Analysis of cancer testis antigens containing BORIS in oral cancer cell lines

Hiroshi Inoue, Yuichi Ohnishi*, Yuki Minamino, Yoshihide Ugaki, Suguru Dateoka, Hirohito Kubo*, Masahiro Inoue**, Mitsuchika Sugitatsu***, Masahiro Nakajima* and Kenji Kakudo*

Graduate School of Dentistry (Second Department of Oral and Maxillofacial Surgery), *Second Department of Oral and Maxillofacial Surgery, **Department of Oral Implantology, Osaka Dental University, 8-1 Kuzuhanazono-cho, Hirakata-shi, Osaka 573-1121, Japan, ***Department of Dentistry and Maxillofacial Surgery, Osaka Red Cross Hospital, Japan

Cancer testis antigens (CTA) are a group of normal testicular proteins. Although their expression appears in cancer cells, it does not appear in somatic cells with the exception of male germ cells. Consequently CTAs are immunogenic in cancer patients, and considered promising target molecules for cancer vaccines. In CTAs, brother of the regulator of imprinted sites (BORIS), which is also known as CCCTC-binding factor-like (CTCFL), is an epigenetic-acting oncogene that suppresses the tumor inhibitor functions of CTCF. For this reason, BORIS is thought to be an ideal molecular target for drugs against various cancers.

In order to detect CTAs in oral cancer, we used the methylation pattern to select six genes containing BORIS, ACTL7B, PPP3R2, SYCP1, DAZL, and TAF7L. We then analyzed the expression of these genes in oral squamous cell carcinoma cell lines and various mice organs. Expression of only BORIS in these genes was detectable in SAS, HSC-3 and HSC-4. Boris expression in adult mice was restricted to the testes. These findings imply that BORIS might be the target of immunotherapy for oral cancer. (J Osaka Dent Univ 2010; 44: 111–117)

Key words: Oral cancer cell lines; Cancer testis antigen; BORIS

INTRODUCTION

The most frequently occurring cancer in the oral and maxillofacial region is squamous cell carcinoma (SCC). Its metastasis and invasive ability result in a poor prognosis. Surgery, radiation and chemotherapy are the primary treatments for oral cancer. Several cancer genes have so far been identified as targets for new, efficient therapies against oral cancer. Our group has researched heparin-binding epidermal growth factor (HB-EGF), which belongs to the EGF family, as a therapeutic target of oral cancer. In addition, we have searched for other genes that might be new therapeutic targets.

Cancer testis antigens (CTA) are a group of normal testicular proteins. Their expression appears in cancer cells, but not in somatic cells with the exception of male germ cells. Therefore, CTA are immunogenic in cancer patients and are considered promising target molecules for cancer vaccines. CTA, which were first identified in human melanoma, are regulated by methylation and represent the most promising immunotherapeutic target for melanoma patients today. CTA, more than 40 of which have been identified, are encoded in many tumors, including melanomas, as well as bladder, lung and liver cancer. Although expression of various oncogenes has been recognized in these cancer cells, CTA have not been studied in the oral and maxillofacial region.

BORIS is a CTA. Although it is normally expressed only in the testes, it is abnormally activated in various cancer cells. BORIS is a mammalian CCCTC-binding factor (CTCF) palalog with central 11 Zn fingers (11 ZF) that mediates specific interactions with multiple DNA sequences, and is be-
lieved to function as an epigenetic-acting oncogene through its ability to induce activation of other oncogenes by inhibiting activity of the CTCF.\textsuperscript{1,11} CTCF, which is ubiquitously expressed, was originally identified for its ability to suppress expression of the oncogene c-myc.\textsuperscript{12} The importance of CTCF as a tumor suppressor gene has been demonstrated in various studies.\textsuperscript{10}

In order to search for new CTA in oral cancer, we selected 6 genes based on their methylation pattern in public data sets: brother of the regulator of imprinted sites (BORIS or CTCL), actin-like 7B (ACTL7B),\textsuperscript{13} protein phosphatase 3 (formerly 2 B) (PPP3R2),\textsuperscript{14} zona pellucida binding protein 2 (ZPB2),\textsuperscript{15} synaptonemal complex protein 1 (SYCP1),\textsuperscript{16} deleted in azoospermia-like (DAZL),\textsuperscript{17} and TAF7-like RNA polymerase II, TATA box binding protein (TBP)-associated factor, 50 kDa 8 (TAF7L).\textsuperscript{18} We expected these genes to express in oral SCC cell lines. In addition, the expression of the gene that expressed in oral SCC cell lines was analyzed in various mice organs.

MATERIALS AND METHODS

Public Datasets

This research is publicly available online as EST (Expressed Sequence Tags) using the Profile Viewer of UniGene from the National Center for Biotechnology Information (NCBI). The public databases used in this research were from the University of California Santa Cruz (UCSC) Human Genome Bioinformatics, and annotation databases were from the human genome assembly freezed in April 2009 (hg19). To identify CTAs in oral cell lines, we searched for testis specific genes by the position of CpG and the CpG value (the ratio of observed to expected CpG) in the public databases from UCSC. The selected CTAs are shown in Table 1.

### Table 1. Testis specific genes selected using Unigene (#212) and UCSC (hg 19)

<table>
<thead>
<tr>
<th>Accession</th>
<th>Symbol</th>
<th>Description</th>
<th>Chromosome</th>
<th>Exon number</th>
<th>CpG island</th>
<th>CpG size</th>
<th>CpG value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_080618</td>
<td>BORIS</td>
<td>Brother of the regulator of imprinted sites</td>
<td>chr 20:55,505,630–55,533,560</td>
<td>11</td>
<td>First exon, first intron</td>
<td>280</td>
<td>0.75</td>
</tr>
<tr>
<td>NM_006686</td>
<td>ACTL7B</td>
<td>Actin-like 7B</td>
<td>chr 9:110,656,692–110,658,031</td>
<td>1</td>
<td>First exon</td>
<td>824</td>
<td>0.79</td>
</tr>
<tr>
<td>NM_147180</td>
<td>PPP3R2</td>
<td>Protein phosphatase 3 (formerly 2B), regulatory subunit B, beta isoform</td>
<td>chr 9:103,393,718–103,397,104</td>
<td>1</td>
<td>First exon</td>
<td>281</td>
<td>0.69</td>
</tr>
<tr>
<td>NM_199321</td>
<td>ZPB2</td>
<td>Zona pellucida binding protein 2</td>
<td>chr 17:35,277,981–35,287,675</td>
<td>7</td>
<td>First exon</td>
<td>492</td>
<td>1.05</td>
</tr>
<tr>
<td>NM_003176</td>
<td>SYCP1</td>
<td>Synaptonemal complex protein 1</td>
<td>chr 1:115,198,978–115,339,513</td>
<td>32</td>
<td>First exon, first intron</td>
<td>494</td>
<td>0.86</td>
</tr>
<tr>
<td>NM_001351</td>
<td>DAZL</td>
<td>Deleted in azoospermia-like</td>
<td>chr 3:16,603,305–16,622,010</td>
<td>11</td>
<td>First Intron</td>
<td>311</td>
<td>0.69</td>
</tr>
<tr>
<td>NM_024885</td>
<td>TAF7L</td>
<td>TATA box binding protein-associated factor, RNA</td>
<td>chr X:100,417,951–100,432,980</td>
<td>13</td>
<td>First exon, first intron</td>
<td>487</td>
<td>1.23</td>
</tr>
</tbody>
</table>

Cell Culture

We used SAS, HSC-3 and HSC-4 to observe the expression of CTA in oral cell lines in this experiment. SAS and HSC-3 are cell lines from poorly differentiated human squamous cell carcinoma of the tongue and HSC-4 is a cell line from a highly differentiated human squamous cell carcinoma of the tongue.\textsuperscript{19,20} SAS derives from the primary tumor while HSC-3 and HSC-4 derive from lymph node metastasis nests. All three human cell lines were purchased from RIKEN BioResource Center, (Ibaraki, Japan). Each cell line was cultured in a Dulbecco’s Modified Eagle’s Medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fe-
tial bovine serum (FBS; Gibco, NY, USA) and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

**Isolation of Total RNA**

We used TRIzol reagent (Gibco) to isolate the total RNA of the three cell lines. The cells that proliferated approximately 80% were rinsed with phosphate buffered saline (PBS) twice. We next added TRIzol reagent to the cells to obtain cell dissolution. Chloroform was further added in the dissolution and the dissolution was separated by centrifugation at 15,000×g for 15 minutes at 4°C. Isopropanol was added at a 1:1 ratio to the supernatant and the mixture was centrifuged at 15,000×g for 10 minutes at 4°C. The pellet was then rinsed in 75% ethanol and dissolved in sterile water.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Reverse transcription was performed with Super-Script III Reverse Transcriptase (Invitrogen, Carlsbad, CA). After isolation of the RNA of 100 ng/μL, Random Primer (New England BioLabs, Massachusetts, USA) was added to 1 μg of the RNA solution. The RNA mixture, 1 μL of random primer, 4 μL of 2.5 mM dNTP mixture (dATP, dCTP, dGTP, dTTP) and sterile water was annealed by incubating at 65°C for 5 minutes and incubated at room temperature for one night. Reverse transcription was then carried out in a volume 20 μL of a solution containing 1 μg of the RNA sample, 1 μL of random primer, 4 μL of 2.5 mM dNTP, 4 μL of 5× First Strand Buffer, 1 μL of 0.1 M DTT, 1 μL of RNase inhibitor (RNaseOUT™ Recombinant RNase Inhibitor; Invitrogen™, Carlsbad, CA), and 1 μL of SuperScript™ III Reverse Transcriptase. RT conditions were for 60 minutes at 50°C, and 15 minutes at 70°C.

PCR was performed using TaKaRa Ex Taq™ Hot Start Version (Takara Biotechnology, Shiga, Japan). The reaction was carried out in 25 μL of solu-

<table>
<thead>
<tr>
<th>Table 2</th>
<th>PCR Primer sequences used in RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene Name</td>
<td>Size</td>
</tr>
<tr>
<td>GAPDH</td>
<td>288</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>BORIS</td>
<td>462</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTL7B</td>
<td>386</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>PPP3R2</td>
<td>324</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>ZPB2</td>
<td>314</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>SYCP1</td>
<td>530</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>DAZL</td>
<td>364</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>TAF7L</td>
<td>212</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>mGapdh</td>
<td>289</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>mBoris</td>
<td>311</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
tion containing 2 μL of cDNA, 2.5 μL of 10 × Ex Taq Buffer (20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween® 20, 0.5% Nonidet P-40®, 50% Glycerol), 2 μL of 2.5 mM dNTP Mixture, 0.5 μL of 10 pM each of forward and reverse primer, and 0.2 μL of TaKaRa Ex Taq® HS. Cycling conditions were 2 minutes at 94°C, 35 cycles of 30 seconds each at 94°C, 30 seconds at 55 °C, 30 seconds at 72°C, and 5 minutes at 72°C. The RT-PCR primers are as shown in Table 2. The obtained PCR products were observed by agarose-gel electrophoresis in 1.6% agarose. Electrophoresis was carried out at 100 V for 45 minutes in 100 × TAE (Tris-acetate EDTA) buffer containing 0.01 μg/ml ethidium bromide. Photographs of gels were taken using UV light.

**Isolation of total RNA from tissues of adult mice**

Three-months-old mice were used to determine the pattern of the Boris expression. Total RNA was isolated from the testes, kidneys, liver, brain, intestines, stomach, heart, pancreas, lungs, ovaries and tongue.

**RESULTS**

**CTA candidates in oral cell lines**

Testis specific genes were selected using Unigene (#212) to identify CTAs in oral cell lines. Moreover, we selected seven genes containing BORIS, ACTL7B, PPP3R2, ZPB2, SYCP1, DAZL, and TAF7L based on the position of the CpG and CpG values. The CpG value was calculated with the formula cited in Gardiner-Garden et al.21 These genes had CpG islands that existed on the first exon, making them near the promoter region. The CpG values of these genes were between 0.69 and 1.23, indicating the possibility that the CpG islands of the seven genes were methylated in somatic cells, and were demethylated in only the testis and cancer cells. Therefore, we expected these genes to express in oral cell lines.

**Expression of testis specific genes in SAS, HSC-3 and HSC-4**

To observe expression of BORIS, ACTL7B, PPP3R2, ZPB2, SYCP1, DAZL, and TAF7L, the total RNA from SAS, HSC-3 and HSC-4 was analyzed by RT-PCR. Although the expression of only BORIS was detectable in all cell lines, that of the other genes was not detectable in any cell line. We found a high incidence of expression of BORIS in SAS and HSC-3 (Fig. 1).

**Expression of Boris in mouse tissues**

Total RNA from the testes, kidneys, liver, brain, intestines, stomach, heart, pancreas, lungs, ovaries, and tongue was analyzed by RT-PCR to observe expression of Boris. The expression was detectable only in the testes. No expression was found in the other tissues (Fig. 2).

**DISCUSSION**

CTA are categorized as cancer-specific genes, ab-
sent in normal adult tissues except in male germ cells. In some cases, CTA are also expressed in the ovaries and in trophoblast. This gene regulation is disrupted in malignancy, resulting in CTA expression in a proportion of various tumors types. Differences in expression are associated with epigenetic alterations in promoter methylation and histone acetylation. Although methylation is considered primarily a mechanism of tumor suppressor gene inactivation, cases of proto-oncogenes activated by promoter demethylation have been reported.

In the genome of adult vertebrate cells, 60-90% of the cytosines in CpG dinucleotides are methylated by DNA methyltransferase. Methylation of CpG islands spanning promoter regions is associated with control of gene expression. CpG islands often include gene promoters and extend further downstream into transcribed regions (pro-CpG). Pro-CpG islands of housekeeping genes are not methylated in any cell type. These genes are expressed ubiquitously. Conversely, although several testis-specific genes are demethylated at pro-CpG islands in the testis, they are methylated in somatic tissues. Some testis-specific genes are demethylated at pro-CpG islands in some cancer cells in the same way as they are in the testis. Therefore some testis-specific genes may express in oral cancer cells. We investigated the methylation status of CpG dinucleotides in seven testis-specific genes: BORIS, ACTL7B, PPP3R2, ZPB2, SYCP1, DAZL and TAF7L.

RT-PCR revealed that although BORIS expression appeared in oral cancer cell lines, it did not appear in the other six genes. The data showed that only BORIS in the genes we selected by methylation status were associated with oral cancer. As shown in Fig. 2, in the mouse, Boris was expressed in the testes but not in the kidneys, liver, brain, intestines, stomach, heart, pancreas, lungs, ovaries, or tongue. Expression of Boris has been well studied in the mouse. Its appearance is restricted to the male germ cells, especially the primary spermatocytes. BORIS located at chromosome 20q 13.2 is a parologue of the transcription factor, CTCF, and has a DNA binding domain similar to that of CTCF. CTCF, a sequence-specific DNA-binding protein that interacts with the chicken c-myc gene promoter, has been identified and characterized. The 11 zinc fingers are distributed in exons E 2 to E 8 of mammalian CTCF. Mammalian CTCF activities are now known to include transcriptional activation and repression, hormone-inducible gene silencing, creation of constitutive chromatin insulators or boundaries, as well as functional reading of imprinted status. CTCF plays a role in transcriptional regulation of many oncogenes. The importance of CTCF as a tumor suppressor gene has been confirmed in various studies. For example, mutation/deletion of CTCF often occurs in tumors.

BORIS, which belongs to the CTA family, shares all the exons coding for 11 ZFs with CTCF, and is associated with epigenetic reprogramming. The similarity of the ZFs shows that mammalian CTCF and BORIS proteins will recognize the same or an overlapping spectrum of DNA sequences, whereas the dissimilar flanking regions indicate that the functional consequences of DNA binding by these two proteins is likely to be different. The function of BORIS is believed to be associated with deletion of methylation patterns during the process of spermatogenesis. Hence the only expression of this gene in normal tissues is in the testis. On the other hand, BORIS in cancer is believed to function as an epigenetic-acting oncogene via its ability to induce derepression of other oncogenes through inhibition of CTCF.

Surgery, radiation and chemotherapy are mainly selected as the treatment for oral cancer. Although cisplatin, carboplatin and paclitaxel are basic agents in chemotheraphy of oral cancer, some patients with advanced oral cancer relapse and finally die due to the development of drug resistance. In breast cancer, it was demonstrated that the inhibition of BORIS expression by siRNA led to the proportional apoptotic death of breast cancer cells but not control cells. Data has confirmed the attractiveness of this epigenetic-acting molecule as a target in therapeutics development.

We confirmed BORIS expression in oral cell
lines. It was suggested that BORIS could activate other genes by demethylation of their promoter sequences. Moreover, CTAs including BORIS might be the target of immunotherapy for oral squamous cell carcinoma because of their restricted expression. It is expected that suppression of BORIS might suppress other oncogenes, leading to selective apoptosis of oral cancer. Because detection of BORIS expression is often the first indication of oral cancer, this might provide a significant opportunity for directed therapeutic intervention that simultaneously targets oral oncogenic pathways. In addition, searching for other CTA genes of in oral cancer cell lines might lead to development of new therapies for oral cancer.

REFERENCES

Analysis of cancer testis antigens containing BORIS in oral cancer cell lines


