TGFB2 and *BCL2L11* methylation in male laryngeal cancer patients

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Abstract. DNA methylation is a major regulatory mechanism of gene expression. The aim of the present study was to test the association of transforming growth factor β2 (*TGFB2*) and B cell lymphoma 2-like 11 (*BCL2L11*) gene methylation with the risk of laryngeal squamous cell carcinoma (LSCC). Using bisulfite pyrosequencing technology, DNA methylation levels of *TGFB2* promoter and *BCL2L11* gene-body CpG cytosines were measured in 90 LSCC tissues and 90 adjacent normal tissues. Analysis of variance and paired sample t-test were used to determine the association of gene methylation and the risk of LSCC. Our results revealed that there were no differences in *TGFB2* and *BCL2L11* methylation levels between the LSCC tissues and the paired normal tissues (P>0.05). Further breakdown analyses demonstrated that the association results of the two gene methylation levels and LSCC remained unchanged with the age, smoking history, histological differentiation or clinical stage of the LSCC patients (all adjusted P>0.05). In conclusion, there is no association of *TGFB2* promoter and *BCL2L11* gene-body methylation with the risk of LSCC in males.

Introduction

Laryngeal squamous cell carcinoma (LSCC) is the second most common head and neck cancer in the world (1). The incidence of LSCC in China has exhibited an increasing trend in the young population (2,3). LSCC is a complex disease, and it is hypothesized that both genetic and environmental factors play a role in it (4). Epigenetic modifications, including DNA methylation, are known as the bridging factors of genetics and the environment (5). However, the role of epigenetic modifications in the pathogenesis and progression of LSCC remains to be explained.

DNA methylation of CpG islands (CGIs) is a significant regulatory mechanism of gene expression (6). Epigenetic silencing of tumor suppressor genes by promoter hypermethylation is considered to be a significant event in the progression of cancers including leukemia (7), lung cancer (8) and prostate cancer (9). Gene-body methylation levels are also demonstrated to be correlated with gene expression (10,11).

TGFB2 is a member of the transforming growth factor β (TGFB) family, which encodes multifunctional peptides in the regulation of cell proliferation, differentiation, adhesion and migration (12). TGFB is able to inhibit tumor growth at the early stages and promote tumor growth at the late stages of disease (13). A previous study has demonstrated that increased *TGFB2* promoter methylation level is associated with prostate cancer progression (14). *BCL2L11* encodes a member of the BCL-2 (B cell lymphoma 2) protein family, which acts as an apoptotic activator that is involved in a wide variety of cellular activities (15). *BCL2L11* methylation has been noted to be associated with the risk of hematological and epithelial cancers (16).

In the current study, the methylation levels of *TGFB2* and *BCL2L11* were investigated in male laryngeal cancer patients to determine whether these epigenetic markers are associated with LSCC risk.

Materials and methods

Tissue samples. LSCC and adjacent tissues were collected from 90 male patients (mean age, 60.5±8.65 years) who had undergone surgical treatment at the Department of Otolaryngology at Ningbo Lihuili Hospital, Zhejiang, China. The 90 tumors consisted of 43 well-differentiated cases, 32 moderately differentiated cases and 15 poorly differentiated cases. Among the LSCC patients, there were 27 stage I, 14 stage II, 11 stage III

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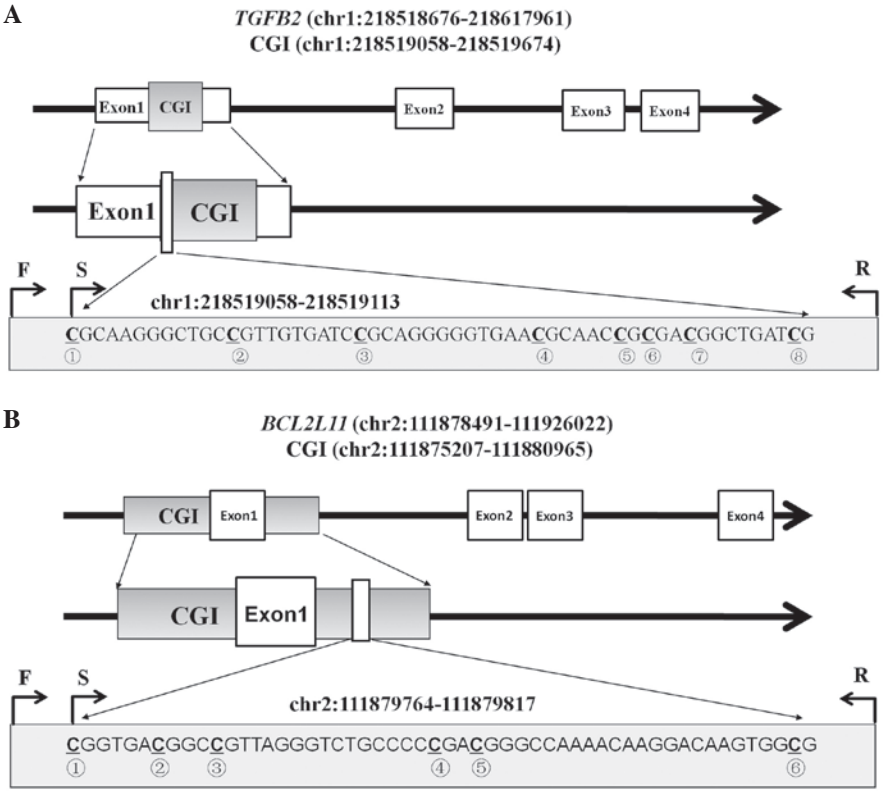

Figure 1. CpG islands (CGIs) within the transforming growth factor β2 (*TGFB2*) promoter and B cell lymphoma 2-like 11 (*BCL2L11*) gene-body. (A) Eight CpG dinucleotides within *TGFB2*; (B) Six CpG dinucleotides within *BCL2L11*. F, forward primer; S, sequencing primer; R, reverse primer.

and 38 stage IV patients. All specimens were obtained freshly during the surgery and stored at -80˚C. Permission was obtained from the bioethics committee at Ningbo Lihuili Hospital. All patients signed informed consent forms.

DNA bisulfite treatment and pyrosequencing analysis. Genomic DNA was isolated from tumor samples and then quantified as previously described (11,17). *TGFB2* and *BCL2L11* methylation levels were measured using bisulfite pyrosequencing technology. DNA conversion was performed using reagents provided in the Zymo EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA, USA). The pyrosequencing procedures were performed as described previously (11,17). The primer sequences of *TGFB2* were 5'-GAGTAATTTTAAGTTGGGGAGAAGTTAGT-3' for the forward primer, 5'-Biotin-ACTCCAAACCCCAACCCA ACCA-3' for the reverse primer, and 5'-CCAACCCAACCA CAA-3' for the sequencing primer. The primer sequences of *BCL2L11* were 5'-GAGGAAGTTGTTGGAGGAGAAT-3' for the forward primer, 5'-Biotin-ACCCTACCCATCCCTATAC-3' for the reverse primer, and 5'-ATCCCCAAACCCAAT-3' for the sequencing primer.

Statistical analyses. Statistical analyses were performed by PASW Statistics 18.0 software (SPSS Inc., Chicago, IL, USA). Logarithmic transformation was applied for the data deviated from normality. Analysis of variance was used to evaluate the association between the risk factors (age, smoking history, histological differentiation and clinical stage) and relative methylation rate difference. A paired sample *t*-test was applied to compare *TGFB2* and *BCL2L11* methylation between the LSCC and adjacent tissues. All *P*-values were adjusted by logistic regression. A two-tailed P<0.05 was considered to indicate a statistically significant difference. All figures were drawn using GraphPad Prism 6 software (GraphPad, San Diego, CA, USA).

Results

In the present study, we assessed the *TGFB2* and *BCL2L11* methylation levels in LSCC and their adjacent non-neoplastic tissues using bisulfite pyrosequencing technology. As shown in Fig. 1, the tested fragments were located in the CGIs of the *TGFB2* promoter (chr1:218519058-218519113) and *BCL2L11* gene-body (chr2:111879764-111879817). A total of eight and six CpG dinucleotides were measured for *TGFB2* and *BCL2L11*, respectively. The mean methylation levels of the tested CpG sites were used to compare their differences between the LSCC tumor and the adjacent normal tissues.

Our results revealed that there were no statistically significant differences of the methylation levels of *TGFB2* promoter (Fig. 2, P=0.653) and *BCL2L11* gene-body (Fig. 3, P=0.311) between LSCC tissues and the paired normal tissues. Further tests demonstrated no change in the results when adjusted for age, smoking history, histological differentiation and clinical stage (all adjusted P>0.05). Moreover, there was no difference in the *TGFB2* promoter or *BCL2L11* gene-body methylation levels between tumor tissues and paired normal tissues in the breakdown analyses by age, smoking history, histological differentiation or clinical stage of the patients (Figs. 2 and 3, all P>0.05, data not shown).

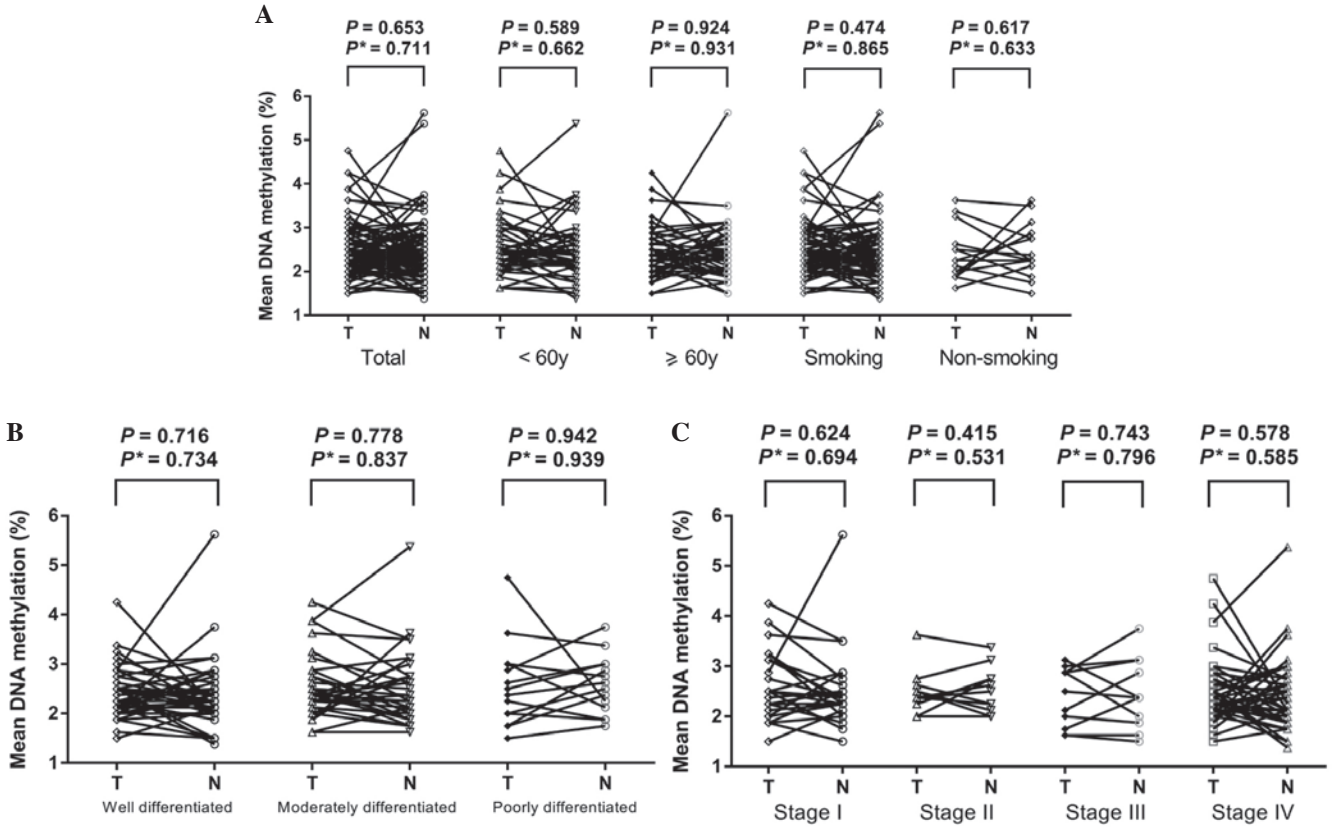

Figure 2. Comparisons of mean methylation level of transforming growth factor β2 (*TGFB2*) gene between tumor tissues and paired adjacent normal tissues. (A) Subgroup tests by age or smoking history; (B) Subgroup tests by histological differentiation; (C) Subgroup tests by clinical stage. T, tumor tissue; N, normal tissue. P*, adjusted for age, smoking history, differentiation and stage.

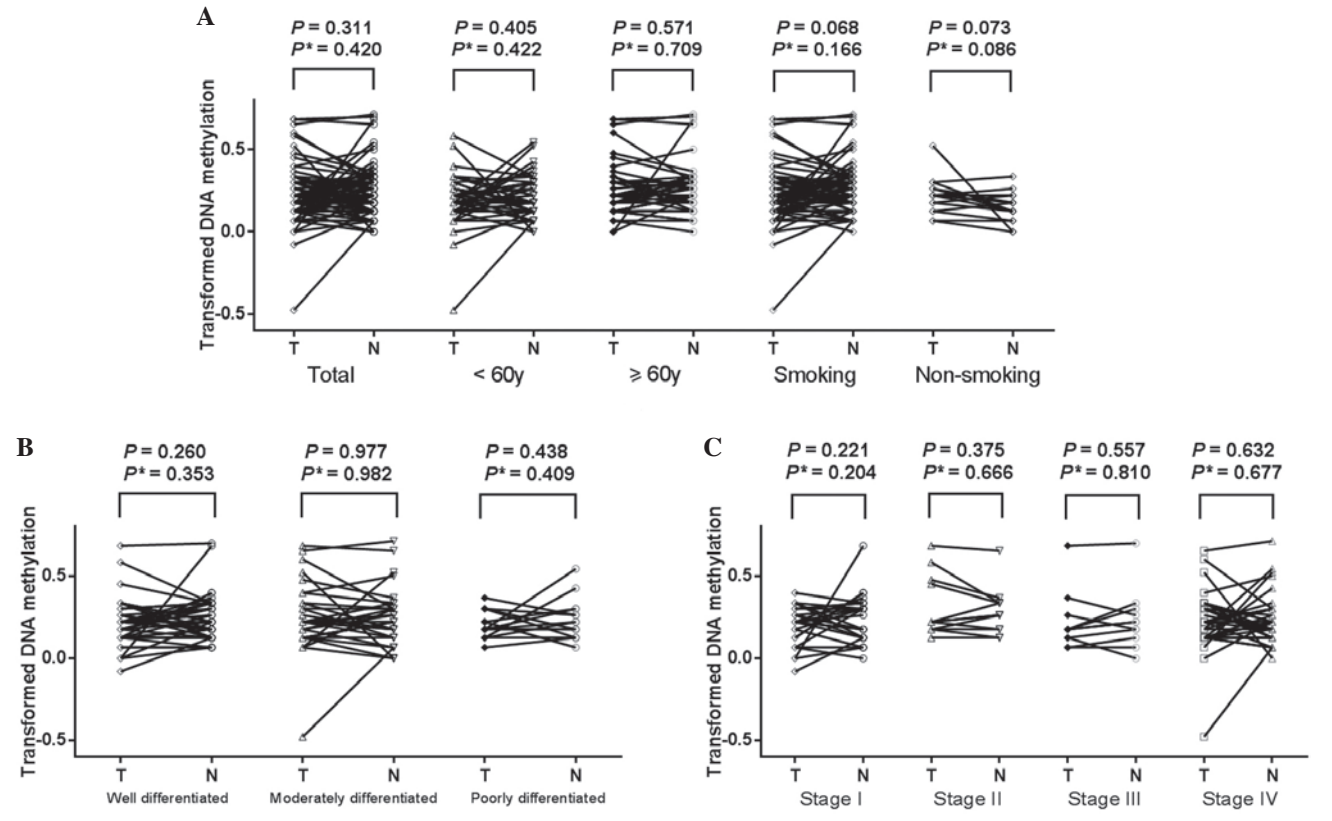

Figure 3. Comparisons of transformed methylation level of B cell lymphoma 2-like 11 (*BCL2L11*) gene between tumor tissues and paired adjacent normal tissues. (A) Subgroup tests by age and smoking history; (B) Subgroup tests by histological differentiation; (C) Subgroup tests by clinical stage. T, tumor tissue; N, normal tissue. P*, adjusted for age, smoking history, differentiation and stage.

Discussion

The aim of the present study was to determine whether DNA methylation of the *TGFB2* promoter and *BCL2L11* gene-body was associated with LSCC risk. Through a series of statistical analyses of these two genes, no clear correlation with laryngeal cancer was observed. Further subgroup tests by age, smoking history, histological differentiation and clinical stage also produced similar results.

Epigenetic modifications are central to tumorigenesis (18). In cancer cells, global hypomethylation is accompanied by hypermethylation of certain gene promoter CGIs (6). DNA methylation of the gene promoter region leads to gene silencing by blocking the binding of transcription factors or methyl-binding proteins (6), while a positive correlation has been observed in a variety of human tissue types between gene-body methylation and gene expression (10,19). As for LSCC, aberrant DNA methylation of tumor suppressor genes, including *MGMT, CHD5*, *p16*, *p14*, *PTEN*, *MYCT1* and *FHIT,* has been reported (4,20-23). DNA methylation levels of the promoter and gene-body are relatively dynamic (24). However, there are no previous studies on gene-body methylation in LSCC.

TGFB2 promoter hypermethylation was first identified as a potential epigenetic marker of prostate cancer in whole genome methylation profiling studies (25). A previous study revealed that the gene suppression of *TGFB2* was not associated with DNA methylation but with chromatin remodeling in breast cancer tissue samples (26). In the present study, we did not observe any association between *TGFB2* methylation and LSCC, suggesting that there were other genetic or epigenetic modifications in LSCC.

The apoptosis-related gene *BCL2LL1* has been noted to be hypermethylated in colon cancer (27), hematological and epithelial cancers (16). In addition, low *BCL2L11* expression in chronic myeloid leukemia was ascribed to *BCL2LL1* hypermethylation at the gene promoter (28). Due to the limitation of not identifying a suitable primer set to assess the promoter CGI of the *BCL2LL1* gene, we selected the CGI in the gene-body region as an alternative. Although our study did not identify any significant association with *BCL2L11* gene-body methylation, the contribution of other CGIs to LSCC risk remains to be explored.

In conclusion, our study suggests a lack of contribution of the *TGFB2* promoter and *BCL2L11* gene-body methylation to LSCC in male patients. However, this study was designed as a pilot study, and further investigations are required to confirm our findings.

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