RESEARCH ARTICLE

Comprehensive Mutation Analysis of PIK3CA, p14ARF, p16INK4a and p21Waf1/Cip1 Genes is Suggestive of a Non-Neoplastic Nature of Phenytoin Induced Gingival Overgrowth

Bhuminathan Swamikannu1, Kishore S Kumar2, Raghavendra S Jayesh1, Senthilnathan Rajendran3, Rajendran Shanmugam Muthupalani4, Arvind Ramanathan5*

Abstract

**Background:** Dilantin sodium (phenytoin) is an antiepileptic drug, which is routinely used to control generalized tonic clonic seizure and partial seizure episodes. A few case reports of oral squamous cell carcinomas arising from regions of phenytoin induced gingival overgrowth (GO), and overexpression of mitogenic factors and \( p53 \) have presented this condition as a pathology with potential to transform into malignancy. We recently investigated the genetic status of \( p53 \) and H-ras, which are known to be frequently mutated in Indian oral carcinomas in GO tissues and found them to only contain wild type sequences, which suggested a non-neoplastic nature of phenytoin induced GO. However, besides \( p53 \) and H-ras, other oncogenes and tumor suppressors such as PIK3CA, p14ARF, p16INK4a and p21Waf1/Cip1, are frequently altered in oral squamous cell carcinoma, and hence are required to be analyzed in phenytoin induced GO tissues to be affirmative of its non-neoplastic nature.

**Methods:** 100ng of chromosomal DNA isolated from twenty gingival overgrowth tissues were amplified with primers for exons 9 and 20 of PIK3CA, exons 1α, 1β and 2 of p16INK4a and p14ARF, and exon 2 of p21Waf1/Cip1, in independent reactions. PCR amplicons were subsequently gel purified and eluted products were sequenced.

**Results:** Sequencing analysis of the twenty samples of phenytoin induced gingival growth showed no mutations in the analyzed exons of PIK3CA, p14ARF, p16INK4a and p21Waf1/Cip1.

**Conclusion:** The present data indicate that the mutational alterations of genes, PIK3CA, p14ARF, p16INK4a and p21Waf1/Cip1 that are frequently mutated in oral squamous cell carcinomas are rare in phenytoin induced gingival growth. Thus the findings provide further evidence that phenytoin induced gingival overgrowth as a non-neoplastic lesion, which may be considered as clinically significant given the fact that the epileptic patients are routinely administered with phenytoin for the rest of their lives to control seizure episodes.

**Keywords:** Cancer from gingival overgrowth - phenytoin induced gingival carcinoma - drug induced gingival carcinoma

Introduction

Seizure episodes associated with generalized tonic clonic or partial seizure disorder is a life style debilitating disease. Several drugs have been developed, which are in use to control seizure episodes include carbamazepine, ethosuximide, felbamate, lamotrigine and phenytoin (Das et al., 2012; Bialer et al., 2013). While a long list of drug exists, phenytoin is preferred drug of choice as: i) can be given intravenously to rapidly control active seizures, ii) is cost effective, and iii) has prolonged duration of action (Brodie et al., 2012). However, administration of phenytoin even at therapeutic dosage for a prolonged period may produce neuro-, hematological and dermatological side effects like horizontal gaze nystagmus, folate deficiency leading to megaloblastic anemia and hypertrichosis respectively (Fuller et al, 2012; Pandey et al., 2012; Tulloch et al., 2012). Phenytoin is also known to cause drug induced lupus and folate deficiency, which is known to be associated with gingival overgrowth (Corrêa et al, 2011). GO is a painful condition that affects about 16% to 94% of patients (Jayaraman et al., 2012) and is often associated with tenderness and bleeding, all of which cumulatively lead to speech disturbances (Lucchesi et al., 2008; Cornacchio et al., 2011). While it has been argued that the GO occurs due to enlargement of...
gingiva by deposition of extracellular matrix components (Kumar et al., 2011), a few cases of oral squamous cell carcinomas (OSCCs) developing in regions of phenytoin induced GO has been reported (McLoughlin et al., 1995). Incidentally OSCC developing from GO tissues associated with administration of two other drugs, cyclosporine - an immunosuppressant and nifedipine - a calcium channel blocker has also been reported (Varga et al., 1991; Pahor et al., 1996), contributing to the possibility of OSCC developing from GO.

An oral lesion with tendency to transform is defined as precancerous lesion based on its presenting histopathological features and/or gene expression profile similar to that of well differentiated OSCC lesions. Consistent with this definition, an expression profile of several mitogenic factors such as c-Myc, PDGF-B, bFGF, and TGF-beta (Corrêa et al., 2011) and cell cycle regulators such as p53 (Saito et al., 1999) has been observed in GO tissues, and which raises the possibility of GO condition as a disease with potential to transform into malignancy (Jayaraman et al., 2012). Since p53 is overexpressed in several carcinoma tissues

In an earlier study, we investigated the genetic status of p53 in GO tissues and found that the p53 gene of these tissues were of wild type in their coding sequence (Jayaraman et al., 2012). We also investigated the status of H-ras gene sequence in the same study, especially since H-ras mutations were found to occur more frequently in Indian OSCCs and found no mutations (Tandon et al., 2010; Murugan et al., 2012). However, it has to be emphasized that the absence of mutations in these two genes as reported in our earlier study rather did not rule out the absence of mutations in other oncogenes and tumor suppressors. It is important to note that phenytoin initiates reactive oxygen species (ROS) to form 8-hydroxy-2'-deoxyguanosine (8-OHdG) adducts that causes G:C to T:A base transversions in chromosomes during DNA replication (Jayaraman et al., 2012). These observations prompted us to extend our investigation to an oncogene, PIK3CA and three tumor suppressors, p14ARF, p16INK4a and p21Waf1/Cip1, all of which have been reported to be mutated in several carcinomas including OSCCs.

The PIK3CA codes for the larger catalytic subunit, 110α (PIK3CA) of the phosphatidylinositol 3 kinase (PI3K) heterodimeric lipid kinase, which plays a pivotal role in transducing signals emanating at the cell membrane to the nucleus to promote cellular proliferation, differentiation, survival and growth. Mutation hot spots within the PIK3CA subunit encoded by exons 9 and 20 of PIK3CA have been reported in (Samuels et al., 2004) OSCCs (Qui et al., 2006; Murugan et al., 2008) that includes E542K, E545K and H1047R with significantly enhanced lipid kinase activity. The INK4a/ARF locus located on chromosome 9p21 region encodes for two distinct cell cycle regulatory genes, p14ARF and p16INK4a, which are produced as a result of alternate splicing of the first exon but are followed by shared downstream exons (Gil et al., 2006). p14ARF binds with the ubiquitin ligase MDM2 and promotes stabilization of wild type p53 molecules, which in turn suppresses unregulated cell division. In contrast, p16INK4a binds with cyclin dependent kinases 4 and 6 (CDK4/6 and CDK6) to inhibit the catalytic activity of CDK4/6-cyclin D complex and prevent cell cycle progression (Kim et al., 2006). Hence loss of function mutations in either one of them, p14ARF or p16INK4a is likely to promote carcinogenesis. Indeed deletions and mutations in both p14ARF and p16INK4a have been observed in several cancers including OSCCs (Kresty et al., 2002; Sailasree et al., 2008). p21Waf1/Cip1 is an universal inhibitor of cyclin dependent kinases and is under transcriptional control of wild type p53. Codon polymorphisms within p21Waf1/Cip1 have been associated with oral premalignant lesions and OSCCs relative to normal tissues (Abbas et al., 2009).

Therefore, in the present study, we have made a comprehensive analysis of PIK3CA, p14ARF, p16INK4a and p21Waf1/Cip1 for the occurrence of genetic alterations in a series of samples of phenytoin induced gingival overgrowth.

### Materials and Methods

Study design, subjects and DNA samples: A cross sectional study was designed with patient DNA samples that had been isolated from twenty phenytoin gingival overgrowth tissues earlier (Jayaraman et al., 2012). 100ng aliquots of the total chromosomal DNA of the above samples were used in the present study after confirming the quality of each of the sample again.

Polymerase Chain Reaction and Direct sequencing: Only exons that are known to be frequently mutated in OSCC lesions were analyzed in the present study. The primer sequences for PIK3CA, p14ARF, p16INK4a and p21Waf1/Cip1 are as mentioned in Table 1. A universal amplification program was developed, which included 35 cycles of the following temperature cycles on 100ng of genomic DNA samples: denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1 min, which was followed by a final extension at 72°C for 5 min. The PCR amplicons were confirmed in comparison to DNA size markers in agarose gel electrophoresis, and were subsequently eluted with Genelute DNA gel elution kit (Sigma Aldrich, cat# NA1111) and subjected to direct sequencing with same set of primers that were used for PCR amplification.

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>SEQUENCE</th>
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<tbody>
<tr>
<td>PIK3CA-ex9f</td>
<td>CTCATGCCTTCGTGTTGTTC</td>
</tr>
<tr>
<td>PIK3CA-ex9r</td>
<td>TAGTGCCCTTTCCAGTGTC</td>
</tr>
<tr>
<td>PIK3CA-ex20f</td>
<td>GGTCAAGGGAAACATCTGGAAAA</td>
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<tr>
<td>PIK3CA-ex20r</td>
<td>AACTGAAACTCAACTCTCTCTG</td>
</tr>
<tr>
<td>P14/16-ex2f</td>
<td>CCTGGCTCTGACATCTTCTG</td>
</tr>
<tr>
<td>P14/16-ex2r</td>
<td>GTGCGCAAGTCATTTCCCCG</td>
</tr>
<tr>
<td>P14ARF-ex1f</td>
<td>CTCAGAGCCGTTCGAGATC</td>
</tr>
<tr>
<td>P14ARF-ex1r</td>
<td>TAGCCTGGGCTAGAGACG</td>
</tr>
<tr>
<td>P16ink-ex1f</td>
<td>TTTGCTAGTGTCGAGAG</td>
</tr>
<tr>
<td>P16ink-ex1r</td>
<td>GGCTCCTCATTTCTCTTCC</td>
</tr>
<tr>
<td>P21/waf-ex2f</td>
<td>ACCAGCTGGAAAGGTAGGAG</td>
</tr>
<tr>
<td>P21/waf-ex2r</td>
<td>GGTTCTTGGCTGCTACTTGC</td>
</tr>
</tbody>
</table>

### Table 1. Primer Sequences of Exons 9 and 20 of PIK3CA, Exon 1α, 1β and 2 of p16INK4a and p14ARF, and Exon 2 of p21Waf1/Cip1 are Shown
Results

In order to understand whether mutations in PIK3CA, p14ARF, p16INK4a and p21Waf1/Cip1 occurred in phenytoin induced GO tissues, selected exons of the above four genes known to be frequently mutated in well differentiated OSCC lesions were analyzed. PCR amplification with exon specific primers, as shown in Table 1, were performed for exons 9 and 20 of PIK3CA, exons 1α, 1β and 2 of p16INK4a and p14ARF, and exon 2 of p21Waf1/Cip1, which was followed by direct sequencing. Though the duration of phenytoin therapy in these patients ranged between 3 to 13 years with an average daily dosage of 184 mg, none of the GO tissue that were analyzed carried mutations in any of the above four genes.

Discussion

Phenytoin induced GO is often a painful condition that requires surgical correction of the overgrowth tissues, which is considered as an additive distress to existing seizure episodes. Under such circumstance, the case reports of OSCCs developing from GO tissues (Varga et al., 1991; McLoughlin et al., 1995; Pahor et al., 1996) were considered as a serious cause of concern especially in those who were being treated with phenytoin for a prolonged period. In the present study, we have examined the GO tissue samples that were identified to be carrying wild type p53 and H-ras genes in earlier study (Jayaraman et al., 2012), for the occurrence of mutations in the oncogene PIK3CA and tumor suppressors p14ARF, p16INK4a and p21Waf1/Cip1. Exons 9 and 20 of PIK3CA subunit of PI3K protein, exons 1α, 1β and 2 of p14ARF and p16INK4a and exon 2 of p21Waf1/Cip1 were investigated as mutations in these regions were found to be highly prevalent in OSCCs (Murugan et al., 2008; Sairasree et al., 2008; Abbas et al., 2009). The finding that none of the analyzed GO samples carried mutation in the above four genes suggests non-neoplastic nature of this condition, which is in agreement with our earlier report. Hence in the case reports, where OSCCs arising from GO tissues were observed it is possible that the OSCC lesion arose due to factors independent of genetic alterations in H-ras, p53, PIK3CA, p14ARF, p16INK4a and p21Waf1/Cip1. Such factors may even be extrinsic such as intraoral trauma for example, that constantly irritated overgrown tissues with consequent dysplasia leading to carcinogenesis (Dayal et al., 2000; Lissowska et al., 2003; Vaccarelzza et al., 2010).

Furthermore, recent investigations have identified phenytoin induced GO as rather a fibrotic condition (Subramani et al., 2012) as the tissues have been found to contain an increase amount of connective tissue stroma along with an elevated presence of collagen fibers (Corrêa et al., 2011). Under normal physiological condition, the degradation of the extracellular matrix is enabled by the enzymatic activities of collagenases and matrix metalloproteinases (MMPs) coupled with a negative regulatory control by tissue inhibitor of MMPs (TIMP), which acts to inhibit the function of MMPs. It has been observed that phenytoin induced GO tissues expressed reduced levels of MMP-1, 2 and 3, while expressing elevated levels of TIMP-1 mRNA, both of which together could disrupt the matrix degradation machinery (Kato et al., 2005; Kanno et al., 2008). Besides, it may also be noted that phenytoin interferes with the cellular calcium influx (Brunet et al., 1996) and hepatic 5,10-methylenetetrahydrofolate reductase activity, both of which consequently affects the metabolism of folates. Folic acid is essential for the production of active collagenases and hence a decrease in the folate metabolism may result in an increase in the level of matrix collagen.

These factors may have acted independently or in concert in disrupting and/or disabling the extracellular matrix degradation machinery leading to GO condition, but nevertheless has been suggested to be one of the leading factors for phenytoin induced GO.

From the analysis of twenty patients with phenytoin induced GO, who were on phenytoin therapy for 3-14 years, it is evident that the drug did not induce mutations in PIK3CA, p14ARF, p16INK4a and p21Waf1/Cip1. The lack of identification of mutation in the oncogenes and tumor suppressor genes that have been analyzed by us so far, H-ras, p53 (Jayaraman et al., 2012), PIK3CA, p53, p14ARF, p16INK4a and p21Waf1/Cip1 identifies phenytoin induced GO as a non-neoplastic condition. However, in the presence of other extrinsic factors such as intra oral trauma, the resultant dysplasia may progress to well differentiated carcinoma. Hence, it is suggested that the oral cavity of patients with GO is prudently evaluated for the possible presence of dysplasia promoting extrinsic factors and rectified.

References


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Lack of Mutations in PIK3CA, p14ARF, p16INK4a and p21Waf1/Cip1 in Phenytoin-Induced Gingival Overgrowth


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