

RESEARCH ARTICLE

Lack of Mutation in p53 and H-ras Genes in Phenytoin Induced Gingival Overgrowth Suggests its Non Cancerous Nature

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Abstract

Background: There have been case reports of oral squamous cell carcinoma arising from gingival overgrowth induced by phenytoin - an antiepileptic drug. However, a detailed analysis for the presence of mutations in p53 and ras genes, which are the two most frequently mutated genes in cancers, in phenytoin induced gingival overgrowth tissues has hitherto not been performed. **Methods:** Cellular DNA isolated from twenty gingival overgrowth tissues collected from patients undergoing phenytoin therapy were amplified using primers for p53 (exons 5-8) and H-ras (exons 1-2) genes. The PCR amplicons were then gel purified and subjected to direct sequencing analysis to screen for mutations. **Results:** Direct sequencing of twenty samples of phenytoin induced gingival growth did not identify mutations in any of the exons of p53 and H-ras genes that were analyzed. **Conclusion:** Our result indicates that mutational alteration of p53 and H-ras genes is infrequent in phenytoin induced gingival growth, which thus suggests a non malignant nature of this pathology. The findings in the present study are clinically significant as a large number of epileptic patients are treated with phenytoin.

Keywords: Gingival overgrowth - phenytoin - gingival carcinoma - squamous cell carcinoma

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Introduction

Phenytoin (sodium 5, 5-diphenylhydantoin) (PHT) has been the drug of choice in grandmal epilepsy because of its potency, low cost and frequency of administration (once a day). Among the side effects of PHT therapy, gingival overgrowth (GO) is one of the most common adverse effect that affects 16-94% of patients (Cornacchio, 2011). Although the exact mechanism underlying the pathogenesis of PHT induced GO remains to be determined, studies have shown an association with other conditions like combinatorial anti-epileptic therapies such as phenytoin in association with phenobarbital and carbamazepine, plaque accumulation and reduced serum folate levels (Lucchesi, 2008).

Studies on the role of alterations in gene expression in the development of PHT induced GO has shown over expression of various mitogenic factors such as c-Myc, PDGF-B, bFGF, and TGF-beta in PHT induced GO tissues (Corrêa, 2011). PHT has also been shown to initiate reactive oxygen species (ROS) mediated 8-hydroxy-2'-deoxyguanosine (8-OHdG) formation in cultured rat skin fibroblasts (Wells, 1997). 8-OHdG is an adduct of ROS mediated reaction that causes G:C to T:A transversion mutations in the genome of cells during DNA

replication. Such mutation events when occurs within the coding region of cell cycle regulatory or mitogenic genes may result in carcinogenesis. Indeed there have been case reports of oral squamous cell carcinoma (OSCC) arising from PHT induced GO (P.McLoughlin, 1995). Consistent with the above finding overexpression of p53 in the nuclei of epithelial cells of PHT induced GO tissues have been observed (Saito, 1999). Both the overexpression of mitogenic factors and p53 has been observed in oral dysplastic and precancerous lesions, and has been frequently associated with the development of a wide range of carcinomas including OSCCs (Haque, 1998; Wakulich, 2002; Abbas, 2007; Angiero, 2008; Bishen, 2008; Pai, 2009;). Incidentally, cyclosporine-an immunosuppressant and nifedepine-a calcium channel blocker induce GO and OSCCs have been reported to arise from these tissues as well (Varga, 1991; Pahor, 1996). These findings present hyperplastic gingival tissue induced by PHT as pathology with a potential to undergo malignant transformation. Hence we sought to identify whether PHT induced genetic changes in p53 and H-ras in the hyperplastic gingival tissues of patients under PHT therapy. We reasoned that investigating the genetic status of p53 and H-ras – the two most common tumor associated markers, which are also altered and overexpressed in

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OSCC lesions (Tandon, 2010; Murugan, 2012) would help to understand and assess the risk potential of malignant transformation of PHT induced gingival hyperplasias. While p53 was included as its overexpression is already known in these tissues, H-ras was included as its mutations have been identified to occur more commonly in Indian patients, and that it has been implicated in the early stages of neoplastic development.

Materials and Methods

Study design and subjects:

A cross sectional study was designed and twenty patients under PHT therapy who visited tertiary dental care centers during the period November 2010 to March 2012 for oral health care procedures to manage GO were included. All patients (n=20) with clinically visible GO irrespective of years under drug treatment were included after obtaining informed consent. However, patients under PHT therapy with no clinically visible GO or those who were not willing for surgical excision of tissues were excluded from the study. GO tissue samples were collected at the time of hyperplastic tissue excision, and were stored at -80°C until being used.

Genomic DNA extraction:

Tissues were washed once with cold 1X PBS (Phosphate Buffered Saline) and were lysed in 500µl of lysis buffer (containing 0.1% SDS, 25 mM EDTA, 75 µg/100 µl Proteinase-K and 200mM Tris-Cl at pH 8 [Sigma-Aldrich, St.Louis, MO, USA]) at 57°C for 12h with intermittent agitation. Cell lysates were treated with equal volume of Tris-saturated phenol and the aqueous phase were extracted by spinning them at 12,000 rpm for 15 min. The aqueous phases were further extracted with equal volume of 24:1 Chloroform/Isoamyl alcohol to remove residual phenol and detergents. Following the extraction steps, 1/10th volume of Sodium Acetate pH 5.2 and 2.5 volume of cold ethanol was added to the aqueous phase, incubated at -20°C overnight and were centrifuged at 12,500 rpm for 15min at room temperature to precipitate the DNA.

Polymerase Chain Reaction and Direct sequencing

Exons 5-8 of the p53 gene and exons 1-2 of H-ras gene- the regions most frequently associated with mutations,

Table 1. Primer Sequences of p53 and H-ras Genes

Exon	Primer Sequence
p53 exon 5 forward	TTCAACTCTGTCTCCTTCC
p53 exon 5 reverse	CAGCCCTGTCGTCTCTCC
p53 exon 6 forward	TGGTTGCCAGGGTCCCC
p53 exon 6 reverse	CGGAGGGCCACTGACAACC
p53 exon 7 forward	AAGGCGCACTGGCCTCATC
p53 exon 7 reverse	CACAGCAGGCCAGTGTGCAG
p53 exon 8 forward	GGACCTGATTTCTTACTGC
p53 exon 8 reverse	TGCACCCTTGGTCTCTCC
H-ras exon 1 forward	CAGGAGACCCTGTAGGAGG
H-ras exon 1 reverse	CTCTATAGTGGGGTCGTATTC
H-ras exon 2 forward	GATTCTACCGGAAGCAGG
H-ras exon 2 reverse	CTGTACTGGTGGATGTCCTC

Table 2. Details of Patients Under Phenytoin Therapy Who Developed Gingival Overgrowth

Patient	Age	Average dosage (mg/day)	Drug treatment (years)
1	14	200	3
2	20	200	3
3	18	100	4
4	16	100	5
5	21	400	5
6	15	100	6
7	20	200	6
8	15	100	7
9	16	300	7
10	16	200	7
11	24	100	7
12	23	50	8
13	18	100	9
14	28	300	9
15	22	200	10
16	24	200	10
17	30	50	10
18	24	300	12
19	32	400	12
20	27	100	13

were amplified with exon specific primers as listed in table 1, using 50ng of genomic DNA under the following conditions: after an initial denaturation at 94°C for 4 min, the exons were amplified for 35 cycles with denaturing at 95°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. Subsequently, the amplified region of exons 5-8 of p53 gene and exons 1-2 of H-ras gene were run in a 1.5% agarose gel and eluted with Genelute DNA gel elution kit (Sigma Aldrich, cat# NA1111). 10 ng of the eluted PCR amplicon was subjected to direct sequencing (Genetics Lab, Sankara Nethralaya, Chennai, India) to identify mutations.

Results

To know whether p53 and ras genes are altered in PHT induced GO, and thus to understand the risk potential of development of oral squamous cell carcinoma, we have screened gingival samples from 20 patients undergoing phenytoin therapy for mutations in p53 exons 5-8 and H-ras exons 1 and 2. The drug history of these patients varied from 3-13 years with dosages that ranged from 50-400 mg/day (Table 2).

DNA from GO tissues were amplified using primer sets for exons 5 to 8 of the p53 gene and exons 1 and 2 of the H-ras gene, followed by direct sequencing of the gel purified PCR amplicons. The results showed no mutations in the amplified fragments of the two genes in the twenty samples that were analyzed.

Discussion

Gingival overgrowth is a common side effect of phenytoin that is frequently associated with patients undergoing this therapy. In this study, we have analyzed

for mutations of p53 and H-ras, in gingival hyperplastic samples obtained from 20 epileptic patients, with a daily phenytoin dosage of 50-400 mg and a treatment history of 3-14 years of duration. We found no mutation based on the direct sequencing analysis, suggesting the expression of wild type p53 and H-ras molecules in these hyperplastic cells.

In the study that reported OSCC lesions arising in an area of PHT induced GO (McLoughlin, 1995), the development of OSCC may have been incidental involving factors independent of phenytoin. However, it is noteworthy that OSCCs have also been reported to develop from hyperplastic gingiva induced by cyclosporine (an immunosuppressor) and nifedipine (a calcium channel blocker) (Varga, 1991; Pahor, 1996). It has also been observed that nifedipine increases the risk of cancers in the aged population (Varga, 1991). Hence it is possible that the transformation factors may have acted in concert with the drugs during the process of carcinogenesis.

Loss of function of tumor suppressor genes and/or constitutive activation of oncogenes due to mutations in their coding region frequently occurs in cancerous lesions. Indeed altered expression of p53 protein (Heah, 2011) and mutations in p53 and H-ras genes have been observed in well differentiated OSCC and oral precancerous lesions such as leukoplakia and submucous fibrosis (Kuo, 1995; Chiang, 2000; Ranganathan, 2011; Nasser, 2011) including those from Indian patients. While the loss of function of p53 (a tumor suppressor) causes unregulated G1 to S phase transitions, hyperactivation of the oncogene H-ras (an oncogene) promotes carcinogenesis by constitutive stimulation of the mitogenic signaling pathway. Although PHT is known to be an inducer of ROS mediated 8-OHdG adduct, which promotes transition and transversion mutations, the lack of mutation in p53 and H-ras in PHT induced GO tissues analyzed in the present study suggests that the human DNA glycosylase/AP lyase may have removed and thus suppressed the mutagenic effect of the 8-OHdG adducts in these tissues. Over expression of p53 with no apparent mutation in its gene regions have been identified in other conditions like epithelial hyperkeratosis/epithelial dysplasia (Chiang, 2000), clinically normal mucosa of heavy smokers with histological changes such as hyperplasia/dysplasia (Ayan, 2000), and inflammatory papillary hyperplasia of the palate (IPHP) (Kaplan, 1998). Hence it is possible that the p53 expression recognized in PHT induced GO could be due to overexpression of wild type p53.

From our analysis of twenty patients with an average daily dosage of 184 mg and a drug history of 3-14 years of duration, it is clear that the PHT did not induce genetic changes in p53 and H-ras. Hence our results indicate that mutational alterations of p53 and H-ras genes are rare in PHT induced GO, which is supportive of the nonmalignant nature of this pathology. However, the alterations of these genes in the subsequent development of carcinoma cannot be ruled out. It is suggested that a longitudinal study on the genetic status of these and other tumor suppressor genes and oncogenes, and expression analysis in a larger number patients under PHT therapy is essential to better understand the pathogenesis of PHT induced GO.

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