Role of Epithelial Mesenchymal Transition in Phenytoin Influenced Gingival Overgrowth in Children and Young Adults. A Preliminary Clinical and Immunohistochemical Study

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Objectives: *To prove the role of epithelial mesenchymal transition (EMT) in the pathogenesis of phenytoin influenced gingival overgrowth (PIGO) in children and young adults.* ***Study design***: *Thirty male individuals who are to start with oral phenytoin therapy were recruited for the study. All the 30 individuals underwent full mouth scaling and root planning and were then followed up for a period of one year at intervals of 3 months each. Based on the clinical gingival status they were divided into group1 (responders) individuals who showed gingival overgrowth (GO) and group 2 (non responders) individuals who do not show any GO. Gingival tissue samples were obtained from both the groups at the end of 1 year and subjected to immuno histochemical analysis for E-cadherin expression and histo-pathological for alteration in the basement membrane and confirmation of the fibrosis.* ***Results***: *Decrease in expression of E cadherin, loss of basement membrane integrity and fibrosis were noted on responder group when compared to non responder group at p<0.001. Fibrosis was seen in the epithelial connective tissue junction.* ***Conclusion***: *Decrease in cell adhesion, degradation of basement membrane and presence of fibrosis could suggest the role of EMT in the pathogenesis of PIGO.*

Keywords: *Epithelial Mesenchymal Transition, Gingival overgrowth, Phenytoin, Collagen, Fibroblast.*

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INTRODUCTION

Phenytoin influenced gingival overgrowth (PIGO) has remained as a problem in developing countries where it is prescribed very commonly, owing to relatively higher cost of alternative antiepileptic medications [1]. PIGO can occur within 3 months of the drug usage and might reach a state of equilibrium often within the first year of the beginning of medication; it is mostly seen on the buccal surface of upper and lower anterior teeth[2]. Despite decades of research, the pathogenesis of PIGO is still unclear and multifactorial[3]. Controversies still exists as to which among the concepts such as fibroblast proliferation, increased collagen synthesis or decreased collagen degradation are responsible for increase in size of the gingiva [4-6]. However studies agree upon the fact that phenytoin is sensitized to collagen degradation. [6-8] Histological analysis of PIGO reveals a redundant tissue of apparently regular composition or with an increased amount of collagen and number of fibroblasts, frequently, the overlying surface epithelium presents rete ridges elongating into the underlying lamina propria [9]. The level of inflammatory cell infiltrate varies significantly. [10]A genetic predisposition could influence variety of factors such as gingival fibroblast functional heterogeneity, collagenolytic activity, drug metabolism and collagen synthesis [11]. Dental plaque as a co-factor in the etiology of PIGO was recognized in the periodontal disease classification system, [12] some studies have found significant

correlations between the incidence and severity of gingival overgrowth and the amount of accumulated dental plaque and calculus [13,14] while others have shown that satisfactory oral hygiene is able to reduce the gingival overgrowth, but not to completely prevent it[15, 16], given these controversial results, one should look for explanation for pathogenesis of PIGO beyond the increased levels of circulating drug and dental plaque and calculus.

Epithelial mesenchymal transition (EMT) is a process in which epithelial cell to cell and cell to extracellular matrix interactions are weakened as epithelial cells transdifferentiate into fibrogenic fibroblast like cells [17]. Molecular matrix remodeling programs are initiated in the epithelial cells that enable these, now motile and transitioning cells to invade through basement membrane and migrate into the connective tissue stroma where they contribute to new extracellular matrix production.[18]

E-cadherin is a calcium binding trans- membrane protein that helps to maintain the integrity of epithelial cell contacts critical for epithelial tissue barrier function.[19, 20] A consistent feature of EMT is reduction in E cadherin expression that is accompanied by loss of cell to cell contacts and compromised barrier integrity and function. In pathology EMT is a well defined concept for fibrosis, cancer progression and metastasis [21] where as in oral condition there are very limited prospective studies on assessing the role of EMT in PIGO. Hence with this background the present study aims:

1. To evaluate the expression of E cadherin in the gingival tissues of responder and non-responder individuals on phenytoin therapy.
2. To assess and compare the basement membrane integrity between responder and non-responder individuals on phenytoin therapy.
3. To confirm the presence of collagen.

MATERIALS AND METHOD

Screening for newly diagnosed epileptic patients from August 2013 to June 2014 in the age group between 13 to 22 years, was done at Victoria Hospital, and the medical outpatient department at the Bangalore Institute of Dental Sciences and Post graduate Research Center, Bangalore, Karnataka; India; approval from the ethics committee of the Bangalore Institute of Dental Sciences and post graduate research center was obtained. Inclusion criteria for the individuals in the study were male patients between the age 13 to 22 years with a minimum of 20 teeth present, and who are to start with oral phenytoin drug; exclusion criteria were females, individuals on other drugs which are known to cause gingival overgrowth, individuals with recent history of periodontal surgery and individuals receiving any long term medication which could interfere with phenytoin metabolism, smokers and alcoholics.

Thirty individuals who met the inclusion and exclusion criteria and are on the same dose of oral phenytoin were explained in detail about the study and the follow up protocol; and informed consent was obtained for the same. Using a structured questionnaire; patient's age, gender, socio-economical status, level of education, oral hygiene practices and habits were recorded. Clinical examination includes full mouth scoring of plaque by Turesky- Gilmore -Glickman modification of Quigley-Hein plaque index (1970)[22], gingival index by Loe and Sillness (1963)[23], and Oral hygiene index by Greene and Vermillion (1960)[24]. To avoid bias in the scoring; the same examiner (PN) did the recordings for all the patients at both the initial and follow up visits. After the recordings, all the individuals received thorough supra gingival and sub gingival scaling, proper oral hygiene instructions were give and reinforced periodically. Follow up visits were scheduled at three months intervals i,e 3,6,9,12 months after the initial visit. At the end of 12 months based on gingival examination, patients were divided into two groups.

Group I – Subjects with GO (Responders) –figure 1

Group II –Subjects without GO (Non Responders)- figure 2

FIGURE 1: Clinical photograph of individuals with gingival overgrowth. (Responder)

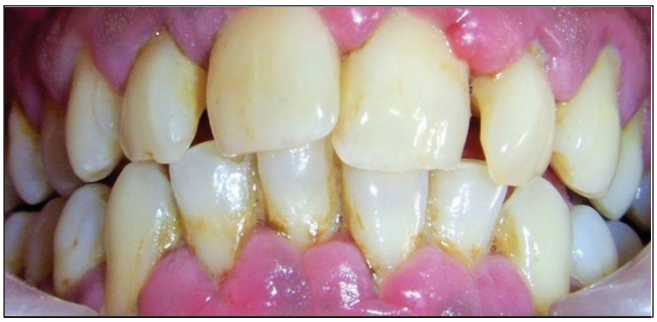
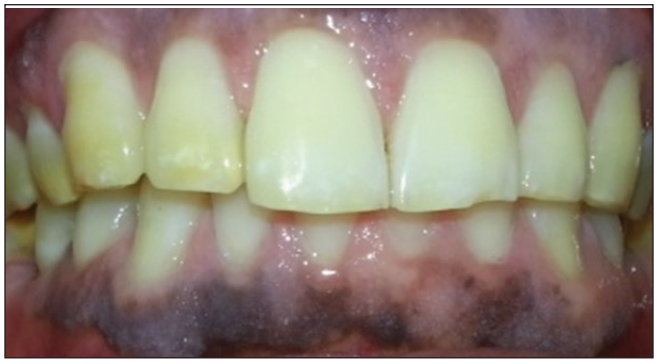


FIGURE 2: Clinical photograph of individuals without gingival overgrowth. (Non responder)



GO was assessed by Bokenkamp and Bohnhorst (1994)[25] index, scoring pattern of which is as follows. Grade 0: No signs of gingival enlargement; Grade 1: Enlargement confined to inter dental papilla; Grade 2: Enlargement involves inter dental papilla and marginal gingiva; Grade 3: Enlargement covers three quarters or more of crown.

One anterior inter dental papilla which showed thick, firm and pale gingiva was selected for incisional biopsy. The site was infiltrated using 0.6 ml of lignocaine 2% with epinephrine at a ratio of 1:100 000 (Warren Indoco, INDIA) around the peripheries of biopsy site; using Bard-Parker scalpel number 15 (Lister surgical blades Kanpur; India), the interdental tissue was excised with a minimal depth of 3 mm, minimal length of 3-6 mm, and minimal width of 1-2 mm. The sample was then rinsed free of blood with saline and was placed immediately into 10 % neutral buffered formalin. Samples from the non responder group were also obtained. After fixation, the tissues was embedded in paraffin wax bath at 56^0-60^0 and the molten wax was poured into leuckarts L pieces mould to form blocks, these

paraffin embedded blocks were then sectioned using tissue microtome. The sections were the subjected to histo-pathological examination using Hemaoxylin and Eosin stain (H and E) for assessment of epithelial hyperplasia, acanthosis and elongation of rete ridges, Periodic Acid Schiff stain (PAS) for basement membrane integrity and Van Gieson's (VG) stain for presence of fibrosis. Another set of sections from the same patient were subjected to immuno – histochemical examination for evaluation of E-cadherin expression under light microscope, the antibody used for the same was E-cadherin 24E10 Rabbit mAb (Cell signaling, Massachusetts, U.S.A)

Slides were observed under light microscope coupled to a digital camera (Labomed inc, LA, U.S.A), the non-responder group were viewed in even higher magnifications than the responder group just to rule out any possibility of subtle changes. The images processed were subjected to evaluation by two experienced examiners (T and D). For statistical purpose the immune histochemical results were scored for presence or absence of E cadherin expression and depending on the depth in expression of the antibody the presence group was subdivided into positive (+), strongly positive (++) and very strongly positive (+++) and similarly the negative group as negative or diffuse weak (-)

Statistical Analysis

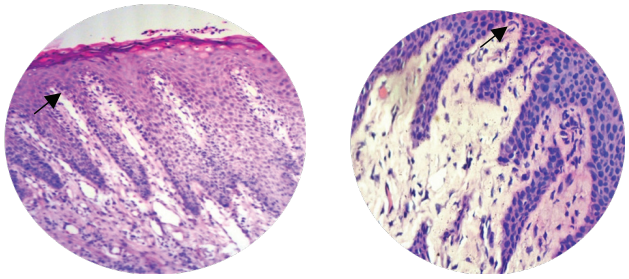
All recorded parameters were analyzed using the statistical package for social sciences (SPSS version20/PC; SPSS, Chicago, IL, USA). Level of significance was set at ≤ 0.01. Chi square test was used to compare the data between the responder and the non responder groups for expression of E cadherin and fibrosis, Fischer's exact test was used to evaluate Basement membrane integrity.

RESULTS

E Cadherin expression between two groups:

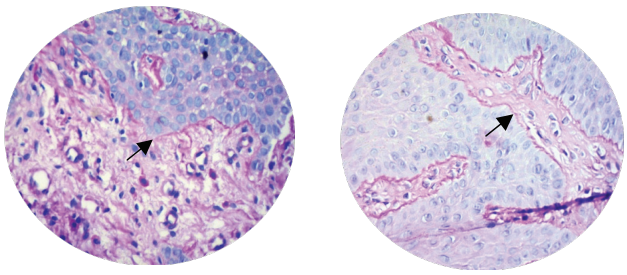
The expression of E cadherin was less in the responder group with only 20 % of the specimens in the very strongly positive group while the non responder group showed 73.3% in the very strongly positive group. Whereas increased expression of E cadherin was seen in the non responder group than the responder group with p value of 0.01 for both the groups. (Table 1, figure 4)

FIGURE 3a: Hematoxylin and Eosin staining of the gingival specimens depicting elongation of rete ridges in the responder group and blunt rete ridges in the non responder group.



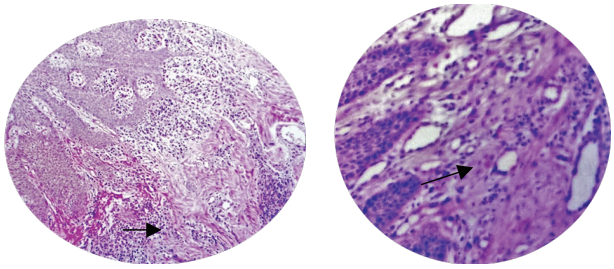
Responder Non responder

FIGURE 3b: Periodic Acid Schiff staining of the gingival specimens depicting breach in the continuity of the basement membrane in the responder group and continuous basement membrane in the non responder group.



Responder Non responder

FIGURE 3c: Van Geison's staining of the gingival specimens depicting presence of fibrosis in the responder group and absence of fibrosis in the non responder group.

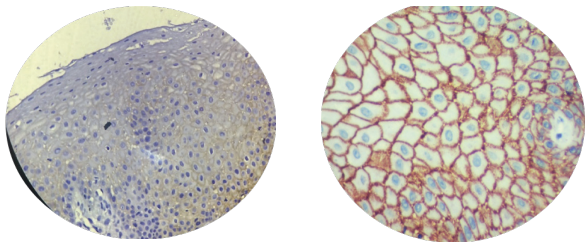


Responder Non responder

Histo-pathological changes:

H and E staining revealed epithelial changes including hyperplasia, acanthosis and elongation of rete ridges which were more pronounced in responder group as compared to non responder group (figure 3a). PAS staining of basement membrane showed discontinuity in responder in contrast to non responder group where it was continuous (Table 3, figure 3b). Fibrosis was noted in the responder group and absent in the non responder group when stained with VG (Table 2, figure 3c).

FIFURE 4: Immuno histochemical analysis for expression of E cadherin depicting decreased response in responder group than non responder group.



Responder Non responder

Table 1: Comparing expression of E cadherin between the responder and non responder groups.

E cadherin	Positive (+)	Strongly positive(++)	Very strongly positive (+++)	p value
Responders n (15)	6	6	3	0.01*
Non responder n (15)	1	3	11	0.01*

Table 2: Distribution of gingival samples stained for fibrosis among responder and non responder groups.

Fibrosis	Present	Absent	Negative	P value
Responder n(15)	12	0	3	0.001*
Non responder n(15)	0	0	15	0.001*

Table 3: Evaluation of basement membrane in the gingival samples between the responder and non responder groups

Basement membrane	Continuous	Discontinuous	P value
Responder n(15)	0	15	0.001*
Non responder n(15)	15	0	0.001*

DISCUSSION

Pathogenesis of PIGO is very complex and multi factorial, studies have related it to long term usage of drug and its levels in serum, [26, 27] mast cells and its products[3], deficiency of folic acid[28] and so forth but till date, the exact mechanism for it to cause a gingival enlargement remains inconclusive [29]. Research from in vitro studies over three decades [4, 30, 28] have stressed the importance of a distinct subpopulation of fibroblast which exists in the gingiva and its reaction to the drug metabolite which could be genetically determined [31,32]. This could be one possible explanation to why only certain patients subjected to the same drug have side effects. Since PIGO are very fibrotic in nature the focus on gingival fibroblast has received lot attention, fibroblast can orchestrate inflammatory, cellular and protein regulation and also has the ability in reversing its role during healing in which it can be collagenolytic [33]. The concept of EMT and its role in cyclosporine induced Gingival overgrowth have been acknowledged, not many longitudinal studies are there in literature to explain this mechanism for PIGO, hence we hypothesized that alteration in basement membrane, loss of E-cadherin and confirmation of fibrosis could be suggestive of the role of Epithelial mesenchymal transition in pathogenesis of PIGO.

Thirty patients completed the study protocol, the study design included the recording of PI, GI, and OHI at all intervals, thorough scaling and root planning was done and reinforced in subsequent intervals of the study so as to evaluate and nullify the role of plaque and inflammation on Gingival overgrowth which could act as a bias, another very important bias ruled out in this study was with the exclusion of females as studies prove that variations in levels of progesterone and estrogens may adversely affect periodontal tissues, inflammation and cause Gingival overgrowth [34]

In our study we first confirmed for gingival overgrowth by histological assessing all the tissue specimens from both the groups using Hemaoxylin and Eosin stain. The responder group individuals showed presence of fibrosis, a dense fibrous connective tissue with an increased amount of collagen fibers arranged randomly, thickening of the stratified squamous epithelium with increase in their numbers, bending of the epithelium deeply into the underlying connective tissue stroma to form elongated rete ridges. These findings were consistent with findings from the studies by Donagri *et al* [35], Kantarci *et al* [36] and Rawisandran *et al*. [37] To justify the role of EMT in the pathogenesis of PIGO we now subjected the tissue specimens after confirmation of overgrowth to immunohistochemical analysis and we chose to study the expression of E cadherin for the same. In the tissue specimens the expression of E cadherin was significantly less in responder group than the non-responder group and the most prominent change in E cadherin expression was seen in the basal layer of the epithelium adjacent to the basement membrane. These findings could be related to the fact that in EMT there is loss of cell to cell contact, compromised barrier integrity and function[38] and also EMT is characterized by the loss of proteins associated with the epithelial phenotype e.g E cadherin; and increased expression of proteins associated with a mesenchymal and migratory cell phenotype e.g vimentin. The present study results were in accordance with Pisoschi [39,] Sume *et al* [40] although these studies did not involve the basement membrane changes in which is also stated to play a role in EMT. Hence along with E cadherin expression we went ahead and studied the changes in the basement membrane in both the responder and non responder group using PAS stain, the finding in our study showed that all the 15 tissue samples of responder group did show loss of integrity in basement membrane. The alterations of basement membrane were closely studied at the interface of epithelium-connective tissue at the higher magnification, all specimens from the responder group showed evidence of disruptions and discontinuities in the basal basement membrane. These finds were supported by the studies done by Kalluri and Weinberg [41], Penarrocha *et al* [42, 36]. Many of these breaks in the basal membrane were accompanied by the presence of cells that we speculate may have migrated from the epithelium. In contrast tissue specimens from non responders showed unspecific epithelial changes, acanthosis and hyperplasia with blunt rete ridges and absence of collagen fibers with decreased number of mast cells and maintaining the continuity of basement membrane. Kantarci *et al* [36] in his study has shown that discontinuous basement membrane is accompanied by a decreased expression of collagen type IV and laminin and contributes to fibrosis and hence to confirm this interesting concept which could suggest the role EMT in fibrosis we stained the tissue samples with Van Gieson's stain which is useful in detecting collagen and in our study it was noted that all the specimens from the non responder group was negative for collagen. However 80% of specimens in the responder group did show positive staining for fibrosis, the remaining 20% in the responder group did not show positive staining for fibrosis even though clinically they showed Gingival overgrowth which can be explained by the fact that Van Geison's stains fails to recognize newly formed collagen and possibly the specimens which failed to take up the stains were from the responders group who showed response to the phenytoin drug metabolite closer to the one year period or the third follow up visit.

Risk factors for PIGO have been identified as age, gender, dental plaque, and genetics, in the present study we included age group of 13 -22 years as clinical studies suggest that children and adolescents appear more susceptible to drug-induced gingival overgrowth than adults.[10, 40] Contradictory reports are also there in literature to age and dental plaque. All though it can be argued that majority of the studies relating to PIGO are either in vitro or observational which are very inconclusive in nature; in our study dental plaque was removed in the initial visit and also in follow up visits whenever necessary and oral hygiene monitored by indices at all follow up visits for one year. Secondly the drug concentration in serum and saliva which could influence the gingival overgrowth was not included in this study as it was account for by the same authors and the result published [43] and the focus mainly in this study were on cell biology. The strength of the present study will be its longitudinal study design, maintenance of gingival health throughout the study period, exclusion of various confounding factors which could act as potential bias, exclusion of female patients which could mislead the hypothesis and confirmation of three key parameters such as loss of epithelial cell contacts, integrity of basement membrane and confirmation of fibrosis at the epithelial connective tissue junction which strongly suggests the role of EMT in pathogenesis of PIGO. Further studies are needed with various other adhesion protein molecules along with E-cadherin, estimation of TGF-β in the tissues and marphometric differentiation of these transformed fibroblasts.

CONCLUSION

Decrease in cell adhesion molecules such E cadherin, alteration of the basement membrane and confirmation of fibrosis could suggest the role of EMT in the pathogenesis of Phenytoin influenced gingival overgrowth.

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