Original Article

Immunohistochemical Evaluation of M30 and M65 in Barrett's Esophagus and Esophageal Cancer

Barrett's Özofagus ve Özofagus Kanserinde M30 ve M65'in İmmünohistokimyasal Olarak Değerlendirilmesi

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ABSTRACT

Introduction: Although many markers have been studied in esophageal adenocarcinomas, there is no marker currently available for clinical use. This study aimed to investigate the role of apoptosis in Barrett's esophagus and adenocarcinoma carcinogenesis, determine whether there is a predictive value of apoptotic-necrotic markers M30 and M65, and examine Barrett's mucosa and cancerous tissue by the immunohistochemical method.

Methods: Esophageal tissue biopsy with an upper gastrointestinal endoscopy was performed on participants, who were older than 18 years and newly diagnosed. There were 20 with Barrett's esophagus, 20 esophageal cancer patients and 20 gastroesophageal reflux disease patients as a control group. Among the tissue samples taken, M30 and M65 were stained with immunohistochemical methods. The samples were examined to see whether there was a significant immunohistochemical difference among the groups in terms of M30 and M65 staining.

Results: There was no statistically significant difference among the groups in terms of M30 expression ($p = 0.329$). When compared to the control and the Barrett's esophagus groups, M65 positivity was significantly higher in the adenocarcinoma group ($p = 0.0001$).

Discussion and conclusion: There was no statistically significant difference in M30 expression among the groups in our study. M65 was found to be significantly high in esophageal adenocarcinoma. This suggests that necrosis is more dominant in the pathogenesis of esophageal adenocarcinoma. M65 can be used as a predictive marker in esophageal adenocarcinoma.

Keywords: Barrett's Esophagus, Esophagus Cancer, M30, M65

ÖZET

Giriş ve amaç: Özofagus adenokarsinomlarında birçok belirteç çalışılmış olmasına rağmen, şu anda klinik kullanım için bir belirteç bulunmamaktadır. Bu çalışmada apoptozun Barrett's özofagusu ve adenokarsinom karsinogenezindeki rolünü araştırmayı, apoptotik-nekrotik belirteçler M30 ve M65'in prediktif değeri olup olmadığını belirlemeyi ve Barrett's mukozasını ve kanserli dokuyu immüno-histokimyasal yöntemle incelemeyi amaçladık.

Yöntem ve gereçler: 18 yaşından büyük ve yeni tanı almış katılımcılara üst gastrointestinal endoskopi ile özofagus doku biyopsisi yapıldı. Kontrol grubu olarak Barrett's özofagusu olan 20, özofagus kanseri hastası ve 20 gastroözofageal reflü hastalığı olan hasta vardı. Alınan doku örneklerinden M30 ve M65 immunohistokimyasal yöntemlerle boyandı. Örnekler, M30 ve M65 boyaması açısından gruplar arasında anlamlı bir immünohistokimyasal farklılık olup olmadığını görmek için incelendi.

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Bulgular: Gruplar arasında M30 ekspresyonu açısından istatistiksel olarak anlamlı fark yoktu (p = 0,329). Kontrol ve Barrett's özofagus grupları ile karşılaştırıldığında, adenokarsinom grubunda M65 pozitifliği anlamlı olarak daha yüksekti (p = 0.0001).
Tartışma ve sonuç: Çalışmamızda gruplar arasında M30 ekspresyonunda istatistiksel olarak anlamlı fark yoktu. Özofagus adenokarsinomunda M65 anlamlı olarak yüksek bulundu. Bu, özofagus adenokarsinomunun patogenezinde nekrozun daha baskın olduğunu göstermektedir. M65, özofagus adenokarsinomunda prediktif bir belirteç olarak kullanılabilir.

Anahtar Kelimeler: Barrett Özofagus, Özofagus Kanseri, M30, M65

Introduction

Esophageal cancer is the eighth most common cancer and the sixth most common cause of death worldwide [1]. The major risk factors for esophageal adenocarcinoma are gastro-esophageal reflux disease (GERD) and Barrett's esophagus (BE). Barrett's esophagus develops through the process of metaplasia, in which columnar cells replace squamous cells due to chronic reflux [2]. Reflux of acid and bile salts can cause oxidative DNA damage and double-strand DNA breaks in Barrett epithelial cells, thereby initiating carcino-genesis [3]. The activation–inhibition steps in the apoptosis process play an important role in the onset and progress of carcinogenesis in Barrett's esophagus [4].

Activation of the caspase enzyme family is one of the events that induce apoptosis. Cytokeratins (CK) are from the intermediate filament protein family. They help support the shape and integrity of cells in epithelial tissues. The secretion of CKs from apoptotic or proliferating cells increases. Cytokeratin 18 (CK18) is secreted extensively from epithelial cells of rapidly growing tumours. Its release increases rapidly due to complicated neo-plastic processes [5]. Janti et al. showed that with the serial gene analysis they performed in BE and esophageal cancers, CK18 is strongly stained in all BE and CK20 is expressed more in adenocancer [6,7]. During epithelial cell death, CK18 is cleaved by caspases at aspartate 238 and aspartate 396, resulting in exposure of the CK18Asp396 neoepitope (M30 antigen). In particular, the resulting monoclonal M30 marker identifies and helps measure the cleaved fragment of CK18 at aspartate 396. However, the monoclonal M65 marker measures both intact CK18 and cleaved CK18. While total CK18 (M65) is secreted in all cell deaths, broken CK18 (M30) occurs during apoptosis and is secreted when cells undergo secondary necrosis. An increase in the M30–M65 ratio favours apoptosis, while a decrease favours necrosis [8]. Thus, monoclonal M30 and M65 antibodies can be used as markers of apoptotic–necrotic epithelial cell death [9].

Serum levels of M30 and M65 have been examined in a number of previous studies that evaluated the apoptosis process in various malignancies [10,11,12]. Although many protein–genetic markers have been studied in esophageal adenocarcinomas such as the caspas family or interleukins, there is no prognostic or diagnostic marker currently available for clinical use [13,14]. This study aimed to investigate the role of apoptosis in Barrett's esophagus and adenocarcinoma carcinogenesis, determine whether there is a predictive value of apoptotic–necrotic markers M30 and M65 and examine Barrett's mucosa and cancerous tissue by the immuno-histochemical method.

Materials and methods

Selection of Patients

This study included 20 patients with GERD who underwent upper gastrointestinal endoscopy, the histopathology of which showed esophageal tissue without Barrett or

cancer, 20 patients with esophageal cancer and 20 patients with Barrett's esophagus, older than age 18, who were admitted to the gastrointestinal clinic with the appropriate medical indications between 2008–2015. Tissue samples taken from the pathology clinic archive were re-evaluated and stained with M30 and M65 by immunohistochemical methods. Patients' demographic data, medical history and endoscopy reports were recorded by file scanning. Those with other known malignancies, active inflammatory diseases or infectious diseases were excluded from the study. Our retrospective study was approved by the local ethics committee (B10.4 İSM.406.68.49 11.3.2015 Keçiören Education and Research Hospital Ankara).

Immunohistochemical Method

For immunohistochemical staining, 4 mm thick sections of epithelial cells from each formalin-fixed, paraffin-embedded uterine horn were used. Tissue sections were deparaffinized in xylene, rehydrated in a graded series of alcohol and immersed in distilled water. Endogenous peroxidase activity was blocked by incubating the sections in 1% hydrogen peroxide (v/v) in methanol for 10 min at room temperature (RT). The sections were subsequently washed in distilled water for 5 min, and antigen retrieval was performed for 3 min using 0.01 M citrate buffer (pH 6.0) in a domestic pressure cooker. The sections were transferred into 0.05 M Tris–HCl (pH 7.6) containing 0.15 M sodium chloride (TBS). After being washed in water, the sections were incubated at RT for 10 min with super block (SHP125) (ScyTek Laboratories, USA) to block nonspecific background staining. The sections were then covered with the primary antibodies diluted 1:100 for M30 and 1:50 for anti-M65 in TBS at 48 C overnight (Santa Cruz Biotech Inc., Europe). After being washed in TBS for 15 min, sections were incubated at RT with biotinylated link antibody (SHP125) (ScyTek Laboratories, USA). This was followed with Streptavidin/HRP complex (SHP125) (ScyTek Laboratories, USA). Diamino-benzidine was used to visualise peroxidase activity in the tissues. Nuclei were lightly counterstained with haematoxylin, and then the sections were dehydrated and mounted. Both positive and negative controls were included in each run. Positive controls comprised sections of reactive lymph nodes for M30 and M65. TBS was used in place of the primary antibody for negative controls. The slides were evaluated with the Olympus Bx 51 light microscope. (Olympus Corp., Japan)

Statistical Method

Statistical analyses were performed using the SPSS 16.0 program. Normally distributed numerical data were presented as mean ± standard deviation (SD); non-normally distributed data were presented as median (minimum–maximum). P-values less than 0.05 were considered statistically significant. The correlation between the categorical variables was analysed by Chi-square analysis and between-group comparisons were made by the Kruskall Wallis H test.

Results

Patient Demographics

Our study was performed with tissue preparations taken from 20 patients with BE without dysplasia and 20 with esophageal adenocarcinoma. The control group of GERD patients comprised 20 samples. Of the 60 individuals included in the study, 26 were female and 34 were male. Demographic data on age and gender of the groups are presented in Table 1. There was no statistically significant difference among the groups in terms of gender distribution (p=0.622). There was a statistically significant difference in age among the groups (p=0.03). The adeno-carcinoma group showed significantly higher age values than the other two groups (Table 1).

Table 1. Demographic comparison of groups

	Barrett	Adenoca	Control	*P*
Patients, *n*	20	20	20	
Age (years) mean (range)	55.4 (32-72)	67 (40-80)	53.8 (35-79)	0.003
Male/Female *n* (%)	11/9 (55/45)	13/7 (65/35)	10/10 (50/50)	0.622

Table 2. Comparison of M30 and M65 staining between groups

		Barrett n(%)	Adeno ca n(%)	Control n(%)	P
Patients, n		20	20	20	
M30					
	+	0 (0%)	2 (10%)	0 (0%)	0,329
	-	20 (100%)	18 (90%)	20 (100%)	
M65					
	+	1 (5%)	13 (65%)	2 (10%)	0,0001
	-	19 (95%)	7 (35%)	18 (90%)	

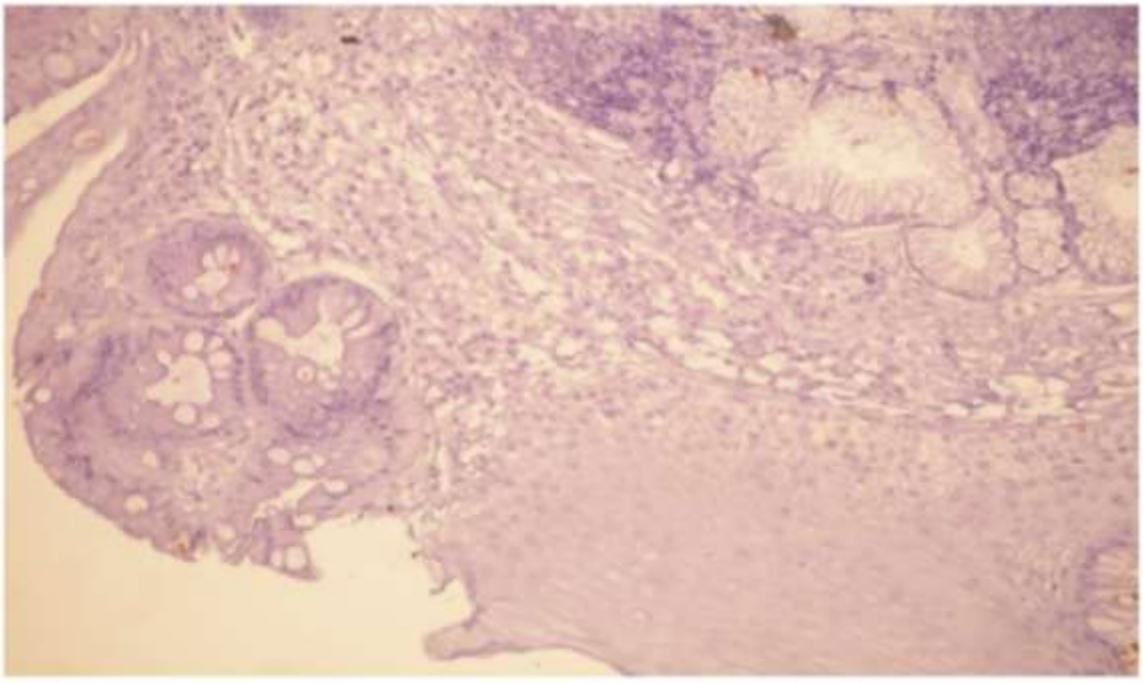

Figure 1. M30 Negative Barrett Esophaguse Area

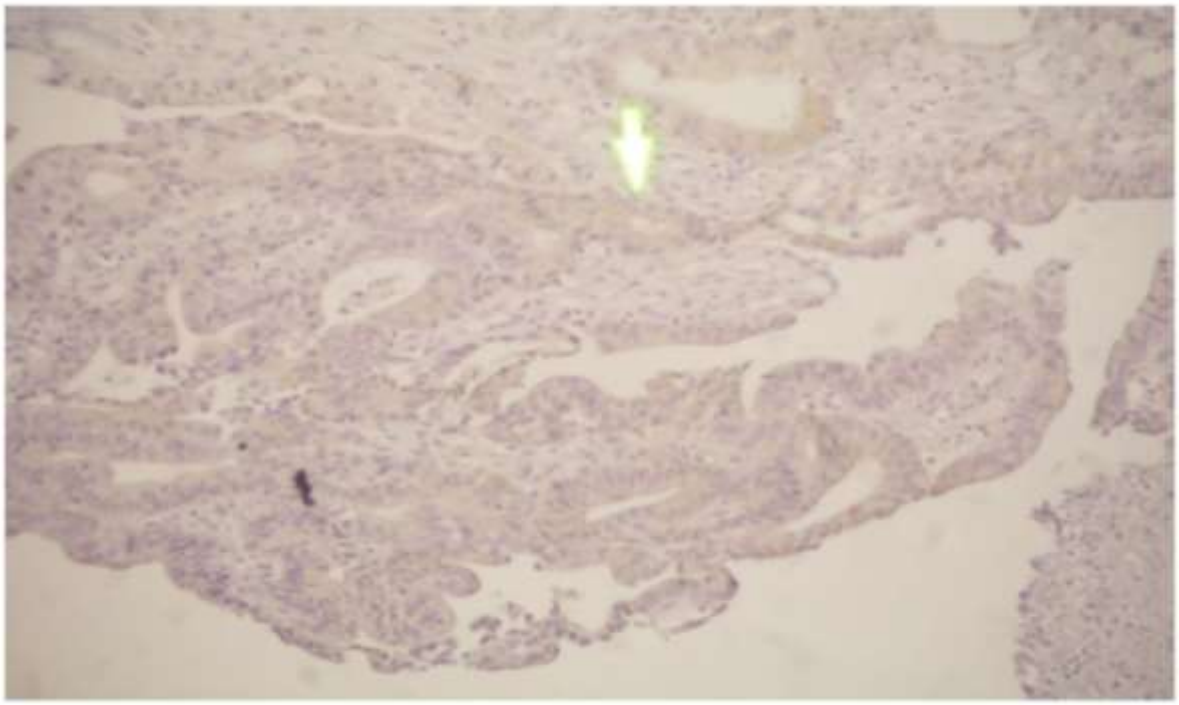

Figure 2. M30 Positive Adenocancer Area

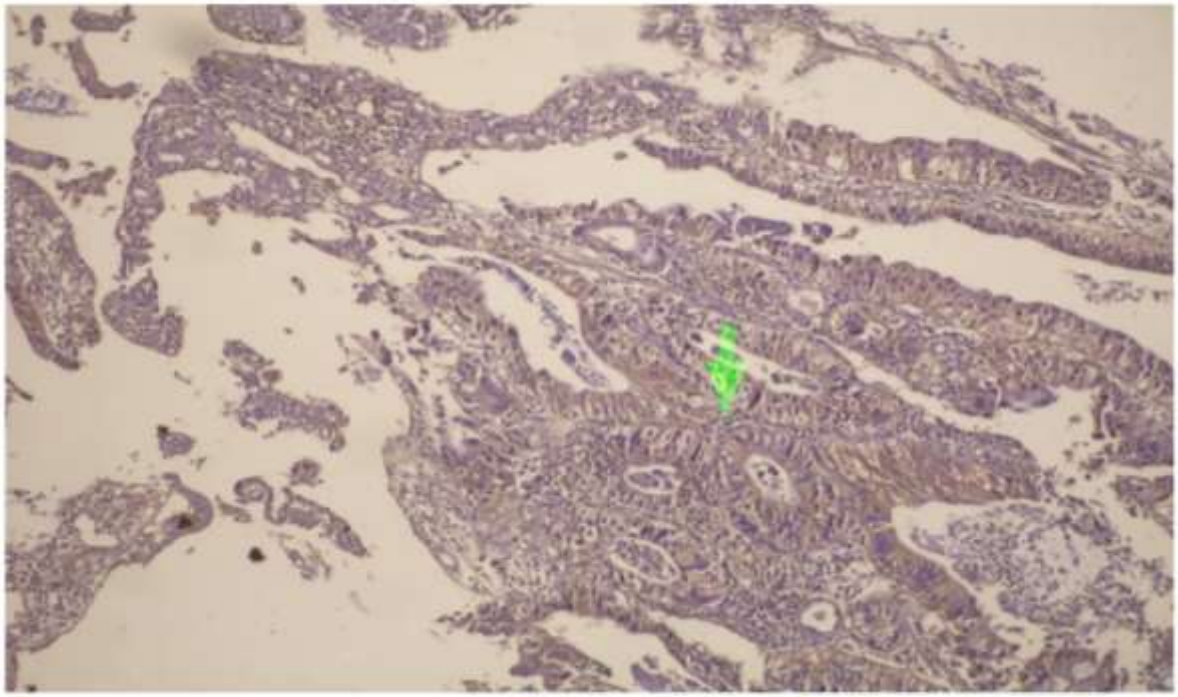

Figure 3. M65 Positive Adenocancer Area

M30 Expression

A total of 3.3% of the cases showed staining with M30. None of the samples in the Barrett's esophagus or the control group were found to be stained, whereas two adenocarcinoma cases (10%) showed staining with M30; there was no statistically significant difference between groups in terms of M30 positivity (p = 0.329) (Figure-1, 2) (Table 2).

M65 Expression

M65 stainings that were evaluated by group showed staining in a total of 26.7% cases. Staining was detected in two control samples (10%) and one Barrett's esophagus sample (5%), and M65 staining was observed in 13 adenocarcinoma cases (65%). M65 positivity was significantly higher in the adenocarcinoma group compared with the control and Barrett's esophagus group (p = 0.0001) (Fıgure-3) (Table 2).

Discussion

M30 expression by the groups showed no statistically significant difference in our study in terms of M30 positivity. M65 expression by the groups was statistically significant in adenocarcinoma cases.

Negative M30 in the control group with GERD patients and Barrett's esophagus suggested that low expression of M30 was present in the esophageal squamous epithelium. M65 was only expressed in one case of GERD and CK18 was usually reported as negative or very low in normal squamous epithelium; this was probably related to the limited use of the M30 pathway in the evaluation of apoptosis in these groups. Also, expression of M30 and M65 might have been limited since the members of the Barrett group did not have dysplasia. In a study in which Dvorakova et al. immunohistochemically investigated apoptosis resistance in BE, biopsy specimens from 10 healthy esophageal squamous epithelium, 13 BE and 4 healthy colon columnar epithelia were examined by seeding the media. Electron microscopy (EM) revealed no apoptotic changes in either the squamous epithelium or the BE epithelium. However, after deoxycholate (DOC), which is both bile acid and an inducer of apoptosis, was added to the media, apoptotic changes were observed in the squamous epithelium in the EM; no changes were observed in BE. When the same tissues were stained with M30, M30 was not expressed in the healthy esophageal squamous epithelium and highly expressed in the DOC-induced healthy colon columnar epithelium; low expression was observed in the DOC-induced BE epithelium. It was thought that M30 is not secreted from the squamous epithelium, which is specific to early apoptotic cells of the glandular tissues; it was also stated that the BE epithelium was resistant to apoptosis [15]. These results are consistent with the absence of M30 positivity in the control group and in the BE cases.

Previous studies showed that the M65 antibody theoretically measures both caspase cleaved and intact CK18; CK18 is usually expressed in glandular epithelium and is found in all BE cases [6]. However, in our study, M65 positivity was detected in only one case of BE. The fact that M65, which reflects the necrotic process, was statistically significant in the adenocarcinoma group compared to the control and the Barrett group can be explained by the fact that the cellular cycles of tumour cells and dysplastic cells are very rapid and high in their production and destruction activities and none of our Barrett patients was dysplastic.

In our study, M30 was only expressed in two patients in the adenocarcinoma group and was not statistically significant; this can be explained by the fact that programmed cell death in cancer cells is inhibited by various mechanisms such as mutations in p53, a tumour suppressor gene, changes in the Fas and FasL molecules that play an important role in the apoptosis mechanism, and

activation of Bcl-2 proto-oncogenin. In a study by Fareed et al. [16], lower M30 staining in the non-chemotherapy group (24.6%) compared with the chemotherapy group (56.7%) and chemotherapy-induced apoptosis that was inhibited in tumour cells also supported this idea. It is also known that cells undergo necrosis instead of apoptosis when cellular Adenosine triphosphate (ATP) production is inadequate [17]. The predominant manner of death might have been necrosis due to the hypoxia of the tumour cells.

In the study by Fareed et al., in which M30 and M65 were examined by the immunohistochemical method to determine the degree of tumour regression by neoadjuvant chemotherapy in patients with gastroesophageal adenocarcinoma, neoadjuvant chemotherapy was not administered to 122 patients with gastric/gastroesophageal carcinoma whereas 97 patients with gastric/ gastroesophageal/ lower esophageal cancer received preoperative platinum-based chemotherapy. M65 was expressed (positive) in most of the patients (92.6%); M30 positivity was detected in 56.7% of patients who received neoadjuvant chemotherapy and 24.6% of those who did not. This was interpreted as the induction of apoptotic cell death in tumour cells exposed to chemotherapy and the expression of M30, an apoptosis marker. Patients who were administered neoadjuvant chemotherapy were found to be correlated with M30 positivity and tumour regression response [16].

M65 may affect carcinogenesis through several signalling pathways, including the phosphoinositide 3-kinase (PI3K)/Akt, Wnt and mitogen-activated protein kinase (MAPK) signalling pathways [18]. Consistent with our study, in previous studies on colonic and gastric cancers, serum M65 was found to be higher than the control group; in gastric and gastroesophageal cancers, M65 was found to be expressed in 92.5% by the immunohistochemical method [8]. In a study by Ausch et al. that examined 62 patients with colorectal cancer and 27 healthy controls, serum M65 levels were higher in patients with cancer, and a significant postoperative decrease was detected in the subgroup of surgery patients (19/31) [19]. In another study by Greystoke et al., M30 and M65 were found to be higher in cancer patients, and M65 tended to increase as the stage progressed [20].

In the literature, there are studies on gastrointestinal cancers that show high serum levels or positively stained M30 by the immunohistochemical method, in contrast to our results. In a meta-analysis that included 11 original studies by Huang et al., the role and prognostic values of M30 and M65 in gastrointestinal cancer were evaluated. Low levels of M30 and M65 were shown to be protective factors for all cancer patients, and low M30 remained a protective factor for metastasized cancer patients [21]. This meta-analysis showed that each cancer has its own specificity. Tumour development, microenvironment, treatment strategies and prognosis are all different in different types of cancer. Also, different measurement techniques for M30 (ELISA, immunohistochemistry) might have caused inconsistent results in different studies. In a study by Brandt et al. on 35 patients with gastrointestinal system cancer, serum M30 levels were found to be higher in cancer patients than in the control group. In the same study, M30 expression was higher in the immunohistochemical examination of tissue samples of patients with colorectal cancer than in the control group. The reason for the positive M30 in serum and tissue in cancer patients is that the apoptosis in the tumour cells continued, though decreased, but apoptosis-resistant cells survived as a result of increased cell turnover [22]. Although not a predominant death pattern in the esophageal adenocarcinoma group, apoptosis was present in varying proportions; however, M30

expression might have been limited to only two patients in our study since the number of patients was not high enough to show this difference and each tumour was at a different stage at the time of histological sampling.

A number of studies have examined the role of serum M30 and M65 in assessing prognosis, survival and response to treatment in gastric cancer. In a study conducted by Oyama et al. that included 54 patients with gastric cancer and 12 healthy controls, M65 was suggested as an independent prognostic indicator [23]. In contrast, some CK18(M65) expressing adenocarcinomas have shown decreased expression with increasing tumour progression, such as breast and colorectal cancer [24,25]. These conflicting results may stem from differences among the types of cancers or among the experimental protocols [20].

There are several potential limitations to this study. The sample size was not adequate and none of our Barrett patients was dysplastic. Additionally this study was a retrospective study with some missing data about patient's tumor stages and treatments protochols.

In conclusion, our study showed that M65 was significantly higher in oesophageal adeno-carcinoma patients. This suggests that necrosis is more dominant in the pathogenesis of oesophageal adenocarcinoma. Despite the fact that many protein–genetic markers have been studied in oesophageal adeno-carcinomas, there is currently no marker for clinical use. M65 can be used as a predictive marker in oesophageal adenocarcinoma. However, there is a need for extensive studies on this topic.

The authors declare that they have no conflict of interest.

Informed consent from patients or families were obtained before the study started.

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