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Differential expression of Tim3 protein in colorectal cancer associated with MSI and Braf mutation

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Summary. Tim3 is a negative immune checkpoint molecule and plays a crucial part in tumor-induced immune suppression. Tim3 is a cell surface molecule expressed on T cells marking dysfunctional CD8+ cells in various kinds of cancers. Tim3 expression was mainly reported in tumor-infiltrating lymphocytes (TILs). There are few studies focusing on the expression of Tim3 in tumor cells. Immunohistochemistry was performed to determine Tim3 expression level. The relationships between Tim3 expression in colorectal cancer cells and in tumor-infiltrating lymphocytes and cilicopathological parameters were statistically analyzed. Tim3 was differentially detected in TILs and in colorectal cancer cells. Positive expression of Tim3 in colorectal cancer cells was associated with tumor location (P=0.001), depth of tumor invasion (P<0.001), lymph node metastasis (P=0.001), TNM stage (P=0.001), MSI (P=0.008), and Braf V600E mutation (P=0.001). On the other hand, positive expression of Tim3 in TILs was only related to depth of tumor invasion (P<0.001). Positive expression of Tim3 in both colorectal cancer cells and TILs was associated with depth of tumor invasion (P<0.001), lymph node metastasis (P=0.002), TNM stage (P=0.002), MSI (P=0.039), and Braf V600E mutation (P=0.009). Kaplan-Meier survival analysis showed that Tim3 expression in colorectal cancer and in TILs was significantly associated with patient overall survival (OS) rate (P=0.039, and 0.001). Tim3 may be a potential prognostic marker and a therapy target for colorectal cancer.

Key words: Tim3, Colorectal cancer, MSI, Braf

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Introduction

The immune system is the mechanism of defense against pathogens and has a relevant role in anticancer response. The activation and proliferation of T lymphocytes are affected by co-inhibitory immune checkpoint molecules that include cytotoxic T-lymphocyte-associated protein 4 (CTLA4), programmed death 1 (PD-1), programmed death ligand 1 (PD-L1) and PD-L2. Immunotherapy has been an evolving treatment option for several types of cancer, but a limited number of patients benefit from such therapy. PD-1/PD-L1 inhibitors are a group of immune checkpoint inhibitors as front-line treatment of multiple types of cancer (Sui et al., 2018; Tan et al., 2019; Aggen et al., 2020; Hayashi and Nakagawa, 2020). Combinations of cytotoxic chemotherapy and PD-1/PD-L1 inhibitors have been approved and are now used in clinical practice for the treatment of advanced non-small cell lung cancer and small lung cancer (Sui et al., 2018; Tartarone et al., 2019; Hayashi and Nakagawa, 2020). However, serious immune-related adverse reactions limit the clinical application of PD-1/PD-L1 inhibitors. It is urgent to develop novel inhibitors to meet the increasing clinical demands. T cell immunoglobulin and mucin domain-containing protein 3 (Tim3), also known as hepatitis A virus cellular receptor 2 (HAVCTR2) belongs to the immunoglobulin superfamily, and TIM family of proteins. It was originally identified as a molecule expressed by interferon-γ -producing $CD4^+$ and $CD8^+$ T cells (Monney et al., 2002). Tim3 has received the most attention because of its association with the regulation of immune responses in autoimmunity and cancer (Ngiow et al., 2011a,b). Tim3 is a negative immune checkpoint molecule and plays a crucial part in tumor-induced immune suppression. It has been shown that tumor cells use checkpoint molecules to evade the immune system. Tim3 and its ligand galectin9 (Gal9) pathway serve a pivotal role in immune regulation, which is similar to the programmed death PD1/PDL1 pathway (Jayaraman et

al., 2010). Tim3 is a cell surface molecule expressed on T cells, and also marks dysfunctional $CD8^+$ T cells in various kinds of cancers. Wang et al detected Tim3 and its ligand Galectin-9 in gastric cancer tissue samples (Wang et al., 2018). They found that Tim3 was mainly expressed in immune cells with minimal expression in gastric cancer cells. Galectin-9 was significantly overexpressed in tumor cells. Tim3 was positively expressed in about 50% of samples of gastric cancer and was related to age of patient, diameter of tumor, pN stage, and TNM stage. Liu et al carried out the immunohistochemistry staining of Tim3 and Galectin9 on head and neck squamous cell carcinoma (HNSCC) (Liu et al., 2018). They demonstrated that Tim3 was specifically expressed on immune cells in the tumor stroma, and Galectin9 was not only expressed on tumor cells of invasive front but also on immune cells in the tumor stroma. They also showed that Tim3 blockade enhanced the antitumor immune response in an HNSCC mouse model.

The expression level of Tim3 in human cancers has not been well documented. Limited studies dealing with Tim3 expression in colorectal cancer were found (Wang et al., 2017; Ma et al., 2018). Though several groups explored Tim3 expression in several types of human cancer, to our knowledge, there are few studies focusing on the expression of Tim3 in both tumor cells and tumor-infiltrating lymphocytes. In this study, we recruited 171 patients with colorectal cancer to examine Tim3 expression level in both tumor cells and TILs, and to analyze its association with clinicopathological parameters.

Table 1. Relationship between Tim3 expression in CRC cells and clinicopatholigical parameters.

	No.	Tim3 expression in CRC		P value
		-	+	
Gender	171	51	120	
Male	117	36	81	0.723
Female	54	15	39	
Age (years)				
<60	42	15	27	0.339
≥60	129	36	93	
Location				
Right	30	3	27	0.001
Left	141	48	93	
Depth				
Tis+T1+T2	33	24	9	<0.001
T3+T4	138	27	111	
Lymph node				
N0	87	36	51	0.001
N1	84	15	69	
Differentiation				
Poor	27	9	18	0.653
Moderate + Well	144	42	102	
TNM stage				
I+II	87	36	51	0.001
III+IV	84	15	69	
MSI				
MSI	31	3	28	0.008
MSS	140	48	92	
Kras mutation				
Yes	62	16	46	0.487
No	109	35	74	
Braf V600E				
Yes	31	2	29	0.001
No	140	49	91	

Materials and methods

Colorectal cancer tissue specimens

All the tissue specimens in our study were collected from 171 patients with colorectal carcinoma, as part of a study approved by the Research Ethics Board of the Jiangyin People's Hospital. This study met the ethical standards of the Helsinki Declaration II. Written informed consent was obtained from every patient enrolled. These patients had undergone surgery in the Jiangyin People's Hospital between Jan, 2015 and Dec, 2017 without any preoperative therapy. These patients consisted of 117 males and 54 females, and the age range was from 29.6 to 84.5 years (median 67.6 years). Tumor staging (TNM) was evaluated based on the WHO classification of Tumors of the Digestive System (5th, 2019). In detail, the clinicopathological parameters of these 171 cases are listed in Table 1.

Immunohistochemistry

Immunohistochemistry was performed using a routine protocol as we described previously (Zhang et al., 2017). Formalin-fixed, paraffin-embedded samples used for immunohistochemistry were sectioned at 2 μm thickness. All the sections were deparaffinized using xylene, dehydrated by gradient ethanol, and then rehydrated with deionized water. Heat-mediated antigen retrieval was run by autoclave treatment (120°C for 2 min in 1 mmol/l EDTA, pH 8.0) and then followed by cooling at room temperature. Incubation with a monoclonal antibody raised against the human Tim3 (dilution 1:200, abcam, 1 Kendall Square, Suite B2304, Cambridge, USA) was performed overnight at 4°C. After washing with phosphate-buffered saline (pH 7.4), the sections were then incubated with secondary anti-body (Dako, UK) for 30 min at room temperature. Color development was performed with 3, 3'-diamino-benzidine. Nuclei were counterstained with hematoxylin.

The immunostaining results were evaluated independently by two pathologists. The different results were unified by consensus. The score of Tim3 expression was made semiquantitatively by assessing the percentage of stained cells and the staining intensity in tumor cells (Fig. 1) and TILs (Fig. 2) according to the scoring system. Briefly, Tim3 cytoplasmic staining was considered as positive staining. The scoring for

percentage of immunoreactive cells was as follows: 0 (0%), 1 (<20%), 2 (20-50%), and 3(>50%). The staining intensity was scored and stratified as follows: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). A final immunoreactivity score (IRS) was obtained for each of the cases by multiplying the percentage score and the intensity score. Protein expression levels were further analyzed by classifying IRS values as negative (IRS value less than 4) or positive (IRS value equal to or greater than 4).

DNA extraction

DNA was extracted from formalin-fixed, paraffin-embedded tissues by using QIAmp DNA FFPE tissue kit (Qiagen, Germen) according to the manufacturer's protocol. Briefly, 10 slides with a thickness of 10 μm from tissues were deparaffinized using xylene, dehydrated by gradient ethanol. The concentration and quality of DNA in elution buffer was determined by measuring the absorbance at 260/280 nm in a spectrophotometer.

MSI

Extracted DNA from tumor and normal mucosa (as control) were amplified by MSI PCR primer sets according to the manufacturer's protocol (Shanghai Yuanqi Biomed.com, Shanghai, China). PCR amplification as: 42°C, 5min; 94°C, 5min; then 94°C, 15sec; 55°C, 25sec, 72°C, 50 sec, for 40 cycles. Genescan was carried out by using ABI3130 Genetic Analyzer. High-frequency microsatellite instability was identified based on the presence of at least two of the

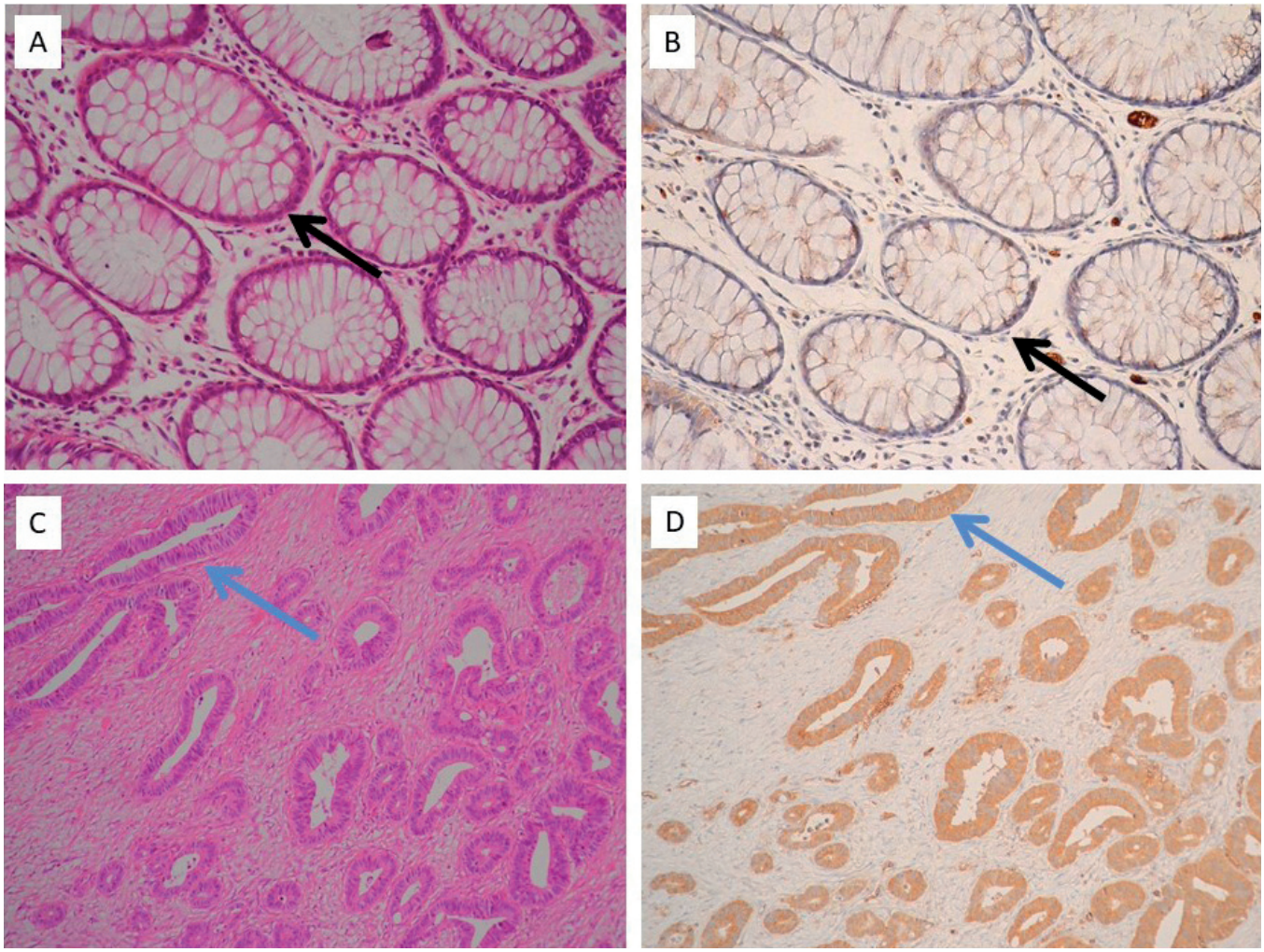

Fig. 1. Tim3 protein expression level in normal mucosa and colorectal cancer cells. **A.** H&E staining of normal mucosa. **B.** Tim3 was negatively expressed in normal mucosa cells. **C.** H&E staining of colorectal cancer. **D.** Tim3 was positively expressed in colorectal cancer cells. Black arrows: normal mucosa cells. Blue arrows: tumor cells. x 400.

five instability markers in the Bethesda microsatellite panel (D5S346, Bat-25, D17S250, Bat-26 and D2S123). Low-frequency microsatellite instability was identified based on the presence of only one instability marker, and MSS was identified based on the absence of instability markers.

Kras mutation

Kras mutations in codon 12 and 13 were checked by TaqMan realtime-PCR method. The kit was purchased from Shanghai Yuanqi Biomed.com. The primer sets and probes were included in kit for detection of mutation G12D, G12A, G12R, G12C, G12V, G12S, G13C, and G13D in codon 12 and 13. The PCR were carried out according to protocol provided by manufacturer.

Braf mutation

Braf V600E mutation was detected by direct Sanger sequencing using primer sets as follows, forward primer sequence is 5'-TGCTTGCTCTGATAGGAAAATG-3' and reverse primer sequence is 5'-AGCATCTCAGGG CCAAAAAT-3'. The PCR product is 228 bps long.

Statistical analysis

The χ^2-test was adopted to determine differences among intergroup variables by use of SPSS 15.0. software (SPSS, Chicago, IL, USA). Kaplan-Meier survival analysis was used to examine the relationship between Tim3 expression and overall survival (OS) for univariate analysis. All the tests were two-sided. A

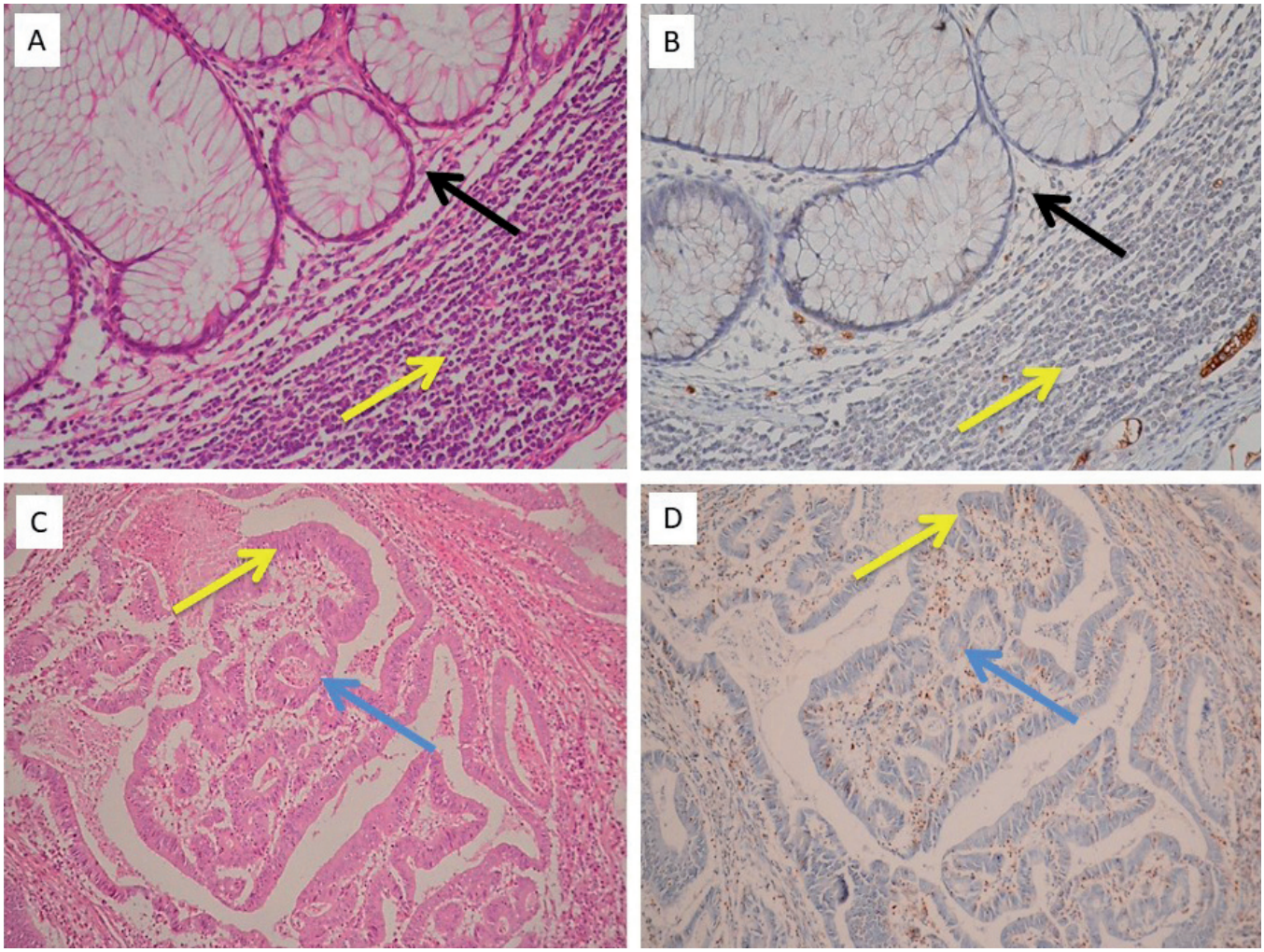

Fig. 2. Tim3 protein expression level in lymphocytes in normal mucosa and colorectal cancer region. **A.** H&E staining of normal mucosa (black arrow) and lymphocytes (yellow arrow). **B.** Tim3 was negatively expressed in normal mucosa cells (black arrow) and lymphocytes (yellow arrow). **C.** H&E staining of tumor cells (blue arrow) and TILs (yellow arrow). **D.** Tim3 was negatively expressed in tumor cells (blue arrow) and positively expressed in TILs (yellow arrow). x 400.

P-value of 0.05 was considered statistically significant.

Results

Expression of Tim3 in colorectal cancer cells and its association with clinicopathological parameters

Tim3 protein was mainly detected in cytoplasm of colorectal cancer cells. Expression of Tim3 was positively detected in 120 out of 171 (70.2%) cases of colorectal cancer, but not in normal intestinal mucosa from the same patient (Fig. 1). Out of 120 cases with positive expression of Tim3 in colorectal cancers, 75 cases (62.5%) had positive expression of Tim3 in TILs. Positive expression of Tim3 was more often found in colorectal tumors located at the right side (P=0.001), and was associated with depth of tumor invasion (P<0.001), lymph node metastasis (P=0.001), advanced TNM stage (P=0.001), MSI (P=0.008), and Braf V600E mutation (P=0.001) (Table 1).

Expression of Tim3 in TILs and its association with clinicopathological parameters

Positive expression of Tim3 in TILs was detected in 81 out of 171 (47.4%) colorectal cancer samples (Fig. 2), and was only related to depth of tumor invasion (P<0.001) (Table 2). Out of 81 cases with positive expression of Tim3 in TILs, 75 cases (92.6%) had positive expression of Tim3 in colorectal cancers.

Expression of Tim3 in colorectal cancer and TILs and its association with clinicopathological parameters

Positive expression of Tim3 both in colorectal cancer cells and TILs was found in 75 out of 171 (43.9%) colorectal cancer patients, and was associated with depth of tumor invasion (P<0.001), lymph node metastasis (P=0.002), advanced TNM stage (P=0.002), MSI (P=0.039), and Braf V600E mutation (P=0.009) (Table 3).

Table 2. Relationship between Tim3 expression in TILs and clinicopatholigical parameters.

	No.	Tim3 expression in TILs		P value
		-	+	
Gender		90	81	
Male	117	63	54	0.742
Female	54	27	27	
Age (years)				
<60	42	27	15	0.109
≥60	129	63	66	
Location				
Right	30	15	15	0.841
Left	141	75	66	
Depth				
Tis+T1+T2	33	30	3	<0.001
T3+T4	138	60	78	
Lymph node				
N0	87	51	36	0.127
N1	84	39	45	
Differentiation				
Poor	27	12	15	
Moderate + Well	144	78	66	0.404
TNM stage				
I+II	87	51	36	0.127
III+IV	84	39	45	
MSI				
MSI	31	15	16	0.687
MSS	140	75	65	
Kras mutation				
Yes	62	32	30	0.874
No	109	58	51	
Braf V600E				
Yes	31	14	17	0.428
No	140	76	64	

Table 3. Relationship between Tim3 expression in both CRC and TILs and clinicopatholigical parameters.

	No.	Tim3 expression (L/T)		P value
		-	+	
Gender	120	45	75	
Male	84	33	51	0.681
Female	36	12	24	
Age (years)				
<60	24	12	12	0.166
≥60	96	33	63	
Location				
Right	18	3	15	0.064
Left	102	42	60	
Depth				
Tis+T1+T2	22	21	1	<0.001
T3+T4	98	24	74	
Lymph node				
N0	66	33	33	0.002
N1	54	12	42	
Differentiation				
Poor	18	6	12	0.795
Moderate + Well	102	39	63	
TNM stage				
I+II	66	33	33	0.002
III+IV	54	12	42	
MSI				
MSI	19	3	16	0.039
MSS	101	42	59	
Kras mutation				
Yes	40	13	27	0.549
No	80	32	48	
Braf V600E				
Yes	19	2	17	0.009
No	101	43	58	

Survival analysis

Kaplan-Meier survival analysis showed that Tim3 expression in colorectal cancer and in TILs was significantly associated with the patient overall survival (OS) rate (P=0.039, and 0.001) (Figs. 3, 4). Expression of Tim3 in colorectal cancer and TILs was not associated with the patient overall survival (OS) rate (P=0.083) (Fig. 5).

Discussion

Immune-checkpoint inhibitors targeted targeting PD-1/PD-L1 have revolutionized the treatment of many cancer types. Generally, the ligand of immune-checkpoint inhibitor such as PD-1 is mainly expressed in immune cells, while receptor (PD-L1) is expressed in tumor cells. The biding binding of PD-1 and PD-L1 generates a net immunosuppressive effect and allows the tumor to evade immune destruction. With the increasing data of PD-1/PD-L1 expression in human cancers, we found that ligand can be differently expressed in certain types of cancer with clinical significance according to the molecular phenotype of the tumor (Chargin et al., 2016; Patel et al., 2017; Thangarajah et al., 2019). For example, PD-1 expression level in non-small lung cancer was associated with Kras mutation (D'Incecco et al., 2015; Ji et al., 2016). PD-1 expression level in tumor cells is of tissue-specificity. In gastric cancer cells, PD-1 mRNA expression level was significantly lower than in normal epithelium, and low PD-1 mRNA level was significantly associated with lymph node metastasis and lymphatic and vascular involvement (Ito et al., 2020). In non-small cell lung cancer cells, PD-1 protein expression ranged from 0 to 10% on immune cells and from 0 to 47% on tumor cells (Chargin et al., 2016).

The identification of new checkpoint targets could allow the field of immuno-oncology to evolve further. Tim3 is a marker of $CD8^+$ T exhaustion and plays critical roles in the negative regulation of T cell response. Therefore, Tim3 is a promising checkpoint target. Till now, the research focused on Tim3 in cancer is limited (Wang et al., 2017, 2018; Wu et al., 2017; Liu et al., 2018; Ma et al., 2018; Martin-Manzo et al., 2019; Zhang et al., 2019; Akagi and Baba, 2020; Sawada et al., 2020). Tim3 expression levels in many types of human cancer are still unknown, especially in colorectal cancer. Previous research on Tim3 expression in human cancers

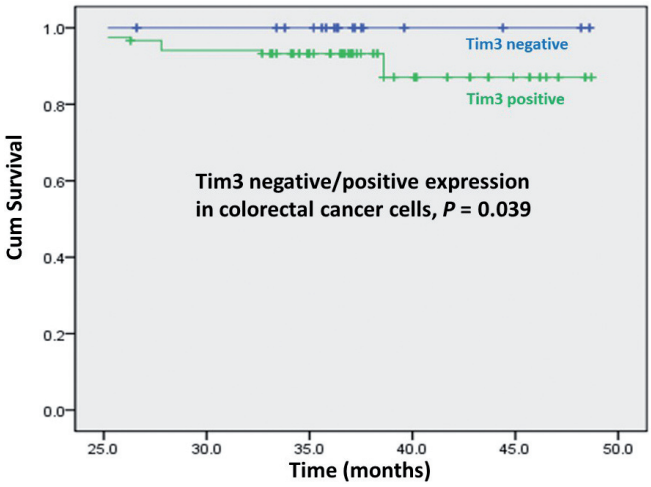

Fig. 3. Kaplan-Meier survival analysis showed that Tim3 expression in colorectal cancer was significantly associated with patient overall survival (OS) rate (P=0.039).

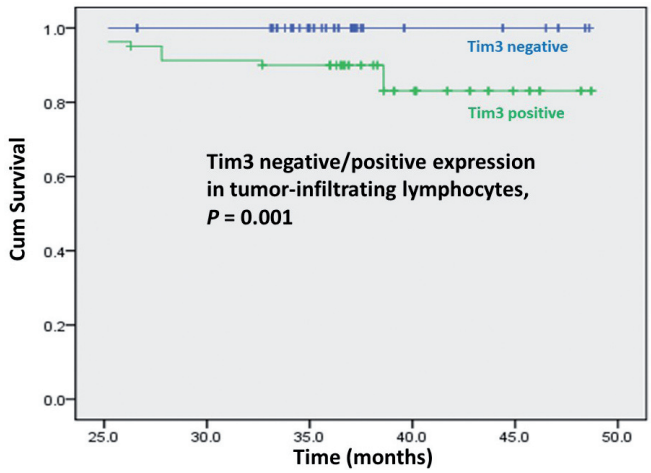

Fig. 4. Kaplan-Meier survival analysis showed that Tim3 expression in TILs was significantly associated with patient overall survival (OS) rate (P=0.001).

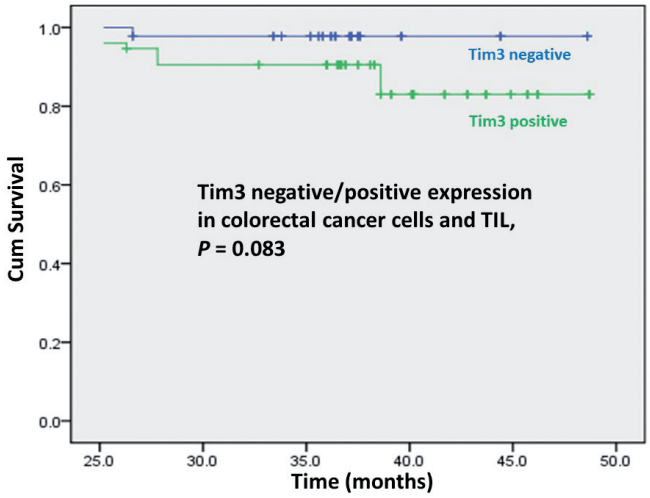

Fig. 5. Kaplan-Meier survival analysis showed that Tim3 expression in colorectal cancer and in TILs was not associated with patient overall survival (OS) rate (P=0.083).

mainly focused on tumor-infiltrating lymphocytes (Wang et al., 2017; Liu et al., 2018; Ma et al., 2018; Sawada et al., 2020), while its ligand Galectin-9 was focused on tumor cells (Li et al., 2017; Wang et al., 2018). There are few studies focusing on the expression of Tim3 in tumor cells. In the present study, we checked Tim3 receptor expression level in colorectal cancer cells and in TILs by using immuno-histochemistry, and analyzed the relationship between Tim3 expression level and clinicopathological parameters and survival time. Our results indicate that Tim3 differentially expressed both in tumor cells and TILs, but was not expressed in normal mucosa cells. Our results are contradictory to that reported by Sun et al. (Sun et al., 2017). Sun et al explored Tim3 mRNA and protein expression in 188 samples of colorectal cancer patients. They found that Tim3 mRNA levels in monocytes from the peripheral blood in normal controls had a positive decreased trend compared to that from colorectal cancer patients. Tim3 mRNA levels were significantly down-regulated in colorectal cancer tissues compared with that in paracancerous tissues and normal colon mucosa tissues. They analyzed the relationship between Tim3 mRNA expression levels in colorectal cancer and clinicopathlogical parameters, and found that it was associated with tumor differentiation, lymph node metastasis, distant metastasis, and clinical stages. In their reports, they did not give the definition of low and high level of Tim3 mRNA. Sun et al detected Tim3 protein expression in colorectal cancer tissues by using western blot and immunohistochemistry. They described the evaluation system for immunohistochemistry staining in detail. However, they did not give the detailed results for immunohistochemistry. In their figure 1, we cannot identify typical normal colon mucosa and colorectal cancer cells. In addition, they did not analyze Tim3 expression level in tumor-infiltrating lymphocytes. Our data show that Tim3 differently expressed in colorectal cancer cells and TILs. Tim3 plays roles as an oncogene in colorectal cancer as indicated by relationships between Tim3 expression and clinicopathological parameters and overall survival. Very interestingly, we analyzed the relationship between Tim3 expression level and MSI status, Kras, and Braf mutations. Our data suggest that Tim3 expression level is associated with MSI and Braf mutation. This is the first time to report that Tim3 expression is molecularly selected in colorectal cancer cells. MSI is the molecular fingerprint of the deficient DNA mismatch repair system (MMR). MMR gene inactivation is a key molecular event when colorectal tumorigensis occurs. In parts of sporadic colorectal cancer, MLH1 hypermethylation or Braf mutation caused MSI (Boland and Goel, 2010; De' Angelis et al., 2018; Evrard et al., 2019; Sun, 2020). Furthermoe, MSI status is associated with the response to 5-fluorouracil based chemotherapy regimens and prognosis of colorectal cancer (Pino and Chung, 2011; Tejpar et al., 2011). The molecular mechanisms of Tim3 in tumorigensis of colorectal cancer are worthy to be intensively studied. We deduced that Tim3 may be a potential prognostic marker and a therapy target for colorectal cancer.

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Ethics approval statement. This study approved by the Research Ethics Board of the Jiangyin People's Hospital.

Conflict of interest disclosure. The authors declare no conflict of interests.

Data availability statement. The data and material are available on reasonable request.

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