Correlation Between THSD7A Expression and Tumor Characteristics of Azoxymethane-Induced Colon Cancer Model in Rats

Oktay Halit Aktepe[1], Taha Koray Sahin[2], Gurkan Guner[1], Deniz Can Guven[1], Haci Hasan Yeter[3], Olcay Kurtulan[4], Ibrahim Hanifi Ozercan[5], Omer Dizdar[6], Suayib Yalcin[1]

[1]Department of Medical Oncology, Hacettepe University Cancer Institute, Ankara, Turkey
[2]Department of Internal Medicine, Hacettepe University Faculty of Medicine, Ankara, Turkey
[3]Department of Nephrology, Gazi University Faculty of Medicine, Ankara, Turkey
[4]Department of Pathology, Hacettepe University Faculty of Medicine, Ankara, Turkey
[5]Department of Pathology, Firat University Faculty of Medicine, Elazığ, Turkey
[6]Department of Preventive Oncology, Hacettepe University Cancer Institute, Ankara, Turkey

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ABSTRACT

Background: *Thrombospondin type 1 domain-containing 7A (THSD7A) has emerged as a new potential molecular tool for multiple tumors since that THSD7A was detected to be expressed in various malignant tumor types including colorectal cancer (CRC). Thus, we investigated the correlation between THSD7A expression and pathologic determinants of azoxymethane (AOM)-induced CRC in a rat model.*

Methods: *A total of 30 rats were included in the study (experimental group; n = 15, control group; n = 15). Azoxymethane was administered to the experimental group weekly as subcutaneous injections at a dose of 15 mg/kg bodyweight for 3 weeks. Five months later, 42 tumors were obtained in the study group and histopathologic evaluation of CRC tumors for THSD7A was performed by immunohistochemical staining. Thrombospondin type 1 domain-containing 7A expression was classified according to staining levels.*

Results: *While 28.6% of the colonic tumors were stained as negative, mild-moderate and strong staining was determined in 61.9% and 9.5% of the tumors, respectively. Thrombospondin type 1 domain-containing 7A expression levels inversely correlated with Ki-67 expression ($P < .001$) and tumor grade ($P = .02$). Receiver operating characteristic analysis showed Ki-67 staining ≥20.5% was determined as a cut-off value for negatively stained THSD7A tumors with 91% sensitivity and 69% specificity ($P = .001$, area under curve: 0.822). Moreover, higher Ki-67 expression was found to be associated with higher tumor grade ($P < .001$), presence of lymphatic invasion ($P = .003$), and higher T stage ($P = .003$).*

Conclusion: *Negative staining for THSD7A seems to be linked to invasive pathologic determinants in AOM-induced CRC in rats.*

Keywords: *AOM, colon cancer, Ki-67, prognosis, THSD7A*

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed tumor causing about 1.8 million new cases and 861 000 deaths annually.[1] While the rate of age-standardized incidence in the United States decreased between 1975 and 2010 because of lifestyle changes and increased screening, this rate is still stable in most developed countries.[2,3] Several determinants have been reported to be associated with the prognosis of patients with CRC including the pathological stage at presentation,[4] Ki-67 expression levels,[4,5] grade,[6] and lymphovascular invasion.[7] However, despite these pathologic features with the prognostic significance, there are only a few parameters that play a role in the selection of specific treatment options including microsatellite instability status, BRAF, and RAS mutations. In recent years, with the addition of biological agents targeting these molecular markers such as cetuximab and panitumumab to the combination chemotherapy regimens in the metastatic setting of CRC patients, a significant breakthrough in the course of the disease has been achieved in terms of progression-free survival and overall survival.[8,9] Despite the use of these innovations in CRC treatment, the reduction in colon cancer mortality is not yet at the desired level. Therefore, there is an urgent need to identify novel prognostic markers and to develop therapeutic agents that target them.

Corresponding author: ***Oktay Halit Aktepe****, e-mail:* ***droktayaktepe@hotmail.com***
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Thrombospondin type 1 domain-containing 7A (THSD7A) was first described in the human brain cDNA, initially called as KIAA0960.[10] Thrombospondin type 1 domain-containing 7A is an N-glycoprotein that presumably plays a role in the pathophysiology of major public health problems such as membranous nephropathy, obesity, osteoporosis, and cancer.[11-13] Thrombospondin type 1 domain-containing 7A has a regulatory function in critical steps of carcinogenesis such as cell adhesion, proliferation, and angiogenesis by inducing endothelial cell migration.[14] However, to our best knowledge, there are only a few reports regarding the correlation between THSD7A expression pattern and human colon cancer characteristics. Loss of THSD7A expression in human colon cancer was found to be associated with adverse tumor characteristics like positive lymph node status, poor differentiation, and vascular invasion.[13] Although THSD7A has been suggested to be involved in the prognosis of CRC, the correlation between the expression of this novel protein with clinicopathological markers of CRC remains unclear.[13,15] Therefore, in the present study, the association between THSD7A expression levels and adverse prognostic features was examined in an AOM-induced rat colon cancer model since THSD7A could act as a novel potential marker in colon cancer pathophysiology.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (n = 30, 8-week old) were obtained from the Experimental Research Center at Firat University. The animals were maintained in cages under standard laboratory conditions with a 12 h light/12 h dark period, at constant temperature (22 ± 2°C). The animals were given free access to water, standard diet ad libitum. The study has the permission of the Animal Experiments Local Ethics Committee, and the procedure for the Care and Use of Laboratory Animals has been seriously followed.

MAIN POINTS

- *Thrombospondin type 1 domain-containing 7A (THSD7A) is an N-glycoprotein that takes part in the pathogenesis of major public health problems including cancer.*
- *Negative staining for THSD7A inversely correlated with invasive pathologic indicators of, azoxymethane-induced colon cancer model in rats, T stage, and tumor grade.*
- *We found for the first time a correlation between the THSD7A staining pattern and Ki-67 expression status.*
- *Thrombospondin type 1 domain-containing 7A expression status could be used as an additive prognostic biomarker combined with other well-known prognostic determinants.*

Experimental Design

All the male Sprague-Dawley rats were randomly categorized into two experimental groups. (i) Control group (n = 15): Rats were fed with standard diet and received saline injections; (ii) azoxymethane (AOM) group (n = 15): Rats injected with AOM were fed with a standard diet.[16] Azoxymethane (15 mg/kg body weight; Sigma Chemicals Co, MO, USA) or saline (saline 0.9%, 15 mg/kg body weight) was administrated subcutaneously once weekly for 3 weeks in 0.9% of saline. After 20 weeks the rats were killed, laparotomy was performed. After the entire colon was removed, it was opened longitudinally, and the contents were drained, and the colon was fixed and tumors were extracted for histopathological analysis. All the rats in the AOM group had more than one colon cancer in the entire colon. Tissues were fixed flat in 10% buffered formalin solution containing 3.6% formaldehyde for 24 h then transferred to 70% ethanol for histological processing.

Immunohistochemistry (IHC)

All tumor sections were blindly examined by two experienced pathologists independently. Immunohistochemistry staining was performed by Thrombospondin Type I Domain-Containing Protein 7A (THSD7A), rabbit polyclonal antibody (orb475355; Biorbyt, Explore Bioreagents, Cambridge, UK), and SP6 domestic rabbit polyclonal antibody Ki-67 (ab15580 Abcam, UK) using an automated method (Ventana, USA). Tissue samples were fixed formalin. Paraffin blocks were sliced into sections with a thickness of 4 μm. Subsequently, the sections were dewaxed and rehydrated with xylene and alcohol. Then tissue sections were stained in the automatic staining machine (Ventana BenchMark XT). The non-tumor tissue adjacent to the tumor was always investigated simultaneously for the detection of THSD7A expression. For assessment of THSD7A expression, both the percentage of stained cells and intensity pattern, designated as 0, 1+, 2+, or 3+, were evaluated. The expression patterns were divided into four groups as described before.[13] In brief, tumors with no staining were regarded as negative. Tumors with positive staining for THSD7A were regarded as weak positive (1+ intensity in ≤70% or 2+ intensity in ≤30% of cancer cells), moderate positive (1+ intensity in >70% or 2+ intensity in >30% but ≤70% or 3+ intensity in <30% of cancer cells), and strong positive (2+ intensity in >70% or 3+ intensity in ≥30%

of cancer cells). Ki-67 labeling index (LI) was determined by light microscopy at the site of the greatest number of Ki-67 positive cells. The sites were identified by scanning tumor sections at low power (×40). For Ki-67 LI, the number of positive cells among approximately 1000 tumor cells was calculated as a percentage.

Statistical Analysis

The data were analyzed using the SPSS statistical program (SPSS version 20.0.0, IBM). Categorical variables are expressed as a percentage. The chi-square test was used to compare categorical variables. Tukey's test was used for post hoc analysis. Spearman correlation test was used to find the correlation between histopathological parameters and characteristics. The receiver operating characteristic (ROC) curve was used to determine the best cut-off value of Ki-67 expression for THSD7A negative staining. *P* values <.05 were considered to indicate statistical significance.

RESULTS

Azoxymethane-Induced Colon Cancer Characteristics

Forty-two malignant tumors were detected in the experimental group. None of the control animals developed colon tumors. An adenocarcinoma is described as a malignant colonic tumor containing invasive glands lined by pleomorphic hyperchromatic epithelium. Neither perineural invasion nor vascular invasion was present in addition to T3-4 stage tumors in the study population. Grade II adenocarcinoma was the most common type [23/42 (55%)] followed by 36% (15/42) grade I tumors and 9% (4/42) grade III tumors. Both lymphatic invasion and T2 tumors were present in the grade III tumors. The mean percentage of Ki-67 expression of the colonic tumors was 23.4% ± 7.03%.

Correlation Between THSD7A Expression Status and Pathologic Determinants of Colonic Tumors

All of the tumor and corresponding intestinal tissue exhibited both membranous and cytoplasmic staining patterns. The different expression patterns of THSD7A are shown representatively in Figure 1.

We found that all samples were stained with THSD7A in the control group. Seventy-three percent (11/15) of control samples had mild-to-moderate THSD7A staining and 27% (4/15) had strong THSD7A staining. While 29% (12/42) of colorectal tumor samples had negative THSD7A staining, 62% (26/42) had mild-to-moderate,

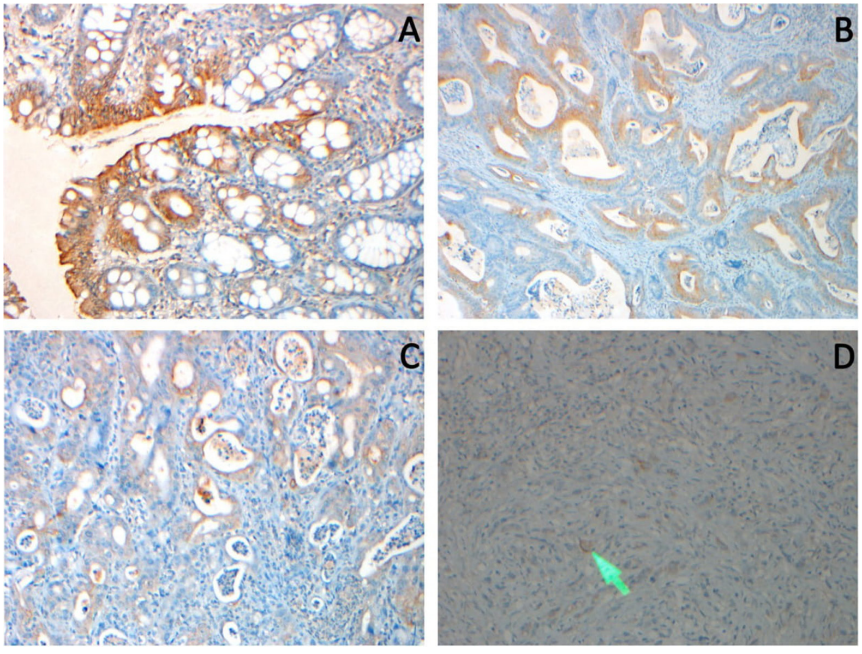

Figure 1. Representative images of different type expressions for THSD7A with magnification 200x. (A) Normal colonic mucosa with strong THSD7A positivity. (B) Well-differentiated adenocarcinoma (Grade 1) with strong THSD7A positivity. (C) Moderate-differentiated adenocarcinoma (Grade 2) with moderate THSD7A positivity. (D) Poor-differentiated adenocarcinoma (Grade 3) with weak THSD7A positivity.

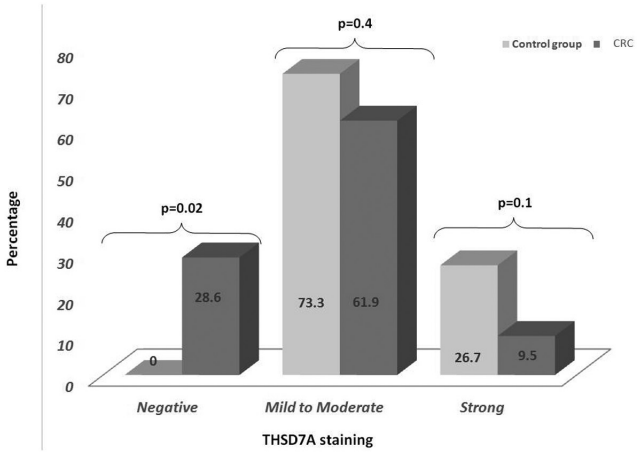

Figure 2. Distribution of THSD7A staining between control and experimental groups.

and 9% (4/42) had strong THSD7A staining. When we compared the staining pattern of THSD7A between the control group and colorectal cancer, we found that the loss of THSD7A staining was significantly higher in the CRC group (P = .02). On the other hand, mild-to-moderate and strong staining for THSD7A were similar between the two groups (P = .4 and P = .1) (Figure 2).

When we evaluated the Ki-67 expression according to the THSD7A staining, we found Ki-67 expression was significantly different between our tumor groups categorized by the amount of THSD7A expression (P =.001). Ki-67 expressions in the normal mucosa and colonic tumor are presented in Figure 3. Post hoc analysis showed that samples with the negative THSD7A had significantly higher Ki-67 expression compared to those with mild-to-moderate (P= .006) and strong (P = .002) THSD7A staining samples. Mild-to-moderate and strong THSD7A staining colonic tumors had similar Ki-67 expression (P = .3) (Figure 4). There was a negative correlation between levels of THSD7A staining and Ki-67 (P < .001) [Correlation coefficiency (CC): −0.54)], and tumor grade (P = .02, CC: −0.35). Furthermore, a strong positive correlation between tumor grade and Ki-67 expression was present (P < .001, CC: 0.84) (Figure 5). ROC analysis was used to find out the best cut-off value of Ki-67 expression for negative THSD7A staining and 20.5% was determined for THSD7A negative staining with 91% sensitivity and 69% specificity (P < .001, AUC: 0.822) (Figure 6). As shown in Table 1, low Ki-67 expression was significantly associated with low tumor grade (P < .001), absence of lymphatic invasion (P = .003), low T stage (P = .003) and positive THSD7A staining (P < .001).

DISCUSSION

Colorectal cancer is one of the most common cancers causing cancer-related death.[1] Colorectal cancer risk has

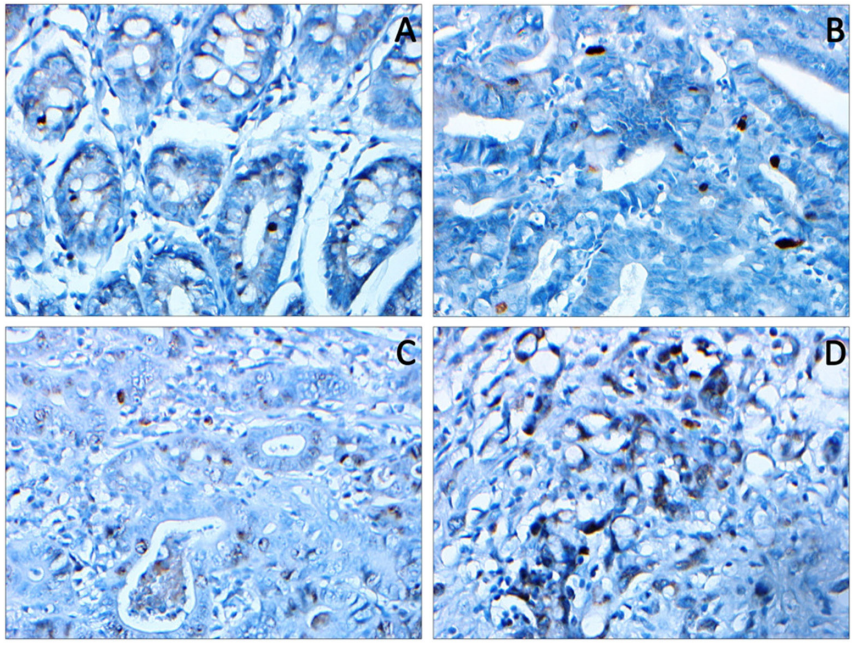

Figure 3. Representative images of Ki-67 expression in normal colonic mucosa and colon adenocarcinoma with magnification 400×. (A) Normal colonic mucosa, (B) Grade 1 adenocarcinoma, (C) Grade 2 adenocarcinoma, (D) Grade 3 adenocarcinoma.

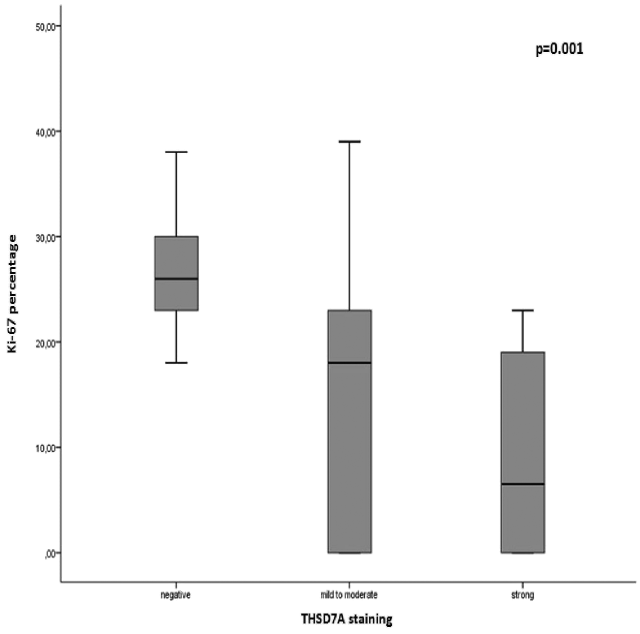

Figure 4. Distribution of Ki-67 expression according to THSD7A staining.

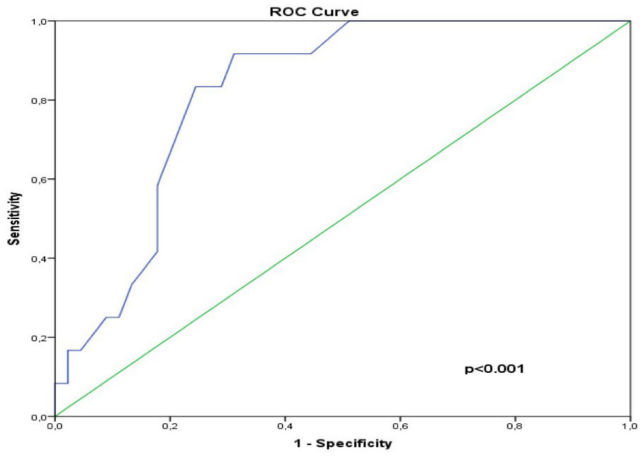

Figure 6. Ki-67 expression level of 20.5% was determined as a cut-off value for negative staining for THSD7A with 91% sensitivity and 69% specificity by ROC analysis (AUC = 0.822).

been associated with various environmental, socioeconomic, genetic, and lifestyle factors.[17] However, it is not easy to fully understand the interplay of all these risk factors and the entire carcinogenesis process. Therefore, it

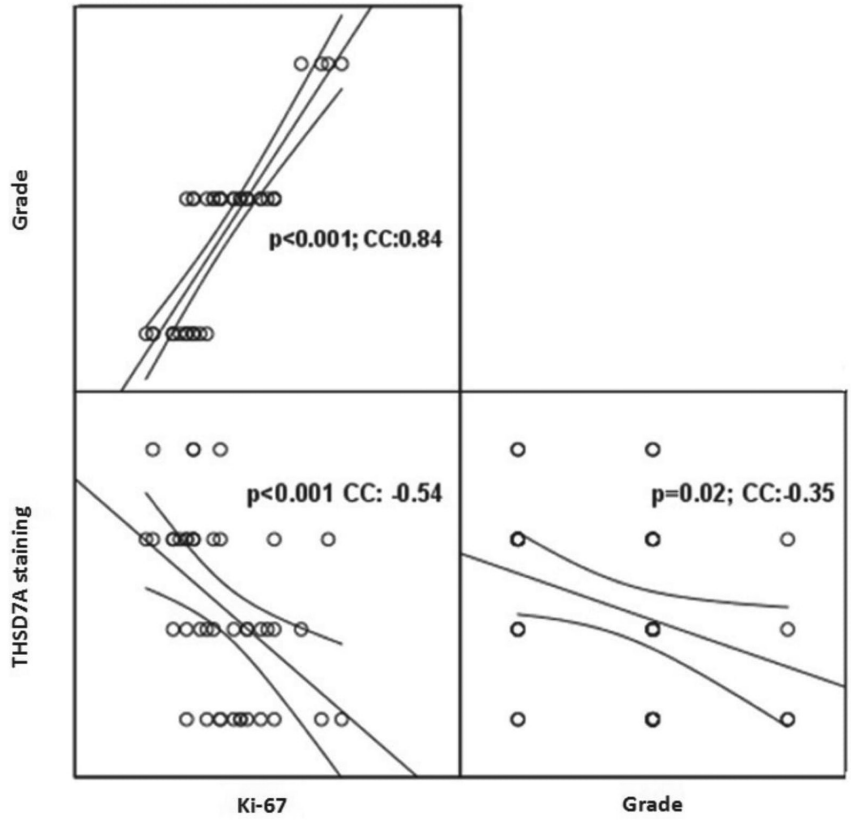

Figure 5. Spearman correlation test showed the negative correlation between THSD7A staining and Ki-67 expression and tumor grade. Also, a positive correlation was determined between Ki-67 expression and tumor grade.

Table 1. Distribution of Histopathological Markers and Findings According to Ki-67 Expression

	Ki67 Expression		
	<20.5%	≥20.5%	*P*
Tumor grade			<.001
G1	14 (93%)	1 (7%)	
G2	3 (13%)	20 (87%)	
G3	0	4 (100%)	
THSD7A staining			<.001
Negative	1 (8%)	11 (92%)	
Weak	7 (44%)	9 (56%)	
Moderate	17 (81%)	4 (19%)	
Strong	7 (88%)	1 (12%)	
Lymphatic invasion	0	4 (100%)	.003
Tumoral invasion	0	4 (100%)	.003

seems imperative to develop effective preventive strategies to alleviate the disease burden and new prognostic markers that will result in the development of novel targeted treatment modalities. The intestinal system pathology of rats has great similarities to humans.[18] Besides, similar sequential histopathological and molecular pathogenesis is observed for the development of malignancy from pre-cancerous lesions in humans and rodent colorectal carcinogenesis models.[19] Therefore, CRC studies in rodents have been used as a model for human colon cancer. Hence, the present study was conducted to elucidate the association between THSD7A expression level, a potential prognostic factor, and the pathological determinants in an experimental rat CRC model. Our results showed that almost 3/4 of the colon tumors were stained with THSD7A and also THSD7A staining pattern was inversely correlated with pathological determinants namely T stage, Ki-67 expression, tumor grade, and lymphatic invasion.

Recently, Thrombospondin type 1 domain-containing 7A (THSD7A) has been identified as an autoantigen that can aid in the diagnosis of membranous nephropathy cases (MN) as well as understanding its pathogenetic processes.[11] THSD7A gene is a large gene (458 kb) located in 7p22 and the chromosome region where the gene is localized is a recurrent breaking point in different types of tumors.[20] In a multi-center study, it was observed that 20% of patients developed malignancy 3 months after the diagnosis of THSD7A-associated MN.[21] THSD7A-associated MN accounts for 1-12% of the overall MN population.[22] Thrombospondin type 1 domain-containing 7A is the soluble form of membrane-associated N-glycoprotein and functions in angiogenesis by regulating endothelial cell migration and tube formation.[14] Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase acting as a mediator involved in the control of proliferation, adhesion, and survival of many cell types.[23] Overexpression and activation of FAK are involved in the development and progression of various malignancies including colon cancer.[24-26] Focal adhesion kinase- associated signal pathways are induced by the soluble form of THSD7A.[27]

To our knowledge, two other studies analyzed the success of THSD7A staining in colon cancer. Stahl et al[13] conducted a study that was focused on the impact of THSD7A on the prognosis of various tumors including CRC. They reported that 56.8% of colorectal tumors were stained negatively although the percentage of weakly, moderately, and strongly staining remained at 20.9%, 16.3%, and 5.9%, respectively, and also THSD7A expression status was inversely associated with T stage ($P = .0487$), vascular invasion ($P < .0001$), tumor grading ($P < .0001$) and the number of metastatic lymph nodes ($P = .0067$). However, Li Xian et al[28] found that 97.5% of CRCs were stained positively and more interestingly most of them had a strong positive staining for THSD7A. The discordant results in these reports may be due to heterogeneity of tumors because tumoral characteristics of patients weren't reported in the second study and strong positivity in this study may result from the selection of tumors having less invasive pathological features.

Based on these contradicting results, the present study was aimed to determine the THSD7A staining pattern and its association with pathological determinants in an experimental rat model. We found that 71.4% of the tumors had positive staining for THSD7A and an inverse correlation was determined between THSD7A staining and adverse prognostic tumor characteristics. In comparison with the study by Stahl et al,[13] our trial demonstrated a higher percentage of THSD7A positivity. This may have resulted from the fact that only T1 and T2 colonic tumors were obtained in our experimental model. Apart from these studies mentioned above, we evaluated for the first time the association between THSD7A expression pattern and Ki-67 proliferation index, and our results demonstrated that a negative correlation was noted between the THSD7A staining pattern and Ki-67 expression status ($P < .001$). We also found that Ki-67 level ≥20.5% was a cut-off value for

negative THSD7A staining with 91% sensitivity and 69% specificity ($P < .001$, AUC: 0.822). From the point of view of this finding, THSD7A can be suggested as a negative proliferation marker.

In summary, our study suggests that the presence of THSD7A expression was closely associated with favorable pathologic determinants in CRC. THSD7A might be regarded as a novel prognostic marker in colon cancer if our results are confirmed by future human trials.

Ethics Committee Approval: This study was approved by the Animal Ethics Committee of Firat University (Date of approval: 14.12.2018 - Decision no: 2018/21-196.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Consept – O.H.A., T.K.S., H.H.Y., G.G., I.H.O., S.Y.; Design – O.H.A., G.G., D.C.G., O.K., O.D., S.Y.; Supervision – O.H.A., D.C.G., S.Y.; Resources – T.K.S, D.C.G.; Materials – O.H.A., D.C.G., O.K., I.H.O.; Data Collection and/ or Processing – T.K.S., O.K., I.H.O.; Analysis and/or Interpretation – O.H.A., I.H.O., O.K., H.H.Y., O.D., S.Y.; Literature Search – O.H.A., H.H.Y., O.K., G.G.; Writing – O.H.A., D.C.G., I.H.O., S.Y.; Critical Reviews – D.C.G., G.G., I.H.O, O.D.

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