Expression of the Brother of the Regulator of Imprinted Sites Gene in the Sputum of Patients with Lung Cancer

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Background: Brother of the regulator of imprinted sites (BORIS) is a putative new oncogene that is classified as a cancer germline gene; however, its role in the development of cancer is unclear. This study investigated the expression of BORIS in lung cancer and its clinical implications. Methods: The expression of BORIS messenger ribonucleic acid (mRNA) in the sputum of 100 patients with lung cancer (50 with squamous cell carcinoma, 36 with adenocarcinoma, and 14 with small-cell carcinoma) was evaluated by reverse transcription polymerase chain reaction. Results: The overall expression rate of BORIS in patients with lung cancer was 36.0%: 19 of 50 squamous cell carcinomas (38.0%), 13 of 36 adenocarcinomas (36.1%), and 4 of 14 (28.6%) small-cell carcinomas. There was no significant difference in the BORIS expression according to age, gender, or histologic type. However, the mRNA expression of BORIS was significantly related to the pathologic cancer stage (p=0.004) and lymph node metastasis (p=0.001). The expression of the melanoma antigen gene family A1–6 was not associated with the expression of BORIS. Conclusion: Our results suggest that the expression of BORIS might be a negative prognostic factor in lung cancers and implicate BORIS as a molecular target for immunotherapy.

Key words: 1. Lung neoplasms 2. Brother of the regulator of imprinted sites 3. Reverse transcription 4. Polymerase chain reaction

INTRODUCTION

Despite advances in diagnostic and therapeutic modalities against lung cancer, little improvement in the prognostic outcome has been accomplished. Therefore, new screening methods and therapeutic approaches, including immunotherapy, are needed for the management of patients with lung cancer. Cancer germline (CG) or cancer testis antigen (CTA) genes are genes that are normally expressed in germline cells but can be expressed in various tumors as a result of demethylation [1,2]. These proteins have been considered to be targets for immunotherapy and to be prognostic factors because their expression is associated with the characteristic features of the neoplastic phenotype, including invasiveness, immune evasion, and metastatic capacity [1]. Brother of the regulator of imprinted sites (BORIS) is a newly described autosomal CG or
CTA that has been proposed to function as an oncogene in human cancer through the dysregulation of the cancer epigenome [1]. However, its role during oncogenesis and the clinical implications of its expression are not clear [2].

The melanoma antigen gene family A (MAGEA) consists of several subtypes, including MAGEA1 to MAGEA12, that are also categorized as CG antigens [3]. During the past several years, the expression of MAGEA genes has been studied for application in cancer diagnosis and targeted immunotherapy [3-5]. The aim of this study was to investigate the expression of BORIS in the sputum of patients with lung cancer and its clinical implications.

### METHODS

1) Materials

The induced sputum of 100 patients with primary lung cancers was obtained within 5 to 10 minutes after inhalation of 3% hypertonic saline aerosols to facilitate expectoration from the deeper parts of the bronchial tracts. The samples were stored immediately after collection in a ribonucleic acid (RNA)-stabilizing solution and frozen at -20°C. Among the 100 patients, 50 patients were diagnosed with squamous cell carcinoma, 36 with adenocarcinoma, and 14 with small-cell carcinoma (Table 1).

2) Methods

(1) Reverse transcription polymerase chain reaction for analysis of BORIS expression: Total messenger RNA (mRNA) was extracted from the sputum, and nested reverse transcription polymerase chain reaction (RT-PCR) was carried out with primers specific for BORIS (Table 2). Total RNA was isolated from samples using RNeasy plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The first round of PCR was carried out in a 20-μL reaction mixture containing 8 μL of the PCR master mixture, 0.5 μL of the outer primers, 2 μL of the RT reaction products, and 9.5 μL of distilled water. The cycling parameters were as follows: denaturation was initiated at 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 65°C for 60 seconds, with a final extension at 65°C for 5 minutes. The PCR products were separated on 1% agarose gels impregnated with ethi-

| Table 1. Patients’ characteristics |
|-----------------------------------|
| Characteristic                  | Value    |
| Total no. of cases             | 100      |
| Gender                         |          |
| Male                           | 78 (62.53)|
| Female                         | 22 (60.25)|
| Histologic type                |          |
| Squamous cell carcinoma        | 50 (62.42)|
| Small-cell carcinoma           | 36 (64.42)|
| Adenocarcinoma                 | 14 (60.42)|
| Pathologic stage by American Joint Committee on Cancer |
| IB                             | 2        |
| IIB                            | 30       |
| IIIA                           | 16       |
| IIIB                           | 46       |
| IV                             | 6        |
| Lymph node metastasis          |          |
| Yes                            | 74       |
| No                             | 26       |

Values are presented as number or number (mean age).

| Table 2. Sequences of primers used for PCR |
|------------------------------------------|
| Primer                  | Sequence of primer       | Size of PCR products |
|-------------------------|-------------------------|---------------------|
| BORIS forward           | Caggccctacaagttacagactgcaaa |                     |
| BORIS reverse           | Gcattgcgaaggctctccctgagggt | 273bp               |
| MAGEA1-6 outer forward  | Cagagaggagagatctgcc       |                     |
| MAGEA1-6 outer reverse  | Ctcagacgtttctcgac         | 831-855bp           |
| MAGEA1-6 inner forward  | Cggagagaggagatctgccw gt   |                     |
| MAGEA1-6 inner reverse  | Ccctgctgcctggtggg         | 469-493bp           |

PCR, polymerase chain reaction; BORIS, brother of the regulator of imprinted sites; bp, base pairs; MAGEA, melanoma antigen gene family A.

*w, A, or T.*
Table 3. Expression of BORIS and MAGE in lung cancer

| Variable (no.) | BORIS | p-value of BORIS |
|----------------|-------|------------------|
| Total (100)    | 36 (36.0) | 64 (64.0) | 0.151 |
| Mean age (yr)  | 64.67±8.277 | 61.80±10.144 | 0.145 |
| Gender         |       |                  |
| Male (64)      | 31    | 47               | 0.557 |
| Female (36)    | 5     | 17               |       |
| Histologic type|       |                  |
| Squamous cell carcinoma | 19 | 31 |       |
| Small-cell carcinoma | 13 | 23 |       |
| Adenocarcinoma  | 4     | 10               |       |
| Pathologic stage by American Joint Committee on Cancer | 0.003 |
| IB (2)         | 0     | 2                |       |
| II B (30)      | 4     | 26               |       |
| IIIA (16)      | 7     | 9                |       |
| IIIB (46)      | 23    | 23               |       |
| IV (6)         | 2     | 4                |       |
| Lymph node metastasis | 0.001 |
| Yes (36)       | 36    | 0                |       |
| No (64)        | 0     | 64               |       |
| MAGE family A1–6 | 0.758 |
| Expression (76) | 28  | 48               |       |
| No expression (24) | 8   | 16               |       |

Values are presented as number (%), mean±standard deviation, or number. BORIS, brother of the regulator of imprinted sites; MAGE, melanoma antigen gene.

dium bromide (0.5 μg/mL) and viewed under ultraviolet illumination.

(2) Melanoma antigen gene family A1–6 expression:
Transcripts of MAGEA1–6 were detected using the Cancer Hunter kit (iC&G Co., Daegu, Korea) [5]. Briefly, reverse transcription reactions were carried out in a 20-μL reaction mixture containing 7 μL of the RT master mixture, 0.5 μL of the RNase inhibitor, 1 μL of RNase, and 11.5 μL of eluted RNA solutions. The reaction mixture was incubated at 42°C for 60 minutes and 95°C for 5 minutes and stored at -20°C until needed for PCR. The first round of PCR was carried out in a 20-μL reaction mixture containing 8 μL of the PCR master mixture, 0.5 μL of the outer primers, 2 μL of the RT reaction products, and 9.5 μL of distilled water. The cycling parameters were as follows: denaturation was initiated at 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 65°C for 60 seconds, with a final extension by incubation at 65°C for 5 minutes. After the first PCR, 20 μL of the nested PCR mixture containing 8 μL of the PCR master mixture, 0.5 μL of the inner primers, and 11.5 μL of distilled water was added to the first PCR tube. Nested PCR was carried out under the same conditions as the first PCR except for the annealing temperature, which was set at 62°C. The nested PCR products were separated on 1% agarose gels impregnated with ethidium bromide (0.5 μg/mL). The DNA sequences of primers for the MAGE are listed in Table 2.

3) Statistical analysis
Statistical analysis was performed using SPSS ver. 12.0 (SPSS Inc., Chicago, IL, USA). Categorical variables were compared by Fisher’s exact test and chi-square analysis with the Yates correction. Differences were considered significant if the p-value was less than 0.05.
The overall expression rate of BORIS in our patients with lung cancer was 36.0% (36/100) (Table 3, Fig. 1). BORIS expression was detected in 19 of the 50 squamous cell carcinomas (38.0%), 13 of the 36 adenocarcinomas (36.1%), and 4 of the 14 (28.6%) small-cell carcinomas. BORIS mRNA expression was not significantly associated with age, gender, or histologic type; however, it was significantly related to the pathologic cancer stage (p=0.004) and lymph node metastasis (p=0.001).

BORIS was expressed in 36 of the 74 patients (48.6%) with lymph node metastasis and in none of the patients without, indicating a significant association with lymph node metastasis (p=0.001). BORIS expression was detected in 92.6% of the cases with pathologic stage IIIb and IV diseases but in none of the patients with pathologic stage Ib disease. Therefore, BORIS expression was positively associated with the cancer stage (p=0.004).

The expression rate of MAGEA1–6 was 76% (76/100) for all lung cancer patients (Fig. 2). MAGE was expressed in 90% (45/50) of the patients with squamous cell carcinoma, 57.1% (8/14) of the patients with small-cell carcinoma, and 36.1% (13/36) of the patients with adenocarcinoma. The expression rate in squamous cell carcinoma was significantly higher than that in the other two histologic types (p=0.001). There was no significant difference in the MAGEA1–6 expression according to the pathologic cancer stage and lymph node metastasis. There was no significant correlation between the expression of BORIS and the expression of MAGE.

**DISCUSSION**

Lung cancer remains one of the most deadly diseases worldwide; therefore, more effective therapeutic approaches are urgently needed. Although resection is a therapeutic option for patients with non-small-cell lung cancer (NSCLC), most patients show a locally advanced or metastatic status at diagnosis. Immunotherapy offers a novel approach for the treatment of patients with NSCLC in both the adjuvant setting as post-primary treatment and as the primary treatment for advanced cancer. A number of promising candidate vaccines based on different types of antigenic stimulus have now been evaluated in clinical studies [6,7], including target protein-specific vaccines designed to induce responses against CGs such as MAGEA [3-7].

BORIS, a novel member of the CG family, is suspected to be a new oncogene with unknown functions [2,8] and is considered a potential molecular target for cancer immunotherapy [8]. In our study, the expression rate of BORIS in lung cancer patients was 36%. The expression of BORIS was not significantly different according to age, gender, or histologic type but was significantly associated with the pathologic cancer stage (p=0.004) and lymph node metastasis (p=0.001). The expression of MAGEA1–6 was not associated with the expression of BORIS.

In the case of breast cancer, the expression rate of BORIS was reported to be 70.7% [9] and BORIS expression was detected in 73 of the 95 (77%) endometrial cancers and 24 of the 31 (77%) uterine-mixed mesodermal tumors [10]. BORIS was also detected in 27% (n=63) of the melanoma tissue samples [11]. BORIS expression was also reported in ovarian cancer patients with advanced stage disease [12]. These reports indicate that the expression of BORIS varies according to the origin or histologic type of tumors. At present, there are no other data on BORIS expression in the sputum or the tissue of patients with lung cancer; therefore, our results cannot be compared or verified. In addition, it is likely that the
expression rate in the sputum is lower than that in the tumor tissue itself.

There have been reports that the functions of BORIS in vitro may involve the expression of other CTAs such as MAGE or other proto-oncogenes [13]. Moreover, the aberrant expression of BORIS is associated with the up-regulation of candidate proto-oncogenes in multiple human malignancies [13,14]. The expression of BORIS might explain how the expression of the human retinoblastoma-related Rb2/p130 gene accelerates the progression and recurrence of lung tumors after treatment [14]. However, findings on the regulatory role of BORIS in the expression of other genes are inconclusive. A previous study reported that CTA expression is dependent on BORIS and BORIS regulates the CTA gene expression through methylation-dependent and -independent mechanisms [15]. In addition, another study suggested that the expression of BORIS and MAGE was not correlated in tumors expressing low levels of BORIS such as melanoma [11]. Our results suggest a similar low expression rate of BORIS (36.0%) in lung cancer. Moreover, the expression of BORIS was not associated with the expression of MAGEA1-6 in our patients.

Our study revealed that lymph node metastasis and pathologic cancer stage were significantly related to the expression of BORIS. This is supported by a report that the BORIS expression is significantly associated with the lymph node metastasis and is an independent poor prognostic factor in esophageal cancer [16].

BORIS has been studied as a target of immunotherapy [17], and our data suggest that 36% of the patients with lung cancer could be considered candidates for immunotherapy using BORIS. However, further studies with larger populations and survival analyses are needed to reach a solid conclusion about the prognostic implications of the BORIS expression in lung cancer.

1) Limitations of this study

Finally, we note a few limitations of this study. We did not quantify the expression of BORIS and MAGE mRNA, while we presented them in the sputum of patients with lung cancer. The survival analysis according to BORIS was not performed. In addition, if the sample size were larger, the study on non-small-cell lung carcinoma would have been more homogenous and valuable. Therefore, a more complete analysis of BORIS, including the survival analysis in larger sample sets, would be the next step to investigate the precise clinical impact of BORIS in the case of lung cancers.

2) Conclusion

BORIS could be a molecular target for immunotherapy in the 36% of patients with lung cancers that express the BORIS gene. The expression of BORIS in sputum might be a prognostic factor associated with a higher pathologic cancer stage and lymph node metastasis.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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