

# 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. [Cancer Res 2007;67(21):10117–22]

## 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).

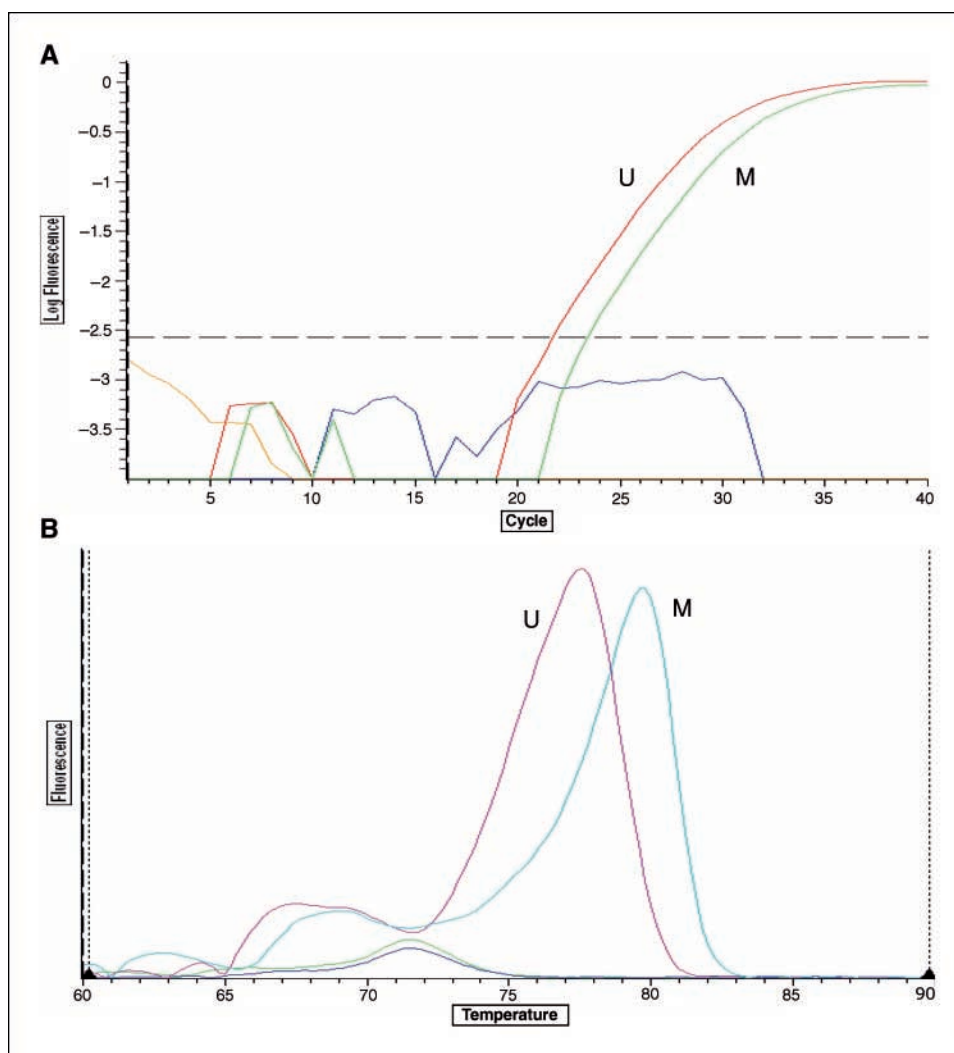
miRNA *let-7* is considered a tumor suppressor in lung cancer as it inhibits the translation of RAS oncogene (7). *let-7a-3* is a member of the *let-7* family and overexpression of *let-7a-3* deregulates the expression of ~200 genes involved in cell adhesion, proliferation, and differentiation (8). The 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%), and the remaining were endometrioid (19.6%), undifferentiated (17.3%), mucinous (8.4%), clear cell (7.5%),

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**Figure 1.** Results of qMSP products and bisulfite sequences for one unmethylated and one methylated sample. *A*, representative qMSP amplification curves for methylated (*M*) and unmethylated (*U*) let-7a-3. *B*, representative qMSP dissociation curves for methylated and unmethylated let-7a-3.

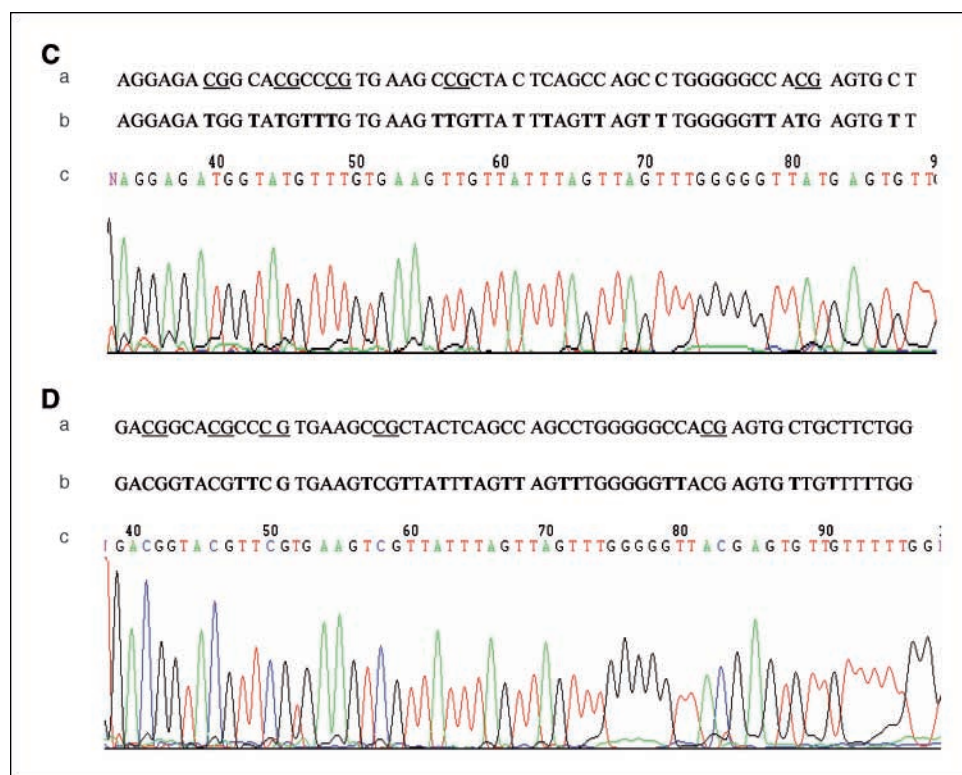
mullerian (6.5%), and other (0.5%). Most of the patients received standard postoperative chemotherapy after cytoreduction surgery and were followed through June 2001. The median follow-up time was 31 months, ranging from 0.6 to 114 months.

**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 (Zymo Research). 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'-GAGGAGATGGTATGTTTGTGAAGTTG (forward) and 5'-AAACATACAAATACCCACCCTACTCA (reverse), where the bold letters indicate bisulfite conversion of C to T. The methylated primers are 5'-GACGGTACGTTTCGTGAAGTCG (forward) and 5'-CATACGAATACCCACCCTACTCG (reverse). In the PCR (20  $\mu$ L), 0.5  $\mu$ L of bisulfite-treated DNA template was mixed with 10  $\mu$ L of 2 $\times$  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. 1*A* 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. 1*C* 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  $\mu$ L of 100 nmol/L deoxynucleotide triphosphate, 1  $\mu$ L of 10 $\times$  reverse transcriptase buffer, 5 units of RNase inhibitor, 100 units of MultiScribe reverse transcriptase, and 1  $\mu$ L of 2.5 $\times$  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 mixtures were incubated at 16°C for 30 min followed by a cycle of 42°C for 30 min and 85°C for 5 min. 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  $\mu$ L), 0.3  $\mu$ L of cDNA template was mixed with 7.5  $\mu$ L of 2 $\times$  Taqman Universal PCR Master Mix (Applied Biosystems), 0.75  $\mu$ L of 20 $\times$  probe/primers 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%.

**Figure 1 Continued.** C, bisulfite DNA sequencing results for unmethylated *let-7a-3* qMSP product (5'-3'). a, original sense strand before sodium bisulfite treatment (CpG sites are underlined); b, bisulfite modified sense (unmethylated) strand (bisulfite modified T from C in bold); c, unmethylated *let-7a-3* qMSP product. D, bisulfite DNA sequencing results for methylated *let-7a-3* qMSP product (5'-3'). a, original sense strand before sodium bisulfite treatment (CpG sites are underlined); b, bisulfite modified sense (methylated) strand (bisulfite modified T from C in bold); c, methylated *let-7a-3* qMSP product.



**Statistical analysis.** An expression index (EI) was calculated for *let-7a* expression as follows,  $1,000 \times 2^{(-\Delta Ct)}$ , where  $\Delta Ct = Ct_{let-7} - Ct_{RNU48}$ . The percentage of *let-7a-3* methylation in a sample was estimated using the following formula:

$$\begin{aligned} \text{methylated } let-7a-3 (\%) &= \frac{M}{M+U} \times 100\% = \frac{1}{1+\frac{U}{M}} \times 100\% \\ &= \frac{1}{1+2^{(-\Delta Ct)}} \times 100\% \end{aligned}$$

where  $M$  is the copy number of methylated *let-7a-3*,  $U$  is the copy number of unmethylated *let-7a-3*, and  $\Delta Ct = Ct_U - Ct_M$ . 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

Variables	Low <i>let-7a-3</i> methylation ( $\leq 42\%$ )		High <i>let-7a-3</i> methylation ( $\geq 90\%$ )		P
	n	Median (5th-95th)	n	Median (5th-95th)	
<i>let-7a</i> miRNA	68	4.9 (0.5-42.0)	13	3.3 (0.5-28.4)	0.763
IGF-II peptide	67	105.6 (23.9-385.4)	13	63.8 (0-127.8)	0.008
IGF-II mRNA	63	20.9 (0-7,637)	13	4.0 (0-68.8)	0.064
IGF-II-P3 mRNA	61	500 (0-42,127)	12	76.7 (2.4-1,170)	0.040
IGF-II-P4 mRNA	61	100 (1.7-2,051)	12	10.5 (0.1-922)	0.008
IGFBP-3 mRNA	63	57.7 (0-835)	13	124.8 (12.2-1,475)	0.143

**Table 2.** Methylation levels of *let-7a-3* and expressions of *let-7a*, IGF-II, and IGFBP-3 in epithelial ovarian cancer

Variables	Low <i>let-7a-3</i> methylation		Medium <i>let-7a-3</i> methylation		High <i>let-7a-3</i> methylation		P
	n	Median (5th-95th)	n	Median (5th-95th)	n	Median (5th-95th)	
<i>let-7a</i> miRNA	51	5.2 (0.5-42.0)	93	4.6 (0.6-49.0)	70	4.5 (0.5-28.4)	0.728
IGF-II peptide	50	106.2 (33.2-434.7)	92	103.8 (34.4-519.7)	70	81.3 (23.4-336.3)	0.020
IGF-II mRNA	47	20.9 (0-9,503)	90	25.9 (0-3,008)	68	3.9 (0-1,112)	0.003
IGF-II -P3 mRNA	45	500 (0-51,387)	88	262.4 (1.7-31,926)	67	85.4 (0-3,767)	0.011
IGF-II -P4 mRNA	45	116.4 (1.7-2,276)	88	41.2 (0.8-2,075)	67	15.4 (0.1-1,551)	0.008
IGFBP-3 mRNA	47	62.3 (0-1,098)	90	101.8 (3.9-5,916)	68	105.0 (10.7-1,491)	0.265

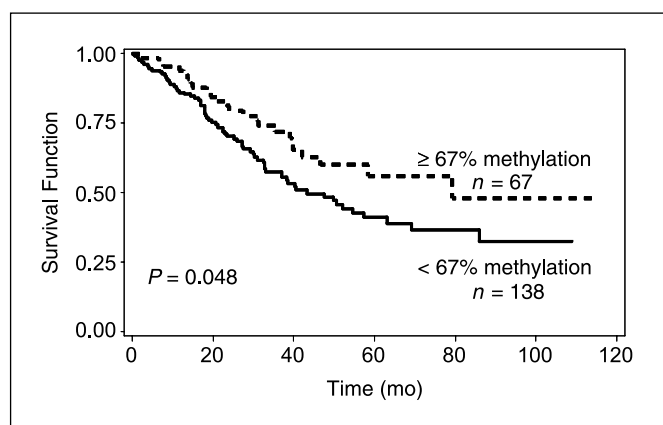
samples. The PCR analysis was verified by DNA sequencing. The results indicated that *let-7a-3* methylation was detectable in epithelial ovarian cancer and the average level of methylation was 56.3%, ranging from 9.1% to 91.2%. There were no associations between *let-7a-3* methylation and clinicopathologic features of ovarian cancer, including disease stage, tumor grade, and patient age at surgery (data not shown).

*In vitro* experiments have found that DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (DAC) could substantially reduce *let-7a-3* methylation from 92% to 42% (8). To assess if these levels of methylation had any effect on expression, we compared *let-7a* expression between patients who had methylation either <42% or >90%. The comparison showed that *let-7a* expression was slightly higher 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-3* expression (8). Adding histone deacetylase inhibitor valproic acid to DAC treatment could further increase the expression of *let-7a-3* (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 ( $P = 0.064$ ), 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, 57.7 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- $\beta$ 2, 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.



**Table 3.** Associations of *let-7a-3* methylation and patient survival

Methylation level	Progression, HR (95% CI)	Death, HR (95% CI)
Unadjusted analysis		
<67%	1.00	1.00
≥67%	0.78 (0.51–1.21)	0.63 (0.39–1.00)
Adjusted analysis*		
<67%	1.00	1.00
≥67%	0.76 (0.48–1.19)	0.58 (0.36–0.94)

\*Adjusted for age at surgery, disease stage, tumor grade, IGF-II, and IGFBP-3.

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 and medium levels of methylation did not differ substantially in progression-free or overall survival but did fare worse than those with high levels of *let-7a-3* methylation (data not shown). Based on this finding, we combined the low and medium groups together as methylation <67% and compared their survival outcomes with those having methylation ≥67%. The comparison 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.

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