# A haplotype of *TGFBR1* is predominantly found in non-small cell lung cancer patients displaying *TGFBR1* allelic-specific expression

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**Abstract.** Non-small cell lung cancer (NSCLC) accounts for ~85% of all cases of lung cancer and TGF-ß refractoriness is very common in NSCLC cells. Constitutively decreased TGFBR1 expression, probably leading to TGF-ß resistance in tumors, is emerging as a novel tumor-predisposing phenotype. However, the precise genetic/epigenetic mechanisms underlying the role of *TGFBR1* in NSCLC carcinogenesis are still largely unknown. In the present study, we performed the SNaPshot method to quantify allelic-specific expression (ASE) of two chosen SNPs that are located in the 3' untranslated region (3' UTR) of the *TGFBR1* gene. We selected seven tagging SNPs (tSNPs) of *TGFBR1* to assess the relationship between ASE of *TGFBR1* and tSNP-reconstructed haplotypes in NSCLC tumors. ASE of *TGFBR1* was detected in 21.1% of NSCLC tumors. One tagging SNP (rs7040869) of *TGFBR1* in the 5' flanking region was found to be significantly associated with *TGFBR1* ASE in NSCLC tumors (P=0.03). A 2-tSNP AT haplotype reconstructed with tSNP rs7040869 and rs4743325, in linkage disequilibrium with each other, was strongly associated with NSCLC cases displaying ASE (P=0.01). In conclusion, our results shed light on the high frequency of *TGFBR1* ASE phenotype in NSCLC tumors, which is associated with the 2-tSNP haplotype of the *TGFBR1* gene. Although this suggests an important role of the *TGFBR1* locus in the etiology of NSCLC, additional studies at the germline level and in various ethnic populations are warranted.

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## Introduction

Lung cancer, predominantly composed of non-small cell lung cancer (NSCLC), is the leading cause of cancer-related death worldwide (1). In the past decade, the morbidity and mortality of lung cancer have dramatically increased in China (2). Although tobacco smoking is considered to be the major cause contributing to lung cancer, <20% of lifetime smokers develop lung cancer (3), indicating that genetic variation could play an important role in lung carcinogenesis.

Transforming growth factor-ß receptor-dependent signaling pathway is critical for cell growth and differentiation. In the pathway, TGF-ß binds directly to TGFBR2, and is then recognized by TGFBR1, which is phosphorylated and activated by TGFBR2 (4). Previous studies have shown that TGF-ß signaling alterations have been identified in development and progression of several epithelial-type human malignancies, including NSCLC (5-8). Accumulating evidence has demonstrated that *TGFBR1* could be a tumor suppressor gene, and constitutively decreased TGFBR1 expression was found to correlate with increased risk of cancer (9-12). Several investigators have focused on the polymorphisms of *TGFBR1* (*TGFBR1**6A and Int7G24A), identifying no association with lung cancer (13-16). Previously, we detected neither genetic aberrations of *TGFBR1* coding sequence nor methylation of *TGFBR1* promoter region in Chinese NSCLC patients (9,17). Interestingly, we found a strong association of a four-marker CTGC haplotype with decreased NSCLC risk, suggesting that interplay between the four SNPs displays stronger association with NSCLC than single SNP (18). However, the precise genetic/epigenetic mechanisms underlying the role of *TGFBR1* in NSCLC carcinogenesis are still elusive.

More recently, germline allelic-specific expression (ASE) of *TGFBR1* was reported to be a major factor for predisposition to colorectal cancer (CRC), and two major haplotypes were predominantly found among cases displaying ASE (19). Two

subsequent studies have supported the idea that expression of allelic imbalance of cancer-related genes, including *TGFBR1* and *CDH1*, plays an important role in carcinogenesis (20,21), albeit Carvajal-Carmona *et al* reported no evidence of greater ASE of *TGFBR1* in CRC cases than controls (22).

Collectively, elucidating the association between ASE of *TGFBR1* and NSCLC may improve understanding of role of *TGFBR1* in NSCLC carcinogenesis. Therefore, we raised the questions whether ASE of *TGFBR1* identified in Caucasian-dominated patients with CRC (19) could make contribution to predisposition to NSCLC and whether particular haplotypes of *TGFBR1* were predominantly associated with ASE of *TGFBR1* in NSCLC. To address the questions, we performed the SNaPshot method to quantify the differential allelic expression of two chosen SNPs that locate in the 3' untranslated region (3' UTR) of the *TGFBR1* gene. Then, we selected seven tagging SNPs (tSNPs) of *TGFBR1* described in our study (18) to assess the relationship between ASE of *TGFBR1* and tSNPs-reconstructed haplotypes in Chinese NSCLC tumors.

## Materials and methods

*Investigation samples*. Tumor tissues specimens were obtained from 126 consecutive patients diagnosed with NSCLC at the First Affiliated Hospital of Soochow University between January 2005 and September 2008. None of NSCLC patients had received either radiotherapy or chemotherapy before sampling. Also, fresh blood specimens were collected from 124 geographically matched controls with the same age range and without a history of cancer at the Second Affiliated Hospital of Soochow University between June and August 2009. All individuals were genetically unrelated ethnic Han Chinese. Tissues and blood samples were obtained after informed consent from all subjects. This study was approved by the Academic Advisory Board of Soochow University. Peripheral blood mononuclear cells (PBMCs) were immediately isolated by Ficoll-Hypaque (PDB, Tianjing, China) density centrifugation with 2-ml venous blood samples and then preserved at -80˚C after sub-packaging.

*DNA/RNA extraction and cDNA synthesis*. Genomic DNA extraction from tissue and blood specimens was performed according to standard proteinase K digestion and phenol-chloroform extraction. Total RNA from tissue and blood samples was isolated and purified with TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was treated with DNase I (Promega, Madison, WI, USA) prior to reverse transcription (RT), which was done using Reverse Transcriptase M-MLV system (Takara, Kyoto, Japan) before RT-PCR.

*SNPs selected for ASE of TGFBR1*. SNPs within cDNA of the *TGFBR1* gene were selected as markers to distinguish and measure the expression of the two alleles. Two SNPs, including rs1590 and rs3739798, located in the 3' UTR sequence of *TGFBR1*, were chosen for ASE analysis. The SNP rs1590 was selected from the group characterized by Valle and colleagues (19); Another rs3739798 was selected on the basis of the information from NCBI genome build 37.1 SNP database. The heterozygosity of both rs1590 and rs3739798 are 0.378 and 0.444 in Han Chinese population, respectively. For genotyping of the rs1590, the primers were designed as follows: forward primer, 5'-CACCCCCAGGA GACTAACAA-3' and reverse primer, 5'-ACATTTTTCCCA AGTGCCAG-3'. For the rs3739798, the primers were included below: forward primer, 5'-ACCATTTGGAGCCA GAACAC-3' and reverse primer, 5'-GGTCTGAAGAGCT GAGCCTG-3'. The PCR reaction was carried out in a total volume of 50 $\mu$l, containing 100 ng of genomic DNA or cDNA, 1 unit of Ex Taq DNA polymerase (Takara), 0.2 $\mu$mol/l of each primer, 1X Ex Taq Buffer ($Mg^{2+}$ Plus) and 0.25 mmol/l of dNTPs. The cycling conditions for PCR consisted of an initial denaturation at 95˚C for 5 min, followed by 30 cycles of 94˚C for 40 sec, 60˚C for 40 sec, and 72˚C for 45 sec. Genotyping for the two SNPs was done by RFLP with restriction endonucleases. The different alleles were visualized by UV illumination of 2% TBE agarose gels containing 0.5 $\mu$g/ml ethidium bromide. The samples heterozygous for the two 3' UTR SNPs, were informative and selected for ASE analysis.

*Determination of ASE for TGFBR1*. PCR products (15 $\mu$l) were incubated with 1 unit of shrimp alkaline phosphatase (SAP) and 10 units of Exo I (MBI Fermentas, Vilnius, Lithuania) for 1 h at 37˚C and for 15 min at 75˚C prior to the primer extension reaction. The extension was performed in a total volume of 10 $\mu$l, containing 5 $\mu$l reaction mix, 1 $\mu$l PCR products, 1 $\mu$l Probe primer and 3 $\mu$l $ddH_2O$, according to the protocol of SNaPshot Multiplex kit (PE Applied Biosystems, Foster City, CA). The reaction conditions for the extension were: 25 cycles of 96˚C for 10 sec, 50˚C for 5 sec, and 60˚C for 30 sec, followed by treatment with 1 unit of SAP at 37˚C for 1 h, and at 75˚C for 15 min. Then, 1 $\mu$l of the product was mixed with 8.8 $\mu$l Hi-di Formamide and 0.2 $\mu$l GS-120LIZ, heated at 96˚C for 5 min and incubated on ice for 5 min before electrophoresis on an Applied Biosystems 3730 DNA analyzer (PE Applied Biosystems). Genemapper V3.7 software (PE Applied Biosystem) was applied to determine peak area of each heterozygous allele. The ratio of allelic expression was calculated at rs1590 and rs3739798 by the formula: cDNA (peak area common allele/peak area rare allele) divided by gDNA (peak area common allele/peak area rare allele). Individuals were considered positive for the presence of ASE if showing an allelic expression ratio of informative (heterozygote) SNPs <0.67 or >1.5, as suggested by two recent studies (19,23).

*Tagging SNPs of TGFBR1 genotyping, LD and haplotype analysis*. Tagging SNPs (tSNPs) of *TGFBR1* selection and genotyping were performed as described previously in our study (18). Briefly, a total of seven tSNPs were selected among 47 SNPs considered across *TGFBR1*, including three in the 5' flanking region (rs7040869, rs4743325 and rs1888223), three in intronic region (rs10819638, rs6478974 and rs10733710) and one in the 3' flanking region (rs597457). The seven *TGFBR1* tSNPs were amplified by PCR and genotyped in the informative NSCLC patients and controls. The LD map of the seven tSNPs was established with the Haploview program (24). Using the genotyping data, we estimated the frequencies of individual haplotypes and

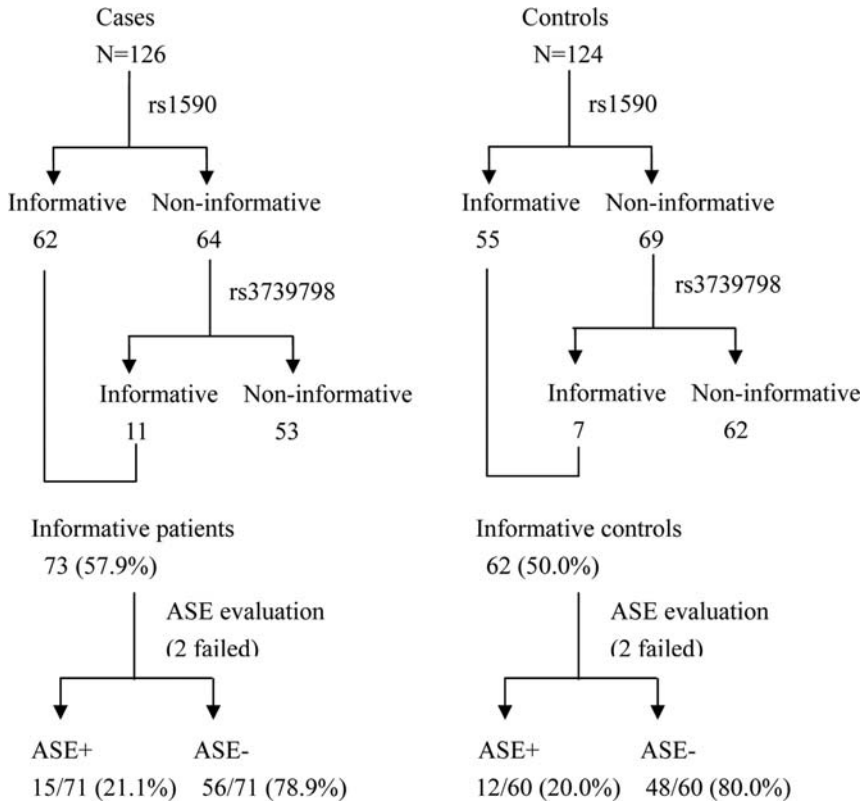


Figure 1. ASE evaluation strategy in NSCLC tumors and controls. Two SNPs of *TGFBR1*, including rs1590 and rs3739798, were chosen for ASE analysis, since rs1590 and rs3739798 have relatively higher heterozygosity (0.378 and 0.444, respectively) in Han Chinese. $ASE^+$ represents ASE <0.67 or >1.5 and $ASE^-$ represents 1.5>ASE>0.67.

reconstructed haplotypes as done in the study (18). Haplotypes with a frequency of <0.05 were not considered in the analysis.

*Statistical analyses*. Comparisons of proportions of ASE for TGFBR1 gene between groups were tested by Chi-square test. All analyses were performed using the statistical software SPSS 15.0. Statistical significant cut-off was P<0.05. The program GraphPad Prism 5.02 was used to plot the ratios of allelic expression.

## Results

*Decreased TGFBR1 allelic expression is a common event in NSCLC tumors*. To determine the presence of ASE of *TGFBR1* in NSCLC patients, we performed the SNaPshot method to quantify the differential allelic expression in Chinese individuals. Two SNPs, including rs1590 and rs3739798, located in the 3' UTR sequence of *TGFBR1*, were genotyped on RNA from NSCLC tumors to choose heterozygous individuals for ASE analysis. Seventy-three among the 126 NSCLC tumors were heterozygous at SNP rs1590 (n=62) and rs3739798 (n=11) (Fig. 1), and their cDNAs were subjected to measuring the relative expression of *TGFBR1* maternal and paternal alleles. Of 62 NSCLC patients heterozygous at SNP rs1590, 9 showed ASE variation ratios <0.67 and 3 showed ratios >1.5 (Fig. 2A), and 3 out of 11 NSCLC patients heterozygous at SNP rs3739798 had ASE values >1.5 (Fig. 2B) and none showed ratios <0.67. Thus, 21.1% (15/71) of informative NSCLC tumors showed ASE of *TGFBR1* (Fig. 1), suggesting that decreased *TGFBR1* allelic expression is common in Chinese NSCLC patients.

To understand whether ASE of *TGFBR1* is tumor-specific in Chinese individuals, we applied the ASE quantification method, established for NSCLC patients, in RNA derived from controls. One hundred and twenty-four controls were selected for genotyping at the two SNPs. Sixty-two individuals were heterozygous at SNP rs1590 (n=55) and rs3739798 (n=7), and ASE analysis in 60 of them presented ratios ranging from 0.54 to 4.57 (Fig. 1). In total, 12 out of 60 informative controls, including 4 with ratios <0.67 and 8 with ratios >1.5, revealed ASE in the *TGFBR1* gene.

The results obtained above showed that decreased *TGFBR1* allelic expression is common in NSCLC patients and controls, suggesting no association between ASE of *TGFBR1* and risk of NSCLC (odds ratio, 0.93; 95% confidence interval, 0.4-2.2, P=0.87) (Fig. 3).

*Relationship between ASE of TGFBR1 and tagging SNPs of TGFBR1 in NSCLC*. Seven tagging SNPs of *TGFBR1*, including three in the 5' flanking region, three in intronic region and one in the 3' flanking region (18), were selected as illustrated in Fig. 4. To assess the association of ASE of *TGFBR1*, frequently present in Chinese individuals, with the tSNPs, we genotyped the seven tSNPs in 71 NSCLC patients and 60 controls who were either ASE-positive or -negative. As summarized in Table I, one tSNP (rs7040869) in the 5' flanking region was significantly associated with decreased

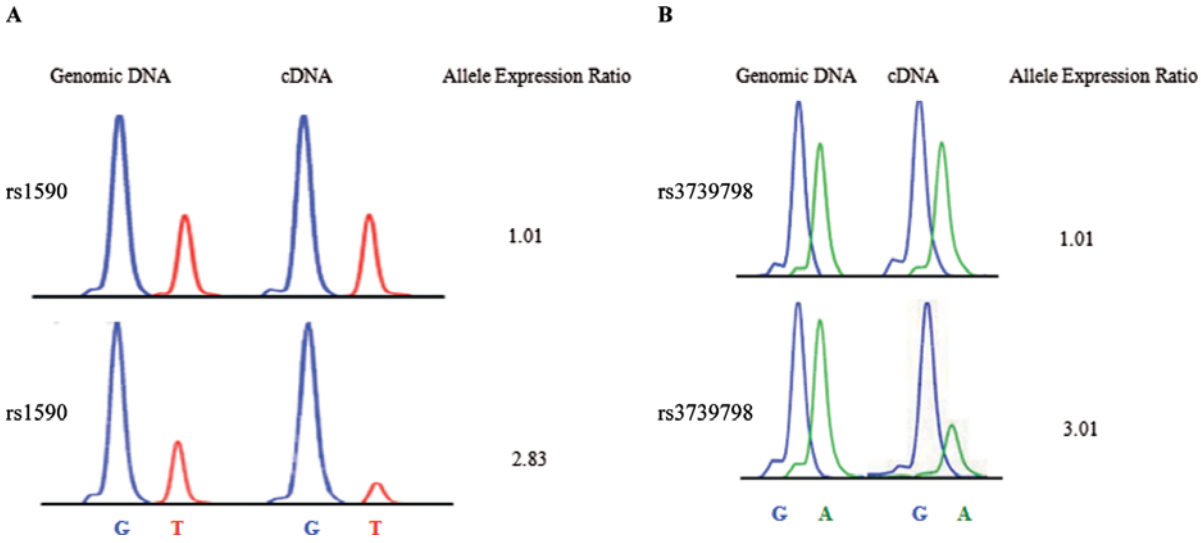


Figure 2. Schematic representatives of ASE detection in four individuals by SNaPshot assays. The ASE ratio was determined by normalizing the ratio between the peak areas of the two alleles (G/T at rs1590 or G/A at rs3739798 of *TGFBR1*) in cDNA with the same parameters in genomic DNA (gDNA). (A) In both exemplified samples heterozygous at rs1590, the transcript from the T allele is preserved and reduced with respect to that from the G allele, respectively. (B) In two exemplified individuals heterozygous at rs3739798, the transcript from the A allele is preserved and reduced with respect to that from the G allele, respectively.

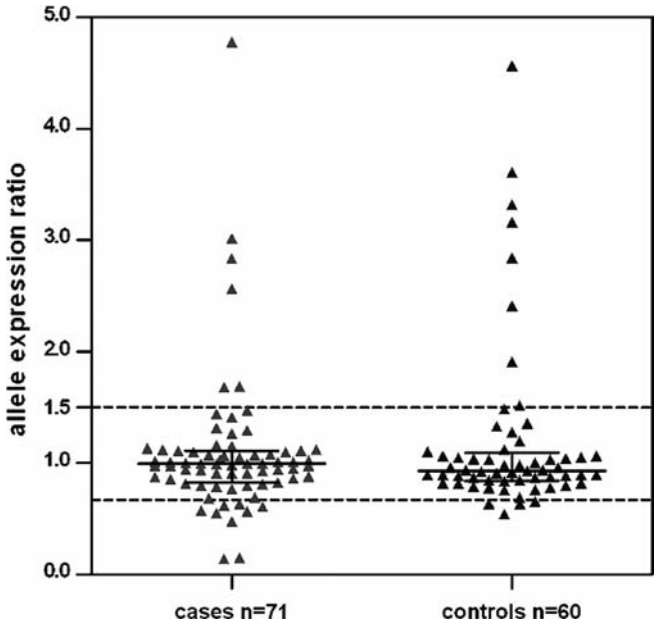


Figure 3. Analysis of *TGFBR1* allelic expression ratios in NSCLC tumors and controls. Allelic expression ratios of *TGFBR1* were shown for each informative individual. Individuals with ratios of >1.5 or <0.67 cut-off values (shown by horizontal dashed lines) were consider positive for ASE. The solid lines within each scatter plot indicate the mean and SE values.

*TGFBR1* allelic expression in NSCLC tumors (P=0.03). However, no significant difference between rs7040869 and *TGFBR1* allelic expression was found in controls (P=0.62).

*Association of 2-tSNP haplotype with TGFBR1 allelic-specific expression*. Although another tSNP (rs4743325) in linkage disequilibrium with rs7040869 (Fig. 4) had no relation to *TGFBR1* ASE (P=0.49) (Table I), a 2-tSNP AT haplotype reconstructed with the two tSNPs presented a significant association with decreased allelic expression of *TGFBR1* in NSCLC tumors (P=0.01) (Table II), suggesting that interplay between the two tSNPs displays stronger association than each of them with *TGFBR1* ASE. Interestingly, four individuals were homozygous for the 2-tSNP haplotype among NSCLC tumors with *TGFBR1* ASE (4 of 15) and two among tumors without *TGFBR1* ASE (2 of 56; P=0.016). We did not observe any association between the 2-tSNP haplotype and the presence of *TGFBR1* ASE in controls (P=0.495).

## Discussion

NSCLC accounts for ~85% of all cases of lung cancer and TGF-ß refractoriness is very common in NSCLC cells (25). Constitutively decreased TGFBR1 expression, probably leading to TGF-ß resistance in tumor (26), is emerging as a novel tumor-predisposing phenotype (12). Valle *et al* reported that ASE of *TGFBR1* gene occurred in 10-20% of CRC patients compared to 1-3% in controls, and showed that this associate with reduced expression of *TGFBR1*, suggesting the possibility that *TGFBR1* ASE might play a role in sporadic CRC (19). Although multiple molecular mechanisms of *TGFBR1* inactivation have been elucidated in NSCLC (9,16-18), little about connection between ASE of *TGFBR1* and NSCLC carcinogenesis was so far known.

As the first attempt to explore the etiological role of *TGFBR1* ASE in NSCLC, the present study provided experimental evidence in two aspects. First, ASE of *TGFBR1* was frequently detected in 21.1% of NSCLC tumors. The results are consistent with the findings that *TGFBR1* ASE is commonly identified in CRC (19,20), suggesting that ASE of *TGFBR1* is a common molecular event and plays a potential role in tumorigenesis. Pinheiro *et al* also reported that ASE presented in the *CDH1* gene among >70% of hereditary

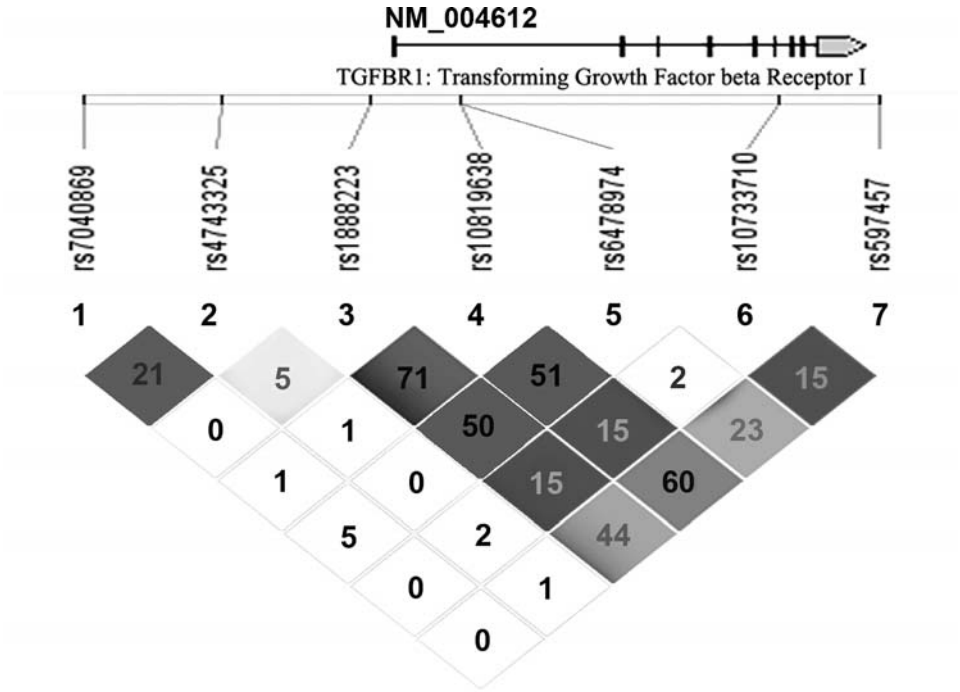


Figure 4. Pairwise LD between seven tSNPs of the *TGFBR1* gene. The value in each diamond indicates pairwise correlation between tagging SNPs (measured as $r^2$) located at the upper left and the upper right sides of the diamond. The shading with a dark grey-to-white gradient reflects higher to lower LD values (measured as D').

Table I. Association of *TGFBR1* SNPs with constitutively decreased *TGFBR1* allelic-specific expression (ASE).[a]

| | Cases | | | | Controls | | | |
|---|---|---|---|---|---|---|---|---|
| SNP | ASE+ | ASE- | P | OR (95% CI) | ASE+ | ASE- | P | OR (95% CI) |
| rs7040869 | 0.47 | 0.26 | 0.03[b] | 2.50 (1.09-5.76) | 0.25 | 0.30 | 0.62 | 0.77 (0.28-2.14) |
| rs4743325 | 0.37 | 0.44 | 0.49 | 0.74 (0.32-1.71) | 0.46 | 0.45 | 0.93 | 1.04 (0.43-2.56) |
| rs1888223 | 0.47 | 0.49 | 0.81 | 0.91 (0.40-2.03) | 0.50 | 0.47 | 0.78 | 1.13 (0.46-2.77) |
| rs10819638 | 0.43 | 0.45 | 0.90 | 0.95 (0.42-2.13) | 0.42 | 0.46 | 0.71 | 0.84 (0.34-2.09) |
| rs6478974 | 0.50 | 0.36 | 0.15 | 1.80 (0.80-4.06) | 0.38 | 0.33 | 0.70 | 1.20 (0.47-3.04) |
| rs10733710 | 0.07 | 0.14 | 0.37 | 0.43 (0.09-1.98) | 0.17 | 0.19 | 1.00 | 0.87 (0.26-2.85) |
| rs597457 | 0.43 | 0.46 | 0.83 | 0.92 (0.41-2.06) | 0.50 | 0.45 | 0.65 | 1.23 (0.50-3.02) |

[a]The variations of ASE+ and ASE- represent minor allele frequency (MAF) of each tagging SNP. [b]Chi-square analysis, P<0.05.

Table II. Association of 2-tSNP haplotypes of *TGFBR1* with constitutively decreased *TGFBR1* allelic-specific expression (ASE).[a]

| | Cases | | | | Controls | | | |
|---|---|---|---|---|---|---|---|---|
| Haplotype[b] | ASE+ | ASE- | P | OR (95% CI) | ASE+ | ASE- | P | OR (95% CI) |
| GT | 0.17 | 0.33 | 0.07 | 0.39 (0.14-1.10) | 0.34 | 0.31 | 0.76 | 1.16 (0.45-2.99) |
| GG | 0.37 | 0.41 | 0.59 | 0.79 (0.35-1.83) | 0.41 | 0.39 | 0.86 | 1.09 (0.44-2.70) |
| AT | 0.47 | 0.23 | 0.01[c] | 2.80 (1.20-6.50) | 0.20 | 0.24 | 0.66 | 0.78 (0.26-2.35) |

[a]Haplotypes with frequencies of >5% were included. The variations of ASE+ and ASE- represent frequency of each haplotype. [b]Two tSNP alleles from left to right (rs7040869 and rs4743325) were used for 2-tSNP reconstruction of haplotypes. [c]Chi-square analysis, P<0.05.

diffuse gastric cancer (21). We failed to find a significant difference in *TGFBR1* ASE between NSCLC tumors and controls. However, considering the fact that we used only NSCLC tumors to assess *TGFBR1* ASE in the present study because the corresponding RNA from blood was not available, we cannot exclude the possibility that germline *TGFBR1* ASE would contribute to predisposition to NSCLC. This could be explained by the notion that either somatically-acquired mutations (27) or epigenetic changes (28) may affect determination of the *TGFBR1* ASE phenotype (20) and may therefore result in lower frequency of *TGFBR1* ASE in epithelium-derived tumors (23). Our previous studies showed that somatic mutations and DNA methylation were rarely observed in exons and promoter region of the *TGFBR1* gene (9,17). Thus, other epigenetic mechanisms, including microRNA expression, may affect *TGFBR1* ASE evaluation of NSCLC tumors. Another explanation for absence of association between *TGFBR1* ASE and NSCLC is that ASE of *TGFBR1* plays an important role in specific types of cancers, i.e. CRC.

One tagging SNP (rs7040869) of *TGFBR1* in the 5' flanking region was found to significantly associate with *TGFBR1* ASE in NSCLC tumors (P=0.03). This is in agreement with the findings that one SNP *TGFBR1**6A (rs11466445) was associated with the *TGFBR1* ASE phenotype of CRC (19). Of note, a 2-tSNP AT haplotype reconstructed with tSNP rs7040869 and rs4743325, in linkage disequilibrium with each other, was strongly associated with NSCLC cases displaying ASE (P=0.01). In support of this, four individuals were homozygous for the 2-tSNP haplotype among 15 NSCLC cases with *TGFBR1* ASE and 2 among 56 cases without *TGFBR1* ASE (P=0.016). The results provide supportive evidence for the idea that several particular haplotypes of *TGFBR1* are predominantly found among CRC cases displaying ASE (19,20). Combined with the data from the two studies, our results suggested that the presence of *TGFBR1* ASE might be caused by specific different haplotypes in different types of cancers. In contrast, no particular haplotype was found to be associated with *CDH1* ASE in hereditary diffuse gastric cancer, due to different molecular mechanisms (21).

In summary, our results shed light on the high frequency of *TGFBR1* ASE phenotype in NSCLC tumor, which is significantly associated with 2-tSNP haplotype of the *TGFBR1* gene. Although this suggests an important role of the *TGFBR1* locus in the etiology of NSCLC, additional studies on germline level and in various ethnic populations are warranted.

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