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ORIGINAL ARTICLE

Quantitative and qualitative analysis of epidermal growth factor receptor expression in pericoronal follicles in predicting proliferative potential

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Abstract

Objective. The odontogenic epithelium in pericoronal follicles (PFs) are known to proliferate to form cysts and tumors. This epithelium is mostly composed of the reduced enamel epithelium (REE) and odontogenic rests (OR). The objective of the present study was to evaluate the epidermal growth factor receptor (EGFR) immunoexpression in these PFs to assess their proliferative potential. ***Study design.*** The immunoexpression of EGFR in 30 PFs were assessed by two independent observers for intensity, percentage and the location of the EGFR staining. ***Results.*** EGFR immunoexpression was noted in 100% of the follicles. A greater proportion of the follicles showed strong intensity (70%). It was noted that nearly 54% of the follicles demonstrated more than 50% of cells with EGFR immunolabelling. EGFR showed combined cytoplasm and membrane staining (40%) and cytoplasm only staining (37%). The analysis of the REE and OR individually for the above-mentioned parameters did not show statistical significance. ***Conclusion.*** The increased intensity and overall positivity of the epithelium in follicles shows that odontogenic epithelium is responsive to EGFR mediated growth factors. The predominant combined staining pattern is suggestive of increased potential for the epithelium to undergo cystic or neoplastic proliferation.

Key Words: *pericoronal follicle, EGFR, odontogenic epithelium, reduced enamel epithelium, odontogenic rests*

Introduction

Odontogenesis is an intricate process and a proper understanding of this sequence gives a valuable insight into the histogenesis of various odontogenic lesions. Once the tooth is fully developed, the dental follicle along with the remnants of the dental lamina and the reduced enamel epithelium persists above the crown of unerupted and impacted teeth, where it is referred to as pericoronal follicle (PF) [1,2]. An increasing trend in the incidence of impacted teeth is noted and various theories like lack of space, hereditary factors, mutations and the change in dietary habits are suggested for this occurrence. The most common teeth to be impacted are the third molars; usually due to lack of space in the jaws [3–5]. It is noted that long-standing cases of impaction at times present with pathologies like odontogenic cysts and tumors [3–7]. The pericoronal follicle and its odontogenic remnants are suspected to play an important role in the histogenesis of various odontogenic cysts, tumors and hamartomas. Due to its known potential to be associated with various odontogenic lesions, many studies have evaluated the PF radiographically, histologically, immunohistochemically and at a molecular level [3–11].

Radiographically the follicle consists of a thin radiolucency surrounding the unerupted tooth. Radiographic pathology is defined by most authors as a pericoronal radiolucency of 2.5 mm or larger [11,12]. Microscopically, most follicles have a lining epithelium and/or isolated nests or cords of odontogenic epithelial cells scattered in a predominantly loose stroma [12]. This lining epithelium is either flattened simple stratified squamous type or reduced enamel epithelium having cuboidal to columnar

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ameloblastic epithelium [2,12,13]. Several proliferative markers have shown that follicles have a higher proliferative potential [14].

Tissue homeostasis is orchestrated by a delicate balance of cellular proliferation and apoptosis and alterations in these are responsible for various pathosis. Growth factors and their receptors play a pivotal role in maintaining this cellular homeostasis [15]. Normal as well as abnormal cell proliferation is associated with the occurrence of growth factors such as Epidermal growth factor (EGF), Transforming Growth Factor–α (TGF-α) acting on Epidermal Growth factor receptors (EGFR). EGFR is a transmembrane glycoprotein that constitutes one of four members of the ErbB family of tyrosine kinase receptors. EGFR with its ligands, are cell signaling molecules involved in diverse cellular functions, that include cell proliferation, differentiation, motility, survival and development [15–17]. Although present in normal cells, EGFR is over-expressed in a variety of tumor cell lines, especially squamous cell carcinoma, and is associated with poor prognosis and decreased survival [15].

Recently several studies have evaluated this growth factor receptor because of the advent of anti-EGFR drugs which could in time reduce the need for surgical measures [18]. However, studies exploring the proliferative behavior of the odontogenic epithelium and its remnants are scarce. Additionally, studies evaluating EGFR in PFs show diverse and contradictory results [9,10]. Hence, the present study was designed to study the follicles for their expression of EGFR and to understand its significance.

Materials and methods

The study was commenced following institutional ethical committee approval. Thirty patients with impacted third molars in the age range of 10–30 years, with absence of symptoms such as pain in the impacted tooth were selected. Radiographic exclusion criteria of patients were cases with pericoronal space of 2.5 mm or more (Figure 1). The pericoronal follicles were collected following the disimpaction of these teeth. Ten sections each of squamous cell carcinoma and normal oral mucosa were taken as controls. Samples were prepared by routine formalin fixation and paraffin processing. Two sections of 4 μm each was obtained from each specimen and stained with Hematoxylin and Eosin and EGFR immunostaining.

Immunohistochemistry

The immunohistochemical analysis for EGFR was performed using a super sensitive one-step polymer-HRP technique (Biogenex Life Sciences, San Ramon, CA). Paraffin-embedded tissue blocks were cut into 4–5 μm thick sections and taken onto 2%, 3-aminopropylethoxysilane solution (APES) (Sigma Aldrich, St. Louis, MO, USA) adhesive coated slides. The sections were then de-paraffinized and rehydrated through xylene and descending grades of alcohol. Antigen retrieval was done using commercial microwave antigen retrieval system where the sections were placed in a container containing TRIS EDTA Buffer (pH-8.0) at 96°C for three cycles of 8 min each (EZ-Retriever System, Biogenex life sciences, San Ramon, CA). After rinsing in PBS, the sections were treated with peroxidase block consisting of 3% H_2O_2 in water for 15 min to block the endogenous peroxidase activity, followed by a 20 min power block to obstruct any non-specific antigenic sites. The sections were then incubated for 1 h at room temperature with optimally pre-diluted antibody against EGFR (Rabbit monoclonal anti human EGFR (PAN): Clone No: EP38Y, Biogenex). After washing with PBS, the sections were then incubated with Polymer-HRP reagent for 30 min. Visualization was performed using freshly prepared DAB (diaminobenzidine tetrahydrochloride). The slides were counterstained with Harris hematoxylin, subsequent to which sections were dehydrated, cleared and mounted with DPX. Squamous cell carcinoma and normal oral mucosa served as the positive controls and the endothelial lining of blood vessels known to be non-reactive to EGFR was used as the internal negative control.

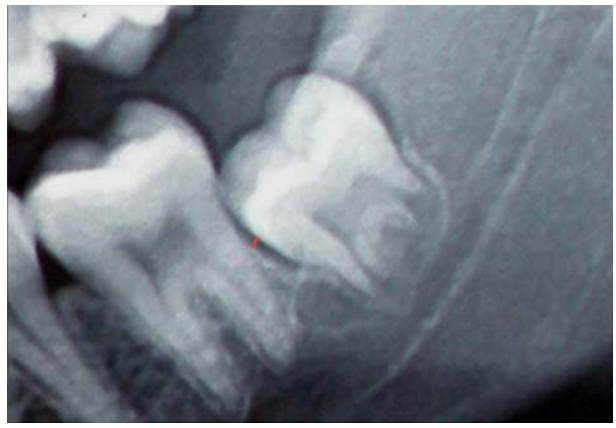

Figure 1. Radiographic evaluation of the impacted third molars for the widest point of the follicular space, i.e. <2.5 mm (Red line) for inclusion as a pericoronal follicle.

Slide analysis

The hematoxylin and eosin stained slides and EGFR immunolabeled slides were analyzed by two independent observers for intensity, location and percentage of EGFR stain. The difference in staining pattern of the odontogenic rests and reduced enamel epithelium in PF was also analyzed. The staining intensity was evaluated on a semi-quantitative three- point scale of

0 = no staining, 1 = weak/mild and 2 = strong/intense staining. The location was studied as C (cytoplasmic staining), M (Membranous staining) and C+M (Cytoplasmic and membranous staining). The percentage of cells stained was analyzed as 1 (1–25% cells); 2 (26–50% cells). The slides were also descriptively studied to understand any subtle differences in the staining patterns.

Statistical analysis

Chi-square test was used to assess the association for EGFR expression between the lining epithelium and odontogenic rests. p-values of less than 0.05 were considered significant.

Results

Among these, 21 (70%) were females and nine (30%) were males. The mean age of the patients was 20.2 years. The follicles were mostly from the mandibular right third molars (60%), mandibular left third molars (15%) and maxillary molars (18%). The remaining two follicles (7%) were collected during the dis-impaction of maxillary right canines for orthodontic purposes. Histopathological analysis of the follicles saw that almost all follicles (93.3%) had both odontogenic rests as well as a thin layer of epithelium with the exception of two follicles, one without rests (3.3%) and the other without epithelium (3.3%). In most cases (78 %) the epithelium was two-to-three layers thick, having flattened stratified squamous epithelium, four cases (15%) showed reduced enamel epithelium with cuboidal to ameloblastic cells (Figures 2A, C and D). Two follicles (7%) showed hyperplastic epithelium and one case demonstrated early cystic changes associated with inflammation. One follicle showed lining epithelium showing mucous metaplasia. The stroma was predominantly (90%) loose and myxomatous interspersed with scant odontogenic rests (Figure 2E). Few cases showed a partially fibrous stroma. Two follicles (7%) showed dense proliferation of odontogenic rests with squamous metaplasia within the odontogenic rests (Figure 2F). Two cases (7%) also showed foci of calcification on histopathological assessment (Figure 2B).

On assessment of the EGFR expression, it was noted that all follicles (100%) demonstrated EGFR positivity. The majority of the PFs showed a predominantly intense staining pattern (70%) with a minor portion showing a mild staining pattern (30%). On assessment of the percentage of cells with EGFR expression it was found that 1–25% of cells were seen in 13% of cases; 33% of cases showed 25–50% of cells with EGFR positivity and the remaining 54% of cases showed greater than 50% of cells with EGFR positivity. On analysis of the location of EGFR staining it was seen that combined staining pattern was seen in 40%, followed by cytoplasmic staining (37%) and membranous staining (23%).

On evaluating the differences between the staining patterns in the reduced enamel epithelium and the odontogenic rests it was seen that both stained predominantly with a strong intensity of EGFR immunostain. The intensity and staining locations of the rests and epithelium were individually assessed and the results were tabulated as follows (Table I). Among the 29 cases with lining epithelium (LE), an intense expression was noted in 55% (Figures 3A–D) of cases followed by mild in 41% of cases and 4% of cases show no staining in the reduced enamel epithelium. In odontogenic rests there was a marginally increased intensity noted, with 66% of cases showing

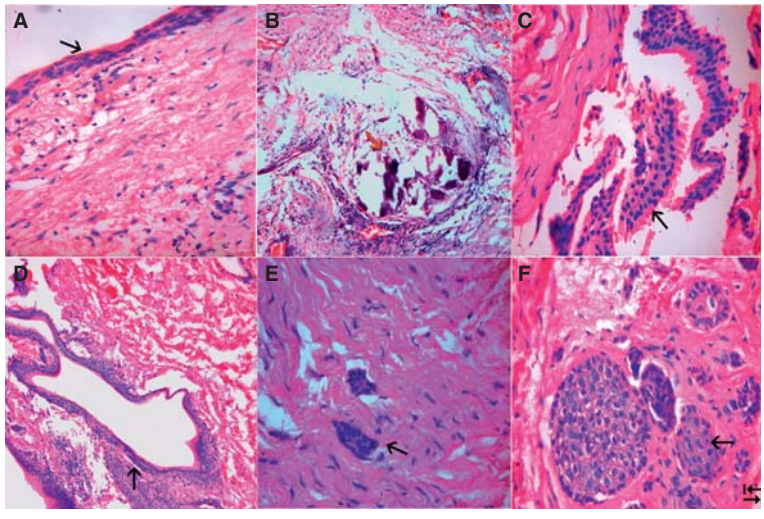

Figure 2. Photomicrograph (H&E) showing pericoronal tissues with reduced enamel epithelium like lining epithelium (A, ×250), Foci of calcification (B, ×250), Presence of ciliated epithelium (C, ×250), Columnar ameloblastic like lining (D, ×250), presence of odontogenic rests (E, ×250) and hyperplastic follicle with extensive squamous metaplasia (F, ×250)

Table I. Intensity and location of EGFR expression in the lining epithelium and the odontogenic rests.

	Intensity			Location		
Criteria scores	0	1	2	C	M	C+M
LE (29)	1 (4%)	12 (41%)	16 (55%)	9 (31%)	5 (17%)	15 (52%)
OR (29)	2 (7%)	8 (27%)	19 (66%)	11 (38%)	6 (21%)	12 (41%)
Chi-square test	p = 0.499 [NS]			p = 0.731[NS]		

LE, Lining Epithelium; OR, Odontogenic Rests; NS, Not significant; C, Cytoplasm; M, Membrane; C+M, Combined cytoplasmic and membrane staining.
0 = no expression; 1 = mild intensity; 2 = Intense expression.

strong immunolabeling (Figures 3E and F). On assessing location the lining epithelium showed predominantly combined (52%) and cytoplasmic staining patterns (31%), with membrane only staining being reported in only 17% of cases. In the rests a similar pattern was noted. The odontogenic rests were marginally more intensely stained as compared to the lining epithelium while the location was predominantly combined in both the groups; however, these differences did not reach statistical significance (Table I).

Discussion

EGFR is an important growth factor receptor whose expression is seen in normal tissues and its amplification is noted predominantly in epithelial tumors especially, the head and neck carcinomas [15]. The increase in EGFR expression in oral epithelium of carcinomas has aroused the interest for exploring EGFR expression in odontogenic epithelium. In long standing cases, certain unknown causes stimulate the follicle to proliferate and give rise to odontogenic lesions like dentigerous cysts, keratinizing odontogenic tumors and ameloblastomas [3–7]. Previous studies have proved that EGFR plays a role in odontogenesis, tooth morphogenesis, ameloblast differentiation, tooth eruption and other cellular functions [16,19]; however, the role played by EGFR in primitive odontogenic epithelium and its role in the histogenesis of odontogenic lesions remains largely obscure.

Several studies have previously assessed follicles histologically to understand their significance in oral pathology [3,11–13,20]. The odontogenic epithelium in the follicles studied were predominantly flattened, about two-to-three layers thick and of stratified squamous type, which is similar to what was observed by some authors [12,20], but, in contradiction to other studies, the reduced enamel epithelium with tall columnar epithelium (ameloblastic type) was more commonly noted [13]. Most previous histopathological assessments on PFs showed a high percentage of odontogenic lesions such as dentigerous cysts and odontogenic tumors [3,11–13,19]; however, in the present study only one case showed definite signs of

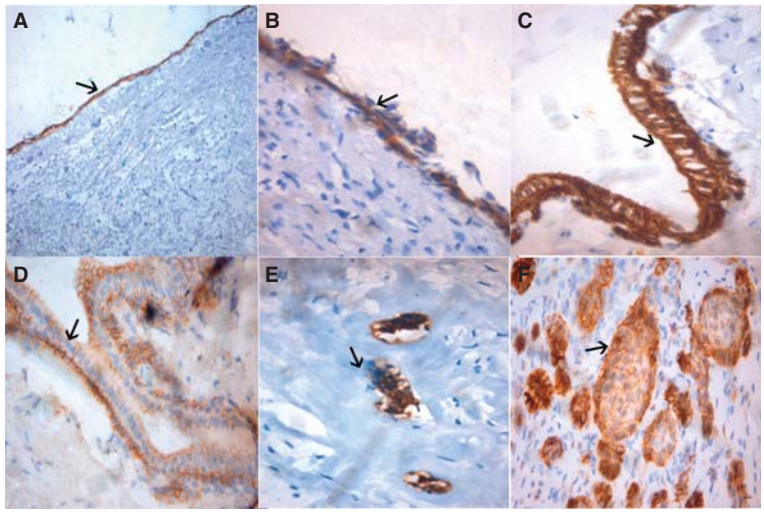

Figure 3. Photomicrograph showing pericoronal follicles demonstrating EGFR immunoexpression in Intense cytoplasmic expression in the reduced enamel epithelium (A; ×100, B; ×250), Lining epithelium with columnar differentiation exhibiting intense mixed (C; ×250) and membranous EGFR positivity (D; ×250). (E) Odontogenic rests showing intense combined membrane and cytoplasmic expression (×250). (F) Mild membranous expression in odontogenic rests with squamous metaplasia.

cyst formation. The hyperplastic dental follicles showed dense proliferation of odontogenic rests, with few showing induction phenomena. Although findings of hyperplastic epithelium, squamous metaplasia and early cystic changes have been reported previously, mucous metaplasia is not commonly noted [3,5,11,13,19].

In the present study, all follicles stained positively for EGFR (100%), showing that the odontogenic epithelium has this receptor and is susceptible to growth factors that act upon EGFR. Similar positivity has been reported in previous studies by Da silva Baumgart et al. [10] and de Oliveira et al. [9] Over-expression of EGFR is strongly associated with the ability to induce proliferation in neoplastic and normal tissues [9,15–17,21].

The follicles showed an overall increased staining intensity (72%), with mild intensity being noted in 28% cases of the follicles, possibly reflecting their potential to undergo proliferation in suitable conditions. To date, there are no reports describing the staining intensity in follicles. This has clinical significance as it clearly suggests that follicles have high growth potential and it is safer to remove impacted teeth and their associated follicles as a precautionary measure. The results further showed a marginally higher intensity in the odontogenic rests.

The next parameter analyzed was the percentage of EGFR positivity. Many studies have combined the staining intensity and percentage of positive lesional cells; however, in the present study, these parameters were exclusively assessed to understand their individual significance [21,22]. It was found that a major portion (54%) of cases showed greater than 50% of cells with EGFR positivity, suggesting that EGFR activity is noted in a major proportion of the odontogenic cells.

The final parameter analyzed was the localization of EGFR. Lots of interest has been focused on this parameter as it is supposed to play a vital role in the biological behavior of neoplasms [9,10]. According to few authors, cytoplasmic EGFR labeling is associated with slower proliferation due to internalization of the EGF receptor. Combined cell and membrane positivity was related to a more physiologic-type response and membrane only response was associated with greater proliferative potential [9,10]. Additionally, recent studies have reported EGFR expression in the nucleus and have concluded that this could be of utmost importance in the understanding of EGFR-related oncogenesis [23]. Evaluation of EGFR in odontogenic lesions by Shrestha et al. [20] showed cell membrane EGFR positivity in all the biopsies. Vered et al. [22] strictly defined membrane and mixed staining as EGFR positive cases. In the present study, analysis of normal oral epithelium used as positive control showed intense combined cytoplasmic and membrane staining in the basal and suprabasal regions, while membrane only staining was visible in the spinous layers, which further decreased to no expression in the upper mature layers. Squamous cell carcinoma controls on the other hand showed mostly intense membrane and combined staining patterns. This expression observed in our study is similar to that obtained by Maiorano et al. [24].

In follicles, it was seen that combined staining pattern was predominant, representing increased proliferative potential. In follicles, cytoplasm only staining could represent the quiescent cells which have the potential to proliferate in the presence of growth factors or they could represent cells which, after odontogenic differentiation, are internalized and degraded [15]. Membrane-only staining, on the other hand, may represent an active proliferative cell with decreased or increased proliferative potential depending on the stimuli. In contrast, recent studies by Da silva Baumgart et al. [10] and de Oliveira et al. [9] showed all normal oral mucosa fields presenting with a combined staining pattern symbolic of slower proliferative response and membrane staining was strictly associated with high proliferation, as seen in squamous cell carcinomas.

Epithelium and rests with squamous metaplasia showed mostly membrane only staining, which indicates the possibility that for squamous metaplasia the EGFR is externalized and used [24]. It was also noted that nests showing squamous differentiation showed more membranous patterns in the center, which is also similar to normal oral mucosa. Thus, in the present study, in physiologic odontogenic epithelium, membrane only staining has been correlated with more mature cells which have low proliferative potential or undergo squamous differentiation. In the reduced enamel epithelium containing tall columnar ameloblastic cells, an intense membrane only staining was observed, suggestive that EGFR is responsible in the ameloblastic differentiation and plays a role in amelogenesis. In other studies on ameloblastomas also, it was noted that the peripheral tall columnar cells and pre-ameloblasts had a membranous staining pattern [25,26].

On detailed examination of EGFR localization in the REE and rests individually, no significant difference was noted between their expressions, which is in contrast to the results obtained by Da silva Baumgart et al. [10]. In follicles, predominantly intense combined staining was noted, suggestive of high proliferative potential; however, in certain nests where induction phenomena was noted, a change to membrane staining was noted. All these finding indicate that EGFR expression is closely related to odontogenic tumor differentiation and squamous differentiation in particular. This was in correlation with the general notion that the squamous tumors have over-expression of EGFR [15,24]. Additionally, several studies have suggested that squamous

metaplasia could represent early changes in the development of odontogenic lesions [4,19,25]. These findings suggest that understanding EGFR stain location plays a vital role in assessing its proliferative potential, biological aggressiveness and treatment options.

Conclusion

The PFs showed consistent EGFR positivity confirming that odontogenic epithelium is an EGFR expressing tissue and EGFR mediated effects play a role in their biological activity. It was seen that the PFs showed predominantly intense combined and cytoplasmic staining patterns, suggestive of an inherent potential for proliferation. Finally, the areas of squamous metaplasia showed a consistent membrane EGFR expression, which highlights the role of EGFR in squamous differentiation which in turn may be related to early pathological changes in dental follicles. Thus, to conclude, EGFR has an immense role in understanding odontogenic pathologies opening vast treatment options to counter them. Further advanced studies must be carried out to confirm their exact role in the pathogenesis of odontogenic lesions.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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