Hypermethylation of let-7a-3 in Epithelial Ovarian Cancer Is Associated with Low Insulin-like Growth Factor-II Expression and Favorable Prognosis

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Abstract
MicroRNAs (miRNA) are endogenous noncoding small RNAs that regulate the activity of mRNAs. Many miRNA genes, including let-7a-3, are located in CpG islands, suggesting possible epigenetic regulation of their expression. Promoter CpG island methylation of tumor suppressor genes is involved in cancer development and progression. Using real-time methylation-specific PCR and real-time reverse transcription-PCR, we analyzed DNA methylation in the let-7a-3 gene and miRNA expression of let-7a in 214 patients with epithelial ovarian cancer to assess the effect of let-7a-3 methylation on the expressions of let-7a as well as a possible target of let-7 regulation, insulin-like growth factor-II (IGF-II). The association of let-7a-3 methylation with patient survival outcomes was also evaluated. let-7a-3 methylation was detected in epithelial ovarian cancer, and the expression of let-7a was slightly affected by the methylation, but the effect was not substantial. The methylation of let-7a-3, however, was inversely correlated with IGF-II expression and positively with insulin-like growth factor binding protein-3 (IGFBP-3) expression. Patients with methylated let-7a-3 seemed to have reduced risk for death compared with those without, and the association was independent of patient age at surgery, tumor grade, disease stage, and IGF-II or IGFBP-3 expression. No association was found for let-7a-3 methylation and disease progression. These results suggest that the let-7a-3 gene is methylated and the methylation may affect IGF-II expression and the survival of ovarian cancer patients. Further investigation of the role of miRNAs and their regulation in cancer is warranted.

Introduction
MicroRNAs (miRNA) are a group of noncoding small RNAs that inhibit gene activities by inducing degradation or repressing translation of mRNAs when the nucleotide sequences of miRNAs are entirely or partially complementary to the 3’-untranslated regions (UTR) of targeted mRNAs (1, 2). It has become clear that miRNAs play pivotal roles in regulating cell activities, including proliferation, differentiation, and apoptosis, and the expression of miRNAs is tightly controlled in a spatial- and temporal-specific manner (1, 2). Aberrant expression of miRNAs has been associated with cancer development and progression. Studies have shown overexpression of pre-miR-155 in Burkitt’s lymphomas and of miR-21 in breast cancer (3, 4), whereas down-regulation of miR-15/16 is found in chronic lymphocytic leukemia (5). Moreover, reduced expression of miRNA let-7 has been linked to poor prognosis of lung cancer (6).

miRNA let-7 is considered a tumor suppressor in lung cancer as it inhibits the translation of RAS oncogene (7). let-7a-3 is a member of the let-7 family and overexpression of let-7a-3 deregulates the expression of ~200 genes involved in cell adhesion, proliferation, and differentiation (8). The mechanisms underlying the regulation of miRNA expression remain largely unknown. The let-7a-3 gene, located on chromosome 22q12.31, is embedded in a CpG island (9). As part of epigenetic regulation, cytosine methylation in CpG islands may inhibit gene expression, and the inhibition, if deregulated, may disrupt normal gene activity causing pathogenic changes that include tumorigenesis (10). Recent research suggests that DNA methylation is also involved in the regulation of miRNA expression, and the methylation can be reversed by DNA methyltransferase inhibitors (8, 11, 12). Moreover, let-7a-3 is found to be less methylated in malignant than in normal lung tissues, and increased expression of let-7a-3 is associated with malignant features of tumor cells (8). However, it remains unclear if let-7a-3 methylation is also present in other forms of cancer and if the methylation is related to tumor progression and patient survival. In a clinical study of epithelial ovarian cancer, we analyzed the methylation of let-7a-3 in tumor tissues using a real-time methylation-specific PCR and evaluated the effect of methylation on let-7a expression, insulin-like growth factor-II (IGF-II), a potential target of let-7a regulation, and patient survival outcomes.

Materials and Methods
Tumor samples and patients. The study analyzed let-7a-3 methylation and let-7a expression in fresh tumor samples of 214 patients who underwent surgery for ovarian cancer in the Department of Gynecology and Obstetrics at the University of Turin in Italy. The specimens were collected for a clinical study of epithelial ovarian cancer that was approved by the university’s ethical review committee. Of the 214 patients who consented to participate, 53 were diagnosed with stage I disease, 12 with stage II, 134 with stage III, and 15 with stage IV. Disease stage and tumor grade were classified according to the International Federation of Gynecology and Obstetrics and WHO criteria, respectively. Tumor grades 1 to 3 were found in 34, 40, and 140 women, respectively. The average age of patient at surgery was 57.7 years (range, 26–82). The most common histology was serous papillary (40.2%), the remaining were endometrioid (19.6%), undifferentiated (17.3%), mucinous (8.4%), clear cell (7.5%), and other types (3.8%).

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mullerian (6.5%), and other (0.5%). Most of the patients received standard postoperative chemotherapy after cytoreduction surgery and were followed through June 2001. The median follow-up time was 31 months, ranging from 0.6 to 114 months.

**Analysis of DNA methylation.** Genomic DNA was extracted from the tumor samples that had been confirmed by pathologists to contain 80% to 90% of tumor cells. The DNA samples were treated with sodium bisulfite using the EZ DNA Methylation-Gold kit (ZymoResearch). DNA methylation of let-7a-3 was analyzed using a SYBR Green-based quantitative methylation-specific PCR (qMSP). Two sets of PCR primers were designed: one for unmethylated and one for methylated DNA sequences. The unmethylated primers are 5'-GAGGAGATTGGTAAAGTGG (forward) and 5'-AAACATTACAAATACCCACCCTACTCA (reverse), where the bold letters indicate bisulfite conversion of C to T. The methylated primers are 5'-GAAGGTAGTTGGTAAAGTGG (forward) and 5'-CATAAAGATACCCACCTACTG (reverse). In the PCR (20 µL), 0.5 µL of bisulfite-treated DNA template was mixed with 10 µL of 2× Power SYBR Green PCR Master Mix (Applied Biosystems) and a pair of primers in a final concentration of 200 nmol/L. The PCR conditions included initial incubation at 50°C for 2 min, denaturing at 95°C for 10 min, and 40 cycles of denaturing at 95°C for 15 s and annealing at 60°C for 1 min. After PCR amplification, a dissociation curve was generated to confirm the size of PCR product. Examples of qMSP amplification and dissociation curves are shown in Fig. 1A and B. To verify the sequence of PCR products, representative methylated and unmethylated PCR products were also purified and sequenced. Sequencing results are shown in Fig. 1C and D. Each sample was tested in duplicate for methylation.

**Analysis of miRNA let-7a and expressions of IGF-II and insulin-like growth factor binding protein-3.** Total RNA was extracted from the same tumor samples; the methods for RNA extraction and analysis of IGF-II and insulin-like growth factor binding protein-3 (IGFBP-3) expression were described elsewhere (13). The miRNA cDNA was prepared using the Taqman miRNA Reverse Transcription kit (Applied Biosystems). In the reaction, 100 ng of RNA sample were mixed with 0.1 µL of 100 nmol/L deoxynucleotide triphosphate, 1 µL of 10× reverse transcriptase buffer, 5 units of RNase inhibitor, 100 units of MultiScribe reverse transcriptase, and 1 µL of 2.5× multiplex stem-loop reverse primers for let-7a and RNU48. RNU48 was used as an internal control for overall miRNA expression in a sample. The cDNA samples were stored at −20°C until analysis. The expression of let-7a was analyzed by the Taqman miRNA assay (Applied Biosystems) using the Chromo4 Real-time PCR System (MJ Research, Inc.). In the PCR (15 µL), 0.3 µL of cDNA template was mixed with 7.5 µL of 2× Taqman Universal PCR Master Mix (Applied Biosystems), 0.75 µL of 20× probe/primer of either let-7a or RNU48, and water. The PCR amplification included initial incubation at 50°C for 2 min, denaturing at 95°C for 10 min, and 40 cycles of denaturing at 95°C for 15 s and annealing at 60°C for 1 min. Each sample was analyzed in duplicate, and the analysis was repeated for those with coefficient of variation of >5%.
Statistical analysis. An expression index (EI) was calculated for let-7a expression as follows, \(1,000 / C_{2}^{2} \times C_{0} - C_{t}\), where \(D_{C_{t}} = C_{t \text{let}-7a} / C_{0} - C_{t \text{RNU48}}\). The percentage of let-7a-3 methylation in a sample was estimated using the following formula:

\[
\text{methylated let-7a-3} \times 100\% = \left( \frac{M}{M + U} \right) \times 100\% = \frac{1}{1 + 2^{(-\Delta Ct)}} \times 100\%
\]

where \(M\) is the copy number of methylated let-7a-3, \(U\) is the copy number of unmethylated let-7a-3, and \(\Delta Ct = Ct_{\text{let}-7a} - Ct_{\text{RNU48}}\). Associations of let-7a-3 methylation with the expressions of let-7a, IGF-II, and IGFBP-3 were analyzed using the Wilcoxon rank sum test. Survival analysis was done to assess the association of let-7a-3 methylation with the risk of disease progression and death using the Cox proportional hazards regression and Kaplan-Meier survival curves. All statistical analyses were done using Statistical Analysis System version 9.1 (SAS Institute).

Results and Discussion

Recent studies have suggested that some miRNA genes are methylated, and the methylation affects miRNA expression (8, 11, 12). Using a bisulfite sequencing approach to analyze the methylation status of 33 CpG sites in the let-7a-3 gene, Brueckner et al. (8) found 90% of the CpG sites being methylated in a human colon cancer cell line, and the gene could be demethylated when the DNA methyltransferase genes were knocked out (DNMT1 and DNMT3B). Furthermore, using the combined bisulfite restriction analysis, the investigators discovered that the let-7a-3 gene was heavily methylated in normal human placenta, brain, bone marrow, colon, skin, and lung tissues but unmethylated in some lung cancer samples (8). To further evaluate DNA methylation and miRNA expression in cancer, we analyzed the methylation status of let-7a-3 in epithelial ovarian cancer. Focusing on the same DNA region as described by Brueckner et al., we developed a qMSP. Using this qMSP, we quantified let-7a-3 methylation in 214 malignant tumor

Table 1. Methylation of let-7a-3 and expressions of let-7a, IGF-II, and IGFBP-3 in epithelial ovarian cancer

<table>
<thead>
<tr>
<th>Variables</th>
<th>Low let-7a-3 methylation (≤42%)</th>
<th>High let-7a-3 methylation (≥90%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Median (5th-95th)</td>
<td>n</td>
</tr>
<tr>
<td>let-7a miRNA</td>
<td>68</td>
<td>4.9 (0.5–42.0)</td>
<td>13</td>
</tr>
<tr>
<td>IGF-II peptide</td>
<td>67</td>
<td>105.6 (23.9–385.4)</td>
<td>13</td>
</tr>
<tr>
<td>IGF-II mRNA</td>
<td>63</td>
<td>20.9 (0–7,637)</td>
<td>13</td>
</tr>
<tr>
<td>IGF-II-P3 mRNA</td>
<td>61</td>
<td>500 (0–42,127)</td>
<td>12</td>
</tr>
<tr>
<td>IGF-II-P4 mRNA</td>
<td>61</td>
<td>100 (1.7–2,051)</td>
<td>12</td>
</tr>
<tr>
<td>IGFBP-3 mRNA</td>
<td>63</td>
<td>57.7 (0–835)</td>
<td>13</td>
</tr>
</tbody>
</table>
found that cells treated with demethylating agent DAC had comparable with the experimental results of Brueckner et al. who inlet-7a expression between low and high methylation groups was high methylation, 4.9 EI versus 3.3 EI (Table 1). Although the high expression in patients with low let-7a-3 methylation than in those with high methylation, 4.9 EI versus 3.3 EI (Table 1). Although the difference was not statistically significant, the 1.5-fold discrepancy in let-7a expression between low and high methylation groups was comparable with the experimental results of Brueckner et al. who found that cells treated with demethylating agent DAC had ~1.3-fold up-regulation of let-7a expression (8). Adding histone deacetylase inhibitor valproic acid to DAC treatment could further increase the expression of let-7a (8), suggesting that histone modification also plays a role in silencing let-7a-3 expression; methylation is not the only mechanism regulating the expression of let-7a-3. Our results suggest that let-7a-3 methylation may result in a small reduction in let-7a expression, but this change is not substantial. Levels of let-7a miRNA reflect the expression of three mature miRNAs: let-7a-1, let-7a-2, and let-7a-3. Specifically measuring let-7a-3 miRNA in tissue samples may allow direct assessment of the effect of methylation on expression. Our analysis of let-7a expression, instead of let-7a-3, may explain in part our incapability of detecting a significant association between let-7a-3 methylation and expression.

A tissue sample normally contains several types of cell, and each cell type may regulate let-7a expression with a distinct mechanism in which DNA methylation may or may not be involved (14–16). Given the cellular complexity of a tissue sample and regulatory diversity of gene expression, a difference in let-7a expression between methylated and unmethylated cells may be readily detectable in specific cells but not in tissue samples. Because miRNAs regulate the translation of proteins or peptides by degrading or repressing their mRNAs, assessing the expression of downstream molecules regulated by let-7a may help evaluation of let-7a activity. To test this possibility, we analyzed let-7a-3 methylation in relation to a growth factor that could be indirectly regulated by let-7a. Predicted by a computer software TargetScan, IGF-II mRNA binding proteins (IMP-1 and IMP-2) are potential targets of let-7. IMPs are known to bind specifically to the 5'-UTR of IGF-II mRNA, blocking its translation. Knocking down IMP-1 expression could significantly increase the expression of IGF-II (17). IGF-II expressions, measured by IGF-II mRNA and peptide, as well as two IGF-II promoter-specific transcripts (IGF-II-P3 and IGF-II-P4), have been analyzed in these tumor samples in our previous studies (13, 18) along with the mRNA expression of a specific IGF binding protein IGFBP-3. Using the existing data, we examined the effect of let-7a-3 methylation on IGF-II activity. As expected, the results showed substantial differences in IGF-II expression between patients with high and low let-7a-3 methylation. Levels of IGF-II peptide, mRNA, and IGF-II-P3 and IGF-II-P4 transcripts were all significantly higher in low than in high methylation groups (Table 1): 105.6 versus 63.8 for IGF-II peptide (P = 0.008), 20.9 versus 4.0 for IGF-II mRNA (P = 0.004), 500.0 versus 76.7 for IGF-II-P3 (P = 0.040), and 100.0 versus 10.5 for IGF-II-P4 (P = 0.008). IGFBP-3 was not significantly different between the two groups, but the low methylation group did have lower expression than the high methylation group, 5.77 versus 124.8 (P = 0.143). It is unclear if let-7a-3 regulates the expression of IGFBP-3, but this miRNA down-regulates transforming growth factor-β2, which may indirectly affect IGFBP-3 expression (8, 19). IGF-II expression may also influence the expression of IGFBP-3.

To assess if there is a dose-response relationship between let-7a-3 methylation and IGF-II expression, we further analyzed the data in all the samples after grouping the methylation levels into three

![Figure 2. Kaplan-Meier overall survival curves by levels of let-7a-3 methylation.](image-url)
categories, low, medium, and high, based on the distribution of methylation at <33%, 33% to 66%, and ≥67% (Table 2). Again, IGF-II expressions, including IGF-II peptide, mRNA, and two promoter-specific transcripts, were significantly correlated with methylation; with increasing levels of methylation, IGF-II expressions were substantially declined at both mRNA and peptide levels (P < 0.05). Although changes in IGFBP-3 with let-7a-3 methylation were not significant (P = 0.265) in the categorical analysis, the Spearman correlation coefficient was significant (r = 0.14, P = 0.039), suggesting a weak positive correlation between let-7a-3 methylation and IGFBP-3 expression. These results show a close correlation between epigenetic regulation of let-7a-3 and IGF-II expression in epithelial ovarian cancer.

In our previous studies, IGF-II expression was found to be an unfavorable indicator of ovarian cancer prognosis (13, 18). High expressions of IGF-II mRNA, especially those from P3 and P4, which were considered fetal promoters, were significantly associated with increased risk of disease progression and death. To evaluate the biological effect of miRNA methylation on ovarian cancer progression, we analyzed the association of let-7a-3 methylation with ovarian cancer survival. Kaplan-Meier survival analysis was done first, and the analysis showed that patients with low let-7a-3 methylation had worse overall survival than those with high methylation (P = 0.048; Fig. 2). However, disease progression-free survival was not affected by let-7a-3 methylation (P = 0.271).

To confirm the Kaplan-Meier analysis and to adjust for confounding factors, we further analyzed the data using the Cox proportional hazards regression. The analysis showed that high let-7a-3 methylation was significantly associated with reduced risk of death, confirming the finding of Kaplan-Meier analysis (Fig. 2). Moreover, the regression analysis indicated that the association was independent of clinical and pathologic features of the disease, including patient age at surgery, disease stage, tumor grade, and expressions of IGF-II and IGFBP-3 (Table 3). Patients with high let-7a-3 methylation had >40% reduction in risk of death compared with those with low methylation [hazard ratio (HR), 0.58; 95% confidence interval (95% CI), 0.36–0.94]. Again, no association was found between disease progression and let-7a-3 methylation (Table 3).

The study suggests that miRNA let-7a-3 may behave in favor of tumor progression and the effect may be mediated in part through the IGF system (i.e., IGF-II). These findings agree with the observations of Brueckner et al. (8) who found that let-7a-3 overexpression increased the malignancy of A549, an adenocarcinoma cell line from lung. These observations, however, seem to be inconsistent with those that lung cancer patients with reduced let-7 expression had a poor prognosis and that let-7 behaved as a tumor suppressor by inhibiting the activity of oncogene Ras in lung cancer (6, 20). Given the fact that let-7a-3 regulates the activities of ~200 genes (8), the inconsistent findings show the complexity of let-7 functions and underscore the importance of studying miRNA on a tissue-specific basis.

In summary, we showed that let-7a-3 was methylated in epithelial ovarian cancer and that low let-7a-3 methylation was associated with high IGF-II and low IGFBP-3 expressions as well as poor prognosis of epithelial ovarian cancer. These findings suggest that epigenetic regulation of let-7a-3 may affect tumor progression probably through its regulatory influence on downstream molecules. Further research of miRNA methylation and expression in cancer is warranted.

### References

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