The regulation of MASPIN expression in epithelial ovarian cancer: Association with p53 status, and MASPIN promoter methylation: A Gynecologic Oncology Group study

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ABSTRACT

Objectives. To elucidate the regulation of MASPIN expression in epithelial ovarian cancer (EOC) and associations with p53 status and MASPIN promoter methylation.

Methods. Seven EOC cell lines and 110 advanced stage EOC specimens were analyzed for MASPIN promoter methylation. The cell lines were treated with 5-azacytidine (5-azaC) and evaluated for MASPIN promoter methylation, protein, and mRNA expression. Wild-type (wt) p53 was transiently transfected into the mutant p53 (m p53) SKOV3 cells which were treated with 5-azaC. Phosphor imager analysis quantified the percent methylation of the MASPIN promoter.

Results. Of the 3 MASPIN-low m p53 cell lines 2 had greater than 5% MASPIN methylation whereas only 1 of 4 MASPIN-high wt p53 cell lines had greater than 5% MASPIN methylation. Despite the presence of aberrant MASPIN promoter methylation in SKOV3 cells, wt p53-transfection alone resulted in a 3.3-fold increase in MASPIN mRNA. The combination of 5-azaC and wt p53-transfection produced a 36% reduction in MASPIN promoter methylation and 4.5-fold increase in MASPIN transcription. Among the 110 ovarian cancer specimens analyzed for methylation of the MASPIN promoter, 81.8% were weakly methylated, 14.5% were heavily methylated and 3.6% were fully methylated. There was no relationship between promoter methylation and p53 status or MASPIN protein expression. However, MASPIN protein was 6 times more likely to be detected in cancer specimens that harbor a p52 mutation relative to cancer specimens with a wt p53 gene.

Conclusion. The regulation of MASPIN is a complex multifactorial process that may be controlled by both p53-dependent and -independent epigenetic mechanisms.

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Introduction

MASPIN, a serine protease inhibitor in the serpin super family of serine protease inhibitors, functions as a tumor suppressor by inhibiting tumor cell motility, invasion, metastasis, and angiogenesis [1–4]. MASPIN has been shown to have differential sub cellular expression in ovarian cancer [5,6] and have prognostic significance for survival [5–7]. Furthermore, transfection of wild-type (wt) MASPIN into aggressive ovarian cancer cell lines has been shown to inhibit the invasive activity of these cells 44–68% [6]. Although the molecular mechanisms regulating MASPIN expression, down-regulation and loss during ovarian cancer progression are as yet undefined, observations from other cancers suggest that these events will likely be under the control of both normal and aberrant transcriptional and epigenetic mechanisms [8–16].

The p53 tumor suppressor pathway has been implicated in the regulation of MASPIN expression in other solid tumors [8–12] and p53 protein overexpression has been shown to be inversely correlated with MASPIN expression in ovarian cancer [17]. In normal and cancerous breast and prostate cells, wt p53 has been shown to activate MASPIN expression by binding directly to the p53 consensus site in the MASPIN promoter [12]. Oshiro et al. demonstrated that mutant p53 (m p53) and aberrant cytosine methylation cooperate to silence expression of MASPIN [15]. The MASPIN promoter is normally associated with unmethylated cytosines and wt p53 may function to protect target promoters from aberrant methylation through its DNA-binding activity. However, upon mutation the wt p53 DNA-binding, activity is lost and the p53 target regions become permissive to aberrant de novo cytosine methylation which subsequently results in the loss of gene expression [15].

The present study was undertaken to determine the relationship of MASPIN expression and p53 status as well as MASPIN promoter methylation in epithelial ovarian cancer (EOC). The manner in which p53 regulates the expression of MASPIN is poorly understood and may include epigenetic mechanisms such as gene silencing via aberrant cytosine methylation of gene promoters. Our primary hypothesis was that inactivation of the p53 tumor suppressor gene pathway leads to MASPIN repression in ovarian cancers through aberrant promoter hypermethylation.

Methods

Ovarian cancer cell lines, DNA methyltransferase inhibition, and p53 transfection

Cell culture

Seven immortalized ovarian cancer cell lines (DOV 13, OVCA 420, OVCA 429, OVCA 432, OVCA 433, SKOV3, OVCAR3) were grown in monolayer culture Type Culture Collection (Manassas, VA) and maintained as recommended by the supplier. The DOV 13, OVCA 420, OVCA 429, and OVCA 433 cell lines contain a wt p53 gene while OVCA 432, OVCAR3, and SKOV3 harbor a m p53 gene [18]. Protein extractions were performed as previously described [18,19] and RNA extractions were performed using the RNeasy Mini Kit following the manufacturer’s recommendations (Qiagen, Inc.; Valencia, CA). For cDNA synthesis, 1 µg of total RNA was incubated for 60 min at 42 °C with oligo (dt) primers and 20 units of AMV reverse transcriptase in 1× reverse transcriptase buffer supplemented with 5 mM of MgCl2, 1 mM of each dNTP, and 25 units of RNase inhibitor in a final volume of 20 µl (Roche Diagnostics Cooperation, Indianapolis, IN). All experiments were performed in triplicate.

DNA methyltransferase inhibition

Ovarian cancer cell lines were grown in six-well plates and treated for 72 h with 5 µM 5-azaCytidine (5-azaC; Sigma Aldrich; St. Louis, MO), a potent inhibitor of DNA methyltransferase (DNMT) activity [20], dissolved in dimethylsulfoxide. Controls were treated with dimethylsulfoxide containing media. Cell pellets were divided to extract both protein and RNA to determine MASPIN protein and mRNA expression, respectively.

Transfection experiments

SKOV3 cells were cultured in 60 mm plates in RPMI 1640 medium (GIBCO®; Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) until semi-confluent. Transient co-transfections of SKOV3 cells were performed using Lipofectamine (GIBCO®) and OptiMEM. Plasmids used for transfection included: 2 µg of pcDNA3 containing wt p53 sequence and corresponding empty vector (provided by Dr. Jeffrey Marks), and 2 µg of pEGFP encoding enhanced green fluorescent protein and corresponding empty vector (provided by Dr. Jeffrey Marks). After a 4 h incubation at 37 °C, the transfection media was replaced with RPMI 1640 and the cells were incubated at 37 °C overnight. Fluorescent microscopy was used to determine transfection efficiency. A subset of the transfected cells was harvested while the remainder underwent treatment for an additional 72 h with 5-azaC prior to harvest for protein and RNA as described above.

Primary ovarian cancer specimens

Frozen tumor specimens were obtained during primary cytoreductive surgery and prior to the initiation of chemotherapy from 110 women with advanced EOC, who participated on GOG specimen banking protocol and randomized phase III first-line treatment protocols [21,22]. One patient enrolled on GOG Protocol 114 was inappropriately classified as having optimally-debulked disease; this case was classified as suboptimally-debulked for this translational research study. Tissues were maintained at −70 °C and histologic evaluation of each sample was performed to confirm that at least 50% of the cellular component was malignant. Data regarding immunohistochemical expression of p53 protein and/or sequencing of exons 2–11 or exons 5–8 of the p53 gene and MASPIN protein expression in the primary epithelial ovarian cancers were previously published [7,23].

Western blot analysis and real-time quantitative PCR

Western blot analysis was performed as previously described [7]. Real-time quantitative PCR (RQ-PCR) was used to analyze mRNA expression in the immortalized ovarian cancer cell lines. Quantification of MASPIN mRNA expression was obtained by RQ-PCR using fluorescent TaqMan methodology (ABI Prism 7700 Sequence Detector; Perkin Elmer Applied BioSystems; Foster City, CA). PCR was performed using 2 µl cDNA, 12 µl Universal PCR Master Mix (Perkin Elmer Biosystems; Branchburg, NJ, USA), 15 pmol forward and reverse primers specific for each marker and 200 nM specific detection probe in a final volume of 25 µl. Primers and probes for MASPIN and GAPDH were obtained from Applied Biosystems (Foster City, CA). All PCR reactions were performed on an ABI Prism 7700 Sequence Detector System (Perkin Elmer Applied Biosystems) with a Gene-amp PCR System 9600. The thermal cycling conditions were: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The comparative cycle threshold method was used to calculate the relative expression of MASPIN mRNA normalized to GAPDH run in parallel [24].

Methylation analyses

Bisulfite sequencing to determine the level of MASPIN promoter methylation was performed as previously described [25,26]. The following primers for the initial reaction 5′-AAGAATGGAGATTAGATTTTGTG-3′ and 5′-CTAAACAGATAATCTATGGGATTGATCC-3′; and second reaction 5′-GAAATTTTGATGTTATTATTATA-3′ and 5′-AAAAACACAAAAACCTAATAAAAAAATA-3′ were utilized. The product, a 368-bp amplicon, was resolved on 1% agarose gels, purified using Sigma GenElute gel purification columns (Sigma-Aldrich, St. Louis, MO) and sequenced using the ThermoSequenase Radiolabeled
MASPIN protein expression. Of the 3 MASPIN-low cell lines, 2
had greater than 5% promoter methylation whereas only 1 of 4 MASPIN-
high cell lines had greater than 5% promoter methylation (Fig. 2D).
In contrast the three ovarian cancer cell lines harboring a p53 mutation
demonstrated low MASPIN protein expression and were more likely to display greater MASPIN promoter methylation. The percent methylation
at individual CpG cytosines of the MASPIN promoter was scored objectively by phosphorimaging for DOV13, OV429, OV433, SKOV3, and OVCAR3. MASPIN promoter methylation was determined subjectively in the OV420 and OV432 cell lines.

Results

MASPIN expression and promoter methylation status in ovarian cancer cell lines

Lower levels of MASPIN protein and mRNA expression were observed in the wt p53 ovarian cancer cell lines relative to those with wt p53 genes (Fig. 1). The 3 ovarian cancer cell lines with wt p53 expressed 7- to 860-fold higher levels of MASPIN protein compared to cell lines with mutant p53. A non-parametric Wilcoxon signed-rank test provided suggestive evidence of a trend between p53 mutation and low levels of MASPIN protein or mRNA (p = 0.057). The ovarian cancer cell lines were divided into 2 groups based on the level of MASPIN protein expression. Of the 3 MASPIN-low m p53 cell lines 2 had greater than 5% MASPIN methylation whereas only 1 of 4 MASPIN-high wt p53 cell lines had greater than 5% MASPIN methylation (Fig. 1). RQ-PCR also demonstrated higher levels of MASPIN mRNA in the wt p53 cell lines compared to the mutant p53 cell lines (Fig. 1). MASPIN protein and mRNA were highly correlated (Kendall's tau-b correlation coefficient = 0.8, p < 0.001).

Effects of demethylating agent 5-azacytidine and p53 transfection on MASPIN promoter methylation and transcription in ovarian cancer cell lines

After treatment with 5-azaC, two MASPIN-low cell lines, OVCAR3 and SKOV3, demonstrated a 2.7- and 2.3-fold induction, respectively, of MASPIN transcription as compared to mock-treated controls (Fig. 2A). Following 5-azaC treatment OVCAR3 was completely demethylated while SKOV3 had only a minimal decrease in promoter methylation (Fig. 2B). In contrast, the MASPIN-high cell lines demonstrated no appreciable change in MASPIN transcription or MASPIN methylation after treatment with 5-azaC (Figs. 2A and B).

Despite the presence of aberrant MASPIN promoter methylation in SKOV3 cells, wt p53-transfection alone resulted in a 3.3-fold increase in MASPIN mRNA (Fig. 2C). However, there was no reduction in MASPIN promoter methylation following wt p53 introduction (Fig. 2D). But when wt p53 transfection was combined with 5-azaC therapy, MASPIN promoter methylation decreased 36% (Fig. 2D) and this was accompanied by a 4.5-fold increase in MASPIN transcription (Fig. 2C).

MASPIN expression and correlation with p53 status and MASPIN methylation status in ovarian cancer specimens

MASPIN expression was not associated with the level of p53 overexpression categorized as no overexpression, limited overexpression (<30% tumor cells exhibiting p53 immunostaining) or extensive overexpression (>30% tumor cells displaying p53 immunostaining) (p = 0.12) or extensive p53 overexpression compared with no or limited overexpression (p = 0.09) (Table 1). However, evidence of an association was observed between MASPIN expression and the type of p53 mutation categorized as no mutation, missense mutation, or a truncation mutation in exons 2–11 (p = 0.032), but not in exons 5–8 (p = 0.123) of the p53 gene. Further exploratory analysis revealed that detectable MASPIN was preferentially associated with a missense mutation compared with no mutation or a truncation mutation in exons 2–11 of p53 (p = 0.012). Eighty-six percent of cancers harboring a missense mutation in exons 2–11 exhibited detectable MASPIN whereas only 53% and 57% of those with wt p53 or a truncation mutation in exons 2–11 demonstrated detectable MASPIN, respectively.

The MASPIN promoter was weakly methylated in 81.8% (90/110), heavily methylated in 14.5% (16/110), and fully methylated in 3.6% (4/110) of the specimens (Fig. 3). Data regarding both MASPIN protein expression and promoter methylation were available for 66 patients and data regarding both MASPIN protein expression and p53 mutation status was available for 104 patients. Fisher’s exact testing was used to
examine the association between relative MASPIN expression categorized as non-detectable or detectable, and MASPIN promoter methylation and alterations in p53 (Table 2). There was no relationship between promoter methylation and p53 status or MASPIN protein expression in these primary ovarian cancers (p-value = 0.55 and 0.265). However, tumors harboring alterations of p53 were more likely to have detectable MASPIN protein expression (Table 3). MASPIN protein was 6 times more likely to be detected in cancer specimens that harbor a p53 mutation relative to cancer specimens with an intact wt p53 gene.

Discussion

Investigators have published evidence to suggest that MASPIN is regulated by p53 in a number of solid tumors including breast, prostate, colon, and ovarian cancers [12,17,29,30]. Our data from immortalized ovarian cell lines, although limited, are consistent with the paradigm that MASPIN is regulated via both p53-dependent and independent pathways. The ovarian cancer cell lines harboring mutant p53 genes expressed low MASPIN protein and mRNA levels and were more likely to have increased MASPIN promoter methylation. In contrast, ovarian cancer cell lines with wt p53 expressed high MASPIN protein levels and were more likely to display decreased or absent MASPIN promoter methylation.

Our finding that MASPIN promoter demethylation occurred upon treatment with 5-azaC in the OVCAR3 cell line with a p53 missense mutation but not in the SKOV3 cell line, which harbors a p53 truncating mutation indicate that the relationship between p53 and
aberrant cytosine methylation may be p53 mutation-type dependent. Furthermore, MASPIN mRNA transcription was independently reactivated through inhibition of DNMT activity or through introduction of wt p53 into SKOV3 cells. However, forced wt p53 introduction was not capable of reversing MASPIN promoter methylation in SKOV3 cells, suggesting that wt p53 may reactivates MASPIN gene transcription via alternative pathways other than promoter demethylation and that wt p53 alone cannot modify the methylation status of the promoter. It is presently unknown what specific percent change in methylation induces a specific quantitative change in transcription. Our data show that a small change in methylation, at least in the SKOV3 cell line, is associated with induction of MASPIN transcription. Whether or not the induction is due entirely to this decrease in methylation and/or to methylation changes at other regions of the MASPIN promoter, or to upstream effects, is not known at this time. Variable responses to DNMT inhibitors (DOV13 and OV432) were seen that may reflect the status of histone modifications and resulting chromatin structure and/or less efficient uptake or incorporation of the cytosine analog into the DNA. Induction of expression in the absence of promoter methylation in the case of OV432 is very likely attributable to indirect effects of the 5-azaC treatment whereby, for example, an upstream transcription factor is reactivated by the treatment, and in turn is able to activate expression of its downstream targets.

Our findings regarding the SKOV3 transfection experiments are consistent with those reported by Oshiro and Murakami [15,31] whereby reintroduction of p53 partially reactivated MASPIN genes expression. However, wt p53 did not affect the methylation status of the promoter, signifying that wt p53 itself can only partially overcome the repressive barrier of DNA methylation. p53 restoration combined with demethylation via 5-azaC synergistically restored MASPIN expression in the breast cancer cell lines to levels of expression approaching the basal levels seen in an immortalized, non-tumorigenic breast epithelial cancer cell line [15]. Similarly, our data revealed an additive effect of demethylation and forced wt p53 reintroduction on MASPIN expression in the SKOV3 ovarian cancer cells. Oshiro also found that wt p53 binding to the MASPIN promoter DNA-binding site stimulated histone acetylation and enhanced accessibility of the promoter site. Changes in histone acetylation can affect the chromatin structure, increasing accessibility for transcription and thus play an important role in gene expression [32]. This mechanism of action may explain how p52 reintroduction induces MASPIN expression despite the presence of DNA methylation. In summary, these results suggest that alterations of p53 and aberrant DNA methylation may control MASPIN gene transcription via independent temporal events but interconnected synergistic events that affect MASPIN expression.

Our findings do differ from that of Rose and colleagues who evaluated MASPIN expression and promoter methylation in normal primary ovarian cancers.

### Table 2
Relationship between MASPIN promoter methylation and MASPIN protein expression and p53 mutations.

<table>
<thead>
<tr>
<th>MASPIN protein expression</th>
<th>Level of MASPIN promoter methylation</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Un/weakly/equally methylated (0,1,2)</td>
<td>Heavily/fully methylated (3,4)</td>
</tr>
<tr>
<td>Non-detectable</td>
<td>15 (23%)</td>
<td>3 (4%)</td>
</tr>
<tr>
<td>Detectable</td>
<td>41 (62%)</td>
<td>7 (11%)</td>
</tr>
<tr>
<td>p53 mutation statusb</td>
<td>59 (57%)</td>
<td>12 (11%)</td>
</tr>
<tr>
<td>Mutated</td>
<td>25 (24%)</td>
<td>8 (8%)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>25 (24%)</td>
<td>8 (8%)</td>
</tr>
</tbody>
</table>

| a Data regarding both MASPIN protein expression and promoter methylation was available on 66 patients. |
| b Data regarding both MASPIN protein expression and p53 mutation status was available on 104 patients. |

### Table 3
Relationship between MASPIN promoter methylation, p53 mutation status or p53 immunohistochemical (IHC) expression and non-detectable MASPIN protein in primary ovarian cancers.

<table>
<thead>
<tr>
<th>MASPIN methylation</th>
<th>p53 mutation</th>
<th>p53 IHC expression</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>No</td>
<td>OR 95% CI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Un/weakly/equally (0, 1 or 2)</td>
<td>15</td>
<td>41</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heavily/fully (3 or 4)</td>
<td>3</td>
<td>7</td>
<td>0.854</td>
<td>0.195-3.736</td>
<td>0.834</td>
</tr>
<tr>
<td>p53 missense mutation in exons 2-11</td>
<td>14</td>
<td>17</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td>4</td>
<td>17</td>
<td>4.941</td>
<td>1.383-17.649</td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>9</td>
<td>32</td>
<td>2.222</td>
<td>0.753-6.558</td>
</tr>
</tbody>
</table>

Mantel–Haenszel common odds ratio (OR) estimate and asymptotic 95% confidence interval (CI).
human ovarian surface epithelium (HOSE) and three ovarian cancer cell lines; two harboring m p53 genes (A222 a silent mutation and OVCA3 a missense mutation) and a SKOV3 cell line with wt p53 gene [33]. They reported that the HOSE and A222 cell lines were MASPIN-negative and had highly methylated MASPIN promoters (60% and 88%, respectively). In contrast, the MASPIN-positive cancer cell lines, OVCA3 and SKOV3, had <0.5% and 40% MASPIN promoter methylation, respectively, which the authors considered undermethylated. The level of MASPIN promoter methylation and protein expression in the SKOV3 cell lines varied between our studies and may be attributable to the disparity in p53 status. Rose and colleagues used SKOV3 cells with wt p53 that may account for the detection of MASPIN protein if p53 was able to transcriptionally induce MASPIN despite the presence of promoter DNA methylation. In contrast, our MASPIN-negative SKOV3 cells also exhibited MASPIN promoter methylation but this was in the context of a m p53 and are consistent with the findings of Zhang et al. [17]. The diverse findings between the studies may be related to differences in methodology, cell lines, historical differences in culture conditions, and the p53 mutational status of the SKOV3 cell line.

Based on our in vitro data, we predicted that ovarian cancers with a p53 mutation would have non-detectable MASPIN protein expression whereas those with no mutation would exhibit detectable MASPIN. In contrast, 86% of the ovarian cancers with a missense mutation within exons 2–11 of p53 displayed detectable MASPIN, while 53% of cancers with no mutation exhibited detectable MASPIN. Further analysis revealed that MASPIN protein was 6 times more likely to be detected in cancer specimens that harbor a p53 mutation relative to cancer specimens with an intact wt p53 gene. In addition there was no association between MASPIN promoter methylation and p53 status or MASPIN protein expression. A lack of association between promoter methylation and protein expression can be seen if mRNA expression and protein levels are dissociated. However, our in vitro data do not support dissociated MASPIN transcription and translation. Disparity in the results obtained in cell lines compared with clinical tumor specimens has been previously reported in studies of other molecular pathways in immortalized ovarian cancer cell lines compared with primary ovarian cancers [18,24,35]. Immortalized ovarian cancer cell lines may not be representative of primary cancers with respect to regulation of growth and angiogenesis. Regardless our results in the tumor specimens do not support that methylation silencing of the MASPIN promoter is controlled by p53 or regulated MASPIN expression. Our results may have been limited by the small number of specimens evaluated.

Others have reported that MASPIN may be regulated via p53-independent mechanisms such as E-twenty six (ETS) and activator protein-1 (AP-1) transcription site activation, hormonal responsive element site repression [9,10], and by the p63 pathway [16]. Our data indicate that the regulation of MASPIN is a complex multifactorial process, that may be controlled by both epigenetic and genetic mechanisms.

Conflict of interest
The co-authors have no conflicts of interest to declare.

References