Differential expression of the epithelial mesenchymal transition factors Snail, Slug, Twist, TGF-β, and E-cadherin in ameloblastoma

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Abstract

Epithelial mesenchymal transition (EMT), the transition of epithelial cells into motile mesenchymal cells, plays an important role in embryogenesis, cancer invasion, and metastasis. Ameloblastomas are common epithelial odontogenic tumors, occurring exclusively in the mandible with locally invasive growth.

Thirty-seven ameloblastoma cases were evaluated for the involvement of EMT by immunohistochemical staining and western blotting using antibodies against Slug, Snail, Twist, TGF-β, and E-cadherin. Double immunostaining was also performed.

Slug and TGF-β were expressed in the nuclei of peripheral and stellate reticulum cells of ameloblastoma nests. Twenty cases of Snail, 36 of Slug, 8 of Twist, and 19 of TGF-β showed strong expression in tumor cells in follicular and plexiform patterns. Expression of Slug and TGF-β increased in regions where the expression of E-cadherin was reduced.

EMT was found to be associated with the local invasive growth of ameloblastoma. These data suggest that reduced expression of E-cadherin and over-expression of Slug, Snail, and TGF-β induce EMT. Given that ameloblastomas are characterized by local invasiveness, EMT might be related to their development. Thus, strong expression of Slug and TGF-β and reduced expression of E-cadherin might be related to the local invasiveness of ameloblastoma.

Introduction

Epithelial mesenchymal transition (EMT) was first reported in 1982 by Greenburg et al. [1]. EMT, the transition of epithelial cells into motile mesenchymal cells, plays an important role in embryogenesis, cancer invasion, and metastasis [2-4]. In addition, EMT is involved in the formation and maintenance of normal tissue architecture, odontogenesis, salivary gland development, palatogenesis, and oral cancer [5].

Slug and Snail, EMT-related proteins, bind to the promoter of E-cadherin, thereby inhibiting the function of cell adhesion. The epithelial cells gain migratory ability and

invade the stromal tissue [6]. Twist is a transcription factor, existing as a heterodimer, and is indirectly suppressed by the expression of E-cadherin. Twist plays an essential role in cancer metastasis and aggressive growth of ameloblastoma [7]. TGF-β promotes the growth of cancer at advanced stages. Snail and Slug, activated by TGF-β, cause an induction of EMT [8, 9].

E-cadherin is a calcium-dependent cell adhesion molecule expressed on the epithelial cell membrane [10]. The correlation between reduction in E-cadherin expression and invasiveness of a malignant tumor via Wnt pathway dysregulation was observed in oral carcinogenesis [11]. The association between EMT and invasiveness of tumors has been reported in various tumors, including meningeal tumors, gastric cancers, oral squamous cell carcinoma, and odontogenic tumors [12-17].

Ameloblastoma is the most common odontogenic benign tumor, exhibiting a biological characteristic of invasiveness in the mandibular bone, along with a high rate of recurrence [18]. The expression of transcription factors Slug, Snail, and Twist is associated with local invasiveness and recurrence [19, 20]. A high risk of recurrence was observed upon increased TGF-β expression in ameloblastoma [21]. In addition, the TGF-β signaling pathway is activated in ameloblastoma, which is believed to be associated with more aggressive biological behavior [22].

The association between EMT-related protein expression and invasiveness has been reported in malignant tumors [4]. Ameloblastoma is a benign tumor, but it shows high invasiveness and recurrence rate.

We examined the relationship between the development of ameloblastomas and involvement of EMT using immunohistochemical staining and western blotting. Some cases were tested by double immunostaining of EMT-related proteins and E-cadherin.

Materials and methods

Specimens obtained from 37 cases of ameloblastoma (20 and 17 cases of the follicular and plexiform types, respectively) were classified based on the WHO Classification of Head and Neck Tumors, 2005; detailed information was collected from the archives of the Osaka Dental University Hospital. The specimens were obtained from 20 male and 17 female patients with ameloblastoma (median age, 39.2 years; range, 9–76 years old; Table 1). The research was approved by the Ethics Committee of Osaka Dental University (Approval Number 110810).

We studied the involvement of EMT by immunohistochemical staining and western blotting using antibodies against Slug, Snail, Twist, TGF-β, and E-cadherin. Double immunostaining was also performed with E-cadherin and other factors.

Immunohistochemical staining

Formalin-fixed paraffin-embedded tissues obtained from biopsies or resection

specimens were cut into 2-μm-thick sections and mounted on silan-coated glass (MAS-GP; Matsunami, Kishiwada, Japan). Sections were deparaffinized in d-limonene (Hemo-De, Falma, Tokyo, Japan), followed by dehydration in a graded alcohol. E-cadherin and TGF-β were retrieved by autoclaving at 121°C for 15 min in the retrieval buffer (Mitsubishi Yatoron, Tokyo, Japan). Slug, Snail, and Twist were recovered after heating at 98°C for 40 min in a water bath. The extracted specimens were cooled at room temperature (25°C). Non-specific reactions were blocked by treatment with 2.5% normal horse serum (VECTOR, Burlingame, CA, USA) at room temperature (25°C) for 30 min. The sections were incubated with the following diluted primary antibodies: rabbit anti-human Slug polyclonal antibody (ab27568) (1:200, Abcam, Cambridge, UK), rabbit anti-human Snail polyclonal antibody (ab180714) (1:200, Abcam, Cambridge, UK), rabbit anti-human Twist polyclonal antibody (ab49254) (1:200, Abcam, Cambridge, UK), rabbit anti-human TGF-β monoclonal antibody (ab124894) (1:200, Abcam, Cambridge, UK), and mouse anti-human E-cadherin monoclonal antibody (#610181) (1:200, BD Transduction Laboratories, San Diego, USA) for 60 min at 37°C. Following antibody incubation, Slug, Snail, Twist, and TGF-β were incubated with alkaline phosphatase-conjugated anti-rabbit antibodies (Nichirei, Tokyo, Japan) for 30 min at room temperature (25°C). The signals were then visualized using the PermaRed (Diagnostic Bio Systems, Pleasanton, CA, USA). Successively, double immunostaining was carried out with E-cadherin. The unreacted primary antibody activity was inactivated in the citrate buffer by autoclaving for 20 min. The sections were incubated with E-cadherin for 60 min at 37°C, followed by incubation with alkaline phosphatase-conjugated antibody for 30 min at room temperature (25°C). The signals were then visualized using the PermaBlue (Diagnostic Bio Systems, Pleasanton, CA, USA) [23].

Evaluation of immunohistochemical staining

Out of 500 tumor cells, positive staining of 50% cells indicated strong expression, less than 50% cells indicated weak expression, and less than 10% cells indicated no expression. Staining for Snail, Slug, and Twist was positive in both the cytoplasm and the nucleus of the tumor cell, whereas TGF-β staining was positive only in the cytoplasm of the striate cell.

Statistical analysis

Correlations between EMT-related protein expression rates and presence of ameloblastomas were analyzed using the Mann Whitney U-test. *P*-values of <0.05 were considered statistically significant.

Western blotting

Proteins were extracted from the biopsies and resected tissues, and subjected to SDS-PAGE, followed by western blotting analysis using the Total Protein Extraction Kit (101 Bio. Com. Palo Alto, CA, USA). The extracted proteins were electrophoresed through a

12% polyacrylamide gel (Bio-Rad Laboratories, CA, USA) for 30 min at 200 V and 0.01A. Subsequently, the proteins were transferred onto a PVDF membrane (Bio-Rad Lab., Hercules, CA, USA) for 30 min at 200 V and 0.01A. Non-specific reactions were blocked by treatment with a blocking solution (Block One, Nacalai Tesque, Kyoto, Japan) at room temperature (25°C) for 30 min. The membranes were incubated with primary antibodies, followed by incubation with an alkaline phosphatase-conjugated antibody (HISTOFINE AP rabbit, Nichirei, Tokyo, Japan). The membranes were then visualized using the BCIP-NBT solution kit (Nacalai Tesque, Kyoto, Japan).

Results

In immunohistochemical staining, Snail, Slug, Twist, and TGF-β were observed in the cytoplasm as well as in the nuclei of peripheral and stellate reticulum cells in the nests of follicular type (Fig. 1) and plexiform type (Fig. 2) ameloblastomas in all the cases. Results of the double immunostaining test showed that expression of Slug, Snail, Twist, and TGF-β were increased in the regions where expression of E-cadherin was decreased in the cell membrane. In particular, these initial findings were remarkable (Fig. 3).

Cases of Slug, Snail, Twist, and TGF-β expression in ameloblastoma

In case of Slug, strong expression was observed in 36 cases, whereas weak expression was observed in a single case. In case of Snail, strong expression was observed in 20 cases, along with weak expression in 12 cases, and no expression in 5 cases. In case of Twist, strong expression was observed in 8 cases, along with weak expression in 12 cases, and no expression in 17 cases. In case of TGF-β, strong expression was observed in 19 cases, along with weak expression in 9 cases, and no expression in 9 cases (Table 2).

In follicular type ameloblastomas, strong expression of Snail was observed in 14 cases, along with weak expression in 3 cases, and no expression in 3 cases. A strong expression of Slug was observed in all 20 cases. In case of Twist, strong expression was observed in 4 cases, along with weak expression in 7 cases, and no expression in 9 cases. A strong expression of TGF-β was observed in 12 cases, along with weak expression in 5 cases, and no expression in 3 cases. In plexiform type ameloblastomas, strong expression of Snail was observed in 6 cases, along with weak expression in 9 cases, and no expression in 2 cases. A strong expression of Slug was observed in 16 cases, along with weak expression in a single case. In case of Twist, strong expression was observed in 4 cases, along with weak expression in 5 cases, and no expression in 8 cases. A strong expression of TGF-β was observed in 4 cases, along with weak expression in 7 cases, and no expression in 6 cases (Table 3).

Western blotting analysis

The expressions of Snail, Slug, Twist, and TGF-β were indicated by bands of 29, 30, 22, and 44 kDa, respectively (Fig. 4).

Statistical analysis

Statistical analysis showed that the expression rate of Snail was significantly higher than that of Twist ($P < 0.05$). The expression rate of Slug was significantly higher than that of Snail, Twist, and TGF-β ($P < 0.01$). There was no significant difference in the expression rate between Snail and TGF-β, and Twist and TGF-β ($P > 0.05$). There was no significant difference in the expression level of Snail, Slug, and Twist between follicular and plexiform patterns ($P > 0.05$). However, the expression of TGF-β in the follicular pattern was significantly higher than that in the plexiform pattern ($P < 0.05$).

Discussion

Strong expression of Slug and TGF-β and the reduced expression of E-cadherin seem to be related to the local invasiveness of ameloblastoma, as detected by single or double immunostaining. The proliferative ability of tumor cells is high in the budding area. The EMT-related proteins were expressed at this site, which showed reduced expression of E-cadherin in the double immunostaining. Snail and Slug were strongly expressed in a recurrence case.

EMT is a phenomenon identified early in ontogenesis, and is known to be associated with wound healing, cancer invasion, and morphogenesis. Slug and Snail are sufficient for the induction of single-cell invasion in an *in vitro* invasion assay [27]. Bolós V et al. reported that reduction of E-cadherin expression induces the detachment of the cells, caused by EMT-related proteins that induce the process of EMT. The region of reduced of E-cadherin expression has been found to be related to cancer invasion [2]. The reduction in expression of E-cadherin and overexpression of Snail increase with malignancy in ovarian epithelial neoplasms [6]. Slug is not a novel invasion-promoting factor, but its role in promoting invasion in lung adenocarcinoma is a novel finding [24]. EMT regulators Snail and SIP1 are strong repressors of the E-cadherin expression gene in gastric cancers [13]. Snail and Slug act as regulators of TGF-β1-trigged EMT in oral squamous carcinoma cells [8]. Costa et al. reported that expression of E-cadherin was notably associated with tumor location and mortality at the invasive front in oral squamous cell carcinoma [15]. The down-regulation of E-cadherin and Twist overexpression is an independent marker for prediction in oral squamous cell carcinoma (OSCC) [16]. Trends in the expression of EMT markers —E-cadherin, β-catenin, APC, and vimentin— suggest their involvement in oral carcinogenesis via Wnt pathway dysregulation [11]. No differences have been found in the expression of E-cadherin or β-catenin between tooth germs and solid and unicystic ameloblastoma [26].

Ameloblastoma is the most common odontogenic benign tumor, exhibiting a biological characteristic of invasiveness in the mandibular bone, along with a high rate of recurrence. The recurrence of the follicular type of ameloblastoma was found to be 39.7% and that

of the plexiform pattern was found to be 12.9%, as observed in a conservative treatment pattern by Hong et al. [18]. In this study, the rate of recurrence of ameloblastoma was 10.8% (4 cases) due to curettage with bone margin, and all these cases showed strong expressions of Slug and Snail.

Chong et al. reported that overexpression of Snail is most likely the prototype transcription factor involved during induction of EMT in ameloblastomas. They also reported that EMT-transcription factors were differentially expressed in ameloblastoma. The percentage differential expression values for Snail, Slug, SIP1, and Twist were 94, 33, 27, and 41%, respectively. These factors also play different roles in mediating local invasiveness in ameloblastoma [19]. However, the present study is markedly different from that of Chong et al. Specifically, Chong et al. examined the unicystic and desmoplastic sub-types of ameloblastoma in 20 subjects, and included only 2 follicular cases. In contrast, the present study included 37 subjects and reports the relationship between follicular and plexiform types.

Furthermore, the reaction time and temperature used in the immunohistochemical analyses in both studies are also different.

The high expression rate of Twist, which might play a role in the pathogenesis of ameloblastoma and odontogenic keratocysts (OKCs), might be one the reasons for the aggressive behavior of ameloblastoma and high recurrence of OKCs, except for dentigerous cysts. The expression of Twist has been found to be higher in ameloblastoma and OKCs than in dentigerous cysts [7]. Twist expression might be associated with invasion in ameloblastoma variants, and stromal cells might play a regulatory role during tumor development [7].

EMT might be involved in the locally aggressive behavior of keratocystic odontogenic tumors [24]. Porto et al. reported that in keratocystic odontogenic tumors, the transcription factors Snail and Slug participate in cadherin switching [25]. Both Snail and Slug act as regulators of TGF-β-1-trigged EMT in OSCC cells as reported by Qiao [8].

TGF-β/Smad signaling pathway is activated in ameloblastomas, adenomatoid odontogenic tumors, and calcifying cystic odontogenic tumors. Reduced TGF-β/Smad activity could be associated with aggressive biological behavior, especially in ameloblastoma, including increased cell proliferation and reduced apoptosis and differentiation [22]. Lezzi et al. reported that higher expression of TGF-β in stromal cells in ameloblastomas showed a higher risk of tumor recurrence [21]. However, TGF-β exerts inhibitory functions in the early stage of cancer and promoting functions in the advanced stages [22]. In addition, the association between TGF-β expression and growth stage of the tumor needs further investigation.

Conclusion

In this study, cases showing strong expression of Slug and TGF-β and reduced E-cadherin expression were observed. Slug- and TGF-β-positive cells were detected in the same region using double immunostaining method. The strong expression of these EMT-transcription factors seems to be related to the local invasiveness of ameloblastomas.

Disclosure of Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

1. Greenburg G, Elizabeth D H (1982) Epithelial suspended in collagen gels can lose polarity and express characteristics of migration mesenchymal cells. J cell Biol 95:333-339

2. Bolós V, Peinado H, Pérez-moreno MA, Fraga MF, Esteller M, Cano A (2002) The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J cell Sci 116:499-511.

3. Hay ED (2005) The mesenchymal cells, its role in the embryo, and the remarkable signaling mechanisms that create it. Dev Dyn 233:706-720.

4. Thiery JP, Acloque H, Huang RTJ, Nieto MA (2009) Epithelial mesenchymal transition in development and disease. Cell 139:871-890.

5. Santosh ABR, Jones TJ (2014) The epithelial-mesenchymal interactions: insight into physiological and pathological aspects of oral tissues. Oncology Rev 8:239-243.

6. Yoshida J, Horiuchi A, Kikuchi N, Hayashi A, Osada R, Ohira S, Shiozawa T, Konishi I (2009) Changes in the expression of E-cadherin repressor, Snail, Slug, SIP1, and Twist, in the development and progression of ovarian carcinoma: the important role of Snail in ovarian tumorigenesis and progression. Med Mol Morphol 42:82-91

7. Andisheh-Tadbir A, Pardis S, Ranjbaran P (2015) Twist expression in dentigerous cyst, odontogenic keratocyst, and ameloblastoma. Oral Maxillofac Surg 19:103-107

8. Qiao B, Johnson NW, Gao J (2010) Epithelia-mesenchymal transition in oral squamous cell carcinoma triggered by transforming growth factor-β1 is Snail family-dependent and correlates with matrix metalloproteinase-2 and -9 expression. Int J Oncol 37:663-668

9. Massagué J (2008) TGFβ in cancer. Cell 134:215-230

10. Liu LK, Jiang XY, Zhou XX, Wang DM, Song XL. Jiang HB (2010) Upregulation of vimentin and the aberrant expression of E-cadherin/β-catenin. Complex in oral squamous cell carcinomas: correlation with the clinicopathological features and patient outcome. Mod Pathol 23:213-224.

11. Chaw SY, Majeed AA, Dalley AJ, Chan A, Stein S, Farah CS (2012) Epithelial to mesenchymal transition (EMT) biomarkers – E-cadherin, β-catenin, APC and vimentin – in oral squamous cell carcinogenesis and transformation. Oral Oncol 48:997-1006

12. Nagaishi M, Nobusawa S, Tanaka Y, Ikota H, Yokoo H, Nakazato Y (2012) Slug, Twist, and E-cadherin as immunohistochemical biomarkers in meningeal tumors. PLOS One 7:1-6.

13. Rosivatz E, Becker, Specht K, Fricke E, Luber B, Busch R, Höfler H, Becker KF (2002) Differential expression of the epithelial-mesenchymal transition regulators Snail, SIP1, and Twist in gastric cancer. Am J Pathol 161:1881-1891.

14. Krisanaprakornkit S and Z Iamaroon A (2012) Review Article Epithelial-mesenchymal transition in oral squamous cell carcinoma. IRSN Oncology Article ID 681469.

15. Costa LCMC, Leite CF, Cardoso SV, Loyola AM, de Faria PR, Souza PEA, Horta

MCR (2015) Expression of epithelial-mesenchymal transition markers at the invasive front of oral squamous cell carcinoma. J Appl Oral Sci 23:169-178
16. Fan CC, Wang TY, Cheng YA, Jiang SS, Cheng CW, Lee AYL, Kao TY (2013) Expression of E-cadherin, twist, and p53 and their prognostic value in patients with oral squamous cell carcinoma. J Cancer Res Clin Oncol 139:1753-1744
17. Zhong WQ, Chen G, Zhang W, Ren JG, Wu ZX, Zhao Y, Liu B, Zhao YF (2015) Epithelial-mesenchymal transcription in keratocystic odontogenic tumor: possible role in locally aggressive behavior. BioMed Res Int Article ID 168089.
18. Hong J, Yun PY, Chung IH, Myoug H, Suh JD, Seo BM, Lee JH, Choug PH (2007) Long-term follow up on recurrence of 305 ameloblastoma cases. Int J Oral Maxillofac Surg 36:283-288
19. Chong HS, Kok HN (2014) Differential expression of transcription factors Snail, Slug, SIP1, and Twist in ameloblastoma. J Oral Pathol Med 43:45-52
20. Feng Y, Zhou YM, Hua CG, Tang XF, He DQ, (2009) Expression of Twist in different subtype of ameloblastoma. Oral Surg, Oral Med, Oral Pathol, Oral Radiol, Endod 108:565-570
21. Lezzi G, Piattelli A, Rubini C, Artrse L, Goteri G, Perrotti V, Carinci F (2008) Expression of Transforming growth factor β1 in ameloblastoma. J Craniofac Surg 19:1618-1621
22. Karathanasi V, Tosios KI, Nikitakis NG, Piperi E, Koutlas I, Trimis G, Sklavounou A (2013) TGF-β1, Smad-2/-3, Smad-1/-5/-8, and Smad-4 signaling factor are expressed in ameloblastomas, adenomatoid odontogenic tumors, and calcifying cystic odontogenic tumors: an immunohistochemical study. J Oral Pathol Med 42:415-423
23. Wato M, Kon-I H, Taguchi Y, Ueda M, Uobe K, Masuno K, Tominaga K, Nishikawa T, Tanaka A (2009) Double-immunostaining of p63 protein and cytokeratin 8 in pleomorphic adenomas. Oral Med Pathol 14:49-54.
24. Shin JY, Tsai MF, Chang TH, Chang YL, Yuan A, Yu CJ, Lin SB, Liou GY, Lee ML, Chen JJW, Hong TM, Yang SC, Su JL, Lee YC, Yang PC (2005) Transcription repressor Slug promotes carcinoma invasion and predicts outcome of patients with lung adenocarcinoma. Clin Cancer Res 11:8070-8078.
25. Porto LPA, dos Santos JN, Ramalho LMP, Figueiredo AL, Júnior BC, Gurgel CA, Paiva KBS, Xavier FCA (2016) E-cadherin regulators are differentially expressed in the epithelium and stroma of keratocystic odontogenic tumors. J Oral Pathol Med 45:302-311
26. Pereira KMA, do Amaral BA, dos Santos BRM, Galvão HC, de Almeida Freitas R, de Souza LB. (2010) Immunohistochemical expression of E-cadherin and β-catenin in ameloblastoma and tooth germs. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 109:425-431.
27. Naber HPH, Drabsch Y, Snaar-Jagalka BE, ten Dijke P, van Laar T. (2013) Snail and Slug, key regulators of TGF-β-induced EMT, are sufficient for the induction of single-cell invasion. Biokem Biophys Res comm 435:58-63.

Table legends

Table 1. Age, gender, and recurrence in patients with ameloblastoma

Table 2. Staining intensity for Snail, Slug, Twist, and TGF-β in ameloblastoma patterns
The intensity of staining was scored as Negative (-), Weak (+), or Strong (+++). Negative (-) expression was indicated by less than 10% of positive reaction, Weak (+) expression was indicated by less than 50% of positive reaction, and Strong (+++) expression was indicated by more than 50% of positive reaction.

Table 3. Staining intensity of Snail, Slug, Twist, and TGF-β in follicular and plexiform patterns
The intensity of staining was scored as Negative (-), Weak (+), or Strong (+++). Negative (-) expression was indicated by less than 10% of positive reaction, Weak (+) expression was indicated by less than 50% of positive reaction, and Strong (+++) expression was indicated by more than 50% of positive reaction.

Figure legends

Fig. 1 Immunostaining of Snail (a), Slug (b), Twist (c), and TGF-β (d) in follicular pattern
Snail, Slug, Twist, and TGF-β were observed in cytoplasm and nuclei of peripheral and stellate reticulum cells in the nests of ameloblastoma (×100)

Fig. 2 Immunostaining of Snail (a), Slug (b), Twist (c), and TGF-β (d) in plexiform pattern
Snail, Slug, Twist, and TGF-β were observed in the cytoplasm and nuclei of peripheral cells of ameloblastoma (×100)

Fig. 3 Double immunostaining of Snail and E-cadherin (a), Slug and E-cadherin (b), Twist and E-cadherin (c), and TGF-β and E-cadherin (d) in ameloblastoma
Snail, Slug, Twist, and TGF-β were stained Red, and E-cadherin was stained Blue
The expression of Snail, Slug, Twist, and TGF-β was increased in the regions where the expression of E-cadherin was reduced (×100)

Fig. 4 Western blot analysis of Snail (a), Slug (b), Twist (c), and TGF-β (d) expression in ameloblastoma
The expressions of Snail, Slug, Twist, and TGF-β were indicated by bands of 29, 30, 22, and 44 kDa, respectively (×100)

Table 1.

Factor (n = 37)		
Gender	Male	20
	Female	17
Age (years)	Mean	39.2
	Range	9–76
Histopathologic pattern	Follicular	20
	Plexiform	17
Recurrence	Recurrence	4
	No Recurrence	33

Table 2.

		Ameloblastomas (n = 37)
Snail	-	5 (14%)
	+	12 (32)
	+++	20 (54)
Slug	-	0
	+	1(3)
	+++	36(97)
Twist	-	17 (46)
	+	12 (32)
	+++	8 (22)
TGF-β	-	9 (24)
	+	9 (24)
	+++	19 (51)

Table 3.

		Follicular (n=20)	Plexiform (n=17)	Statistics
Snail	-	3 (15%)	2 (12)	P > 0.05
	+	3 (15)	9 (53)	
	+++	14 (70)	6 (35)	
Slug	-	0	0	P > 0.05
	+	0	1 (6)	
	+++	20(100)	16 (94)	
Twist	-	9 (45)	8 (47)	P > 0.05
	+	7 (35)	5 (29)	
	+++	4 (20)	4 (24)	
TGF-β	-	3 (15)	6 (35)	P < 0.05
	+	5 (25)	7 (41)	
	+++	12 (60)	4 (24)	

Fig.1

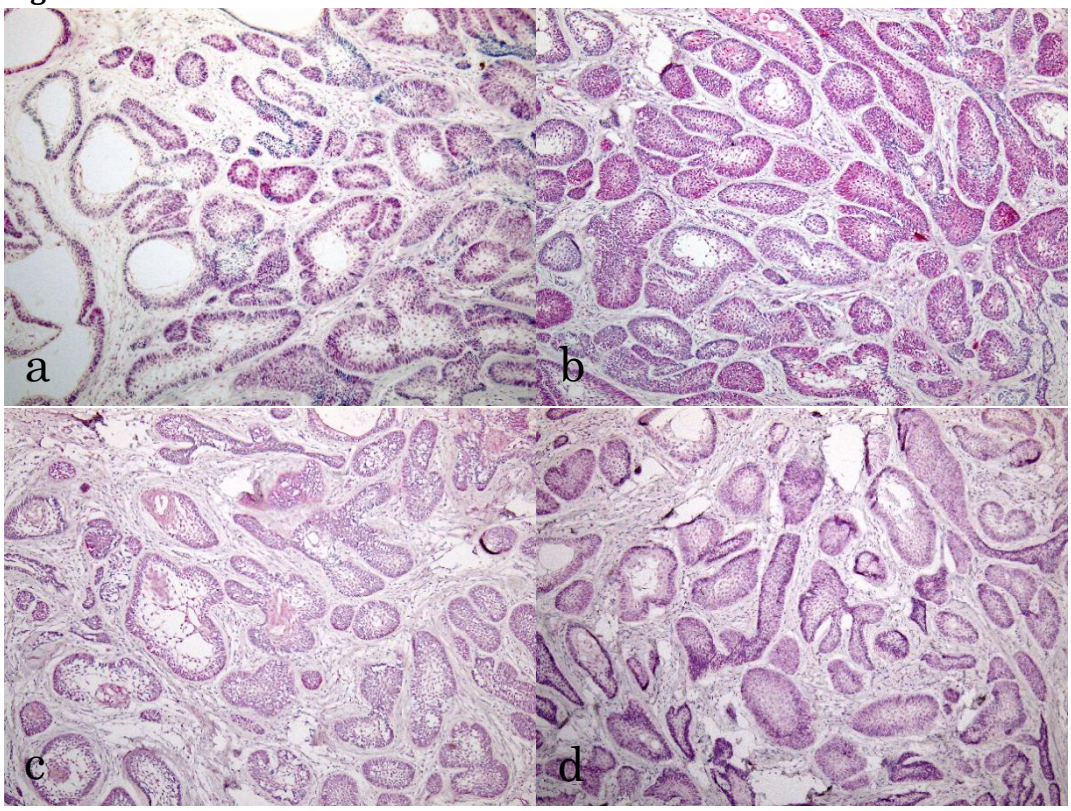

Fig.2

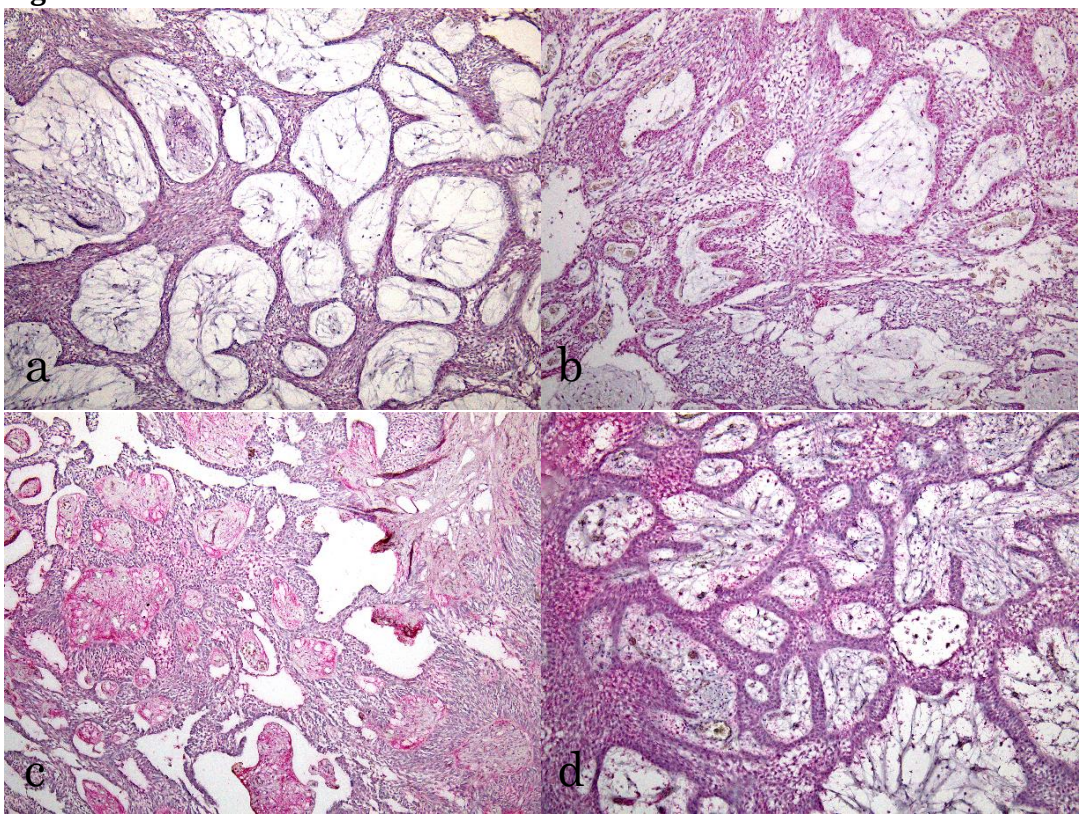

Fig3.

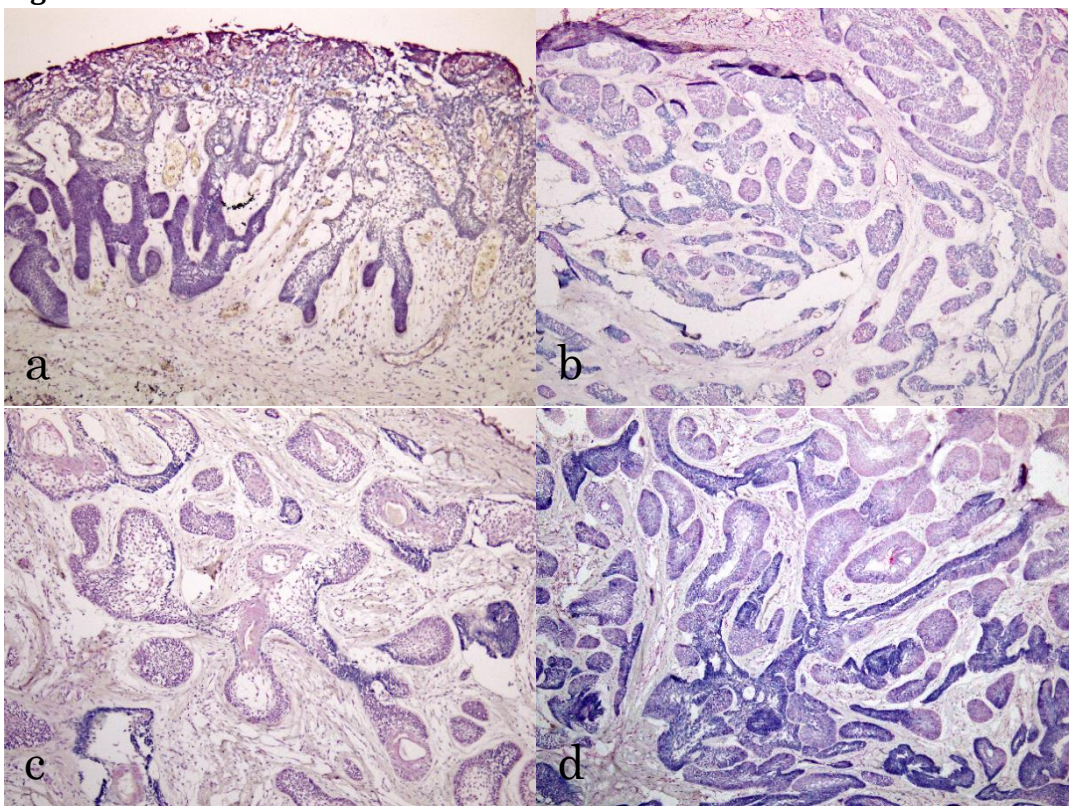

Fig4.