Immunoexpression of stem cell markers SOX-2, NANOG AND OCT4 in ameloblastoma

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Abstract

20 **Background.** Ameloblastoma (AME) is characterized by a locally invasive growth pattern. In an
21 attempt to justify the aggressiveness of neoplasms, the investigation of the role of stem cells has
22 gained prominence. The SOX-2, NANOG and OCT4 proteins are important stem cell biomarkers.
23 **Methodology.** To verify the expression of these proteins in tissue samples of AME, dentigerous
24 cyst (DC) and dental follicle (DF), immunohistochemistry was performed and indirect
25 immunofluorescence were performed on the human AME (AME-hTERT) cell line.
26 **Results.** Revealed expression of SOX-2, NANOG and OCT4 in the tissue samples and AME-
27 hTERT lineage. Greater immunostaining of the studied proteins was observed in AME compared
28 to DC and DF (p < 0.001).
29 **Conclusions.** The presence of biomarkers indicates a probable role of stem cells in the genesis and
30 progression of AME.

Introduction

32 Ameloblastoma (AME) is an odontogenic tumour of epithelial origin (Effiom et al., 2018)
33 and, although classified as a benign tumour, it is characterized by a locally invasive growth pattern,
34 which can reach large proportions and promote facial deformities in patients (Rioux-Forker et al.,
35 2019).

36 Surgical removal is the treatment of choice, but when conservative techniques are applied,
37 small islands of tumour are not completely removed, leading to local recurrence in 60–80% of
38 cases of solid AME (Gomes et al., 2010).

39 In the search for cellular mechanisms that justify the local aggressiveness of this benign
40 lesion, the investigation of the role of stem cells (SCs) and cancer stem cells (CSCs) has gained
41 prominence in tumour biology, with research identifying their participation in growth,
42 angiogenesis, progression, tumour recurrence and self-renewal potential (Reya et al., 2001; Silva
43 et al., 2016; Pardal et al., 2003).

44 The SOX-2, NANOG and octamer-binding protein 4 (OCT4) are important biomarkers in
45 the analysis of the presence of SCs. They act as critical regulators of embryonic self-renewal and
46 pluripotency capable of mediating tumour proliferation and differentiation (Chambers et al., 2003;
47 Luo et al., 2013; Ren et al., 2016).

48   Furthermore, the relation of these proteins seems to play an oncogenic role, considering

49 studies that point to the presence of these factors in different types of cancers, such as lung

50 adenocarcinoma, breast, colorectal and gastric cancer (Luo et al., 2013; Ren et al., 2016).

51   The SOX-2 protein (HMG-box gene 2 related to SRY) acts as an important transcription

52 factor in maintaining the self-renewal capacity of SCs. Previous studies have shown it to be

53 associated with a pro-oncogenic function (Wilbertz et al., 2011), in AME (Juuri et al., 2013) and

54 ameloblastic carcinoma (Khan et al., 2018; Lei et al., 2014), with a differentiated expression

55 between the last two.

56   NANOG (homeodomain protein) is another transcription factor that plays a central role in

57 maintaining cell pluripotency during embryonic development, in addition to being associated with

58 cell proliferation and renewal (Fan et al., 2017). The high expression of NANOG has also been

59 identified in patients with some types of cancer (Luo et al., 2013; Yu et al., 2020), but it has not

60 yet been studied in AME.

61   OCT4 also acts in pluripotency and cell self-renewal (Schulenburg et al., 2006), and has

62 been associated with cell proliferation and tumour progression (Hu et al., 2020).

63   The expression of OCT4 in AME (Monroy et al., 2018) was related to cell development

64 and differentiation, and an initial study on the expression of this protein in lesions of odontogenic

65 origin showed divergence in expression between AME and ameloblastic carcinoma (Khan et al.,

66 2018).

67   SOX-2 and OCT4 are considered essential regulators for the maintenance and early

68 development of SCs (Chambers et al., 2003). Although they have independent roles in different

69 cell types, they present a synergistic interaction that leads to the transcription of target genes

70 (Avilion et al., 2003), with NANOG as one of the targets of this interaction (Rodda et al., 2005).

71   When together, SOX-2, NANOG and OCT4 bind to the promoters of their own genes,

72 forming interconnected self-regulatory loops (Boyer et al., 2005). It is believed that this self-

73 regulation network can provide advantages for SCs, such as reduced response time to

74 environmental stimuli and greater stability of gene expression, thus maintaining cell fate (Rodda

75 et al., 2005). These characteristics are important for cell survival, stability and tumour progression.

76   It has been reported that odontogenic neoplasms, such as AME, originate from remaining

77 SCs from the dental lamina (Harada et al., 2002). However, the true contribution of SCs to the

78 molecular mechanism involved in the pathogenesis of AME still needs clarification (Silva et al.,

79   2016; Jurri et al., 2013). This is the first work to study these three proteins (SOX-2, NANOG and

80   OCT4) in AME. In this sense, identifying the proteins that are related to cellular self-renewal and

81   pluripotency may justify the biological behaviour of AME and becomes extremely important to

82   the development of better treatments and prognosis.

83   **Materials & Methods**

84   **Sample**

85   This was an experimental laboratory study. For the *in vivo* study, 23 AME samples were

86   used, retrieved from the archives of the Department of Oral Pathology, Faculty of Dentistry,

87   University Centre of the State of Pará (CESUPA). DC, as well as AME, is derived from the

88   odontogenic epithelium, but has a less aggressive behaviour. Thus, ten samples of DC were used

89   as a control, added to 10 samples of DF (tissue without neoplastic alterations of odontogenic

90   origin), obtained from the Laboratory of Pathological Anatomy and Immunohistochemistry,

91   Faculty of Dentistry, Federal University of Pará (UFPA). The clinical data of the AME samples

92   were acquired through medical records with reports present in the files, collected manually, and

93   histologically classified by two oral pathologists. For the *in vitro* study, the cell line derived from

94   human AME, called AME-hTERT, established at the Cell Culture Laboratory of the Faculty of

95   Dentistry, Federal University of Pará (UFPA) (Cruz et al., 2021), was used. This study was

96   registered and approved by the Human Research Ethics Committee of the Health Sciences Institute

97   of the Federal University of Pará - CEP/ICS/UFPA (CAAE: 30647720.6.0000.0018).

98   **Immunohistochemistry**

99   Immunohistochemical analysis was performed according to Mitre et al. 2021, where AME,

100   DC and DF tissues were incubated with Anti-SOX-2 (1:50 Sigma®), Anti-Nanog (1:150

101   Millipore, Burlington, MA, USA) and Anti-Oct4 (1:25 Millipore ®) antibodies for 1 h. As a

102   positive control, samples of oral squamous cell carcinoma were used and as a negative control, the

103   primary antibody was replaced by BSA and fetal bovine serum in TRIS buffer.

104   **Immunohistochemical evaluation**

105   Five brightfield images were randomly acquired from each AME, DC and DF of regions

106   with intact epithelium, and in the case of AME that was representative of the lesion, a sample on

107 an AxioScope microscope (Carl Zeiss, Oberkochen, DEU) equipped with an AxioCam HRC
108 colour CCD camera (Carl Zeiss®) was obtained. Images were taken at 400x magnifications and
109 saved in TIFF format. The areas stained with diaminobenzidine were analysed using the
110 "Immunohistochemistry (IHC) Image Analysis Toolbox" of the ImageJ software (National
111 Institute of Mental Health (NIMH), National Institute of Health (NIH), Bethesda, MD, USA).
112 Semi-automatic image analysis was then performed by detecting DAB staining. The mean
113 percentages of marking obtained in the tumour parenchyma, of five fields per sample, were
114 analysed using the GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA).

115 **Cell cultivation**
116   The ameloblastoma cell line was cultured and maintained in culture bottles according to
117 Valladares et al, 2022.

118 **Indirect immunofluorescence**
119   The AME-hTERT strain was seeded on glass coverslips in 24-well plates and submitted to
120 an indirect immunofluorescence protocol to detect the expression of SOX-2, NANOG and OCT4.
121 This process was initiated by fixing the cells in 2% paraformaldehyde for 10 minutes, followed by
122 washing with PBS, permeabilization of the membrane with 0.5% Triton X-100 (Sigma®) solution
123 for 5 min, a second washing with PBS, and incubation in PBS/BSA (BSA, Sigma®) at 1% for 30
124 min. Subsequently, primary antibodies diluted in PBS/BSA at 1% were incubated for a maximum
125 of 18 h in a humid chamber at 4 ºC. The primary antibodies used were: Anti-SOX-2 (1:50
126 Sigma®), Anti-Nanog (1:50 Millipore, Burlington, MA, USA), and Anti-Oct4 (1:50 Millipore®).
127 To detect the primary antibody, incubation in a solution containing the secondary antibody
128 conjugated to AlexaFluor 488 (Invitrogen, Carlsbad, CA, USA) for 1 h in a dark, humid chamber
129 at room temperature was performed. For better visualisation of the cytoskeleton, Alexa Fluor 568
130 Phalloidin (Life Technologies®, Carlsbad, CA, USA) was used. The nuclei were labelled with
131 DAPI coupled to ProLong® Gold antifade reagent (Invitrogen®). After mounting, the coverslips
132 were analysed in a fluorescence microscope (AxioScope.A1, Zeiss®), equipped with a digital
133 camera (AxioCamMRc, Zeiss®), with which images were obtained from the slides to record
134 immunoexpression. All images were acquired at the same magnification (40x lens). The slides
135 were examined under a fluorescence microscope (Axio Scope.A1, Zeiss. Jena, TH, DEU) equipped

136   with a digital camera (AxioCam MRc - Zeiss®), in which five images were randomly obtained

137   from each slide for the acquisition of 50 cells per group. All images were acquired at the same

138   magnification (40x objective). The immunostaining evaluation was performed using the ImageJ

139   software.

140   **Statistical analysis**

141   Clinicohistological data were then tabulated and analysed using descriptive statistics. To

142   analyse the expression of the three proteins (SOX-2, NANOG and OCT4), comparing the AME

143   samples with those of DC and DF, the statistical tests analysis of variance (ANOVA; samples with

144   parametric distribution) and Kruskal-Wallis were used, followed by Dunn's test multiple

145   comparisons (samples with non-parametric distribution). To verify correlation, the Pearson

146   correlation test (samples with parametric distribution) was used. A significance level of $\alpha = 0.05$

147   was adopted.

148   # Results

149   **Clinicohistological data of patients with AME**

150   In the sample studied, the average age was 39 years, with 61% of individuals below this

151   average and the remaining 39% above. The male gender was observed in 56% of the cases and

152   the female in 44%. The location of greatest involvement was the mandible, totalling 91% of the

153   cases. Regarding the histological type, 10 cases were of the follicular type, eight of plexiform,

154   three acanthomatous and two of granular cells (Table 1).

155   **AME presents a begreater expression of stem cell markers when compared to dentigerous**

156   **cyst and dental follicle**

157   AME samples showed higher expressions of SOX-2, NANOG and OCT4 proteins when

158   compared to dentigerous cyst (DC) and dental follicle (DF) ($p < 0.001$). There was no statistical

159   difference between DC and DF ($p > 0.05$) (Figure 1).

160   **Variations in the immunomarking of SOX-2, NANOG and OCT4 in the neoplastic cell**

161   **compartment of AME**

162   Immunohistochemical staining for SOX-2, NANOG and OCT4 was mainly located in the
163   cords and islands of the odontogenic tumour epithelium. SOX-2 labelling was present
164   predominantly in the nucleus, while NANOG and OCT4 were found in the cell nucleus and
165   diffusely in the cytoplasm of tumour parenchymal cells. Subtle nuclear markings of SOX-2, OCT4
166   and NANOG were observed in the DC cystic epithelium, and the same occurred in the DF
167   epithelial islands. NANOG was diffusely labelled in the connective tissue of the DF (Figure 1).

168   **AME-hTERT lineage presents immunoexpression of stem cell markers**

169   The AME-hTERT strain was verified to express SOX-2, NANOG and OCT4 proteins.
170   Predominantly nuclear expression of SOX-2, NANOG and OCT4 was observed in neoplastic cells
171   (Figure 2).

172 ## Discussion

173   This study verified the expression of SOX-2, NANOG and OCT4, SC biomarkers, in AME
174   parenchyma, and subtle expression in the odontogenic epithelium of DC and DF,
175   immunoexpression of the studied proteins was observed in the AME-hTERT cell.

176   The clinical and histological data of the studied samples corroborate the data found in the
177   literature, in which the tumour shows a predominance during the third to seventh decade of life,
178   and the mandible is the location of the highest involvement (Effiom et al., 2018).

179   In our study, we observed a high expression of transcription factors SOX-2, NANOG and
180   OCT4 in the parenchyma of AME samples. On the other hand, there was a weak expression of
181   these factors in DC and DF.

182   Cunha et al., 2013, performed immunohistochemical analyses of the expression of SCs
183   transcription factors (OCT4, SOX-2, NANOG and Stat-3) in tooth germs, suggesting a wide
184   potential for development and differentiation. In our immunohistochemical analysis, the
185   expression of SOX-2, NANOG and OCT4 was observed both in the periphery and central cells of
186   the nests and epithelial cords, which may be related to the broad expression of these factors in the
187   development of AME, considering that the SCs of the dental lamina are possible targets of
188   carcinogenic agents (Harada et al., 2002).

189   The expression of SOX-2 in this study was predominantly nuclear in the tissue samples of
190   AME and AME-hTERT lineage, as observed in nasopharyngeal carcinoma, gastric, colorectal,

191   lung and breast cancer (Luo et al., 2013; Ren et al., 2016). Juuri et al., 2013, also observed the
192   presence of SOX-2 in AME and related it to the proliferation and embryonic origin of this tumour.
193   In our work, a greater expression of SOX-2 was observed in AME parenchyma in relation to the
194   DC lining epithelium and DF epithelial nests.

195   Although smaller, the nuclear expression of SOX-2 in the odontogenic epithelium of DC
196   and DF is interesting. Boumahdi et al., 2014, indicate that SOX-2 plays an essential role in tumour
197   genesis, pointing out the role of SOX-2 in regulating functions in the initiation and progression of
198   squamous cell carcinoma. It was indicated that the expression of this protein in cells originating
199   from the odontogenic epithelium of the dental blade could lead to the development of AME (Juuri
200   et al., 2013). It was also speculated whether the same could not happen with DC and DF, since
201   their epithelia express SOX-2.

202   High OCT4 expression was detected in lung adenocarcinoma CSCs (Hu et al., 2020) and
203   tumour initiator cells in a mouse model with tumour p53 -/- (Darini et al., 2012), depicting that
204   OCT4 expression plays a critical role in tumour cell survival. Khan et al., 2018, did not detect
205   OCT4 expression in 20 cases of aggressive multicystic solid AME, stating that this protein could
206   be used as a useful indicator to histologically distinguish ameloblastic carcinoma from aggressive
207   multicystic solid AME; whereas, in our study, we observed high OCT4 expression in 23 cases of
208   solid AME. However, we cannot fail to consider the differences found when comparing the
209   methodologies used.

210   OCT4 was also observed in epithelial and mesenchymal components during tooth
211   development and is believed to participate in the ameloblast differentiation process (Kero et al.,
212   2014). In both the AME samples and the AME-hTERT strain, OCT4 staining was predominantly
213   nuclear. According to Monroy et al., 2018, the presence of OCT4 in the nucleus is linked to cellular
214   "stemness", which indicates that in ameloblastoma the nuclear expression of this protein may be
215   related to tumour cell survival.

216   NANOG was found expressed in the tumour parenchyma of the tissue samples of AME
217   with cytoplasmic and nuclear marking, as well as in the AME-hTERT lineage, in this study. The
218   epithelial expression of NANOG has been described in head and neck cancers (Lee et al., 2015)
219   (Rodrigues et al., 2017). Its expression was recently investigated in the stroma of odontogenic
220   lesions, including AME, by Chacham et al., 2020, in which the presence of mesenchymal cells

221 positive for NANOG was verified. Thus far, the present study is the only one to observe the
222 expression of this protein in AME parenchyma.

223   In general, the expression pattern identified for SOX-2, NANOG and OCT4 is in
224 accordance with their biological functions as transcription factors. The literature indicates that
225 these markers are crucial transcription factors that are capable of allowing cancer cells to obtain
226 properties similar to those of SCs (Yu et al., 2020). CSCs, in turn, manifest properties similar to
227 those of SCs. These properties include the oncogenic reprogramming of different self-renewal
228 genes, presenting characteristics of immortality, which persist in tumours, usually in nests,
229 representing the source of expansion of growth and tumour maintenance, metastasis formation and
230 tumour recurrence (Liu et al., 2021).

231   There are difficulties in the literature regarding naming the AME neoplastic cells that
232 express these proteins. It seems inappropriate to call them SCs, given the various genetic and
233 proteomic alterations described in AME neoplastic cells (Gomes et al., 2010). Even more
234 misleading would be to call them CSCs. The term "tumour stem cells" seems to be the most
235 appropriate, since describing them as cells that have characteristics of SCs (Monroy et al., 2018)
236 or cells that express SC (Chacham et al., 2020) markers seems somewhat vague.

237   Rodda et al., 2005, showed, using chromatin immunoprecipitation, that OCT4 and SOX-2
238 interact synergistically and bind to NANOG in human and live mouse embryonic stem cells,
239 driving the expression of target genes related to pluripotency. According to Boyer et al., 2005,
240 transcription factors together bind to the promoters of their own genes, forming interconnected
241 autoregulatory loops and an autoregulatory network that is capable of providing advantages for the
242 SCs, which are important for cell survival, stability and tumour progression. In comparison to the
243 non-tumour epithelium, we observed that the expression levels of SOX-2, NANOG and OCT4
244 were increased in AME parenchyma, suggesting that these molecules may be involved in the
245 pathogenesis of AME. It is noteworthy that in the literature, there are no studies that jointly assess
246 the expression of these three markers both in the parenchyma of this neoplasm, as well as in cell
247 lines originating from AME.

248   It is worth highlighting the need for further studies, such as mechanistic assays, which can
249 suppress the expression of stem cell biomarkers and see the influence of this lock, given the
250 limitations that the immunohistochemical study and the sample size may have. Furthermore, some
251 steps in the use of the method used (IHC) aim to increase the specificity of the primary antibody,

252 such as blocking with BSA and using a positive control. However, this does not guarantee the full

253 specificity of the antibody used.

Conclusions

255   Based on the obtained results, the high expression of SOX-2, NANOG and OCT4 markers

256 in AME neoplastic cells using immunohistochemistry, and in the AME-hTERT cell line using

257 immunofluorescence, was verified. The methods used confirm the presence and probable

258 participation of these proteins in the origin and progression of AME. It is suggested that this

259 tumour has cells with characteristics of CSs that could be related to the progression and recurrence

260 of this odontogenic tumour.

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Tabela 1. Clinicohistological data of patients with AME

Case	Genre	Age	Localisation	Histological type
1	Male	45	Upper jaw	Plexiform
2	Female	31	Mandible	Follicular
3	Male	31	Mandible	Plexiform
4	Female	27	Mandible	Plexiform
5	Male	34	Mandible	Plexiform
6	Female	32	Mandible	Follicular
7	Male	34	Mandible	Follicular
8	Female	55	Mandible	Plexiform
9	Female	20	Mandible	Plexiform
10	Male	32	Mandible	Plexiform
11	Male	33	Mandible	Acanthomatous
12	Female	59	Mandible	Follicular
13	Female	47	Mandible	Acanthomatous
14	Female	14	Mandible	Follicular
15	Male	42	Mandible	Granular Cells
16	Male	43	Mandible	Granular Cells
17	Male	28	Mandible	Follicular
18	Male	27	Mandible	Acanthomatous
19	Female	62	Mandible	Follicular
20	Male	84	Upper jaw	Follicular
21	Male	49	Mandible	Follicular
22	Male	22	Mandible	Plexiform
23	Female	38	Mandible	Follicular

Figure 1

Figure 1. Comparative immunoexpression of SOX-2, NANOG and OCT4 in AME, DC and DF samples.

Intense imunoexpression of SOX-2 (A), NANOG (D) and OCT 4 (G) were observed in AME samples. There is a predominant nuclear location of immunostaining for the three markers in the parenchymal cells of ameloblastoma. There is a low intensity immunoexpression of the three proteins in the nucleus of some epithelial cells in both DC (B, E and H) and DF (C, F and I) samples. Statistical analysis of percentage of labelling area of the three markers (J, K and L) between AME and DC, and AME and DF. Statistically, a significant difference was observed when comparing the expression of all proteins in AME cells in relation to the expression in DC and DF. (***p<0,001).

Scale bar:20μm.

AME = ameloblastoma; DC = dentigerous cyst; DF = dental follicle.
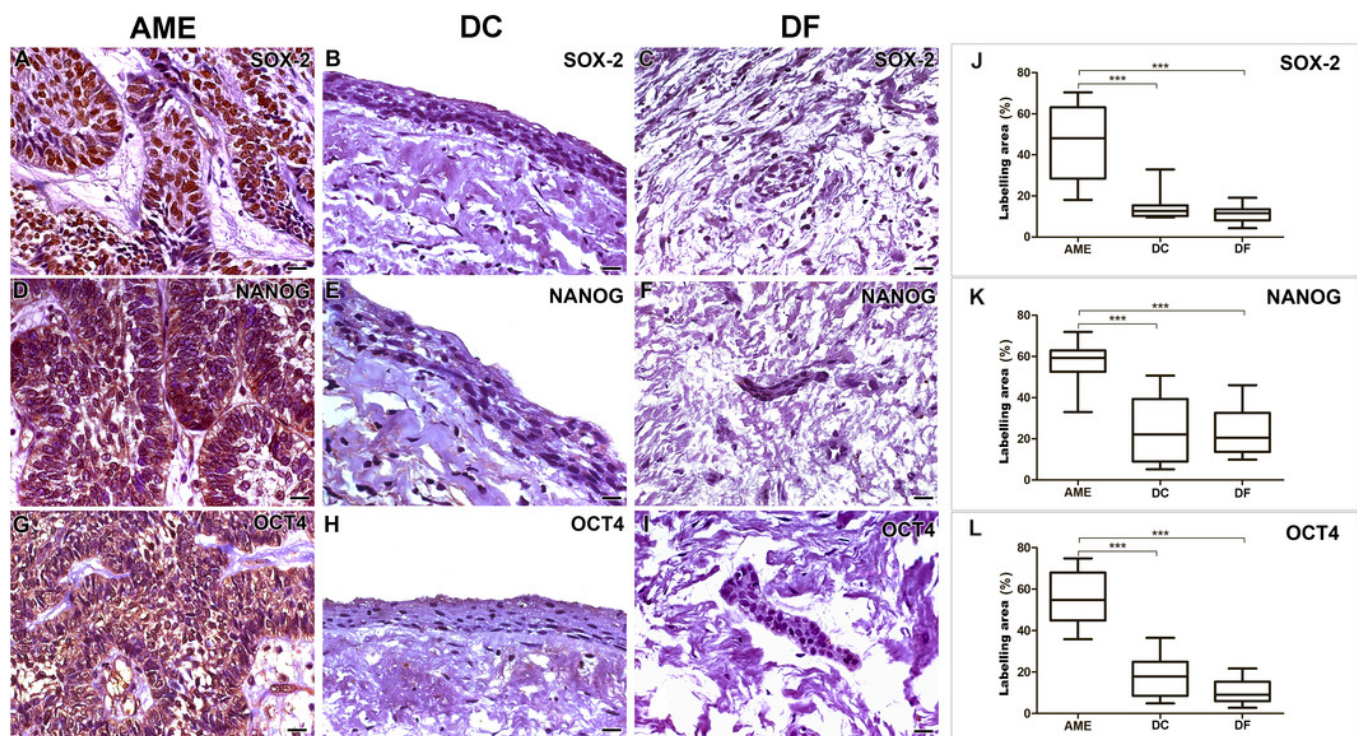
Figure 2

Figure 2. AME-hTERT cells immunoexpress SOX-2, NANOG and OCT4.

SOX-2 was observed as an intense granular immunostaining (green) distributed mainly in the nuclei (A). Granular expression (green) of NANOG (C) was also observed throughout the nuclei and a weak stain in cytoplasm. OCT4 (E) was observed as granular and dot-like (green) predominantly in the nuclei, but staining in cytoplasm, was also visualized. Cytoskeleton is stained with phalloidin (red) and nuclei is stained with DAPI (blue). Merged staining for SOX-2 (B), NANOG (D), OCT4 (F). Statistical analysis of fluorescence intensity of the three markers studied (G, H and I) between nuclei and cytoplasm of AME-hTERT cells, was carried out. A strong labelling was observed in nuclei when compeer with cytoplasm. Statistically, a significant difference was observed when comparing the expression of all proteins in AME-hTERT cells (****$p<0,0001$). Scale bar: 20μm.