RESEARCH ARTICLE

BRD7 Promoter Hypermethylation as an Indicator of Well Differentiated Oral Squamous Cell Carcinomas

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Abstract

Background: Promoter hypermethylation mediated gene silencing of tumor suppressor genes is considered as most frequent mechanism than genetic aberrations such as mutations in the development of cancers. BRD7 is a single bromodomain containing protein that functions as a subunit of SWI/SNF chromatin-remodeling complex to regulate transcription. It also interacts with the well know tumor suppressor protein p53 to trans-activate genes involved in cell cycle arrest. Loss of expression of BRD7 has been observed in breast cancers and nasopharyngeal carcinomas due to promoter hypermethylation. However, the genetic status of BRD7 in oral squamous cell carcinomas (OSCCs) is not known, although OSCC is one of the most common among all reported cancers in the Indian population. Hence, in the present study we investigated OSCC samples to determine the occurrence of hypermethylation in the promoter region of BRD7 and understand its prevalence. Materials and Methods: Genomic DNA extracted from biopsy tissues of twenty three oral squamous cell carcinomas were digested with methylation sensitive HpaII type2 restriction enzyme that recognizes and cuts unmethylated CCGG motifs. The digested DNA samples were amplified with primers flanking the CCGG motifs in promoter region of BRD7 gene. The PCR amplified products were analyzed by agarose gel electrophoresis along with undigested amplification control. Results: Methylation sensitive enzyme technique identified methylation of BRD7 promoter region seventeen out of twenty three (74%) well differentiated oral squamous cell carcinoma samples. Conclusions: The identification of BRD7 promoter hypermethylation in 74% of well differentiated oral squamous cell carcinomas indicates that the methylation dependent silencing of BRD7 gene is a frequent event in carcinogenesis. To the best of our knowledge, the present study is the first to report the occurrence of BRD7and its high prevalence in oral squamous cell carcinomas.

Keywords: Oral carcinoma - BRD7 expression - hypermethylation - differentiation marker

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Introduction

Squamous Cell Carcinoma of head and neck (HNSCC) is one of the most prevalent forms of cancer in India that accounts for upto 45% of all cancers and hence, poses a major health problem (Mishra and Meherotra, 2014). The incidence of OSCC has increased over the past decades as has the mortality associated with it. OSCC may develop as a result of malignant transformation of precancerous lesions such as leukoplakia and erythroplakia, and precancerous conditions such as oral submucous fibrosis (OSMF) and lichen planus (LP) (Tushar et al., 2013; Mehta and Meherotra, 2014). During malignant transformation, the cells accumulate genetic aberrations such as deletion, mutation and duplications, and epigenetic abnormalities that modify bases without altering the DNA sequence. Chemical modification of cytosine residues by addition of methyl group and posttranslational modification of histone proteins by acetylation of lysine residues constitute the two major epigenetic modifications (Gunn et al., 2014). Of the two, hypermethylation of promoter region of genes was the first modification to be recognized for its role in transcriptional silencing during development and carcinogenesis (Jiang et al., 2014; Kanherkar et al., 2014). The modification occurs as a result of addition of methyl group at the 5' position of the cytosine ring within CpG dinucleotides catalyzed by the de novo methyltransferases, DNMT3a and DNMT3b that preferentially targets unmethylated CpGs. Most of the CpG dinucleotides (~50%-to 70%) in the heterochromatin regions are methylated, while those in euchromatin regions especially those in gene promoters remain locally unmethylated (Martin et al., 2013).

As hypermethylation of promoter region causes transcriptional silencing, methylation is used as a natural regulatory mechanism during growth and development

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to suppress the expression of non-essential genes. Examples include imprinting of genes and X-chromosome inactivation in females (Park et al., 2011). However, when methylation occurs in promoters of essential genes that are otherwise not methylated in fully differentiated cells, the abnormal silencing affects the functional state of cells culminating in the development and progression of tumors (Ehrlich et al., 2009). As these genomic changes are heritable, methylation is maintained through cell division, which as a result provides a selective advantage for growth and survival of cancerous cells during pathogenesis (Bakhtiar et al., 2015). Hypermethylation mediated transcriptional silencing of tumor suppressor genes such as p16INK4a, p15INK4b, p14ARF, p73, APC and BRCA1 have been reported in many types of tumors (Bodoor, 2014; Kanherkar, 2014).

BRD7 (bromodomain containing 7) is a novel tumour suppressor gene (Drost et al., 2007), the protein product of which acts as a transcriptional co-factor for the tumour suppressor p53 (Drost et al., 2007; Sharma et al., 2014). The p53-BRD7 complex negatively regulates transcription of P21, HDM2, cyclin G1 (CCNG1), RRM2B, ZMAT3 (WIG1) and FGF2 genes (Drost et al., 2007). Loss of expression of BRD7 in BRD7KD (Knock Down) cells also affects the expression of these genes even in the presence of wild type p53, and fails to suppress p53 dependent oncogene induced senescence (Drost et al., 2007). These findings clearly established the essential role of BRD7 in regulating uncontrolled proliferation and in oncogenesis. A few reports have confirmed the in vitro observations of oncogenic potential of BRD7 by identifying loss of expression of BRD7 due to deletion of the entire BRD7 gene locus in human breast tumour cells, and promoter hypermethylation in nasopharyngeal carcinomas (Mantovani et al., 2010). Although HNSCCs are known to be the most frequent cancers affecting the Indian population, the occurrence of hypermethylation of BRD7 promoter remains yet to be elucidated. Finding the occurrence will enable to understand its, (1 prevalence and 2) potential of being used as a transformation predictive biomarker. Based on these aspects, we designed the present study and investigated twenty three histopathologically defined well differentiated oral squamous cell carcinomas by methylation sensitive enzyme based technique.

Materials and Methods

Patient samples: Biopsy tissue samples obtained from twenty three patients with well differentiated oral squamous cell carcinoma were included in the study after obtaining informed consent from patients. Tissue samples were collected in 2ml microfuge tubes containing 1.5ml of RNA Save reagent (Biological Industries Israel Beit-Haemek Ltd. Kibbutz Beit-Haemek, 25115, Israel) and stored at -20°C until being processed for DNA extraction.

DNA extraction and Methylation Sensitive Restriction Digestion: Tissue genomic DNA was extracted as described earlier (Mehta, 2014). The digestion of total genomic DNA from all tumor samples were performed with 10U of methylation sensitive type II restriction endonuclease, HpaII (New England Biolabs, Ipswich, MA, USA) at 37°C for 4h in a 20μl reaction volume followed by inactivation by incubation of the reaction mix at 65°C for 20m.

PCR amplification of HpaII digested DNA: The Hpa II digested tumor DNA samples were amplified with BRD7 promoter specific primers designed to amplify only the -400 base pairs upstream of transcriptional start site, which encompasses seven CCGG motifs. The following primers were used: Forward primer: ACGGGAAGAGGAGAAGGAAG, Reverse primer: GATTAAAATCGGGGCTCTCC. After an initial denaturation at 94°C for 5 minutes, the samples were subjected to 35 cycles of denaturation at 94°C for 45 seconds, primer annealing at 55°C for 45 seconds, primer extension at 72°C for 45 seconds.

Results

Promoter analysis and Primer designing for BRD7 promoter region

The sequence of promoter region of BRD7 gene

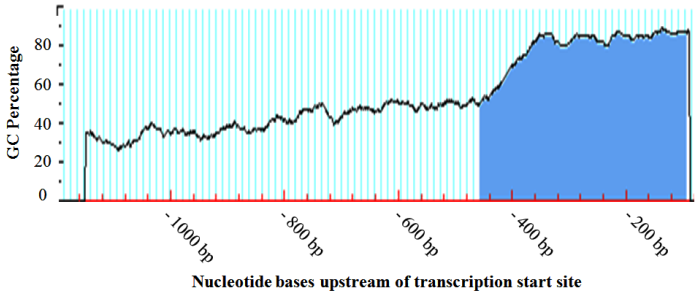

Figure 1. CpG Island Prediction Analysis of BRD7 Promoter. The methylation finder program identified CpG rich island in the -400 base pair region upstream of transcription start site. CpG rich island is shaded in blue

agacaaattgaggggaaagggaggaaggaagaaagacgagagtctgagcggtggatcccgccCCGGccccg
ccctcggccccgcccCCGGcccgcgagctgcagcaccacctCCGGtcgccagggccgccacccgcagc
cgCCGGcaccctgtgcgcgcggcgggccttctttcccgcctctgcgCCGGccgcgcgcccccccgcgccc
gctctcgggctCCGGCCGGgtctcgcgcgccgtcttctc→ **transcriptional start site**

Figure 2. Prediction Analysis of BRD7 Promoter. The methylation finder program identified CpG rich islands in the -400 base pair region upstream of transcription start site. CpG rich islands are shaded in blue

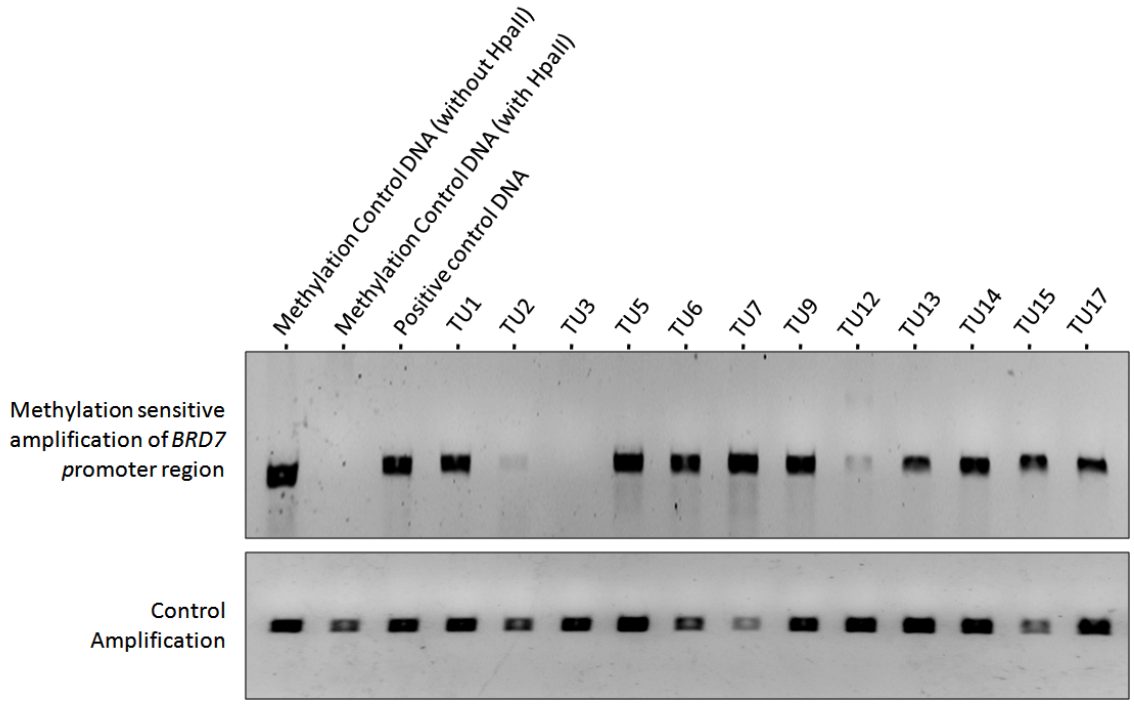

Figure 3. Representative Images of Gel Electrophoresis of HpaII Enzyme Based Methylation Sensitive PCR. Top panel: Amplification of BRD7 promoter region. Note that amplification is seen only when the promoter is methylated. Bottom panel: Control amplification of undigested tumor and control DNA samples

acquired from the public database (www.ncbi.nlm.nih.gov/gene), was analyzed with the methylation finder software (http://www.urogene.org). The criteria used to predict the CpG rich region was as follows: 1) that the CpG island size should be greater than 100 base pairs, 2) GC percentage within the predicted CpG island should be greater than 50.0, and 3) observed versus expected ratio of the CpG islands predicted based on the above two criteria should be greater than 0.60. Sequence analysis with the methylation finder indicated a single CpG rich island spanning -400 base pairs upstream of transcriptional start site (Figure 1).

Following prediction of the CpG rich island, the 400 base pair sequence was scanned for the presence of tetranucleotide recognition motif CCGG for HpaII restriction enzyme, which identified seven CCGG motifs within the 400 base pair region (Figure 2).

HpaII digestion of genomic DNA and PCR amplification to detect BRD7 promoter hypermethylation

In order to confirm the presence of hypermethylation in BRD7 gene, PCR was performed on twenty three HpaII digested tumor DNA samples under conditions mentioned in the methods section. A 20μl aliquot of the reaction was run in a 1.5% agarose gel to analyze the amplification of BRD7 promoter region. Gel analysis showed BRD7 promoter specific amplification in 17 out of 23 (74%) tumor DNA samples (Figure 3).

In the absence of methylation, the HpaII enzyme cuts all the seven CCGG motifs present in the promoter of BRD7and fragments them. The fragmented promoter region fails to get amplified by PCR as the primers were designed to amplify unfragmented promoter only. However, when the CCGG motifs are methylated, they become resistant to Hpa II mediated digestion and the DNA region remains unfragmented, which as a result serves as an ideal template for amplification in PCR. The presence of BRD7 promoter specific PCR band in the tumor samples hence indicated hypermethylation of BRD7 promoter in these samples. Undigested tumor genomic DNA was amplified in independent PCR assay as control for presence of DNA in all samples, which showed positive amplification in all the samples confirming for the presence of DNA (Figure 3).

Discussion

Gene silencing caused by aberrant methylation of promoter regions in tandem with global hypomethylation of genome act as one of the "hits" in Knudson's 2-hit hypothesis for malignant transformation. In present study, we analyzed CpGs within the promoter region of BRD7 to understand its occurrence and associate it with prevalence in well differentiated oral squamous cell carcinomas (OSCC). Of the twenty three OSCC samples that were analyzed BRD7 promoter hypermethylation was observed in 74% of the samples, which indicated that the methylation mediated silencing of BRD7 may be one of the frequent events in well differentiated OSCCs. The HpaII methylation sensitive enzyme based technique that was used in the detection is unlikely to have influenced the higher detection of methylation as the technique has been established to be sensitive and widely reported (Bhatia et al., 2014). Besides the enzyme digestion and PCR were performed in triplicates with identical data output. Based on these facts, we believe that the higher percent prevalence observed to be a true event. While 74% occurrence is a significant finding, the lack of methylation in rest of the OSCCs strongly suggest that the methylation

pattern to be heterogenous, i.e., CpG sites methylated in one patient need not be so in another patient. Such heterogeneity has been reported for other genes in OSCCs. For example, earlier studies have shown varying percent occurrence of hypermethylation of the following genes in OSCCs from Indian patients: 1) 23% of cell cycle regulators-p15 and p16, 2) 8% and 41% of the mismatch repair genes-hMLH1 and MGMT respectively, and 3) 35% of the homotypic cell-cell adhesion gene - E-Cadherin (Asokan et al., 2014).

BRD7 protein is a transcriptional co-factor of p53 tumor suppressor protein (Drost et al., 2010), which plays an essential role in transcriptional activation of genes involved in cell cycle arrest (Sen et al., 2012). In the absence of BRD7 but in the presence of wild type p53, these genes fail to be induced and respond to oncogene induced senescence demonstrating their combined role in transcriptional activation. Genetic aberrations in both partner molecules have been reported in cancerous lesions (Drost et al., 2010). While deletion of BRD7and hypermethylation associated downregulation of BRD7 have been observed in breast cancers and nasopharyngeal carcinomas (Mantovani et al., 2010), mutations in its partner - p53 have been extensively reported in several cancers including OSCCs tissues. Earlier studies have identified prevalence of loss of function p53 mutations in OSCC tissues of patients from India to be about 21% (Sharan et al., 2012). The identification of higher occurrence of BRD7 promoter methylation in the present study with samples that were also obtained from Indian patients indicates that loss of expression of BRD7 may be a more frequent event than loss of function of p53 in OSCCs. This observation gains significance especially since loss of expression of BRD7 has been reported in human breast tumour cells harboring wild-type, but not mutant p53 suggesting that the two genetic aberrations may be occur and affect independently (Drost et al., 2010; Mantovani et al., 2010). Further evidence in this regard comes from BRD7KD cells carrying wild type p53, which when exposed to DNA-damaging signals bypasses growth arrest. Based on these established observations, we believe that the genetic status of BRD7 may serve as a better biomarker in early diagnosis of OSCCs than p53. Further investigations, however, are required in OSCCs with established clinical findings, habits of patients and histopathology based stages of differentiation to positively associate the occurrence of BRD7 methylation and p53 mutations, and evaluate the usage as a biomarker.

The finding of present study has considerable clinical significance as the hypermethylation profile may also be used to track the behavior of malignant lesions as in case of metastasis and response to chemo-/radio- therapy (Miao et al., 2014; Zhou et al., 2014). Most of all hypermethylation is a reversible event in the cells, which means those genes silenced by hypermethylation may be reactivated by drugs designed to de-methylate the hypermethylated regions. *In vitro* studies have shown that genes silenced by methylation may be re-expressed by using demethylating agents. Two such agents, 5-azacytidine (Vidaza) and 5-aza-2- deoxycytidine (decitabine) have been recently approved to treat myelodysplastic syndrome and leukemia (Hagemann et al., 2011). Taken together, the present study has identified for the first time a higher prevalence of BRD7 promoter hypermethylation in OSCCs, which suggests that BRD7 may be potentially used as a cancer predictive biomarker and explored for the efficacy of demethylating drugs in therapeutic management of OSCCs.

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