

Epigenetic and Genetic Modifications of BRG1, A Candidate Tumor Suppressor Gene in Oral Cancer

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Abstract: To detect deletions of specific chromosome regions, loss of heterozygosity (LOH) analysis is a sensitive method. In different types of human cancer, BRG1, a member of SWI/SNF complex proteins, located at 19p13.2, suggested to be a candidate tumor suppressor gene (TSG). We detected allelic deletion in 25 of 39 (64%) samples at 19p13 by using six microsatellite markers. The BRG1 specific microsatellite marker showed the highest LOH in tumor samples. As we couldn't detect any mutation of the BRG1 gene in oral cancers, we examined the mRNA expression level. During expression analysis we detected an alternative in frame splicing form of BRG1, which includes exon 26 is selectively decreased or lost in most tumor samples. This 33 amino-acid sequence of BRG1 protein shows very high homology with heterogenous nuclear ribonucleoprotein E. Thus may affect the function and level of BRG1 through modifications on post-transcriptional control.

Key words: SWI/SNF, BRG1, BRM, Oral Cancers, 19p13, LOH, Alternative Splicing

Introduction

Allelotype studies suggested 19p13 region as a hot spot in different kinds of human cancers and existence of at least one TSG^{1,2)}. Brahma related gene 1 (BRG1) (also called SMARCA4 or SNF2 beta), a member of SWI/SNF complex proteins, was localized into 19p13.2 region. We therefore redefined the map of chromosome 19p13.1-13.2 region and examined the BRG1 gene as a candidate TSG in oral squamous cell carcinomas. Our data suggest that frequent allelic deletion and epigenetic alterations of BRG1 promote oral cancer development.

Materials and methods

Tissue Samples

Paired normal and tumor samples were obtained from 39 patients with primary oral squamous cell carcinoma, Okayama University Hospital after acquisition of informed consent from each patient.

DNA and RNA extractions

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 (ISOGEN; Nippon Gene Co., Tokyo, Japan).

Microsatellite Analysis

Primers for amplification of microsatellite markers D19S1034, D19S586, D19S906, D19S584, and D19S221 are available through the internet genome database (<http://www.gdb.org>). We also designed and used a BRG1 specific microsatellite marker, forward BRG1-MS1 (5'-GAGGGGATAGACAAGAGCCG), reverse, BRG1-MAS1 (5'-TCCGCCGAGAAATCAGTGGC), which is localized approximately at 200 kb of the telomeric side of the BRG1 gene. PCR was carried out in 20 ml of reaction mixture with 20 pmol of

each primer, 100 ng of genomic DNA, 1X PCR buffer, 200 mM of each deoxynucleotide triphosphate, and 0.5 unit of Taq DNA polymerase (Takara, Kyoto, Japan). Initial denaturation at 94°C for 3 min was followed by 25 cycles of a denaturation step at 94°C for 30s, an annealing step at 54 °C for 30s, and an extension step at 72°C for 1 min. A final extension step at 72°C for 7 min was added. After amplification, 2-4 ml of the reaction mixture were mixed with 8 ml of loading dye (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol), heat denatured, chilled on ice, and then electrophoresed through an 8% polyacrylamide gel containing 8M urea. The DNA bands were visualized by silver staining. LOH was scored if one of the heterozygous alleles showed at least 50% reduced intensity in tumor DNA as compared with the corresponding normal DNA.

Mutation analysis of BRG1

We examined all coding exons of BRG1 at genomic level by using intron spanning primers. The PCR mixture contained 100 ng of genomic DNA or cDNA, 1.2 mM MgCl₂, 1X PCR buffer, 200 μM of each deoxynucleotide triphosphate, 20 pmol of each primer, and 1 unit of rTth DNA polymerase XL (Perkin-Elmer Applied Biosystems, Foster City, CA) in a 50 ml volume. Initial denaturation at 94°C for 3 min was followed by 33 cycles of a denaturation step at 94°C for 30s, an annealing step at 56°C for 1 min, and extension step at 72°C for 1 min. A final extension step at 72°C for 7 min was added. The resultant PCR products were purified using GeneClean III kit (BIO 101 systems, Qiogene, Carlsbad, CA). Purified PCR products were sequenced on an automated sequencer (ABI prism 377, Applied Biosystems, Foster City, CA) using the same primers for PCR [with BigDye terminator sequencing kit (Applied Biosystems)].

RT-PCR analysis

Total RNA was reverse-transcribed with the Toyobo pre-amplification system (Toyobo ReverTra Ace Kit, Osaka, Japan)

starting with 2 µg of total RNA from each sample, according to the procedures provided by the supplier. One ml of each RT reaction was amplified in 50 µl mixture containing 1.2 mM MgCl₂, 1X PCR buffer, 200 µM of each deoxynucleotide triphosphate, 20 pmol of each primer, and 1 unit of rTth DNA polymerase XL (Perkin-Elmer Applied Biosystems, Foster City, CA). Thirty-three PCR cycles for the mRNA expressions of BRG1, and 25 cycles for GAPDH mRNA expression by using their specific primers were performed. Appropriate cycling number for non-saturating measure was determined empirically by a quantitative PCR system (ABI GeneAmp 5700, Applied Biosystems). PCR amplification was performed as described in microsatellite analysis except that the annealing step was at 60°C for 1 min.

Quantitation of the RT-PCR products

RT-PCR products were separated through 2% agarose gel and stained with ethidium bromide. The sizes of the RT-PCR products were 223, 279, and 456 bps for BRG1, BRM and GAPDH, respectively. The intensity of ethidium bromide staining of each band was measured by a CCD image sensor (GelPrint 2000/VGA, Toyobo, Osaka, Japan), and analysed by a computer program for band quantification (Quantity One, Toyobo). The values of tumor specific BRG1 expressions were determined by calculating the ratio of the expression level in the tumor and that in the matched normal sample, each of which was normalized for the corresponding GAPDH expression level. Decreased and increased expression levels were defined when this ratio was ≤ 0.75 (25% or more decrease) and ≥ 1.25 (25% or more increase), respectively. Reproducibility was confirmed by independent PCR repeated twice.

Results

Loss of heterozygosity analysis of chromosome 19p13 region

Including the BRG1 specific microsatellite marker, we selected 6 microsatellites markers on chromosome 19p13 to examine allelic loss in the BRG1 locus, demonstrated the highest LOH score reaching 57% allelic deletion in informative tumor samples. Overall, LOH was detected at least one locus in 25 of 39 (64%) samples.

Mapping of chromosome 19p13 region

Most of the chromosome 19 regions have been sequenced and contiguous sequence results are available through the genome database on National Center for Biotechnology Information home page (<http://www.ncbi.nlm.nih.gov/genome/guide/human>).

Genomic structure of the BRG1 gene

Genomic contiguous sequence (NT_011295) included the genomic DNA covering the entire region of the BRG1 gene. The BRG1 gene was composed of at least 35 exons spanning over 100 kb genomic interval. Full-length mRNA (5681 bps) of BRG1 (NM_003072) encodes a protein with 1647 amino acids. At least 2 different in frame alternate splicing forms, which excluded either exon 26 or exon 29, have been reported.

Mutation analysis of the BRG1 gene

No somatic change was detected in any of the tumor samples except some synonymous single nucleotide polymorphisms. As all alterations were detected at corresponding normal tissues, they were considered as polymorphisms.

BRG1 mRNA expression analysis

Since somatic mutation of the BRG1 gene was not detected in tumor samples, we looked for other possible inactivation mechanisms. For this aim, we examined expression level of BRG1

mRNA in tumor samples comparing with the paired normal tissue. Twenty-one out of 34 tumor tissues (62%) showed increased expression of BRG1 mRNA (Figure 1).

Preferential Loss of Alternatively Spliced Isoform of BRG1 mRNA with exon 26

During mutation analysis of BRG1 gene at RNA level, we noticed that expression of an alternatively spliced isoform of BRG1, which includes exon 26 in frame is selectively decreased or lost in most tumor samples. All normal tissues except one with RNA available also had an alternative splicing form of BRG1 with exon 26 other than the major form devoiding exon 26. However, 30 out of 33 tumor samples (91%) showed either total deletion or extremely decreased expression of the isoform with exon 26 (Figure 1). Amino-acid sequence of this unique part of BRG1 protein (NP_003063) displays 65% homology with an isoform of heterogenous nuclear ribonucleoprotein (hnRNP) E2 (NP_005007), which plays important roles in the regulation of gene expression (Figure 1).

Discussion

All previous allelotyping analyses and functional assays of BRG1 strongly suggested it a potential targeted TSG in this region. Our detailed LOH analysis showed that BRG1 is frequently deleted also in oral cancers. Surprisingly, analysis of BRG1 mRNA showed increased expression in 62% of primary tumors instead of decreased expression, which is most likely to happen in most TSGs. In another study, similar to our findings, increased BRG1 mRNA expression was detected in 61% of gastric carcinomas³⁾

Most interesting and potentially important finding that we found is that an alternatively spliced form of BRG1 with exon 26 selectively showed either total absence or extremely reduced expression in most tumor samples (91%). This splicing variant with exon 26 is in frame with the major form of BRG1 mRNA. This part of BRG1 gene shows 65% homology with an isoform of heterogenous nuclear ribonucleoprotein-E2 (hnRNP-E2) a highly abundant subset of RNA-binding proteins, play prominent roles in the regulation of eukaryotic gene expression via post-transcriptional control of cellular mRNAs. These post-transcriptional controls may enhance the complexity of nuclear RNAs via alternative splicing and editing, may modulate information flow from nucleus to cytoplasm and vice versa or may alter levels and sites of protein synthesis through controls on mRNA stability, translation efficiency, and subcellular localization⁴⁾. By this way, BRG1 with exon 26 may be necessary for basic functions of it in the nucleus. During tumor development, the loss of this splicing variant may affect the subcellular location as well as expression level of BRG1 major product, which in turn may alter the expressions of downstream genes including BRG1 itself. As a conclusion, our study suggested that BRG1 might have an important role in oral carcinogenesis through allelic loss and subsequent epigenetic alterations.

References

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