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Susceptibility for breast cancer in young patients with short rare minisatellite alleles of *BORIS*

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In this study, we characterized two blocks of minisatellites in the 5′ upstream region of the *BORIS* gene (*BORIS*-MS1, -MS2). *BORIS*-MS2 was found to be polymorphic; therefore, this locus could be useful as a marker for DNA fingerprinting. We assessed the association between *BORIS*-MS2 and breast cancer by a case-control study with 428 controls and 793 breast cancers cases. Rare alleles in the younger group (age, <40) were associated with a statistically significant increased risk of breast cancer (odds ratio, 4.84; 95% confidence interval, 1.06-22.22; and *P* = 0.026). A statistically significant association between the short rare alleles and cancer was identified in the younger group (8.02; 1.01-63.83; *P* = 0.021). Kaplan-Meier estimates showed that poor prognosis was associated with patients who contained the rare alleles. Our data suggest that the short rare alleles of *BORIS*-MS2 could be used to identify the risk for breast cancer in young patients. [BMB reports 2010; 43(10): 698-703]

INTRODUCTION

BORIS is a member of the cancer-testis antigen (CTA) family that is present only in the testis during spermatogenesis, (1) but is abnormally expressed in multiple cancers, including female cancers such as uterine (endometrial) and breast tumors (2-4). Furthermore, the expression of *BORIS* in normal cells induces activation of several cancer-testis genes (MAGE-A1, NY-ESO-1, and others) (2, 5).

While the BORIS protein is present at variable levels in the nucleus and the cytoplasm in several breast cancer cell lines, it is not expressed in normal breast cells (4). Moreover, high levels of the BORIS protein in the leukocyte fraction of patients with breast cancer was detected, which suggests that *BORIS* can be used as a valuable marker for early detection of breast cancer in blood leukocytes (6). Because of this abnormal expression of *BORIS* in several cancers including breast cancer, the regulation of its expression deserves much study.

Breast cancer is the most common cancer found in women worldwide (7), and the incidence has been continuously increasing in Korea (8). Interestingly, the age distribution of breast cancer patients in Korea is quite different from that of Western countries; premenopausal breast cancer patients in Korea constitute about 60% of newly diagnosed breast cancer patients, while only 25% of all breast cancer patients are premenopausal in Western countries (9-11). Many studies have reported that young patients with breast cancer showed poor prognosis compared with older patients and it develops more aggressively (12-15). In spite of the fact that *BORIS* is not normally expressed in females, BORIS protein was detected in 70.7% of breast tumors (4).

Some minisatellite alleles are associated with human disorders and with differential expression of nearby genes (16-19). Recently, we demonstrated a relationship between cancer predisposition and minisatellite (VNTR, variable number of tandem repeats) variants. Rare alleles of minisatellites are associated with a high risk for various types of cancer (20-24). These data lend support to the concept that biologically significant consequences might result from variations in a minisatellite locus and suggests a biological basis for some cancer predisposition.

In this study, we characterized the entire genomic region of the *BORIS* locus including the promoter region and identified two minisatellites loci (*BORIS*-MS1, *BORIS*-MS2) upstream of *BORIS*. We examined the multiallelic properties of these minisatellite loci. To determine whether allelic variation in *BORIS* minisatellites influences susceptibility for breast cancer, a case control study was performed using a PCR-based method. To genotype the *BORIS* polymorphisms, genomic DNA obtained from 428 cancer-free controls and 793 breast cancer patients were analyzed. Here, we report that allelic variations in the minisatellites of *BORIS* are related to susceptibility in young patients for breast cancer in the Korean population.

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RESUTS AND DISCUSSION

Identification and analysis of the minisatellite polymorphisms in *BORIS*

Two minisatellites (*BORIS*-MS1 and *BORIS*-MS2) and a CpG island (−1,096 to −762 upstream of the first ATG) were identified through the characterization of genomic DNA sequence in the *BORIS* upstream region that was found in the NCBI database under accession number AL035541.15 (Genbank no. AL035541/1546043). *BORIS*-MS1 is located −5,936 to −5,734 bp upstream, and *BORIS*-MS2 is located −2,586 to −1,812 bp upstream of the first ATG of the *BORIS* gene. A search of the GenBank database using the BLASTN program revealed that there was no significant similarity between the two novel minisatellites and other regions of the genome. Therefore, all of the minisatellites examined in this study are unique to *BORIS*, and the properties they confer may be directly related to the function of *BORIS*.

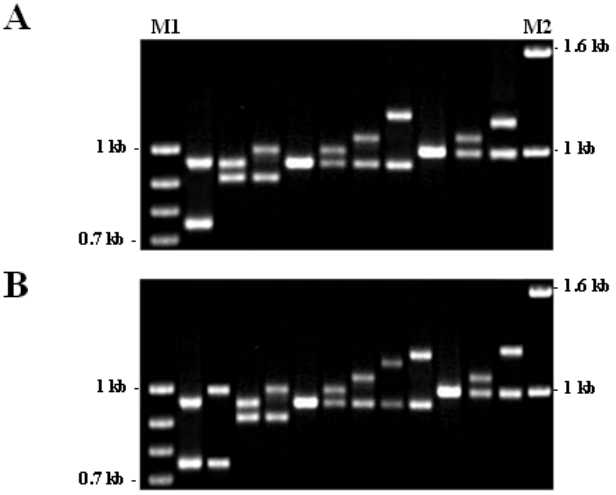
Using PCR amplification with diagnostic primers against human genomic DNA samples isolated from the unrelated controls, the degree of polymorphism within the minisatellites was examined. We analyzed 200 controls to determine if they were polymorphic or not; however, after it was determined that the minisatellite was a polymorphic locus, we increased the number of samples. *BORIS*-MS1 showed a monomorphic pattern in the 200 controls with a 236 bp length which contained 13 copies of the repeat unit (16 bp; CACACCAGTG CAGGCT). *BORIS*-MS2 was found to be polymorphic with a 56 bp repeat unit (GGGGGAATGG ATAAGGAGGG GAGG AGGGCC CTGCAGGGGG CGCTGGGAGA CCTGGG) in cancer-free controls (Fig. 1A).

Subsequently, we increased the number of samples for *BORIS*-MS2 and seven alleles were identified from the 428 female control samples. The seven alleles in *BORIS*-MS2 ranged from 785 to 1,220 bp in length and contained 10-18 copies of the repeat unit, with 14 copies being present in the most common allele (61.3%). Ten different genotypes with seven alleles were found in BORIS-MS2 (Fig. 1A, C) with 0.489 heterozygosity in female controls.

The correlation between breast cancer and genetic susceptibility at *BORIS*-MS2

Several breast cancer cell lines and a significant portion of the breast tumors express high levels of *BORIS* (4). This suggests the potential of *BORIS* as a valuable marker for early detection of breast cancer and a candidate for the development of a future breast cancer vaccine (4, 6). Because of the correlation between breast cancer and *BORIS* expression, we investigated whether *BORIS*-MS2 may influence breast cancer development. For assessment of the contribution to genetic susceptibility of *BORIS*-MS2 to breast cancer, we compared the distribution and frequency of the polymorphic *BORIS*-MS2 alleles between controls and patients with breast cancer.

A case-control study was conducted to compare DNA obtained from 428 controls and from 793 patients with breast cancer (Table 1). The *BORIS*-MS2 had twelve types of haploid patterns (Fig. 1B) and the heterozygosity was 0.487 in breast cancer patients. Table 1 summarizes the frequency of minisatellite alleles for *BORIS*-MS2 between the controls and the breast cancer groups. For analysis, each minisatellite allele was grouped into two sets (common and rare alleles) based on their frequency in the control population. The expected frequency for rare alleles was ≤1%. Seven alleles of *BORIS*-MS2



C

Genotype	Controls [N(%)]		Cases [N(%)]	
10/14	3	(0.7)	2	(0.3)
10/15	0	(0.0)	1	(0.1)
13/14	3	(0.7)	12	(1.5)
13/15	4	(0.9)	11	(1.4)
14/14	165	(38.6)	314	(39.6)
14/15	184	(43.0)	340	(42.9)
14/**16**	2	(0.5)	1	(0.1)
14/**17**	0	(0.0)	2	(0.3)
14/**18**	3	(0.7)	7	(0.9)
15/15	62	(14.5)	99	(12.5)
15/**16**	1	(0.2)	1	(0.1)
15/**17**	1	(0.2)	0	(0.0)
15/**18**	0	(0.0)	3	(0.4)

Bolds showed the rare alleles.

Fig. 1. Allele typing of *BORIS*-MS2 in cancer-free female controls and breast cancer patients. (A) Electrophoretic patterns of PCR products of *BORIS*-MS2 in female controls. Seven *BORIS*-MS2 alleles and ten haplotype patterns were detected in DNA from 428 cancer-free female controls. (B) Electrophoretic patterns of PCR products of *BORIS*-MS2 in breast cancer patients. Seven *BORIS*-MS2 alleles and twelve haplotype patterns were detected in DNA from 793 patients with breast cancer. The first and last lanes correspond to a 100-bp (M1; Invitrogen Co., CA, USA) and a 1-kb size marker (M2; Invitrogen). (C) Allelic genotypes and frequency in female controls and patients with breast cancer. Bold numbers represent the rare alleles of *BORIS*-MS2.

were grouped into two common alleles (14 and 15 repeats) and five rare alleles (10, 13 16, 17 and 18 repeats). Furthermore, we divided the rare alleles into short (10 and 13) and long (16, 17 and 18), according to their tandem repeat lengths (Table 1). Analysis of these data revealed no significant association between rare alleles and odds for cancer. (*BORIS*-MS2 and breast cancer OR, 1.27; 95% CI, 0.720-02.27; $P = 0.402$). Short rare alleles showed a slight tendency of 1.41 (CI: 0.68-2.94; $P = 0.357$) for breast cancer (Table 1), but it did not show statistical significance.

Breast cancer has been continuously increasing in Korea, but the age distribution of breast cancer patients in Korea is younger than Western countries (9-11). Breast cancer cases were grouped as young or older, using a cut-off year of 40 according to previous reports (25-27). Then, we examined the effects of age at diagnosis and also divided the rare alleles into short and long alleles according to the number of tandem repeats. Interestingly, younger patients (<40 years) had an increased ratio (OR: 2.53, CI: 1.25-5.13; $P = 0.008$) of correlation between the rare *BORIS*-MS2 alleles and breast cancer, while there was no significant difference in female controls (OR: 0.43, CI: 0.10-1.89; $P = 0.251$). Furthermore, a comparison between controls and cancer patients within the same age group verified a statistically significant difference in the association ratio (OR: 4.84, CI: 1.06-22.22; $P = 0.026$) between rare *BORIS*-MS2 alleles and breast cancer in younger females.

We also determined the effect of rare alleles by length: Table 2 summarizes the frequency of the short rare *BORIS*-MS2 alleles according to age at diagnosis. In the control group, we also found that there was no difference in the frequencies of short rare alleles between younger and older individuals. In comparison to older patients, however, we found that younger individuals with breast cancer had an increased ratio (3.69, CI: 1.63-8.35; $P = 0.0008$) of association between short rare *BORIS*-MS2 alleles and breast cancer (Table 2). Specifically, a comparison of the normal controls and the cancer cases showed the following differences in the association ratio between breast cancer and short rare *BORIS*-MS2 alleles in younger- and older-patients: younger, 8.02 (CI: 1.01-63.83.5; $P = 0.021$) vs. older 0.73 (CI: 0.33-1.58; $P = 0.417$) (Table 2). However, the frequency of long rare alleles did not show such differences in this analysis. We also determined the significance between short rare alleles and breast cancer by Fishers exact test (Table 2). These results suggest that rare *BORIS*-MS2 alleles may be genetically related to breast cancer in Korea.

Relation between rare *BORIS*-MS2 alleles and prognosis for breast cancer patients

The functional role that the *BORIS*-MS2 minisatellite plays is not clear; however, polymorphisms may relate to cancer prognosis. We used additional clinicopathological information

Table 1. The rare *BORIS*-MS2 alleles associated with breast cancer

Subjects	No. of alleles	Common alleles			Short rare alleles			Long rare alleles				Total rare alleles
		14	15	Total	10	13	Total	16	17	18	Total	Short + Long
Female controls	856	525	314	839	3	7	10	3	1	3	7	17
	(%)	(61.3)	(36.7)	(98.0)	(0.35)	(0.82)	(1.17)	(0.35)	(0.12)	(0.35)	(0.82)	(1.99)
Breast cancer	1,586	992	554	1,546	3	23	26	2	2	10	14	40
	(%)	(62.6)	(34.9)	(97.5)	(0.19)	(1.45)	(1.6)	(0.13)	(0.13)	(0.63)	(0.88)	(2.52)
OR (95% CI)		1 (reference)			1.41 (0.68-2.94)			1.08 (0.43-2.69)				1.27 (0.72-2.27)
P		—			*P*= 0.357			*P* = 0.868				*P* = 0.402

Table 2. Frequency of *BORIS*-MS2 and risk of breast cancer by age and allele length

Age at diagnosis	Female controls		Breast cancer cases		Reference (controls of the same age)	
	Total cases	Short rare alleles	Total cases	Short rare alleles	OR (95% CI) *P*	Fisher exact test
Younger (<40)	90	1 (1.11%)	121	10 (8.26%)	8.02 (1.01-63.83) $P = 0.021$*	7.39 $P = 0.02942$*
Older (≥40)	338	11 (3.25%)	672	16 (2.38%)	0.73 (0.33-1.58) $P = 0.417$	0.34 P =0.5598
Reference (older group)	0.33 (0.04-2.62); $P = 0.27$		3.69 (1.63-8.35); $P = 0.0008$*			
Fisher exact test	2.92; $P = 0.4736$		8.434; $P = 0.00368$*			

*Statistically significant ($P < 0.05$).

obtained between 1997 and 2004 from Dong-A University. Tumor, node, and metastases (TNM) stages were analyzed according to the World Health Organization (WHO) system. Breast tumors were grouped into the appropriate class, and we then estimated the frequency of each class in the total cancer group and in the rare allele group by Pearson's chi-squared test. There are no associations between short rare alleles and cancer according to tumor size, T stages, N stages, M stages and hormonal receptor status. The frequency of short rare alleles in the younger group (20-39 years; 38.4%) was higher than in the total breast cancer group (20-39 years; 15.2%). The mean age of short rare allele cases was significantly younger than total cases (P = 0.032). However, we found a similar proportion of tumor size, stage and hormonal receptor status between the short rare alleles group and the cancer group.

To examine whether frequency of short rare *BORIS*-MS2 alleles can reflect different prognosis in breast cancer patients, we followed the survival time between the younger group (P = 53) and the older group (P = 225) in breast cancer patients. However, survival time of each subgroup by Kaplan-Meier plots with log-rank test showed no significant difference between two groups in our data (Fig. 2A). Kaplan-Meier analysis was also performed on another two groups with rare and common alleles of *BORIS*-MS2 (Fig. 2B). The patients in the group of rare alleles had a poorer prognosis with a 78.6% 5-year survival rate while the group of common alleles with better prognosis had a 94.7% 5-year survival rate. The log-rank test revealed that these two subgroups had significant differences in survival, with a P = 0.048. This result suggested that the frequency of rare *BORIS*-MS2 alleles may indeed have the potential to provide a novel prognostic model that can predict breast cancer patients' prognosis more precisely. To verify the association between rare *BORIS*-MS2 alleles and age-dependent susceptibility to breast cancer, we compared the survival rate of common and rare alleles in the older group and the younger group. While there was no significant difference in the older group (Fig. 2C), the younger group exhibited a suggestive difference in the survival rate between rare and common alleles in that the survival rate of the younger group had a P value of 0.174 (Fig. 2D). Because of the small group size of younger patients, we could not find statistical significance in the survival time of younger patients with rare *BORIS*-MS2 alleles. However, we suggest there may be a possible risk factor of rare *BORIS*-MS2 alleles for poor prognosis in younger patients.

This suggests a potential association of rare minisatellite alleles at *BORIS* and cancer. In this study, we could find a statistically significant elevated frequency of rare alleles in the younger age group of breast cancer patients compared with cancer-free female controls. In addition, short rare alleles exhibited higher susceptibility in younger breast cancer patients.

Therefore, we suggest that the short rare *BORIS*-MS2 alleles have a genetic influence on breast cancer. This finding may prove useful as a diagnostic biomarker of increased risk for breast cancer and cancer prognosis, though the short rare alleles group is too small to be common in breast cancer cases.

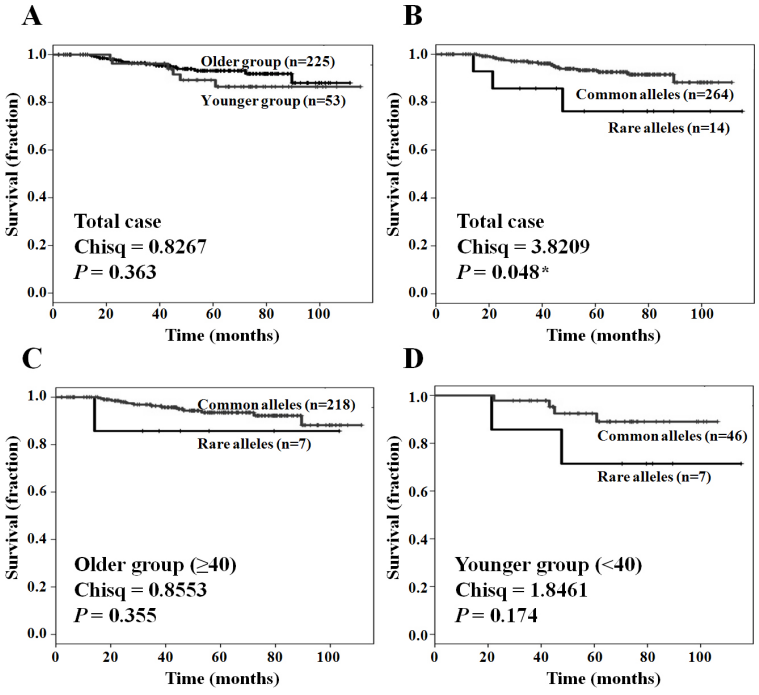


Fig. 2. Kaplan-Meier plots of overall survival and *BORIS*-MS2 alleles with breast cancer (A) Survival time of younger group (<40 years; n = 53) and older group (≥40 years; n = 225) in breast cancer patients. (B) Survival time of the patients in the group with rare alleles (n = 14) and common alleles (n = 264). (C) Survival time of the patients with rare alleles (n = 7) and common alleles (n = 218) in the older group. (D) Survival time of the patients with rare alleles (n = 7) and common alleles (n = 46) in the younger group.

MATERIALS AND METHODS

Identification of the tandem repeats of *BORIS*

BORIS genomic sequences that are analyzed in this study for tandem repeats have been assembled by the UCSC (>hg19_dna range = chr20 : 55,000,001-56,500,000 and the NCBI (>ref |AL035541|NC000020|AL160176 Homo sapiens chromosome 20, GRCh37 primary reference assembly). To find the minisatellites and other repeated regions, the Tandem Repeats Finder software (28) was used. Repeat units between 10 and 100 bp in length that scored > 300 in the program algorithm were selected for further analysis.

Preparation of Genomic DNA

To assess the degree of minisatellite polymorphism of *BORIS*, unrelated healthy individuals were analyzed. The case-control study of this work included 428 cancer free female controls and 793 breast cancer cases and the controls had a similar proportion of sex and age to the cases (control average age, 47.6 yr, range 23-78 yr; patient average age, 48.9 yr, range 22-78 yr). Controls were selected from the Department of Preventive Medicine and Internal Medicine of Dong-A University hospitals between 1997 and 2004 (Busan, Korea). The control group, who has no personal history of cancers or current cancer, was recruited and completed an interview. Cases with breast cancer and controls were recruited from Dong-A University Hospital of Busan, Korea. Prior to collection, each participating subject provided her informed consent. For the PCR experiments, genomic DNA was isolated from the peripheral leukocytes, which were isolated from 400 μl of whole blood using a Blood and Cell Culture DNA Mini Kit (Qiagen, CA, USA).

Genotyping assay for the minisatellite polymorphism of *BORIS*

The genotyping assay of the minisatellite polymorphism was described previously (22) with the PCR primer pairs of 5'-CGG CAGCTCTAGCACACCAG-3' (forward) and 5'-CCTCCCACAC TCGGTCCCATT-3' (reverse) for *BORIS*-MS1 and 5'-CTTGGGA GACCTGGGGGATGAATAG-3' (forward) and 5'-GCACCCCA TTCCCCATCCTC-3' (reverse) for *BORIS*-MS2. The PCR products were analyzed on a 1.2% agarose gel at 80 V for 4 hours and stained with ethidium bromide.

Statistical analysis

The degree of polymorphism, which ranges from 0 to 1, generally increases with the number of alleles. To evaluate the probability of two randomly-chosen alleles being different (heterozygosity) at a given locus, a measure of genetic diversity was calculated using the method described by Chakravarti and Lynn (29). Regression analyses were performed to determine the odds ratios (ORs) of association between control and case groups. ORs were estimated using the natural logarithm and its standard error. Where relevant, a chi-squared test was used with one degree of freedom to assess statistical significance. Differences were considered significant for confidence intervals (CIs) of 95%. All tests were two-sided, with $P < 0.05$ being considered statistically significant. Statistical analyses were performed using MS Excel with CHITEST and R statistical software (v2.5.1, www.r-project.org) with chisq.test for the calculation of chi-squared values. The Kaplan-Meier plot was used in R program version 2.10.0. An estimate of the survival effect from life-time data that related to rare alleles at the *BORIS* locus was determined.

Acknowledgments

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