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 Swamikannu[1], Kishore S Kumar[2], Raghavendra S Jayesh[1], Senthilnathan Rajendran[3], Rajendran Shanmugam Muthupalani[4], Arvind Ramanathan[5]*

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

Asian Pacific J Cancer Prev, **14 (5)**, 2743-2746

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 it: 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-, hemato- 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

*[1]Department of Prosthodontia, [2]Department of Orthodontia, [4]Department of General Medicine, [5]Human Genetics Laboratory, Sree Balaji Dental College and Hospital, Bharath University, Chennai, [3]Department of Oral and Maxillofacial Surgery, Meenakshiammal Dental College and Hospital, Maduravoyal, Tamil Nadu, India *For correspondence: drarvindram@yahoo.co.in*

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 nifedepine - 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 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 $p21^{Waf1/Cip1}$, 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 (Qiu 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 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). $p21^{Waf1/Cip1}$ is an universal inhibitor of cyclin dependent kinases and is under transcriptional control of wild type *p53*. Codon polymorphisms within $p21^{Waf1/Cip1}$ have been associated with oral premalignant lesions and OSCCs relative to normal tissues (Abbas et al., 2009).

Table 1. Primer Sequences of Exons 9 and 20 of *PIK3CA*, Exon 1α, 1β and 2 of *p16INK4a* and *p14ARF*, and Exon 2 of $p21^{Waf1/Cip1}$ are Shown

Primer ID	SEQUENCE
PIK3CA-ex9f	CTCATGCTTGCTTTGGTTC
PIK3CA-ex9r	TAGTGCCTTTCCCAGTGC
PIK3CA-ex20f	GGTCAGGGAACATCTGGAAA
PIK3CA-ex20r	ACTGCAACCTCAACCTCCTG
P14/16-ex2f	CCTGGCTCTGACCATTCTG
P14/16-ex2r	GTGCGCAAGTCCATTTCGGG
P14ARF-ex1f	CTCAGAGCCGTTCCGAGATC
P14ARF-ex1r	TAGCCTGGGCTAGAGACG
P16ink-ex1f	TTCGCTAAGTGCTCGGAG
P16ink-ex1r	GGCTCCTCATTCCTCTTCC
P21/waf-ex2f	ACCAGCTGGAAGGAGTGAG
P21/waf-ex2r	GGTCTTTGCTGCCTACTTGC

Therefore, in the present study, we have made a comprehensive analysis of *PIK3CA*, *p14ARF, p16INK4a* and $p21^{Waf1/Cip1}$ 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 $p21^{Waf1/Cip1}$ 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.

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; Sailasree 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; Vaccarezza 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 collagens. 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

Abbas T, Dutta A (2009). p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer*, **9**, 400-14.

Bialer M, Johannessen SI, Levy RH, et al (2013). Progress report on new antiepileptic drugs: a summary of the Eleventh Eilat Conference (EILAT XI). *Epilepsy Res*, **103**, 2-30.

Brodie MJ, Kwan P (2012). Current position of phenobarbital in epilepsy and its future. *Epilepsia*, **53**, 40-6.

Brunet L, Miranda J, Farré M, Berini L, Mendieta C (1996). Gingival enlargement induced by drugs. *Drug Saf*, **15**, 219-31.

Carl GF, Smith DB (1983). The effect of chronic phenytoin treatment on tissue folate concentrations and on the activities of the methyl synthetic enzymes in the rat. *J Nutr*, **113**, 2368-74.

Cornacchio AL, Burneo JG, Aragon CE (2011). The effects of antiepileptic drugs on oral health. *J Can Dent Assoc*, **77**, b140.

Corrêa JD, Queiroz-Junior CM, Costa JE, Teixeira AL, Silva TA (2011). Phenytoin-induced gingival overgrowth: a review of the molecular, immune, and inflammatory features. ISRN Dent, 2011, 497850.

Das N, Dhanawat M, Shrivastava SK (2012). An overview on antiepileptic drugs. *Drug Discov Ther*, **6**, 178-93.

Dayal, Reddy R, Anuradha Bhat K (2000). Malignant potential of oral submucous fibrosis due to intraoral trauma. *Indian J Med Sci*, **54**, 182-7.

Fuller KL, Wang YY, Cook MJ, Murphy MA, D'Souza WJ (2013). Tolerability, safety, and side effects of levetiracetam versus phenytoin in intravenous and total prophylactic regimen among craniotomy patients: A prospective randomized study. *Epilepsia*, **54**, 45-57.

Gil J, Peters G (2006). Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. *Nat Rev Mol Cell Biol*, **7**, 667-77.

Kanno CM, Oliveira JA, Garcia JF, Castro AL, Crivelini MM (2008). Effects of cyclosporin, phenytoin, and nifedipine on the synthesis and degradation of gingival collagen in tufted capuchin monkeys (Cebus apella): histochemical and MMP-1 and -2 and collagen I gene expression analyses. *J Periodontol*, **79**, 114-22.

Kato T, Okahashi N, Kawai S, et al (2005). Impaired degradation of matrix collagen in human gingival fibroblasts by the antiepileptic drug phenytoin. *J Periodontol*, **76**, 941-50.

Kim WY, Sharpless NE (2006). The regulation of INK4/ARF in cancer and aging. *Cell*, **127**, 265-75.

Kresty LA, Mallery SR, Knobloch TJ, et al (2002). Alterations of p16(INK4a) and p14(ARF) in patients with severe oral epithelial dysplasia. *Cancer Res*, **62**, 5295-300.

Lissowska J, Pilarska A, Pilarski P, et al (2003). Smoking, alcohol, diet, dentition and sexual practices in the epidemiology of oral cancer in Poland. *Eur J Cancer Prev*, **12**, 25-33.

Lucchesi JA, Cortelli SC, Rodrigues JA, Duarte PM (2008). Severe phenytoin-induced gingival enlargement associated with periodontitis. *Gen Dent*, **56**, 199-203

McLoughlin P, Newman L, Brown A (1995). Oral squamous cell carcinoma arising in phenytoin-induced hyperplasia. *Br Dent J*, **178**, 183-4.

Murugan AK, Hong NT, Fukui Y, Munirajan AK, Tsuchida N (2008). Oncogenic mutations of the *PIK3CA* gene in head and neck squamous cell carcinomas. *Int J Oncol*, **32**, 101-11.

Murugan AK, Munirajan AK, Tsuchida N (2012). Ras oncogenes in oral cancer: the past 20 years. *Oral Oncol*, **48**, 383-92.

Pahor M, Guralnik JM, Ferrucci L, et al (1996). Calcium-channel blockade and incidence of cancer in aged populations. *Lancet*, **348**, 493-97.

Pandey AK, Gupta S (2012). Psychiatric symptomatology, scholastics, and phenytoin. *Indian J Psychiatry*, **54**, 286-7.

Qiu W, Schönleben F, Li X, et al (2006). *PIK3CA* mutations in head and neck squamous cell carcinoma. *Clin Cancer Res*, **12**, 1441-6.

Sailasree R, Abhilash A, Sathyan KM, et al (2008). Differential roles of *p16INK4A* and *p14ARF* genes in prognosis of oral carcinoma. *Cancer Epidemiol Biomarkers Prev*, **17**, 414-20.

Saito K, Mori S, Tanda N, Sakamoto S (1999). Expression of *p53* protein and Ki-67 antigen in gingival hyperplasia induced by nifedipine and phenytoin. *J Perio*, **70**, 581-86.

Samuels Y, Wang Z, Bardelli A, et al (2004) High frequency of mutations of the *PIK3CA* gene in human cancers. *Science*, *304*, 554.

Subramani T, Senthilkumar K, Periasamy S, Rao S (2012). Expression of angiotensin II and its receptors in cyclosporine-induced gingival overgrowth. *J Periodont Res*, doi: 10.1111/jre.12020

Tandon S, Tudur-Smith C, Riley RD, Boyd MT, Jones TM (2010). A systematic review of *p53* as a prognostic factor of survival in squamous cell carcinoma of the four main anatomical subsites of the head and neck. *Cancer Epidemiol Biomarkers Prev*, **19**, 574-87.

Tulloch JK, Carr RR, Ensom MH (2012). A systematic review of the pharmacokinetics of antiepileptic drugs in neonates with refractory seizures. *J Pediatr Pharmacol Ther*, **17**, 31-44.

Vaccarezza GF, Antunes JL, Michaluart-Júnior P (2010). Recurrent sores by ill-fitting dentures and intra-oral squamous cell carcinoma in smokers. *J Public Health Dent*, **70**, 52-7.

Varga E, Tyldesley WR (1991). Carcinoma arising in cyclosporin-induced gingival hyperplasia. *Br Dent J*, **171**, 26-7.