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Original article

## Analysis of tumour markers in esophageal carcinoma with different age groups



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### ABSTRACT

**Background:** Esophageal cancer has been reported in various countries, including Saudi Arabia, India and United States. Many biomarkers have been used to detect cancers at an early stage to improve the survival rate of esophageal cancer.

**Methods:** The present study investigated the biomarkers, biglycan, myeloperoxidase, cytokine (TNF- $\alpha$ , IFN- $\gamma$ , IL-10 and IL-12) and carcinoembryonic antigen (CEA), squamous cell carcinoma antigen (SCC) and carbohydrate antigen L1 cell adhesion molecule from the tumour and non-tumour samples.

**Results:** The increased amount of circulatory biglycan level was detected using the ELISA method in tumour cases compared to control samples ( $p < 0.001$ ). The expression of biglycan was high in certain tumour cases and was statistically significant ( $p < 0.01$ ). The variation of myeloperoxidase markers in control and experimental subjects were statistically significant ( $p < 0.01$ ). Among cancer patients, elevated levels of carcinoembryonic antigen ( $28.3 \pm 1.2$  ng/ml and  $30.3 \pm 1.1$  ng/ml), squamous cell carcinoma antigen ( $5.3 \pm 1.1$  ng/ml and  $4.9 \pm 0.5$ ) and carbohydrate antigen 19-9 ( $18.3 \pm 1.1$  ng/ml and  $15.1 \pm 2.6$  ng/ml) were detected in male and female cases ( $p < 0.001$ ). In the normal tissues, the expression of L1 cell adhesion molecule was high and was expressed in low amount in tumour tissues in both male and female individuals ( $< 0.001$ ).

**Conclusion:** These biomarkers are useful for the early diagnosis of esophageal cancer.

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## 1. Introduction

Esophageal cancer accounts for about 1 % of the total number of cancer patients in various developed countries. Its prevalence in other countries varies, and maximum incidences are reported in Africa, Asia, and Iran. Moreover, the total incidence of esophageal cancer increases continuously and is mainly associated with the increasing incidence of adenocarcinoma (AC) (Ishibashi et al., 2020). In the United States, the incidence of AC and squamous cell carcinoma (SCC) are equal. Furthermore, increased SCC incidence was registered in the 1960's. The types of carcinoma varied widely based on demographics. SCC and AC types are almost common in

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men; moreover, SCC has increase prevalence in urban cases in African-Americans, whereas AC is a major disease in Caucasians. Despite the development of new treatment strategies, the change in survival rate is very small. The five-year survival for both SCC and AC was less than 29 % (Qin et al., 2022). The incidence of AC increased considerably over the past three decades, and this sudden rise showed much interest in studying various risk factors associated with esophageal cancer. Smoking is one of the important risk factors associated with AC and SCC, and increased consumption of alcohol is mainly associated with SCC. Dietary factors such as consumption of betel nuts, *N*-nitroso compounds, beverages, and hot foods are associated with the development of SCC. Obesity is another reported factor and a body mass index (BMI) > 30 was associated with esophageal AC (Zhao and Lim, 2020).

Many esophageal cancers are diagnosed at later stages. Early stage carcinomas are frequently treated with multimodality therapy to improve the survival of individuals. Despite continuous effort, >50 % of recurrent cases have been reported. Abdominal, thoracic, and cervical tumours can be treated with multimodality strategies (Crosby et al., 2009). Treatment output from neoadjuvant chemoradiotherapy and neoadjuvant chemotherapy is not effective in improving the survival rate of patients (Li et al., 2016). There is an urgent need to develop a non-invasive, low-cost, convenient method for the early detection of esophageal cancer. The determination of biomarkers in serum samples is one of the simple and convenient methods for some tumours and in monitoring metastasis or recurrence. The serum biomarkers are defined as the molecules in the serum whose levels can be applied to determine certain cancers, prognosis, diagnosis and clinical management of malignant diseases (Li et al., 2022). In recent years, various biomarkers have been proposed in the diagnosis and treatment of esophageal cancer, including, squamous cell cancer antigen (SCC-Ag), carcinoembryonic antigen (CEA), etc. (Zhang et al., 2015a,b).

Genetic markers are frequently used for the determination of somatic tumours. However, it has certain limitations due to decreased detection limit in the case of low tumour load in early stages of cancer (Shain et al., 2015). Hence, it is very clear that the genetic markers are considered to lack of sensitivity and specificity for cancer types. Epigenetic markers, mainly DNA methylation are considered one of the important markers for early determination of cancer. This method is useful to study the occurrence, cancer-specific methylation patterns, technical repeatability, and biological stability (Koch et al., 2018). Methylation-based biomarkers are helpful for the diagnosis and prediction of survival of solid tumours. DNA methylation markers are widely used to differentiate tumour tissues and normal tissues in various types of cancers, including, liver, lung, colon, and breast cancer (Hao et al., 2017). Moreover, type-specific epigenomic markers for prognosis and diagnosis of esophageal cancer were not completely elucidated. Aberrant methylation has been frequently reported in various types of cancers, including esophageal cancer, which greatly contributes to carcinogenesis (Kou et al., 2019). In this study, cancer biomarkers were used for the early determination of esophageal tumours.

## 2. Materials and methods

### 2.1. Blood samples

The blood samples were collected from cancer patients with written informed consent. The sample details, such as sex, age, type of specimen, and non-tumour specimens, were collected from

the individuals. Control samples were collected from healthy individuals matched with a pre-determined age.

### 2.2. Tissues

Tissue samples were collected from patient's tumour specimens and control non-tumour specimens from the adjacent places. These tissue samples were collected from patients who underwent treatment with written consent. Ethical approval was obtained from the Institute Ethical Committee (Approval number: SDB-152/2022).

### 2.3. Determination of biglycan from the sample

An enzyme-linked immunosorbent assay (ELISA) was used to determine biglycan from the blood samples using an ELISA kit as suggested by the manufacturer. The concentration of biglycan was determined and the intensity of the reaction was measured at 450 nm against appropriate controls (Zaidi et al., 2014).

### 2.4. Isolation of RNA from the tissues and real time reverse transcription polymerase chain reaction (RT-PCR)

The collected frozen tissues were used for the isolation of RNA using a RNA extraction kit as suggested by the manufacturer's instructions. The extracted RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific™, USA). A total of 2 mg of total RNA from the tissue sample was used to convert cDNA. The quantitative real-time reverse transcription polymerase analysis was performed by a polymerase chain reaction machine using the forward and reverse primers. The primers used were, 5'AGGTGCCCAAGGGAGTGTC 3' (forward) and 5'TGGTCTAGGTGGAGTTCATTCAGG 3' (reverse) primers. PCR reactions were performed and the products were analyzed. B-actin was used as the positive control (Johnson et al., 2000).

### 2.5. Western blotting analysis

Tumour (experimental) and non-tumour (adjacent control) tissues were lysed using radioimmunoprecipitation assay lysis buffer (RIPA lysis buffer). RIPA buffer composed of (1 % Triton X-100, 0.15 M NaCl, 0.1 % sodium dodecyl sulphate (SDS), 0.5 % deoxycholate, 0.05 M Tris, pH 7.4) containing appropriate quantities of protease inhibitors and ethylenediaminetetraacetic acid. The total protein content of the lysed sample was determined by the Bradford method using bovine serum albumin standard. The protein sample from control and experimental tissue was resolved using the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (11 % separating gel and 5.5 % stacking gel). The separated protein was blotted from the gel onto a nitrocellulose membrane using electro blotting method. The nitrocellulose membrane of the remaining unbound regions was blocked using bovine serum albumin (5 %) and incubated in buffer B (Tris-buffered saline, pH 7.4, 0.1 % Tween 20) for 3 h. Anti-β actin primary and secondary antibodies were used for the determination of β actin. Biglycan primary antibodies were incubated for overnight at 4 ± 1 °C and horse-radish peroxidase conjugated secondary antibody were incubated for 2 h (Sinkala et al., 2017).

### 2.6. Myeloperoxidase activity in plasma

The amount of myeloperoxidase was analysed using an ELISA assay method as suggested by the manufacturer (Bio Rad). To perform the assay, anti-MPO autoantibodies were inactivated from the plasma samples. Experiments were performed in duplicates and the results were expressed as mean ± SD (Goiffon et al., 2015).

### 2.7. Cytokine analysis

Blood samples were used for the determination of cytokine levels. The collected blood was centrifuged at 3000 rpm for 10 min, and the serum sample was used. An ELISA kit was used for the determination of IL-10, IFN- $\gamma$ , TNF- $\alpha$  and IL-12 (Asselin-Paturel et al., 1998).

### 2.8. Analysis of serum carcinoembryonic antigen (CEA), squamous cell carcinoma antigen (SCC) and carbohydrate antigen (CA) 19-9

Serum samples were used for the determination of tumour makers such as CEA, SCC and CA 19-9. The serum antigens (ng/ml) were estimated using a commercial immunoassay kit. The amount of CEA above 5 ng/mL and CA 19-9 and SCC above 1.5 ng/mL was considered as CEA positive, CA 19-9 were positive (Mino-Miyagawa et al., 1990).

### 2.9. L1 cell adhesion molecule (L1CAM) assay

An Enzyme-linked immunosorbent assay was used for the determination of L1 cell adhesion molecule. Briefly, ELISA plate was coated with antibody and incubated at 4 °C. After 1 h of incubation, the microtitre plate was washed and incubated with blocking buffer for 1 h. After clear washing, 100  $\mu$ l was incubated for 2 h at 28  $\pm$  1 °C. L1CAM was used for the preparation of the standard curve at various concentrations. The colour intensity was read at 450 nm against control as described by the manufacturers protocol (Ichikawa et al., 2019).

### 2.10. Statistical analysis

The results were expressed as mean  $\pm$  SD. The statistical significance between non-tumour and tumour was analysed using Student's *t*-test. The *p* value < 0.01 was considered statistically significant.

**Table 1**  
Biglycan level from the normal and cancer cases.

Characters	Biglycan (ng/ml)		
	Cancer patients (n = 60)	Control (n = 12)	
Sex			
Male (n = 50)	0.873 $\pm$ 0.031	0.61 $\pm$ 0.04	<0.001
Female (n = 22)	0.764 $\pm$ 0.02	0.562 $\pm$ 0.03	<0.001
Age group-Male			
45–60	0.951 $\pm$ 0.05	0.54 $\pm$ 0.01	<0.001
61–75	0.892 $\pm$ 0.0	0.57 $\pm$ 0.04	<0.002
76–90	0.876 $\pm$ 0.07	0.62 $\pm$ 0.05	<0.003
Age group-Feale			
45–60	0.748 $\pm$ 0.28	0.538 $\pm$ 0.04	<0.005
61–75	0.737 $\pm$ 0.16	0.541 $\pm$ 0.02	<0.006
76–90	0.738 $\pm$ 0.0	0.549 $\pm$ 0.05	<0.007

The result was expressed as mean  $\pm$  standard deviation.

## 3. Results

### 3.1. Biglycan level in blood samples

ELISA test was used for the determination of biglycan from the non-tumour and tumour samples. The circulatory biglycan level was higher in diseased patients than in control samples (*p* < 0.001). The amount of biglycan level was elevated in tumour tissues (Table 1).

### 3.2. Western blotting analysis

Western blotting analysis was performed to analyse the expression of biglycan in tumour and non-tumour tissues. Fig. 1 depicted the expressed level and the biglycan and control  $\beta$ -actin. The expression of biglycan was high in certain tumour cases and was significant (*p* < 0.01). The expressed biglycan was low in non-tumour cells than tumour tissues (Fig. 1).

### 3.3. Myeloperoxidase activity of plasma in tumour and non-tumour cases

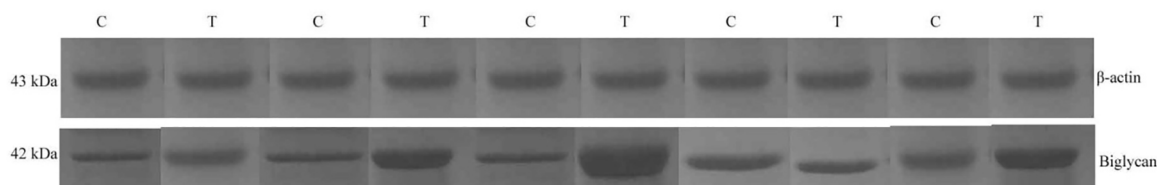
The amount of myeloperoxidase was decreased in cancer cases than non-tumour individuals. In the non-tumour group, myeloperoxidase levels ranged from 114.1  $\pm$  1.2 ng/ml to 118.4  $\pm$  10.4 ng/ml. In control subject myeloperoxidase levels were considerably lower. The variation of myeloperoxidase in control and experimental subjects were statistically significant (*p* < 0.01). The difference in myeloperoxidase level as a function of age was not significant (*p* > 0.01) (Table 2).

### 3.4. The cytokine level in tumour and non-tumour tissues

TNF- $\alpha$  level was elevated over tenfold in tumour group and statistical significance was observed between two groups. The Student's 't' test revealed a significant difference between the control and the experimental group (*p* < 0.0001). In serum, the amount of IFN- $\gamma$  was increased considerably than non-tumour control (*p* < 0.0001). The mean concentrations of IL-10 and IL-12

**Table 2**  
Myeloperoxidase profile of tumour and control group.

Characters	Myeloperoxidase (ng/ml)	
	Tumour (n = 60)	Non-Tumour (n = 12)
Male (n = 40)	20.3 $\pm$ 1.1	118.4 $\pm$ 10.4
Female (n = 22)	27.3 $\pm$ 2.5	117.1 $\pm$ 1.2
Age group-Male		
45–60	35.4 $\pm$ 2.2	114.1 $\pm$ 1.2
61–75	34.8 $\pm$ 2.3	117.1 $\pm$ 2.7
76–90	33.8 $\pm$ 1.1	116.1 $\pm$ 2.2
Age group-Female		
45–60	37.3 $\pm$ 1.3	112.3 $\pm$ 8.8
61–75	31.2 $\pm$ 2.2	116.2 $\pm$ 1.7
76–90	30.4 $\pm$ 1.9	115.7 $\pm$ 2.9



**Fig. 1.** Western blotting analysis of biglycan in oesophageal carcinoma and adjacent normal tissues.

elevated about 2-fold in tumour group than control ( $p < 0.001$ ) (Table 3).

3.5. Determination of carcinoembryonic antigen (CEA), squamous cell carcinoma antigen (SCC) and carbohydrate antigen (CA) 19-9 in the serum sample

The positive esophageal cancer was determined based on the amount of CEA, SCC and CA in the serum sample. CEA was  $95 \pm 2.3 \%$ ,  $68.9 \pm 4.7 \%$ , respectively for the male and female patients. CA 19-9 was considered as one of the important markers and was detected in the blood of  $85.4 \pm 2.8 \%$  and  $80.1 \pm 3.1 \%$ , male and female cases. Among cancer patients, elevated levels of CEA ( $28.3 \pm 1.2$  ng/ml and  $30.3 \pm 1.1$  ng/ml), SCC ( $5.3 \pm 1.1$  ng/ml and  $4.9 \pm 0.5$ ) and CA 19-9 ( $18.3 \pm 1.1$  ng/ml and  $15.1 \pm 2.6$  ng/ml) were detected in male and female cases ( $p < 0.001$ ). Moreover, the amount of these antigens did not vary significantly based on age group and the results (Table 4).

3.6. L1 cell adhesion molecule as a marker for early diagnosis of cancer

Expression of L1CAM protein in the serum sample of cancer tissue and normal tissues were studied. In the normal tissue, the amount of L1CAM protein was high and was expressed in a low amount in the cancerous tissue in both male and female individuals ( $<0.001$ ). Moreover, the amount of L1CAM was not varied significantly between male and female cases. L1CAM expression changes were observed between cancer and normal tissue in all selected age-groups (Table 5).

4. Discussion

In cancer patients, inflammation plays a critical role in each stage of the cancer development. Inflammatory cells or tumour cells are able to indirectly or directly stimulate or inhibit tumour growth. In our study, cytokines levels were examined in the control and tumour groups and an elevated level were observed. TNF- $\alpha$ , IFN- $\gamma$ , IL-10 and IL-12 level increased in the tumour cases. IFN- $\gamma$  and IL-10 were played critical role in tumour cell growth. IFN- $\gamma$  induced apoptosis and inhibited tumour cell growth (Wall et al., 2003). IL-10 regulated cell-mediated immunity and it induced tumour growth (Ueda et al., 1994). In this study, elevated level of IL-20 was registered in cancer patients than in non-tumour control and the enhanced level was positively correlated. IL-12 activated antigen-specific and innate adaptive immunity against tumour tissues and inhibited angiogenesis via IFN- $\gamma$ . In tumour samples, the maximum level of IL-12 indicated the sign of good survival. The amount of MPO was found to be less in tumour cases than non-

Table 3  
The cytokine level in tumour and control patients.

Cytokines (pg/ml)	Tumour group (n = 60)	Non tumour group (n = 12)
TNF- $\alpha$	64.2 $\pm$ 4.8	6.18 $\pm$ 0.09
IFN- $\gamma$	72.6 $\pm$ 1.6	4.02 $\pm$ 0.08
IL-10	24.7 $\pm$ 0.7	10.3 $\pm$ 0.65
IL-12	32.4 $\pm$ 0.28	16.2 $\pm$ 0.14

Table 4  
Analysis of carcinoembryonic antigen, squamous cell carcinoma antigen and carbohydrate antigen 19-9 in the serum sample.

Patients and age group	CEA		SCC		CA 19-9	
	%	ng/ml	%	ng/ml	%	ng/ml
Male (n = 50)	95 $\pm$ 2.3	28.3 $\pm$ 1.2	98.4 $\pm$ 2.7	5.3 $\pm$ 1.1	85.4 $\pm$ 2.8	18.3 $\pm$ 1.1
Female (n = 22)	68.9 $\pm$ 4.7	30.3 $\pm$ 1.1	99.3 $\pm$ 1.1	4.9 $\pm$ 0.5	80.1 $\pm$ 3.1	15.1 $\pm$ 2.6

Table 5  
Expression of L1CAM protein in the serum sample of cancer tissue and normal tissues. The result was expressed as mean  $\pm$  standard deviation and the expressed protein level was compared with control.

	Cancerous tissue	Normal tissue	p-value
Patients and age group			
Male (n = 50)	31.2 $\pm$ 2.4	48.3 $\pm$ 2.2	<0.001
Female (n = 22)	30.9 $\pm$ 2.7	47.9 $\pm$ 1.3	<0.001
Age group			
45-60	28.2 $\pm$ 1.4	49.1 $\pm$ 2.1	<0.001
61-75	31.9 $\pm$ 2.2	48.4 $\pm$ 1.3	<0.002
76-90	34.2 $\pm$ 1.5	49.3 $\pm$ 1.2	<0.003

tumour healthy cases. Myeloperoxidase deficiency has been linked with cancer disease and has been reported previously (Aratani et al., 1999).

Myeloperoxidase catalyses aromatic amines and polycyclic aromatic hydrocarbons and is involved in the biosynthesis of carcinogenic reactive metabolites (Kadlubar et al., 1992). In our study, the inflammatory marker, MPO level decreased and the analysis of this marker may provide a good understanding of oesophagus cancers as tumour markers. MPO is generally present in the neutrophils and diffuses into inflamed tissues.

Carbohydrate antigens are widely used to determine breast, pancreatic, and colon cancers. The amounts of CEA, SCC and CA in the blood sample were quantified. Many studies have conducted to analyse the tumour and prognostic markers in cancer. Niho and Shinkai (2001) and Charalabopoulos et al. (2007) elevated CEA level in BAL fluid in patients have been determined. Moreover, Lazarev et al. (2010) revealed no significant changes of CEA in normal and tumour cases. In diagnosed tumour cases, elevated levels of CA125 and CEA have been reported (Cedrés et al., 2011). The mortality and morbidity of cancer increased in recent times due to delayed diagnosis. In gastric cancer, environmental factors and Helicobacter pylori infection were determined as the main causes of infection (Kusano et al., 2017). In recent years, serum tumour markers were widely used to determine types of cancers. Tumour markers directly reflected cancer and the amount of markers revealed the severity of disease. L1CAM promoted the remodelling of various extracellular matrixes and improved the chemotaxis of tumour cells to the extracellular matrix, promoting tumour invasion and metastasis (Raveh et al., 2009). The expression of L1CAM protein changes has also been studied to analyse the malignancy and prognosis of melanoma, endometrial and ovarian tumours (Geels et al., 2016). In this study, the level of L1CAM protein in oesophageal cancer was analysed in cancer tissues and adjacent normal tissues of patients with oesophageal cancer and the results revealed a moderately elevated level of L1CAM in cancer tissues were compared to normal tissues. The present finding reveals that L1CAM is fairly involved in the development of oesophageal cancer. Recent findings revealed that L1CAM level was elevated in certain tumours than normal tissues, the level of L1CAM was elevated in ovarian, uterine and gastrointestinal stromal tumors, moreover reduced level of expression was observed in esophageal squamous cell carcinoma, which was consistent with the present study (Zhang et al., 2015a,b). In this study, the amount of L1CAM was less in patients diagnosed with esophageal cancer than



in healthy controls ( $p < 0.001$ ). The variation of L1CAM was mainly associated with variations in the expression patterns of L1CAM in various types of cancer (Xu et al., 2018).

## 5. Conclusions

In this study, we observed expression changes of biglycan, myeloperoxidase, cytokines, carcinoembryonic antigen (CEA), squamous cell carcinoma antigen (SCC) and L1 cell adhesion molecule in esophageal cancer. Analysis of one tumour marker is not recommended to differentiate the cancer cells. Expression of biglycan is one of the classical methods for the determination of esophageal cancer and an elevated level was observed. The amount of myeloperoxidase, cytokine (TNF- $\alpha$ , IFN- $\gamma$ , IL-10 and IL-12) in blood influenced significant variation in normal tissues and cancer cells. In normal tissue, the amount of expression of L1 cell adhesion molecule protein was high and was expressed in a low amount in cancerous tissues.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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