Functional study of risk loci of stem cell-associated gene lin-28B and associations with disease survival outcomes in epithelial ovarian cancer

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Several single-nucleotide polymorphisms (SNPs) of the stem cell-associated gene lin-28B have been identified in association with ovarian cancer and ovarian cancer-related risk factors. However, whether these SNPs are functional or might be potential biomarkers for ovarian cancer prognosis remains unknown. The purposes of this study were to investigate the functional relevance of the identified lin-28B SNPs, as well as the associations of genotype and phenotype with epithelial ovarian cancer (EOC) survival. We analyzed five SNPs and mRNA levels of lin-28B in 211 primary EOC tissues using Taqman® SNP genotyping assays and SYBR green-based real-time PCR, respectively. The RNA secondary structures at the region of a genome-wide association-identified intrinsic rs314276 were analyzed theoretically with mfold and experimentally with circular dichroism spectroscopy. We found that rs314276 was a cis-acting expression quantitative trait locus (eQTL) in both additive and dominant models, while rs7759938 and rs314277 were significant or of borderline significance in dominant models only. The rs314276 variant significantly affects RNA secondary structure. No SNPs alone were associated with patient survival. However, we found that among patients initially responding to chemotherapy, those with higher lin-28B expression had higher mortality risk (hazard ratio = 3.27, 95% confidence interval: 1.63–6.56) and relapse risk (hazard ratio = 2.53, 95% confidence interval: 1.41–4.54) than those with lower expression, and these associations remained in multivariate analyses. These results suggest that rs314276 alters RNA secondary structure and thereby influences gene expression, and that lin-28B is a cancer stem cell-associated marker, which may be a pharmaceutical target in the management of EOC.

Abbreviations: EOC, epithelial ovarian cancer; eQTL, expression quantitative trait locus; SNP, single-nucleotide polymorphism.

Introduction

Several common single nucleotide polymorphisms (SNPs) in the gene lin-28B have been identified in association with the risk of ovarian cancer as well as its risk factors such as age at menarche, body mass index and height (1–7). Of these lin-28B variants, two (rs314276 and rs314277) are intronic polymorphisms, while two others (rs7759938 and rs12194974) are located in the promoter region of lin-28B. Thus far, only two studies have conducted functional assessment of these SNPs. One study performed expression quantitative trait loci (eQTL) analyses with publicly available data from lymphoblastoid cell lines of 400 children, and did not find associations between lin-28 expression levels and the SNPs (3). Another study showed that the SNP rs12194974 was functional by constructing lin-28B G>A reporters to measure promoter activities of wild-type and variant constructs in eight cell lines (1). It would be useful to investigate whether these SNPs are functional and have clinical implications in ovarian cancer prognosis.

Lin-28B is a homolog of RNA-binding protein lin-28 (8). Both of these proteins are able to reprogram somatic cells into stem cells, and they are considered stem cell-associated genes (9–11). Lin-28B was first characterized in human liver cancer, containing RNA-binding domains of a cold-shock domain and two retroviral-type CCHC zinc finger domains (8,12). Gain- and loss-of function experiments show that lin-28B acts as an oncogene, playing important roles in regulating cell proliferation and apoptosis (8). Like lin-28, lin-28B represses miRNA let-7 maturation, thereby activating many oncoproteins including k-ras, c-myc, HMGA2, cyclin D1 and IGF-II (13–16). Animal models suggest that cancer growth depends on lin-28B protein; loss of lin-28B significantly inhibits Myc-dependent cell proliferation (17,18). Abnormal alteration of lin-28B is very common in human cancer, and activation of lin-28B has been observed in subsets of tumors that were poorly differentiated or that had the worst prognosis (13,19,20). Our previous study showed that patients with high lin-28 levels had an aggressive disease and increased risks of prognosis in epithelial ovarian cancer (EOC) (14). Thus, the purposes of this study were to: (i) perform eQTL analyses for the selected SNPs of lin-28B in a clinical cohort study on EOC; (ii) investigate the effects of the variant rs314276 on the RNA secondary structure; (iii) examine the associations of the lin-28B genotypes with disease outcomes and (iv) investigate whether lin-28B is a potential cancer stem cell marker by evaluating the associations lin-28B mRNA with disease outcomes in patients who initially responded to chemotherapy.

Materials and methods

Study patients

A clinical study, which was approved by the university’s human subjects review committee, consecutively enrolled 211 patients who underwent surgery for primary EOC in the Gynecologic Oncology Unit at the University of Turin, Italy, between October 1991 and February 2000. Fresh tumor samples were collected from the patients during surgery. The specimens were snap-frozen in liquid nitrogen immediately after resection, and then transferred to a −80°C freezer for storage until analysis. Clinical and pathology information on these patients was abstracted from the medical charts and pathology reports. The average age of patients at surgery was 57.9 years (range: 26–82). Of the 211 patients, 34 (16.1%) had Grade 1 tumors, 40 (19.0%) Grade 2, and 137 (64.9%) Grade 3. Based on the criteria of the International Federation of Gynecology and Obstetrics Classification (21), Disease stages I–IV were found in 52 (24.6%), 12 (5.7%), 133 (63.0%) and 14 (6.6%) patients, respectively. The tumor histology was papillary serous in 40.3% of cases, followed by endometrioid (19.4%), undifferentiated (17.1%), mucinous (8.5%), clear cell (7.6%), mixed mullerian (6.6%) and other epithelial (0.5%) classified according to World Health Organization guidelines (22). After cytoreductive surgery, patients were followed through June 2001. Median follow-up time was 31 months (range: 0.6–114 months). At the end of the study follow-up, 92 patients died, and 95 had a progressive disease.

Of the 211 patients, 178 received standard post-operative platinum-based chemotherapy, one had chemotherapy without platinum, and 32 did not receive chemotherapy due to early stage disease, progressive tumor, advanced age or severe comorbidities. Each patient was evaluated for treatment response, classified into four categories based on the clinical criteria as described previously elsewhere (23). Briefly, (i) complete response, indicating resolution of all evidence of disease for at least 1 month, (ii) partial response, a decrease of ≥50% or an increase of ≥25% in the product of the diameters of all measurable lesions or the development of new lesions for at least 1 month, (iii) stable disease, a decrease of <50% or an increase of <25% in the product of the diameters of all measurable lesions for at least 1 month, (iv) progressive disease, an increase of ≥50% in the product of the diameters of all radiologically measurable lesions or the development of new lesions. Of the 176 patients with information on treatment response, the 128 (72.7%) with complete responses were considered responders in the data analysis, while the 48 (27.3%) in the other three categories were grouped as non-responders.

Study patients

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Genomic DNA extraction, lin-28B SNP selection and genotyping

Fresh frozen tumor specimens were pulverized manually in liquid nitrogen. Genomic DNA was extracted from approximately 100 mg of tissue powder following a standard phenol–chloroform protocol, and the quality and quantity of the extracted DNA samples was determined by using a spectrophotometer.

Four of the five SNPs from the lin-28B gene were the identified risk loci of either ovarian cancer (rs12194974) or the ovarian cancer-related factor age at menarche (rs7759938, rs314277 and rs314276), and the other one (rs17065417) is located in the predicted binding site of transcription factors in the promoter region of lin-28B. Genotyping of the selected SNPs was performed using Taqman® SNP genotyping assays (Applied Biosystems, Foster City, CA) following the manufacturer’s instructions. Ten percent of samples were run in duplicate for quality control; the concordance was 100%.

Analysis of lin-28B mRNA levels

Total RNA was extracted from the tumor samples by conventional methods; complementary DNA was prepared using cloned AMV First-Strand complementary DNA synthesis kits (Invitrogen, Carlsbad, CA). The SYBR green-based real-time PCR was performed to determine the levels of lin-28B mRNA using the Chromo4™ Real-time PCR System (MJ Research, Waltham, MA). The sequences of primers were as described previously (14). In a volume of 20 μl PCR reaction, 1 μl of complementary DNA template was mixed with 10 μl of 2× Power SYBR® PCR master mix (Applied Biosystems), 200 nM of paired primers of either target gene or RNU48 as an internal control, and dis-tilled water. PCR amplification included initial incubation at 50°C for 2 min, annealing at 60°C for 1 min. Melting curves were analyzed after each run.

SNP upstream and downstream DNA sequences (approximately 500 bp each) were retrieved based on the accession number in the SNP genebank (build 132, http://www.ncbi.nlm.nih.gov/snp/). RNA secondary structures of the retrieved sequences with either the wild-type or variant SNP were predicted using the internet-based computer-modeling program mfold (24), we found that the variation of the SNP might affect the RNA secondary structure. Using the CD spectroscopy analysis, the differences in secondary structure folding were compared. The graphs of the CD spectra show ellipticity (Θ) as a function of the wavelength (λ). The intensity (ellipticity, which is proportional to the difference between absorbance of left circularly polarized and right circular polarized light) of peaks reflects thermodynamic stability for a structure; the larger the absolute value of a maximum peak, the more stable the structure is likely to be. However, CA had a lower but not statistically significant expression than those with either CA or AA (P = 0.097), respectively. When the genotypes were treated as categorical variables of three categories, patients with the rs314276 CC genotype had significantly higher lin-28B levels among the three genotypes (P = 0.009). No significant differences in lin-28B expression were found among different genotypes.

Statistical analysis

Expression index was calculated for lin-28B using the formula: 10^x × 2^−ΔCt, where ΔCt = C_{lin-28B}−C_{RNU48}. The Wilcoxon rank-sum test was performed to analyze eQTL. In the spearman correlation analyses, genotypes of each SNP were coded in an additive model as 0, 1 and 2, respectively, based on the number of minor alleles. The χ² or Fisher’s exact test was used to determine the associations of lin-28B SNPs with clinical and pathologic variables. Survival analysis was performed to assess the associations of lin-28B phenotype and genotypes with the risk of disease progression and death using the Cox proportional hazards regression model and Kaplan–Meier survival curves, in which the samples were classified into three groups (low, medium and high) based on the levels of lin-28B expression; the samples with undetectable expression of lin-28B were classified as low, while those with detectable expression were divided into the medium and high categories using the median as the cutoff. All statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC).

Results

Lin-28B genotype and cis-acting eQTL analysis in EOC tissues

The results of genotype distributions and cis-acting eQTL of each SNP are shown in Table I. For one patient (0.5%), the genotype of rs314277 was undetermined. In an additive model, when we coded the genotypes as numerical variables, the Spearman correlation analysis showed that an inverse correlation was observed between the number of minor alleles of rs314276 and lin-28B mRNA levels; the Spearman correlation coefficient was −0.16 (P = 0.024) (Table I). No associations were found between the number of minor alleles of either rs17065417 or rs12194974 and lin-28B expression. SNP rs314277 and rs7759938 were borderline significant; the Spearman correlation coefficient was −0.13 (P = 0.060) and −0.12 (P = 0.071), respectively. When the genotypes were treated as categorical variables of three categories, patients with the rs314276 CC genotype had significantly higher lin-28B levels than those with either CA or AA (P = 0.029). However, CA had a lower but not statistically significant expression level as compared with AA using Bonferroni adjustment test (data not shown). For rs7759938, there were borderline significantly different lin-28B levels among the three genotypes (P = 0.069). No significant differences in lin-28B expression were found among different genotypes for rs17065417, rs314277 and rs12194974.

Consequently, since the minor allele frequency was very low, we focused only on the dominant besides additive models in the analyses of genotype and lin-28B levels. Significant associations of lin-28B levels were found with genotypes at rs314276 and rs7759938 and borderline significant at rs314277, but not at rs17065417 and rs12194974.

Influence of the SNP in lin-28B on its RNA secondary structure

Since rs314276 is an intronic eQTL, we questioned whether the variation of the SNP might affect the RNA secondary structure. Using the internet-based computer-modeling program mfold (24), we found that the predicted RNA secondary structure changes according to SNP genotype (Figure 1A and 1B). The mutant AA genotype had a structure with a ΔG of −80.84, while the wild-type CC had a ΔG of −81.82. We validated the differences in the secondary structures of the wild-type and mutant RNA transcripts experimentally, we analyzed by CD spectroscopy in vitro transcribed wild-type and mutant...
RNA transcripts from the PCR fragments with identical sequences except for a single-nucleotide change at rs314276 (CA). We found a spectrum characteristic of the RNA helical conformation secondary structure (25–27), including two intense peaks, a positive peak at wavelength approximately 260 nm, and a negative peak at approximately 240 nm and 225 nm, respectively, were also observed. The CD spectra showed that the wild-type (CC) RNA transcript had a maximum intensity (mean = 29.92 millidegrees, SD = 0.211 millidegrees) at a wavelength of 264 nm, while the mutant (AA) had a maximum intensity (mean = 28.59 millidegrees, SD = 0.233 millidegrees) at wavelength 211 nm. A 0.5-nm wavelength shift of the trough was observed for the mutant compared to the wild-type. The difference in the minimum intensity was also statistically significant (P = 1.7 × 10⁻³). The alteration spectrum (Figure 1D) showed the difference of both unstacked bases of the region at rs314276 (Figure 1), by which gene expression may be significantly associated with lin-28B mRNA levels. These findings indicate that patients with high lin-28B levels had significantly worse overall survival (P = 0.002) and disease progression-free survival (P = 0.003), and the associations were independent of founding variables, we performed Cox proportional hazards regression analyses. As expected, the results showed that among patients initially responding to chemotherapy, those with high lin-28B levels had significantly worse overall survival (P = 0.001) and disease progression-free survival (P = 0.007) than those with low levels (Figure 2A and 2B). To adjust for potential confounding variables, we performed Cox proportional hazards regression analysis. Again, we found that patients with high lin-28B levels had higher risks of death and relapse in both univariate and multivariate analyses (Table II). There were statistically significant linear trends between lin-28B levels and both overall (P = 0.001) and disease progression-free survival (P = 0.003), and the associations were independent of patient age at surgery, disease stage, tumor grade, residual tumor size and histological type.

**Table I.** Associations between lin-28B SNPs and lin-28B mRNA levels in epithelial ovarian cancer

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Lin-28B mRNA (EI)</th>
<th>Spearman correlation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>Range</td>
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<td>rs17065417</td>
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<td></td>
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<td>AA</td>
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<td>0–469</td>
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<tr>
<td>CA</td>
<td>80</td>
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<tr>
<td>CA/AA</td>
<td>116</td>
<td>0.16</td>
<td>0–1167</td>
</tr>
</tbody>
</table>

Notes: Spearman correlation coefficient.

In Spearman correlation analyses, genotypes of each SNP were coded as 0, 1 and 2 based on the number of minor alleles, respectively.

**Associations of lin-28B genotype and phenotype with ovarian cancer outcomes**

Lin-28B and its paralog lin-28 have been shown to reprogram somatic cells into stem cells and are considered as stem cell-associated transcription factors (9–11,28,29). Based on the cancer stem cell hypothesis, we hypothesized among patients initially responding chemotherapy regimes, those with high lin-28B mRNA had worse prognosis than those with low one. To test this hypothesis, we performed Kaplan-Meier survival curves analyses. As expected, the results showed that among patients initially responding to chemotherapy, those with high lin-28B levels had significantly worse overall survival (P = 0.002) and disease progression-free survival (P = 0.007) than those with low levels (Figure 2A and 2B). To adjust for potential confounding variables, we performed Cox proportional hazards regression analysis. Again, we found that patients with high lin-28B levels had higher risks of death and relapse in both univariate and multivariate analyses (Table II). There were statistically significant linear trends between lin-28B levels and both overall (P = 0.001) and disease progression-free survival (P = 0.003), and the associations were independent of patient age at surgery, disease stage, tumor grade, residual tumor size and histological type.

**Discussion**

In this study, we genotyped five selected SNPs of the lin-28B gene in 211 primary EOC tissue samples, and evaluated their associations with lin-28B mRNA levels as well as patient survival. In the additive model, we found a significantly negative correlation between the number of minor alleles of rs314276 and lin-28B expression levels, suggesting the expression levels decrease with the increasing number of minor alleles. In the dominant model, we also found rs314276 variation was significantly associated with lin-28B levels and both overall (P = 0.001) and disease progression-free survival (P = 0.003), and the associations were independent of patient age at surgery, disease stage, tumor grade, residual tumor size and histological type.
Fig. 1. Alterations of computationally predicted and experimentally observed RNA secondary structures of the region surrounding rs314276 in lin-28B. Theoretical models (predicted by mfold) of the RNA secondary structures in this region at rs314276 are shown for the (A) mutant genotype AA and (B) the wild-type CC. (C) Circular dichroism spectra and (D) differential circular dichroism spectrum are shown for the mutant (genotype AA) and wild-type (CC) RNAs. The spectra were obtained from three individual runs of each of the two RNA preparations for each sample.
be regulated. Almost equal free energy (ΔG) predicted using mfold suggests that the two different secondary structures have a similar thermodynamic stability, and that large kinetic barriers may exist to override in the rearrangement of base pair stacking for the transition between them. Evidence has shown that the alteration of RNA (mRNA precursor) secondary structure may affect the formation of a spliceosome, a large protein–RNA complex consisting of five small nuclear ribonuclear proteins and more than 100 other protein molecules (30). If the altered structure blocks the access of proteins to the RNA sequence, splicing will be inhibited and mRNA levels will be downregulated. In some circumstances, the altered structure in pre-mRNA can facilitate splicing through bringing important splicing sequences closer together (31,32). It has also been reported that multiple cryptic splice-site sequences may occur in many introns. These cryptic sequences match the consensus of either 5' or 3' splice site sequences which are normally sequestered in certain RNA structures to avoid misuse by spliceosomes (33,34). However, if alterations in RNA secondary structure occur due to point mutations that unmask these signal sequences, the splicing machinery can use these cryptic sites rather than the appropriate splice sites (33,35–37). Rogic and colleagues (38) experimentally validated the effects of intronic mutations on correlations between RNA secondary structures and splicing efficiency in yeast. They found that intronic mutations causing RNA secondary structure alteration affected splicing efficiency, consequently leading to the differences in gene expression.

Among the other three selected SNPs located in the promoter region of lin-28B, we found a borderline significant correlation between rs7759938 and lin-28B expression in the additive genetic model, while in the dominant model there was a statistically significant correlation between rs7759938 and lin-28B. The homozygous wild-type gene tends to have higher lin-28B levels than the variant, suggesting that rs7759938 is also an eQTL, although it is located far from the transcription site of lin-28B. Using the genome browser (http://genome.ucsc.edu), we found that rs7759938 is located in a region of the lin-28B promoter having characteristics of an enhancer (H3K4Me1) on eight cell lines (data not shown). ENCODE broad chromatin state segmentation results show that in human stem cells, the enhancer is weak where rs7759938 is located. This difference between the wild-type and variant may be caused by the disruption of consensus transcription-factor-binding sites in the predicted enhancer. Similarly, Harismendy and colleagues (39) demonstrated that coronary artery disease risk variants (rs10811656 and rs10757278) located in the enhancer ECAD9 were associated with CDKN2A/B expression; the variants disrupt the binding site of STAT1.

Recently, Permuth-Wey and colleagues (1) reported that rs12194974 was associated with risk of ovarian cancer in a recessive model; individuals with the homozygous variant had lower risk as compared with those with the common allele. These authors also used an in vitro luciferase assay to show that the activities of the wild-type (~727G) allele were significantly higher than the variant (~727A) in seven different ovarian cancer cell lines. However, in our study, we did not find a significant correlation between rs12194974 and lin-28B expression levels in ovarian cancer tissues. Additionally, we found that patients with the homozygous wild-type (GG) had lower, but not significant, lin-28B levels than those with the variant allele (P = 0.41). This discrepancy suggests that the regulation of lin-28B in vivo is more complicated than experimentally in vitro. Aside from the disruption of transcription-factor-binding sites by variants in the promoter region, gene expression is also governed by high-order chromatin structures, which may be cell-specific and temporal-specific. The results of ENCODE Broad chromatin state segmentation on this region of rs12194974 (http://genome.ucsc.edu) showed the features of an active promoter in human stem cells, K562 and HepG2 cells, but of repressed status in other cells (data not shown).

Based on the findings reported by Chang and colleagues (17), rs17065417 in the promoter region of lin-28B is located in the binding region of the c-Myc oncogenic transcription factor (Myc). The expression of lin-28B was significantly impaired when the Myc-bound region was deleted from 265 bp upstream to 1.4 kb downstream of the transcription start site (TSS) (17). However, in our study we did not find a significant correlation between rs17065417 and lin-28B levels. Although the heterozygote (AC) had much lower lin-28B levels than the wild-type (0.04 for AC vs. 2.20 for AA), patients with the
homzygous variants had higher lin-28B levels (4.21 for CC vs. 2.20 for AA). One possibility for the discrepancy here is due to a minor allele frequency of less than 10%, and our low power to distinguish the difference because of the relative small sample size. Another possibility is that a single base pair variant may not be sufficient to affect the binding of e-Myc appreciably. Myc proteins belong to the basic-helix-loop-helix-zipper (bHLHZ) superfamily, directly binding to a canonical CACGTG E-box (40). Moreover, the regulation of Myc on gene expression is not very strong, generally modest, in the area of 2-fold magnitude (41–44).

In our previous study, we showed that lin-28B may enhance ovarian cancer progression. However, none of the SNPs was associated with patient survival, although four of the five selected SNPs have been observed elsewhere in genome-wide association studies in association with risk of ovarian cancer and with the ovarian cancer-related risk factors, age at menarche and timing of puberty (1–6). This finding suggests that the risk loci may not function as prognostic markers.

As expected, we found among the subgroup of patients who initially had complete response to chemotherapy, those with high lin-28B levels had higher risks of death and disease progression as compared with those with low levels. Patients with high lin-28B levels had median overall and disease progression-free survival of 32.7 and 18.4 months, respectively, while for those with medium lin-28B levels the medians were 58.5 and 38.9 months. These associations with lin-28B level seem to be independent of other clinical and pathologic variables including patient age at surgery, disease stage, tumor grade, histological types and residual tumor size. Our results support the hypothesis that lin-28B is a cancer stem cell-associated gene; patients initially respond well to conventional chemotherapeutic agents, which kill differentiated cancer cells but not cancer stem cells, the culprit of the aggressiveness of the disease, metastasis and resistance to therapy. Hosomuina and colleagues (45) recently showed that ovarian cancer patients with cancer stem cell-like side populations had worse prognoses than those without; all 8 cases of relapse or death within 2 years from initial therapy were side population positive, while 5 of 11 cases of disease progression-free survivors were side population negative, and 6 were positive. Experiments with in vitro cell lines and in vivo animal models show that stem and progenitor-like cells make the behavior of human EOC aggressive (46). Szotek and colleagues (47) reported that side populations of ovarian cancer cell lines were more significantly resistant to the lipophilic chemotherapeutic agent doxorubicin than non-side population cells. Similar results that knockdown of lin-28B expression could sensitize cancer cell lines to radiotherapy have also been reported in lung cancer (48). Additionally, King and colleagues (19) showed that lin-28B can upregulate the intestinal/colonic epithelial stem cell markers LGR5 and PROM1, and promote cell migration and invasion and transform immortalized colonic epithelial cells. Their results suggest that lin-28B may play a role in the establishment or maintenance of intestinal stem cells. Another previous study also showed that downregulation of lin-28B could repress the self-renewal capability of prostate cancer cell lines (11). Thus, it is postulated that target therapy against lin-28B may be able to enhance the effect of chemotherapy on survival. In summary, we have demonstrated associations of selected risk SNPs in the lin-28B gene with expression levels and patient survival in EOC. Three genome-wide association-identified SNPs were significantly (rs314276) or borderline significantly (rs314277 and rs7759938) associated with lin-28B levels, but two (rs17065417 and rs12194974) were not. The intronic SNP rs314276 identified by the genome-wide association studies significantly altered the predicted RNA secondary structure in its local region. These SNPs alone were not significantly associated with patient survival. To our knowledge, this is the first study to perform in vivo eQTL analysis of lin-28B SNPs. We found that patients with high lin-28B levels had higher risks of death and relapse than those with low levels, even though they initially responded to chemotherapy. These findings suggest that intronic SNPs may affect RNA secondary structure, thereby regulating gene expression, but that the SNPs alone may not be sufficient to predict prognosis of EOC. The results also indicate that lin-28B may be a cancer stem cell marker, which could be of significant clinical implications in the management of epithelial ovarian cancer.

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References


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