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

Lingeng Lu[1], Dionyssios Katsaros[2], Evan Risch[1], Qian Deng[3], Nicoletta Biglia[4], Elisa Picardo[2], Marco Mitidieri[2], Harvey A Risch[1], Herbert Yu[5]

[1]Department of Chronic Disease Epidemiology, School of Public Health, School of Medicine, Yale Cancer Center, Yale University, New Haven, CT 06520-8034, USA; [2]Department of Surgical Science, A O Città Della Salute E Della Scienza Di Torino, S. Anna Hospital, 10126 Turin, Italy; [3]National Center for Chronic and Non-communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China; [4]Department of Surgical Science, Division of Obstetrics and Gynecology, University of Torino School of Medicine, Mauriziano Hospital, 10128 Turin, Italy; [5]Cancer Epidemiology Program, University of Hawaii Cancer Center, Honolulu, HI 96813, USA

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]. Dysregultion 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 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], *let-7a*-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 2XTaqMan® 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

Table 1. Genotype distributions and their associations with gene expression and patient survival in epithelial ovarian cancer

Genotype	N	Frequency (%)	n	Gene expression Median (5th-95th)	Death HR[1] (95% CI[2])	Relapse HR (95% CI)
rs731085	211			*let-7a*	0.89 (0.66-1.21)	1.10 (0.83-1.45)
CC	85	40.3	85	4.47 (0.71-35.28)		
CG	91	43.1	91	4.63 (0.39-23.93)		
GG	35	16.6	35	4.63 (0.48-58.52)		
P value for *let-7a* expression				0.671		
P value for HWE		0.211				
rs731085	211			pri-/pre-let-7a-3		
CC	85	40.3	85	0.06 (0-5.68)		
CG	91	43.1	91	0.05 (0-1.91)		
GG	35	16.6	35	0.05 (0-41.67)		
P value for pri-/pre-*let-7a-3* expression				0.829		
rs314276	211			*LIN-28B*	1.13 (0.84-1.51)	1.08 (0.81-1.44)
CC	95	45	95	0.05 (0-6.99)		
AC	80	37.9	90	0.001 (0-5.27)		
AA	36	17.1	36	0.01 (0-3.44)		
P value for *LIN-28B* expression				0.029		
P value for HWE		0.01				
rs4320932	211			*IGF-II*	1.64 (1.19-2.26)	1.80 (1.31-2.47)
TT	124	58.8	117	14.3 (0-3008)		
CT	73	34.6	71	8.8 (0-9503)		
CC	14	6.6	14	12.7 (0-5661)		
P value for *IGF-II* expression				0.982		
P value for HWE		0.469				

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.

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 $\Delta Ct = Ct_{target\ gene} - Ct_{internal\ control}$. 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-

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, let7a3 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 clinico-pathologic features

Eight haplotypes were predicted based on a Bayesian statistical method, and their estimat-

ed 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

Variable	N	Haplotype frequency (%)							
		C-A-C[1]	C-A-T	C-C-C	C-C-T	G-A-C	G-A-T	G-C-C	G-C-T
Frequency		0.055	0.183	0.108	0.273	0.023	0.099	0.053	0.206
95% CI[2]		0.033-0.077	0.146-0.220	0.078-0.137	0.230-0.316	0.009-0.037	0.071-0.128	0.032-0.075	0.167-0.244
Disease stage									
I-II	64	0.036	0.217	0.123	0.241	0.044	0.061	0.023	0.254
III-IV	147	0.060	0.162	0.109	0.288	0.017	0.121	0.059	0.184
P value		0.312	0.172	0.582	0.318	0.082	0.058	0.116	0.103
Tumor grade									
I-II	74	0.047	0.241	0.089	0.234	0.021	0.090	0.074	0.200
III	137	0.057	0.156	0.117	0.292	0.025	0.102	0.046	0.206
P value		0.634	0.030	0.372	0.247	0.785	0.689	0.220	0.863
Histological type									
Serous	85	0.051	0.224	0.125	0.277	0.00002	0.078	0.048	0.198
Non-serous	126	0.056	0.153	0.099	0.272	0.043	0.114	0.053	0.212
P value		0.808	0.063	0.399	0.908	0.004	0.224	0.734	0.735
Residual tumor size									
0	91	0.052	0.213	0.087	0.291	0.026	0.077	0.055	0.199
> 0	116	0.059	0.152	0.122	0.270	0.024	0.114	0.054	0.205
P value		0.741	0.111	0.255	0.614	0.855	0.210	0.906	0.811
Debulking results									
Opitmal	108	0.056	0.207	0.090	0.286	0.021	0.091	0.046	0.203
Suboptimal	100	0.058	0.148	0.125	0.269	0.027	0.107	0.061	0.205
P value		0.939	0.121	0.248	0.683	0.710	0.562	0.495	0.899
Response to chemotherapy									
Yes	128	0.049	0.200	0.090	0.286	0.024	0.106	0.044	0.201
No	48	0.086	0.139	0.139	0.271	0.036	0.082	0.062	0.185
P value		0.192	0.193	0.184	0.782	0.531	0.497	0.473	0.727

1. SNPs were arranged in the order of rs731085-rs314276-rs4320932. 2. CI: confidence interval.

Table 3. Multivariate analyses for haplotype-specific associations with death and relapse in epithelial ovarian cancer

Haplotype[1]	Death			Relapse		
	HR[2]	95% CI[3]	P value	HR	95% CI	P value
Unadjusted[4]						
C-A-C	3.55	0.85-14.8	0.082	5.87	1.48-23.2	0.012
C-A-T	1.00	0.40-2.52	0.995	0.52	0.21-1.29	0.155
C-C-C	2.63	0.97-7.14	0.057	2.20	0.87-5.58	0.097
C-C-T	0.85	0.40-1.81	0.678	0.61	0.34-1.40	0.305
G-A-C	4.39	0.44-44.3	0.210	13.80	1.66-114	0.015
G-A-T	1.16	0.42-3.22	0.779	1.14	0.40-3.24	0.803
G-C-C	5.31	1.49-18.9	0.010	3.74	1.10-12.8	0.035
G-C-T	0.25	0.11-0.62	0.003	0.60	0.28-1.26	0.174
Adjusted[5]						
C-A-C	0.25	0.03-2.47	0.238	1.56	0.18-13.6	0.689
C-A-T	0.87	0.16-4.62	0.868	046	0.07-1.76	0.204
C-C-C	0.74	0.13-4.37	0.738	0.57	0.11-2.94	0.498
C-C-T	2.69	0.58-12.3	0.204	3.05	0.74-12.7	0.124
G-A-C	0.35	0.01-9.17	0.530	2.17	0.12-39.2	0.600
G-A-T	2.87	0.63-13.1	0.174	1.82	0.38-8.62	0.451
G-C-C	7.48	1.01-55.7	0.049	1.11	0.18-6.65	0.912
G-C-T	0.14	0.03-0.70	0.016	0.46	0.11-2.02	0.305

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).

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

carry it. The conditional effects of both rs4320932 and rs314276 SNPs on patient survival of epithelial ovarian cancer were in consistence 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 pre-miRNAs 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.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Lingeng Lu, Department of Chronic Disease Epidemiology, School of Public Health, School of Medicine, Yale Cancer Center, Yale University, 60 College Street, New Haven, CT 06520-8034, USA. Tel: 203-737-6812; Fax: 203-785-2207; E-mail: lingeng.lu@yale.edu

References

[1] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.

[2] Vasudevan S, Tong Y and Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. Science 2007; 318: 1931-1934.

[3] Turner M, Galloway A and Vigorito E. Noncoding RNA and its associated proteins as regulatory elements of the immune system. Nat Immunol 2014; 15: 484-491.

[4] Ambros V. microRNAs: tiny regulators with great potential. Cell 2001; 107: 823-826.

[5] Rougvie AE. Control of developmental timing in animals. Nat Rev Genet 2001; 2: 690-701.

[6] Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR and Ruvkun G. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 2000; 403: 901-906.

[7]   Johnson CD, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, Ovcharenko D, Wilson M, Wang X, Shelton J, Shingara J, Chin L, Brown D and Slack FJ. The let-7 MicroRNA Represses Cell Proliferation Pathways in Human Cells. Cancer Res 2007; 67: 7713-7722.

[8]   Chu YD, Wang WC, Chen SA, Hsu YT, Yeh MW, Slack FJ and Chan SP. The receptor of activated C kinase RACK-1 regulates let- 7 microRNA expression and terminal cell differentiation in Caenorhabditis elegans. Cell Cycle 2014; 13: 1995-2009.

[9]   Hunter SE, Finnegan EF, Zisoulis DG, Lovci MT, Melnik-Martinez KV, Yeo GW and Pasquinelli AE. Functional genomic analysis of the let-7 regulatory network in Caenorhabditis elegans. PLoS Genet 2013; 9: e1003353.

[10]   Boyerinas B, Park SM, Hau A, Murmann AE and Peter ME. The role of let-7 in cell differentiation and cancer. Endocr Relat Cancer 2010; 17: F19-36.

[11]   Zhu H, Shyh-Chang N, Segre AV, Shinoda G, Shah SP, Einhorn WS, Takeuchi A, Engreitz JM, Hagan JP, Kharas MG, Urbach A, Thornton JE, Triboulet R, Gregory RI, Altshuler D and Daley GQ. The Lin28/let-7 axis regulates glucose metabolism. Cell 2011; 147: 81-94.

[12]   Kumar MS, Erkeland SJ, Pester RE, Chen CY, Ebert MS, Sharp PA and Jacks T. Suppression of non-small cell lung tumor development by the let-7 microRNA family. Proc Natl Acad Sci U S A 2008; 105: 3903-3908.

[13]   Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D and Slack FJ. RAS is regulated by the let-7 microRNA family. Cell 2005; 120: 635-647.

[14]   Tsonis PA, Call MK, Grogg MW, Sartor MA, Taylor RR, Forge A, Fyffe R, Goldenberg R, Cowper-Sal Lari R and Tomlinson CR. MicroRNAs and regeneration: Let-7 members as potential regulators of dedifferentiation in lens and inner ear hair cell regeneration of the adult newt. Biochem Biophys Res Commun 2007; 362: 940-945.

[15]   Frost RJ and Olson EN. Control of glucose homeostasis and insulin sensitivity by the Let-7 family of microRNAs. Proc Natl Acad Sci U S A 2011; 108: 21075-21080.

[16]   Park SM, Shell S, Radjabi AR, Schickel R, Feig C, Boyerinas B, Dinulescu DM, Lengyel E and Peter ME. Let-7 prevents early cancer progression by suppressing expression of the embryonic gene HMGA2. Cell Cycle 2007; 6: 2585-2590.

[17]   Shell S, Park SM, Radjabi AR, Schickel R, Kistner EO, Jewell DA, Feig C, Lengyel E and Peter ME. Let-7 expression defines two differentiation stages of cancer. Proc Natl Acad Sci U S A 2007; 104: 11400-11405.

[18]   Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, Mitsudomi T and Takahashi T. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 2004; 64: 3753-3756.

[19]   Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu CG, Croce CM and Harris CC. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 2006; 9: 189-198.

[20]   Dahiya N, Sherman-Baust CA, Wang TL, Davidson B, Shih Ie M, Zhang Y, Wood W 3rd, Becker KG and Morin PJ. MicroRNA expression and identification of putative miRNA targets in ovarian cancer. PLoS One 2008; 3: e2436.

[21]   Lu L, Schwartz P, Scarampi L, Rutherford T, Canuto EM, Yu H and Katsaros D. MicroRNA let-7a: a potential marker for selection of paclitaxel in ovarian cancer management. Gynecol Oncol 2011; 122: 366-371.

[22]   Tang Z, Ow GS, Thiery JP, Ivshina AV and Kuznetsov VA. Meta-analysis of transcriptome reveals let-7b as an unfavorable prognostic biomarker and predicts molecular and clinical subclasses in high-grade serous ovarian carcinoma. Int J Cancer 2014; 134: 306-318.

[23]   Boyerinas B, Park SM, Murmann AE, Gwin K, Montag AG, Zillhardt M, Hua YJ, Lengyel E and Peter ME. Let-7 modulates acquired resistance of ovarian cancer to Taxanes via IMP-1-mediated stabilization of multidrug resistance 1. Int J Cancer 2012; 130: 1787-1797.

[24]   Mezzanzanica D, Bagnoli M, De Cecco L, Valeri B and Canevari S. Role of microRNAs in ovarian cancer pathogenesis and potential clinical implications. Int J Biochem Cell Biol 2010; 42: 1262-1272.

[25]   Lu L, Katsaros D, de la Longrais IA, Sochirca O and Yu H. Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis. Cancer Res 2007; 67: 10117-10122.

[26]   Sugimura K, Miyata H, Tanaka K, Hamano R, Takahashi T, Kurokawa Y, Yamasaki M, Nakajima K, Takiguchi S, Mori M and Doki Y. Let-7 expression is a significant determinant of response to chemotherapy through the regulation of IL-6/STAT3 pathway in esophageal squamous cell carcinoma. Clin Cancer Res 2012; 18: 5144-5153.

[27]   Oh JS, Kim JJ, Byun JY and Kim IA. Lin28-let7 modulates radiosensitivity of human cancer cells with activation of K-Ras. Int J Radiat Oncol Biol Phys 2010; 76: 5-8.

[28]   Hummel R, Hussey DJ and Haier J. MicroRNAs: predictors and modifiers of chemo- and radio-

therapy in different tumour types. Eur J Cancer 2010; 46: 298-311.

[29] Lu L, Katsaros D, Shaverdashvili K, Qian B, Wu Y, de la Longrais IA, Preti M, Menato G and Yu H. Pluripotent factor lin-28 and its homologue lin-28b in epithelial ovarian cancer and their associations with disease outcomes and expression of let-7a and IGF-II. Eur J Cancer 2009; 45: 2212-2218.

[30] Heo I, Joo C, Cho J, Ha M, Han J and Kim VN. Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. Mol Cell 2008; 32: 276-284.

[31] Viswanathan SR, Daley GQ and Gregory RI. Selective blockade of microRNA processing by Lin28. Science 2008; 320: 97-100.

[32] Newman MA, Thomson JM and Hammond SM. Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing. RNA 2008; 14: 1539-1549.

[33] Brueckner B, Stresemann C, Kuner R, Mund C, Musch T, Meister M, Sultmann H and Lyko F. The human let-7a-3 locus contains an epigenetically regulated microRNA gene with oncogenic function. Cancer Res 2007; 67: 1419-1423.

[34] Vrba L, Munoz-Rodriguez JL, Stampfer MR and Futscher BW. miRNA gene promoters are frequent targets of aberrant DNA methylation in human breast cancer. PLoS One 2013; 8: e54398.

[35] Ko YC, Fang WH, Lin TC, Hou HA, Chen CY, Tien HF and Lin LI. MicroRNA let-7a-3 gene methylation is associated with karyotyping, CEBPA promoter methylation, and survival in acute myeloid leukemia. Leuk Res 2014; 38: 625-631.

[36] Lu L, Katsaros D, Mayne ST, Risch HA, Benedetto C, Canuto EM and Yu H. Functional study of risk loci of stem cell-associated gene lin-28B and associations with disease survival outcomes in epithelial ovarian cancer. Carcinogenesis 2012; 33: 2119-2125.

[37] Guo Y, Chen Y, Ito H, Watanabe A, Ge X, Kodama T and Aburatani H. Identification and characterization of lin-28 homolog B (LIN28B) in human hepatocellular carcinoma. Gene 2006; 384: 51-61.

[38] Viswanathan SR, Powers JT, Einhorn W, Hoshida Y, Ng TL, Toffanin S, O'Sullivan M, Lu J, Phillips LA, Lockhart VL, Shah SP, Tanwar PS, Mermel CH, Beroukhim R, Azam M, Teixeira J, Meyerson M, Hughes TP, Llovet JM, Radich J, Mulligan CG, Golub TR, Sorensen PH and Daley GQ. Lin28 promotes transformation and is associated with advanced human malignancies. Nat Genet 2009; 41: 843-848.

[39] Wang YC, Chen YL, Yuan RH, Pan HW, Yang WC, Hsu HC and Jeng YM. Lin-28B expression promotes transformation and invasion in human hepatocellular carcinoma. Carcinogenesis 2010; 31: 1516-1522.

[40] Zhang L, Volinia S, Bonome T, Calin GA, Greshock J, Yang N, Liu CG, Giannakakis A, Alexiou P, Hasegawa K, Johnstone CN, Megraw MS, Adams S, Lassus H, Huang J, Kaur S, Liang S, Sethupathy P, Leminen A, Simossis VA, Sandaltzopoulos R, Naomoto Y, Katsaros D, Gimotty PA, DeMichele A, Huang Q, Butzow R, Rustgi AK, Weber BL, Birrer MJ, Hatzigeorgiou AG, Croce CM and Coukos G. Genomic and epigenetic alterations deregulate microRNA expression in human epithelial ovarian cancer. Proc Natl Acad Sci U S A 2008; 105: 7004-7009.

[41] Mayr C, Hemann MT and Bartel DP. Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science 2007; 315: 1576-1579.

[42] Kallen AN, Zhou XB, Xu J, Qiao C, Ma J, Yan L, Lu L, Liu C, Yi JS, Zhang H, Min W, Bennett AM, Gregory RI, Ding Y and Huang Y. The imprinted H19 lncRNA antagonizes let-7 microRNAs. Mol Cell 2013; 52: 101-112.

[43] Xie R, Wang Y, Nie W, Huang W, Song W, Wang Z and Guan X. Lin28B Expression Correlates with Aggressive Clinicopathological Characteristics in Breast Invasive Ductal Carcinoma. Cancer Biother Radiopharm 2014; 29: 215-20.

[44] Hovestadt V, Jones DT, Picelli S, Wang W, Kool M, Northcott PA, Sultan M, Stachurski K, Ryzhova M, Warnatz HJ, Ralser M, Brun S, Bunt J, Jager N, Kleinheinz K, Erkek S, Weber UD, Bartholomae CC, von Kalle C, Lawerenz C, Eils J, Koster J, Versteeg R, Milde T, Witt O, Schmidt S, Wolf S, Pietsch T, Rutkowski S, Scheurlen W, Taylor MD, Brors B, Felsberg J, Reifenberger G, Borkhardt A, Lehrach H, Wechsler-Reya RJ, Eils R, Yaspo ML, Landgraf P, Korshunov A, Zapatka M, Radlwimmer B, Pfister SM and Lichter P. Decoding the regulatory landscape of medulloblastoma using DNA methylation sequencing. Nature 2014; 510: 537-41.

[45] Wu T, Jia J, Xiong X, He H, Bu L, Zhao Z, Huang C and Zhang W. Increased expression of Lin28B associates with poor prognosis in patients with oral squamous cell carcinoma. PLoS One 2013; 8: e83869.

[46] Cheng SW, Tsai HW, Lin YJ, Cheng PN, Chang YC, Yen CJ, Huang HP, Chuang YP, Chang TT, Lee CT, Chao A, Chou CY, Chan SH, Chow NH and Ho CL. Lin28B is an oncofetal circulating cancer stem cell-like marker associated with recurrence of hepatocellular carcinoma. PLoS One 2013; 8: e80053.

[47] Guo L, Chen C, Shi M, Wang F, Chen X, Diao D, Hu M, Yu M, Qian L and Guo N. Stat3-coordi-

nated Lin-28-let-7-HMGA2 and miR-200-ZEB1 circuits initiate and maintain oncostatin M-driven epithelial-mesenchymal transition. Oncogene 2013; 32: 5272-5282.

[48] Madison BB, Liu Q, Zhong X, Hahn CM, Lin N, Emmett MJ, Stanger BZ, Lee JS and Rustgi AK. LIN28B promotes growth and tumorigenesis of the intestinal epithelium via Let-7. Genes Dev 2013; 27: 2233-2245.

[49] Copley MR, Babovic S, Benz C, Knapp DJ, Beer PA, Kent DG, Wohrer S, Treloar DQ, Day C, Rowe K, Mader H, Kuchenbauer F, Humphries RK and Eaves CJ. The Lin28b-let-7-Hmga2 axis determines the higher self-renewal potential of fetal haematopoietic stem cells. Nat Cell Biol 2013; 15: 916-925.

[50] Lee YT, de Vasconcellos JF, Yuan J, Byrnes C, Noh SJ, Meier ER, Kim KS, Rabel A, Kaushal M, Muljo SA and Miller JL. LIN28B-mediated expression of fetal hemoglobin and production of fetal-like erythrocytes from adult human erythroblasts ex vivo. Blood 2013; 122: 1034-1041.

[51] Alajez NM, Shi W, Wong D, Lenarduzzi M, Waldron J, Weinreb I and Liu FF. Lin28b promotes head and neck cancer progression via modulation of the insulin-like growth factor survival pathway. Oncotarget 2012; 3: 1641-1652.

[52] Lu L, Katsaros D, Wiley A, Rigault de la Longrais IA, Risch HA, Puopolo M and Yu H. The relationship of insulin-like growth factor-II, insulin-like growth factor binding protein-3, and estrogen receptor-alpha expression to disease progression in epithelial ovarian cancer. Clin Cancer Res 2006; 12: 1208-1214.

[53] Lu L, Katsaros D, Wiley A, Rigault de la Longrais IA, Puopolo M, Schwartz P and Yu H. Promoter-specific transcription of insulin-like growth factor-II in epithelial ovarian cancer. Gynecol Oncol 2006; 103: 990-995.

[54] Huang YF, Cheng WF, Wu YP, Cheng YM, Hsu KF and Chou CY. Circulating IGF system and treatment outcome in epithelial ovarian cancer. Endocr Relat Cancer 2014; 21: 217-229.

[55] Lu Y, Lu P, Zhu Z, Xu H and Zhu X. Loss of imprinting of insulin-like growth factor 2 is associated with increased risk of lymph node metastasis and gastric corpus cancer. J Exp Clin Cancer Res 2009; 28: 125.

[56] Trajkovic-Arsic M, Kalideris E and Siveke JT. The role of insulin and IGF system in pancreatic cancer. J Mol Endocrinol 2013; 50: R67-74.

[57] Gebeshuber CA and Martinez J. miR-100 suppresses IGF2 and inhibits breast tumorigenesis by interfering with proliferation and survival signaling. Oncogene 2013; 32: 3306-3310.

[58] Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer 2008; 8: 915-928.

[59] Jelinic P and Shaw P. Loss of imprinting and cancer. J Pathol 2007; 211: 261-268.

[60] Lu L, Katsaros D, Zhu Y, Hoffman A, Luca S, Marion CE, Mu L, Risch H and Yu H. Let-7a regulation of insulin-like growth factors in breast cancer. Breast Cancer Res Treat 2011; 126: 687-694.

[61] Chang TC, Zeitels LR, Hwang HW, Chivukula RR, Wentzel EA, Dews M, Jung J, Gao P, Dang CV, Beer MA, Thomas-Tikhonenko A and Mendell JT. Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation. Proc Natl Acad Sci U S A 2009; 106: 3384-3389.

[62] Park JH, Wacholder S, Gail MH, Peters U, Jacobs KB, Chanock SJ and Chatterjee N. Estimation of effect size distribution from genome-wide association studies and implications for future discoveries. Nat Genet 2010; 42: 570-575.

[63] Park JH, Gail MH, Weinberg CR, Carroll RJ, Chung CC, Wang Z, Chanock SJ, Fraumeni JF Jr and Chatterjee N. Distribution of allele frequencies and effect sizes and their interrelationships for common genetic susceptibility variants. Proc Natl Acad Sci U S A 2011; 108: 18026-18031.

[64] Nishimura R, Okumura Y and Arima N. Trastuzumab monotherapy versus combination therapy for treating recurrent breast cancer: time to progression and survival. Breast Cancer 2008; 15: 57-64.

[65] Hu JK, Wang X and Wang P. Testing gene-gene interactions in genome wide association studies. Genet Epidemiol 2014; 38: 123-134.

[66] Pang H, Hauser M and Minvielle S. Pathway-based identification of SNPs predictive of survival. Eur J Hum Genet 2011; 19: 704-709.

[67] Locke AE, Kahali B, Berndt SI, Justice AE, Pers TH, Day FR, Powell C, Vedantam S, Buchkovich ML, Yang J, Croteau-Chonka DC, Esko T, Fall T, Ferreira T, Gustafsson S, Kutalik Z, Luan J, Magi R, Randall JC, Winkler TW, Wood AR, Workalemahu T, Faul JD, Smith JA, Hua Zhao J, Zhao W, Chen J, Fehrmann R, Hedman AK, Karjalainen J, Schmidt EM, Absher D, Amin N, Anderson D, Beekman M, Bolton JL, Bragg-Gresham JL, Buyske S, Demirkan A, Deng G, Ehret GB, Feenstra B, Feitosa MF, Fischer K, Goel A, Gong J, Jackson AU, Kanoni S, Kleber ME, Kristiansson K, Lim U, Lotay V, Mangino M, Mateo Leach I, Medina-Gomez C, Medland SE, Nalls MA, Palmer CD, Pasko D, Pechlivanis S, Peters MJ, Prokopenko I, Shungin D, Stancakova A, Strawbridge RJ, Ju Sung Y, Tanaka T, Teumer A, Trompet S, van der Laan SW, van Setten J, Van Vliet-Ostaptchouk JV, Wang Z, Yengo L, Zhang W, Isaacs A, Albrecht E, Arnlov J, Arscott GM, Attwood AP, Bandinelli S, Barrett

A, Bas IN, Bellis C, Bennett AJ, Berne C, Blagieva R, Bluher M, Bohringer S, Bonnycastle LL, Bottcher Y, Boyd HA, Bruinenberg M, Caspersen IH, Ida Chen YD, Clarke R, Daw EW, de Craen AJ, Delgado G, Dimitriou M, Doney AS, Eklund N, Estrada K, Eury E, Folkersen L, Fraser RM, Garcia ME, Geller F, Giedraitis V, Gigante B, Go AS, Golay A, Goodall AH, Gordon SD, Gorski M, Grabe HJ, Grallert H, Grammer TB, Grassler J, Gronberg H, Groves CJ, Gusto G, Haessler J, Hall P, Haller T, Hallmans G, Hartman CA, Hassinen M, Hayward C, Heard-Costa NL, Helmer Q, Hengstenberg C, Holmen O, Hottenga JJ, James AL, Jeff JM, Johansson A, Jolley J, Juliusdottir T, Kinnunen L, Koenig W, Koskenvuo M, Kratzer W, Laitinen J, Lamina C, Leander K, Lee NR, Lichtner P, Lind L, Lindstrom J, Sin Lo K, Lobbens S, Lorbeer R, Lu Y, Mach F, Magnusson PK, Mahajan A, McArdle WL, McLachlan S, Menni C, Merger S, Mihailov E, Milani L, Moayyeri A, Monda KL, Morken MA, Mulas A, Muller G, Muller-Nurasyid M, Musk AW, Nagaraja R, Nothen MM, Nolte IM, Pilz S, Rayner NW, Renstrom F, Rettig R, Ried JS, Ripke S, Robertson NR, Rose LM, Sanna S, Scharnagl H, Scholtens S, Schumacher FR, Scott WR, Seufferlein T, Shi J, Vernon Smith A, Smolonska J, Stanton AV, Steinthorsdottir V, Stirrups K, Stringham HM, Sundstrom J, Swertz MA, Swift AJ, Syvanen AC, Tan ST, Tayo BO, Thorand B, Thorleifsson G, Tyrer JP, Uh HW, Vandenput L, Verhulst FC, Vermeulen SH, Verweij N, Vonk JM, Waite LL, Warren HR, Waterworth D, Weedon MN, Wilkens LR, Willenborg C, Wilsgaard T, Wojczynski MK, Wong A, Wright AF, Zhang Q, LifeLines Cohort S, Brennan EP, Choi M, Dastani Z, Drong AW, Eriksson P, Franco-Cereceda A, Gadin JR, Gharavi AG, Goddard ME, Handsaker RE, Huang J, Karpe F, Kathiresan S, Keildson S, Kiryluk K, Kubo M, Lee JY, Liang L, Lifton RP, Ma B, McCarroll SA, McKnight AJ, Min JL, Moffatt MF, Montgomery GW, Murabito JM, Nicholson G, Nyholt DR, Okada Y, Perry JR, Dorajoo R, Reinmaa E, Salem RM, Sandholm N, Scott RA, Stolk L, Takahashi A, Tanaka T, Van't Hooft FM, Vinkhuyzen AA, Westra HJ, Zheng W, Zondervan KT, Consortium AD, Group A-BW, Consortium CAD, Consortium CK, Glgc, Icbp, Investigators M, Mu TC, Consortium MI, Consortium P, ReproGen C, Consortium G, International Endogene C, Heath AC, Arveiler D, Bakker SJ, Beilby J, Bergman RN, Blangero J, Bovet P, Campbell H, Caulfield MJ, Cesana G, Chakravarti A, Chasman DI, Chines PS, Collins FS, Crawford DC, Cupples LA, Cusi D, Danesh J, de Faire U, den Ruijter HM, Dominiczak AF, Erbel R, Erdmann J, Eriksson JG, Farrall M, Felix SB, Ferrannini E, Ferrieres J, Ford I, Forouhi NG, Forrester T, Franco OH, Gansevoort RT, Gejman PV, Gieger C, Gottesman O, Gudnason V, Gyllensten U, Hall AS, Harris TB, Hattersley AT, Hicks AA, Hindorff LA, Hingorani AD, Hofman A, Homuth G, Hovingh GK, Humphries SE, Hunt SC, Hypponen E, Illig T, Jacobs KB, Jarvelin MR, Jockel KH, Johansen B, Jousilahti P, Jukema JW, Jula AM, Kaprio J, Kastelein JJ, Keinanen-Kiukaanniemi SM, Kiemeney LA, Knekt P, Kooner JS, Kooperberg C, Kovacs P, Kraja AT, Kumari M, Kuusisto J, Lakka TA, Langenberg C, Le Marchand L, Lehtimaki T, Lyssenko V, Mannisto S, Marette A, Matise TC, McKenzie CA, McKnight B, Moll FL, Morris AD, Morris AP, Murray JC, Nelis M, Ohlsson C, Oldehinkel AJ, Ong KK, Madden PA, Pasterkamp G, Peden JF, Peters A, Postma DS, Pramstaller PP, Price JF, Qi L, Raitakari OT, Rankinen T, Rao DC, Rice TK, Ridker PM, Rioux JD, Ritchie MD, Rudan I, Salomaa V, Samani NJ, Saramies J, Sarzynski MA, Schunkert H, Schwarz PE, Sever P, Shuldiner AR, Sinisalo J, Stolk RP, Strauch K, Tonjes A, Tregouet DA, Tremblay A, Tremoli E, Virtamo J, Vohl MC, Volker U, Waeber G, Willemsen G, Witteman JC, Zillikens MC, Adair LS, Amouyel P, Asselbergs FW, Assimes TL, Bochud M, Boehm BO, Boerwinkle E, Bornstein SR, Bottinger EP, Bouchard C, Cauchi S, Chambers JC, Chanock SJ, Cooper RS, de Bakker PI, Dedoussis G, Ferrucci L, Franks PW, Froguel P, Groop LC, Haiman CA, Hamsten A, Hui J, Hunter DJ, Hveem K, Kaplan RC, Kivimaki M, Kuh D, Laakso M, Liu Y, Martin NG, Marz W, Melbye M, Metspalu A, Moebus S, Munroe PB, Njolstad I, Oostra BA, Palmer CN, Pedersen NL, Perola M, Perusse L, Peters U, Power C, Quertermous T, Rauramaa R, Rivadeneira F, Saaristo TE, Saleheen D, Sattar N, Schadt EE, Schlessinger D, Slagboom PE, Snieder H, Spector TD, Thorsteinsdottir U, Stumvoll M, Tuomilehto J, Uitterlinden AG, Uusitupa M, van der Harst P, Walker M, Wallaschofski H, Wareham NJ, Watkins H, Weir DR, Wichmann HE, Wilson JF, Zanen P, Borecki IB, Deloukas P, Fox CS, Heid IM, O'Connell JR, Strachan DP, Stefansson K, van Duijn CM, Abecasis GR, Franke L, Frayling TM, McCarthy MI, Visscher PM, Scherag A, Willer CJ, Boehnke M, Mohlke KL, Lindgren CM, Beckmann JS, Barroso I, North KE, Ingelsson E, Hirschhorn JN, Loos RJ and Speliotes EK. Genetic studies of body mass index yield new insights for obesity biology. Nature 2015; 518: 197-206.

[68] Lu L, Risch E, Deng Q, Biglia N, Picardo E, Katsaros D and Yu H. An insulin-like growth factor-II intronic variant affects local DNA conformation and ovarian cancer survival. Carcinogenesis 2013; 34: 2024-2030.

[69] Pearce CL, Doherty JA, Van Den Berg DJ, Moysich K, Hsu C, Cushing-Haugen KL, Conti DV,

Ramus SJ, Gentry-Maharaj A, Menon U, Gayther SA, Pharoah PD, Song H, Kjaer SK, Hogdall E, Hogdall C, Whittemore AS, McGuire V, Sieh W, Gronwald J, Medrek K, Jakubowska A, Lubinski J, Chenevix-Trench G, Beesley J, Webb PM, Berchuck A, Schildkraut JM, Iversen ES, Moorman PG, Edlund CK, Stram DO, Pike MC, Ness RB, Rossing MA and Wu AH. Genetic variation in insulin-like growth factor 2 may play a role in ovarian cancer risk. Hum Mol Genet 2011; 20: 2263-2272.

[70] Permuth-Wey J, Kim D, Tsai YY, Lin HY, Chen YA, Barnholtz-Sloan J, Birrer MJ, Bloom G, Chanock SJ, Chen Z, Cramer DW, Cunningham JM, Dagne G, Ebbert-Syfrett J, Fenstermacher D, Fridley BL, Garcia-Closas M, Gayther SA, Ge W, Gentry-Maharaj A, Gonzalez-Bosquet J, Goode EL, Iversen E, Jim H, Kong W, McLaughlin J, Menon U, Monteiro AN, Narod SA, Pharoah PD, Phelan CM, Qu X, Ramus SJ, Risch H, Schildkraut JM, Song H, Stockwell H, Sutphen R, Terry KL, Tyrer J, Vierkant RA, Wentzensen N, Lancaster JM, Cheng JQ and Sellers TA. LIN28B polymorphisms influence susceptibility to epithelial ovarian cancer. Cancer Res 2011; 71: 3896-3903.

[71] Elks CE, Perry JR, Sulem P, Chasman DI, Franceschini N, He C, Lunetta KL, Visser JA, Byrne EM, Cousminer DL, Gudbjartsson DF, Esko T, Feenstra B, Hottenga JJ, Koller DL, Kutalik Z, Lin P, Mangino M, Marongiu M, McArdle PF, Smith AV, Stolk L, van Wingerden SH, Zhao JH, Albrecht E, Corre T, Ingelsson E, Hayward C, Magnusson PK, Smith EN, Ulivi S, Warrington NM, Zgaga L, Alavere H, Amin N, Aspelund T, Bandinelli S, Barroso I, Berenson GS, Bergmann S, Blackburn H, Boerwinkle E, Buring JE, Busonero F, Campbell H, Chanock SJ, Chen W, Cornelis MC, Couper D, Coviello AD, d'Adamo P, de Faire U, de Geus EJ, Deloukas P, Doring A, Smith GD, Easton DF, Eiriksdottir G, Emilsson V, Eriksson J, Ferrucci L, Folsom AR, Foroud T, Garcia M, Gasparini P, Geller F, Gieger C, Gudnason V, Hall P, Hankinson SE, Ferreli L, Heath AC, Hernandez DG, Hofman A, Hu FB, Illig T, Jarvelin MR, Johnson AD, Karasik D, Khaw KT, Kiel DP, Kilpelainen TO, Kolcic I, Kraft P, Launer LJ, Laven JS, Li S, Liu J, Levy D, Martin NG, McArdle WL, Melbye M, Mooser V, Murray JC, Murray SS, Nalls MA, Navarro P, Nelis M, Ness AR, Northstone K, Oostra BA, Peacock M, Palmer LJ, Palotie A, Pare G, Parker AN, Pedersen NL, Peltonen L, Pennell CE, Pharoah P, Polasek O, Plump AS, Pouta A, Porcu E, Rafnar T, Rice JP, Ring SM, Rivadeneira F, Rudan I, Sala C, Salomaa V, Sanna S, Schlessinger D, Schork NJ, Scuteri A, Segre AV, Shuldiner AR, Soranzo N, Sovio U, Srinivasan SR, Strachan DP, Tammesoo ML, Tikkanen E, Toniolo D, Tsui K, Tryg-

gvadottir L, Tyrer J, Uda M, van Dam RM, van Meurs JB, Vollenweider P, Waeber G, Wareham NJ, Waterworth DM, Weedon MN, Wichmann HE, Willemsen G, Wilson JF, Wright AF, Young L, Zhai G, Zhuang WV, Bierut LJ, Boomsma DI, Boyd HA, Crisponi L, Demerath EW, van Duijn CM, Econs MJ, Harris TB, Hunter DJ, Loos RJ, Metspalu A, Montgomery GW, Ridker PM, Spector TD, Streeten EA, Stefansson K, Thorsteinsdottir U, Uitterlinden AG, Widen E, Murabito JM, Ong KK and Murray A. Thirty new loci for age at menarche identified by a meta-analysis of genome-wide association studies. Nat Genet 2010; 42: 1077-1085.

[72] Zeng Y, Qu LK, Meng L, Liu CY, Dong B, Xing XF, Wu J and Shou CC. HIWI expression profile in cancer cells and its prognostic value for patients with colorectal cancer. Chin Med J (Engl) 2011; 124: 2144-2149.

[73] Ong KK, Elks CE, Li S, Zhao JH, Luan J, Andersen LB, Bingham SA, Brage S, Smith GD, Ekelund U, Gillson CJ, Glaser B, Golding J, Hardy R, Khaw KT, Kuh D, Luben R, Marcus M, McGeehin MA, Ness AR, Northstone K, Ring SM, Rubin C, Sims MA, Song K, Strachan DP, Vollenweider P, Waeber G, Waterworth DM, Wong A, Deloukas P, Barroso I, Mooser V, Loos RJ and Wareham NJ. Genetic variation in LIN28B is associated with the timing of puberty. Nat Genet 2009; 41: 729-733.

[74] Sulem P, Gudbjartsson DF, Rafnar T, Holm H, Olafsdottir EJ, Olafsdottir GH, Jonsson T, Alexandersen P, Feenstra B, Boyd HA, Aben KK, Verbeek AL, Roeleveld N, Jonasdottir A, Styrkarsdottir U, Steinthorsdottir V, Karason A, Stacey SN, Gudmundsson J, Jakobsdottir M, Thorleifsson G, Hardarson G, Gulcher J, Kong A, Kiemeney LA, Melbye M, Christiansen C, Tryggvadottir L, Thorsteinsdottir U and Stefansson K. Genome-wide association study identifies sequence variants on 6q21 associated with age at menarche. Nat Genet 2009; 41: 734-738.

[75] He W, Wang Z, Wang Q, Fan Q, Shou C, Wang J, Giercksky KE, Nesland JM and Suo Z. Expression of HIWI in human esophageal squamous cell carcinoma is significantly associated with poorer prognosis. BMC Cancer 2009; 9: 426.

[76] Perry JR, Stolk L, Franceschini N, Lunetta KL, Zhai G, McArdle PF, Smith AV, Aspelund T, Bandinelli S, Boerwinkle E, Cherkas L, Eiriksdottir G, Estrada K, Ferrucci L, Folsom AR, Garcia M, Gudnason V, Hofman A, Karasik D, Kiel DP, Launer LJ, van Meurs J, Nalls MA, Rivadeneira F, Shuldiner AR, Singleton A, Soranzo N, Tanaka T, Visser JA, Weedon MN, Wilson SG, Zhuang V, Streeten EA, Harris TB, Murray A, Spector TD, Demerath EW, Uitterlinden AG and Murabito JM. Meta-analysis of genome-wide associa-

tion data identifies two loci influencing age at menarche. Nat Genet 2009; 41: 648-650.

[77] Bearfoot JL, Choong DY, Gorringe KL and Campbell IG. Genetic analysis of cancer-implicated MicroRNA in ovarian cancer. Clin Cancer Res 2008; 14: 7246-7250.

[78] Hu Z, Chen J, Tian T, Zhou X, Gu H, Xu L, Zeng Y, Miao R, Jin G, Ma H, Chen Y and Shen H. Genetic variants of miRNA sequences and non-small cell lung cancer survival. J Clin Invest 2008; 118: 2600-2608.

[79] Jazdzewski K, Murray EL, Franssila K, Jarzab B, Schoenberg DR and de la Chapelle A. Common SNP in pre-miR-146a decreases mature miR expression and predisposes to papillary thyroid carcinoma. Proc Natl Acad Sci U S A 2008; 105: 7269-7274.

[80] Hu Y, Liu CM, Qi L, He TZ, Shi-Guo L, Hao CJ, Cui Y, Zhang N, Xia HF and Ma X. Two common SNPs in pri-miR-125a alter the mature miRNA expression and associate with recurrent pregnancy loss in a Han-Chinese population. RNA Biol 2011; 8: 861-872.

[81] Ribic CM, Sargent DJ, Moore MJ, Thibodeau SN, French AJ, Goldberg RM, Hamilton SR, Laurent-Puig P, Gryfe R, Shepherd LE, Tu D, Redston M and Gallinger S. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N Engl J Med 2003; 349: 247-257.

[82] Scully R, Sobin L and Serov S. Histological typing of ovarian tumors. Berlin (NY): Springer; 1999.

[83] Niu T, Qin ZS, Xu X and Liu JS. Bayesian haplotype inference for multiple linked single-nucleotide polymorphisms. Am J Hum Genet 2002; 70: 157-169.

[84] Song C, Chen GK, Millikan RC, Ambrosone CB, John EM, Bernstein L, Zheng W, Hu JJ, Ziegler RG, Nyante S, Bandera EV, Ingles SA, Press MF, Deming SL, Rodriguez-Gil JL, Chanock SJ, Wan P, Sheng X, Pooler LC, Van Den Berg DJ, Le Marchand L, Kolonel LN, Henderson BE, Haiman CA and Stram DO. A genome-wide scan for breast cancer risk haplotypes among African American women. PLoS One 2013; 8: e57298.

[85] Fallin D and Schork NJ. Accuracy of haplotype frequency estimation for biallelic loci, via the expectation-maximization algorithm for unphased diploid genotype data. Am J Hum Genet 2000; 67: 947-959.

[86] Yang R, Schlehe B, Hemminki K, Sutter C, Bugert P, Wappenschmidt B, Volkmann J, Varon R, Weber BH, Niederacher D, Arnold N, Meindl A, Bartram CR, Schmutzler RK and Burwinkel B. A genetic variant in the pre-miR-27a oncogene is associated with a reduced familial breast cancer risk. Breast Cancer Res Treat 2010; 121: 693-702.

[87] Slaby O, Bienertova-Vasku J, Svoboda M and Vyzula R. Genetic polymorphisms and microRNAs: new direction in molecular epidemiology of solid cancer. J Cell Mol Med 2012; 16: 8-21.