presence of the C-allele significantly delays ovarian carcinoma progression and increases sensitivity to chemotherapy. On the basis of these data, we propose that miRNA-mediated downregulation of *MDM4-C* has a significant impact on cancer progression and chemoresistance. Unexpectedly, we find that the frequency of *p53* mutations is not significantly lower in A/A patients, raising the possibility that these effects are mediated via *p53*-independent mechanism(s).

Materials and Methods

Patient population and clinical data

Paraffin-embedded tissue samples from 113 invasive ovarian carcinomas that were diagnosed at the Institute of Pathology, Martin-Luther-University Halle-Wittenberg, between 1997 and 2005 were selected on the basis of the availability of tissue. This study has been approved by the local ethical committee. All histologic slides were reevaluated using a multihead microscope by 2 pathologists (E.G. and S.H.). Tumor patients and tissue samples were in part described elsewhere (6, 20). Additional 49 samples from ovarian carcinoma patients were provided by the Tumor Bank Ovarian Cancer (TOC), Department of Gynecology and Obstetrics of the Charité (Berlin, Germany). These samples were diagnosed and reevaluated at the Institute of Pathology of the Charité by C.D. and S.D.-E. Histology was classified according to the World Health Organization, and grading was assessed according to Silverberg (21). Data retrieved from clinical files included the patient age, amount of residual tumor, FIGO stage, adjuvant chemotherapy, and follow-up (Supplementary Table 1). Genomic DNA from healthy, age-matched volunteers was received from the Institute of Medical Immunology of the Martin-Luther-University Halle-Wittenberg. All volunteers and patients enrolled in this study were Caucasian women.

Cell lines and DNA isolation

The ovarian cancer cell lines A2780, EFO-21, and OAW-42 cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Hyclone, Perbio), Napyruvate (0.4 mmol/L; Sigma), nonessential amino acids (100×; Sigma), penicillin (100 U/mL), and streptomycin (0.1 mg/mL). The MCF-7 cell line was maintained with RPMI medium (Gibco) supplemented with FBS, Napyruvate, nonessential amino acids as described previously with bovine insulin at 10 μg/mL (Sigma). The cell lines used in this study were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany); they have been authenticated by analysis of short tandem repeats. Genomic DNA was isolated using the QIAamp DNA Micro kit (Qiagen, Hilden Germany) according to the manufacturer's instructions.

Sequence analysis of *MDM4 and p53*

We PCR-amplified all coding exons of the *MDM4* gene. Because of its size, exon 11 was separated in 3 overlapping fragments. The sequence of the primers used and PCR conditions are available on request. PCR products were gel purified and subsequently analyzed by direct sequencing in both sense and antisense directions, using the BigDye Terminator Cycle Sequencing 3.1 kit (Applied Biosystems, Darmstadt, Germany). The sequencing reactions have been carried out according to the manufacturer's instructions. The *p53* mutation analysis was performed as previously described (6).

Reporter constructs, transfection and luciferase assays

The amplification of 224-bp fragments of the 3′UTR of *MDM4* centered around SNP34091 was achieved by PCR using genomic DNA from EFO-21 (C/C) and OAW-42 (A/A) cell lines. Both PCR products were subcloned into the psicheck2 luciferase reporter vector (Promega) to generate the *MDM4-200A* and *-200C* constructs. These constructs and the empty psicheck2 vector were transfected either alone or together with oligonucleotides in triplicate according to the manufacturer's instructions (Lipofectamin 2000; Invitrogen). Cells were harvested 24 hours posttransfection and luciferase activity was measured using the "dual glo luciferase assay" (Promega). We designed a target site blocker that is predicted to bind selectively to a sequence overlapping the miR-191 site in the 3′UTR of *MDM4* with the sequence 5′-GTGGTAAGTGAACGGAAT-3′ and a mismatch control blocker with the sequence 5′-GTGGTCAGTTCAGCGATT-3′ (changes underscored). The blockers were synthesized as fully phosphorothiolated DNA/LNA mixmers, with an LNA content of 33.3%, and purified by preparative HPLC before use.

Affinity purification of miR191 targets

The pull-out assays were carried out as previously described (22). Briefly, a biotin-tagged miR-191 mimic was transfected (2 nmol/L) into the cells and total RNA was extracted using Trizol (Invitrogen) 24 hours later. The RNA (10 μg) was then incubated with BSA-blocked streptavidin-sepharose beads in pull-out buffer for 2 hours. The beads were then washed 4 times and the RNA was extracted using Trizol (Invitrogen).

MDM4 mRNA expression levels

RNA was isolated using Trizol (Invitrogen, Karlsruhe, Germany) or miRNeasy kit (Qiagen) according to the

manufacturer's instructions. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) or the Superscript II reverse transcriptase using random hexamers (Invitrogen, Karlsruhe, Germany). *MDM4* levels were assessed using the TaqMan Gene Expression Assay (Hs00159092_m1) or a custom-designed assay (AJ Roboscreen AG, Leipzig, Germany) or using Fast SYBR green mastermix (Applied Biosystems) and the following primers: *MDM4* forward: 5′-TGAACAT TTCACCTTGCGCACCTG; *MDM4* reverse: 5′-CAACATCTGA-CAGTGCTTGCAGGA. Expression levels were normalized to the levels of several housekeeping genes including GAPDH (AJ Roboscreen AG), β-actin or RPL13a, and TBP by using both the $2^{-\Delta\Delta}C_t$ method and the real-time PCR data analysis software qBase (Ref). The *MDM4* transcript levels in each patient sample are given as zmol of MDM4 mRNA (zeptomol, 10^{-21} mol) per amol of GAPDH-mRNA (attomol, 10^{-18} mol). Differential expression levels of the *MDM4-A* and *-C* transcripts were assessed using the Plexor qPCR system kit (Promega) and the following primers: 5′-HEX-*iso*-dC-CATGATCCTGGTAAGTGAACTGA-3′ for the A-allele and 5′-FAM-*iso*-dC-GGATCGATGTAAGTGAACGGA-3′ for the C-allele. 5′-AGTACGAACATAAAAATG CATTTAT-3′ was used as the anchor primer. The primers have been designed using the Plexor Primer design software (Promega).

hsa-miR-191 expression levels

RNA was isolated from cell lines and five 4-μm sections of FFPE material, using the miRNeasy and the miRNeasy FFPE kits, respectively (Qiagen). Ten nanograms of total RNA of each sample was used for cDNA synthesis of the microRNAs hsa-miR-191, RNU24, RNU38, and snoU18, using stem-loop primers (Applied Biosystems). Reverse transcription reactions were carried out with the TaqMan Reverse Transcription kit according to manufacturer's protocol (Applied Biosystems). Real-time PCR measurements with specific TaqMan primers were carried out in triplicates, using the Rotorgene 3000 Realtime PCR System (Qiagen).

Immunohistochemistry

The analysis of the ER expression was carried out using the anti-ER antibody SP1 (Labvision, Germany), as described elsewhere (6). MDM4 protein expression was analyzed on a tissue microarray composed of 2 cores per specimen, using a polyclonal serum [rabbit polyclonal against human recombinant glutathione-*S*-transferase-MDM4Δ109-198 protein (15)]. The serum was a kind gift from G.G. Lozano (Houston, TX). The immunoreactivity was scored as the percentage of stained cells by counting 150 tumor cells on average as follows: less than 10% of positive cells, no expression; between 10% and 50% of positive cells, moderate expression; and more than 50% of positive cells, high expression.

Western blot analysis

Cells were lysed on ice in 1% Triton X-100 [50 nmol/L of HEPES (pH 7.5), 150 nmol/L of NaCl, 1 mmol/L of EDTA, 2.5 mmol/L of EGTA] and a mixture of protease inhibitors [1 mmol/L of NaF, 10 mmol/L of β-glycerophosphate, phosphatase inhibitor (Sigma), phosphatase inhibitor cocktail (Sigma)]. Protein lysates (50 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto hybond-PVDF membrane (GE Healthcare). Membranes were blocked with 10% nonfat dry milk (0.2% TBST) for 40 minutes at room temperature and incubated overnight at 4°C with the primary antibodies: polyclonal anti-MDM4 antibody (1:5,000; Bethyl Laboratories Inc.), monoclonal anti-p53 antibody (1:1,000, DO-1; Santa Cruz), and Vinculin (1:1,000; Sigma). The membranes were then incubated with the secondary HRP-linked antibodies (dilution 1:10,000; Cell Signaling) and signals were visualized by using SuperSignal West Femto Maximum Sensitivity Substrate (ThermoScientific).

Statistical evaluation

All statistics, including the Cox's proportional regression hazard model and the Kaplan–Meier survival estimates, were carried out using the SPSS 17 software (SPSS Science, Chicago, IL).

Results

MDM4 somatic mutations and allelic variants in ovarian cancer

To identify putative somatic mutations and/or allelic variants of *MDM4*, we sequenced all coding exons of the *MDM4* gene and flanking intronic regions in 8 ovarian cancer cell lines as well as in MCF-7 and HeLa cells. Three SNPs that have previously been listed in the NCBI SNP database were detected (Supplementary Table 2). Interestingly, one of them lies in the 3′UTR, 32 nucleotides downstream of the stop codon, at position 34,091 (A > C; rs4245739; GenBank accession no: AY207458) (Fig. 1A). Of the 8 ovarian cancer cell lines analyzed, 2 were homozygous for SNP34091 (C/C), 2 were heterozygous (A/C), and 4 were homozygous for the wild-type allele (A/A) (Supplementary Table 2). In addition, a single-nucleotide change in codon 387 (Pro > Ala) was detected in the cell line A2780. Codon 387 is located in the C-terminal region outside of any known *MDM4* functional domains.

We also sequenced exon 11, encoding for the regulatory RING-finger domain from 113 ovarian cancer patients. In addition to the SNP in the 3′-UTR, only 2 missense mutations and 2 silent mutations were identified. The missense mutation in codon 421 (AGA > GGA; Arg > Gly) was identified in 3 patients. One patient also harbored a silent mutation in codon 305 (CAG > CAA, Gln > Gln). Two additional mutations were detected in another patient: a missense mutation in codon 439 (GTC > ATC; Val > Ile) and a silent mutation in codon 477 (TTA > TTG, Leu > Leu). We concluded that somatic mutations in *MDM4* are rare in ovarian cancer.

hsa-miR-191 differentially regulates expression of the allelic variants of rs4245739

SNPs that reside within 3′-UTRs can potentially affect mRNA stability and/or translation efficiency by perturbing miRNA-mediated gene regulation (23). We therefore searched for putative miR-binding sites spanning the SNP34091, using algorithms that predict miRNA-binding sites. hsa-miR-191 is

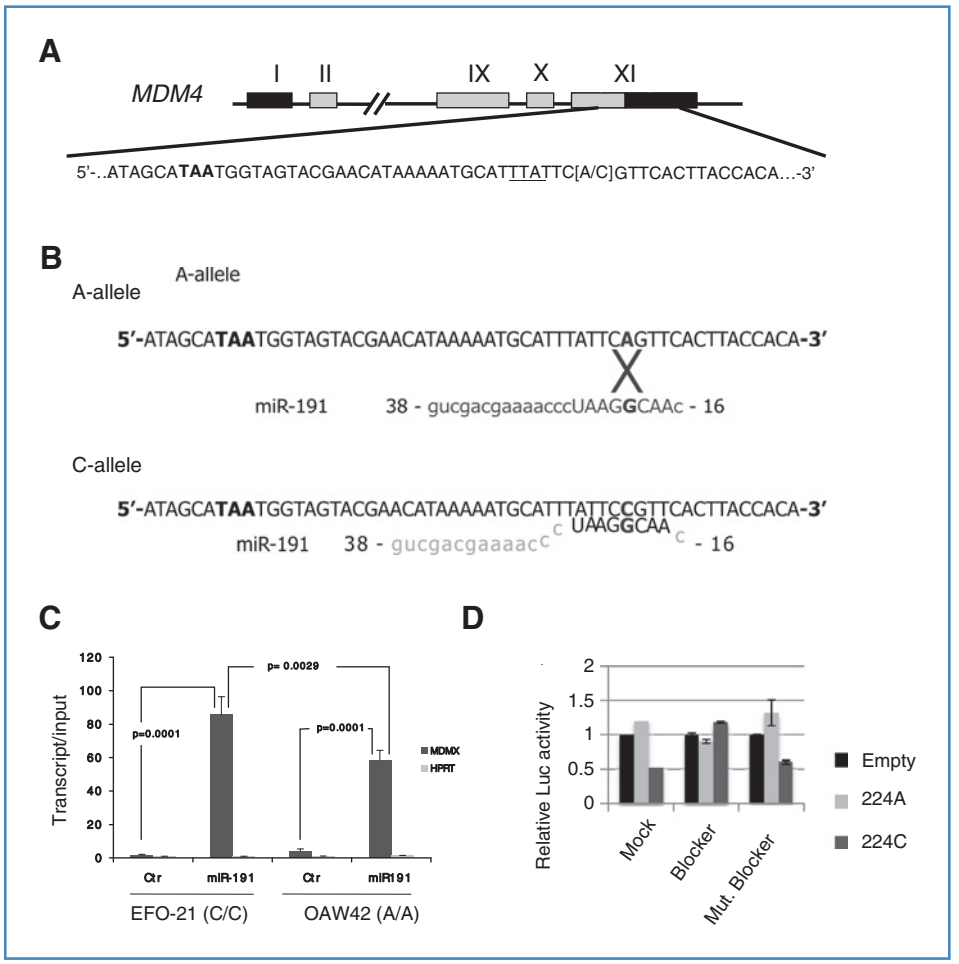

Figure 1. hsa-miR-191 differentially regulates the *MDM4* allelic variants of rs4245739. A, schematic representation of the *MDM4* locus. The coding and noncoding exons are indicated by gray and black boxes, respectively. Partial sequence of the 3′UTR of *MDM4* is shown; the SNP rs4245739 is located 32 nucleotides downstream the Stop codon (in bold). B, seed pairing of hsa-miR-191 (nucleotides 2–8 at 5′ end) with A (top sequence) and C (bottom sequence) alleles of rs4245739. C, biotinylated miR-191 or nontagged miR-191 (Ctr) was transfected in the indicated cell lines. Pull-out assays were carried out as described in Materials and Methods and the amount of *MDM4* and HPRT transcripts recovered was normalized to the total amount of transcripts expressed in these cells (Input). D, empty psicheck2 vector (empty) or fused to 224th nucleotide of the 3′UTR of *MDM4-A* (224A) or *MDM4-C* (2224-C) were transfected alone (Mock) or with the miR-191 Blocker or mutant blocker (Mut Blocker) in the A2780 ovarian cancer cell line. The data are normalized to the luciferase activity observed with the empty psicheck2 vector, which is set to 1. The data represent the mean ± SD of 4 independent biological replicates.

predicted by TargetScan (24), miRanda (25), and MicroInspector (26) to target the *MDM4-C* variant. Strikingly, SNP34091 resides within the critical region for miRNA binding, a region known as the "seed" that lies 2 to 8 nucleotides from the 5′ end of the miRNAs (27) (Fig. 1B). Because there is a tendency of miRNAs to form complete base pairings with the target mRNA at the seed (27), we hypothesized that hsa-miR-191 might differentially bind the transcript produced by the *MDM4-C* allele as compared with the one produced by the A-allele.

To experimentally validate this prediction, we used a direct affinity purification method described previously (28). Cells of A/A or C/C genotype were transfected with biotin-tagged miR-191 and miR-RISC-mRNA complexes were purified using streptavidin-sepharose beads. After recovery of the purified RNA, reverse transcription quantitative PCR (RT-qPCR) demonstrated a specific and significant enrichment for *MDM4* transcripts in C/C cells compared with A/A cells; the data are normalized to the levels of total endogenous *MDM4* transcripts (Fig. 1C). In contrast, there was no specific enrichment of nonrelevant transcripts, such as HPRT. Moreover, only negligible amounts of *MDM4* transcripts were recovered after transfection of the cells either with a nontagged version of hsa-miR-191 duplexes or with a tagged version of an irrelevant miRNA, hsa-miR-10A (Fig. 1C; data not shown). These data indicate that hsa-miR-191 directly interacts with *MDM4-C*, and it does so with a significantly higher affinity than *MDM4-A*.

miRNA-targeted mRNAs either are translationally repressed and/or directly cleaved or are subjected to enhanced degradation (29). To examine whether the A to C transversion affects *MDM4* translation efficiency and/or mRNA stability, we cloned a 224-bp fragment of the 3′-UTR of *MDM4* centered around SNP34091 from A/A and C/C cells into the 3′-UTR of the psicheck2 luciferase reporter vector. We transfected the

resulting reporter constructs (224A and 224C) into the A/C ovarian cancer cell line A2780, which express high levels of endogenous hsa-miR-191. We consistently observed a significant reduction of signal in cells transfected with 224C, equivalent to 40% to 60% of the signal observed in cells transfected with 224A. Notably, the signal obtained with the latter vector did not differ from the signal obtained with the unmodified psicheck2 (Empty, Fig. 1D). These data suggest that the C variant at rs4245739 reduces translation efficiency and/or decreases mRNA stability of *MDM4*, possibly due to the creation of an illegitimate miR-191 binding site.

To substantiate this hypothesis further, we used a novel approach in which we used target site blockers. Target site blockers are oligonucleotides, 18 to 20 nucleotides long, designed to bind selectively to a single site within a specific 3′-UTR at a location overlapping an miRNA recognition site. The blockers are synthesized as fully phosphorothiolated DNA/LNA mixmers both to increase their affinity and selectivity for the target and to inhibit RNAseH recognition and cleavage. We designed a target site blocker (18 nucleotides) to bind selectively to a sequence overlapping the hsa-miR-191 site in the 3′-UTR of *MDM4-C*. The blocker has no significant match to any human annotated 3′-UTR except for *MDM4*. We also designed a mismatch control blocker (Mut. Blocker) that has no significant match to 3′-UTR of *MDM4-C* or to any other human annotated 3′-UTR. In agreement with our prediction, transfection of the has-miR191 target site blocker, but not of the mutant blocker, significantly rescued the reduction of the MDM4-C luciferase signal in a dose-dependent manner (Fig. 1D). Similar results were obtained upon transfection in several other cancer cell lines including 293T, HCT116, MCF-7, and EFO-21, all of which express high and comparable levels of endogenous hsa-miR-191 (data not shown).

Expression levels of *MDM4* and hsa-miR-191 in ovarian cancers

hsa-miR-191 was previously found ubiquitously and highly expressed across dozens of normal and cancerous human solid tissues (30). In fact, it is one of the very few miRNAs that is expressed at high and stable levels in all tissues analyzed (30). We confirmed that hsa-miR-191 is highly and stably expressed in a panel of ovarian cancer cell lines (data not shown). Expression levels of miR-191 were also examined in 61 ovarian carcinoma samples. The average expression levels of miR-191 were 1.41 relative to the snoU18 expression levels. There was no difference between tumors of patients with A/A and A/C + C/C genotypes (A/A: 1.38; A/C + C/C: 1.47; $P = 0.79$, Student's t test). Furthermore, we found comparable miR-191 expression levels in both low-grade and high-grade carcinomas ($P = 0.411$; Student's t test). There was also no correlation between miR-191 expression levels and the ER expression status of the tumors.

If rs4245739A-C causes differential miR-191–mediated degradation and/or inhibition of translation of *MDM4* transcripts, C/C tumors and cells derived from these tumors should have reduced MDM4 protein levels. Accordingly, *MDM4* expression levels were extremely low in all C/C ovarian cancer cell lines analyzed (3/3) (Fig. 2A). In contrast, MDM4 was readily detectable in the A/C (3/3) and all but one (OVCAR-3) A/A (2/3) cell lines analyzed. Notably, *p53* is mutated in OVCAR-3 (Supplementary Table 2); there is, therefore, no need for alternative mechanism of *p53* inhibition, such as high *MDM4*, in these cells. The ability of miR-191 to selectively target *MDM4-C* was next assessed in a larger data set by examining MDM4 protein expression in 66 different primary ovarian carcinoma samples by immunohistochemistry on tissue microarrays. There was no detectable MDM4 protein expression in 48% (31/66) of the cases. *MDM4* was found at moderate and high expression levels in 14% (10/66) and 38% (25/66), respectively. Importantly, the A/A genotype of SNP34091 was correlated with increased MDM4 protein expression levels ($P = 0.096$; χ^2 test) (Fig. 3A and B).

To determine whether miR-191 has the ability to promote selective degradation of *MDM4-C*, we analyzed MDM4-C and MDM4-A mRNA levels in our panel of ovarian cancer cell lines by RT-qPCR (Fig. 2B). In contrast to *MDM4-A*, the *MDM4-C* transcript was virtually undetectable in all A/C cell lines (3/3) analyzed (Fig. 2C; data not shown). Importantly, transfection of increasing amounts of the hsa-miR-191 target site blocker in A2780 A/C cells increased the levels of the C-transcript in a dose-dependent manner until reaching levels comparable with the A-transcript. This experiment indicates that hsa-miR-191 can selectively target the *MDM4-C* transcript for degradation (Fig. 2C). This view was further supported by the analysis of MDM4 mRNA expression in 112 ovarian cancer patients. Indeed, the A/A genotype of SNP34091 was correlated with increased MDM4 mRNA ($P = 0.038$; chi-square test) expression levels (data not shown).

Surprisingly, MDM4 mRNA levels are relatively high in 2 of the 3 C/C cell lines analyzed (EFO-27 and HOSE; Fig. 2B). Because MDM4 protein levels are very low in these cells, these data indicate that in addition to its ability to promote degradation, miR-191 is also likely to block *MDM4-C* translation. Together, these analyses indicate that *MDM4-C* is selectively targeted by hsa-miR-191 for inhibition of translation and/or degradation depending on the cellular context.

rs4245739 status and disease-free survival

Our biochemical data are consistent with a model in which rs4245739 influences *MDM4* expression. As *MDM4* is a key regulator of *p53* tumor suppressor function (13), these data predict that rs4245739 might influence cancer risk and/or progression. To test this possibility, we assessed the status of rs4245739 in a total of 154 ovarian carcinoma patients. The distribution of the genotypes was comparable between patients with ovarian cancer (A/A, 55.8%; A/C, 39.6%; and C/C, 4.5%) and age-matched healthy volunteers (A/A, 57.4%; A/C, 36.6%; and C/C, 6%). The percentage of cases with the wild-type genotype (A/A) was slightly lower in low-grade carcinomas (47.5%) and higher in high-grade carcinomas (57.8%) than in controls. However, this difference was not statistically significant ($P = 0.263$; chi-square test). Patients with the A/A genotype also showed a decreased overall survival time compared with heterozygous (A/C) or homozygous (C/C) patients (63 months vs. 72 months for A/A and A/C + C/C, respectively), although this difference was not statistically significant ($P = 0.091$; log-rank test). However, in a

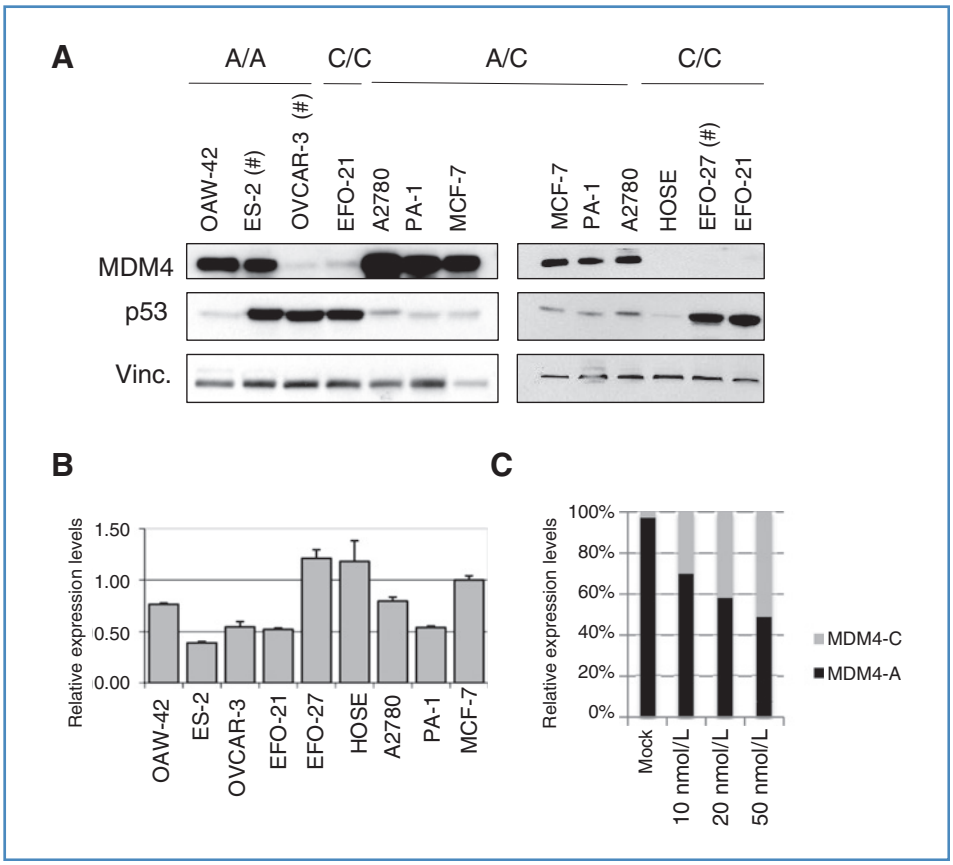

Figure 2. *MDM4* expression levels in ovarian cancer cell lines. A, MDM4 and p53 protein expression levels in ovarian cancer cell lines as assessed by Western blot analysis. Vinculin (Vinc.) serves as loading control. B, MDM4 mRNA expression levels in various ovarian cancer cell lines as assessed by RT-qPCR. Data are normalized to the levels observed in MCF-7, which is set to 1. The data represent the mean (±SD) of 4 independent biological replicates. C, relative expression levels of *MDM4-A* versus *MDM4-C* transcripts in A2780 that are either untreated or trans-fected with the indicated amounts of the miR-191 target site Blocker. The data represent the mean of 3 independent biological replicates. #, cells harboring *p53* mutations.

previous study, we showed that the impact of the *MDM2* SNP309 on patient survival was dependent on the ER expression status (6). We find the same to be true in the case of *MDM4*. The median overall survival time for *MDM4-A* homozygous patients with no ER expression was indeed significantly lower (52 months vs. 82 months) than for patients who carried at least 1 C-allele ($P = 0.042$; log-rank test). A multivariate Cox's regression model (Fig. 3C; Supplementary Table 3), adjusted according to the residual tumor, revealed that the A/A genotype is correlated with a significantly increased risk of tumor-related death (hazard ratio [HR] = 5.5; 95% CI = 1.5–20.5; $P = 0.01$) in patients with ER-negative tumors. In contrast, the overall survival of patients with ER expression was comparable across genotypes (A/A vs. A/C + C/C; $P = 0.89$; log-rank test).

We next investigated whether rs4245739 was associated with the progression of the disease in patients who received adequate chemotherapeutic treatments. Consistent with the already described synergy between *MDM4* polymorphism and ER status, the A/A genotype was correlated with a strikingly decreased progression-free survival time in ER-negative patients (18.7 months vs. 50.6 months for A/A and A/C + C/C, respectively; $P = 0.012$, log-rank test). In a multivariate Cox's regression model, we calculated the risk of recurrence and found that it was 4.1-fold increased for ER-negative patients who are homozygous for the A-allele (95% CI = 1.2–13.5; $P = 0.02$) (Fig. 3D) whereas ER-overexpressing patients (A/A genotype) had no elevated risk of relapse regardless of their rs4245739 genotype (HR = 1.06; $P = 0.885$). Together, these observations indicate that the A-allele is associated with an increased *MDM4* expression (mRNA and protein) and subsequently accelerated tumor progression and chemoresistance in ER-negative tumors.

Because *MDM4* is thought to affect cancer development by inhibiting *p53* function (13), we determined the mutational status of *p53* in our cohort of ovarian cancer samples. Unexpectedly, the A/A genotype did not significantly correlate with a lower frequency of *p53* mutations. The frequency of *p53* mutations in A/A patients (~50%) was comparable with that in A/C and C/C patients (37%; $P = 0.33$, chi-square test). These data, therefore, raise the possibility that variation in *MDM4* expression affects tumorigenesis (overall survival and chemosensitivity), at least partly, via mechanism(s) that are *p53* independent.

Discussion

Most common variants that affect cis transcript levels in have been found to modulate the activity of promoters and enhancers. However, recent studies have shown that genetic variants can influence organismal phenotypes by perturbing miRNA-mediated gene regulation (31). Our data now provide evidence that this new class of regulatory mutations are also important modifiers of cancer risk. We show that an SNP

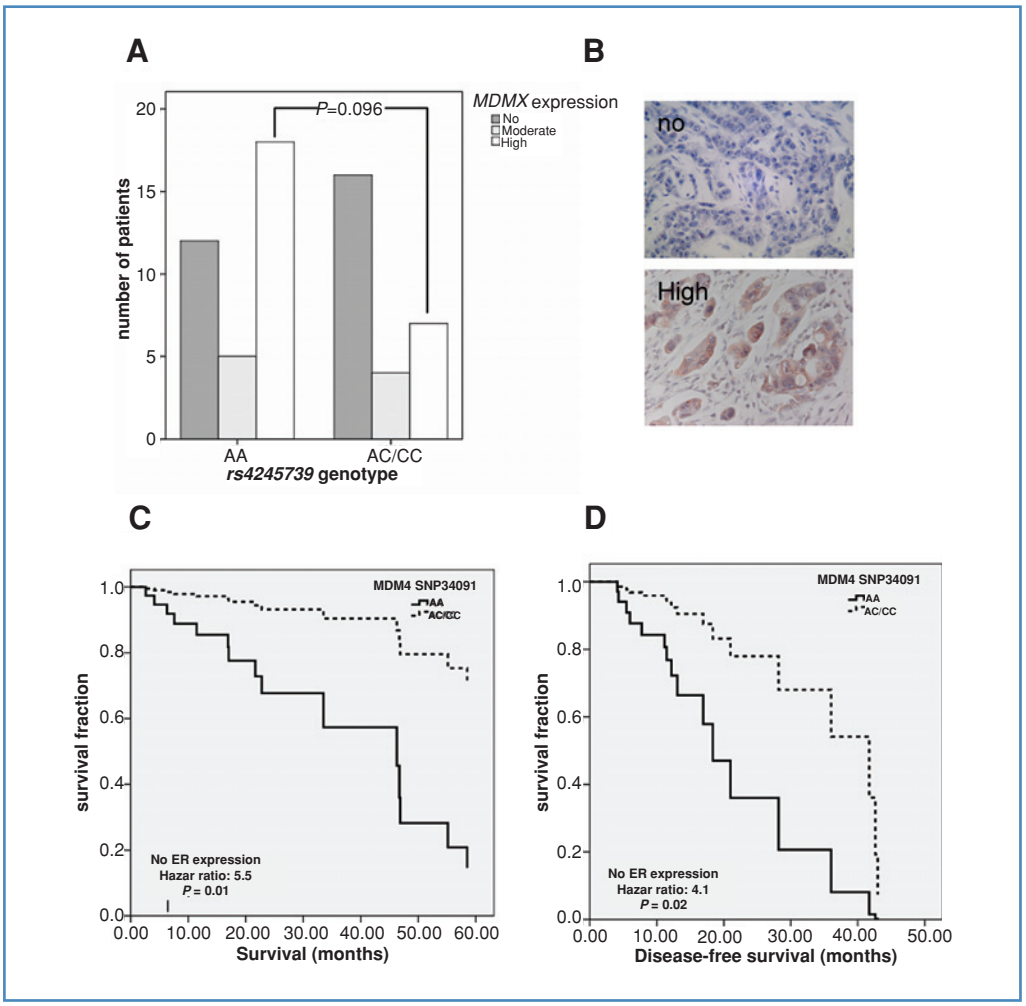

Figure 3. The A-allele of SNP34091 is correlated with poorer disease-free and overall survival in ER-negative ovarian carcinoma patients. A and B, A/A genotype correlates with increased MDM4 protein levels. *MDM4* expression levels were assessed by immunohistochemistry in 66 primary ovarian carcinomas. A, immunoreactivity was scored as follows: less than 10% of positive cells, no expression; between 10% and 50% of positive cells, moderate expression; and more than 50% of positive cells, high expression. B, representative immunostaining for tumors with no (top) or high (bottom) *MDM4* expression. C, graph displays the survival of ER-negative ovarian carcinoma patients plotted against the survival in months. The multivariate Cox regression, which was adjusted to the residual tumor, revealed that patients homozygous for the A-allele have a 5.5-fold increased risk of tumor-related death ($P = 0.01$). D, A-allele is also correlated with a significantly decreased disease-free survival of ovarian carcinoma patients who were treated with adequate chemotherapy after surgical removal of the tumor and a 4.1-fold increased risk of relapse ($P = 0.02$). The multivariate Cox regression was adjusted according to the residual tumor.

(rs4245739) in the 3′UTR of the *MDM4* oncogene creates an illegitimate has-miR-191 target site and is correlated with both disease-free and overall survival of ovarian cancer patients.

The functionality of the *p53* tumor suppressor pathway is frequently attenuated in ovarian cancer either by somatic or germline genetic events within *TPR53* itself or within its upstream and downstream regulators (32, 33). We and others have previously shown that a naturally occurring polymorphism in the *p53*-sensitive MDM2-P2 promoter is associated with an accelerated tumor formation, especially in female patients with soft tissue sarcoma (34, 35) and in ovarian cancer, in an ER-dependent manner (6). Notably, the reason why the ER expression status is an important determinant for this association remains to be elucidated. Our biochemical data indicate that the *MDM4* A-allele, which cannot be regulated by hsa-miR-191, correlates with an overall increase in *MDM4* level of expression. As there is overwhelming evidence supporting a key role for *MDM4* in the regulation of *p53* tumor suppression function, it is tempting to speculate that the increased cancer risk observed in A/A patients result, at least in part, from an attenuation of the *p53* tumor suppressor activity. However, our unexpected observation that A/A patients did not carry *p53* mutations significantly more frequently raises a possibility that variations in *MDM4* levels can affect tumorigenesis in a manner that is independent of *p53*. Recent biochemical and genetic data have indeed highlighted *p53*-independent activities for Mdm4/*MDM4* under specific experimental conditions. For instance, *MDM4*-mediated

proteasomal degradation of p21, one of the key downstream *p53* effectors, is independent of *Mdm2* and *p53* (36). *MDM4* also seems to exert a positive effect on stress-activated *p53* proapoptotic function (37) and modulates tumorigenesis and chromosomal stability in *p53*-null cells and mice (38). These, or other yet unknown *p53*-independent activities, may explain why rs4245739 is not correlated with a decreased frequency in *p53* mutations.

A negative effect of the *MDM4* A/A genotype was observed on the progression-free and overall survival in ER-negative patients. However, in contrast to recently reported data on an SNP (rs1563828) located in intron 10 of *MDM4* (39), we did not observe an effect of the rs4245739 genotypes on the age of onset. This suggests that increased *MDM4* levels are correlated with the clinical course and the tumor response to chemotherapy and not with the development of ovarian cancer in ER-negative patients (as was previously seen by us for the *MDM2* G-allele of SNP309 (6)). In contrast to *MDM2*, *MDM4* does not contain ER-binding sites; therefore, the expression of *MDM4* is not regulated by estrogen in a *p53*-independent manner (40). We previously showed that the *MDM2* G-allele of SNP309 requires an intact ER signaling (6, 35). Therefore, the effect of the MDM2 promoter polymorphism is especially pronounced in ER-positive patients and this overrides the effect of SNP34091 that can be observed in ER-negative patients. In line with our observations, Kulkarni et al. have recently shown that rs1563828 is associated with accelerated age of onset only in ER-negative breast cancer patients (41).

A causative link between high levels of *MDM4* and tumorigenesis has already been proposed (17, 18). Our data further underscore the clinical relevance of *MDM4* genetic alterations in human cancer and indicate that *MDM4* is a promising therapeutic target for the treatment of ER-negative ovarian cancer patients. Moreover, although our current work is restricted to ovarian cancer, given that hsa-miR-191 is expressed at high levels in all normal tissues and primary tumor samples analyzed (27 and our unpublished data), our findings can most likely be extended to other tumor types. It will, therefore, be of interest to assess the SNP status of 3′-UTR in other tumor types in order to determine whether the creation of the miR-191 illegitimate target site plays a broader role in tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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