

## Identification of A Novel Hotspot and A Candidate Tumor Suppressor Gene at 10q21, *RHOBTB1*, in Head and Neck Cancer

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**Abstract:** We previously defined human chromosome 10q21 as a hotspot of regional loss in head and neck squamous cell carcinomas (HNSCC) by genome-wide loss of heterozygosity (LOH) analysis. Aims of this study are to narrow-down the target area by using new microsatellite markers and to define candidate tumor suppressor genes (TSG). LOH analysis on 10q21 in 52 HNSCC by 8 highly polymorphic markers indicated distinctive and frequent allelic loss at D10S589 (42%). Among flanking genes, we found the *RHOBTB1* gene as a candidate TSG, since an intragenic marker demonstrated the highest LOH (44%). Semi-quantitative expression analysis revealed down-regulation of *RHOBTB1* mRNA in 37% of tumors. Interestingly, all tumors that showed decreased expression of *RHOBTB1* were accompanied with LOH, supporting the haploinsufficiency and class 2 TSG characteristics of *RHOBTB1*. Although no pathogenic mutation of *RHOBTB1* was found, frequent allelic loss and decreased expression of *RHOBTB1* suggested that this gene has a role in tumorigenesis of a subset of HNSCC.

### Introduction

Loss of heterozygosity (LOH) analysis is a strong tool to define the location of putative tumor suppressor genes (TSG). LOH method reveals whether any of the alleles is retained or lost in a specific chromosomal region. Loss of one allele by deletion means one hit of the “two-hits theory” hypothesized by Knudson<sup>1</sup>) to explain the mechanism of the inactivation of the genes functioning as tumor suppressor, whereas mutation, as another “hit” destroys the remaining allele.

Objectives of the present study are to analyze the hotspot regions of previous genome-wide LOH analysis in detail to narrow down the target areas. At first, we selected 10q21 and analyzed in detail which defined minimally deleted region at 10q21.3. In the second step, after reviewing flanking genes considering TSG potential, the *RHOBTB1* gene located at 10q21.3 was selected for further analysis. We assessed for the first time, alterations in sequence and expression of *RHOBTB1*, as well as correlation between the expression level and LOH status in HNSCC.

### Materials and Methods

Tumor samples were obtained at the Okayama University Hospital. Squamous cell carcinoma was confirmed by histological examinations. Genomic DNAs were isolated from frozen tissues by SDS/proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation. Total RNAs were prepared by using a modified acid guanidium phenol chloroform method.

LOH analysis was performed with highly polymorphic microsatellite markers. After sense primers were labeled with 5-iodoacetamide fluorescein, PCR was carried out. PCR products were applied on ABI Prism 3100 DNA sequencer and analyzed by Genescan analysis.

### Mutation analysis of *RHOBTB1*

Nine coding exons of the *RHOBTB1* gene were amplified by PCR and treated with ExoSAP, labeled with the Big Dye sequencing kit and analyzed on ABI PRISM 3100.

### Semi-quantitative RT-PCR

Total RNA was reverse-transcribed with Toyobo pre-amplification system starting with 2 µg of total RNA from each sample. Amplification was performed using specific primers for *RHOBTB1*, RS1 (5'-GCT CTC TTA CTT GGA ATT GGC T) and *RAS1* (5'-CGC TGG TAG TGA TCT TCT TCC). RT-PCR products were separated through 2% agarose gel and stained with ethidium bromide. The sizes of RT-PCR products were 204 and 456 bps for *RHOBTB1* and *GAPDH*, respectively. The intensity of ethidium bromide staining of each band was measured by a CCD image sensor and analyzed by a computer program for band quantification. The values of tumor specific *RHOBTB1* expressions were determined by calculating the ratio of the expression level in tumor and matched normal samples, each of which was normalized for corresponding *GAPDH* expression level. Expression levels were defined as decreased (ratio d'0.50) or increased (ratioe'1.50). Fisher's exact test (two-sided) was used for statistical analysis. *P* values less than 0.05 were considered as statistically significant.

### Results

#### *LOH analysis*

Allelic loss was analyzed in 52 pairs of HNSCC samples matched with adjacent normal tissue. Among microsatellite markers (D10S1221, D10S196, D10S1790, D10S1756, D10S589, D10S1225, D10S581, D10S210), D10S589 showed a prominent result: 14 out of 33 (42%) informative samples displayed allelic loss as the highest LOH frequency of the region (fig 1A). Moreover, 8 samples have distinctive loss compared with flanking markers. Fig. 1B shows representative examples with LOH and retention cases. The genes located around D10S589 were defined and reviewed in detail regarding TSG potential. *RHOBTB1* gene as a TSG candidate was selected for further analysis. To confirm high LOH frequency in regard to the *RHOBTB1* gene, two additional new microsatellite markers, one (MS-1) located in intron 7 of the gene, the other (MS-2) 50 kbp telomeric side of the gene, were designed. LOH analysis of the markers D10S589 and MS-1 showed higher LOH frequencies (42% and 44%, respectively)

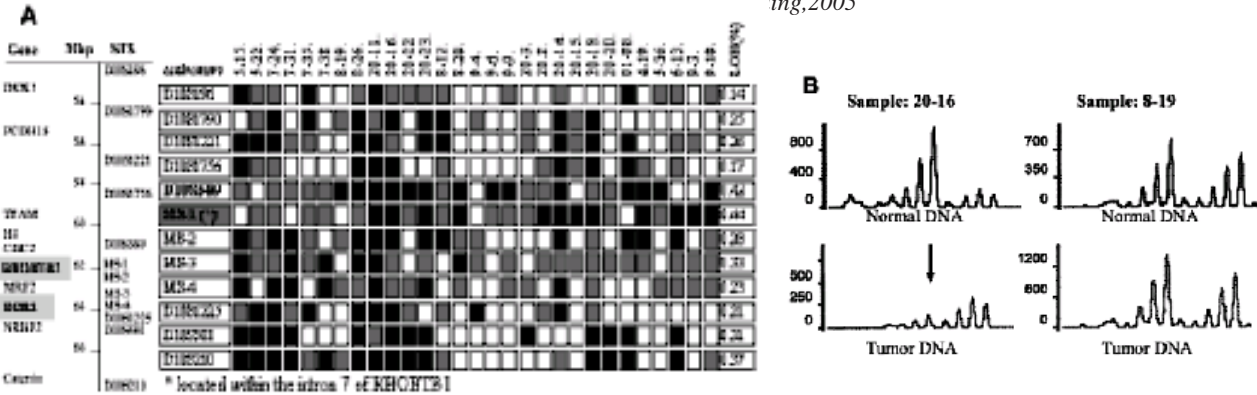


Fig. 1. LOH analysis on chromosome 10q21 in HNSCC. (A) Schematic representation of LOH distribution with physical map. Case numbers are shown at the top. Only cases with at least one LOH were shown. Microsatellite markers are shown to the left and LOH frequencies to the right. Filled box, LOH; open box, retention of heterozygosity; shaded box, not informative. (B) Representative electropherograms of LOH analysis on the *RHOBTB1* gene locus (MS-1) by microcapillary electrophoresis. LOH was scored by comparing the peak heights of tumor and matched normal gene allele. The arrows mark the lost allele in sample 20-16, while sample 8-19 represents retention.

among all markers whereas flanking microsatellites displayed only 17% and 28% deletions.

**Genomic structure of *RHOBTB1***

The *RHOBTB1* gene (KIAA0740) is mapped 1 Mbp telomeric to D10S589 at 10q21.3 band as a member of *RHOBTB* subfamily. The human *RHOBTB1* gene has 11 exons encoding 696 amino acid residues and spans over 131 Kbp genomic region. *RHOBTB* proteins are characterized by modular organization consisting GTPase region immediately followed by a short proline-rich region, a tandem of two BTB domains and a carboxyl-terminal region. The precise function of the *RHOBTB* proteins is unknown at present.

**Mutation analysis**

All coding 9 exons and exon-intron junctions of *RHOBTB1* were screened for mutation by PCR-direct sequencing in 52 primary HNSCC samples. Sequence analysis revealed no presumably pathogenic mutations of *RHOBTB1* in HNSCC.

**Expression analysis**

*RHOBTB1* expressions were analyzed by semi-quantitative RT-PCR (using primer set covering exons 9-10-11) in RNA available 46 HNSCC samples and corresponding normal tissues of the same patient (Fig. 2). After quantitation of RT-PCR products normalized for GAPDH expression, comparison between normal and tumor tissues revealed decreased expression in 37% of tumor specimens (17 of 46), increased expression in 35% of tumor specimens (16 of 46) and no change in 28% of specimens (13 of 46). When the relationship between mRNA expression and LOH analysis of the *RHOBTB1* gene was examined, a significant result appeared that all low-expression samples displayed allelic loss of the gene for informative cases ( $p = 0.01$ ), although number of the non-informative cases for the marker was fairly high.

We analyzed the relationship between LOH - semi-quantitative mRNA expression result of *RHOBTB1* gene and various clinical characteristics including age, gender, smoking status, tumor localization, nodal status, TNM stage, histological grade and mutation status of *p53*. We did not find significant relation between genetic-epigenetic modifications and clinicopathological markers.

**Discussion**

We found for the first time high and distinctive LOH focus of the chromosomal 10q21 band in HNSCC<sup>2)</sup>. Only a few studies exist evaluating the LOH status of chromosome 10q<sup>3)</sup> showed two separate hotspots at 10q22-26 interval in HNSCC.

As continuation of the previous screening genome-wide LOH work, 10q21 was analyzed in detail which revealed D10S589

marker (10q21.3) with high and distinctive LOH results (42%) in HNSCC as a novel minimally deleted region. In the second step, we reviewed genes flanking D10S589 with respect to TSG potential and selected *RHOBTB1* as a target for further molecular analysis considering two factors. First, BTB/POZ domain proteins are found in transcriptional repressors and influence cellular development<sup>4)</sup>. Moreover, other putative TSGs carrying BTB domain such as *HIC1*, *BPOZ* and *APM1* have been reported. Second, the *RHOBTB2* gene of the same subfamily (or *DBC2* gene: deleted in breast cancer) was suggested as a strong candidate TSG in breast cancer<sup>5)</sup>. Comparison of sequences showed that *RHOBTB1* and *RHOBTB2* are closely related to each other. Recently, increasing amount of evidence indicates that several members of the Rho GTPase are important players in tumor biology. However, there is still little information available on the clinical significance of Rho GTPase expression in human cancer specimens. *RHOBTB* subgroup (*RHOBTB1* and *RHOBTB2*) was suggested to have a function distinct from classical Rho GTPases, without obvious role in organizing the actin filament system<sup>6)</sup>.

Semi-quantitative RT-PCR analysis was performed to evaluate expression pattern of *RHOBTB1*, because one of the mechanisms for gene inactivation is suppression of expression. Overall 17/46 of the samples (37%) showed tumor specific reduction of *RHOBTB1* expression. When expression levels were compared with LOH patterns, all 5 informative cases with the low expression (<50%) showed LOH. That association is a significant result defining the role of allelic loss in down-regulation of the gene product.

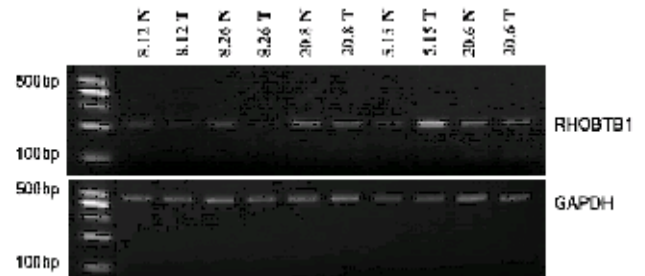


Fig. 2. Expression levels of the *RHOBTB1* genes in HNSCC and adjacent normal tissues. Lower panel represent the expression of GAPDH

Sequencing analysis of *RHOBTB1* to determine existence of inactivating mutations revealed no somatic mutations in the same samples. However, when down-regulated samples with LOH are taken into consideration, loss of one allele may result in haploinsufficiency, which may be sufficient to accelerate tumor progression especially when exposed to carcinogens such as uv or smoking. Such an affect was demonstrated for a group of TSGs including *p53*<sup>7)</sup> and *PTEN*<sup>8)</sup>.

In conclusion, our study demonstrated high and distinctive LOH at the *RHOBTB1* locus and attenuated expression levels of the gene in about 40% of HNSCC samples. All cases with low expression had LOH indicating the haploinsufficiency effect of the lost allele of the gene. Further functional analysis is warranted to define the mechanisms for loss of the gene function and fine roles in controlling cell proliferation.

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