Identification of ANXA1 as a diagnostic and lymphatic metastasis factor in colorectal cancer

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Abstract

Objective: To determine whether ANXA1 can be served as biomarker for diagnosis in colorectal cancer (CRC) and analyse the correlation of ANXA1 and clinicopathological parameters of CRC patients.

Methods: Tissue array (n=95) CRC specimens were utilised in this study. ANXA1 in cancer and non-cancerous tissues were detected immunohistochemically. The correlations between ANXA1 and clinicopathological parameters of CRC were evaluated. Concentrations of ANXA1 in peripheral blood were detected by enzyme-linked immunosorbent assay (ELISA).

Results: ANXA1 was upregulated in CRC tissues than that in non-cancerous tissues (P <0.05). The mean value for ANXA1 level in peripheral blood of CRC patients was significantly higher than that of healthy individuals (P <0.05). Increased ANXA1 was observed in deeper tumor invasion, lymphatic metastasis and late TNM stage of CRC patients.

Conclusions: ANXA1 increased in CRC tissues and in peripheral blood of CRC patients. In addition, increased ANXA1 was correlated with deeper tumor invasion, lymph node metastasis and late TNM stage of CRC patients. Our findings would provide new insights into significant of ANXA1 in diagnosis and lymphatic metastasis of CRC. The underlying mechanism of ANXA1 in CRC progress will be further explored.

Introduction

CRC is the third most common cancer in male and the second in female worldwide. In addition, CRC is the fourth leading cause of cancer mortality worldwide [1]. The treatment of CRC patients with metastases through combination chemotherapy helps improving the prognosis, but overall prognosis for CRC patients remains poor and the 5-year survival rate is less than 19% in stage IV patients with distant metastases [2]. Therefore, studying the molecular mechanism of CRC cell growth, metastasis and finding key markers is very important for early diagnosis, prognosis and may give insight into clinic targeted therapy of CRC patients.

Annexin a1 (ANXA1), the first characterized member of annexin superfamily contributes to variety cellular biological activities: anti-inflammatory effects, cell differentiation and most importantly involved in the process of cancer [3]. ANXA1 has been recognized down regulated in head neck cancer and esophageal cancer, up regulated in pancreatic cancer and mammary adenocarcinoma [4,5]. Researchers are hampered by conflicting roles of ANXA1 in cancers and by lack of demonstrating the specific expression of it.

We therefore systematically investigate the expression of ANXA1 in human CRC solid cancers and matched non-cancerous tissues. Immunohistochemistry were established to detect the tissue-specific expression of ANXA1. Furthermore, we detected the concentrations of ANXA1 in peripheral blood of CRC patients and healthy individuals by ELISA. Our data may provide useful information for ANXA1 used as a diagnostic marker and/or further used as therapeutic targets for CRC patients.

Materials and methods

Patients and samples

CRC tissues and non-cancerous tissues (control) were obtained from 95 CRC patients undergoing resection in the Affiliated Hospital, Xuzhou Medical University, China, between 2007 and 2011. These CRC patients were comprised of 54 males and 41 females, aged from 34 to 69 years old (Table 1). No chemotherapy was used in these patients prior to resection. This study was approved by the Human Ethical Committee, the Institutional Review Boards of Affiliated Hospital of Xuzhou Medical University, and written informed consents were obtained.

Between 2016 and 2017, 20 patients with CRC and 20 healthy individuals were included in this study. The peripheral blood samples from CRC patients were collected before resection of primary tumour. This study was approved by the Human Ethical Committee, the Institutional Review Boards of Affiliated Hospital of Xuzhou Medical University, and written informed consents were obtained.

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Table 1. Expression of ANXA1 in CRC tissues and in adjacent non-cancerous tissues

Groups	n	Positive rate (%)	Negative rate (%)	χ^2	P
CRC	95	71 (74.74)	24 (25.26)	11.896	0.001
Non-cancer	95	48 (50.53)	47 (49.47)		

Immunohistochemistry

Tissue microarrays had been used the paraffin-embedded specimens of 95 CRC tissues and non-cancerous tissues by a Beecher Instrument (Silver Spring, MD, USA). The sections (5 μm) from the tissue microarray blocks were labelled with anti-ANXA1 polyclonal antibody (TA500967, OriGene Technologies, Inc., USA) at dilution of 1:200. HRP- conjugated secondary antibody (12127A07, Beijing Sequoia Jinqiao Biological Technology Co., Ltd.) was used according to the manufacturer's instructions. The specific target(s) was visualized with 3, 3'-diaminobenzidine (DAB) detection kit (Beijing Sequoia Jinqiao Biological Technology Co., Ltd.) and counterstained with hematoxylin. All the slides were examined and scored independently by two pathologists. ANXA1 was stained brown in cytoplasm with or without in nucleus of CRC.

ELISA

In our study, we prospectively enrolled 20 CRC patients in the Affiliated Hospital, Xuzhou Medical University, China, in 2016-2017. Patients who had received preoperative chemotherapy were excluded. And 20 healthy persons from non-cancer patients were collected as controls in our study. 5ml peripheral blood was collected from subjects and centrifuged at 3000rpm for 15min at 4°C, and then the supernatants were stored at -80°C until analysis. According to the manufacturers's instructions of Human ANXA1 (SEE787Hu, Cloud-Clone Corp, Wuhan, China), we determined concentrations of ANXA1 by ELISA kit (Bio-Rad 550, US) at 450nm. All samples were checked in duplicates.

Statistical analysis

SPSS16.0 statistical software package was used for our statistical analysis. Association between the expression of ANXA1 and the clinicopathological characteristics had been analyzed by using Chi-square test. Student's t test was used for comparing variable between groups in ELISA assay. $P<0.05$ was considered as statistically significant.

Results

Expression of ANXA1 in CRC tissue

ANXA1 in CRC tissues was stained in brown mainly located in cytoplasm with or without nuclear staining in immunohistochemistry assay (Figure 1). Additionally, the expression of ANXA1 was significantly increased in CRC compared to non-cancerous tissues ($P<0.05$) (Table 2 and Figure 2A).

Correlation between ANXA1 in CRC and clinicopathological parameters

Associations between clinicopathological parameters and ANXA1 are listed in Table 1 and Figures 2B-2D. There was significantly higher expression of ANXA1 in the group of patients with lymph node metastasis, deeper tumour invasion (invasion depth) and late TNM stage. Moreover, no significant differences were observed in ANXA1 with age, gender, differentiation and diameter of CRC.

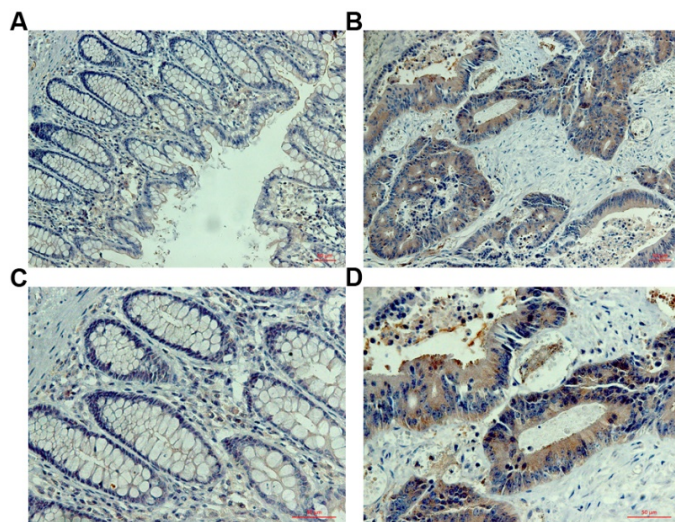## ANXA1 in peripheral blood of CRC patients and in healthy individuals

The mean value for ANXA1 in peripheral blood of CRC patients was 5.184 ± 1.070 ng /ml, which was significantly higher than that of healthy individuals (1.031 ± 0.600 ng/ml) ($P<0.05$) (Figure 3).

Discussion

Although ANXA1 was an attracting protein in cancer research, the contradictory expression levels in solid tumors and different roles limited its usage as biomarker in diagnosis and therapeutic of cancer in clinic [6-8]. Furthermore, previous studies had reported that ANXA1 was also altered in the same type tumors, e.g., large number reports of contradictory results of ANXA1 in breast cancers [9-11]. In our present study, the expression of ANXA1 in human CRC tissues was explored

Table 2. Comparision of clinicopathological features in CRC patents (n=95)

| Variables	ANXA1 staining			
	Positive (%)	Negative (%)	Total	P
Age				
>55	35(72.92)	13(27.08)	48	0.68
≤ 55	36(76.60)	11(23.40)	47	
Gender				
Male	43(79.63)	11(20.37)	54	0.208
Female	28(68.29)	13(31.71)	41	
Tumor differentiation				
Well	14(70.00)	6(30.00)	20	0.86
Moderate	48(76.19)	15(23.81)	63	
Poor	9(75.00)	3(25.00)	12	
Lymph node metastasis				
Yes	38(90.48)	4(9.52)	42	0.001
No	33(62.26)	20(37.74)	53	
Tumour size (diameter)				
<5 cm	38(76.00)	12(24.00)	50	0.765
≥ 5cm	33(73.33)	12(26.67)	45	
Serosa invasion (invasion depth)				
Yes	56(81.16)	13(18.84)	69	0.019
No	15(57.69)	11(42.31)	26	
TNM stage				
I-II	32(64.00)	18(36.00)	50	0.011
III-IV	39(86.67)	6(13.33)	45	

Figure 1. Immunohistochemistry assay
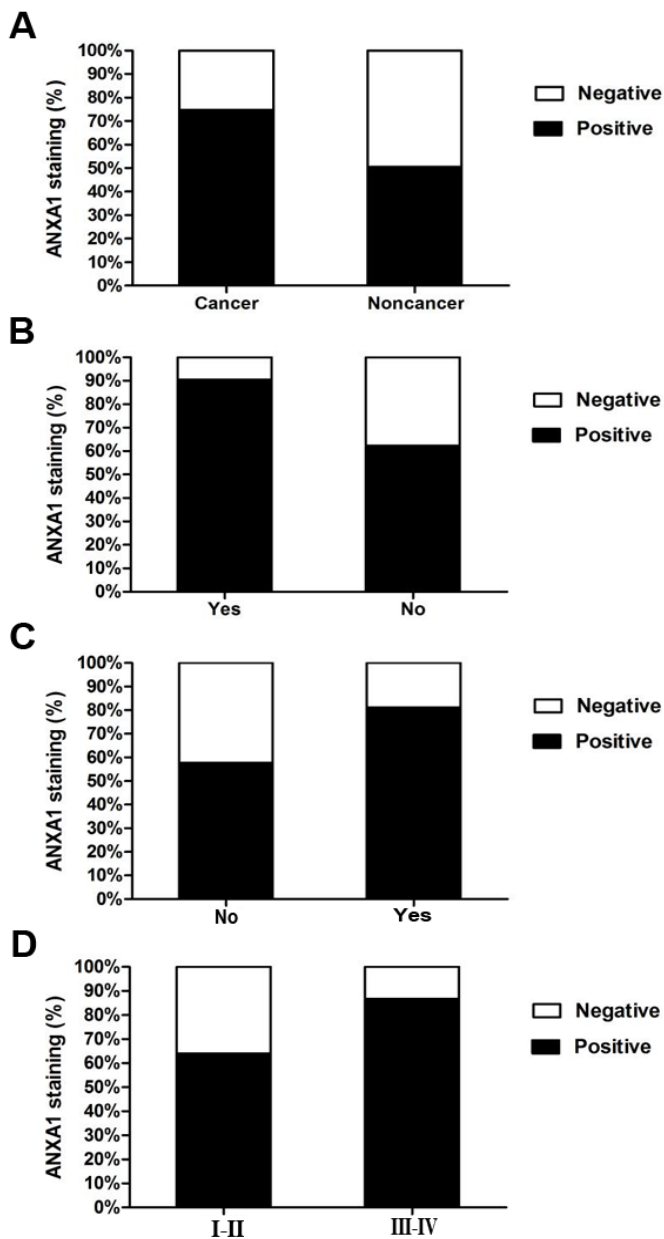
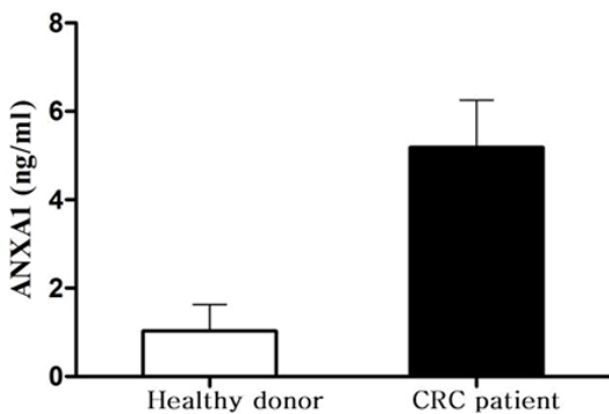**Figure 2.** Increase in ANXA1 in CRC compared to non-cancerous tissues

Figure 3. ANXA1 in peripheral blood of CRC patients and in healthy individuals

by immunohistochemistry. In addition, the expression level of ANXA1 in peripheral blood of CRC patients was detected by ELISA. Our results showed that ANXA1 was increased in CRC tissues and upregulated in the peripheral blood of CRC patients. We provided evidences that ANXA1 upregulated in CRC and it may promote the development and growth of CRC.

Depth of tumor invasion has been assessed as an important prognostic marker for tumor progression and prognosis [12,13]. Tumor depth is a well-known prognostic factor in gastric cancer patients and was demonstrated that deeper tumor invasion was significantly associated with poor prognosis [13]. In CRC, pT1+pT2+pT3 ≤ 5mm were subdivided in low risk group and pT3>5mm +pT4 were subdivided in high risk group. Furthermore, the sensitivity and specificity of the two groups were 0.90 and 0.75 [14]. Our results demonstrated ANXA1 upregulation in CRC was related to deeper tumour invasion depth which suggested that ANXA1 may promote the invasion of CRC. Many experiments have demonstrated that tumor invasion depth and tumor size correlate closely with positive lymph node metastasis [15,16].

Invasion and metastasis are the main causes of death for cancer patients and lymph node are the common sites of cancer metastasis [17]. Meanwhile, lymphatic metastasis predicts increased mortality of many cancer types. Although, the role of ANXA1 in tumor progress and metastasis also had inconsistent results [9,18], there were more consistently results showed that upregulated ANXA1 related to cancer metastasis. Higher ANXA1 expressions were showed in lung metastases cell line compared with cell line from primary tumour and often higher stained in the lymph node metastases [10]. Our results demonstrated ANXA1 upregulation in CRC was related to lymph node metastasis. Our data suggested that ANXA1 may promote the metastases of CRC.

Increased migratory ability is one key step for tumour metastasis. Overexpression of ANXA1 could enhance cell migration of breast cancer cell line MCF-7 through ERK-RhoA-NFκB activation loop [19]. ANXA1 also could regulate mTOR signalling and the activity of MMP9 for regulating the migration activity of breast cancer cells, but which was not been uniformly supported [20]. EMT (epithelial mesenchymal transition), had been recognised playing vital role in tumour metastasis and there were evidences of relationships between ANXA1 and EMT in different subtypes of breast cancers [21-23]. The mechanism of ANXA1 regulating metastasis of CRC need further explored.

In most tumours, lymphatic metastasis plays an important role in tumour diffusion. The TNM (TNM Classification of Malignant Tumors) system underlines the importance of the depth of tumor invasion, lymphatic metastasis assessment and distant metastasis both in pathologic and in clinical setting [24]. The prognostic significance of lymphatic nodal involvement and TNM stage have been confirmed by many experiments that the outcome of tumor patients strongly depends on lymphatic nodal involvement and the stage of tumor at the time of diagnosis [25-27]. Our data also demonstrated ANXA1 upregulated in CRC was related to late TNM stage. Our data suggested that ANXA1 might have prognostic significance in CRC.

In summary, ANXA1 was upregulated in CRC tissues, and ANXA1 was correlated with deeper tumor invasion and lymph node metastasis of CRC. Furthermore, ANXA1 was correlated with late TNM stage of CRC patients. These results suggested that ANXA1 may promote the progression of CRC. In addition, high ANXA1 in peripheral blood may be considered as a biomarker for diagnosis of CRC. Our findings would provide new insights into significant of ANXA1 in diagnosis and prognosis of CRC. The underlying mechanism of ANXA1in CRC progression will be further explored.

Conflict of interest

Qing-Hua Liu declares that she has no conflict of interest. Mei Ding declares that she has no conflict of interest. Jin-Min Sun declares that she has no conflict of interest. Zu-An Zhu declares that he has no conflict of interest.

Acknowledgements

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