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Expression of Thymidylate Synthase and Thymidine Phosphorylase as Prognostic Markers in Advanced Esophageal Squamous Cell Carcinoma

[1,2]Arbabi Bidgoli, Sepideh, [3]Minaee, Bagher, [1]Ghahremani, H. Mohammad, [4]Djamali Zavarhei, Mansoor, [1]Ostad, Seyed Nasser, [1]Fouladdel Shamileh and [1]Azizi, Ebrahim
[1]Molecular Research Laboratory, Department of Pharmacology and Toxicology,
Faculty of Pharmacy, Tehran University of Medical Sciences,
[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Islamic Azad University,
[3]Department of Anatomy and Embryology, [4]Department of Pathology,
Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran

Abstract: There are controversial data regarding the correlation between gene expression of Thymidylate Synthase (TS) and Thymidine Phosphorylase (TP) with response to chemotherapy and patients' survival in gastrointestinal malignancies. Therefore, the prognostic role of TS and TP in GI malignancies and in particular, the Esophageal Squamous Cell Carcinoma (ESCC) needs to be more clarified. The TS and TP status in ESCC was determined and correlated molecular alterations with clinicopathological findings. Tumor samples of 30 surgically resected patients were analyzed by immunohistochemical techniques using primary antibodies for TS and TP and Dako labeled Streptavidin-Biotin® 2 (LSAB2) System detection kit. TS and TP were positive in 63.3 and 89.7% of tumor samples, respectively. Unlike TS, expression of TP was correlated with tumor size ($p = 0.013$). Although the most prevalent immunophenotype was TS^+/TP^+ (16/30; 53.33%) but the other immunophenotype (TS^-/TP^+; 10/30; 33.32%) also showed significant relationship with lymphatic invasion ($p = 0.035$) and histological differentiation ($p = 0.001$). Despite low number of metastatic cases, four cases out of five had positive expression of both TS and TP in their primary tumor samples. The high TP expression (89.1%) and its established role in tumor angiogenesis could be the reason for its correlation with tumor size and also progression of early stage of ESCC to the advanced stage. Based on present data as well as others, the overexpression of both TS and TP would indicate a poor prognosis for patients with ESCC. On the other hand, our data showed that 36.7 and 10.3% of patients were TS and TP negative, respectively, that obviously affect their response to 5-FU based chemotherapy. In conclusion, the present data showed the importance of TS and TP as valuable prognostic markers that are in correlation with clinicopathological findings.

Key words: Esophageal squamous cell carcinoma, thymidylate synthase, thymidine phosphorylase, prognostic markers

INTRODUCTION

Esophageal Squamous Cell Carcinoma (ESCC) is the predominant histological subtype of esophageal cancers and characterized by high mortality rate and geographical differences in incidence[1]. Despite marked advances in surgical therapy of ESCC the overall prognosis and survival of these patients have not markedly improved during the past decades[2]. Recent improvements in molecular biology have refined the mechanisms of action and resistance to therapy for a number of therapeutic regimens including 5FU, but little data exist in this regard for esophageal cancer[3]. Therefore, future improvements in the chemotherapy response and overall survival of patients with ESCC could only be achieved by individualized therapeutic strategies based on the status of prognostic and predictive markers.

At present, fluoropyrimidines are one of the standard regimens for the treatment of esophageal carcinoma[4]. Several molecules have been under investigation as potential predictors of response to fluoropyrimidines including Thymidylate Synthase (TS) and Thymidine Phosphorylase (TP). The gene expression levels of Thymidylate Synthase (TS) and Thymidine

Corresponding Author: Ebrahim Azizi, Director of Molecular Research Laboratory,
Department of Pharmacology and Toxicology, Faculty of Pharmacy,
Tehran University of Medical Sciences, Tehran, P.O. Box 14155-6451, Iran
Tel/Fax: (+98 21) 695 9100, (+98 21) 646 1178 E-mail: aziziebr@sina.tums.ac.ir

Phosphorylase (TP) are associated with controversial responses to chemotherapy and also patients' survival in gastrointestinal malignancies[5] but their prognostic roles in ESCC remained unclear[1]. Expression of TS protein is associated with poor response to 5-fluorouracil (5-FU) in human colorectal, gastric, head neck and breast carcinomas[6], while there is no data supporting the predictive role of TS expression in esophageal cancers. Thymidine Phosphorylase (TP)/platelet-derived endothelial growth factor (PD-ECGF), is also involved in transformation of 5FU and fluoropyrimidinic agents into active cytotoxic metabolites[7-10]. Higher expression of TP is associated with an increase of Intratumoral Micovessel Density (IMVD), p53 expression and an unfavorable patient prognosis[11]. Since TS and TP could be identified as two critical enzymes in patient clinical evaluation and tumor biology of ESCC, in this study we have immunohistochemically assessed the TS and TP expression as single or double markers and determined their relationships to the available clinicopathological parameters.

MATERIALS AND METHODS

Patients and sample characteristics: Tissue samples of 30 patients with esophageal squamous cell carcinoma were randomly selected for this study. Samples of esophageal tumors were obtained from patients who underwent surgery at two different university hospitals (Shohadaye Tajrish and Imam Khomeini) during the year 2000-2003. The patients included were Iranian from different geographical locations within Iran. Based on the designed questionnaire, data were collected for age at surgery, sex and pathological diagnosis. Histopathological data contained tumor anatomical location, tumor pathological type, tumor size, histological differentiation (malignancy grade), stage and lymphatic invasion.

TS and TP immunohistochemical analyses: As previously described[12], dewaxed and rehydrated tissue sections were subjected to antigen retrieval using microwave oven and boiling citrate buffer (pH = 6.0). Endogenous peroxidase activity and nonspecific binding sites were blocked by incubating sections in 3% hydrogen peroxide in methanol for 30 min and 5% BSA for 60 min, respectively. Sections were then incubated overnight at 4°C with TS mouse monoclonal antibody (TS 106, Lab Vision Corporation,USA) that recognizes nuclear and cytoplasmic expression of the human TS protein. In case of TP we used the mouse monoclonal antibody (P-GF.44C, LabVision Corporation, USA) that reacts with wild type TP protein and recognizes nuclear and cytoplasmic expression of the human TP protein. The primary antibodies were used at dilution of 1:40 for TS and 1:100 for TP. The results were visualized using the Dako labeled Streptavidin-Biotin® 2 System detection kit (LSAB2; Dakocytomation-Denmark) and DAB chromogen (Dakocytomation-Denmark) based on the manufacturer's instruction with necessary modifications. Sections were also counterstained with Meyer's hematoxyline. In each series, a section in which incubation with the primary antibody was omitted used as negative control. The ideal staining conditions were established in our preliminary experiments. The areas of highest protein expression evident at low-power scanning were taken for analysis. Staining was considered negative only after careful examination of the entire tissue section. Quantitation of the intensity and number of positive tumor cells was performed by two independent pathologists (B.M. and M.D.) blinded to the clinical outcome. In cases in which the investigators disagreed, the immunohistochemical scoring was repeated to agree on same scoring by both observers. Tumor samples were then classified into four categories based on the nuclear and cytoplasmic expression of TS and TP. Tumor cells were scored as 3+ if they had strong staining (>50%) and nuclear/cytoplasmic expression of makers, 2+ if they had moderate staining (25-50%) and only cytoplasmic expression, 1+ if they had mild cytoplasmic staining (5-25%) and 0 if the expression was negative or <5% staining.

Statistical analyses: For the statistical analyses, descriptive data were expressed as the mean±SD. In order to compare the immunostaining patterns of TS and TP in relation to categorical variables, chi-square (χ^2) test was applied using SPSS10 software[13]. The significant relationships between TS and TP expression and between each marker expression with tumor anatomical location, size, histological differentiation, lymphatic and secondary organ metastasis, as well as patients' gender and age at surgery were statistically evaluated.

RESULTS

Patients' characteristics: This study included 30 post-operative esophageal squamous cell carcinoma, 17 (56.7%) males and 16 (43.3%) females. The mean age of these patients was 59 yr±12.6709 (range: 34-80). These patients had received no prior chemotherapy or radiotherapy before surgery. No one had familial history of esophageal cancer, history of background diseases and/or history of addiction to alcohol and abuse-substances.

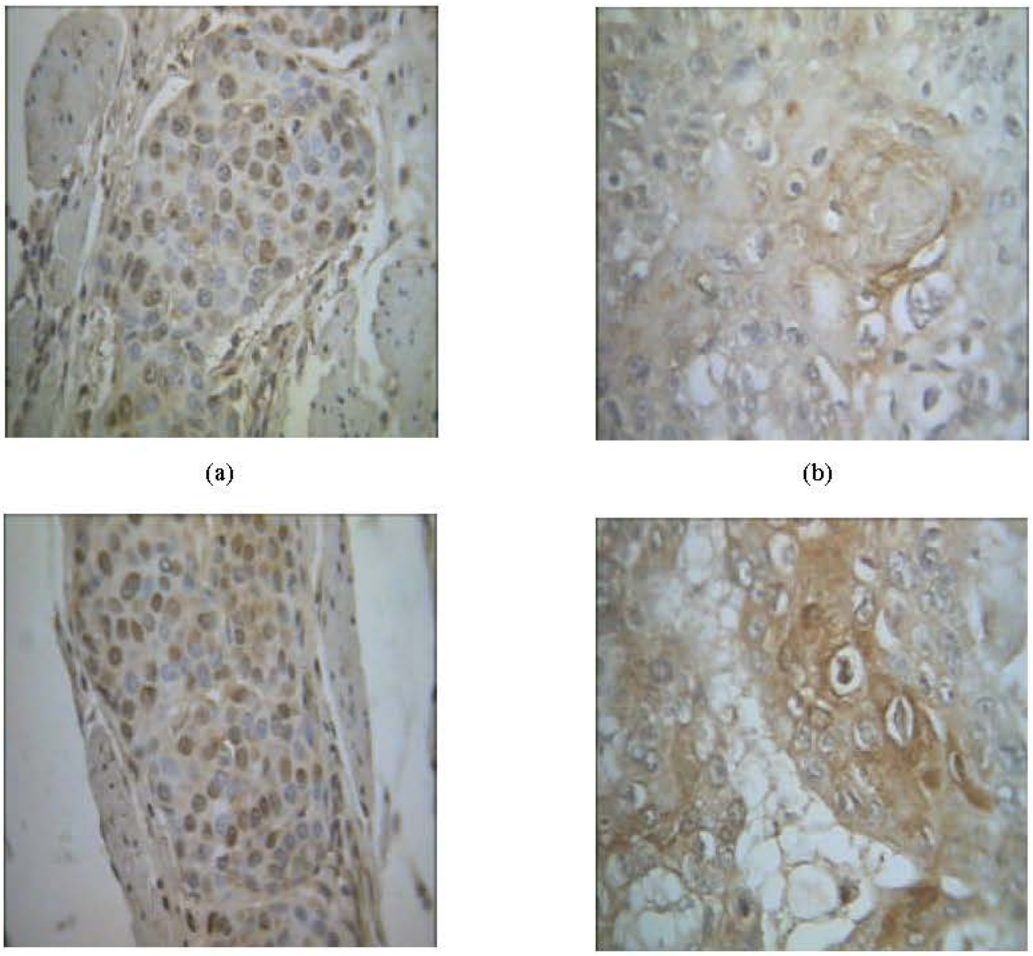

Fig. 1: Immunohistochemical staining of TS and TP in tumor samples of patients with ESCC. Tumor sections were stained using primary monoclonal antibodies for TS and TP as described in materials and methods. a) Strong nuclear staining of TS, b) Moderate cytoplasmic staining of TS, c) Strong nuclear staining of TP and d) Moderate cytoplasmic staining of TP (magnification 400x)

Table 1: Clinicopathological features of ESCC patients

Age: (mean±SD)	59 yr±12.67 (range: 34-80)	Tumor size: (mean±SD)	4.9 cm±2.07 (range:1.5- 8.5)
Sex:		Metastasis:	
M	17 (56.7%)	Positive	5(16.7%)
F	16 (43.3%)	Negative	25(83.3%)
H. G[1]:			
W	13 (43.3%)	Nodal state:	19(63.3%)
M	10 (33.3%)	Positive	11(36.7%)
P	6 (20.0%)	Negative	
U	1 (3.3%)		
T.A.L[2]:			
U	2 (6.7%)		
M	8 (26.7%)		
L	20(66.7%)		

1: Histological Grade: (W) Well differentiated, (M) moderately differentiated, (P) poorly differentiated, (U) Undifferentiated; 2: Tumor anatomical level: (U) upper third, (M) middle third, (L) lower third. Data are from 30 patients with ESCC. Numbers in the parenthesis are percentage of patients for each parameter

Clinicopathological features: The distribution of tumor anatomical level was 2 (6.7%) in the upper third, 8 (26.7%) in the middle third and 20 (66.7%) in the lower third of esophagus. The mean macroscopic size of the resected tumors was 4.9 cm (range: 1.5- 8.5). The carcinomas were 13 (43.3%) well differentiated, 10 (33.3%) moderately differentiated, 6(20%) poorly differentiated and 1 (3.3%) undifferentiated. More than half of the samples 19 (63.3%) were also lymph node positive and 11(36.7%) of these samples had negative lymphatic invasion (Table 1). Only five patients (16.7%) had also secondary organ metastasis. All of tumors were in advanced stage of esophageal carcinoma due to observed adventitia invasion according to the guidelines for clinical and pathological studies on carcinoma of the esophagus[14].

Table 2: Clinicopathological significance of TS (+/-) and TP (+/-) cases of ESCC patients

Variable	No. of patients	TS (+)	TS (-)	P -value	TP (+)	TP (-)	P-value
Age:							
Over 59	16	10	6	0791.000	15	1	0.0060**
Under59	14	8	6		11	3	
Sex:							
Male	17	11	6	1.000	13	4	0.0490*
Female	10	7	6		13	0	
Tumor size:							
>4.9 cm	16	12	4	0.754	14	2	0.0130*
<4.9 cm	14	6	8		12	2	
Metastasis:							
Positive	5	4	1	0.001***	4	1	0.0001***
Negative	25	14	11		22	3	
H.G[1]:							
W	13	8	5	0.388	10	3	1.0000
M	10	7	3		8	2	
P	6	3	3		5	1	
U	1	1	0		1	0	
Nodal state:							
N(+)	19	11	8	1.000	16	3	0.092
N(-)	11	7	4		10	1	
T.A.L[2]:							
U	2	1	1	0.889	1	1	0.730
M	8	4	4		0	8	
L	20	8	12		3	17	

1: Histological Grade: (W) Well differentiated, (M) moderately differentiated, (P) poorly differentiated, (U) Undifferentiated; 2: Tumor anatomical level: (U) upper third, (M) middle third, (L) lower third. Data are from 30 patients with ESCC. * denotes significant relationship with $p<0.05$. ** denotes significant relationship with $p\leq0.01$. *** denotes significant relationship with $p\leq0.001$

Results of staining with each antibody: Out of 30 postoperative esophageal squamous cell carcinoma, 19 (63.3%) showed cytoplasmic TS expression. The distribution of positive staining for TS was 12 (40%) with strong nuclear and cytoplasmic staining (3+) (Fig. 1a), 6 (20%) with moderate cytoplasmic staining (2+) (Fig. 1b) and 1 (3.3%) with mild cytoplasmic staining (1+). For TP, its staining with cytoplasmic localization was found in 26 (89.7%) of tumor samples. The distribution of positive staining was 14 (46.7%) with strong nuclear and cytoplasmic staining (3+) (Fig. 1c), 7 (23.3%) with moderate cytoplasmic staining (2+) (Fig. 1d) and 5 (16.7%) with mild cytoplasmic staining (1+).

Relationship between TS and TP expression: The prevalence of TP overexpression was significantly ($p = 0.039$) greater in TS (+) tumors (53.33%) compared with TS (-) tumors (33.32%). Tumors were classified to four immunophenotypes: a) TS^-/TP^- (2/30; 6.67%), b) TS^+/TP^- (2/30; 6.67%), c) TS^-/TP^+ (10/30; 33.32%) and d) TS^+/TP^+ (16/30; 53.33%).

Relationship between TS and TP expression and clinicopathological findings: The clinicopathological features of TS positive/negative and TP positive/negative cases were compared in Table 2. Based on the results of IHC staining, the percent of patients did not differ significantly between positive and negative group of markers with respect to malignancy grade and tumor anatomical level. Unlike TS, TP immunostaining was correlated with age ($p = 0.006$), gender ($p = 0.049$), tumor size ($p = 0.013$) and relatively to lymphatic invasion ($p = 0.092$). We also observed a significant relationship between TS ($p = 0.001$) and TP ($p = 0.0001$) expression and secondary organ metastasis. Despite low number of metastatic cases in the present study, 4 cases out of 5 had positive expression of TS and TP in their primary tumor samples.

Relationship between TS and TP coexpression and clinicopathological findings: The present results showed significant relationship between TS and TP expression ($p = 0.039$). The relationship between coexpression patterns of markers with clinicopathological data were compared with other mentioned immunophenotypes (Table 3). Positive TS and TP immunophenotype differed significantly with respect to secondary organ metastasis but not with respect to other parameters when compared with other immunophenotypes.

Relationship between TS-/TP+ immunophenotype and clinicopathological findings: Our data showed a high frequency of TS-/ TP+ expression pattern (10/30). The clinicopathological features of TP positive and TS negative cases were compared in Table 3. Only this combination of expression pattern showed significant relationship with lymphatic invasion ($p = 0.035$) and histological grade ($p = 0.001$).

Table 3: Clinicopathological significance of TS and TP coexpression and TS-/TP+ cases of ESCC patients

Variable	No. of patients	TS+/TP+	Other phenotypes	P-value	TS-/TP+	Other phenotypes	p-value
Age:							
Over 59	16	8	8	1.000	7	9	0.146
Under59	14	8	6		3	11	
Sex:							
Male	17	8	9	1.000	5	12	0.143
Female	10	8	2		5	5	
Tumor size:							
>4.9 cm	16	10	6	1.000	4	12	0.238
<4.9 cm	14	6	8		6	8	
Metastasis:							
positive	5	3	2	0.007**	1	4	0.267
negative	25	13	12		9	16	
H.G[1]:							
W	13	7	6	0.118	5	8	0.001***
M	10	5	5		2	8	
P	6	3	3		2	4	
U	1	1	0		0	1	
Nodal state:							
N(+)	19	9	10	0.629	7	12	0.035*
N(-)	11	7	4		3	8	
T.A.L[2]:							
U	2	0	2	0.158	1	1	0.213
M	8	4	4		4	4	
L	20	12	8		5	15	

Histological Grade: (W) Well differentiated, (M) moderately differentiated, (P) poorly differentiated, (U) Undifferentiated; 2: Tumor anatomical level: (U) upper third, (M) middle third, (L) lower third. Data are from 30 patients with ESCC. * denotes significant relationship with $p<0.05$. ** denotes significant relationship with $p\leq0.01$. *** denotes significant relationship with $p\leq0.001$

Table 4: Clinicopathological significance of TS and TP strong staining (3+) cases of ESCC patients

Variable	No. of patients	TS(3+) N = 12	Others	p-value	TP(3+) N = 14	Others	p-value
Age:							
Over 59	16	5	9	0.804	7	9	0.8040
Under59	14	7	7		7	7	
Sex:							
Male	17	6	11	0.332	6	11	0.6480
Female	10	6	4		8	2	
Tumor size:							
>4.9 cm	16	8	8	0.338	6	10	0.8150
<4.9 cm	14	4	10		8	6	
Metastasis:							
positive	5	4	1	0.039*	2	3	0.035*
negative	25	8	17		12	13	
H.G[1]:							
W	13	5	8	0.013*	6	7	0.006**
M	10	4	6		7	3	
P	6	2	4		1	5	
U	1	1	0		0	1	
Nodal state:							
N(+)	19	8	11	0.118	9	10	0.302
N(-)	11	4	7		5	6	
T.A.L[2]:							
U	2	0	2	0.791	0	2	0.424
M	8	4	4		5	3	
L	20	8	12		9	11	

Histological grade: (W) Well differentiated, (M) moderately differentiated, (P) poorly differentiated, (U) Undifferentiated; 2: Tumor anatomical level: (U) upper third, (M) middle third, (L) lower third. Data are from 30 patients with ESCC. * denotes significant relationship with $p<0.05$. ** denotes significant relationship with $p\leq0.01$

Relationship between TS and TP strong staining and clinicopathological findings: Based on present scoring method that nuclear and cytoplasmic expression of markers considered to be 3+, we analyzed the clinicopathological significance of these strong stainings. Data showed (Table 4) that nuclear and cytoplasmic staining of TS and TP were significantly correlated with histological grade ($p = 0.013$ and 0.006, respectively) and metastasis ($p = 0.039$ and 0.035, respectively).

DISCUSSION

ESCC is one of the most malignant tumors with a poor prognosis. A considerable number of patients die from

cancer recurrence or relapse within a few years of surgery[15]. In view of high mortality rate and the geographical differences in incidence of esophageal cancer, many studies have been performed which bear the objective to increase understanding of the behavior of esophageal cancer and to improve the management of patients with this cancer[1]. The analysis of TS and TP expression may also provide new biological information about ESCC. Due to the clinical values of TS and TP in GI cancers chemotherapy and lack of comprehensive study on the clinical significance of these markers in ESCC, the status of TS and TP expression and their clinicopathological significance were analyzed. The clinicopathological significance of TS in ESCC, prevalence and clinicopathological significance of TS/TP immunophenotypes and their nuclear/cytoplasmic expressions were also evaluated on which no report is available to the best of our knowledge. The present study revealed significant relationships between TS and TP and several clinicopathological features of ESCC. A major finding of this study seems to be the importance of high level expression of TP (89.1%) in ESCC. Igarashi *et al.*[15] reported that in ESCC, angiogenesis is essential for the development of early stage ESCC to the advanced stage. Therefore, TP overexpression may be an early event in ESCC progression. Since the studied cases were in advanced stage of ESCC, therefore this finding is coincided well with their report. Other findings stressed that TP expression is an independent, unfavorable prognostic factor for ESCC patients[16,17]. Okamoto *et al.*[17] indicated TP expression might enhance invasiveness and the metastatic ability of cancer cells by their angiogenic potency. However, the present study could not confirm the association between TP expression and patients prognosis, but we observed a relative significant relationship between lymphatic invasion and TP expression ($p = 0.092$). In addition, the present data showed a positive correlation between TP strong staining and secondary organ metastasis ($p = 0.039$). However, we didn't find any correlation between TP expression and histological grade which is in agreement with other studies[15-17], but our data showed a significant association between TP strong staining and histological grade. Among the evaluated cases 10.3% had negative TP expression. Although the present study could not confirm the association between TP expression and patients prognosis but Koide *et al.*[18] have reported the survival rate after surgery was better in patients with TP- negative ESCC. Therefore, favorable survival could possibly be predictable in only 10.3% of our patients.

The TS immunostaining using the TS 106 antibody, has been shown to correlate with TS mRNA expression assessed by the reverse transcriptase-polymerase chain reaction (RT-PCR) technique in a series of gastrointestinal tumor samples[19]. In the present study, we analyzed ESCC samples by IHC method using TS 106 antibody. Although there is no report about the TS expression in ESCC using TS106, but reports on the expression of TS in patients with all stages of gastric cancer have shown that prognoses were poorer for groups with higher TS expression[20]. Most studies of advanced cancers have also reported worse prognoses and decreased responses to chemotherapy with higher levels of TS expression[7]. Overexpression of TS appears to be a major method of resistance o 5-FU and data from colorectal cancer patients suggest an association with TS and resistance to 5-FU[3]. Harpole *et al.*[3] observed TS overexpression in 56% of esophageal cancer patients who had decreased survival. The present data demonstrated TS expression in 19/30 (63.3%) of patients which is in agreement with Harpole *et al.*[3] study. Although in this study, no association was found between TS immunoreactivity in the primary tumors and other clinicopathological features of patients, but others have reported similar data in other GI cancers[21]. We found a close relationship between histological grade and high intensity of TS expression. Among five metastatic cases in this study, four cases had strong nuclear and cytoplasmic expression of TS. These findings could also suggest the prognostic role of strong staining of TS expression in ESCC.

The present study recognized two significant immunophenotypes in ESCC cases. The most prevalent immunophenotype was TS+/TP+ with 53.3% of expression ($p = 0.039$). Although we did not observe its association with clinicopathological features, but one study supported this combination with least favorable survival in gastric cancers[20]. Therefore, lower survival is expectable in this group and needs further investigations. The present results also showed that 33.3% of tumors were TP positive and TS negative. This immunophenotype indicate another type of alterations with significant relationship to histological grade ($p = 0.001$) and lymph node involvement ($p = 0.035$). These results suggest that alterations in both TS and TP protein expression may be correlated with tumor progression in esophageal cancer and further emphasize on their importance as prognostic markers.

In conclusion, our data on TS and TP overexpression, observed in squamous cell carcinoma of esophagus could suggest the aggressiveness of ESCC and possibility of drug resistance to fluoropyrimidines in these patients. On the other hand, our data showed that 36.7 and 10.3% of patients were TS and TP negative, respectively that obviously affect their response to 5-FU based

chemotherapy. Our results also showed the importance of TS and TP as valuable prognostic markers and their correlation with clinicopathological findings. Therefore, it is advisable to analyze tumor samples of ESCC patients for determining the expression patterns of important molecular markers such as TS and TP proteins to identify tumor biological behavior and to select patients who will most likely benefit from adjuvant therapy after esophagectomy.

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