A polymorphic minisatellite region of BORIS regulates gene expression and its rare variants correlate with lung cancer susceptibility

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Exceptional expression of BORIS/CTCFL (Brother of the Regulator of Imprinted Sites/CTCF-like protein) is reported in different malignancies. In this study, we characterized the entire promoter region of BORIS/CTCFL, including the CpG islands, to assess the relationship between BORIS expression and lung cancer. To simplify the construction of luciferase reporter cassettes with various-sized portions of the upstream region, genomic copies of BORIS were isolated using TAR cloning technology. We analyzed three promoter blocks: the GATA/CCAAT box, the CpG islands and the minisatellite region BORIS-MS2. Polymorphic minisatellite sequences were isolated from genomic DNA prepared from the blood of controls and cases. Of the three promoter blocks, the GATA/CCAAT box was determined to be a critical element of the core promoter, while the CpG islands and the BORIS-MS2 minisatellite region were found to act as regulators. Interestingly, the polymorphic minisatellite region BORIS-MS2 was identified as a negative regulator that repressed the expression levels of luciferase reporter cassettes less effectively in cancer cells compared with normal cells. We also examined the association between the size of BORIS-MS2 and lung cancer in a case–control study with 590 controls and 206 lung cancer cases. Rare alleles of BORIS-MS2 were associated with a statistically significantly increased risk of lung cancer (odds ratio, 2.04; 95% confidence interval, 1.02–4.08; and P=0.039). To conclude, our data provide information on the organization of the BORIS promoter region and gene regulation in normal and cancer cells. In addition, we propose that specific alleles of the BORIS-MS2 region could be used to identify the risk for lung cancer.

INTRODUCTION

The BORIS/CTCFL (Brother of the Regulator of Imprinted Sites/CTCF-like protein) gene is classified as a member of the cancer-testis antigen (CTA) family; it is expressed in the testis during spermatogenesis,12 as well as in multiple cancers, including uterine (endometrial), breast, lung and gastric cancers.3–6 High levels of the BORIS protein in the leukocytes of patients with breast cancer were detected, which suggests that BORIS can be used as a valuable marker.7 Moreover, a CpG island in the promoter region of BORIS is known to be a key factor regulating gene expression,4,8 and BORIS expression in human cancer cells has been proposed to lead to epigenetic deregulation. Overexpression of BORIS has been correlated with the hypomethylation of its promoter in prostate and ovarian cancers in several reports,9,10 and partial demethylation of the BORIS promoter has been detected in ovarian,11 colon11 and lung cancer8 and in leukemic cell lines.11 However, in our previous study, no correlation between methylation status and BORIS gene expression in gastric cancer tissues was detected.6

To elucidate the regulation of BORIS during tumorigenesis, in a previous study, we characterized the entire genomic region of the BORIS locus including the promoter region in gastric cancer cells.12 A CpG island (∓1096 to –762 from the first ATG) and two minisatellites (variable number of tandem repeats; BORIS-MS1 and BORIS-MS2) were identified through the characterization of the genomic DNA sequence upstream of

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the gene-coding region. In this study, we assessed the expression and methylation status of the BORIS gene in lung cancer tissues and re-analyzed the regulatory elements, that is, the CpG island and two minisatellites, in its promoter region to determine whether these elements are necessary for expression of a reporter gene in lung cancer cells. In addition, we described two well-positioned elements that contained the binding sequences for GATA-1 and the CCAAT box. 

Lung cancer is the most commonly diagnosed cancer and the leading cause of death in males. In addition, it is the fourth most common cancer in females worldwide, based on GLOBOCAN 2008 estimates, and it is a major public health problem in Korea. Because some minisatellite alleles are associated with human diseases, we investigated the relationship between cancer predisposition and minisatellite variants in BORIS and revealed increased susceptibility in young patients with breast cancer containing short rare minisatellite alleles of BORIS-MS2. To determine whether allelic variation in BORIS minisatellites influences susceptibility to lung cancer, a case–control study was performed using a PCR-based method. Here, we report that rare allelic variants of BORIS-MS2 are correlated with lung cancer susceptibility in a Korean population. In addition, to assess whether this minisatellite region has a role in gene regulation, the transcriptional levels of a reporter gene linked to the minisatellites and driven by the BORIS promoter in lung cell lines were examined.

MATERIALS AND METHODS

Study population and genotyping assays for BORIS-MS2

To examine the minisatellite polymorphisms in BORIS-MS2, a case–control study was performed with 590 cancer-free controls and 206 lung cancer cases. The controls and cases had similar characteristics regarding sex and age (control average age, 60.5 years, range 30–83 years; patient average age, 63.0 years, range 32–82 years) (Table 1). Controls were selected from the Department of Preventive Medicine and Internal Medicine at Dong-A University hospitals between 1997 and 2004 (Busan, Korea). The control group, with no personal history of cancer or current cancer, was recruited and completed an interview. The biospecimens from lung cancer patients in this study were provided by the Chungbuk National University Hospital, a member of the National Biobank of Korea, which is supported by the Ministry of Health, Welfare and Family Affairs and Dong-A University Hospital in Busan, Korea. All samples from the National Biobank of Korea were obtained with informed consent and institutional review board-approved protocols. The Committee of Bioethics of Dong-A University approved the procedure and design of this study (#IRB-06-10-02 & IRB-07-10-7; Busan, Korea).

For genotyping, genomic DNA was isolated from peripheral leukocytes, which were isolated from 400 μl of whole blood using a Blood and Cell Culture DNA Mini Kit (Qiagen, Valencia, CA, USA). The genotyping assay of the minisatellite polymorphism was described previously with the PCR primer pair 5′-CTTGGGAGACCTGGGGGATGAATAG-3′ (forward) and 5′-GCACCCCCATCCCCATCCTC-3′ (reverse) for BORIS-MS2. The PCR products were separated on a 1.2% agarose gel at 80 V for 4 h and then stained with ethidium bromide.

RNA isolation and reverse transcription

Total RNA was isolated from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The Qiagen RNeasy Mini kit (Qiagen) was used for isolation of total RNA from cell lines and lung cancer tissue specimens. For the reverse transcription reaction, a mixture of total RNA (3 μg), an oligo(dT)20 (50 nm) and 10 mM dNTP were incubated at 65°C for 5 min. A mixture of 1× RT buffer, 25 mM MgCl2, 0.1 mM DTT and RNaseOUT were incubated at 42°C for 2 min. After incubation, Invitrogen SuperScript III (200 U μl−1) was added. This mixture was then incubated at 42°C for 50 min followed by 70°C for 15 min. RNase H (2 U μl−1) was then added, and the sample was incubated at 37°C for 20 min in a 9700 Thermocycler (Perkin-Elmer, Waltham, MA, USA) as described previously.

Bisulfate sequencing of the BORIS promoter regions

Genomic DNA was subjected to bisulfite modification using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA). Each sequence was amplified using G-Taq polymerase (Cosmo Genetech, Seoul, Korea) as previously described. PCR fragments were gel extracted using a Gel Extraction kit (Zymo Research, Irvine, CA, USA) as previously described. The PCR products were separated on a 1.2% agarose gel at 80 V for 4 h and then stained with ethidium bromide.

Table 1 Age and sex distribution of cases and controls

| Characteristic | Males (%) | Females (%) | Total |
|----------------|-----------|-------------|-------|
| **Age (years)** |           |             |       |
| 30–39          | 7 (2.1)   | 21 (8.0)    | 28 (4.7)|
| 40–49          | 21 (6.4)  | 43 (16.3)   | 64 (10.8)|
| 50–59          | 71 (21.8) | 71 (26.9)   | 142 (24.1)|
| 60–69          | 153 (46.9)| 81 (30.7)   | 234 (39.7)|
| 70–79          | 68 (20.9) | 42 (15.9)   | 110 (18.6)|
| ≥ 80           | 6 (1.8)   | 6 (2.3)     | 12 (2.0) |
| **Average**    | 62.9      | 57.5        | 60.5  |
| **Median**     | 64        | 56          | 62    |
| **N**          | 326       | 264         | 590   |

Lung cancer cases, N (%)

| Characteristic | Males (%) | Females (%) | Total |
|----------------|-----------|-------------|-------|
| **Age (years)** |           |             |       |
| 30–39          | 2 (1.2)   | 2 (5.7)     | 4 (1.9)|
| 40–49          | 10 (5.8)  | 4 (11.4)    | 14 (6.8)|
| 50–59          | 37 (21.6) | 7 (20.0)    | 44 (21.4)|
| 60–69          | 84 (49.1) | 11 (31.4)   | 95 (46.1)|
| 70–79          | 34 (19.9) | 9 (25.7)    | 43 (20.9)|
| ≥ 80           | 4 (2.3)   | 2 (5.7)     | 6 (2.9) |
| **Average**    | 63.4      | 61.1        | 63.0  |
| **Median**     | 64        | 64          | 64    |
| **N**          | 171       | 35          | 206   |
as follows: open circles indicate non-methylated CpG and filled circles indicate methylated CpG (Figure 1).

**Plasmid construction**

To generate the luciferase reporter vectors with various fragments of the BORIS 5′-promoter region, fragments were amplified from a bacterial artificial chromosome clone (Supplementary Figure S1) containing the BORIS genomic sequence by PCR and inserted into the KpnI/NheI sites of the luciferase reporter vector pGL3-Basic (Promega, Madison, WI, USA). The primers and the plasmids in this study are listed in Supplementary Table S1. To characterize the effect of BORIS-MS2 on BORIS expression, part of the BORIS promoter p700 (−1108 to −425) was cloned and introduced into the BgIII/HindIII sites of the luciferase reporter vector pGL3-Basic to generate the p700 fragment (Figure 2). Mutated constructs (GATA-1Δ1, GATA-1Δ2 and CCAATΔ) were prepared by substitution of the GATA-1 site and CCAAT box in the p700 construct as shown in Figure 3. Two common (TR14 and TR15) alleles and five rare alleles (TR10, TR13, TR16, TR17 and TR18) were amplified from genomic DNA derived from controls and cases and inserted into the KpnI/BglII sites of the p700 fragment to generate the reporter plasmids p700+TR10, p700+TR13, p700+TR14, p700+TR15, p700+TR16, p700+TR17 and p700+TR18 (Figure 4). All constructs were confirmed by DNA sequencing.

**Cells and luciferase assays**

The following human cell lines were tested for the effect of BORIS-MS2 on BORIS expression: 293T/HEK293T (human embryonic kidney cell line obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea), H1299 (lung cancer cell line from the American Type Culture Collection (ATCC, Manassas, VA, USA) and MDA-MB-231 (breast cancer cell line from KCLB). For the luciferase assay, cells

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**Figure 1** Gene expression and methylation status of the 5′ flanking non-coding region of BORIS. (a) Gene expression of BORIS in tumor tissues (T1–T10) of lung cancer patients. (b) Results of bisulfite sequencing analysis of a CpG island in the 5′ flanking region of BORIS in tumor tissues (T) and lung cancer cells (H1299 cells and H1299 cells treated with 1 and 5 μM 5-azad-C). Open circles (○) indicate non-methylated CpG; filled circles (●) indicate methylated CpG; percentages indicate the ratio of methylation.
(1 × 10^5) were seeded in 12-well plates, cultured overnight and transfected with the BORIS promoter-luciferase plasmids (0.5 μg per well) using the FuGENE6 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) at a ratio of DNA/FuGENE6 of 1:3. Analysis of the cells was performed using a dual-luciferase reporter assay system (Promega) 48 h after completion of the transfection procedure. Firefly luciferase activities were normalized to Renilla luciferase values, and activity was expressed in relative luciferase units to reflect the promoter activity. Triplicate transfections of each construct were tested for each experiment, and the final results were calculated from four independent experiments.

Statistical analysis

Regression analyses were carried out to determine the odds ratios (ORs) of association between the control and case groups. ORs were predicted using the natural logarithm and its s.e. Where relevant, a χ²-test was performed with one degree of freedom for statistical significance. Differences were regarded as significant with confidence intervals (CIs) of 95%. All tests were two-sided, with P<0.05 considered statistically significant. Statistical analyses were performed using MS Excel with CHITEST and R statistical software (v2.5.1, www.r-project.org) with χ²-test for the χ²-calculation values. The Kaplan–Meier plot was used in the R program version 2.10.0 (https://www.r-project.org/).

RESULTS

BORIS expression and methylation status in lung cancer tissues

BORIS gene expression was examined in 20 cell lines, which included normal fibroblast, osteosarcoma, melanoma, breast cancer, prostate cancer, colon cancer, lung cancer and gastric cancer cell lines from a previous study. Compared with the
normal cells, various cancer cells, including osteosarcoma, melanoma, breast cancer, prostate cancer, colon cancer, lung cancer and gastric cancer showed abnormal expression of BORIS. Among the lung cancer cell lines (A549, H358, H460, H1299), H1299 cells showed the highest expression. Then, we investigated BORIS gene expression in lung tumor tissues (Figure 1). In 10 lung tumor tissues, the BORIS levels were different; the lowest levels were observed in 2 cancer tissues (T2 and T10), moderately increased levels were observed in 4 cancer tissues (T1, T3, T4 and T5) and the highest levels were detected in 4 cancer tissues (T6, T7, T8 and T9) at the same level as the H1299 cancer cell line (Figure 1a). To analyze the possible relationship between BORIS gene activity and epigenetic changes in lung cancer, we assessed DNA methylation levels in these tumor tissues. As previously reported in gastric cancer tissues, methylation (89.1–95.7%) of the BORIS promoter region in lung cancer tissues was almost the same despite the different degree of BORIS expression (Figure 1b). The lung cancer cell line H1299 also showed similar levels of methylation (94%), but cells treated with 5-azad-C (1 and 5 μM) showed decreased methylation levels (31.7–47%). We next extensively analyzed the BORIS promoter region to elucidate the regulation of this gene during tumorigenesis.

Cloning of the 5′-regulatory region of the BORIS gene and characterization of transcriptional activities of the promoter

We cloned the genomic fragment containing the entire BORIS gene region (Supplementary Figure S1) from human genomic DNA using the TAR cloning method. Two clones containing a ~70-kb genomic fragment were isolated and found to carry the entire BORIS gene (Supplementary Figures S1B and C).

The YAC clone was identified by screening a mini-YAC library with PCR using a set of diagnostic primers (Supplementary Table S1). The YAC includes the BORIS-coding region, the ~30 kb 5′ upstream region and the ~8 kb 3′ flanking regions. TAR cloning of BORIS in yeast greatly simplifies PCR amplification of the promoter regions enriched by repeated and CG-rich sequences, which are poorly amplified from human genomic DNA. We constructed the various reporter vectors (see Materials and Methods, Supplementary Table S1, Figures 2, 3, 4) using this clone (BORIS I, Supplementary Figures S1B and C).

Transient transfection of 293T cells with the cloned −2909/+5 genomic fragment (p2914) using the luciferase reporter construct resulted in a significant (P < 0.001) increase in promoter activity, which was >18-fold greater than the activity observed with the pGL3-Basic vector (Figure 2a). In contrast, a larger 5115 bp genomic fragment (p5115; −5110/+5) of the BORIS 5′-regulatory region cloned into the pGL3-Basic plasmid and transfected in 293T cells did not show any promoter activity (Figure 2a).

To determine the core BORIS promoter, we performed 5′-deletion analysis to determine the minimal region required for BORIS promoter activity (Figure 2b). The promoter activity of each of the deletion constructs was assessed by measuring luciferase activities in transiently transfected 293T cells. The results showed that luciferase activity from the shorter 700-bp promoter fragment (−1108/−425, p700) had approximately threefold greater activity than the full-length fragment (p2914); this p700 vector contained the CpG and GATA-1 regions. This observation suggests the possible existence of a strong suppressor element between −2909 and −1108 nt of the
A previous study\textsuperscript{11} reported three transcription start sites in the BORIS sequence—1447, 899 and 658 bp upstream of the first ATG codon. This observation suggests the presence of three alternative promoter regions that may have cell type-specific activity. We constructed three expression cassettes (PrA, PrB and PrC) containing the predicted promoters\textsuperscript{11} and compared levels of luciferase expression in HEC293 cells (Supplementary Figure S2). The p700 vector containing the CpG and GATA-1 regions showed the highest promoter activity, which was over 60-fold greater than the activity observed with the pGL3-Basic vector (Supplementary Figure S2).

To obtain information on putative regulatory sites in the p700 promoter region of the BORIS gene, we analyzed the DNA fragment (−1108/−425). As shown in Figure 2a, a deletion of 23 bp from the 3′-end of the p700 vector (vector p678) resulted in a dramatic reduction in promoter activity. The deleted region (−447/−425 region, Figure 2) includes the GATA-1-binding site and the CCAAT box.\textsuperscript{11} To assess the involvement of these putative regulatory sites in regulating the promoter activity of the BORIS gene, we introduced specific mutations in the core sequences of these sites and examined the effect of these mutations on promoter activity (Figure 3a). The results show that mutating these elements led to a significant ($P<0.01$) reduction in promoter activity compared with the p700 promoter (Figure 3b). These results suggest that the region including the GATA-1 site and the CCAAT box has a role in activating BORIS expression.

**Effect of minisatellites (BORIS-MS2) on BORIS expression**

The comparative promoter activities of p2914 and p700 in Figure 2b suggests the presence of a suppressor element located in the −2909 to −1108 region; this region corresponds to the minisatellites of BORIS-MS2. Therefore, we constructed seven different vectors (Figures 4a; p700+TRs) consisting of the p700 fragment and the BORIS-MS2 minisatellites (TR10, TR13, TR14, TR15, TR16, TR17 and TR18), which were genotyped using genomic DNA from lung cancer cases (Figure 5). For all seven constructs harboring minisatellites, the luciferase activities were significantly reduced in 293T cells compared with the p700 reporter vector (5- to 10-fold reductions) (Figure 4b). Notably, when the same BORIS-MS2-containing constructs were transfected into lung cancer cells (H1299) or breast cancer cells (MDA-MB-231), we observed only a approximately twofold reduction in promoter activity (Figures 4b and c). This result suggests that the constructs containing the minisatellites showed different promoter activity in normal and cancer cell lines. In addition, we also constructed seven variants of the p2298 vector, which included the p700 promoter region, the region between the p700 promoter and BORIS-MS2, and different fragments of BORIS-MS2 (10–18 copies of a 56 bp repeat) (Supplementary Figure S3). These constructs were also transfected into three different cell lines, and similar results were observed. Therefore, BORIS-MS2 minisatellites may have a role in aberrant activation of BORIS during carcinogenesis. Next, we investigated a possible link between the minisatellite variants and lung cancer in a Korean population.

**Analysis of minisatellite variants in lung cancer patients**

Several lung cell lines and many lung tumor specimens express high levels of BORIS.\textsuperscript{6,8} Because BORIS is a potential new marker for the detection of lung cancer, we investigated whether BORIS-MS2 may correlate with lung cancer development. In a previous study, BORIS-MS2 was found to possess
genetically variable alleles. In this study, we investigated BORIS of a Haplotyping of Figure 5 satellite repeat on another chromosome) was significantly more frequent in cases (2.9%) than in controls (0.5%) (P<0.005), and for the frequency of the allelic distribution (Table 2) 13 repeated alleles of BORIS-MS2 were significantly more frequent in cases than in controls (0.5 vs 1.7%). Analysis of these data revealed a significant association between 13 tandem repeat alleles and the odds for lung cancer (BORIS-MS2 and lung cancer OR: 3.38, CI: 1.13–10.12; P = 0.021).

For further analysis, each BORIS-MS2 allele was divided into common or rare alleles (the frequency for rare alleles was <1%) based on their frequency in the control population. Seven alleles of BORIS-MS2 were grouped into 2 common alleles (14 and 15 repeats) and 5 rare alleles (10, 13, 16, 17, and 18 repeats) in this study (Table 3). There was a significant difference in the frequency of rare alleles found in controls and cancer cases (OR: 2.04, CI: 1.02–4.08; P = 0.039). Specifically, the frequency of rare alleles in female cases with lung cancer showed a statistically significant difference between controls and cases (OR = 3.14; P = 0.047). Furthermore, we divided the rare alleles into short (10 and 13) and long (16, 17 and 18) groups based on their tandem repeat lengths (Table 4). The analysis of the short rare alleles (10 and 13 repeats) group, comparing controls and cases, also showed an association, with a relative lung cancer OR of 2.37 (P = 0.049). There was a more significant difference in the frequency of short rare alleles between male controls and male cases (OR = 3.86; P = 0.041) (Table 4).

We used clinicopathological information obtained in 2009 from the Korea University Anam Hospital in Korea and between 2002 and 2007 from Dong-A University and Chungbuk National University Hospital in Korea (Supplementary Table S2). Tumors, nodes and distant metastasis stages were analyzed according to the World Health Organization (WHO) system. Lung tumors were categorized into the appropriate class, and we then assessed the frequency of each class in the entire cancer group and the rare allele group. There was no association between short rare alleles and cancer classification corresponding to stage, T stage, N stage, M stage or cell type.

**DISCUSSION**

BORIS is a CTA gene family member, and its transcription is abnormally activated in various tumors and cancer cells. It was also reported that the expression of BORIS is predominantly controlled by DNA methylation and that BORIS mediates the epigenetic regulation of other genes (NY-ESO-1, NOTCH3, hTERT and so on) in cancer cells. In our previous study, however, no correlation between methylation status and BORIS gene expression was detected in tissues from gastric cancer. Similar data were obtained in this work based on analysis of tissues from lung cancer. Based on these results, we suggest that the expression of BORIS is regulated by more complex epigenetic mechanisms.

To elucidate the regulation of BORIS beyond DNA methylation, we physically characterized the entire BORIS locus. A CpG island (−1096 to −762 from the first ATG codon) and two minisatellites (variable number of tandem repeats; BORIS-MS1 and BORIS-MS2) were identified through the
characterization of the genomic DNA sequence upstream of the gene. In this study, we examined the ~5.5-kb region upstream of the start codon of BORIS. A set of luciferase reporter cassettes carrying fragments from the upstream region of various sizes were constructed and tested (Figure 2). The highest luciferase activity was detected with a 700-bp promoter fragment (~1108–425, p700) which contained CpG islands, a GATA-1 site and CCAAT box regions. This finding is in

Table 2 Comparison of allelic sizes and frequency of Boris–MS2

| TR | Size (bp) | Male N = 652 | Female N = 528 | Total N = 1180 | Male N = 342 | Female N = 70 | Total N = 412 | OR (95% CI) | P-value |
|----|-----------|--------------|----------------|---------------|--------------|--------------|--------------|------------|---------|
| 10 | 785       | 1 (0.002)    | 4 (0.008)      | 5 (0.004)     | 1 (0.003)    | 1 (0.014)    | 2 (0.005)    | 1.15 (0.22–5.93) | 0.871   |
| 13 | 935       | 2 (0.003)    | 4 (0.008)      | 6 (0.005)     | 5 (0.015)    | 2 (0.029)    | 7 (0.017)    | 3.38 (1.13–10.12) | 0.021*  |
| 14 | 990       | 392 (0.601)  | 331 (0.627)    | 723 (0.613)   | 219 (0.640)  | 47 (0.671)   | 266 (0.646)  | 1.15 (0.91–1.45) | 0.236   |
| 15 | 1050      | 250 (0.383)  | 187 (0.354)    | 437 (0.370)   | 113 (0.330)  | 19 (0.271)   | 132 (0.320)  | 0.80 (0.63–1.01) | 0.069   |
| 16 | 1100      | 2 (0.003)    | 1 (0.002)      | 3 (0.003)     | 3 (0.009)    | —            | 3 (0.007)    | 2.88 (0.58–14.31) | 0.177   |
| 17 | 1160      | 1 (0.002)    | —              | 1 (0.001)     | —            | —            | —            | —          | 0.554   |
| 18 | 1220      | 4 (0.006)    | 1 (0.002)      | 5 (0.004)     | 1 (0.003)    | 1 (0.014)    | 2 (0.005)    | 1.15 (0.22–5.93) | 0.871   |

Abbreviations: CI, confidence interval; OR, odds ratio; TR, tandem repeat.

*P<0.05.

Table 3 The frequency of rare Boris–MS2 alleles in controls and lung cancer cases

| No. of alleles | 14 | 15 | Total |
|----------------|----|----|-------|
| Controls       |    |    |       |
| Male           | 652 (%) | 392 (60.1) | 250 (38.4) | 642 (98.5) |
| Female         | 528 (%) | 331 (62.7) | 187 (35.4) | 518 (98.1) |
| Total          | 1180 (%) | 723 (61.3) | 437 (37.0) | 1160 (98.3) |
| Lung cancer cases |    |    |       |
| Male           | 342 (%) | 219 (64.0) | 113 (33.0) | 332 (97.0) |
| Female         | 70 (%)   | 47 (67.2)  | 19 (27.1)  | 66 (94.3)  |
| Total          | 412 (%)   | 266 (64.6) | 132 (32.0) | 398 (96.6) |

Abbreviations: CI, confidence interval; OR, odds ratio.

*P<0.05.

Table 4 The frequency of short rare Boris–MS2 alleles in controls and lung cancer cases

| No. of alleles | 14 | 15 | 16 | 17 | 18 | Total |
|----------------|----|----|----|----|----|-------|
| Controls       |    |    |    |    |    |       |
| Male           | 652 (%) | 392 (60.1) | 250 (38.3) | 2 (0.3) | 1 (0.2) | 4 (0.6) | 649 (99.5) |
| Female         | 528 (%) | 331 (62.7) | 187 (35.4) | 1 (0.2) | 0 (0.0) | 1 (0.2) | 520 (98.5) |
| Total          | 1180 (%) | 723 (61.3) | 437 (37.0) | 3 (0.3) | 1 (0.1) | 5 (0.4) | 1169 (99.1) |
| Lung cancer cases |    |    |    |    |    |       |
| Male           | 342 (%) | 219 (64.0) | 113 (33.0) | 3 (0.9) | 0 (0.0) | 1 (0.3) | 336 (98.2) |
| Female         | 70 (%) | 47 (67.2) | 19 (27.1) | 0 (0.0) | 0 (0.0) | 1 (1.4) | 67 (95.7)  |
| Total          | 412 (%) | 266 (64.6) | 132 (32.0) | 3 (0.7) | 0 (0.0) | 2 (0.5) | 403 (97.8) |

Abbreviations: CI, confidence interval; OR, odds ratio.

*P<0.05.
agreement with a previous study, where three transcription start sites were mapped within the same 5'-upstream region. However, our analysis revealed a potential suppressor between -2909 and -1108 nt of the BORIS 5'-regulatory region containing the minisatellite region of BORIS-MS2 (Figure 2b). This finding suggests that the transcriptional regulation of BORIS is more complicated and that other types of regulation may exist in addition to DNA methylation. Moreover, there are also several reports indicating that minisatellite polymorphisms can influence gene expression.16,17,24-28

In separate experiments, we investigated how polymorphic variants of BORIS-MS2 minisatellite affect the transcriptional activity of BORIS in cell lines using a luciferase reporter system (Figure 4). In 293T normal cell lines, all seven minisatellite constructs (TR10, 13, 14, 15, 16, 17, and 18) significantly decreased (5-10 times reduction) the activity of the p700 BORIS promoter, but there was no clear relationship between the tandem repeat copy number and level of gene expression. In principle, this result may be suggestive of the restriction of in vivo experiments. However, when the same constructs were transfected in lung cancer cells (H1299) or breast cancer cells (MDA-MB-231), the promoter activity was reduced only approximately two times compared with the control. This result suggests that the expression cassettes carrying BORIS-MS2 are controlled in a different way in normal and cancer cell lines. Similar results for transcriptional regulation by minisatellite fragments were previously reported with H-Ras, IL-1α, IGF2, MUC6 and TERT genes.16,17,24-26 Minisatellites of IL-1α, MUC6 and TERT may control gene expression in cancer cell lines, although these are located in the intronic regions of genes.16,17 We suggest that BORIS-MS2, located in the promoter region, differentially regulates the gene transcription in cancer cells and normal cells. This suggests a potential association between BORIS-MS2 and cancer. For example, specific alleles of BORIS-MS2 may affect susceptibility to lung cancer. In this study, we found a significantly elevated frequency of rare alleles of the minisatellite in lung cancer cases compared with cancer-free controls. In addition, short rare minisatellite alleles were associated with higher susceptibility in lung cancer patients. A similar result has been shown in a report examining the relationship between short rare alleles of BORIS-MS2 and breast cancer in a younger group.12 Other studies on genetic susceptibility due to specific minisatellites have also been reported.17,29-31

It is commonly accepted that genomic instability has a crucial role in the accumulation of genetic alterations that are responsible for cancer cell development.32 It has also been reported that genomic instability in minisatellite regions is related to loss of heterozygosity and rearrangements in cancer tissues.33,34 Thus, the BORIS-MS2 minisatellites may be involved in tumorigenesis not only via gene regulation but also via genomic instability. Further analysis is required to elucidate the correlation between rare minisatellite alleles and the susceptibility to lung cancer observed in this study.
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