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# Genetic variation in a miR-335 binding site in BIRC5 alters susceptibility to lung cancer in Chinese Han populations

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ABSTRACT

Polymorphisms in 3′ untranslated region (UTR) of cancer-related genes might affect their regulation by microRNAs (miRNAs) and thereby contribute to carcinogenesis. In this study, we screened single nucleotide polymorphisms (SNPs) in 3′ UTR of cancer-related genes and investigated their effects on risk of lung cancer. First, we genotyped seven SNPs in a Chinese Han population with 600 lung cancer patients and 600 matched healthy controls and found that compared with the TT genotype of rs2239680 in 3′ UTR of baculoviral IAP repeat containing 5 (BIRC5), C allele was associated with a significantly increased risk of lung cancer and advanced pathologic stage, with the odds ratio for participants carrying the CT or CC genotype being 1.50 [95% confidence interval (CI) 1.20–1.89, $P < 0.01$] and 2.29 (95% CI 1.64–3.18, $P < 0.01$), respectively. These results were further replicated and confirmed in another independent population with 1000 lung cancer cases and 1000 matched healthy controls. In support of the postulation that the 3′ UTR SNP may directly affect miRNA-binding site, reporter gene assays indicated BIRC5 was a direct target of miR-335, and the rs2239680 T > C change resulted in altered regulation of BIRC5 expression. Moreover, BIRC5 was over expressed in lung cancer tissues compared with the normal lung tissues, and the protein levels of BIRC5 correlated with SNP genotypes in normal lung tissues. Our findings defined a 3′ UTR SNP in human *BIRC5* oncogene that may increase individual susceptibility to lung cancer probably by attenuating the interaction between miR-335 and BIRC5.



## 1. Introduction

Lung cancer is the most frequently occurring malignancy with increasing incidence and is the leading cause of mortality in cancer-related deaths in China and worldwide [1,2]. Epidemiologic and biologic studies have confirmed an etiologic link between tobacco smoking and lung cancer risk [3]. However, only a small number of those exposed to tobacco develop lung cancer, suggesting that genetic heterogeneity may also play a role [4]. In addition, lung cancer patients with similar stages or histological classifications have dramatically different responses to anticancer therapies and distinct survival outcomes, likely due to heterogeneity of gene/protein expression profiles [5]. Therefore, novel molecular biomarkers and genetic variants of the key process genes may assist in identifying high-risk subpopulations for lung cancer.

MicroRNAs (miRNAs) are non-protein-coding RNA molecules that can regulate the translation or degradation of about 30% human gene messenger RNAs (mRNAs) by binding with the 3′ untranslated regions (3′ UTRs) of the genes [6–8]. They can also function as tumor suppressors and/or oncogenes [9]. It has been proposed that single nucleotide polymorphisms (SNPs) located in 3′ UTR of genes might affect miRNA-mediated gene regulation and thus contribute to individual susceptibility to cancers [10–13]. Therefore, we hypothesized that SNPs located in the 3′ UTR of cancer-related genes might be associated with the susceptibility and progression of lung cancer. Seven miRNA-binding SNPs in cancer-related genes were chosen by literature review and bioinformatics tools. Among the SNPs, rs2239680 in baculoviral IAP repeat containing 5 (*BIRC5*) was included. *BIRC5*, also named survivin, is a member of the inhibitor of apoptosis (IAP) family. BIRC5 can inhibit caspase activation and negatively regulate the apoptosis or programmed cell death. BIRC5 is highly expressed in most human tumors and fetal tissues, but is completely absent in terminally differentiated cells [14]. Abnormal activation of BIRC5 can contribute to malignancy via distinct signal pathways [15].

*Abbreviations:* 3′ UTR, 3′ untranslated region; BIRC5, baculoviral IAP repeat containing 5; CI, confidence interval; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; MiRNA, microRNA; OR, odds ratio; SNP, single nucleotide polymorphism.

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However, the molecular mechanisms underlying the regulation of BIRC5 in carcinogenesis remain largely unknown.

In this study, we detected the association between SNPs in the 3′ UTRs of cancer-related genes and risk of lung cancer in two independent Chinese populations. We also validated the functions of the rs2239680 SNP in the miRNA-related regulation of BIRC5.

## 2. Materials and methods

### 2.1. Study population and tissue samples

All participants were Chinese Han from Hubei, Wuhan city. Patients in panel I were enrolled from Tongji hospital, and patients in panel II were from Liyuan hospital. Patients with medical history of radiotherapy or chemotherapy were excluded. Healthy controls were matched with cases by age, sex, and residential area, and they had no medical history of cancers. The study was approved by the ethical committee of Tongji Medical College, and informed consent was obtained from each participant. Questionnaires were used to collect the demographic data of each participant. Tumor and adjacent normal tissues were collected from patients who underwent surgery in Liyuan Hospital.

### 2.2. Cell lines and plasmids

A549 (a human lung cancer cell line) and 16HBE (a human bronchial epithelia cell line) were purchased from Cell Bank of Chinese Academy of Sciences. HEK293T (human embryonic kidney 293) and NCI-H446 (a human lung cancer cell line) were obtained from Chinese Academy of Medical Sciences. The pMIR-REPORT luciferase miRNA expression reporter vector was purchased from ABI (Foster City, CA).

### 2.3. SNP selection

Cancer-related genes were screened from databases and literature review. A 3′ UTR dataset and a miRNA target dataset of human genes were obtained from the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgTables). We used TargetScan (http://www.targetscan.org/), miRBase (http://www.mirbase.org/), miRSNP (http://compbio.uthsc.edu/miRSNP/) and PicTar (http://pictar.mdc-berlin.de/) to choose SNPs in miRNA targeting sites. SNPs with a minor allele frequency (MAF) >5% in Chinese population were selected based on the HapMap CHB database, as listed in Supplementary Table 1.

### 2.4. Genotyping

Genotyping of all subjects was carried out by the 5′-nuclease TaqMan assay using the ABI 7900HT Sequence Detection System (Applied Biosystems). The primers and probes were designed by ABI and were provided in the kits. Genotypes of the selected SNPs in cell lines were determined by direct sequencing.

### 2.5. Luciferase reporter assay

The 3′ UTRs of *BIRC5* gene containing different alleles of rs2239680 were amplified and cloned into the pMIR-REPORT Firefly luciferase reporter vector (Ambion, Grand Island, NY). The accuracy of the plasmid constructs was examined by DNA sequencing. Human cell lines HEK293T and A549 were co-transfected with 100 ng of luciferase reporter construct, 5 ng of the β-gal control plasmid and 10 pmol of miRNAs using 1 μl of lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, NY). MiR-335 mimics and negative control (NC) were purchased from GenePharma (Shanghai, China). The luciferase assays were carried out in triplicates using the luciferase reporter assay system (Promega, Madison, WI) after transfection for 48 h. Measurements of luminescence and absorbance of β-gal were performed on a luminometer (Glomax 20/20; Promega) and enzyme-linked immunosorbent assay (Bio-Rad, Hercules, CA) individually.

### 2.6. RT-PCR

Total RNA was isolated using the RNAiso Plus Kit (TaKaRa, Otsu, Shiga Japan). A total of 100 ng RNA was reverse-transcribed (RT) and subjected to conventional PCR in triplicates using a forward primer (5′-TGACGACCCCATAGAGGAACA-3′) and a reverse primer (5′-CGCACTTTCTCCGCAGTTTC-3′). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control for normalization.

### 2.7. Western blotting

Protein levels were detected using anti-BIRC5 (1:500; Abcam, Cambridge, UK) and anti-GAPDH (1:500; Abcam, Cambridge, UK) antibodies. Quantity One Software (Bio-Rad) was used to quantify the intensity signals.

### 2.8. Statistical analysis

Differences in the general characteristics between cases and controls were evaluated by the Student's *t*-test for continuous variables and the $\chi^2$ test for categorical variables. The associations between SNPs and risk of lung cancer were evaluated by the multivariate logistic regression model. The Hardy–Weinberg equilibrium of SNPs among controls was evaluated by the $\chi^2$ test. Comparisons of BIRC5 expression levels between groups were analyzed by the *t* test or one-way analysis of covariance. All statistical analyses were performed by Statistical Package for Social Sciences software (SPSS version 11.0, Chicago, IL). A value of $P < 0.05$ was considered significant (two-tailed).

## 3. Results

### 3.1. Association between SNPs and risk of lung cancer

The general clinical characteristics of panel I and panel II populations are listed in Table 1. No significant difference in age, sex, and drinking status ($P > 0.05$) was found between patients and controls in both panels. However, 72.0% and 80.0% of the cases were smokers in the two panels respectively, which are much higher than that in the controls ($P < 0.01$). Most patients were divided into stage II lung cancer (55.0% in panel I, and 60.0% in panel II). As presented in Table 2, the genotype frequencies of all SNPs conformed to the Hardy–Weinberg equilibrium in panel I (data not shown). Subjects with the rs2239680 CT and CC genotype had a 1.32-fold ($P = 0.025$) and a 3.43-fold ($P < 0.01$) increased risk of lung cancer respectively, while the combined CT + CC genotype had a 1.50-fold increased lung cancer risk ($P < 0.01$) compared with the TT genotype. No significant association was found between rs6573, rs1476215, rs473698, rs1057035, rs1049931, or rs3757 frequencies and lung cancer risk ($P > 0.05$ for all). In panel II, the genotype frequencies of rs2239680 also conformed to the Hardy–Weinberg equilibrium (data not shown). Subjects with CT, CC, or CT + CC genotypes (rs2239680) also had a significantly increased risk of lung cancer (adjusted odds ratio (OR), 1.27, 1.63, and 1.33, respectively; all $P < 0.01$), indicating that C allele is a risk factor for lung cancer.

**Table 1**
The clinical characteristics of lung cancer patients and controls.

| Variables | Panel I | | | Panel II | | |
|---|---|---|---|---|---|---|
| | Control ($n$ = 600) | Lung cancer ($n$ = 600) | $P$ | Control ($n$ = 1000) | Lung cancer ($n$ = 1000) | $P$ |
| Age, years | 66.3 ± 8.0 | 65.7 ± 8.9 | 0.82[a] | 65.3 ± 5.2 | 66.3 ± 4.5 | 0.86[a] |
| Males, % | 83.7 | 82.6 | 0.85[b] | 80.5 | 82.6 | 0.61[b] |
| Smoking, $n$ (%) | | | <0.01[b] | | | <0.01[b] |
| Never | 295 (49.2) | 168 (28.0) | | 500 (50.0) | 200 (20.0) | |
| Former | 25 (4.2) | 40 (6.7) | | 50 (5.0) | 70 (7.0) | |
| Current | 280 (46.7) | 350 (65.3) | | 450 (45.0) | 730 (73.0) | |
| Alcohol, $n$ (%) | | | 0.67[b] | | | 0.47[b] |
| Yes | 364 (60.7) | 350 (58.3) | | 600 (60.0) | 550 (55.0) | |
| No | 236 (39.3) | 250 (41.7) | | 400 (40.0) | 450 (45.0) | |
| Stage, $n$ (%) | | | | | | |
| I | | 10 (1.6) | | | 50 (5.0) | |
| II | | 330 (55.0) | | | 600 (60.0) | |
| III | | 130 (21.7) | | | 200 (20.0) | |
| IV | | 130 (21.7) | | | 150 (15.0) | |

Data are expressed as mean ± SD or percentages.
[a] Student's $t$ test for the differences between lung cancer patients and controls;
[b] Chi-square tests for the differences between lung cancer patients and controls.

**Table 2**
Association between SNPs genotypes and risk of lung cancer.

| Genotype | Control, $n$ | Lung cancer, $n$ | OR (95% CI) | $P$[a] |
|---|---|---|---|---|
| Panel I | 600 | 600 | | |
| rs2239680 | | | | |
| TT | 360 | 300 | 1.00 (reference) | |
| CT | 219 | 240 | 1.32 (1.04–1.67) | 0.025 |
| CC | 21 | 60 | 3.43 (2.04–5.77) | <0.01 |
| CT + CC | 240 | 300 | 1.50 (1.20–1.89) | <0.01 |
| rs6573 | | | | |
| CC | 440 | 444 | 1.00 (reference) | |
| AC | 150 | 140 | 0.92 (0.70–1.20) | 0.53 |
| AA | 10 | 16 | 1.70 (0.76–3.70) | 0.20 |
| AC + AA | 160 | 156 | 0.97 (0.75–1.25) | 0.80 |
| rs1476215 | | | | |
| TT | 480 | 460 | 1.00 (reference) | |
| AT | 110 | 120 | 1.14 (0.85–1.52) | 0.38 |
| AA | 10 | 20 | 2.08 (0.97–4.51) | 0.06 |
| AT + AA | 120 | 140 | 1.22 (0.92–1.60) | 0.16 |
| rs473698 | | | | |
| CC | 460 | 430 | 1.00 (reference) | |
| GC | 130 | 155 | 1.28 (0.98–1.67) | 0.08 |
| GG | 10 | 15 | 1.61 (0.71–3.61) | 0.25 |
| GC + GG | 140 | 170 | 1.25 (1.00–1.68) | 0.054 |
| rs1057035 | | | | |
| TT | 350 | 380 | 1.00 (reference) | |
| CT | 210 | 190 | 0.83 (0.65–1.06) | 0.14 |
| CC | 40 | 30 | 0.69 (0.42–1.13) | 0.14 |
| CT + CC | 250 | 220 | 0.81 (0.64–1.02) | 0.08 |
| rs1049931 | | | | |
| AA | 340 | 350 | 1.00 (reference) | |
| AG | 220 | 213 | 0.94 (0.74–1.20) | 0.62 |
| GG | 40 | 37 | 0.90 (0.56–1.44) | 0.66 |
| AG + GG | 260 | 250 | 0.93 (0.74–1.17) | 0.56 |
| rs3757 | | | | |
| GG | 410 | 400 | 1.00 (reference) | |
| AG | 170 | 180 | 1.09 (0.85–1.40) | 0.52 |
| AA | 20 | 20 | 1.03 (0.54–1.93) | 0.94 |
| AG + AA | 190 | 200 | 1.08 (0.85–1.37) | 0.54 |
| Panel II | 1000 | 1000 | | |
| rs2239680 | | | | |
| TT | 570 | 500 | 1.00 (reference) | |
| CT | 360 | 400 | 1.27 (1.05–1.53) | <0.01 |
| CC | 70 | 100 | 1.63 (1.17–2.26) | <0.01 |
| CT + CC | 430 | 500 | 1.33 (1.11–1.58) | <0.01 |

BIRC5, baculoviral IAP repeat containing 5; RAP1A, member of RAS oncogene family; FGF2, fibroblast growth factor 2; TGFA, transforming growth factor, alpha; DICER, ribonuclease type III; COL4A2, collagen, type IV, alpha 2; DGCR8, DiGeorge syndrome critical region gene 8.
[a] Adjusted for age, sex, smoking status, and stage.

**Table 3**
Association between rs2239680 genotypes and the stage of lung cancer.

| Genotype | I/II, *n* (%) | III/IV, *n* (%) | OR (95% CI) | *P*[a] |
|---|---|---|---|---|
| Panel I | 340 | 260 | | |
| TT | 200 | 100 | 1.00 (reference) | |
| CT + CC | 140 | 160 | 2.29 (1.64–3.18) | <0.01 |
| Panel II | 650 | 350 | | |
| TT | 350 | 150 | 1.00 (reference) | |
| CT + CC | 300 | 200 | 1.56 (1.20–2.02) | <0.01 |

OR: odds ratio; CI: confidence interval.
[a] Adjusted for age, sex, and smoking.

In addition, the stratification analysis by TNM (Tumor Node and Metastasis) stages showed a significant association between rs2239680 genotypes and lung cancer clinical status ($P < 0.01$, OR = 2.29; 95% CI: 1.64–3.18 in panel I; $P < 0.01$, OR = 1.56; 95% CI: 1.20–2.02) in panel II, respectively; Table 3).

### 3.2. SNP rs2239680 affects the interaction between miR-335 and the 3′ UTR of BIRC5

In silico analysis showed that the rs2239680 SNP generated a binding site for the seed region of miR-335. The T allele matches the predicted miR-335 seed-binding domain, whereas the C allele represents a C: A mismatch base pairing (Fig. 1A). We hypothesized that miR-335 would bind BIRC5 mRNA transcripts containing the T allele, negatively regulating BIRC5 expression. On the other hand, binding of miR-335 to mRNA containing the C allele would be disrupted, allowing upregulation of BIRC5 expression. To test the postulation, the reporter plasmid (Fig. 1B) was transfected into 293T and A549 cell lines along with miR-335 mimics or the NC. We found that cells transfected with vectors containing the T allele and miR-335 mimics yielded a significantly decreased luciferase activity compared with cells transfected with T allele and NC (Fig. 1C). However, no significant difference in the luciferase activity was found between the two groups of cells transfected with vectors containing the C allele and miR-335 mimics or NC. Thus, the T > C change might abolish the negative regulation of BIRC5 expression by miR-335.

To further verify whether the rs2239680 SNP may affect the ability for BIRC5 3′ UTR to interact with miR-335, the mRNA and protein levels of BIRC5 were detected in cells transfected with miR-335 mimics or NC. As shown in Fig. 2, HEK293T and A549 cells are CT homozygote, while 16HBE and NCI-H446 cells are TT heterozygote. Transfection of miR-335 mimics significantly decreased the expression levels of BIRC5 compared with NC in both 293T and A549 cells (Fig. 2A). However, no significant difference in BIRC5 expression levels was found between the two transfection groups in 16HBE and NCI-H446 cells (Fig. 2B).

### 3.3. Effect of SNP rs2239680 on the expression of BIRC5 in lung tissues

BIRC5 was highly expressed in lung cancer tissues compared with the adjacent normal tissues (Fig. 3A, upper), suggesting that BIRC5 may act as a molecular signature in lung cancer. In addition, the BIRC5 protein levels in normal lung tissues were significantly lower in patients carrying rs2239680 TT genotype than those carrying CC genotype ($P = 0.04$, as shown in Fig. 3A lower and Fig. 3B). However, the difference in BIRC protein levels in tumor tissues was not significant among the patients with different genotypes ($P > 0.05$).

## 4. Discussion

In this study, we evaluated the association between genetic variants in 3′ UTRs of the cancer-related genes and risk of lung cancer in two independent case-control Chinese populations. C allele of the rs2239680 SNP in 3′ UTR of BIRC5 conferred a significantly increased risk of lung cancer and a higher stage of lung cancer. The reporter assay suggested that BIRC5 is a direct target of

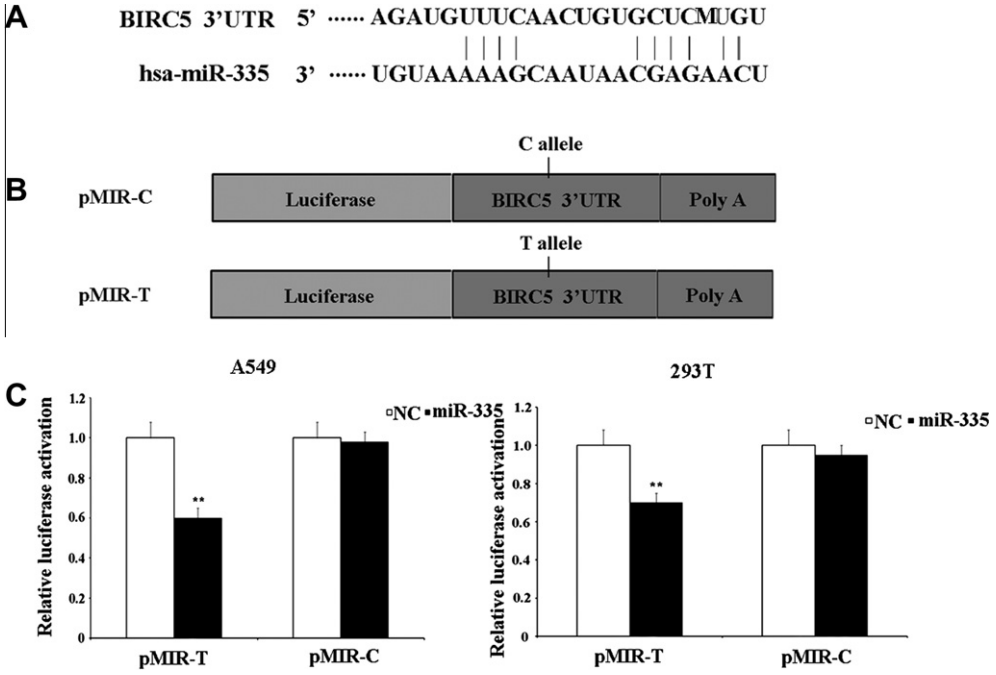


**Fig. 1.** Luciferase reporter gene expression assays with constructs containing pMIR-T and pMIR-C. (A) A schematic shows the potential binding site of baculoviral IAP repeat containing 5 (BIRC5) 3′ untranslated region (UTR). M: SNP rs2239680 T/C. (B) Schema of the constructs harboring different alleles of miRNA binding sites. (C) MiR-335 mimics or its negative control (NC) was co-transfected with the pMIR-REPORT constructs containing T or C allele into A549 or 293T cell lines. Data shown are the mean fold increase ± SD from three independent experiments. $^{**}P < 0.01$ compared with NC group.

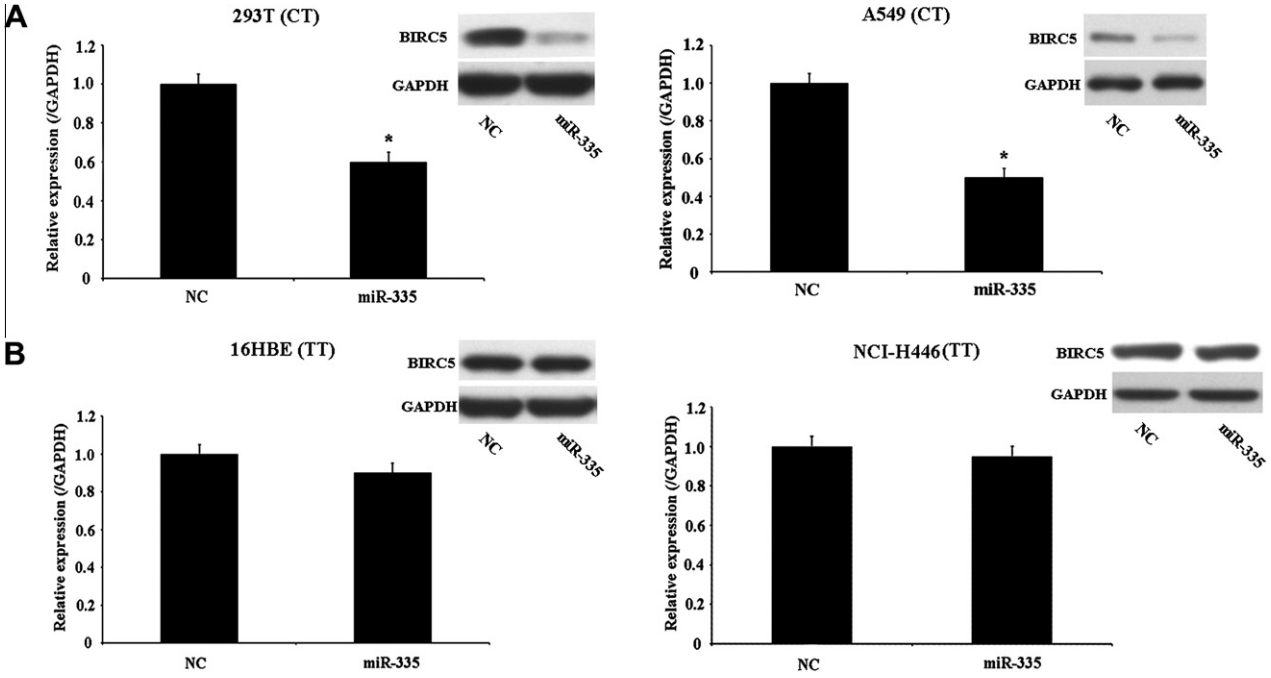


**Fig. 2.** The mRNA and protein expression levels of baculoviral IAP repeat containing 5 (BIRC5) after transfection of miRNA-335 mimics or negative control (NC) into cells with different genotypes. (A) Cells with CT heterozygote transfected with miRNA-335 mimics or NC. After 48 h of transfection, the cells were harvested and determined the mRNA and protein levels of BIRC5 by reverse transcription- polymerase chain reaction (RT-PCR) and western blot analysis, respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. $^{*}P < 0.05$ compared with NC. (B) Cells with TT homozygote transfected with miRNA-335 mimics or NC. After 48 h of transfection, cells were harvested and determined the mRNA and protein levels of BIRC5 using RT-PCR and western blot analysis, respectively. GAPDH served as a loading control.

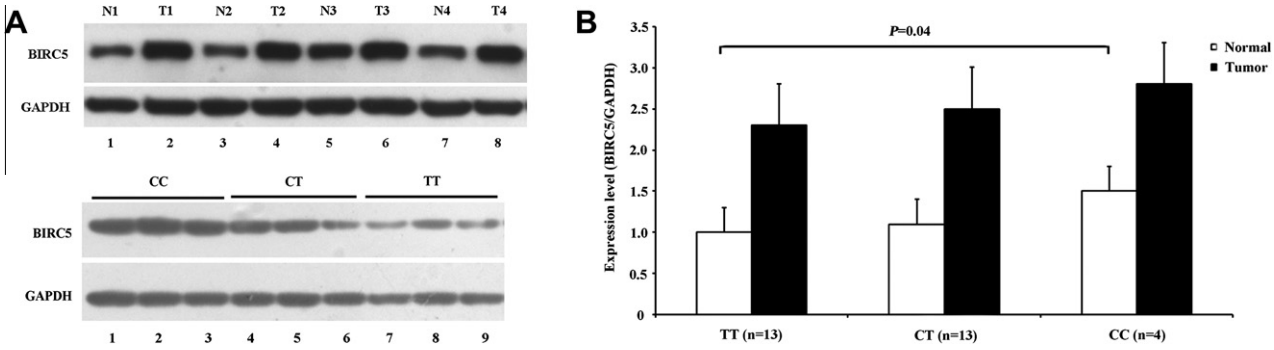


**Fig. 3.** Effect of T/C polymorphisms on baculoviral IAP repeat containing 5 (BIRC5) protein levels in lung tissues. (A) A representative western blotting demonstrated that BIRC5 protein levels in pair wise normal and tumor lung tissues (upper) or in normal lung tissues by BIRC5 T/C genotypes (lower). (B) Densitometric analysis of western blotting of BIRC5 protein levels in 30 pairs of normal and tumor lung tissues by BIRC5 T/C genotypes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was normalized as loading control. Data are the mean ± SD of band density ratios of BIRC5/GAPDH for each genotype in triplicate.

miR-335, while the rs2239680 T > C change could interfere the interaction between miR-335 and BIRC5. In addition, CC carriers showed a higher BIRC5 expression levels in normal lung tissues than TT carriers.

To our knowledge, this is the first study to evaluate the relationship between the rs2239680 SNP and lung cancer risk. The findings are biologically plausible, especially in light of the cytoprotective roles of BIRC5 in cell death and survival. BIRC5 functions as an anti-apoptotic protein interacting with the key components of both intrinsic and extrinsic apoptosis signaling pathways, and is also the downstream molecule of the Bax and Fas signaling pathways [15]. The change of particular IAPs expression levels could induce spontaneous cell death or increase the sensitivity to death stimuli [15]. As a result, the attenuation of BIRC5 levels associated with the T allele may confer a higher individual susceptibility to tumorigenic stimuli like apoptotic factors and cytotoxic agents. Tamm et al. found that BIRC5 was highly expressed in the lung, breast cancer lines [15] and the metastatic tissues [16], which again confirm the role of BIRC5 in lung cancer progression and metastasis.

Consistent with the previous findings that the C allele of rs2239680 is associated with an increased risk of lung cancer, our reporter gene assays revealed that the T > C change could affect the interaction between miR-335 and BIRC5. Among the genes evaluated in our study, RAP1A, BIRC5 and TGFA are highly expressed in human cancers [14,17]. DICER and DGCR8, the miRNA processing genes, play critical roles in the development and prognosis of cancers [18]. FGF2 can mediate the formation of new blood vessels in tumor development [19], while COL4A2 is an inhibitor of angiogenesis and tumor growth [20]. However, we did not find any significant association between the SNPs in 3′ UTR of these genes and risk of lung cancer. The rs6573 SNP in RAP1A was significantly associated with risk and metastasis of esophageal squamous cell

carcinoma [17]. In addition, the SNP affected the binding between miR-196a and RAP1A. The rs1057035 SNP in DICER was previously identified to be significantly associated with the survival of breast cancer [21]. Moreover, rs3757 in DGCR8 was associated with the susceptibility to Schizophrenia and Depression [22,23], while its association with cancer has not been reported yet. Therefore, the roles of other SNPs in lung cancer remain to be evaluated in future studies.

Increasing evidence has show that the 3′ UTR SNPs can influence the susceptibility to certain diseases including cancers by regulating the expression levels of genes. In the present study, we referred to the gene expression profile databases to identify SNPs located in 3′ UTRs of cancer-related genes. Among the putative SNPs, only 7 SNPs showed a MAF $\geqslant$5% in Chinese population. Previous studies have also shown that the majority of SNPs in miRNA target sequences appear to be at relatively low population frequencies [24,25]. This might partially explain why none of the SNPs in miRNA target sequences have ever been identified as susceptibility locus for common diseases in the genome-wide association studies. Inclusion of rare SNPs in miRNA target sequences in future genome-wide association studies would help to reveal low-penetrance susceptibility loci of the diseases.

In conclusion, the rs2239680 SNP in miR-335 binding site of oncogene *BIRC5* is associated with risk and stage of lung cancer. The T > C change could prevent miR-335 from binding to *BIRC5* mRNA, and result in altered regulation of *BIRC5* expression. In addition, CC genotype showed a higher protein level of BIRC5 in normal lung tissues. It is reasonable to speculate that miR-335 may play a role in lung carcinogenesis as a tumor suppressor, while warrants further investigations. Our study provides a new insight into lung cancer tumorigenesis and shows potential implications for the early detection and targeted treatment of lung cancer.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.001.

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