Original Article

Associations of LIN-28B/let-7a/IGF-II axis haplotypes with disease survival in epithelial ovarian cancer

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Abstract: The axis of LIN-28B, let-7a and IGF-II has been shown to be involved in human diseases. Several functional intronic single nucleotide polymorphisms (SNPs) rs314276 in LIN-28B and rs4320932 in IGF-II are found to be associated with epithelial ovarian cancer (EOC) or its risk factors. SNPs in pri- or pre-miRNAs may affect miRNA expression, but it is unclear whether rs731085 in let-7a-3 influences let-7a or pri-/pre-let-7a-3 levels. It is also not known if these SNPs in combination have a joint effect on EOC. Here we analyzed the LIN-28B/let-7a/IGF-II axis haplotype-specific association with EOC survival by genotyping these SNPs, and mainly assessed the effect of rs731085 genotype on let-7a and pri-/pre-let-7a-3 expression in 211 primary EOC samples. No statistically significant association was found between the genotype of rs731085 and both let-7a and pri-/pre-let-7a-3 expressions. Multivariate Cox regression analyses showed that rs4320932, but neither rs314276 in LIN-28B nor rs731085 in let-7a-3, was significantly associated with patient overall and progression-free survival. Furthermore, the haplotype G-C-C was associated with increased risk of death, while the haplotype G-C-T was a favorable prognostic indicator. The adjusted hazard ratios for death (HRs) were 1.64 (95% CI: 1.19-2.26) for rs4320932, 1.13 (95% CI: 0.84-1.51) for rs314276, 0.89 (95% CI: 0.66-1.21) for rs731085, 7.48 (95% CI: 1.01-55.7) for the haplotype G-C-C and 0.14 (95% CI: 0.03-0.70) for the haplotype G-C-T, respectively. These results suggest that the haplotype-specific effects are stronger compared to the sum of three individual SNPs, and that the haplotype G-C-C of the axis has an unfavorable effect on patient overall survival.

Keywords: Epithelial ovarian cancer, haplotype, LIN-28B/let-7a/IGF-II axis, prognosis, single nucleotide polymorphism (SNP)

Introduction

MicroRNAs (miRNAs), a group of small non-coding RNA, are important regulators in gene expression [1, 2]. It has been shown that miRNAs play important roles in a variety of physiological processes and biological functions [1-3]. Let-7 is a well-characterized miRNA [4-9], and in human there are 13 family members (let-7a to let-7i) located on 9 different chromosomes [10]. Dysregulation of let-7 has been suggested to be involved in human diseases including cancer and metabolic disorders [7, 11-15]. In vitro experiments have shown that let-7a acts as a tumor suppressor by repressing oncogenes including the embryonic gene high mobility group A2 (HMGA2), and RAS [7, 16]. Poorly differentiated ovarian cancer cells showed the deficiency in let-7 [17], and patients who had reduced let-7 expression had poor survival [13, 18, 19]. Accumulating evidence also indicates that let-7 may affect the response of patients to chemotherapy or radiotherapy and disease outcomes [20-28]. DNA methylation and post-transcriptional modification are two major mechanisms contributing to the reduced let-7a expression. 
LIN-28B/let-7a/IGF-II axis haplotypes and ovarian cancer survival

expression in cancer [25, 29-32]. The promoter of let-7a-3 contains rich CpG sites, and their methylation was associated with the downregulation of let-7a and its biological activity in human cancer [25, 33-35]. RNA binding proteins LIN-28A and LIN-28B have also been demonstrated to be involved in post-transcriptional modification of let-7a transcripts, blocking its maturation, and are negatively associated with let-7a expression [29-32].

LIN-28A and its homolog LIN-28B are important transcription factors in maintaining stem cell-like properties and tumorigenicity, and thus termed as stem cell-associated proteins [25, 36-39]. They share similar molecular structures in several RNA-binding domains, a cold-shock domain (CSD) and two retroviral-type CCHC zinc finger domains (ZFMs) [31, 40]. With the RNA-binding domains, LIN-28A and LIN-28B are able to post-transcriptionally modify let-7a, affecting let-7a maturation and its abundance [29-32]. Down-regulation of let-7a releases its inhibitory effects on many oncogenes including k-RAS, c-MYC, HMGA2, cyclin D1 and insulin-like growth factor-II (IGF-II) [25, 29, 38, 41, 42]. LIN-28B was first characterized to be overexpressed in human liver cancer [37]. Dysregulation of LIN-28B was subsequently found in other human cancers including brain, breast, ovary and head and neck, and the condition was associated with prognosis and treatment response [29, 43-45]. High LIN-28B expression in peripheral blood mononuclear cells was also shown to be positively associated with the risk of relapse and unfavorable features of hepatocellular carcinoma [46]. In vitro experiments show that LIN-28B/let-7a axis may also be involved in epithelial-mesenchymal transition (EMT) [47], tumorigenesis [48], and the programming of hematopoietic stem cells [49, 50]. Moreover, there was a positive correlation reported between the expression of LIN-28B and IGF-II in cancer tissues [29, 51].

Insulin-like growth factor II is a mitogen, playing an important role in the development and cell proliferation. IGF-II overexpression in relation to increased risk of human cancer including ovarian cancer has been reported previously [52-56]. Loss of imprinting (LOI), DNA methylation and miRNAs (small and large) are underlying mechanisms regulating IGF-II expression [57-59]. Enforced let-7a overexpression could lead to elevated levels of IGF-II transcripts [60]. The associations among LIN-28B, let-7a and IGF-II expression suggest that the LIN-28B/let-7a/IGF-II axis as a whole may have biological implications [25, 29]. It has been reported that the LIN-28B/IGF axis is linked to the progression of head and neck cancer [51], and the LIN-28B/let-7 axis promotes transformation, proliferation and invasion of human cancer [39, 61].

Gene-environment interaction plays an important in cancer development and tumor progression. Genome-wide association studies (GWAS) have identified thousands of genetic susceptibility variants for complex traits in human including risks for developing various types of cancer. However, the effect sizes of these genetic factors are small (less than 20%) [62, 63]. Due to limited study power, most of the low-penetrance common variants may have been missed by GWAS. On the other hand, many complex traits or diseases like cancer are affected by a number of genetic variants collectively, and each variant has a small effect on a trait or disease. The advantage of combination therapy over monotherapy as well as stronger SNP prediction of patient survival by the pathway-based approach also suggest that multiple polymorphisms in a complex biological network may synergistically lead to a biological phenotype [64-66]. Even though some low-penetrance variants individually have very subtle effect size, not reaching statistical significance, they may have biological effects collectively on human health [67]. Haplotype is one of those collective approaches to investigate the combined effects of multiple variants on complex diseases. Previous studies have shown that rs4320932 in IGF-II was functionally associated with ovarian cancer risk and progression [68, 69], and that rs314276 in LIN-28B was a GWAS-identified expression quantitative trait locus (eQTL) and a risk factor in ovarian cancer [36, 70-76]. The variant of rs731085 in let-7a-3 was not found to be associated with ovarian cancer [77], but the study was relatively small (n = 90). In addition, genetic variants in pre-miRNA genes have been shown to affect miRNA expression [78-80]. The purposes of this study were to investigate the combined effects of multiple SNPs on ovarian cancer through the haplotype approach and the associations between rs731085 and both let-7a and pri/pre-let-7a-3 expression. To achieve the goal, we genotyped 3 candidate SNPs (rs314276, rs731085 and rs4320932) in the LIN-28B/let-
7a/IGF-II axis to evaluate their prognostic values, and analyzed the expression of the genes to determine eQTL in epithelial ovarian cancer.

Materials and methods

Patients and tumor samples

This study was approved by the ethical review committee of University of Turin in Italy. With informed consents from patients, 211 fresh tissues of epithelial ovarian cancer were collected at surgery in the Department of Gynecology and Obstetrics at University of Turin between October 1991 and February 2000. The specimens were snap-frozen in liquid nitrogen immediately after resection, and then transferred to a -80°C freezer for storage. Clinical and pathology information on these patients was retrieved from the medical charts and pathology reports. According to FIGO and WHO criteria for disease stage and tumor grade [81], of the 211 patients, 52 were diagnosed with stage I disease, 12 with stage II, 133 with stage III, and 14 with stage IV. Tumor grades 1-3 were found in 34, 40, and 137 patients, respectively. Patient age at surgery averaged 57.9 years (range: 26-82). Based on the WHO guidelines for ovarian tumor histology [82], papillary serous was 40.3%, followed by endometrioid (19.4%), undifferentiated (17.1%), mucinous (8.5%), clear cell (7.6%), müllerian (6.6%), and other (0.5%). Most of the patients received standard post-operative platinum-based chemotherapy after cytoreduction surgery, and were subsequently followed through June 2001 for disease progression. The median follow-up time was 31 months with the range from 0.6 to 114 months. At the end of the study follow-up, 92 patients died and 95 had a progressive disease.

Each patient was evaluated for chemotherapy response, which was classified into four categories: (a) complete response, resolution of all evidence of disease for at least 1 month; (b) partial response, a decrease of ≥ 50% in the product of the diameters (maximum and minimum) of all measurable lesions without the development of new lesions for at least 1 month; (c) stable disease, a decrease of < 50% or an increase of < 25% in the product of the diameters of all measurable lesions; and (d) progressive disease, an increase of ≥ 25% in the product of the diameters of all measurable lesions or the development of new lesions.

Genomic DNA and total RNA extraction

The frozen tumor specimens, which had been examined by two independent pathologists to confirm greater than 80% of tumor cells contained in each specimen, were pulverized manually in liquid nitrogen, and approximately 100 mg of tissue powder were used for the extraction of genomic DNA and total RNA using a standard phenol-chloroform approach. The quality and quantity of the extracted DNA and RNA samples was determined by a spectrophotometer.

Genotyping of LIN-28B, let7a3 and IGF-II SNPs

Genotypes of the LIN-28B SNP (rs314276, C/G) [36], let7a-3 SNP (rs731085, C/A) [77] and IGF-II SNP (rs4320932, T/C) [68] were determined using the TaqMan® SNP genotyping assay (Applied Biosystems, Foster City, CA) following the manufacturer’s protocols as described previously elsewhere. Briefly, in a volume of 8 μl PCR reaction, 4 μl of 2X iTaq™ Fast Supermix with ROX (Bio-Rad, Hercules, CA) was mixed with pre-designed TaqMan® primers/probes (Applied Biosystems), approximately 10-50 ng of genomic DNA, and distilled water. The PCR conditions were initial denaturing at 95°C for 10 min followed by 50 cycles of denaturing at 92°C for 15 seconds and annealing/extension at 60°C for 1 min. The reactions were carried out in an ABI 7500 Real-time PCR system (Applied Biosystems). Ten percent of samples were run in duplicate for quality control, with 100% concordance.

Analysis of let-7a, pri-/pre-let-7a-3, lin-28B and IGF-II expression

Analysis of let-7a expression in tumor tissue was performed using the TaqMan™ microRNA assay (Applied Biosystems) following the manufacturer’s instruction as described elsewhere [25]. Briefly, levels of let-7a and RNU48 (an internal control for normalization) expression in the samples were determined with the TaqMan® miRNA assay (Applied Biosystems) using the Chromo 4 Real-time PCR System (MJ Research Inc., Waltham, MA). In the PCR reaction (15 μl), 0.3 μl of cDNA template was mixed with 7.5 μl of 2X TaqMan® Universal PCR master mix (Applied Biosystems), 0.75 μl of 20X probe/primers (Applied Biosystems) of either let-7a or RNU48, and water. The PCR amplification conditions were initial denaturing at 95°C for 10
min followed by 40 cycles of denaturing at 92°C for 15 seconds and annealing/extension at 60°C for 1 min.

Expressions of pri-/pre-let-7a-3, LIN-28B, and IGF-II were analyzed using SYBR green-based RT-qPCR on the Chromo4™ Real-time PCR System (MJ Research Inc., Waltham, MA), and the sequences of the primers pri-/pre-let-7a-3, LIN-28B, and IGF-II, as well as the internal controls RNU48 (for pri-/pre-let-7a-3 and LIN-28B) and GAPDH (for IGF-II) were as described previously elsewhere [29, 52]. In the PCR reaction (20 μl), 1 μl of cDNA template was mixed with 10 μl of 2X Power SYBR® PCR master mix (Applied Biosystems), 200 nM of paired primers, and water. The PCR amplification included initial incubation at 50°C for 2 minutes, denaturing at 95°C for 10 minutes, and 40 cycles of denaturing at 95°C for 15 seconds and annealing at 60°C for 1 minute. Melting curves were analyzed after each run to verify the size of PCR product.

Each sample was analyzed in duplicate, and the analysis was repeated for those with CV above 5%.

### Statistical analysis

Expression of let-7a was quantified as an expression index (EI), which was calculated based on the formula $1000 \times 2^{-\Delta Ct}$, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{internal control}}$. A Bayesian approach was applied to reconstruct haplotypes and estimate their frequencies [83]. Hardy-Weinberg equilibrium (HWE) and the associations between clinicopathologic features and the haplotypes were analyzed by the Chi-square test. Survival analyses were performed to assess the associations of haplotypes and risks of disease progression and death using the Cox proportional hazards regression models, treating each haplotype as a continuous variable. Both haplotype-specific associations in a 1-degree-of-freedom test and a global test simultaneously fitting all haplotypes with pa-

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Frequency (%)</th>
<th>n</th>
<th>Gene expression</th>
<th>Death</th>
<th>Relapse</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs731085</td>
<td>211</td>
<td></td>
<td></td>
<td>let-7a</td>
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<td></td>
</tr>
<tr>
<td>CC</td>
<td>85</td>
<td>40.3</td>
<td>85</td>
<td>4.7 (0.71-35.28)</td>
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<td>CG</td>
<td>91</td>
<td>43.1</td>
<td>91</td>
<td>4.63 (0.39-23.93)</td>
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<td>GG</td>
<td>35</td>
<td>16.6</td>
<td>35</td>
<td>4.63 (0.48-58.52)</td>
<td>1.00</td>
<td>1.09</td>
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<td>211</td>
<td></td>
<td></td>
<td>pri-/pre-let-7a-3</td>
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<tr>
<td>CC</td>
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<td>40.3</td>
<td>85</td>
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<td>16.6</td>
<td>35</td>
<td>0.05 (0-41.67)</td>
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<td></td>
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<tr>
<td>P value for pri-/pre-let-7a-3 expression</td>
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<td>LIN-28B</td>
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<td>45</td>
<td>95</td>
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<td>AC</td>
<td>80</td>
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<td>90</td>
<td>0.001 (0-5.27)</td>
<td></td>
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<td>36</td>
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<td>36</td>
<td>0.01 (0-3.44)</td>
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<tr>
<td>rs4320932</td>
<td>211</td>
<td></td>
<td></td>
<td>IGF-II</td>
<td>1.64</td>
<td>1.80</td>
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<td>TT</td>
<td>124</td>
<td>58.8</td>
<td>117</td>
<td>14.3 (0-3008)</td>
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<td></td>
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<td>CT</td>
<td>73</td>
<td>34.6</td>
<td>71</td>
<td>8.8 (0-9503)</td>
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<td></td>
</tr>
<tr>
<td>CC</td>
<td>14</td>
<td>6.6</td>
<td>14</td>
<td>12.7 (0-5661)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value for IGF-II expression</td>
<td>0.982</td>
<td></td>
<td></td>
<td></td>
<td>0.469</td>
<td></td>
</tr>
</tbody>
</table>

1. HR: hazard ratio obtained from a multivariate Cox proportional hazard regression model based on the additive model of the SNPs (major allele homozygote = 0, heterozygote = 1, and minor allele homozygote = 2) and adjusted for patient age at surgery, disease stage, tumor grade, residual tumor size and histological type. 2. CI: confidence interval.
tient survival were performed [84]. The overall survival time and progression-free survival time were calculated as the time from surgery to death, relapse, or the last follow-up, respectively. For chemotherapy response in our data analyses, we grouped patients into two categories, ‘responders’, which included complete response, and ‘non-responders’, which included partial response, stable disease and progressive disease. All statistical analyses were carried out using SAS version 9.3, and a p value less than 0.05 was considered as statistical significance.

Results

Genotypes of SNPs in LIN-28B, let-7a-3 and IGF-II and their associations with patient survival

Genotyping of the rs314276, rs731085 and rs4320932 polymorphisms in LIN-28B, let-7a-3 and IGF-II, respectively, was successfully achieved in 211 epithelial ovarian cancer tissues. The frequency distributions of the genotypes are shown in Table 1. SNP rs314276 (P = 0.010) but neither rs731085 (P = 0.211) nor rs4320932 (P = 0.469) was deviated from HWE. The minor allele A frequency of rs314276 was 0.36, and more homozygotes were observed than the theoretical expectation.

Multivariate Cox proportional hazard regression analysis showed that the SNP rs4320932 in IGF-II, but neither rs731085 in let-7a-3 nor rs314276 in LIN-28B, was significantly associated with the risks of both death and disease progression (Table 1). The adjusted hazard ratios (HRs) of rs4320932 in an additive model were 1.64 (95% CI: 1.19-2.26) for death (P = 0.002) and 1.80 (95% CI: 1.31-2.47) for disease progression (P = 0.0003), respectively.

Table 1 also shows no statistically significant associations between the genotype of rs-731085 and both let-7a expression (P = 0.671) and pri/-pre-let-7a-3 (P = 0.829), and between rs4320932 and IGF-II transcripts (P = 0.982), but a significant one between rs314276 and LIN-28B transcripts (P = 0.029).

Associations of the haplotypes and clinicopathologic features

Eight haplotypes were predicted based on a Bayesian statistical method, and their estimated frequencies and 95% CIs were shown as in Table 2. The highest frequency of haplotypes was C-C-T (0.273, 95% CI: 0.230-0.316), followed by G-C-T, C-A-T, C-C-C, G-A-T, C-A-C, G-C-C and G-A-C.

Associations of haplotypes with clinicopathologic features are summarized in Table 2. Patients with a serous ovarian cancer had significantly lower frequencies of G-A-C haplotype than those with a non-serous type (0.00002 vs 0.043, P = 0.004). In contrast, patients with a serous type had higher frequencies of C-A-T than those with a non-serous one (0.224 vs. 0.153, P = 0.063), respectively. Similarly, patients with a grade I-II tumor had significantly higher frequencies of C-A-T than those with a grade III disease (0.241 vs. 0.156, P = 0.030). In addition, patients with an advanced stage disease had higher frequencies of G-A-T haplotype compared to those with an early stage (0.121 vs. 0.061, P = 0.058), whereas the frequency of G-A-C haplotype was lower in patients with an advanced stage disease than those with an early stage (0.017 vs 0.044, P = 0.082). However, none of the haplotypes were found in significant association with other clinicopathologic features, including residual tumor size, debulking results and response to chemotherapy.

Associations of haplotypes and patient survival

Multivariate Cox proportional hazard regression models were developed, in which each haplotype was treated as a continuous variable, and patient age at surgery, disease stage, tumor grade, residual tumor size and histological types were included as covariates. We first performed a global test simultaneously fitting all haplotypes in one model, and the P value for this test was less than 0.0001 (data not shown).

To study the haplotype-specific association with patient survival, we developed multivariate Cox proportional hazard regression models, in which each individual haplotype and the covariate variables of patient age, disease stage, tumor grade, residual tumor size and histological type were included, with or without the genotypes of the SNPs. The results are shown in Table 3. Before adjusting for the genotypes, two haplotypes were significantly associated with the risk of death. Patients who carried one
Table 2. Distribution of predicted haplotypes and their associations with clinical and pathological features in epithelial ovarian cancer

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>C-A-C(^1)</th>
<th>C-A-T</th>
<th>C-C-C</th>
<th>C-C-T</th>
<th>G-A-C</th>
<th>G-A-T</th>
<th>G-C-C</th>
<th>G-C-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency</td>
<td></td>
<td>0.055</td>
<td>0.183</td>
<td>0.108</td>
<td>0.273</td>
<td>0.023</td>
<td>0.099</td>
<td>0.053</td>
<td>0.206</td>
</tr>
<tr>
<td>95% CI(^2)</td>
<td></td>
<td>0.033-0.077</td>
<td>0.146-0.220</td>
<td>0.078-0.137</td>
<td>0.230-0.316</td>
<td>0.009-0.037</td>
<td>0.071-0.128</td>
<td>0.032-0.075</td>
<td>0.167-0.244</td>
</tr>
<tr>
<td>Disease stage</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>64</td>
<td>0.036</td>
<td>0.217</td>
<td>0.123</td>
<td>0.241</td>
<td>0.044</td>
<td>0.061</td>
<td>0.023</td>
<td>0.254</td>
</tr>
<tr>
<td>III-IV</td>
<td>147</td>
<td>0.060</td>
<td>0.162</td>
<td>0.109</td>
<td>0.288</td>
<td>0.017</td>
<td>0.121</td>
<td>0.059</td>
<td>0.184</td>
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<tr>
<td>P value</td>
<td></td>
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<td>0.582</td>
<td>0.318</td>
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<td>Tumor grade</td>
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<tr>
<td>I-II</td>
<td>74</td>
<td>0.047</td>
<td>0.241</td>
<td>0.089</td>
<td>0.234</td>
<td>0.021</td>
<td>0.090</td>
<td>0.074</td>
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<tr>
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<td>0.117</td>
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<td>0.025</td>
<td>0.102</td>
<td>0.046</td>
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<tr>
<td>P value</td>
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<td>0.785</td>
<td>0.689</td>
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<td>0.863</td>
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<td>Histological type</td>
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<tr>
<td>Serous</td>
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<td>0.051</td>
<td>0.224</td>
<td>0.125</td>
<td>0.277</td>
<td>0.00002</td>
<td>0.078</td>
<td>0.048</td>
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<tr>
<td>Non-serous</td>
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<td>0.153</td>
<td>0.099</td>
<td>0.272</td>
<td>0.043</td>
<td>0.114</td>
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<tr>
<td>P value</td>
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<td>0.808</td>
<td>0.063</td>
<td>0.399</td>
<td>0.908</td>
<td>0.004</td>
<td>0.224</td>
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<td>&gt; 0</td>
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<td>0.152</td>
<td>0.122</td>
<td>0.270</td>
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<td>0.114</td>
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<tr>
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<td>0.255</td>
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<td>0.207</td>
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<td>0.286</td>
<td>0.021</td>
<td>0.091</td>
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<tr>
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<td>0.024</td>
<td>0.106</td>
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<td>0.139</td>
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<tr>
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<td>0.531</td>
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<td>0.473</td>
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1. SNPs were arranged in the order of rs731085-rs314276-rs4320932. 2. CI: confidence interval.
copy of the haplotype G-C-C had an increased risk of death compared to those who did not have it; the HR was 5.31 (95% CI: 1.49-18.9). In contrast, patients who carried one copy of the haplotype G-C-T had a decreased risk of death compared to those who did not carry it; the HR was 0.25 (95% CI: 0.11-0.62). These associations remained significant after adjusting for the genotypes. Their HRs conditional on the genotypes were 7.48 (95% CI: 1.01-55.7) for the G-C-C carriers, and 0.14 (95% CI: 0.03-0.70) for the G-C-T carriers, respectively. By comparing the Wald chi-square statistics of both the full and reduced models, we found the haplotype G-C-C-specific association with the death risk was significant (P = 0.008), while the haplotype G-C-T-specific association with the death risk was borderline significant (P = 0.069). We also found that three haplotypes (C-A-C, G-A-C, and G-C-C) individually were significantly associated with disease progression before the adjustment for genotypes; the carriers of each individual of the three haplotypes had increased risk of relapse compared to those who did not carry them. Their HRs were 5.87 (95% CI: 1.48-23.2), 13.8 (95% CI: 1.66-114), and 3.74 (95% CI: 1.10-12.8), respectively. However, the significances of these associations with disease progression turned null after the adjustment. Similarly, none of haplotype-specific associations with relapse risk were significant by comparing the full and reduced models.

### Discussion

In this study we demonstrated the associations of the LIN-28B/let-7a/IGF-II axis haplotypes with patient survival in epithelial ovarian cancer. With the genotypes of three candidate SNPs in three genes, eight haplotypes were predicted using a Bayesian model. Although there was a deviation of rs314276 in LIN-28B from HWE, this may not affect the haplotype estimation given that more homozygosities were found in this study. It has been reported that excess homozygosity could improve estimation accuracy using the Expectation-Maximization (EM) algorithm [85], and that the Bayesian algorithm holds robust estimation even at the violation of HWE [83]. The haplotype of C-C-T, which is composed of each major allele of the three SNPs, is the most common one with an estimated frequency of 0.273. In contrast, the minor allele-composed haplotype G-A-C is the least common one with an estimated frequency of 0.023.

By examining the effects of the genotypes on patient survival of epithelial ovarian cancer, we found in this study only rs4320932 in IGF-II, neither rs314276 in LIN-28B nor rs731085 in let-7a-3, was associated with the risks of death and relapse in multivariate analysis. Patients carrying one copy of minor allele C had a 64% and 80% increases in risk of death and relapse, respectively, compared to those who did not

<table>
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<th>Haplotype</th>
<th>Death</th>
<th>Relapse</th>
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<td>Unadjusted</td>
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1. SNPs were arranged in the order of rs731085-rs314276-rs4320932. 2. HR: hazard ratio obtained from a Cox proportional hazard regression model adjusted with patient age at surgery, disease stage, tumor grade, residual tumor size and histological type, and with or without rs731085, rs314276 and rs4320932. 3. CI: confidence interval. 4. The Cox models were conditionally adjusted without rs731085, rs314276 and 4320932 in an additive model. 5. The Cox models were conditionally adjusted with rs731085, rs314276 and 4320932 in an additive model (major allele homozygote = 0, heterozygote = 1, and minor allele homozygote = 2).
The conditional effects of both rs4320932 and rs314276 SNPs on patient survival with epithelial ovarian cancer were in consistency with the previous studies, in which the SNPs were individually analyzed [36, 68]. The SNP rs731085 is located at the 63 bp downstream of pre-let-7a-3, which was not associated with the risk of ovarian and breast cancer [86]. In contrast, several SNPs in premiRnas were significantly associated with cancer risk [87]. These findings suggest that the effect size of rs731085 in let-7a-3 on ovarian cancer may be too small to detect. We also did not find an association of rs731085 with the expressions of let-7a and pri-/pre-let-7a-3 in this study.

When analyzing the combined effects of the three SNPs on patient survival with epithelial ovarian cancer, we found that patients with one copy of the haplotype G-C-C had an over 7-fold increase in risk of death compared to those who did not carry it, while patients with one copy of the haplotype G-C-T had an over 80% but borderline significant reduction in risk of death compared to those who did not carry it. These findings suggest that the combined effects of these SNPs in haplotype are much stronger than the effects of each individual SNP or the sum of their effects. It seems that the allele of IGF-II SNP determines the direction of effect, risk or protection. The underlying mechanism(s) of this modified direction by the alleles of IGF-II SNP rs4320932 is still unclear. In our previous report, it was shown that the allele C of IGF-II SNP rs4320932 significantly increased risk of death in epithelial ovarian cancer [68]. Although the IGF-II SNP rs4320932 is intronic, the variant may alter local DNA conformation [68]. Studies also have reported that high LIN-28B expression is associated with increased risk of death and unfavorable malignancies, and patients with the allele C of LIN-28B SNP rs413276 has significantly higher LIN-28B expression than those with the allele A [29, 36, 38]. Given that the expressions of LIN-28B and IGF-II are positively correlated, the secondary structures of IGF-II RNA may also be affected by the allele C and T of rs4320932, thereby influencing the phenotype. Moreover, given the relative small sample size, our study results should be interpreted with caution. The study finding warrants further validation in independent studies with larger sample sizes.

In summary, our study showed that the haplotype G-C-C in the LIN-28B/let-7a/IGF-II axis was an unfavorable prognostic indicator, while the haplotype G-C-T was a favorable one in epithelial ovarian cancer, and that their combined effects were much stronger than the sum or each individual SNP effect. This finding suggests that if combining them, the effects of SNPs with a small effect size may be amplified, and we should not overlook the joint effect of multiple common SNPs which have low penetrance. The effect of the LIN-28B/let-7a/IGF-II axis haplotype on overall survival of EOC warrants confirmation in additional studies.

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Disclosure of conflict of interest

None.

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