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“Cell Cycle Aberration in Ameloblastoma: As Evidenced by an Immunohistochemical Expression of p53 and Survivin”

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Abstract- Evasion from apoptosis by aberrations of apoptotic regulatory factors has been found to cause accumulation of neoplastic cells in various tumours. Survivin, an inhibitor of apoptosis protein localizes to the nucleus as well as in the cytoplasm depending on its function such as cell division and inhibiting apoptosis. The regulation of Survivin seems to be linked to p53, a tumour suppressor gene. Though there are studies showing the role of p53 and Survivin in oral carcinogenesis, but the field of odontogenic tumours yet needs to be explored. The aim of this study was to correlate the expression of p53 and Survivin in normal tissues (tooth germ) and Ameloblastoma as well as to assess differential localization of Survivin. Qualitative and quantitative assessment of immunexpression of p53 and Survivin (nuclear and cytoplasmic) was evaluated in a total of 35 cases which included 10 tooth germs and 25 Ameloblastoma. The expression levels of p53 and nuclear Survivin were significantly higher in Ameloblastoma quantitatively but there was no significant correlation between p53 and cytoplasmic expression of Survivin.

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Apoptosis and Cell Cycle Aberration in Ameloblastoma – Evidenced by Immunohistochemical Study

Dr. Zulfin Shaikh^α & Dr. Niranjana K C^ο

Abstract- Evasion from apoptosis by aberrations of apoptotic regulatory factors has been found to cause accumulation of neoplastic cells in various tumours. Survivin, an inhibitor of apoptosis protein localizes to the nucleus as well as in the cytoplasm depending on its function such as cell division and inhibiting apoptosis. The regulation of Survivin seems to be linked to p53, a tumour suppressor gene. Though there are studies showing the role of p53 and Survivin in oral carcinogenesis, but the field of odontogenic tumours yet needs to be explored. The aim of this study was to correlate the expression of p53 and Survivin in normal tissues (tooth germ) and Ameloblastoma as well as to assess differential localization of Survivin. Qualitative and quantitative assessment of immunexpression of p53 and Survivin (nuclear and cytoplasmic) was evaluated in a total of 35 cases which included 10 tooth germs and 25 Ameloblastoma. The expression levels of p53 and nuclear Survivin were significantly higher in Ameloblastoma quantitatively but there was no significant correlation between p53 and cytoplasmic expression of Survivin. There was up-regulation of both p53 and Survivin in Ameloblastoma. This study highlights the nuclear and cytoplasmic expression of Survivin and p53 in tumorigenesis of Ameloblastoma.

Keywords: apoptosis, survivin, p53, ameloblastoma, immunohistochemical expression, tumorigenesis, aberrations, tumour suppressor gene, inhibitor of apoptosis protein, apoptotic regulatory factors.

1. INTRODUCTION

Odontogenic tumours comprise a complex group of lesions exhibiting considerable variations in clinical and histological behavior [1]. A series of genetic and molecular alterations appear to promote the development and progression of these tumours [2]. Ameloblastoma is an epithelial odontogenic tumour characterized by a benign but locally invasive behavior, with a high risk of recurrence. Ameloblastoma shows considerable variation with different types such as

solid/multicystic, unicystic, peripheral and desmoplastic [3].

Regulation of the cell cycle and control of apoptosis are thought to be intimately linked processes in maintaining homeostasis and developmental morphogenesis [4]. *TP53* is one of the most frequently altered tumour suppressor genes and its gene products play an important role in response to genomic damage by inducing cell cycle arrest and apoptosis [2]. *TP53* encompasses 16-20kb of DNA on human chromosome 17p13.1 [5].

Apoptosis, also known as programmed cell death or physiological cell death, has diverse roles in development and normal homeostasis as well as in a variety of pathological conditions [6]. Survivin, a member of inhibitor of apoptosis protein family (IAP), has a molecular weight of 16.5kDa and the gene is located on chromosome 17q25 [7]. It is a unique bifunctional protein which suppresses apoptosis by inhibiting caspase-3 and caspase-9 and regulates G2/M phase of the cell cycle by associating with mitotic spindle microtubules. It is highly expressed in embryonic tissues and neoplasm's but is absent in terminally differentiated cells [8]. Several studies have proposed that the sub-cellular distribution of Survivin is regulated by active import into the nucleus and CRM1-mediated export to the cytoplasm [9-11]. Recent studies have suggested that the nuclear pool of Survivin is involved in promoting cell proliferation, whereas the cytoplasmic pool of Survivin controls cell survival [9, 10, 12,13].

Mutation of p53 in tumours causes over expression of Survivin which rescues cells from p53 induced apoptosis [14]. Survivin is highly expressed in most human tumours of the oral cavity, lung, colon, breast, liver, gastrointestinal and prostate [15-21]. The expression of Survivin in odontogenic epithelial cells has already been described [22-24], but to date, no study has investigated the relationship between nuclear and cytoplasmic expression of Survivin in odontogenic tumours. The role of p53 in various tumours has been extensively studied but there are few reports about its association with anti-apoptotic proteins in odontogenic tumours. Therefore, the present study was performed to

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determine the role of p53 and Survivin, in addition to assess nuclear and cytoplasmic localization of Survivin in Ameloblastoma to gain a better insight into its tumorigenesis.

II. MATERIALS AND METHODS

Twenty five formalin fixed paraffin embedded tissue blocks with histological diagnosis of Ameloblastoma were retrieved from the archives of the Department of Oral and Maxillofacial Pathology, SDM College of Dental Sciences and Hospital, Dharwad. The study sample being an odontogenic tumour, the control tissue included ten tooth germs taken from the postmortem fetal oral tissues fixed in 10% formalin. All the tooth germs taken were between 20th to 25th weeks of gestation.

The study was approved by the institutional ethics committee. Immunostaining was performed using the super sensitive polymer – Horseradish Peroxidase (HRP) detection system, a biotin free detection system supplied by Biogenex life sciences limited [California, USA]. The paraffin-embedded tissues were cut into four-micrometer thick sections, mounted on amino-propyltriethoxysilane (APES) coated slides, deparaffinized in xylene, rehydrated in alcohol and then treated with 3% hydrogen peroxide for 10 minutes.

The tissue sections were then incubated with primary antibodies against p53 [Mouse monoclonal, Clone DO7, IgG2b immunoglobulin; Biogenex Ltd, USA; diluted at 1:150] and Survivin [Rabbit monoclonal, Clone EP28880Y, IgG immunoglobulin; Biogenex Ltd, USA; diluted at 1:40] as per the instructions given by the manufacturer. Next, the tissue sections were incubated with the secondary antibody and immunoreactivity was visualized with 3, 3'-diaminobenzidine solution. Finally, the sections were counterstained with Harri's hematoxylin.

Two independent observers scored all samples in a blinded manner and no interobserver variability was observed. An immunoreactivity scoring system was applied as follows [25]: (i) the staining intensity was graded on a four point scale such as '0' for no staining,

'1' for mild staining, '2' for moderate staining and '3' for intense staining, and (ii) the number of positively stained cells with brown colour were counted among 400 tumour cells using an oculometer eye-piece grid at the objective magnification x40. The results were multiplied by 100 and the score was expressed as the percentage of positive cells. Nuclear (A) and cytoplasmic (B) expression of Survivin was assessed separately. The results were subjected to statistical analysis. Mann Whitney U test was performed to determine the significance of p53 and Survivin expression between tooth germ and Ameloblastoma. Wilcoxon Signed Ranks test was used to assess the significance between p53 and Survivin expression in each group. Pearson's correlation test was done to determine the correlation between p53 and Survivin expression in each group. A p-value of <0.05 was considered as statistically significant.

III. RESULTS

The study sample of 25 Ameloblastoma included 12 follicular, 4 plexiform and 9 cases of unicystic subtypes. Out of ten tooth germs five showed hard tissue formation.

a) p53 expression in tooth germ and Ameloblastoma

p53 expression was localized to the nucleus [Figure 1] with predominant intense staining compared to that of tooth germ [Table 1]. Out of ten tooth germs, p53 expression was seen in six tooth germs and was more evident in inner enamel epithelial cells than in stellate reticulum & outer enamel epithelial cells [Figure 1a]. In Ameloblastoma, p53 was expressed in all the tissues. In follicular and plexiform subtypes, p53 expression was seen in peripheral ameloblast-like and few central stellate reticulum-like cells [Figure 1b & 1c] and in unicystic variant, expression was seen in basal and suprabasal cells [Figure 1d]. p53 expression was statistically significant between tooth germ and Ameloblastoma in the number of positive cells but no significance was seen in relation to the staining intensity [Table 2].

Table 1 : Qualitative and quantitative analysis of p53 expression in tooth germ and Ameloblastoma

Group	Total no. of cases	Negative staining	Mild staining	Moderate staining	Intense staining	Average % of positive cells
Tooth germ	10	1	1	6	2	24.89%
Ameloblastoma	25	0	1	9	15	75.97%
Follicular	12	0	1	5	6	79.37%
Plexiform	4	0	0	0	4	81.62%
Unicystic	9	0	0	4	5	68.91%

Table 2 : Pair wise comparison of p53 expression in tooth germ and Ameloblastoma by Mann Whitney U test

	N	Sum of Ranks	Mean Rank	U-value	Z-value	p-value
Qualitative analysis						
Tooth germ	10	123.50	12.35	68.50	-2.296	0.038
Ameloblastoma	25	506.50	20.26			
Quantitative analysis						
Tooth germ	10	74.00	7.40	19.00	-3.874	0.000*
Ameloblastoma	25	556.00	22.24			

*Statistically significant

b) Survivin expression in tooth germ and Ameloblastoma

Survivin expression was predominantly localized to the cytoplasm [Figure 2]. The staining intensity was predominantly moderate in Ameloblastoma compared to tooth germ [Table 3]. Out of ten tooth germs, Survivin was expressed in five tooth germs with cytoplasmic expression in inner enamel epithelial cells, stellate reticulum & outer enamel epithelial cells with focal nuclear expression in inner enamel epithelial cells [Figure 2a]. In follicular and plexiform ameloblastoma,

Survivin showed cytoplasmic expression both in peripheral ameloblast-like and central stellate reticulum-like cells with focal nuclear expression in peripheral cells [Figure 2b & 2c]. Among unicystic ameloblastoma, cytoplasmic expression of Survivin was seen in basal and suprabasal cells with focal nuclear staining in basal cells [Figure 2d]. Similar to p53, Survivin expression was statistically significant between tooth germ and Ameloblastoma but no significance was seen with respect to staining intensity [Table 4].

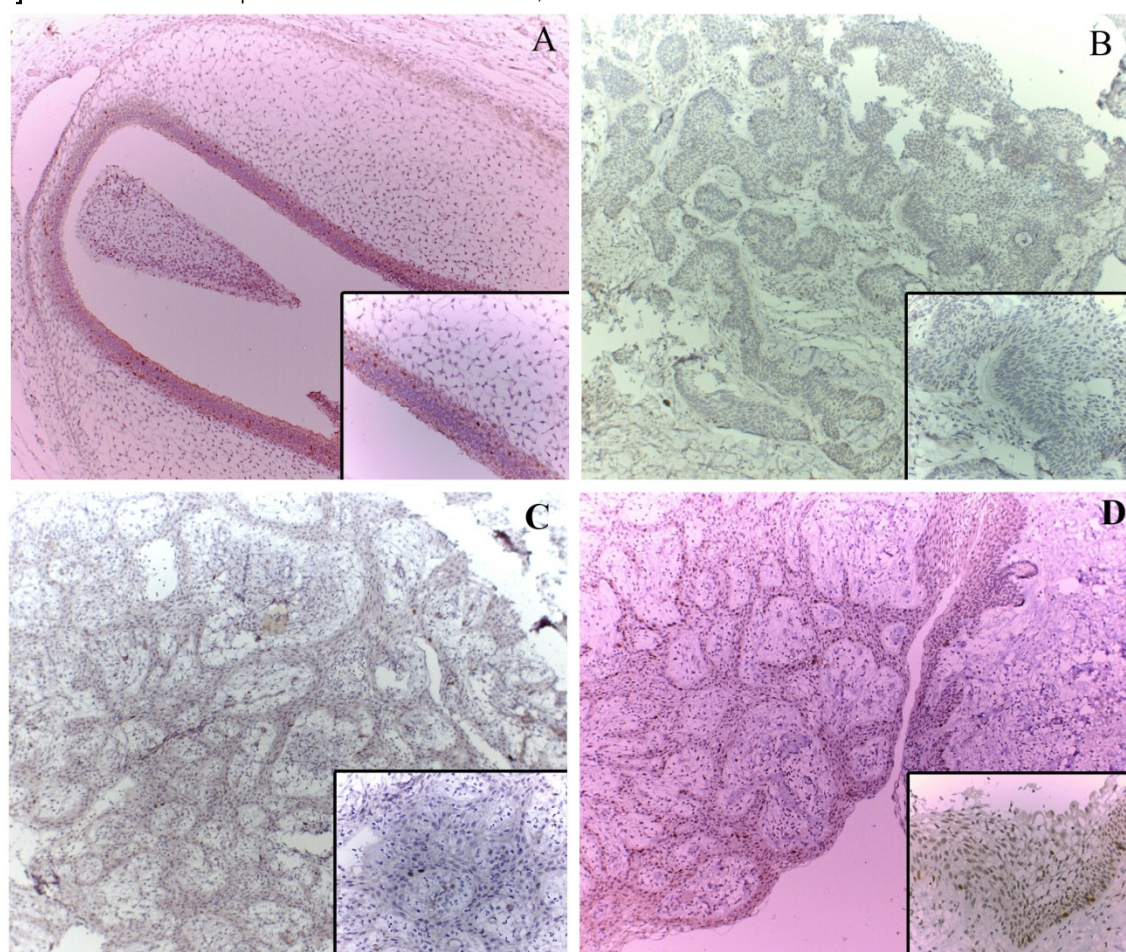


Figure 2 : Expression of Survivin in tooth germ and Ameloblastoma

- a) Intense Survivin expression in tooth germ (Original magnification x10). Survivin cytoplasmic expression in inner enamel epithelial cells, stellate reticulum and outer enamel epithelial cells with focal nuclear expression in inner enamel epithelial cells (Inset-Original magnification x40) (Immunostaining: DAB chromogen, Survivin monoclonal antibody).

- b) Mild Survivin expression in follicular ameloblastoma (Original magnification x10). Survivin cytoplasmic expression in ameloblast-like cells & stellate reticulum-like cells with focal nuclear expression in peripheral cells (Inset-Original magnification x40) (Immunostaining: DAB chromogen, Survivin monoclonal antibody).
- c) Moderate Survivin expression in plexiform ameloblastoma (Original magnification x10). Survivin cytoplasmic expression in peripheral and central neoplastic cells with focal nuclear expression in the peripheral cells (Inset-Original magnification x40) (Immunostaining: DAB chromogen, Survivin monoclonal antibody).
- d) Intense Survivin expression in unicystic ameloblastoma showing intraluminal proliferation in plexiform pattern (Original magnification x10). Survivin nuclear and cytoplasmic expression in basal and suprabasal cells (Inset-Original magnification x40) (Immunostaining: DAB chromogen, Survivin monoclonal antibody).

Table 3 : Qualitative and quantitative analysis of Survivin expression in tooth germ and Ameloblastoma

Group	Total no. of cases	Negative staining	Mild staining	Moderate staining	Intense staining	Average % of positive cells
Tooth germ	10	0	4	4	2	29.10%
Ameloblastoma	25	0	6	13	6	78.25%
Follicular	12	0	5	7	0	78.06%
Plexiform	4	0	0	4	0	79.12%
Unicystic	9	0	1	2	6	56.66%

Table 4 : Pair wise comparison of Survivin expression in Tooth germ and Ameloblastoma by Mann Whitney U test

	N	Sum of Ranks	Mean Rank	U-value	Z-value	p-value
Qualitative analysis						
Tooth germ	10	161.00	16.10	106.00	-0.752	0.506
Ameloblastoma	25	469.00	18.76			
Quantitative analysis						
Tooth germ	10	73.00	7.30	18.00	-3.913	0.000*
Ameloblastoma	25	557.00	22.28			

*Statistically significant

c) p53 and Survivin expression in tooth germ and Ameloblastoma

Four tooth germs showed both p53 and Survivin expression, three showed negative staining for both p53 and Survivin, two tooth germs had only p53 expression and one tooth germ showed only Survivin expression. A positive correlation was found between p53 and Survivin expression in Ameloblastoma [$r=0.251$, $p=0.226$]. We found that p53 and Survivin expression was statistically

significant in Ameloblastoma with respect to staining intensity [Table 5]. Quantitatively, p53 and nuclear Survivin was statistically significant in Ameloblastoma [$p=0.000$] but no significance was seen in p53 and cytoplasmic expression of Survivin in Ameloblastoma [$p=0.330$]. Due to focal expression of Survivin in the nucleus of normal and neoplastic cells, a statistical correlation could not be obtained between cytoplasmic and nuclear Survivin.

Table 5 : Analysis of p53 and Survivin expression in tooth germ and Ameloblastoma by Wilcoxon Signed Ranks test

	N	Sum of Ranks	Mean Rank	Z-value	p-value
Qualitative analysis					
Tooth germ	10	28.00	8.00	-0.378	0.705
Ameloblastoma	25	171.00	18.2	-2.854	0.004*
Quantitative analysis					
Tooth germ	10	28.00	8.60	-0.676	0.499
Ameloblastoma	25	276.00	24.25	-0.821	0.411

*Statistically significant

IV. DISCUSSION

Various concepts have been proposed which explain the pathogenesis of odontogenic tumours, but what causes odontogenic cells to transform into an odontogenic tumour is yet to be explored [23].

p53, a tumour suppressor gene, acts as a 'molecular policeman' by monitoring the integrity of the genome. If DNA is damaged, p53 accumulates and switches off cell replication to allow extra time for repair mechanisms to act. If the repair fails, p53 triggers cell suicide by apoptosis [26]. Survivin, an inhibitor of

apoptotic protein (IAP) inhibits caspase activation. This has been shown by disruption of Survivin induction pathways leading to increase in apoptosis and decrease in tumour growth. Besides its role as an IAP, Survivin acts as a subunit of the chromosomal passenger complex (CPC) and as a regulator of microtubule dynamics [8, 27]. The CPC also composed of the Aurora-B kinase, Borealin and INCENP corrects attachment errors between chromosomes and the mitotic spindle, regulates the quality-control checkpoint and ensures the correct completion of cytokinesis [10]. The typical chromosomal passenger localization pattern of Survivin can be observed not only in normal but also in tumour cells [27, 28]. Li *et al.* have suggested that nuclear Survivin is involved in the promotion of cell proliferation, whereas cytoplasmic Survivin may help control cell survival [29]. Thus, Survivin exists in two subcellular pools (cytoplasmic and nuclear) in response to its function in the regulation of both cell survival and cell division.

Experiments have revealed that transient expression of wild-type p53 results in marked repression of Survivin at both the mRNA and protein levels [14]. Up-regulation of Survivin and mutation of p53 occurring concomitantly in many human tumours implicates that these two events are related [30, 33]. p53 expression in tooth germs in contrast to previous studies [34,35], can be explained by the fact that p53 activation is not limited to DNA damage; it is also activated in response to myriad stresses like oxidative stress, osmotic shock, heat shock, hypoxia, ribonucleotide depletion and deregulated oncogene expression leading to accumulation of p53 in stressed cells [36]. Absence of p53 staining in other tooth germs correlates with the literature that p53 does not normally accumulate to amounts detectable by immunohistochemical staining because of its short half-life (6-20 minutes) [34, 35, 37].

EL-SISSY NA *et al.* reported faint p53 staining and attributed this to slow tumour growth, expansion and correlated with the benign nature of Ameloblastoma resulting from relatively quiescent tumour cells [34]. Thus, the presence of predominant intense p53 staining in the present study may be suggestive of rapid growth, expansion and locally aggressive behavior of Ameloblastoma [38].

Survivin is chiefly expressed in the undifferentiated proliferating cells like stem cells, basal cells of the oral epithelium, embryonic and fetal tissues but becomes undetectable in most terminally differentiated cells [39]. Absence of Survivin expression in five tooth germs may be due to differentiation of inner enamel epithelial cells into ameloblast because hard tissue formation was evident in these tooth germs.

KUMAMOTO *et al.* suggested that cells neighboring the basement membrane are proliferative cells and those away from the basement membrane are apoptotic [23]. Studies in the literature have described cytoplasmic expression of Survivin to be associated with

inhibition of cell death [9-13]. Thus, in the present study predominant cytoplasmic expression of Survivin in the peripheral ameloblast-like cells and stellate reticulum-like cells with focal nuclear expression is suggestive of its role in evading apoptosis rather than in the proliferation of neoplastic cells in Ameloblastoma.

Co-expression of p53 and Survivin in the present study implicates that these two proteins may have a combined role in inhibiting apoptosis, where mutant p53 led to up-regulation of Survivin. But the expression of only p53 or Survivin in few tissues also cannot be ignored. Though possible explanation was obtained through literature review, the significance of clinical and other molecular factors needs to be explored. The present study was done to throw light on nuclear and cytoplasmic expression of Survivin in relation to p53 in Ameloblastoma which would add-up to the existing understanding about its biological behavior and explore new and better therapeutic results. Further molecular studies with large sample size are required to confirm these findings.

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FIGURE LEGENDS

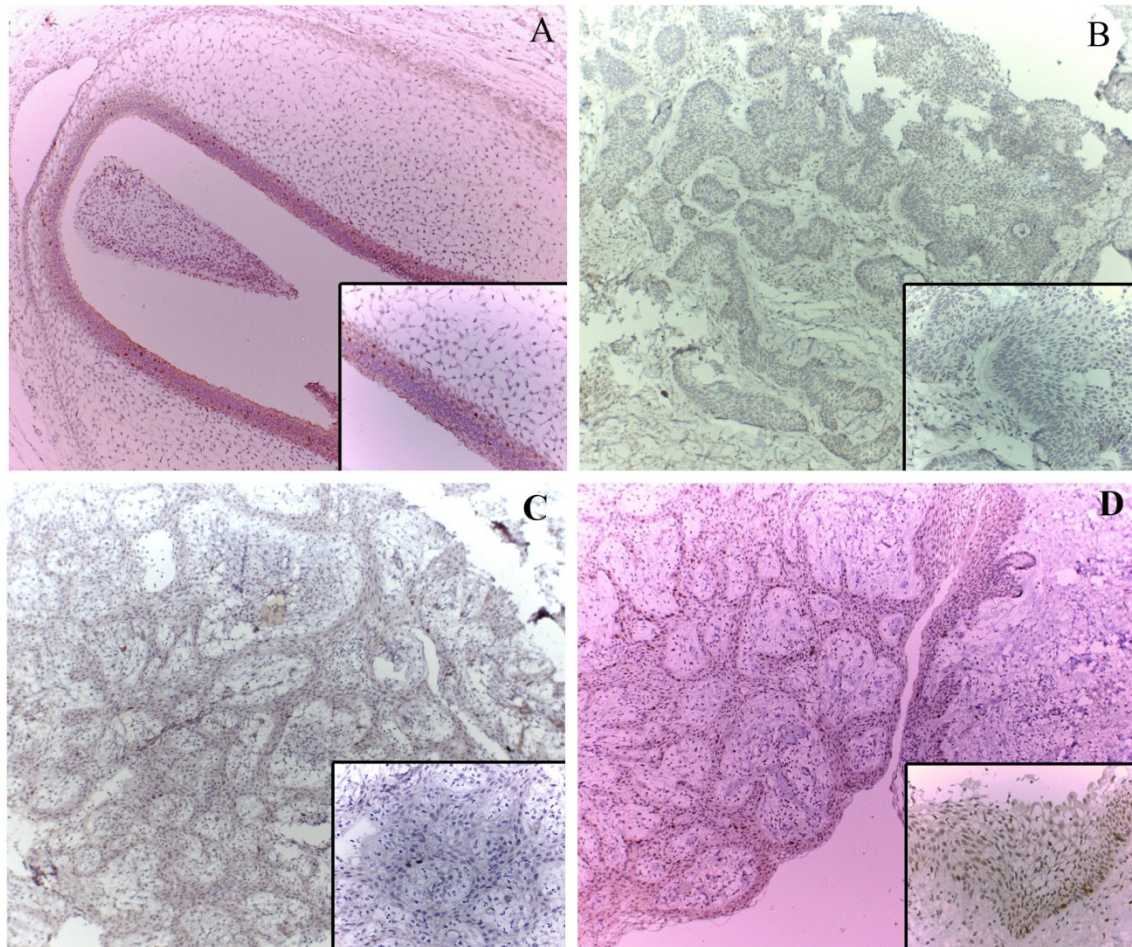


Figure 1 : Expression of p53 in tooth germ and Ameloblastoma

- Intense p53 expression in tooth germ (Original magnification x10). p 53 nuclear expression in inner enamel epithelial cells (Inset-Original magnification x40) (Immunostaining: DAB chromogen, p53 monoclonal antibody).
- Intense p53 expression in follicular ameloblastoma (Original magnificationx10). p 53 nuclear expression in ameloblast-like cells and stellate reticulum-like cells (Inset-Original magnificationx40) (Immunostaining: DAB chromogen, p53 monoclonal antibody).
- Intense p53 expression in plexiform ameloblastoma (Original magnificationx10). P 53 nuclear expression in peripheral and central neoplastic cells (Inset-Original magnificationx40) (Immunostaining: DAB chromogen, p53 monoclonal antibody).
- Intense p53 expression in unicystic ameloblastoma showing intraluminal proliferation in plexiform pattern (Original magnification x10). P 53 nuclear expression in basal and suprabasal cells (Inset-Original magnificationx40) (Immunostaining: DAB chromogen, p53 monoclonal antibody).