Expression of p63 in tooth germ, dentigerous cyst and ameloblastoma

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Abstract

Context: To assess the role of p63, a p53 homolog, in the cytodifferentiation (odontogenesis) and oncogenesis of odontogenic epithelium.

Aim: The present study aimed to compare the expression pattern of p63 in the epithelium of tooth germ, dentigerous cyst (DC) and ameloblastoma (AB).

Materials and Methods: Tissue specimens of thirty tooth germs, thirty ABs and thirty DCs were examined by immunohistochemistry for the expression of p63.

Results: p63 labeling index (LI) was observed in descending order in epithelial cells of ABs, tooth germs and DCs. p63 LI was statistically nonsignificant among all the three groups. ABs revealed the highest p63 expression, but, surprisingly, tooth germs showed higher expression than DCs.

Conclusion: p63 plays a role in the cytodifferentiation and proliferation of odontogenic epithelial cells irrespective of the tissue (normal developing or lesional tissue). This implies that p63 cannot be used as a diagnostic marker. However, our results indicate p63 overexpression as a mark of increased proliferation. Thus, it can be stipulated that p63 can be used as a prognostic marker in odontogenic lesions with more aggressive and invasive phenotype. Our results also suggest the differential function of p63 in both developing and lesional odontogenic tissues, which, however, depends on p63 isoform predominantly being expressed. Therefore, identification of p63-predominant isoform in a particular lesion is more important than the presence or absence of p63. Consequently, we suggest the performance of polymerase chain reaction analysis along with immunohistochemical evaluation in further studies.

Keywords: Cytodifferentiation, labeling index, odontogenic lesion, oncogenesis

INTRODUCTION

Odontogenic lesions are remarkable among oral lesions because of their clinical and histological heterogeneity. This diversity is reflected as there is complex development of dental structures. Odontogenic lesions are derived from aberrations in odontogenesis or the cell remnants of odontogenic apparatus are common source of odontogenic lesions within the jawbones. Among odontogenic lesions, dentigerous cysts (DCs) are one
of the most common developmental odontogenic cysts, making up to 16.6% of all jaw cysts, and ameloblastomas (ABs) are the neoplasms that account for ~1% of all other oral tumors. ABs deserve special attention because of their particular complex biologic behavior, exhibiting great infiltrative potential and high recurrence. Several recent studies have detected genetic and cytogenetic alterations in these epithelial odontogenic lesions; however, the detailed mechanism of aberration in odontogenesis, oncogenesis, cytodifferentiation and tumor progression remains unknown. p53 gene is a well-recognized tumor suppressor gene that is frequently altered in cysts and tumors. p63 gene, a p53 homolog, has been identified at loci 3q27–29. p63 gene encodes multiple proteins that have a significant degree of sequence homology, particularly in the transactivation, DNA binding and oligomerization domains. Isoforms derived from two different promoters are named TA and Δ N isoforms. TA isoforms (TAp63) containing the transactivation domain are capable of transactivating p53 target genes and inducing growth arrest or cell death, whereas Δ N isoforms (Δ Np63) lacking the transactivation domain exert a dominant negative effect on their TA isoforms and wild-type p53 by blocking their transactivation. Although p63 might have a tumor suppressor function, genetic alterations, such as mutation and loss of heterozygosity, are less frequent than those associated with p53.

Previous immunohistochemical studies done in tooth germs and ABs using p53 antibody suggest that the p53 signaling cascade has an important role in the cytodifferentiation (odontogenesis) and oncogenesis of odontogenic epithelium. Several syndromes associated with p63 gene mutations have shown tooth abnormalities of both primary and permanent dentition as one of the features. Keeping two things in mind that p63 belongs to p53 tumor suppressor gene family and has association with syndromes showing tooth abnormalities, the present study was planned to know whether p63 is also having a similar role as p53 gene. In recent studies, the expression of p63 has been demonstrated in odontogenic cysts. In one study, the expression of p63 was examined in ABs. No immunohistochemical study has been performed in tooth germs, DCs and ABs together using p63 antibody. In the present study, the expression of p63 in tooth germs, DCs and ABs was examined by immunohistochemistry (IHC) to clarify its role in normal odontogenesis and in cases of aberration in odontogenesis, i.e., in odontogenic lesions.

### MATERIALS AND METHODS

A descriptive observational study was carried out from August 2009 for 2 years in the Department of Oral Pathology and Microbiology, Sharad Pawar Dental College and Hospital, Datta Meghe Institute of Medical Sciences (deemed to be university), Sawangi (M), Wardha, Maharashtra, India. The study protocol was approved by the Institutional Ethical Committee. The study included histopathologically diagnosed thirty cases of tooth germs, thirty cases of DCs and thirty cases of ABs. ABs constituted of histological subtypes of 10 cases of follicular ABs and 10 cases of plexiform ABs, five cases of acanthomatous ABs, three cases of basal cell ABs and two cases of desmoplastic ABs. IHC was performed on paraffin-embedded tissues fixed in 10% neutral-buffered formalin. The sections were cut serially to 5 µm of thickness for immunohistochemical staining. After immunostaining, p63 immunolabeling was assessed in all the tissue sections for immunonegativity, type of staining (whether weak or strong) and analyzing layers showing dense immunonegativity.

### Immunohistochemical method for the detection of p63 antigen

The tissue sections were deparaffinized. For IHC, Streptavidin-Biotin Detection System HRP-DAB (product code: RE7110K, Novo-castra kit) was employed as peroxidase detection system. Blocking of endogenous peroxidase activity was done by treating hydrated sections with 3% H2O2 in methanol for 30 min. Then, antigen retrieval was done by applying 0.01 M sodium citrate buffer (pH 6.0) over the sections. Then, the slides were heated in a microwave oven for 10 min and bench cooled for 20 min, and again the same cycle was repeated. To prevent nonspecific reactions, sections were incubated with 10% serum for 10 min. Prediluted p63 antibody (clone p63 BC4A4; product code: Sc-56188, Santa Cruz Biotechnology Inc.,) was incubated at room temperature in a humidifying chamber for 60 min and then at 4°C overnight. One section from each positive control was used as the negative control by incubating with nonimmune serum instead of primary antibody. Then, incubation with secondary biotinylated antibody and streptavidin-peroxidase reagent at room temperature in a humidifying chamber for 30 min was done. Freshly prepared substrate/chromogen solution of 3,3’-diaminobenzidine (mix 5 ml of concentrated 3,3’-diaminobenzidine in 50 ml of substrate buffer) was applied to visualize the antigen–antibody reaction. Finally, the sections were counterstained with Mayer’s hematoxylin. Sections were examined by conventional light microscopy (Leica microscope with image analyzer LEICA QWIN standard).
Assessment of immunohistochemically stained sections

The cells were scored p63 immunopositive only if nucleus stained brown, i.e., only when intranuclear DAB staining was observed. Cells that lacked nuclear staining or a clear nucleus were excluded. All the stained nuclei were scored positive regardless of the intensity of their staining. A minimum of 1000 cells were counted in each section. Tissue sections were scanned at ×100 magnification for most heavily immunolabeled p63-positive cells in the epithelium. The cell counts were made in ten randomly selected fields on captured image at ×400 magnification with conventional light microscope. The counting was done by two observers, and the mean was taken as a final count. The number of positively stained nuclei was counted and expressed as a percentage of the total number. The index of positivity, i.e., labeling index (LI), was obtained for p63 protein expression for all the groups as follows:

\[ \text{p63 LI} = \frac{\text{Number of IHC-positive cells for p63}}{100} \times \text{total number of cells observed} \]

Statistical analysis

Group mean for p63 LI was derived for each group [Table 1]. The data were analyzed statistically using SPSS software version 16.0 for Windows. Pearson's correlation test was applied. The level of statistical significance was at \( P < 0.05 \).

RESULTS

p63 labeling index

p63 antigen was expressed in 100% of tooth germs, 100% of DCs and 100% of ABs. In tooth germs, almost complete epithelium showed dense p63 immunolabeling [Figure 1a and b]. In DCs, strong and dense p63 immunolabeling was seen in the basal and parabasal layers, and the absence of staining was seen in most part of the superficial layer of epithelium [Figure 1c]. In ABs, p63 immunoreactivity was dense predominantly in the peripheral cells of the ameloblastic follicle among all of the histologic variants [Figure 1d-g]. Fewer central cells of ameloblastic follicle of all variants showed staining. Keratinizing cells showed markedly decreased reactivity for p63 in acanthomatous ABs [Figure 1h]. Basal cell ABs exhibited p63 reactivity in most neoplastic cells [Figure 1i].

Statistically, a nonsignificant difference of p63 LI was noted among ABs, DCs and tooth germs [Table 2].

Table 1: p63 labeling index in all groups: Descriptive statistics

| Groups         | Mean (SD) | p63LI |
|----------------|-----------|-------|
| p63 in AB      | 91.11 (4.92) |       |
| p63 in tooth germ | 90.96 (5.86) |       |
| p63 in DC      | 89.64 (4.55) |       |

The mean difference is significant at 0.05 level. SD: Standard deviation, DC: Dentigerous cyst, p63LI: p63 labeling index, AB: Ameloblastoma

Table 2: Pearson’s correlation coefficient

|                         | p63 in tooth germ | p63 in DC |
|-------------------------|-------------------|-----------|
| Pearson’s correlation   | −0.149            | −0.121    |
| \( P \)                 | 0.431             | 0.525     |
|                         | NS, \( P > 0.05 \) | NS, \( P > 0.05 \) |

DC: Dentigerous cyst, ABs: Ameloblastomas, NS: Not significant

Figure 1: Photomicrograph of immunostaining for p63 (a) Bud stage – p63 immunostaining (×400). (b) Advanced bell stage – p63 immunostaining (×40). (c) Dentigerous cyst – p63 immunostaining (×400). (d) Follicular ameloblastoma – p63 immunostaining (×100). (e) Follicular ameloblastoma – p63 immunostaining (×400). (f) Plexiform ameloblastoma with areas of follicular ameloblastoma – p63 immunostaining (×40). (g) Plexiform ameloblastoma – p63 immunostaining (×400). (h) Acanthomatous ameloblastoma – p63 immunostaining (×400). (i) Basal cell ameloblastoma – p63 immunostaining (×100)
p63 LI was observed in descending order in ABs, tooth germs, and DCs [Table 1]. However, the correlation of p63 LI was higher in AB than DC (P = 0.525) and tooth germs (P = 0.431). Surprisingly, the correlation of p63 LI was higher in tooth germs than DC (P = 0.795) [Table 2].

**DISCUSSION**

The discovery of p63, a p53 homolog, has fueled new insights and exposed enigmas in the understanding of the dramatic tumor suppressor gene family. p63 plays a major role in the maintenance of epithelial stem cells, as well as in their terminal differentiation rather than tumor suppression.\(^{18,19}\) p63 protein is found restricted to cells with high proliferative potential and is absent from cells undergoing terminal differentiation.\(^{22}\)

Recently, p63 has been proposed to be a specific marker for precursor/stem cells in many epithelial tissues and could be considered as a marker for assessing the proliferative activity of the cell.\(^{18}\) It was seen that, in the absence of p63, stem cells and their progenies die by apoptosis, and the crippled stem cells are unable to carry cell proliferation and self-renewal function.\(^{19}\) Mutations of p63 gene have recently been shown to cause several inherited human syndromes with abnormal limb development and/or ectodermal dysplasia, such as ectrodactyly, facial cleft syndrome, ankyloblepharon and clefting syndrome, acro-dermato-ungual-lacrimal-tooth syndrome and limb-mammary syndrome, which often accompany abnormal tooth development ranging from enamel dysplasia to a tooth loss.\(^{13,16,17}\) The authors of the present study report that tooth germ develops from both epithelial and mesenchymal components, and aberration in odontogenesis leads to the emergence of the odontogenic lesions. The authors further state that the extent of the penetrance of aberrancy in odontogenic apparatus gives rise to the prevailing heterogeneity in odontogenic lesions, i.e., odontogenic cyst or odontogenic tumor. The exact mechanism for aberrancy or how many factors are involved or what decides that whether a cyst will be formed or a tumor is still unknown. However, we hypothesize that mild aberrations to odontogenic apparatus lead to the formation of odontogenic cyst such as DC, whereas greater degree of aberration leads to the development of odontogenic tumors such as AB, on the basis of their clinical behavior. An extensive research in this context is needed to be taken up. In the present study, IHC for p63 was employed among tooth germs, DCs and ABs to evaluate its role in the differentiation, proliferation and oncogenesis of odontogenic epithelial cells. Our study also aimed to evaluate the proliferative potential and aggressive behavior of odontogenic lesions (DC and AB) when compared with tooth germs.

In the present study, tooth germs immunohistochemically showed p63 expression, in epithelial components, suggesting that p63 is involved in epithelial differentiation during tooth development. Almost the complete epithelium of tooth germ showed p63 expression, reflecting high proliferative activity occurring during its development [Figure 1a and b]. Our finding is in accordance with the concept that tooth germ is dynamic with regard to its higher epithelial turnover rate and subsequent differentiation process.

In DC, strong and dense p63 immunolabeling is seen in the basal and parabasal layers, and absence of staining is seen in most part of superficial layer; these results were in accordance with those of other studies [Figure 1c].\(^{18,20}\) p63 is critical for the stem cell compartment, so strong staining is seen in basal and parabasal layers. p63 is least or not present in the differentiated cells, so no expression could be seen in the superficial layer of DC which is central for terminal differentiation. This type of staining in DC could indicate the normal wild-type p63 protein expression, related to apoptosis and cell proliferation, thus maintaining the regular 2–3 cell layer thickness of epithelium. Thus, it can be concluded that the mechanism of expansion of DC could be some distinct factors other than the epithelial proliferation which is often the probable cause of expansion for other cysts. This is confirmed by the lack of increased proliferative activity in lining the epithelium of DC.

In ABs, densely p63-positive cells were mainly located in the peripheral cells of neoplastic islands, whereas fewer central cells showed immunoreactivity; these results were in accordance with those of a previous study.\(^{21}\) The results of the present study might indicate that p63 abnormalities (i.e., overexpression) can play a crucial role in early neoplastic transformation (oncogenesis) during the development of ABs, a similar concept was put forth in a previous study.\(^{21}\) Increased immunoreactivity of p63 only in the peripheral cells of neoplastic islands of ABs could suggest its locally invasive biological behavior.
along with aggressive infiltrating nature of ABs as well as different morphology between DCs and ABs facilitates more difficulty in surgical accessibility in ABs than DCs though both are odontogenic lesions. This could suggest the recurrence and worse prognosis for ABs than DCs. In the present and previous studies, the keratinizing cells of acanthomatous AB did not show p63 immunoreactivity, whereas p63 immunoreactivity was slightly higher in basal cell AB than that in other subtypes [Figure 1h and i].

In acanthomatus ABs, there was absence of staining in the keratinizing cells as they are the differentiated compartment. Basal cell ABs show higher expression of some apoptotic inhibitory factors, including Bcl-2, Bcl-x and survivin, than other subtypes, and this AB variant is considered to possess a high potential for cell survival.

As for ABs, the present study results were in accordance with those of other studies which have found nonsignificant differences of proliferative activity among the different histological subtypes of ABs. This may suggest that the histological subtype of Ameloblastoma does not have any significant effect on prognosis. Future studies should be carried out by using a large number of cases of each histological subtype of Ameloblastoma.

In the present study, a nonsignificant difference of p63 LI was observed between tooth germ and DC. Surprisingly, in tooth germ, p63 LI was found higher than that of DC but lower than that of AB. As tooth germ is a developing odontogenic tissue, it has higher proliferative activity which is indicated by an increased p63 LI. Although tooth germ is dynamic, it maintains regular thickness of fetal epithelium, suggesting balanced cellular proliferation and apoptosis. Lower p63 LI in DC indicates lack of increased proliferative activity in lining epithelium, and this is confirmed by the fact that DC maintains regular 2–3 cell layer thickness of epithelium.

However, the highest p63 LI in AB than tooth germ and DC indicates the greatest proliferative potential of ABs because of its neoplastic nature. Hence, it could be stipulated that, in ABs, apart from the proliferative compartment deciding the p63 LI, more importantly, p63 might have a role in the oncogenesis or malignant transformation of odontogenic epithelium, thus increasing the p63 immunoreactivity.

While p53 gene is mutated in more than 50% of human cancers, mutations of its homolog p63 gene are infrequent. Thus, it could be stipulated that not the mutated form but one of the isoforms of wild-type p63 may play oncogenic role. Recently, Δ Np63 amplification and/or overexpression has been identified in human malignancies, including nasopharyngeal, bladder, prostate, skin and esophageal carcinomas. These features support the notion that Δ Np63 plays an oncogenic role in human tumors. Thus, it could be hypothesized that Δ Np63 isoform might play a role in the oncogenesis of odontogenic epithelium, which was in accordance with the previous results.

To confirm this hypothesis, extensive study with larger sample size and all kinds of odontogenic lesions must be taken into account for IHC along with the other advanced diagnostic modalities, for example, polymerase chain reaction (PCR).

**CONCLUSION**

The expression of p63 in tooth germs, DCs and ABs suggests that this p53 homolog plays a role in the differentiation and proliferation of odontogenic epithelial cells. Hence, it could be stipulated that p63 can be used as a prognostic marker in odontogenic lesions. In addition, variations of expressed p63 isoforms suggest that p63 might differentially function in both developing and neoplastic odontogenic tissues, which further needs to be assessed by PCR technique.

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**Conflicts of interest**

There are no conflicts of interest.

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