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ORIGINAL ARTICLE

Immunohistochemical Assessment of Transthyretin Association with Colorectal Adenocarcinoma

Bei Wang [1], Huihui Hu [2], Honglei Zhang [1], Dingrong Zhong [1]

[1] *Department of Pathology, China-Japan Friendship Hospital, Beijing, China*
[2] *Department of Clinical Laboratory, Beijing Shijitan Hospital, Capital Medical University, Beijing, China*

SUMMARY

Background: **To explore the association of transthyretin (TRR) with colorectal cancer (CRC) development and progression.**
Methods: **This study was conducted on 12 normal colorectal tissue samples, 15 colorectal adenomas, and 39 colorectal adenocarcinoma tissue specimens. TTR expression was assessed by immunohistochemistry, and the results were correlated to clinicopathological characteristics of CRC patients.**
Results: **TTR staining was detected in 16.7% (2/12) of normal colon tissues, 46.7% (7/15) of colorectal adenomas, and 89.7% (35/39) of colorectal adenocarcinoma tissues. TTR staining scores in normal colon tissues, adenoma, and adenocarcinoma were 0.58, 2.27, and 5.40, respectively. G3 grade adenocarcinoma had a higher TTR staining score compared with G2 and G1 grades (8.40, p = 0.0009). Lower TTR expression was significantly associated with metastasis (p = 0.043).**
Conclusions: **TTR expression is positively correlated with adenoma to CRC progression. Thus, TTR has the potential to serve as a predictive marker in CRC.**
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Correspondence:
Dingrong Zhong
2 East Ying Hua Yuan Street
Chao Yang District
Beijing
China
Phone: +86 010 84205191
Email: txzzzryy@163.com

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KEY WORDS

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide [1]. Although CRC incidence and mortality rates have decreased since the early 1980s [2], it is still the fourth type of cancer by incidence in China, and the number of newly diagnosed cases has been continuously rising in recent years. Improvement in surgical treatment has improved the survival of CRC patients without metastases at the time of diagnosis. However, about 20 - 25% of patients are diagnosed with synchronous metastases at the time of diagnosis [3,4]. Tumor stage, tumor grade, weight loss, and anatomic sites of metastatic disease all influence the survival of CRC patients. Nevertheless, despite advancements in CRC therapy, metastatic CRC patients still have cancer recur-

rence, and the median survival is less than 2 years [5-7]. It has been reported that 5-year recurrence rates for patients with stage I, stage II, and stage III cancer are 10%, 20%, and 30 - 50%, respectively [8]. The survival rate is lower in patients with advanced CRC, especially those with metastatic disease, compared to patients with well-differentiated tumors. Therefore, identifying good biomarkers that can indicate the clinical characteristics of CRC would be of great clinical benefit.

Transthyretin (TTR) was originally named "prealbumin" as it was the only plasma protein that migrated anodal to albumin during electrophoresis [9]. It was first detected in human cerebrospinal fluid and serum samples. It was identified as a thyroid hormone-binding protein. TTR is synthesized in the liver, choroid plexus, and intestine [10]. It mainly exists as a homotetrameric protein of about 55 kDa, and has a role in the transport of retinol by binding to retinol-binding protein [11]. Many studies have reported the relationship between TTR expression and human cancer. For instance, TTR was found to be overexpressed in pancreatic cancer juice by two-dimensional gel electrophoresis, matrix-assisted laser desorption/ionization-time of flight (TOF) mass spectrometry (MS), and western blot analysis [12]. TTR is reportedly a novel serum biomarker in lung cancer [13]. In addition, a previous study showed that TTR expression was downregulated in the serum of patients with cholangiocarcinoma (CC) [14]. It has also been shown that TTR may be used as a complementary marker with carbohydrate antigen 19-9 (CA19-9) for the diagnosis of CC [15]. The relationship between TTR expression and CRC has also been examined. In the study by Fentz et al. [16], the surface-enhanced laser desorption/ionization-TOF MS approach was applied to serum samples from 83 CRC patients with stage III cancer. According to their results TTR expression was lower in the serum of CRC patients compared to patients with adenomas [16]. Furthermore, proteomic analysis of TTR expression showed that it was decreased in the serum of CRC patients with lymph node metastasis compared to those with non-metastatic CRC [17].

More than 90% of colorectal carcinomas are adenocarcinomas originating from epithelial cells of the colorectal mucosa. We examined TTR protein expression using immunohistochemistry in normal colorectal tissues, colorectal adenomas, and colorectal adenocarcinoma tissues.

MATERIALS AND METHODS

Sample Characteristics

This study included 39 colorectal adenocarcinoma patients, 12 patients with colorectal adenomas, and 12 healthy individuals from the China-Japan Friendship Hospital (Beijing, China) from September 2011 through December 2012. All cancer patients underwent initial surgery with no prior radiotherapy and chemotherapy. Patients with a history of other malignant tumors or abnormal metabolism-related diseases were excluded from the study. Cancer tissue samples were classified according to tumor-node-metastasis staging, cancer cell differentiation (grade), and metastases. In addition, cancer patients were also subdivided into four groups depending on the depth of tumor invasion (T1, T2, T3, T4), three groups depending on cancer cell differentiation (Grade 1, highly differentiated tumor; Grade 2, moderately differentiated; Grade 3; poorly differentiated) [18,19] and two groups depending on the presence or absence of metastases. Normal colorectal tissue from the same patient was obtained. The samples were taken during surgery at least 5 cm away from the tumor. Each patient provided signed informed consent and the study was approved by the Ethics Committee of China-Japan Friendship Hospital (No. 2018-72-K52).

Immunohistochemistry

For immunohistochemical analysis, formalin-fixed, paraffin-embedded tissue sections (4 µm) were dried, deparaffinized, and rehydrated. Heat-mediated antigen retrieval was performed using steamer treatment for 20 minutes in Target Retrieval Solution (Dako S1699). The slides were incubated with the monoclonal antibody against TTR (rabbit anti-prealbumin antibody [EP29-29Y]; Abcam, Cambridge, MA, USA) at a dilution of 1:200. Secondary antibody and chromogenic agent were purchased from Roche Diagnostic Products Co., Ltd. (Shanghai, China). Immunohistochemical staining was carried out according to the manufacturer's instructions, and the corresponding negative and positive controls were established. All pathological sections were read independently by two senior pathologists.

Assessment of TTR immunohistochemical staining

To determine TTR protein level in tissue samples, semi-quantitative immunohistochemical detection was used. In brief, cytoplasmatic immunoreactivity for TTR protein was scored by evaluating the percentage of positive enterocytes or tumor cells and the staining intensity. The percentage of positive cells was scored as follows: "0" (negative staining, no positive cells), "1" (1 - 10% of positive cells), "2" (11 - 50% of positive cells), "3" (51 - 80% of positive cells), and "4" (81 - 100% of positive cells). Staining intensity was scored as "0" (no staining), "1" (weakly stained), "2" (moderately stained), and "3" (strongly stained). The final TTR staining scores were calculated by multiplying the two scores: TTR staining scores = percentage of positive cells x staining intensity. Both scores were determined under double-blind conditions by two independent pathologists. TTR staining scores were classified as follows: 0 - 4, low TTR expression; and 5 - 12, high expression.

Statistical analysis

Software SPSS 16.0 was used for the statistical analysis. The difference in TTR expression between groups (stage, grade, and metastases) was examined using one-way analysis of variance and Student's *t*-test, after the

validation of normal distribution and homogeneity of variance. Two-tailed $p < 0.05$ was accepted as a statistically significant difference.

RESULTS

TTR expression and TTR scores were significantly increased in normal colorectal tissues, colorectal polyps, and colorectal adenocarcinoma

In our study, TTR was mostly localized in the cytoplasm. Compared with normal colorectal tissue and colorectal adenoma tissue, positive TTR staining was detected in only 16.7% (2/12) of normal colorectal tissues, 46.7% (7/15) of colorectal adenomas, and 89.7% (35/39) of colorectal adenocarcinomas. The TTR staining scores (mean ± SD) of normal colon tissues, colorectal adenomas, and colorectal adenocarcinoma were 0.58 ± 0.34, 2.27 ± 0.49, and 5.40 ± 0.40, respectively (Table 1). A difference in TTR expression was observed between normal colon tissue and adenomas ($p = 0.02$). The difference in TTR expression between colorectal adenoma and colorectal adenocarcinoma tissues was also statistically significant ($p = 0.03$), as was the difference in TTR expression between normal colon tissue and colorectal adenocarcinoma tissues ($p = 0.002$). High TTR expression was detected in 66.7% of colorectal adenocarcinoma tissues. Furthermore, TTR expression in colorectal adenocarcinoma tissue was 9.31-fold higher than that in normal colorectal tissue. The protein expression in colorectal adenocarcinoma tissues was 2.38-fold higher than that in colorectal adenoma tissue (Table 2).

TTR staining scores in colorectal adenocarcinoma tissue are associated with tumor grade

The tumor grade is determined according to the degree of differentiation, the size of atypia, and the number of mitotic images. In recent years, the concise three-level classification method has been widely used in pathological grading diagnosis [20]. Although this classification method is significant for clinical treatment and prognosis, the lack of quantitative standards is greatly affected by subjective factors. In our study, we used the TTR staining scores and the results were related to tumor grading. Our findings have shown that the lower the tumor grading, the higher the TTR staining scores. In our study, G3 grade with a mean 8.40 had higher TTR staining scores compared with G2 and G1 ($p = 0.0009$). In addition, a difference in TTR expression was observed in G1 and G3 ($p < 0.001$) and G2 and G3 ($p < 0.01$). Therefore, TTR staining scores may be a more objective quantitative indicator to establish tumor grading standards (Figure 1).

TTR expression in colorectal adenocarcinoma tissue does not seem to be closely associated with local invasion

Tumor staging is by far the most important prognostic predictor of clinical outcome for patients with colorectal carcinoma. Histological examination of surgically resected specimens has an irreplaceable role in determining the depth of tumor invasion (T) [21]. In this study, we stratified colorectal adenocarcinoma tissue patients according to tumor stage (T1, T2, T3, and T4). High TTR expression was detected in 14 (63.6%) of 22 stage T1 and T2 patient samples, whereas only 7 (41.1%) of 17 stage T3 and T4 patients had high TTR expression. This suggests that lower TTR expression was associated with a more advanced stage of colorectal adenocarcinoma. However, TTR expression showed no statistically difference in tumor stages.

Low TTR expression is associated with metastatic colorectal adenocarcinoma

Early metastasis is an essential feature of malignant tumors [22]. Thus, identifying metastasis-related markers is significant for tumor evaluation. Therefore, we assessed the association of TTR with metastatic colorectal adenocarcinoma. In our study, 16.7% (3/18) of metastatic colorectal adenocarcinoma tissues had no TTR expression. In addition, metastatic colorectal adenocarcinoma had a lower TTR staining score, compared with non-metastatic tumors (4.41 vs. 6.38, $p = 0.043$) (Figure 3).

DISCUSSION

Patient survival rate mostly depends on early tumor detection [23]. The adenoma-carcinoma theory has been widely accepted as an explanation for the pathogenesis of CRC [24]. It takes about 10 years from adenoma to progress to CRC [25], which provides a favorable time frame for prevention and screening. Fecal occult blood tests, detection of tumor biomarkers, and endoscopic check-ups are important methods of screening for CRC [26]. Finding a new biomarker for the early detection of CRC is necessary to improve the survival rate of patients. In recent years, many molecular biomarkers in different biological samples reportedly correlate with CRC development and progression. Indeed, DNA, RNA, and protein biomarkers have all been used to diagnose this disease [27,28]. Still, the most widely used biomarker for CRC is carcinoembryonic antigen (CEA). However, CEA has low sensitivity, especially in early-stage patients [29]. In addition, carbohydrate antigen 19-9 (CA199), CA50, and CA724 are also used to establish clinical diagnosis [30]. Despite many types of molecular biomarkers, very few specific biomarkers can be used for the early clinical diagnosis and monitoring of CRC. Our study showed that a progressive increase in TTR staining score from normal colorectal tissue to adenoma occurred during tumorigenesis of CRC. In ad-

Table 1. Characteristics of patients with normal colorectal tissue and colorectal adenoma.

Variables	Normal colorectal tissue	Colorectal adenoma
Median age (range)	62 (43 - 78)	60.5 (46 - 83)
Gender, n (%)		
Men	9 (75%)	10 (66.7%)
Women	3 (25%)	5 (33.3%)

Table 2. Characteristics of patients with colorectal adenocarcinoma.

Variables		n (%)	TTR staining scores (mean ± SD)
Age	Median (range)	67 (54 - 91)	
Gender	men	27 (69.2%)	5.11 ± 0.59
	women	12 (30.8%)	5.68 ± 1.12
Site	right	16 (41%)	5.37 ± 0.85
	left	23 (59%)	5.42 ± 0.68
Tumor Grade	G1	8 (20.5%)	3.25 ± 0.78
	G2	24 (61.5%)	4.54 ± 0.46
	G3	7 (18.0%)	8.40 ± 0.68
T Stage	T1	10 (25.6%)	5.60 ± 0.97
	T2	12 (30.8%)	6.00 ± 1.16
	T3	9 (23.1%)	4.90 ± 1.12
	T4	8 (20.5%)	5.08 ± 1.07
Metastasis	metastatic sites		
	lymph node	8 (20.5%)	4.41 ± 0.62
	liver	4 (10.2%)	
	peritoneum	2 (5.1%)	
	lung	4 (10.2%)	
	none	21 (53.8%)	6.38 ± 0.68

dition, in our study, TTR was positively correlated with histological tumor grading [31], which may be used as an effective indicator for early screening.

Conventional adenocarcinoma is characterized by glandular formation, which is the basis for histological tumor grading [31].The prognosis of patients with poorly differentiated colorectal adenocarcinomas is typically reported to be poor and more unfavorable than that of those with well or moderately differentiated adenocarcinomas [32]. Poorly differentiated adenocarcinomas are mostly solid with < 50% gland formation and high TTR expression (mean score, 8.40) compared with well-differentiated adenocarcinoma, which have a > 95% gland formation and mean TTR staining score of 3.25 ($p < 0.001$); and moderately differentiated adenocarcinoma, which show 50 - 95% gland formation with a mean TTR staining score of 4.54 ($p < 0.001$). The TTR staining score results showed that TTR expression was closely related to the degree of tumor differentiation, in accordance with tumor evolution.

TTR expression is decreased in a variety of tumors compared to the corresponding normal tissue, precancerous diseases, and early-stage tumors. In a recent study, TTR was significantly lower in lung cancer sera than in sera from normal individuals ($p < 0.001$), which had a 78.5% sensitivity and 77.5% specificity for lung cancer versus normal [33]. In another study, TTR expression in gastric tumors was lower than that of the benign lesions, which to a certain degree was correlated with clinical stage, lymph node metastasis, and differentiation [34]. The mean levels of TTR in the serum showed a tendency to decrease with the severity of ovarian cancer and was lower in affected women whose C-reactive protein levels were > 40 mg/mL [35], which

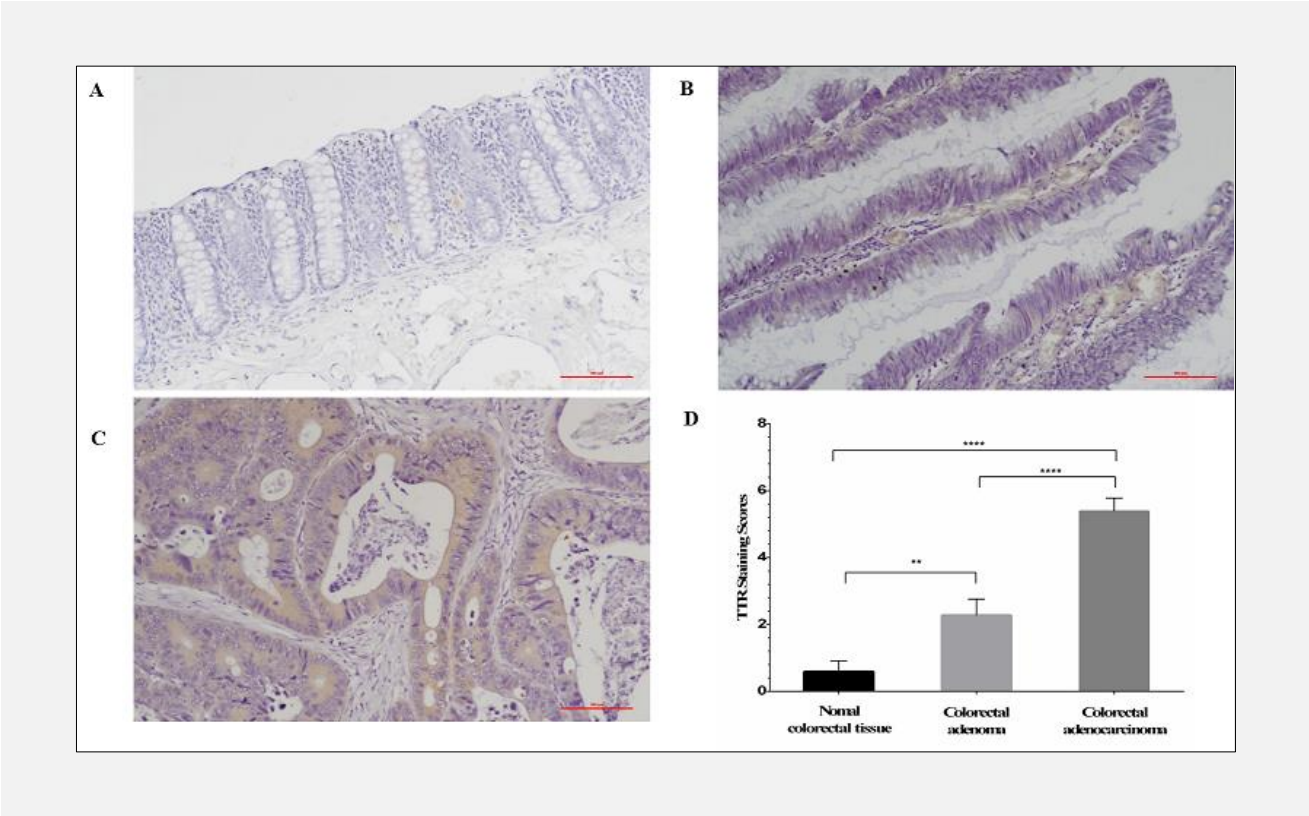

Figure 1. TTR expression in normal colorectal tissue, colorectal adenomas, and colorectal adenocarcinoma tissues.

TTR immunostaining is mostly localized in the cytoplasm of enterocytes or tumor cells: (A) normal intestinal epithelium was almost negative, (B) adenomas showed weakly positive staining, tubulovillous adenoma had high-grade intraepithelial lesions, and (C) staining of colorectal adenocarcinomas was positive and more intense, moderately differentiated adenocarcinoma. (200 x magnification). (D) TTR expression level of normal colorectal tissue (n = 12), colorectal adenomas (n = 12), and colorectal adenocarcinoma tissues (n = 39). Values are expressed as the mean ± SD. ** $p < 0.01$, and **** $p < 0.0001$.

combined with CA125, can be monitored in early-stage epithelial ovarian cancer [36]. Our previous study confirmed that the expression of TTR in CRC was downregulated in the serum, especially during the T4 stage, whereas TTR was highly expressed in adenomas. In this study, we found that TTR decreased in the CRC serum as the tumor progressed. However, we did not find this in the T stage of the colorectal adenocarcinoma tissue, possibly due to the small sample size of the subgroup. It is worth mentioning that metastatic colorectal adenocarcinoma has low TTR expression with a score of 4.41. We also previously reported that the serum levels of TTR in CRC patients are associated with lymph node metastasis. Low TTR levels may be indicators for poor prognosis among cancer patients in palliative care settings [37]. In a recent study, patients with NSCLC serum TTR levels < 22 mg/dL exhibited a worse overall ($p = 0.008$) and recurrence-free survival ($p = 0.027$) compared with those with serum TTR levels ≥ 22 mg/dL [38]. Together, these results suggest that low TTR expression in advanced tumors may indicate a poor prognosis and can be used as a prognostic biomarker. According to these study results, TTR is continuously increasing before or even early in the occurrence of malignant tumors, while TTR expression is significantly decreased once metastasis occurs, similar to the study results in serum. Current studies have shown that in patients with metastatic CRC, TTR expression is significantly reduced in tumor tissues or serum tests, which may predict a poor prognosis. However, in terms of the depth of invasion or the size of the tumor, the results of serum and tissue were inconsistent and further studies in large cohorts are needed. In conclusion, the continuous increase of TTR can assist in the diagnosis of adenoma and colorectal adenocarcinoma grading, and may be useful for monitoring the status of early-stage tumors. Decreased TTR may predict disease progression and poor prognosis. Based on these findings, we conclude that TTR has the potential to be used as a biomarker for the diagnosis and treatment of this disease.

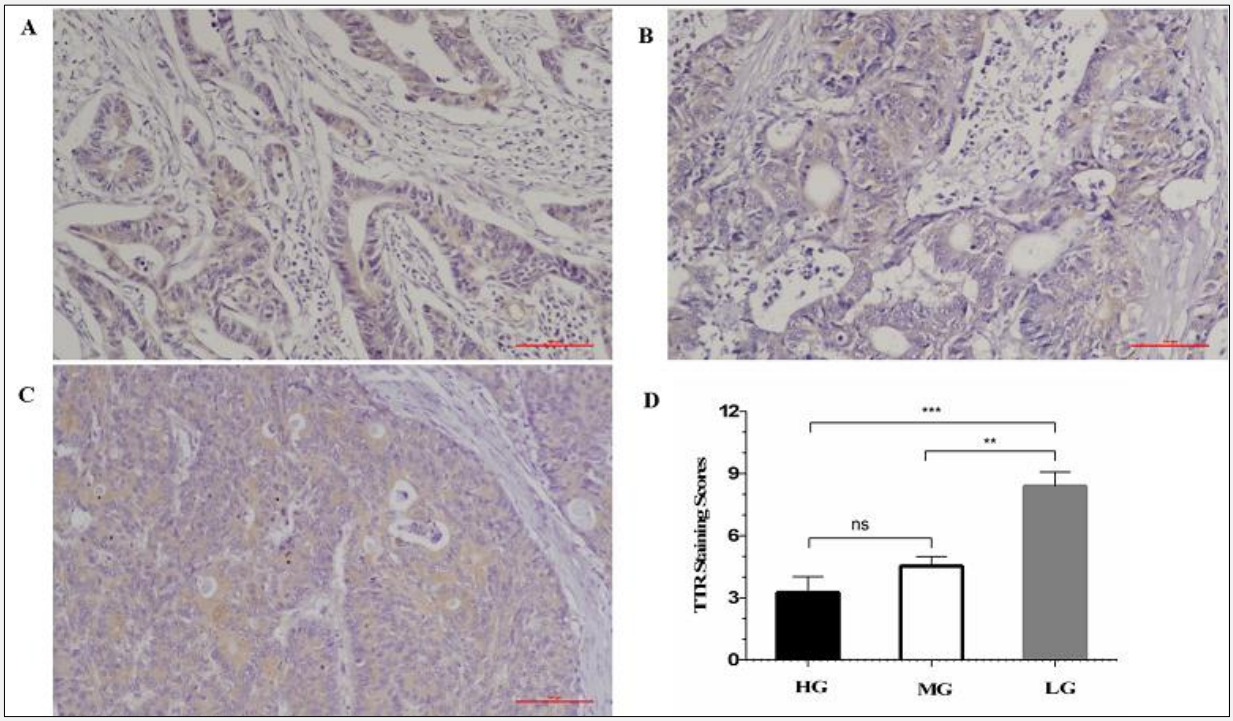

Figure 2. TTR expression is associated with histologic tumor grading.

(A) TTR is weakly expressed in the cytoplasm of some tumor cells of well-differentiated adenocarcinoma (G1). (B) The number of TTR-expressing tumor cells in moderately differentiated adenocarcinoma (G2) is close to that of well-differentiated adenocarcinoma (G1), but the staining intensity is a little stronger. (C) Poorly differentiated adenocarcinoma tumor cells often showed diffuse strong TTR-positive staining (G3). (200 x magnification). (D) TTR expression level of G1 (n = 8), G2 (n = 24), and G3 (n = 7). Values are expressed as the mean ± SD. ** $p < 0.01$, *** $p < 0.001$, ns indicates not significant.

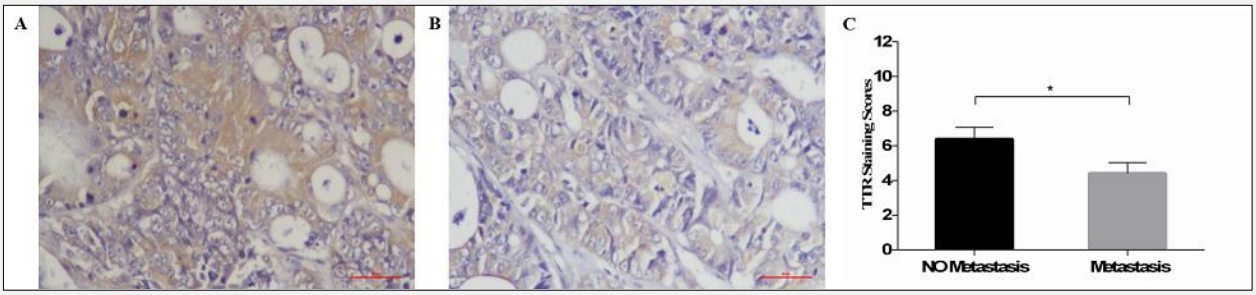

Figure 3. TTR expression is associated with the presence of metastases in colorectal adenocarcinoma.

(A) In adenocarcinoma with G3 histological grade, more tumor cells expressed TTR and the staining intensity was strong in the cases without metastasis; (B) the proportion of tumor cells expressing TTR was less in the metastatic cases and the staining intensity was slightly lower (400 x magnification). (C) TTR expression level of metastases (n = 18) and no metastases (n = 21). Values are expressed as the mean ± SD, * $p < 0.05$.

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The authors declare that they have no competing interests.

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