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Original research article

The role of P2X7 receptor in prognosis and metastasis of colorectal cancer

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ABSTRACT

Purpose: Colorectal cancer (CRC) is one of the leading causes of cancer mortality in the world. P2X7 receptor (P2X7R), encoded by the *P2rx7* gene, is a trimeric ion channel activated by extracellular Adenosine triphosphate and is widely expressed in various types of tissues and tumors to regulate inflammation, cell proliferation, or death. The discovery of new biomarkers and understanding the role of P2X7R in CRC are therefore critical to improving the prognosis and treatment of CRC.

Materials and methods: P2X7R expression was analyzed in CRC tumor samples and normal colorectal tissues from 97 patients and various colon cancer cell lines. The correlation of tumor antigens, survival periods, and P2X7R expression were documented.

Results: $P2X7R^{High}$ and $P2X7R^{Low}$ populations were observed in CRC patients. $P2X7R^{High}$ patients had relatively shorter survival periods, higher levels of serum carcinoembryonic antigen, and greater numbers of advanced tumors. In addition, P2X7R expression had a significant up-regulation in metastatic CRC and metastatic CRC cell lines, which indicates that P2X7R expression is positively associated with metastasis.

Conclusions: P2X7R expression might be a potential biomarker for prognosis and metastasis of CRC.

1. Introduction

Colorectal cancer (CRC) is one of the most aggressive cancers worldwide. Approximately 5% of people will develop CRC during their lifetime in the United States. Over 40,000 adults in the United Kingdom are diagnosed with CRC each year [1]. Metastatic CRC (mCRC) patients generally receive systemic chemotherapy, including targeted therapies aimed at vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), or KRAS [1]. However, many patients with advanced and metastatic tumors will still succumb to the disease. Further screening and discovery of new biomarkers or analyzing genomic information in CRC is therefore needed to improve detection and mortality statistics [2].

The P2X7 receptor (P2X7R) is encoded by the *P2rx7* gene, located on human chromatin 12, and belongs to the P2X receptor family, which is activated by extracellular Adenosine triphosphate (ATP) [3]. P2X7R is expressed in various cell types, including stem, blood, glial, neural, endothelial, muscle, and renal cells, and regulates inflammation, cell proliferation and death, metabolism, and phagocytosis [4]. P2X7R activation by high concentrations of ATP is generally associated with dramatic cellular events, such as membrane permeabilization and blebbing, loss of asymmetric distribution in phosphatidyl serine, cell swelling, increase of internal Ca^{2+}, or loss of mitochondrial potential [5,6]. Recent study has shown that P2X7R is highly associated with tumor progression, migration, and invasion [7]. P2X7R was also reported to regulate cell survival, migration in pancreatic ductal adenocarcinoma, since P2X7R allosteric inhibitor reduces proliferation, and cell migration [8]. Thus P2X7R might be a candidate cancer biomarker or pharmacological intervention target.

In this work, we collected 97 paired human tumor samples form CRC patients and compared their P2X7R expression. We tried to identify the relationship between P2X7R and CRC prognosis. We also analyzed P2X7R expression in metastatic CRC and colon cancer cell lines. Finally, we attempted to determine whether P2X7R is a possible biomarker for CRC prognosis and metastasis.

2. Materials and methods

2.1. CRC clinical tissue specimens

According to the criteria of the National Comprehensive Cancer Network classification system, a total of 97 surgical resection samples of paraffin specimens of primary colon cancers and matched adjacent normal colorectal tissues (at least more than 5 cm away from the tumor

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Table 1
Primer sequences of qRT-PCR.

Gene name	Forward primer	Reverse primer
P2rx7	GACGCTCTGTTCCTCTGACC	CACCAGGCAGAGACTTCACA
Gadph	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

and histologically confirmed) were obtained from the Department of Pathology, Shengjing Hospital affiliated with China Medical University between 2013 and 2014. In addition, each 10 paraffin specimens of inflammatory polyp, tubular adenoma, lymph node metastasis and liver metastasis were collected as control. Patients were not treated with radiotherapy or chemotherapy prior to surgery. The patients were followed up, and their complete clinical data was collected. Overall survival (OS) was defined as the interval between the dates of surgery and death. Histological types were assigned according to the criteria of the National Comprehensive Cancer Network classification system.

2.2. Cell culture

Colon cancer and normal cell lines NCM460, HCT116, SW480, SW620 were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China), and cultured in RPMI 1640 or DMEM medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf

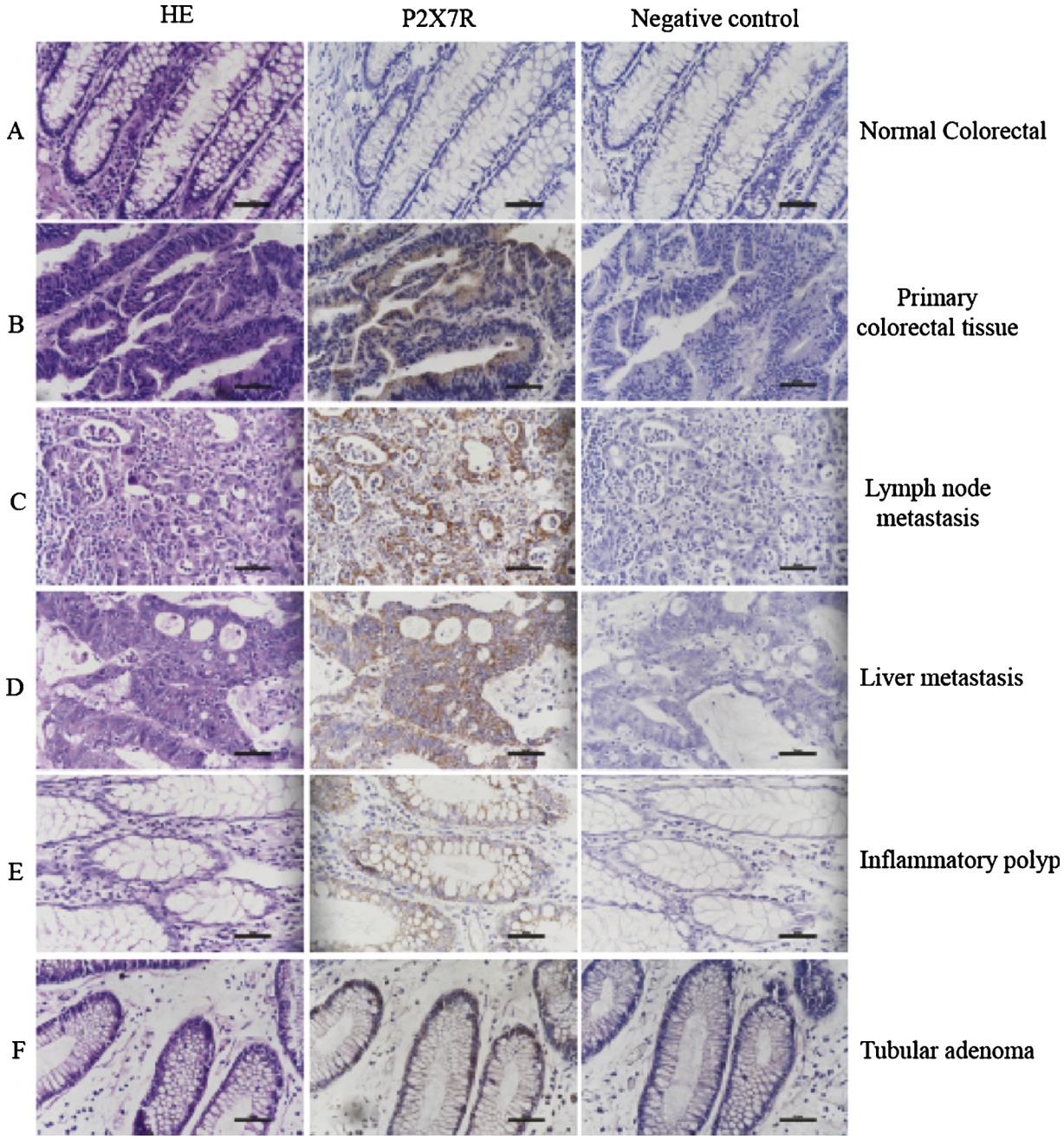

Fig. 1. IHC analysis of P2X7R expression. IHC of P2X7R was performed in (A) normal colorectal tissue, (B) primary colorectal tissue, (C) lymph node metastasis, (D) liver metastasis, (E) inflammatory polyp, and (F) tubular adenoma. IHC performed without the P2X7R antibody was defined as a negative control. HE staining was performed as a counter stain.

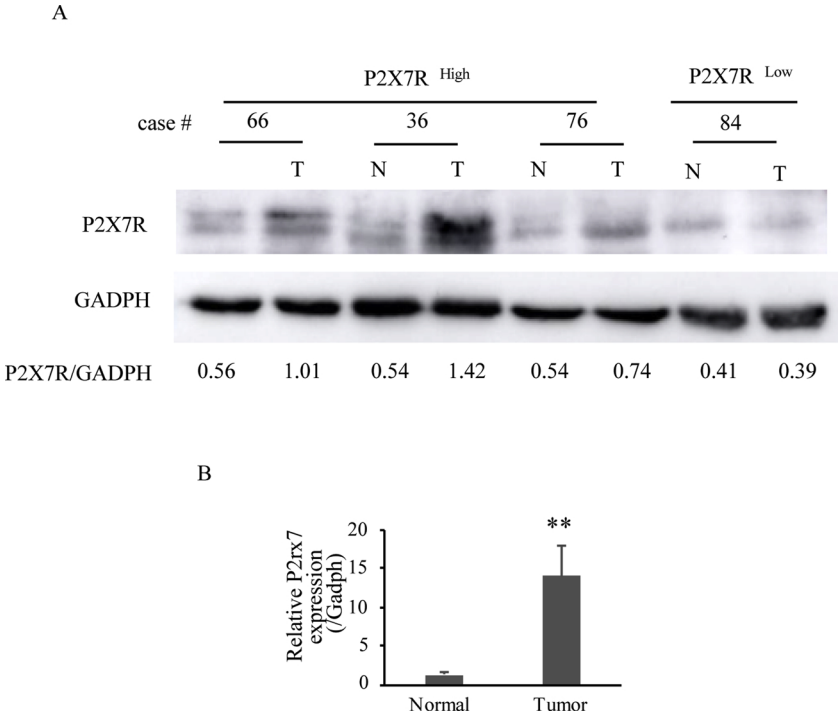

Fig. 2. P2X7R was increased in some primary CRCs. (A) Western blot was used to analyze the expression level of P2X7R in paired CRC tissues (T) and adjacent normal tissues (N). Four individual samples from cases No. 66, 36, 76, and 84 were used. GAPDH was measured as internal control. The densitometry ratio between P2X7R and GAPDH is listed below. (B) P2rx7gene expression was quantified by qRT-PCR in P2X7R + population.Results are expressed as mean ± SD, n = 3. **p < 0.01.

serum, 2 mM glutamine (Gibco, Grand Island, NY, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml), and maintained at 37 °C with CO_2 in a humidified atmosphere.

2.3. Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously reported [9]. Briefly, 4 mm formalin-fixed paraffin-sections were deparaffinized in xylene, rehydrated in a graded ethanol series, and submerged in EDTA antigenic retrieval buffer for 15 min in a microwave oven. After blocking by 5% bovine serum albumin, a rabbit polyclonal antibody against P2X7R (1:100; Proteintech, Rosemont, IL, USA) was incubated overnight at 4℃. After the incubation with secondary antibody, the visualization signal was developed with 3,3′-diaminobenzidine tetrachloride. The negative control was performed without antibody. The images were obtained for analysis. Five random fields at magnification ×400 in each section was scored based on the H-score (histochemistry score) system as previous reported [10]. The median value was used as the cutoff criterion in those specimens.

2.4. Western blot analysis

All the tissue samples and cells were homogenized and lysed in lysis buffer (50 mM Hepes pH 7.4, 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin) at 4 °C for 60 min. The supernatants were collected for Western blot analysis, and protein concentration was determined by the Folin assay. Equal amounts of protein samples were run in SDS-PAGE and trans-bolting to PVDF membranes. Antibodies against P2X7R (Proteintech, Rosemont, US), GADPH (KangChen, Shanghai, China) and horseradish peroxidase (HRP)-conjugated secondary antibody were used, and the specific protein level was visualized by using ECL as the HRP substrate. The densitometry of each band was measured by NIH ImageJ. The ratio between P2X7R and GADPH of each sample was calculated and showed in the below of each figure.

2.5. qRT-PCR

RNA was purified from tissue samples or cells by TRIzol Reagent (Invitrogen, Waltham, US). *P2rx7* expression was examined by qRT-PCR using SYBR green real-time PCR Master Mix (TOYOBO, Tokyo, Japan). *Gadph* was quantified as internal control. The primers used are described in Table 1.

2.6. Statistical analysis

Results are presented as the mean ± standard deviation (SD). Differences between groups were examined for statistical significance using Student's t-test. The statistical significance of the relationship between P2X7R expression and the clinicopathologic features of patients was evaluated using Mann-Whitney *U* test. Cox multivariate analysis was used to determine the independent prognostic factors.

2.7. Ethical issues

Informed consent was obtained from all patients prior to tissue acquisition. This study was approved by the Ethics Committee of the Affiliated Shengjing Hospital of China Medical University (ethics approval number: 2016PS274K).

3. Results

3.1. CRC patients can be classified using P2X7R expression

To analyze the relationship between P2X7R and CRC, IHC was

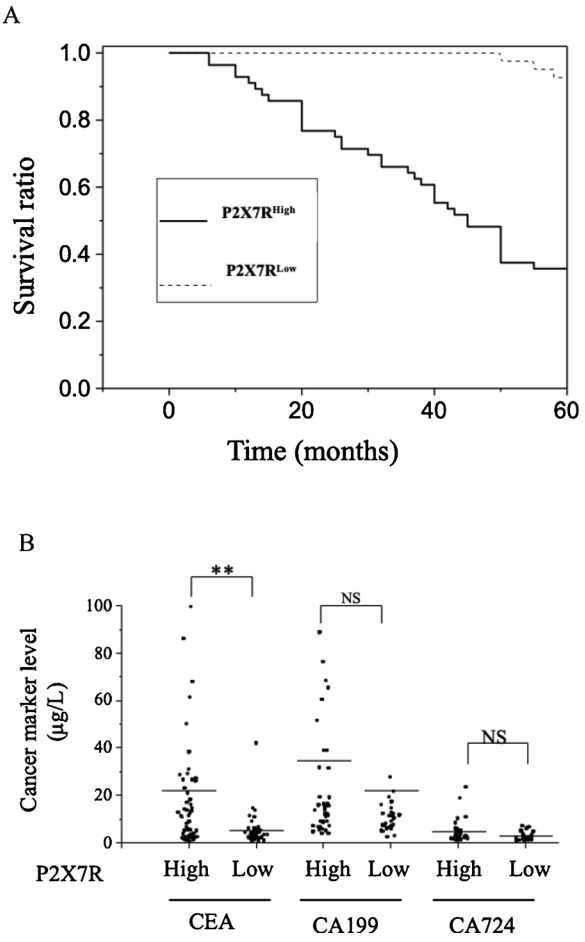

Fig. 3. P2X7RHighpopulation can be associated with poor prognosis. (A) The P2X7RHigh population had relatively shorter survival periods. Kaplan–Meier curves for each population were generated to understand the survival periods. (B) The P2X7RHigh population had relatively higher levels of blood CEA. The cancer markers CEA, CA 199, and CA 724 were quantified by ELISA using serum samples. Dot plots show the cancer marker amount for individual patients in P2X7RHigh andP2X7RLow populations. Middle lines indicate the mean, n = 55, 37; 46, 34; 33, 26. **p < 0.01.

Table 2
The relationship between P2X7R expression and clinicopathological features of colorectal cancer.

Variable	Total	P2X7R expression No. of patients (%)		P
		Low	High	
Age (years)				
< 60	31	14(14.4)	17(17.5)	0.833
> =60	63	27(27.8)	36(37.1)	
Gender				
Male	59	25(25.8)	34(35.1)	0.820
Female	38	17(17.5)	21(21.6)	
Depth of invasion				
T1	5	1(1)	4(4.1)	0.126
T2	15	9(9.3)	6(6.2)	
T3	67	31(32)	36(37.1)	
T4	10	1(1)	9(9.3)	
Lymphatic invasion				
N−	49	30(30.9)	19(19.6)	0.001***
N+	48	12(12.4)	36(37.1)	
TNM Stage				
I	16	7(7.2)	9(9.3)	0.001***
II	30	23(23.7)	7(7.2)	
III	41	11(11.3)	30(30.9)	
IV	10	1(1)	9(9.3)	
Distant metastasis				
M0	87	41(42.3)	46(47.4)	0.026*
M1	10	1(1)	9(9.3)	
Histopathological type				
Well differentiated	11	6(6.2)	5(5.2)	0.126
Moderately differentiated	68	31(32)	37(38.1)	
Poorly differentiated	18	5(5.2)	13(13.4)	
Tumor site				
Right side	28	12(12.4)	16(16.5)	0.971
Left side	16	7(7.2)	9(9.3)	
Rectum	53	23(23.7)	30(30.9)	
Vascular invasion				
Yes	24	7(7.2)	17(17.5)	0.109
No	73	35(36.1)	38(39.2)	
Nerve invasion				
Yes	26	8(8.2)	18(18.6)	0.134
No	71	34(35.1)	37(38.1)	
Total	97	42	55	

*p < 0.05, ** p < 0.01, ***p < 0.001.

performed in 97 paired paraffin-embedded samples to measure the P2X7R expression. Compared to adjacent normal column tissues, tumor specimens from 56 patients showed an up-regulation of the P2X7R signal, which was classified as a P2X7R^{High} population (Fig. 1A and B). P2X7R expression in the remaining 41 tumor specimens did not increase, which was defined as a P2X7R^{Low} population. P2X7R expression was then confirmed in the CRC tumor cells using a western blot assay and qRT-PCR. In the P2X7R^{High} tumor samples (No. 66, 36, and 76; Fig. 2A), P2X7R expression was relatively higher than in the adjacent normal colorectal tissues. However, P2X7R expression did not change in the P2X7R^{Low} tumor sample (No. 84; Fig. 2A). Furthermore, P2X7R mRNA levels were detected by qRT-PCR, showing P2X7R expression to be statistically significantly higher than that in the adjacent normal colorectal tissues in the P2X7R^{High} samples. These data suggest that two groups can be observed based on their P2X7R expression in CRC.

3.2. P2X7R^{High} patients are associated with short survival time

To analyze the effect of P2X7R expression on prognosis of CRC patients, we calculated survival periods in the P2X7R^{High} and P2X7R^{Low} populations. Using Kaplan-Meier curves, it was evident that survival periods of the P2X7R^{High} population were much shorter than those in the P2X7R^{Low} patients. This indicates that the P2X7R^{High} patients have poorer prognoses (Fig. 3A). We also compared the clinicopathological features of the two patient groups using the Mann-Whitney U test. There were no statistically significant differences in gender, age, tumor sites, differentiated histopathological type, and vascular and nerve invasion between the two groups (Table 2). Based on TNM staging, the percentage of stage III and IV diagnoses in the P2X7R^{High} patients was statistically significantly increased. mCRC was mainly observed in the P2X7R^{High} patients. The percentages of lymphatic invasion were clearly increased in the P2X7R^{High} patients compared to the P2X7R^{Low} patients (Table 2). Moreover, Cox multivariate analysis was performed to clarify the role of the clinicopathological features in the survival periods. P2X7R expression level was found to be one of the contributors to poor survival periods (Table 3).

Since cancer markers are important directors of tumor progression [11], we analyzed the levels of different cancer markers in the two groups. Serum CEA levels were statistically significantly higher in the P2X7R^{High} group (Fig. 3B). CA 199 and CA 724 cancer markers were not statistically significantly changed in these two groups (Fig. 3B), demonstrating that the P2X7R^{High} population could have a poorer prognosis.

Table 3
Univariate and multivariate analyses of individual parameters for correlation with OS.

Characteristics	Univariate			Multivariate		
	HR	CI (95%)	P	HR	CI (95%)	P
Age (≥60/ < 60)	1.000	0.964, 1.037	0.993	0.964	0.925, 1.003	0.073
Gender (Male/Female)	0.844	0.415, 1.716	0.640	1.011	0.447, 2.287	0.979
Location (right/left/rectum)	0.850	0.580, 1.246	0.405	0.807	0.520, 1.252	0.338
Lymphatic invasion (0/ > 0)	7.754	2.985, 20.140	< 0.001***	0.856	0.166, 4.425	0.853
Distant metastasis	7.665	3.488, 16.846	< 0.001***	0.405	0.041, 3.955	0.437
TNM stage (I/II/III/IV)	4.847	2.875, 8.170	< 0.001***	5.593	1.107, 28.257	0.037*
P2X7R expression (high/low)	34.501	4.707, 252.856	< 0.001***	24.476	3.196, 187.475	0.002**

OS - overall survival; HR - Hazard ratio; CI - confidence interval.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

3.3. P2X7R expression is associated with CRC metastasis

To understand the relationship between P2X7R expression and mCRC, we analyzed the P2X7R expression in mCRC tumor samples. P2X7R expression in some metastatic tumors, including lymph node and liver tumors, was much higher compared to primary colorectal tissues (Fig. 1C and D). In addition, a weak signal was observed in the inflammatory polyp and tubular adenoma, which might be due to P2X7R's reported association with inflammation (Fig. 1E and F). To confirm the IHC results, we analyzed the P2X7R protein levels in primary tumor cells and metastatic tumor cells. In the lymph node metastasis (No. 70), the expression of P2X7R was higher than in the primary tumor tissue (Fig. 4A). P2X7R expression in liver metastasis (No. 77) was also higher than that in the primary tumor tissue (Fig. 4A). These data indicate that P2X7R expression level might play an essential role in CRC development and metastasis.

3.4. P2X7R is highly expressed in metastatic cell lines

To further understand whether the expression of P2X7R is associated with metastasis of CRC, we compared a normal colon cell line (NCM 460) and CRC cell lines (HCT 116, SW 480, and SW 620). P2X7R protein and mRNA levels were analyzed using western blot (Fig. 4B) and qRT-PCR, respectively (Fig. 4C). Compared to the normal colon cell line NCM460, CRC cell lines showed a higher expression of P2X7R. Among them, SW 620 cell line demonstrated the highest level. Interestingly, SW 480 and SW 620 were derived from a single patient. While SW 480 is a primary tumor-derived cell line, SW 620 cells are metastasis-derived CRC cells [12]. Compared with the SW 480 cells, the expression of P2X7R in SW 620 cells was statistically significantly increased. This suggests that the increase in P2X7R expression might be a director of CRC metastasis.

4. Discussion

In the present study we demonstrated that P2X7R is highly expressed in some CRC tumors and that its expression level is negatively associated with survival periods in CRC patients. Moreover, the expression of cancer marker CEA is associated with short survival periods and advanced tumor stages in CRC patients [13]. Therefore, the up-regulation of CEA in the $P2X7R^{High}$ populations might be indicative of a poor prognosis. High P2X7R expression was also reported to induce worse clinical outcomes in patients with clear-cell renal cell carcinomas, including shortened cancer-specific survival and high TNM stage [14]. Another report showed that high P2X7R expression statistically significantly correlated with tumor size, lymph node metastasis, TNM stage, and poor overall survival in CRC patients [15]. On the contrary, P2X7R was reported as a biomarker in various tumor cells, including uterine epithelial [16], breast [17], and prostate [18] cancers. Thus, P2X7R could be a good biomarker for CRC, which might be associated with CRC progress.

We found the expression of P2X7R to be statistically significantly up-regulated in metastatic tumor cells and cell lines. As an ATP receptor, P2X7R was reported to contribute to cancer cell invasiveness. In prostate cancer cells, PI3K/AKT and ERK1/2 pathways were enhanced by the activation of P2X7R after ATP addition, with the downstream invasiveness-associated genes Snail, E-cadherin, Claudin-1, IL-8, and MMP-3 serving as further inductors [18]. MMP-3-dependent tumor cell invasion was also observed in mCRC [19]. This indicates that an increase in P2X7R may also induce MMP-3-dependent tumor cell invasion in CRC. Moreover, expression of P2X7R increases tumor growth *in vivo*. Human embryonic kidney cells with P2X7R-inoculated mice exhibited a more tumorigenic phenotype, such as high proliferation and low apoptosis, which might be associated with high levels of activated transcription factor NFATc1 and VEGF secretion [20]. In addition, the growth and proliferation of mCRC depends on VEGF signaling pathways [21]. Antiangiogenic agents - bevacizumab and aflibercept - statistically significantly increased the overall survival times in mCRC patients [22]. An increase in P2X7R expression could induce VEGF secretion and active VEGFR. The crosstalk between P2X7R and VEGF signaling pathways might contribute to CRC metastasis. This suggests that P2X7R could regulate CRC metastasis and proliferation via crosstalk with other CRC biomarkers.

In the 1920s, Nobel Prize winner Otto Warburg demonstrated that high glycolytic flux is a core metabolic signature of cancer cells, which has been named the Warburg effect [23,24]. Strategies to target cancer by disrupting the flow of ATP and NADH have been tested, since high levels of ATP and NADH are required for tumor metastasis, proliferation, and even survival. Various reagents against glucose metabolism and ATP production have been tested as anti-cancer drugs [25,26]. In CRC, high glucose was reported to induce the migration and invasion of CRC cells [27]. P2X7R is a key player in tumor metabolism. In human neuroblastoma cell line - ACN, P2X7R induced cell metabolism reprograming to meet the needs imposed by adverse environmental conditions through up-regulation of glucose metabolism transporter Glut1, the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase, phosphofructokinase, and pyruvate kinase M2 [28]. Therefore, up-regulation of P2X7R expression in some CRC cells could contribute to

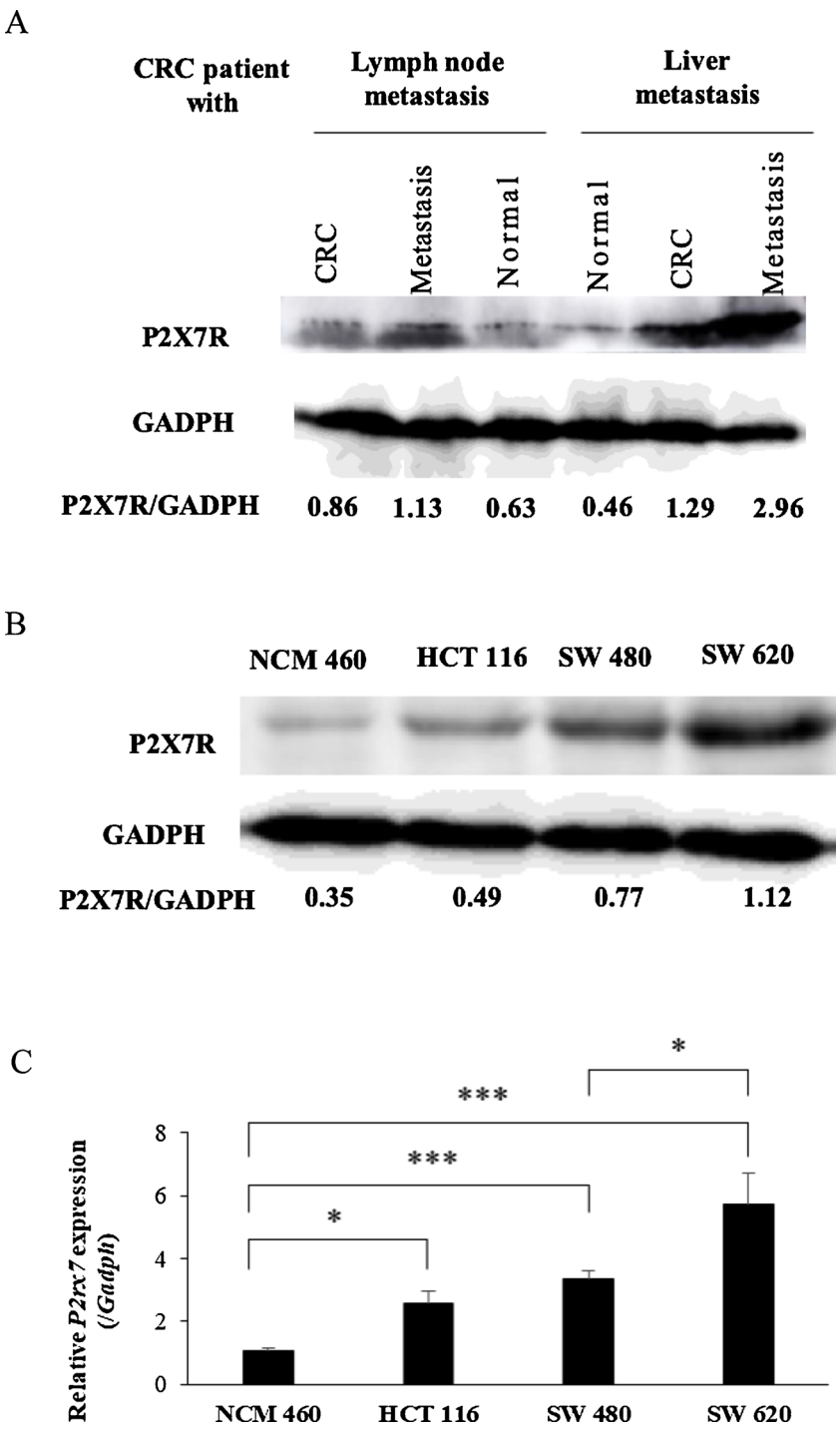

Fig. 4. P2X7R highly expressed in metastatic CRC tissues and cell lines. (A) P2X7R is highly expressed in metastatic tissues. Western blot was performed to measure the P2X7R expression in primary colorectal tissues (CRC), matched adjacent normal tissues (Normal), and metastatic tissues (Metastasis) from the same patient. Two individual CRC patients with lymph node and liver metastasis were used. (B) The P2X7R expression was analyzed in colon cell lines by western blot. The densitometry ratio between P2X7R and GAPDH is listed below. (C) P2rx7gene expression was quantified by qRT-PCR in colon cell lines. Results are expressed as mean ± SD, n = 3. *p < 0.05, ***p < 0.001.

tumor metabolism and promote CRC tumor proliferation and metastasis.

Furthermore, inhibition of P2XR7 might be a good strategy for treatment of CRC or other cancers. Even though there is currently no successful anti-P2X7 receptor drug for human use, an inhibitor of P2X7R AZ10606120 was recently tested and showed some anti-cancer activity in pancreatic cancer and stellate cells [29]. Because of high levels of P2X7R expression in CRC tumors and metastatic cells, we speculated that inhibition of P2X7R might be a good approach for CRC treatment. Using a P2X7R inhibitor might improve survival times and quality of life in CRC patients.

5. Conclusions

In this study we demonstrated that CRC patients with a high expression of P2X7R had a poor prognosis and that the expression of P2X7R is associated with CRC progress and metastasis. Therefore, P2X7 receptor might be a good biomarker and drug target for CRC.

Conflict of interest

The authors declare no conflicts of interest regarding the content herein.

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The author contribution

Study design: Ying Zhang, Lili Wang
Data collection: Ying Zhang, Jingjing Ding
Statistical analysis: Ying Zhang, Jingjing Ding
Data interpretation: Ying Zhang, Jingjing Ding, Lili Wang
Manuscript preparation: Ying Zhang, Lili Wang
Literature search: Ying Zhang, Jingjing Ding, Lili Wang
Funds collection: Lili Wang

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