Advances in Medical Sciences 63 (2018) 296–300

Contents lists available at ScienceDirect

Advances in Medical Sciences

journal homepage: www.elsevier.com/locate/advms

Original research article

Expression profile and cellular localizations of mucin proteins, CK7, and cytoplasmic p27 in Barrett's esophagus and esophageal adenocarcinoma

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ARTICLE INFO

Keywords:
Prognostic marker
Mucin
Esophageal carcinoma
Barrett`s esophagus
Dysplasia

ABSTRACT

Purpose: Barrett's esophagus is one of the main risk factors for increased incidence of esophageal adenocarcinoma. In this study, we studied protein expression levels and cellular localizations of MUC-1, MUC-2, MUC-5AC, CK7, and cytoplasmic p27 to assess the relationship between the expression of each of these proteins and the disease progression on endoscopic biopsies.

Materials and methods: Immunohistochemical analyses were performed using antibodies produced against MUC-1, MUC-2, MUC-5AC, CK7, and p27. Endoscopic specimens of esophageal mucosa were obtained from 72 patients who underwent esophagectomy for Barrett's esophagus, metaplasia, dysplasia, or esophageal adenocarcinoma developed from Barrett's esophagus.

Results: Multilayer squamous epithelium showed only MUC-1 positivity in the EAC group while MUC-2 and MUC-5AC staining could not be detected in this group. Strong and diffused membranous or cytoplasmic staining of CK7 was observed at squamous, ductal, surface columnar and/or glandular epithelium. c-p27 staining was diffused and moderate in the cellular membranes observed in all groups except for esophageal epithelial metaplasia without intestinal metaplasia. Additionally, weakly focal cytoplasmic staining in squamous epithelium of p27 in EAC was detected.

Conclusions: Barrett's esophagus, which has a heterogeneous epithelium, might yield different diagnosis based on endoscopic evaluation and immunohistological investigation. Thus, the use of MUC1, p27, and CK7 might strengthen the truthful diagnosis. MUC-1, CK7, and c-p27 immunostaining can be used as the predictive markers for esophageal cancer progression from Barrett's esophagus.

1. Introduction

Barrett's esophagus (BE) is abnormal condition in the cells of the lower portion of the esophagus that may dispose to the development of esophageal adenocarcinoma (EAC) in a multistep fashion due to mainly long-term gastroesophageal acid reflux and various genetic and molecular alterations [1–3]. These damaged cells may transform; and as a result, rapid and uncontrolled growth and invasion into the deeper layers of the esophagus may occur [1].

BE is often considered and described by its resemblance of intestinal metaplasia and contains a variety of glands and cells including columnar of both intestinal and gastric cell lineages in addition to a high number of goblet cells [4,5]. Gastric columnar cells express MUC-1 and MUC-5AC while gastric mucus secreting cells express MUC-6. Goblet cells express MUC-2 and MUC-3 which resemble intestinal goblet cells. Abnormal mucin expression in the epithelium of esophagus may be associated with sustained tissue injury, and stimulated cell proliferation and metastasis [6].

The markers selected for this study of immunohistochemical (IHC) analysis were cytoplasmic p27 (c-p27) for mobility, CK7 increase with Barrett's progression and unique to esophageal cells, MUC-1 marker specific for gastric epithelial cells, MUC-5AC which is specific for gastric foveolar epithelial cells and MUC2 is selective marker for intestinal epithelial cells. As BE progresses toward dysplasia, it becomes very complicated to diagnose using currently available markers [7–11]. In this study, the protein expression profile and cellular localizations of CK7, c-p27, MUC-1, MUC-2, and MUC-5AC were investigated in esophageal biopsies. We hypothesized that determination of the expression levels of these markers will reflect the type of cell combination as well as the rate of disease progression.

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https://doi.org/10.1016/j.advms.2018.04.002
Received 22 August 2017; Accepted 18 April 2018
Available online 24 May 2018

2. Materials and methods

2.1. Biopsies

This study was approved by Kafkas University, Medical School Ethics Committee (approval number: 80576354-050-99/81, date: 15.10.2014). In this study, BSOG-2005 criteria, Modified Savary Miller grading, AGAI- histological typing, Vienna and ACG-2008 dysplasia criteria that are specific for BE have been used for grading the biopsies which were taken by endoscopy. Indication of endoscopy were indigestion, dysphagia, vomiting, abdominal pain, upper gastrointestinal bleeding, and anemia. Histopathologic confirmation of the biopsies were done for the assessment of the grading of 72 cases [12–14]. Most of the tissues (67) are endoscopic biopsy tissues. The other 5 (containing adenocarcinomas) were obtained from resection. Tissues were fixed in formaldehyde for 72 h and stained with hematoxylin and eosin. BE related EAC specimens have been typed, staged and graded as previously described [15,16]. BE related EAC specimens have been typed, staged and graded by dedicated pathomorphologists including the first author, as previously described [15,16].

Cases were grouped as Group 1 CLO (columnar lined esophageal epithelial metaplasia without intestinal metaplasia, n = 5), Group 2 CLO-IM (columnar lined esophageal epithelial metaplasia with intestinal metaplasia, n = 20), Group 3 CLO-SD (columnar lined esophageal epithelial metaplasia with suspicious dysplastic region, n = 27), Group 4 CLO-LGD (columnar lined esophageal epithelial metaplasia with low grade dysplastic region, n = 14), and Group 5 BE associated EAC, n = 6. There were not BE cases with high grade dysplasia in our study.

PAS/Alcian blue staining (pH: 2.5) has been used to stain mucin which was used for intestinal metaplasia examination and typing [17]. All specimens which were taken from the patients showed short segment BE. Histopathologic characteristics of the specimens are presented in Table 1.

2.2. Immunostaining

The following primary antibodies were used for immunostaining of the specimens: CK7 (Biocare Medical,Clone:OV-TL 12/30), MUC1 (Biocare Medical, Clone:Mouse monoclonal), Rabbit polyclonal MUC2 (Gene Tex), MUC5AC (Biocare Medical, Clone:45M1) and monoclonal p27 (Transduction Laboratories, Lexington, KY) antibodies were used with Streptavidin-Biotin Peroxidase procedure on paraffin embedded tissue sections of 2 µm thickness. Prior to application of the primary antibodies, endogenic peroxidase activity was blocked by 3% hydrogen peroxide for 10 minutes, followed by blocking the tissue sections using Ultra V Block solution (Thermoscientific, Fremont, CA, U.S.A). In order to make the antigen-antibody connections visible, chromogen, (3, 3 −diaminobenzidine) was applied for 5 minutes. Tissues washed with distilled water were applied Mayer's hematoxylin for 5 minutes for counterstain. Positive and negative controls were also run in parallel to the patient tissue sections. There are different theories for the development of Barrett's esophagus and each represents different types of cells. Recent studies have been trying to determine which cell type is more specific to this disease in histopathological diagnosis. Therefore, in this study we have chosen and investigated four cell types of mucosa which we think they closely represent this situation for histopathological diagnosis. Immunohistochemically stained slides were evaluated microscopically by examining four different areas consisting of squamous epithelia, metaplastic surface columnar epithelia, ductal and glandular epithelia in each group of patients.

The number of stained cells were counted under the light microscope at a magnification of 400X and the staining intensity of the cells were determined arbitrarily as “weak”, “moderate”, and “strong”. H-scoring was used for semiquantitative assessment.

Table 1
Histological characterization of cases.

	Stage	Histological type	Grade	Intestinal metaplasia
Group 1: CLO(n = 5)	2(n = 5)	1(n = 3)	0(n = 5)	–
	–	3(n = 2)	–	–
Group 2: CLO-IM (n = 20)	1(n = 12)	1(n = 12)	–	CIM(n = 4)
	2(n = 7)	3(n = 8)	2(n = 20)	Mixed type(n = 2)
	3(n = 1)	–	–	ICIM(n = 14)
Group 3: CLO-SD (n = 27)	1(n = 20)	1(n = 16)	2(n = 26)	CIM(n = 4)
	2(n = 7)	3(n = 11)	3(n = 1)	Mixed type(n = 1)
	–	–	–	ICIM(n = 22)
Group 4: CLO-LGD (n = 14)	1(n = 10)	1(n = 7)	1(n = 1)	CIM(n = 1)
	2(n = 4)	2(n = 1)	2(n = 13)	Mixed type(n = 1)
	–	–	–	ICIM(n = 12)
Group 5: EAC(n = 6)	IB(n = 1)	Tip-3(n = 6) carcinoma	3(n = 2)	–
	IIB(n = 1)	–	2(n = 4)	ICIM(n = 6)
	IIIA(n = 2)	1(n = 4)	–	–
	IV(n = 2)	3(n = 2) CLO regions	–	–

2.3. Statistics

H-score is a scoring algorithm calculated by the formula I x PC (I: the intensity of staining; PC: the percentage of the cells in each intensity). It is a number between 0-300. The value of H-scoring was evaluated statistically using Kruskal-Wallis test and Chi-Square analysis. The outcome of scores were plotted graphically. The criterion for statistical significance was set at $p < 0.05$.

3. Results

3.1. MUC-1, MUC-2, MUC-5AC, CK7, and c-p27 expressions in squamous epithelia

Squamous epithelial cells showed a conspicuous increase in MUC-1 expression as the cells transformed and progressed to esophageal adenocarcinoma. MUC-1 expression was observed mainly in the EAC group ($P < 0.05$). In parallel, c-p27 expression was mainly seen in intestinal metaplasia and EAC groups. P27 staining for CLO-IM and EAC was cytoplasmic and appeared to be increased two-fold in esophageal adenocarcinoma. In all groups, CK7 was present at low levels. MUC-2 and MUC-5C expressions were very little or absent in the squamous epithelium (Fig. 1).

3.2. MUC-1, MUC-2, MUC-5AC, CK7, and c-p27 expressions in surface columnar lined esophageal epithelia

The positivity of all antibodies were widespread in surface columnar lined epithelium more than squamous epithelium. CK7 expression was determined at middle levels in all groups except for CLO. CK7 is rod-like and extends from the nuclear membrane towards the cellular membrane (Fig. 5F). c-p27 expression was middle levels in all groups except for CLO and CLO-SD. MUC-1 was seen at middle and low levels in CLO-IM and CLO-SD, respectively. MUC-2 and MUC-5AC were widespread, mostly at high levels in all groups except for CLO. MUC2 and MUC5AC expressions were prevalent in the surface columnar lined esophageal epithelium ($P < 0.05$ for both) (Fig. 2).

3.3. MUC-1, MUC-2, MUC-5AC, CK7, and c-p27 expressions in ductal epithelia

The expression of these proteins were widespread at high levels in

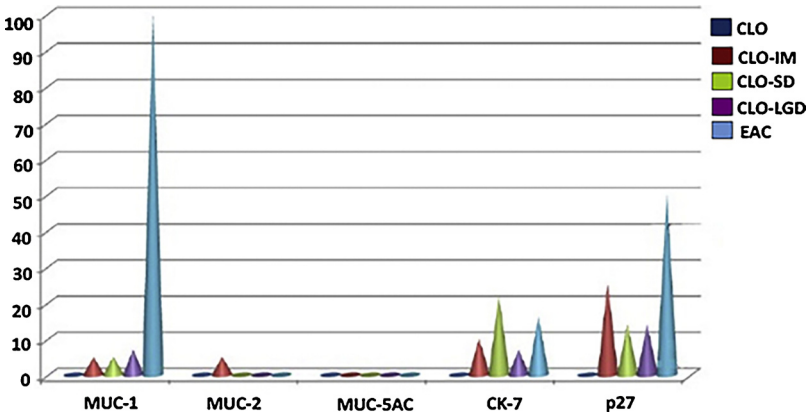

Fig. 1. Squamous epithelial CK7, P27, MUC1, MUC2, and MUC5AC protein expressions. CLO: columnar lined esophageal epithelial metaplasia without intestinal metaplasia; **CLO-IM:** CLO with intestinal metaplasia; **CLO-SD:** CLO with suspicious dysplastic region; **CLO-LGD:** CLO with low grade dysplastic region; and **EAC:** Barrett's esophagus associated esophageal adenocarcinoma.

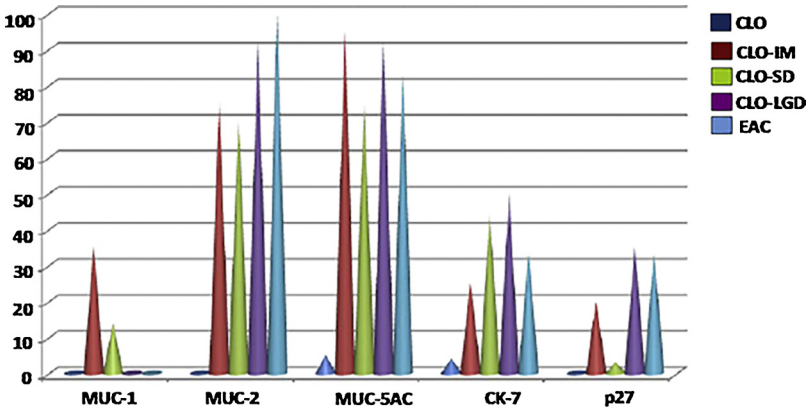

Fig. 2. Surface columnar lined esophageal epithelial MUC1, MUC2, MUC5AC, CK7, and cP27 expressions. CLO: columnar lined esophageal epithelial metaplasia without intestinal metaplasia; **CLO-IM:** CLO with intestinal metaplasia; **CLO-SD:** CLO with suspicious dysplastic region; **CLO-LGD:** CLO with low grade dysplastic region; and **EAC:** Barrett's esophagus associated esophageal adenocarcinoma.

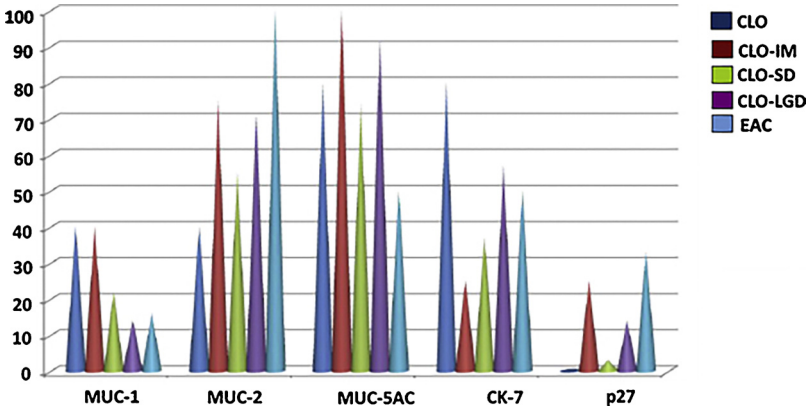

Fig. 3. Ductal epithelial MUC1, MUC2, MUC5AC, CK7, and cP27 expressions. CLO: columnar lined esophageal epithelial metaplasia without intestinal metaplasia; **CLO-IM:** CLO with intestinal metaplasia; **CLO-SD:** CLO with suspicious dysplastic region; **CLO-LGD:** CLO with low grade dysplastic region; and **EAC:** Barrett's esophagus associated esophageal adenocarcinoma.

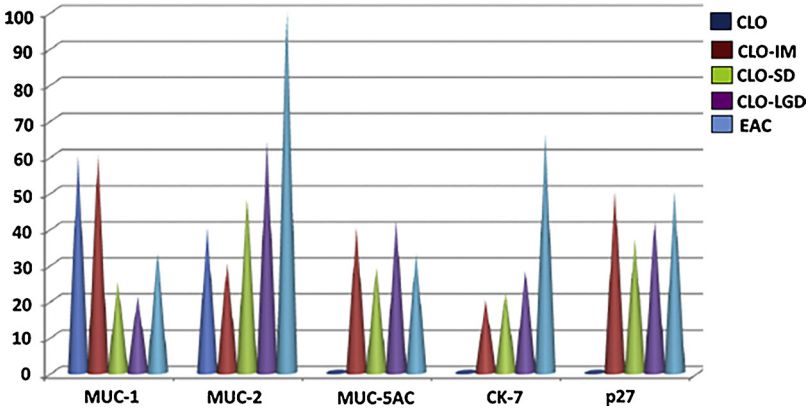

Fig. 4. Glandular epithelial MUC1, MUC2, MUC5AC, CK7, and cP27 expressions. CLO: columnar lined esophageal epithelial metaplasia without intestinal metaplasia; **CLO-IM:** CLO with intestinal metaplasia; **CLO-SD:** CLO with suspicious dysplastic region; **CLO-LGD:** CLO with low grade dysplastic region; and **EAC:** Barrett's esophagus associated esophageal adenocarcinoma.

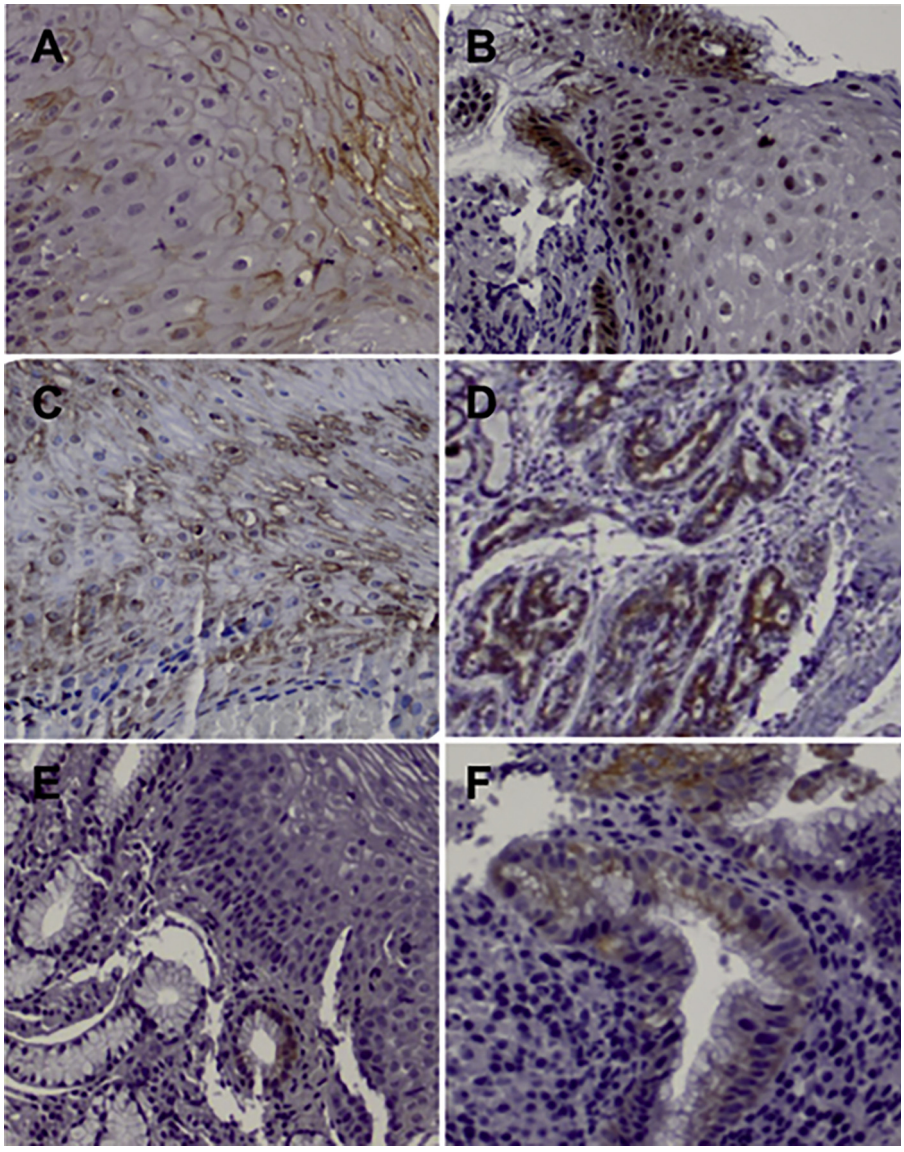

Fig. 5. Cellular localization of Muc-1, Muc-2, p27, and CK7. A) MUC-1 staining in EAC. **B)** MUC-2 staining on squamous epithelia in CLO-IM. **C)** p27 staining in squamous epithelia in EAC group. **D)** CK7 staining in glandular epithelia in EAC. **E)** CK7 staining in ductal epithelia in CLO. **F)** CK7 staining in surface columnar epithelia in CLO-LGD.

all groups, resembled those observed in surface columnar lined epithelium. MUC-1 were prevalent at middle levels in CLO and CLO-IM; and gradually decreased in carcinoma. In beginning of disease (CLO), MUC-5AC was at high levels mostly in ducts. Additionally, distributions of MUC2, MUC5AC and CK7 expression were similar to surface columnar line epithelium. c-p27 was widespread at low levels all groups except for CLO group (Fig. 3).

3.4. MUC-1, MUC-2, MUC-5AC, CK7, and c-p27 expressions in glandular epithelia

In the glandular epitheium, all proteins expression profiles were somewhat different than the other epithelium. All proteins were prevalent at low-to-middle levels in all groups except for CLO. MUC-2 and CK7 were at their highest levels in EAC group. MUC-1 protein was highly expressed in CLO group in glandular epithelium ($P < 0.05$). Its distribution was variable and was somewhat increased in EAC group. c-p27 expression was also high in glandular epithelium except for CLO ($P < 0.05$) (Fig. 4).

3.5. Cellular localization of MUC-1, MUC-2, CK7, and c-p27

MUC-1 immunostaining was determined to be diffused, weakly-moderate and cytoplasmic-membranous in EAC group (Fig. 5A). As for, MUC-2 staining, it was weak and cytoplasmic close to squamo-columnar transition area in CLO-IM group (Fig. 5B). p27 expression was determined to be weak and mainly cytoplasmic on squamous epithelia in EAC group (Fig. 5C). Strong, diffused membranous and cytoplasmic CK7 staining was observed in glandular epithelia in EAC group (Fig. 5D). Strong, diffused membranous CK7 staining was seen in ductal epithelia in CLO group (Fig. 5E). Lastly, strong, diffused cytoplasmic and membranous CK7 staining was seen in surface columnar epithelia of the CLO-LGD group (Fig. 5F).

4. Discussion

In this study, we examined the expression patterns of mucins, CK7 and c-p27 to address if the expression profile of this proteins could be a valuable diagnostic tool in the setting of Barrett's disease and BE associated esophageal adenocarcinoma.

Squamous epithelial MUC-1 positivity has previously been reported in BE [18,19]. Upregulation in MUC-1 expression is seen during the progression from dysplasia to EAC, and one of the most important prognostic markers among other mucins [18]. In this study, we noticed that diffused and weakly-moderate cytoplasmic-membranous MUC-1 staining in all squamous epithelial layers in the EAC group. Molecular and genetic alterations in Barrett's epithelium, such as in c-erbB2 and p53 have been reported [3]. The association between these molecules may lead to overexpression of MUC-1 and this may increase the instability of epithelial cells towards EAC [18].

Premalignant intestinal metaplasia is very important for BE [7]. Upregulation of MUC-2 is frequently observed in intestinal metaplasia in BE [18]. Consistent to these, we determined MUC-2 in the squamous epithelia of CLO-IM and it reaches its highest levels in the EAC. As for MUC5-AC, positive cell clones with this mucin were widespread in ducts and glands on the surface columnar epithelia.

We found that CK7 immunostaining was under the squamo-columnar transition of submucosal ductal structure and the expression was widespread from ducts to glands. Its expression increases as the disease progresses. The results we observed are supported by de novo stem cell-clonal development-crypt fission- laterally cancerization theories [7]. We detected strong and diffused membranous CK7 staining in epithelia in EAC and we believe that the staining pattern of CK7 can be an important diagnostic marker value.

c-p27 plays a role in cell-cell interactions and cell mobility in BE [20]. We found that c-p27 is widespread and high levels in EAC group and glands in CLO-IM group. Cellular localization of p27 is present weakly-focally in the cytoplasm of squamous epithelia and we believe that c-p27 is the second most important prognostic marker after MUC1.

5. Conclusions

We found using immunostaining that MUC1, p27 and CK7 are important markers indicating progression towards adenocarcinoma. Among them, the strongest marker that makes a difference in our study is MUC1. Regardless of the groups, weak or moderate cytoplasmic/membranous staining of MUC1 in squamous epithelium might point out for progression to adenocarcinoma. For p27, we only evaluated the cytoplasmic accumulation and staining of p27 because we intended to determine oncoprotein property of p27. p27 showed cytoplasmic staining in the squamous and glandular epithelium in the EAC group. Observation of this kind of staining in a case might not be an indicator for carcinoma but might be in favor of progression toward it. Lastly, our results show that CK7 staining in columnar surface epithelium in CLO-SD and CLO-LGD groups could be an indicator for abnormal proliferation, and consequently disease progression.

The Author Contribution

Study conception and design: H.E.A.O.; acquisition of data: H.E.A.O.; analysis and interpretation of data: H.E.A.O., T.A., O.O.; drafting of manuscript: H.E.A.O., T.A., O.O.; critical revision: H.E.A.O., T.A., O.O. Authors give final approval of the version: H.E.A.O., T.A., O.O.

Conflict of interests

The authors declare no conflict of interests.

Financial disclosure

The authors have no funding to disclose.

Acknowledgements

We thank Prof. Dr. Huseyin Ustun for helping with histological examination and Prof. Dr. Ahmet Arslan for helpful discussion on ultrastructural findings.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.advms.2018.04.002.

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