**Rs2853677 modulates Snail1 binding to the TERT enhancer and affects lung adenocarcinoma susceptibility**

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**Keywords:** rs2853677, Snail1, TERT, enhancer

**Received:** December 15, 2015  **Accepted:** April 28, 2016  **Published:** May 13, 2016

**ABSTRACT**

Genome wide association studies (GWAS) have shown that SNPs in non-coding regions are associated with inherited susceptibility to cancer. The effect of one single SNP, however, is weak. To identify potential co-factors of SNPs, we investigated the underlying mechanism by which SNPs affect lung cancer susceptibility. We found that rs2853677 is located within the Snail1 binding site in a TERT enhancer. This enhancer increases TERT transcription when juxtaposed to the TERT promoter. The binding of Snail1 to the enhancer disrupts enhancer-promoter colocalization and silences TERT transcription. The high risk variant of rs2853677 disrupts the Snail1 binding site and derepresses TERT expression in response to Snail1 upregulation, thus increasing lung adenocarcinoma susceptibility. Our data suggest that Snail1 may be a co-factor of rs2853677 for predicting lung adenocarcinoma susceptibility and prognosis.

**INTRODUCTION**

Cancer is frequently characterized by genetic alterations accompanied by epigenetic changes. These changes result in the aberrant activation and/or silencing of tumor related genes and confer premalignant epithelial cells with the capacity for unrestrained proliferation, resistance to cell death, evasion of immune destruction, and progression to frank malignancy [1–3]. Genetic alterations include somatic mutations, gene amplification, gene deletions, and SNPs. Whereas somatic mutations contribute to sporadically arising cancers, SNPs, which are found throughout the genome and can be inherited, play important roles in both sporadic and familial forms of cancer. A better understanding of the SNPs underlying the development of cancer is strongly needed to elucidate the etiology of the disease and to identify high-risk individuals for targeted screening and/or prevention.

GWAS have identified more than 150 loci associated with increased susceptibility to cancer, according to the Catalog of Published Genome-wide Association Studies [4]. Some SNPs are located in the protein-coding region and affect cancer susceptibility by changing protein function. Most SNPs, however, are located outside of known protein-coding sequences [5]. When present in the promoter region, SNPs can create or delete transcription factor binding sites and have appreciable effects on both gene transcription and protein yield [6, 7]. When present in the 3′ UTR region, SNPs can affect the binding affinity of miRNA [8]. However, how SNPs that are located in introns or inter-gene regions alter cancer susceptibility is largely uncharacterized.

Lung cancer is the leading cause of cancer-related death in most countries. Lung cancer associated SNPs were analyzed by GWAS in patients of various ethnic backgrounds. A Chinese group identified two intronic SNPs (rs2736100 at 5p15.33 and rs4488809 at 3q28) and two intergenic SNPs (rs753955 at 13q12.12 and rs12296850 at 12q23.1) with MAFs > 0.25 that are associated with lung cancer in a Chinese population [9].
Another two SNPs (rs2853677 at 5p15.33 and rs2741354 at 8q21.1) have been reported to be associated with lung cancer in Japanese and European populations, respectively [10, 11]. However, how these SNPs affect lung cancer susceptibility is completely unknown. Here, we investigated these 6 previously reported risk loci and confirmed that rs2853677 in the second intron of the telomerase reverse transcriptase (TERT) gene is associated with a high risk of lung adenocarcinoma in the Han Chinese population. A DNA fragment encompassing rs2853677 functions as an enhancer, which increases TERT transcription when colocalizing with TERT promoter. Snail1 binds to the enhancer, reconfigures the chromatin structure within the TERT gene, and represses TERT transcription. The high risk allele of rs2853677 disrupts the Snail1 binding site, causing derepression of TERT transcription in response to Snail1 upregulation. Our data implicate that rs2853677 may be a potential biomarker for prognosis in Snail1 associated cancer.

RESULTS

rs2853677 is associated with an increased risk of lung adenocarcinoma

We successfully genotyped 6 SNPs using SBE assay. All SNPs conformed to HWE in the controls (Table 1). After adjustment for gender and age, none of the SNPs reached statistical significance (Table 2).

Following subgroup analysis stratified by tumor histology, rs2853677 was associated with adenocarcinoma in a dominant model [OR\textsubscript{CC/CT vs. TT} = 2.33 (1.33–4.06), \textit{P} = 0.0024] (Table 3). It was not associated with either squamous cell carcinoma or small cell lung cancer (SCLC). The other SNPs were not associated with any subtype of lung cancer (Table 3).

The region encompassing rs2853677 functions as an enhancer of TERT

Rs2853677 is located in the second intron at + 7969 base pairs (bp) from the transcription start site of the TERT gene. The TERT gene encodes the catalytic subunit of telomerase, the ribonucleoprotein complex that maintains telomere length. To test whether rs2853677 resides in a cis-regulatory element of TERT, a 600 bp fragment encompassing the rs2853677 was amplified from human A549 cellular DNA that carries the T/C genotype of rs2853677. PCR products with the T or C allele were inserted into a construct containing a reporter gene and the TERT promoter in the original or inverted orientation. Luciferase reporter constructs were transfected into HEK293 cells, and the shifted band was supershifted when FLAG antibody was added (Figure 2A). This result indicates that Snail1 binds to the

rs2853677-C disrupts a binding site for Snail1 and abolishes the suppressive role of Snail1 in TERT transcription

Bioinformatic analysis revealed that CG[C/T]CTG is a potential binding site of the Snail family of proteins. We first evaluated the interaction between this consensus sequence and Snail proteins (Snail1, Snail2 and Snail3) and its modulation by rs2853677 \textit{in vitro} using an EMSA assay. The 25 bp oligonucleotides encompassing the rs2853677-T or -C allele were labeled with biotin at their 3' termini and incubated with nuclear extract derived from Snail1-FLAG, Snail2-FLAG or Snail3-FLAG overexpressing H446 cells. Supershift assays were performed to confirm the association of the tagged Snail proteins with the respective oligonucleotide probes. A shifted band was observed when the oligos containing the T allele were incubated with nuclear extract containing Snail1-FLAG, but not Snail2-FLAG or Snail3-FLAG. The shifted band was supershifted when FLAG antibody or Snail1 antibody was added (Figure 2A). The C allele oligos, however, were not shifted by any nuclear extract (Figure 2A). This result indicates that Snail1 binds to the
Figure 1: Rs2853677 is located inside of an enhancer. (A) Luciferase reporter studies with serial extensions of the TERT promoter containing DNA fragment encompassing rs2853677 with T or C allele. The DNA fragment carrying T or C allele was amplified from A549 cells and placed directly upstream of the TERT promoter in forward or inverted orientation. The constructs were transiently transfected along with pRL-CMV Renilla luciferase reporter into HEK293 cells. luciferase activity was measured after 24 hrs’ transfection. Mean ± SD indicates three independent transfections. (B) 3C was used to calculate cross linking frequency between chromatin segments to assess proximity in A549 cells. Vertical lines represent Pst I restriction sites. Arrows indicate PCR primer sites and direction. Anchor symbol marks anchoring primer. Cross linking frequency between the TERT promoter and different segments is shown. Top panels show representative PCR products. Mean ± SD of 3 independent chromatin preparations is shown. (C) 3C assay was performed with the DNA fragment harboring rs2853677 as an anchor fragment. Top panels show representative PCR products. Mean ± SD indicate 3 independent chromatin preparations.
### Table 1: HWE tests in controls

| SNP ID     | HWE P value in controls |
|------------|-------------------------|
| rs753955   | 0.62                    |
| rs2853677  | 0.63                    |
| rs2736100  | 0.58                    |
| rs4488809  | 1                       |
| rs2741354  | 0.71                    |
| rs12296850 | 0.056                   |

### Table 2: Associations between 6 SNPs with lung cancer risk

| SNP   | Genotype | Control, n (%) | Case, n (%) | OR   | 95% CI      | P   |
|-------|----------|----------------|-------------|------|-------------|-----|
| rs753955 | T/T      | 159 (47.2)     | 158 (40.4)  | Reference | Referent   | 1   |
|        | C/T      | 142 (42.1)     | 174 (44.5)  | 1.18  | 0.74–1.88   | 0.493 |
|        | C/C      | 36 (10.7)      | 59 (15.1)   | 1.74  | 0.87–3.47   | 0.116 |
|        | Dominant |               |             | 1.29  | 0.83–2.00   | 0.26  |
|        | Recessive|               |             | 1.6   | 0.84–3.07   | 0.15  |
| rs2853677 | T/T      | 143 (42.4)     | 137 (35)    | Reference | Reference   | 1   |
|        | C/T      | 157 (46.6)     | 194 (49.6)  | 1.56  | 0.97–2.51   | 0.66  |
|        | C/C      | 37 (11)        | 60 (15.3)   | 1.86  | 0.92–3.78   | 0.085 |
|        | Dominant |               |             | 1.62  | 1.03–2.54   | 0.035 |
|        | Recessive|               |             | 1.45  | 0.76–2.80   | 0.26  |
| rs2736100 | T/T      | 117 (34.7)     | 109 (27.9)  | Reference | Reference   | 1   |
|        | G/T      | 159 (47.2)     | 201 (51.4)  | 1.27  | 0.77–2.09   | 0.359 |
|        | G/G      | 61 (18.1)      | 81 (20.7)   | 1.29  | 0.69–2.42   | 0.421 |
|        | Dominant |               |             | 1.27  | 0.79–2.05   | 0.32  |
|        | Recessive|               |             | 1.12  | 0.65–1.93   | 0.69  |
| rs4488809 | T/T      | 100 (29.7)     | 115 (29.4)  | Reference | Reference   | 1   |
|        | C/T      | 168 (49.9)     | 179 (45.8)  | 0.98  | 0.58–1.63   | 0.927 |
|        | C/C      | 69 (20.5)      | 97 (24.8)   | 0.97  | 0.52–1.82   | 0.934 |
|        | Dominant |               |             | 0.98  | 0.60–1.59   | 0.93  |
|        | Recessive|               |             | 0.99  | 0.58–1.68   | 0.97  |
| rs2741354 | G/G      | 159 (47.2)     | 175 (44.8)  | Reference | Reference   | 1   |
|        | G/A      | 143 (42.4)     | 187 (47.8)  | 1.46  | 0.92–2.31   | 0.104 |
|        | A/A      | 35 (10.4)      | 29 (7.4)    | 0.83  | 0.37–1.86   | 0.644 |
|        | Dominant |               |             | 1.33  | 0.86–2.06   | 0.2   |
|        | Recessive|               |             | 0.69  | 0.32–1.50   | 0.35  |
| rs12296850 | A/A      | 171 (50.7)     | 210 (53.7)  | Reference | Reference   | 1   |
|        | G/A      | 148 (43.9)     | 159 (40.7)  | 1.03  | 0.66–1.62   | 0.894 |
|        | G/G      | 18 (5.3)       | 22 (5.6)    | 0.91  | 0.34–2.42   | 0.843 |
|        | Dominant |               |             | 1.02  | 0.66–1.57   | 0.94  |
|        | Recessive|               |             | 0.89  | 0.34–2.33   | 0.82  |

**NOTE:** Statistically significant ($P < 0.0083$) associations are in bold. Logistic regression was adjusted for gender and age.
oligos carrying the T allele, but not the C allele. Neither Snail2 or Snail3 binds to either oligo. We then examined the effect of polymorphism rs2853677 on the in vivo occupancy of Snail1 to the consensus binding site using ChIP analysis. We analyzed the genotype of rs2853677 in 5 human lung cancer cell lines, including A549, H1229, H69, H209 and H446. H446 and H209, which carry the T/T and C/C genotypes, respectively, were selected for further analysis. Snail1 conjugated with FLAG at its C-terminus was transfected into these two cell lines. ChIP was performed using FLAG antibody. Enrichment of Snail1 in the CDH1 promoter was set to 1.0. Occupancy of Snail1 with the TERT enhancer was detected in H446 cells, but not in H209 cells (Figure 2B). These data indicate that the rs2853677-C allele disrupts the Snail1 binding site.

We next tested whether this differential binding could modulate TERT gene expression in response to Snail1 upregulation. Luciferase assays of HEK293 cells showed that cotransfection of SNAI1 with the luciferase reporter partially repressed enhancer activity when the enhancer harbored the T allele but had no effect on the enhancer carrying the C allele (Figure 2C), indicating that Snail1 inhibits enhancer activity upon association.

| SNP       | Genotype | Squamous OR | 95% CI | P      | SCLC OR | 95% CI | P      | Adenocarcinoma OR | 95% CI | P      |
|-----------|----------|-------------|--------|--------|---------|--------|--------|-------------------|--------|--------|
| rs753955  | T/T      | Reference   | Reference | Reference   | 0.50–2.19 | 0.903 | 0.81 | 0.38–1.72 | 0.578 | 1.03 | 0.60–1.77 | 0.922 |
|           | C/T      | 1.05        |         |        | 2.38    | 0.85–6.68 | 0.098 | 2.7 | 0.99–7.33 | 0.052 | 1.16 | 0.48–2.78 | 0.741 |
|           | C/C      | 1.27        | 0.64–2.55 | 0.49 | 2.33    | 0.90–6.01 | 0.082 | 2.99 | 1.17–7.62 | 0.026 | 1.14 | 0.50–2.63 | 0.75 |
|           | Dominant | 1.05        | 0.50–2.09 | 0.945 | 1.03    | 0.50–2.09 | 0.954 | 1.07 | 0.52–2.19 | 0.859 | 2.27 | 1.26–4.06 | 0.006 |
|           | Recessive| 1.14        | 0.38–3.41 | 0.82 | 1.14    | 0.38–3.41 | 0.82 | 0.86 | 0.27–2.75 | 0.8 | 1.58 | 1.75–3.29 | 0.23 |
| rs2853677 | T/T      | 1.03        | 0.50–2.09 | 0.945 | 1.07    | 0.50–2.19 | 0.954 | 1.07 | 0.52–2.19 | 0.859 | 2.27 | 1.26–4.06 | 0.006 |
|           | C/C      | 1.15        | 0.36–3.68 | 0.811 | 0.89    | 0.26–3.02 | 0.858 | 2.55 | 0.55–2.19 | 0.78 | 1.05 | 0.63–1.76 | 0.85 |
|           | Dominant | 1.05        | 0.53–2.07 | 0.89 | 1.04    | 0.52–2.07 | 0.92 | 2.33 | 1.33–4.06 | 0.0024 |
|           | Recessive| 1.14        | 0.38–3.41 | 0.82 | 1.14    | 0.38–3.41 | 0.82 | 0.86 | 0.27–2.75 | 0.8 | 1.58 | 1.75–3.29 | 0.23 |
| rs2736100 | T/T      | 1.03        | 0.50–2.09 | 0.945 | 1.07    | 0.50–2.09 | 0.954 | 1.07 | 0.52–2.19 | 0.859 | 2.27 | 1.26–4.06 | 0.006 |
|           | G/T      | 0.85        | 0.40–1.82 | 0.672 | 0.75    | 0.34–1.64 | 0.475 | 1.65 | 0.89–3.05 | 0.111 |
|           | G/G      | 0.77        | 0.28–2.07 | 0.602 | 0.84    | 0.32–2.19 | 0.715 | 1.74 | 0.83–3.63 | 0.142 |
|           | Dominant | 0.83        | 0.40–1.70 | 0.61 | 0.78    | 0.37–1.61 | 0.5 | 1.67 | 0.94–2.99 | 0.08 |
|           | Recessive| 0.85        | 0.36–2.04 | 0.72 | 0.99    | 0.42–2.31 | 0.98 | 1.26 | 0.68–2.33 | 0.47 |
| rs4488809 | T/T      | 1.03        | 0.50–2.09 | 0.945 | 1.07    | 0.50–2.19 | 0.859 | 2.27 | 1.26–4.06 | 0.006 |
|           | G/T      | 0.85        | 0.40–1.82 | 0.672 | 0.75    | 0.34–1.64 | 0.475 | 1.65 | 0.89–3.05 | 0.111 |
|           | G/G      | 0.77        | 0.28–2.07 | 0.602 | 0.84    | 0.32–2.19 | 0.715 | 1.74 | 0.83–3.63 | 0.142 |
|           | Dominant | 0.83        | 0.40–1.70 | 0.61 | 0.78    | 0.37–1.61 | 0.5 | 1.67 | 0.94–2.99 | 0.08 |
|           | Recessive| 0.85        | 0.36–2.04 | 0.72 | 0.99    | 0.42–2.31 | 0.98 | 1.26 | 0.68–2.33 | 0.47 |
| rs741354  | G/G      | 1.41        | 0.61–3.25 | 0.426 | 0.67    | 0.30–1.48 | 0.319 | 1 | 0.55–1.81 | 0.99 |
|           | G/A      | 1.18        | 0.45–3.13 | 0.733 | 0.81    | 0.31–2.07 | 0.656 | 0.89 | 0.43–1.84 | 0.747 |
|           | Dominant | 1.33        | 0.60–2.94 | 0.48 | 0.71    | 0.34–1.48 | 0.37 | 0.96 | 0.55–1.69 | 0.89 |
|           | Recessive| 0.94        | 0.43–2.05 | 0.72 | 1.03    | 0.45–2.34 | 0.94 | 0.89 | 0.48–1.66 | 0.71 |
| rs12296850| A/A      | 1.71        | 0.84–3.50 | 0.142 | 1.45    | 0.70–2.98 | 0.317 | 1.72 | 1.00–2.95 | 0.049 |
|           | G/A      | 1.56        | 0.48–5.06 | 0.458 | 1.3 | 0.37–4.49 | 0.681 | 0.79 | 0.28–2.23 | 0.663 |
|           | Dominant | 1.68        | 0.85–3.32 | 0.13 | 1.42    | 0.71–2.82 | 0.32 | 1.53 | 0.91–2.57 | 0.1 |
|           | Recessive| 1.2        | 0.39–3.68 | 0.75 | 1.1    | 0.33–3.61 | 0.88 | 0.61 | 0.23–1.66 | 0.33 |

NOTE: Statistically significant (P < 0.0028) associations are in bold.
with the binding site. Accordingly, RT-PCR with one-, two-, or three-fold template DNA concentrations showed that transient expression of Snail1 repressed TERT transcription in H446 cells, but not in H209 cells (Figure 2D). Furthermore, TERT transcription was inhibited in H446 cells after 24 hr treatment with 10 µg/ml TGF-β, but not in TGF-β treated H209 cells, although both exhibited enhanced Snail1 expression upon TGF-β treatment (Figure 2E). These data indicate that Snail1 can bind to the enhancer and repress TERT transcription by inhibiting enhancer activity. The C allele of rs2853677 disrupts the association of Snail1 and prevents TERT downregulation in response to Snail1 upregulation.

The well-known function of Snail1 is to promote the epithelial-mesenchymal transition (EMT), a process that is critical for cancer malignancy and metastasis. We accessed The Cancer Genome Atlas (TCGA) public database to analyze any correlation among Snail1, TERT and E-cadherin (an important cell-cell adherent junction protein whose downregulation is a golden mark of the EMT) in lung adenocarcinoma. Expression of E-cadherin correlated negatively with Snail1 ($r = -0.169, p = 4.545 \times 10^{-5}$), but not with TERT ($r = -0.041, p = 0.321$) (Figure 2F). Accordingly, rs2853677 is not associated with lung adenocarcinoma metastasis [OR $0.90 (0.45–1.83), P = 0.78$] (Table 4). These results suggest that the C allele of rs2853677-C allele has no effect on the EMT of lung adenocarcinoma.

Snail1 binds to the TERT enhancer and disrupts long range communication between the enhancer and the promoter

We next investigated the mechanism by which Snail1 regulates the activity of the intronic enhancer. We have previously shown that the intronic enhancer physically interacts with the TERT promoter by looping out the intervening sequences. We examined whether Snail1 could change this chromatin structure using 3C. Transient expression of Snail1 caused delocalization of the intronic enhancer from the TERT promoter in H446 cells (Figure 3A), but not in H209 cells (Figure 3B). Notably, the TERT gene in both H446 and H209 cells exhibited physical interaction between the promoter and the intronic enhancer (Figure 3A and 3B), and the intronic enhancer carrying either the C or the T allele exhibited the same effect on TERT promoter activity in a luciferase assay in HEK293 cells. This leads us to postulate that Snail1 may not be expressed in these cells. We then examined the expression of Snail1 in HEK293, H446, and H209 cells. As expected, these three cell lines and A549 do not express Snail1 (Figure 3C). These data support a model whereby TERT transcription requires long-range physical interaction between the enhancer and the TERT promoter. Snail1 associates with the enhancer, disrupting enhancer-promoter physical interaction, downregulating TERT transcription. The C allele of rs2853677 disrupts the binding site of Snail1, thereby abolishing the suppressive effect of Snail1 on TERT transcription (Figure 3D).

DISCUSSION

Genetic factors contribute to cancer susceptibility. Here, we confirmed that rs2853677, which is located in the second intron of TERT, is associated with a high risk of lung adenocarcinoma in the Han Chinese population. The high risk variant disrupts the Snail1 binding site and prevents repression of TERT in response to the upregulation of Snail1, thus resulting in a high level expression of TERT in the presence of Snail1.

TERT expression level determines telomere length, and overexpression of TERT immortalizes normal cells [20–22]. Several lines of evidence have shown that immortalized human cells are susceptible to transformation via the introduction of an oncogene [23–25]. There is also evidence showed that cotransfection of TERT with H-ras can transform immortalized cells [26]. Therefore, high levels of TERT expression in oncogene (for example, Snail1) expressing cells promote tumorigenesis. TERT promoter mutations at sites – 57, – 256, or – 228 are associated with melanoma, non-small cell lung cancer and bladder cancer, respectively [7, 27, 28]. Due to its

| Model      | Genotype | OR (95% CI)    | P     |
|------------|----------|----------------|-------|
| Codominant | T/T      | Reference       | 0.6   |
|            | C/T      | 0.80 (0.38–1.69)|       |
|            | C/C      | 1.29 (0.47–3.50)|       |
| Dominant   | T/T      | Reference       | 0.78  |
|            | C/T-C/C  | 0.90 (0.45–1.83)|       |
| Recessive  | T/T-C/T  | Reference       | 0.4   |
|            | C/C      | 1.46 (0.60–3.60)|       |

NOTE: Logistic regression was adjusted for gender and age.
Figure 2: Rs2853677 affects TERT transcriptional regulation by Snail1. (A) EMSA shows mobility super shift (S.S.) of DNA fragment containing rs2853677. SNAI1-FLAG or SNAI2-FLAG or SNAI3-FLAG were overexpressed in H446 cells. Oligos carrying T or C allele were labeled with biotin in its 3' terminus and incubated with nuclear extract that was purified from H446 expressing Snail1/2/3-FLAG. The antibody against Snail1/2/3 or FLAG was added to confirm the binding of Snail proteins to the oligos. Oligos harboring T allele but not the C allele can be bound by Snail1. (B) ChIP shows the occupancy of Snail1 in the TERT intronic enhancer in vivo. SNAI1-FLAG was overexpressed in H446 or H209 cells. ChIP was performed with FLAG antibody. Enrichment fold was evaluated by real-time PCR. Upper panels showed genotype of rs2853677 in H446 and H209 cells. Lower panels showed relative fold enrichment of Snail1 in the TERT intronic enhancer. CDH1 promoter was used as a positive control and its enrichment was set to 1.0. Actin was used as a negative control. Association of Snail1-Flag with the TERT intronic enhancer was shown. (C) Luciferase reporter study shows effect of Snail1 cotransfection on the TERT intronic enhancer activity. Snail1 or empty vector was cotransfected with the enhancer-TERT promoter constructs into HEK293 cells. Luciferase activity was measured after 24 hrs' transfection. Mean ± SD indicate three independent transfections. (D) Semiquantitative RT-PCR was performed to measure TERT transcription in control or Snail1 expressing cells. 1 ul, 3 ul, 9 ul of reverse transcribed cDNA was analyzed by PCR. (E) Expression of Snail1 and E-cadherin was assessed for correlation in lung adenocarcinoma using TCGA databases. SNAI1 negatively correlated with CDH1 (r = -0.169, p = 4.545 × 10⁻⁵). (G) Expression of TERT and E-cadherin was assessed for correlation in lung adenocarcinoma using TCGA databases. TERT did not correlate with CDH1 (r = -0.041, p = 0.321).
importance, transcriptional regulation of TERT has been studied, and multiple cis-regulatory elements have been found within or upstream of the TERT promoter [29, 30]. In this report, we identified a new cis-regulatory element residing within the second intron. This cis-regulatory element attenuates silencer activity when juxtaposed to the TERT promoter by looping out the intervening DNA and increasing TERT transcription. An oncogenic transcription factor, Snail1, binds to the enhancer and disrupts this physical interaction, repressing TERT transcription.

Snail1 is a member of the Snail superfamily of zinc-finger transcription factors, which also includes Snail2 (Slug) and Snail3 (Smuc). Although the three members share a conserved DNA binding domain, and their regulated genes overlap, only Snail1 can bind to the sequence harboring rs2853677. Elevated Snail1 expression is detected in many types of cancer and correlates with tumor malignancy [31, 32]. Although Snail1 is generally considered to be an oncogene, our results clearly indicate that in addition to repressing the expression of E-cadherin, the enhanced expression of Snail1 also downregulates TERT transcription, which inhibits tumorigenesis. Therefore, Snail1 executes dual functions in tumorigenesis. The C allele of rs2853677, however, disrupts the Snail1 binding site and derepresses the expression of TERT by Snail1, blocking the inhibitory effect of Snail1 on tumorigenesis and thus increasing lung cancer susceptibility. Although the well-known function of Snail1 is to promote the EMT, rs2853677 does not seem to affect the EMT.

Figure 3: Snail1 disrupts long range communication between the intronic enhancer and the TERT promoter. (A) Snail1 was overexpressed in H446 cells that harbor T/T genotype of rs2853677. 3C was performed to test the effect of Snail1 overexpression on proximity of the TERT enhancer and the promoter in H446 cells. Upper panels show representative PCR products. The lower panel shows the relative cross-linking frequencies between the TERT promoter and other DNA fragments. Mean ± SD indicate 3 independent chromatin preparations. (B) Snail1 was overexpressed in H209 cells that harbor C/C genotype of rs2853677. 3C was performed to test the effect of Snail1 overexpression on proximity of the TERT enhancer and the promoter in H209 cells. (C) Western blot showing the expression of Snail1 in variant cells. H446 expressing Snail1 was a positive control. (D) Schematic showing formation of a transcriptionally active complex containing the TERT promoter (TERT P) and the enhancer (E). Upon binding of Snail1 (shaded circles) to enhancer (T allele), the interaction is disrupted and TERT is downregulated.
Unexpectedly, rs2853677 is associated only with lung adenocarcinoma and is not related to SCLC, although SCLC expresses more TERT than lung adenocarcinoma [33, 34]. This may be due to other transcription factors or signaling pathways that are activated in SCLC and may block the effect of Snail1 on TERT transcriptional regulation. It is known that nearly all patients with SCLC are missing RB1 and have more frequent mutations in TP53 than patients with lung adenocarcinoma [35]. The p53 protein is a potent inhibitor of the TERT promoter [36]. Mutation of p53 can relieve repression of the TERT promoter and therefore lead to elevated TERT expression in SCLC. In addition, the PI3-kinase (PI3K) pathway, another positive TERT transcriptional regulator, is also activated in SCLC [37, 38]. The mechanism by which these signaling pathways activate the TERT promoter is not clear. It is possible that Snail1 induced TERT transcriptional inhibition is not feasible in the presence of these signaling pathways.

In summary, we identified Snail1 as a co-factor of rs2853677 for predicting susceptibility to and the prognosis of lung adenocarcinoma. Given that Snail1 expression is enhanced in many types of cancers, the association of rs2853677 with other cancer types needs to be studied.

MATERIALS AND METHODS

Cell lines

HEK293, A549, H1229, H69, H209, and H446 cell lines were from ATCC, and were cultured in RPMI1640 containing 10% FBS, 100 U of penicillin/ml, and 100 μg of streptomycin/ml.

Study population

This case control study consisted of 391 lung cancer cases and 337 controls. The 391 patients with histologically confirmed lung cancer were recruited from Tianjin Medical University Cancer Institute & Hospital in China. The 337 controls were randomly selected from volunteers enrolled for physical examination at Tianjin General Hospital. All the controls did not have a history of cancer. Informed consent was obtained from all participants. This study was approved by Ethics Committee of Tianjin Medical University. The detail of the subject characteristics are listed in Table 5.

SNP selection and genotyping

6 previously reported risk loci located in the intron or inter-gene region were selected [9–14]. The information of six SNPs was listed in Table 6. The genotyping of 6 SNPs were examined in one PCR reaction. 2 μl of the PCR product was cleaned up with 1 μl of ExoSAP-IT (Amersham Biosciences, USA). Multiplex SBE reactions were performed in a total volume of 5 μl comprising 2 μl of SNaPshot ready reaction mix (Applied Biosystems, USA), 1.5 μl of cleaned PCR products and 1 μl of SBE primer mix. The SBE products were purified again using ExoSAP-IT. Genotyping for SNPs was performed by ABI 3130 Genetic Analyzer (Applied Biosystems, USA) according to the supplier’s instructions. Primers were listed in Table 7.

Data analysis

Differences of gender and age between cases and controls were compared by χ2 tests and student t tests respectively. Hardy-Weinberg Equilibrium (HWE) was assessed in the control samples by applying an exact test and then genotype frequencies were compared using χ2 tests. The association between lung cancer and each SNP was examined using logistic regression under Codominant, dominant and recessive models after adjusting for sex and age. The significance level was set at 0.018. These analyses were carried out using SNPStats [15] and SPSS 16.0.

Cloning and transduction

Human SNAI1 was amplified from H1155 cDNA. SNAI2 or SNAI3 was amplified from Beas-2B cDNA. FLAG was conjugated to the C-terminus. SNAI1-FLAG or SNAI2-FLAG or SNAI3-FLAG was ligated into the lentiviral shuttle pCCL.PPT.hPGK.IRES.GFP/pre. The plasmid was used to produce lentivirus in 293T cells with the packaging plasmids pMD2.BSBG, pMDLg/pRRE and pRSV-REV.

Luciferase assay

A 528 bp and 1051 bp DNA fragment upstream of TERT TSS were amplified from A549 cells DNA using primers listed in Table 7. The DNA fragments were inserted into the pGLbasic. Two analogous 600 bp DNA fragments encompassing the rs2853677 site were subsequently inserted in forward or inverted orientation into upstream of the TERT promoter. Plasmids were cotransfected into HEK293 cells using Lipofectamine 2000 (Invitrogen) with pRL-CMV Renilla luciferase reporter, which was used for normalization (Promega).

Chromatin immunoprecipitation

SNAI1-FLAG was overexpressed in H446 and H209 cells. ChIP was performed using M2-argarosese as described previously [16]. Fold enrichment was analyzed.
Table 5: Characteristics of cases and controls

|                      | Controls, n (%) | Cases, n (%) | P     |
|----------------------|-----------------|--------------|-------|
| Total subjects       | 337 (100%)      | 391 (100%)   |       |
| Gender               |                 |              | 0.969 |
| Male                 | 228 (67.7%)     | 264 (67.5%)  |       |
| Female               | 109 (32.3%)     | 127 (32.5%)  |       |
| Age                  |                 |              | 0.000 |
| Mean ± SD            | 38.8 ± 10.7     | 58.63 ± 8.8  |       |
| Tumor type           |                 |              |       |
| Squamous             | 107 (27.4%)     |              |       |
| SCLC                 | 64 (16.4%)      |              |       |
| Adenocarcinoma       | 183 (46.8%)     |              |       |
| Other                | 37 (9.5%)       |              |       |

Table 6: Information of six SNPs

| SNPs   | Allele | Locus      | Location   |
|--------|--------|------------|------------|
| rs753955 | C/T   | 13q12.12   | intergenic |
| rs2853677 | C/T   | 5p15.33    | intron of TERT |
| rs2736100 | G/T   | 5p15.33    | intron of TERT |
| rs4488809 | C/T   | 3q28       | intron of TP63 |
| rs2741354 | G/A   | 8q21.1     | intergenic |
| rs12296850 | G/A   | 12q23.1    | intergenic |

by PCR assays. β-actin promoter was treated as a negative control and CDH1 promoter was treated as a positive control. Primers used in this study are listed in Table 7.

EMSA

Nuclear proteins from H446 expressing Snail1 or Snail2 or Snail3 cells were extracted using a NucBuster protein extraction kit (Novagen) according to the manufacturer’s instructions. Double-stranded oligonucleotides corresponding to the potential Snail1 binding sites 5′-ACTAGAGACCCG[C/T]CTGGTGCACTCTG was end-labeled with biotin in its 3′ terminus. Binding assays were performed in 10 μl of reaction mixture with 2 μg of nuclear protein extracts and 1 nM labeled probes at room temperature for 30 min in binding buffer (10 mM Tris-Cl, 55 mM KCl, 2.5 mM MgCl₂, 0.25 mM EDTA, 1 mM DTT, 0.05% NP-40, 5% Glycerol and 1 μg poly dl-dC). In supershift assay, Snail1 or Snail2 or Snail3 antibodys or FLAG antibody was added. Reactions were analyzed by electrophoresis on a 6.0% non-denaturing polyacrylamide gel at 100 V for 1 h. After transfer, the membrane was immediately cross-linked and bands detected by chemiluminescence (Pierce, USA).

Chromosome conformation capture

3C assays were performed as described previously [17]. Briefly, 10⁶ cells were cross-linked, lysed, and nuclei were digested with Pst I. After ligation and subsequent DNA purification, the cross-linking frequencies between the anchor and test fragments were estimated by PCR reactions using primers listed in Table 7. To create a standard for normalization of relative PCR efficiencies, three PCR products together containing all the sequence from the TERT gene TSS to10 Kb downstream were amplified, digested with Pst I, and ligated at high concentrations to generate equimolar mixtures of all possible ligation products. The cross-linking and ligation efficiencies between different samples were normalized by setting the highest cross-linking frequency to 1.0.
### Table 7: Primers and probes used in experiment

| Primers used for genotyping of SNPs |  
|-----------------------------------|---|
| rs753955-F 5′- AATATAGGTGGGCTGTCCGTCC-3′ |  
| rs753955-R 5′- GGGAAAGACAATTGGTGTG-3′ |  
| rs2853677-F 5′- CCAATCCAGTGCTGACATCG-3′ |  
| rs2853677-R 5′- GAAAACAGGACAAGGACA-3′ |  
| rs2736100-F 5′- GTGCTGTGTTCCTGCTGAC-3′ |  
| rs2736100-R 5′- GGGAAACAAGGAGGAAAAG-3′ |  
| rs4488809-F 5′- ATGCAAGCATCTCGCTCTTG-3′ |  
| rs4488809-R 5′- TGTCATTCTCCTGTTTCTCTT-3′ |  
| rs2741354-F 5′- GGCCAAACAGGAAGAAGCT-3′ |  
| rs2741354-R 5′- ATTTCTGCTGAGCTTTTCTT-3′ |  
| rs12296850-F 5′- AGGATTCCATGGGAACTG-3′ |  
| rs12296850-R 5′- GTAGGTCCCACAGGAGT-3′ |  
| rs753955-SBE 5′- (GACT)1ATCATGTGAAGGCTTGAA-3′ |  
| rs2853677-SBE 5′- (GACT)3TTTGTCACTAGAGACCC-3′ |  
| rs2736100-SBE 5′- (GACT)5TCCGTGTGGTGTTTCT-3′ |  
| rs4488809-SBE 5′- (GACT)7TGCTTGGAGGAGCTA-3′ |  
| rs2741354-SBE 5′- (GACT)9GGTATCACCCAATACCAAG-3′ |  
| rs12296850-SBE 5′- (GACT)10CACATATAAAGAAAAGGGCTTAC-3′ |  

| Primers used for cloning |  
|--------------------------|---|
| SNAI1-CDS-F-Nflag 5′- CCGGTGCCACCATGAAGGACGAGCA CGATGAAAAGCAGGACGACG |  
| SNAI1-CDS-R 5′- CGGGATCCATCCAGGGCAGAAGGAGTGGCCAGGA-3′ |  

| Primers used for luciferase assay |  
|----------------------------------|---|
| TERT-A 5′- AGGAAGCTTCCGCAGGATTGCTCAGTCG-3′ |  
| TERT-S500 5′- TCACTCGGACATCGTGGTCGAGCC-3′ |  
| TERT-S1000 5′- GTCCCTGAGTTCGCTTGGATTGCGGC-3′ |  
| SNP-F 5′- CGGGGTACCATGTGAAGGACAGGAGCAGCG |  
| SNP-R 5′- CGGGAGCTTCAAGTGCAGGAGGAAAGCAGCA-3′ |  

| Primers used for EMSA |  
|----------------------|---|
| Snail-EMSA-F1-biotin 5′- ACTAGGACCCGGCCTGGCAGACTCTG-3′ |  
| Snail-EMSA-R1-biotin 5′- CAGAGTGCAACAGGCGTCTCTTAGT-3′ |  
| Snail-EMSA-F2-biotin 5′- ACTAGGACCCGGCTTGCTGCACTCTG-3′ |  
| Snail-EMSA-R2-biotin 5′- CAGAGTGCAACAGGCGTCTCTTAGT-3′ |  

| Primers used for ChIP |  
|----------------------|---|
| ChIP-S 5′- ACTTTTGGAAGACTCAAGGGATC-3′ |  
| ChIP-A 5′- ACACCTGGCAGGAAACAG-3′ |  
| E-Cad-S 5′- CAACCTCGAGCTGACGGATGACCAGC-3′ |  
| E-Cad-A 5′- AACTGACTTCGCAGAAGCTCAG-3′ |  
| β-actin-S 5′- GCCAAACGGCGAGAAGTAGCCAGA-3′ |  
| β-actin-A 5′- GAGTCCATCAGCAGATGCGCT-3′ |  

| primers used for RT-PCR | sequence |
|------------------------|----------|
| TERT-Q-F               | 5'-CCGATTGTGAACATGGACTACG-3' |
| TERT-Q-R               | 5'-CACGCTGAACGTTGCCTTC-3'   |
| SNAI1-Q-F              | 5'-ACTGCAACAAGGAAATACCTCAG-3' |
| SNAI1-Q-R              | 5'-GCACCTGTAACCCTTGGACATCTG-3' |
| GAPDH-F                | 5'-GTCAACGGATTTGGTCTGTATT-3' |
| GAPDH-R                | 5'-AGTCTTCTGGGTTGCACTGTAT-3' |
| primers used for 3C    | sequence |
| 3C-1                   | 5'-CCAGCCCCTCCCTTCTTTT-3'   |
| 3C-2                   | 5'-GCTCCAGGCACAAACGACGC-3'  |
| 3C-3                   | 5'-GGAGATGAGCAGTCGGGACT-3'  |
| 3C-4                   | 5'-CGGTGCTCGCCAAGCTATAT-3'  |
| 3C-5                   | 5'-CCCCAGGTGTCTTTGGCTTTG-3' |
| 3C-6                   | 5'-GGTPGGTATTAGTTATGGAAGCATG-3' |
| 3C-7                   | 5'-TGGTGCAGGTGTCTGGGATAG-3' |
| control 1-S            | 5'-GGGGAACCACGGACATGC-3'   |
| control 1-A            | 5'-CCCTGAAACCCACAAAACACT-3' |
| control 2-S            | 5'-GGCTGCTTCTGGAATGTTGGT-3' |
| control 2-A            | 5'-TGTTGAGAAACAGGGAACGAG-3' |
| control 3-A            | 5'-CTCCATACATCCAGCTACC-3'  |
| control 3-S            | 5'-TGGGAAACCAGGACAAAGG-3'  |

**Abbreviations**

The abbreviations used are: SNP, single nucleotide polymorphism; GWAS, genome-wide association studies; TERT, telomerase reverse transcriptase; HWE, Hardy-Weinberg Equilibrium; SBE, single base extension; 3C, chromosome conformation capture; ChIP, chromatin immunoprecipitation; EMT, epithelial-mesenchymal transition; RT-PCR, reverse transcription-polymerase chain reaction; SCLC, small cell lung cancer.

**ACKNOWLEDGMENTS**

We thank Dr. Weidong Li at Tianjin Medical University for his critical review.

**CONFLICTS OF INTEREST**

The authors declare no conflicts of interest.

**GRANT SUPPORT**

This work was supported by the National Natural Science Foundation of China (91519331, 31371295, 81572271, 81372307, 81572882), Ministry of Science and Technology of China (2014CB910104), the Tianjin Municipal Science and Technology Commission (15JCZDJC34800), the specialized Fund for the Doctoral Program of Higher Education (20121202110001).

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