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• BASIC RESEARCH •

Genomic determination of CR1 CD35 density polymorphism on erythrocytes of patients with gallbladder carcinoma

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Abstract

AIM: To study the changes of quantitative expression, adhering activity and genomic density polymorphism of complement types in erythrocytes (CR1) of patients with gallbladder carcinoma and the related clinical significance.

METHODS: Polymerase chain reaction (PCR), *Hind*III restriction enzyme digestion, quantitative assay of CR1 and adhering activity assay of CR1 in erythrocytes were used.

RESULTS: The number and adhering activity of CR1 in patients with gallbladder carcinoma (0.738±0.23, 45.9±5.7) were significantly lower than those in chronic cholecystitis and cholecystolithiasis (1.078±0.21, 55.1±5.9) and healthy controls (1.252±0.31, 64.2±7.4) (P<0.01). The number and adhering activity of CR1 in patients with chronic cholecystitis and cholecystolithiasis (1.078±0.21, 55.1±5.9) were significantly lower than those in healthy controls (1.252±0.31, 64.2±7.4) (P<0.05). There was a positive correlation between quantitative expression and adhering activity of CR1 (r = 0.79, P<0.01). Compared with those on preoperative day (0.738±0.23, 45.4±4.9), the number and adhering activity of CR1 in patients with gallbladder carcinoma decreased greatly on the third postoperative day (0.310±0.25, 31.8±5.1) (P<0.01), and on the first postoperative week (0.480±0.25, 38.9±5.2) (P<0.01), but they were increased slightly than those on the preoperative day (P>0.05). The number and adhering activity of CR1 recovered in the second postoperative week (0.740±0.24, 46.8±5.9) (P<0.01) and increased greatly in the third postoperative week (0.858±0.35, 52.7±5.8) (P<0.01) in comparison with those on the preoperative day and in the first postoperative week. The number and adhering activity of CR1 of gallbladder carcinoma patients with infiltrating, adjacent lymphogenous and distant organ metastases were significantly lower than those of gallbladder carcinoma patients without them (P<0.01). No difference was observed between the patients with gallbladder carcinoma and healthy individuals in the spot mutation rate of CR1 density gene (χ^2 = 0.521, P>0.05). The distribution of expression was 67.8% in high expression genomic type, 24.8% in moderate expression genomic type, and 7.4% in low expression genomic type. The number and adhering activity of CR1 high expression genomic type gallbladder carcinomas (0.749±0.22, 42.1±6.2) were significantly lower than those of healthy individuals(1.240±0.29, 63.9±7.2), and were also significantly lower than those of healthy individuals (0.921±0.23, 54.8±7.1), but no difference was observed between the number and adhering activity of CR1 lower expression genomic type gallbladder carcinomas (0.582±0.18, 44.3±5.5) and those of healthy individuals (0.610±0.20, 45.8±5.7) (P>0.05).

CONCLUSION: Defective expression of CR1 in gallbladder carcinoma is mostly acquired through central peripheral mechanisms. The changes in CR1 quantitative expression and adhering activity are consanguineously related to the development and metastasis in gallbladder carcinoma.

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INTRODUCTION

Carcinoma of the gallbladder is the most common neoplasm in biliary tract, and its incidence has been rising in recent years[1-4]. The C3b receptors (CR1, Cd35) on erythrocytes serve as the primary transport system for immune complexes from peripheral blood of the liver[5-8], and CR1 plays an important role in this system, the rate of clearance of immune complexes from the circulation is directly related to the number of CR1 molecules expressed on erythrocytes[9-11]. However, there are few reports on the changes of genomic density polymorphism of complement types in erythrocytes of patients with gallbladder carcinoma. We previously demonstrated the altered levels of cellular immunity and humoral immunity in patients with gallbladder carcinoma, and the effects of radical cholecystectomy on nutritional and immune status in patients with gallbladder carcinoma[12-15]. In this study, we reported the changes in quantitative expression, adhering activity and genomic density polymorphism of complement types in erythrocytes (CR1) of patients with gallbladder carcinoma.

MATERIALS AND METHODS

Patients

A total of 33 patients with gallbladder carcinoma were admitted to the First Affiliated Hospital of Sun Yat-Sen University from August 2000 to August 2002.Those were 16 men and 17 women aged from 50 to 70 years (median, 52.7 years). Nineteen patients were preoperatively diagnosed as polypois, cholecystolithiasis and chronic cholecystitis, and diagnosed as gallbladder carcinoma by frozen sections after surgery. Fourteen patients were diagnosed as gallbladder carcinoma preoperatively, of them, 4 patients had radical cholecystectomy, 7 patients had U-tube drainage operation, 3 patients cholecystectomy. Direct invasion or distant metastasis was found in 10 patients during operation, simple gallbladder carcinoma in 15 patients, both gallbladder carcinoma and cholecystolithiasis in 18 patients. The patients with gallbladder

carcinoma were grouped according to the staging system of Nevin[16], the number of cases on stages I, II, III, IV and V was 7, 11, 5, 7 and 11, respectively. Tumor size ranged from 1.8 cm to 6 cm in diameter (median, 2 cm), 23 patients had tumors smaller than 2 cm in diameter, 10 patients had tumors larger 2 cm in diameter.

No therapy was administered to patients preoperatively. Meanwhile, 90 patients with cholecystits or cholecystolithiasis had cholecystectomy, including 44 patients with cholecystitis and 46 patients with choleithiasis. No severe systemic diseases were found, such as, myocardial infarction, cerebral vascular accidents, uncontrollable diabetes mellitus, Hypertension. Their disease was stable in a period of one month. All the surgical specimens were examined histologically, and classified into hyperplasia, atypical hyperplasia (mild, moderate and severe) according to Gong *et al*.[17]. A total of 59 patients exhibited epithelial hyperplasia, 10 patients had mild dysplasia, 10 patients moderate dysplasia, 11 patients severe dysplasia. Normal controls were 30 healthy individuals (18 men and 12 women) aged from 56 years to 72 years (median 61.7 years).

Methods

Quantitative expression, adhering activity and genomic density polymorphism of complement types in erythrocytes (CR1) patients with gallbladder carcinoma were assessed preoperatively (1 wk before surgery), and on d 3, 7, 14 and 21 postoperatively.

Genomic determination of CR1 (Cd35) density polymorphism on erythrocytes[18]

DNA samples DNA was extracted using the following simplified protocol. Following lysis of erythrocytes, nucleated cells from 10 mL of blood were incubated in 3.5 mL of 10 mmol/L Tris, 2 mmol/L EDTA buffer pH7.5, containing 0.4 mol/L NaCL, 7 g/L sodium dodecylsulfate and proteinase K (30 mg/L) for 16 h at 37 ℃, 1 mL of 6 mol/L NaCL was added. After vortexed and ethanol-precipitated, the pellets were washed three times in 700 mL/L ethanol and solubilized in 10 mmol/L Tris, 1 mmol/L EDTA buffer pH7.5.

Sequencing

A construct in pUC-18 containing *Hin*d III-kpn fragments from the introns located within the first and second exons of the short consensus repeat d2 from the long homologous repeat D of CR1 was sequenced. Sequencing was performed using the modified Sanger method with a commercial T_7 DNA polymerase kit.

Primers

Primers were designed based on the analysis of internal homologies within the CR1 gene and homologies with other human sequences obtained from the EMBL databank using the Bisance software. The 5' primer was from base 4 415 to base 4 435 from the CR1 cDNA sequence. The 3' primer was 75 to 55 from the *Hin*d III monomorphic restriction site in the introns. The latter primer was chosen due to the presence of five mismatches between the CR1 intron sequence and the consensus *Hin*d III 1.9 ku repetitive sequence. Primers were synthesized on a 380 Applied Biosystem apparatus. Twenty base oligonucleotides were recovered from the solid phase by rinsing the column with 250 g/L ammonium. After incubation at 55 ℃ for 16 h, ammonium was expelled through evaporation using a Speed-Vac apparatus. Primers were used without further purification.

Amplification and agarose gel electrophoresis

PCR was performed using a Techne PHC-I apparatus with the Taq polymerse.

Amplified DNA was precipitated and then digested using the *Hin*d III restriction enzyme, and separated by electrophoresis in 20 g/L agarose gels. Gels were analyzed under UV illumination. The genomic density polymorphism of complement type 1 (CR1) in erythrocytes was classified into three types: high expression genomic type (1.8 Kb1), moderate expression type (1.8 kb, 1.3 Kb and 1.5 Kb) and low expression genomic type (1.3 Kb, 0.5 Kb).

Quantitative expression of CR1 in erythrocytes of patients with gallbladder carcinoma

EBC enzyme-linked immunosorbent assay This assay was based on the method of Cornillet *et al*.[18]. All RBCs in the assay were routinely glutaraldchyde-fixed. Finally, 20 μL of S/BSA were added to each well, followed by 20 μL of substrate. The plates were incubated at 37 ℃ for 90 min with gentle agitation every 15-20 min to ensure the maximum contact of RBC with substrate. Subsequently, 100 μL of S/BSA was added to each well, the plate was centrifuged at 1 800 r/min for 90 s and 100 μL of supernatant was transferred to a clean microtitre plate for readings in a plate reader at 405 nm (Dynatech MR5000; DynatchmBillingshurst, Suaaex, UK). In each case, a mean of the readings obtained for the RBC without first antibody (the blank controls) was subtracted from the other readings obtained, in order to correct nonspecific phoshatase-like activity within RBC membranes. Duplicate values were obtained for each RBC sample with each antibody. Black control values were obtained for both the antimouse conjuates and for the anti-rabbit conjuates.

Adhering activity of CR1 in erythrocytes of patients with gallbladder carcinoma

A 50 μL of 1×10^8/mL RBC suspensions was added to each well, then added and 50 μL of plasma itself and 100 μL of 1×10^6/mL tumor cells, mixed completely, stained. Fields were examined (×640), 5 RBC combined each tumor cell were labeled as a flower, tumor cell flowers were assessed.

Statistical analysis

For each variable, mupltiple analysis of variance for repeated measurements was used to compare the values measured before operation with those measured at four subsequent time points. The results were presented as mean±SE based on the mixed model of repeated measurement analysis. Statistical analysis was performed with SAS software. $P<0.05$ was considered statistically significant.

RESULTS

The number and adhering activity of CR1 in patients with gallbladder carcinoma, chronic cholecystitis and cholecystolithiasis and healthy individuals

As shown in Table 1, the number and adhering activity of CR1 in patients with gallbladder carcinoma (0.738±0.23, 45.9±5.7) were significantly lower than those in patients chronic cholecystitis and cholecystolithiasis (1.078±0.21, 55.1±5.9) and healthy individuals controls (1.252±0.31, 64.2±7.4) ($P<0.01$). In Table 2 the number and adhering activity of CR1 in patients with chronic cholecystitis and cholecystolithiasis were significantly lower than those in healthy controls ($P<0.05$). There was a positive correlation between quantitative expression and adhering activity of CR1($r = 0.79$, $P<0.01$).

In the series of epithelial pathologic changes including hyperplasia, atypical hyperplasia, carcinoma *in situ* or invasive carcinoma, the number and adhering activity of CR1 decreased gradually (Table 3), and there was a positive correlation between quantitative expression and adhering activity of CR1 ($r = 0.77$, $P<0.01$). In Table 4 compared with those on the preoperative day (0.738±0.23, 45.4±4.9), the number and adhering activity of CR1 in patients with gallbladder carcinoma decreased greatly on the third postoperative day (0.310±0.25, 31.8±5.1) ($P<0.01$) and in the first postoperative week (0.480±0.25, 38.9±5.2) ($P<0.01$), but they were increased slightly than those on the preoperative

day (P>0.05). In comparison with those on the preoperative day and in the first postoperative week, the number and adhering activity of CR1 recovered in the second postoperative week (0.740±0.24, 46.8±5.9) (P<0.01), and increased greatly in the third postoperative week (0.858±0.35, 52.7±5.8) (P<0.01).

The number and adhering activity of CR1 in gallbladder carcinoma patients with infiltrating, adjacent lymph node and distant organ metastases were significantly lower than those in gallbladder carcinoma patients without metastasis (Table 5, P<0.01).

Table 1 The number and adhering activity of CR1 in different groups before operation (mean±SE)

Group	Cases	CR1 (A_{405})	Adhering activity (%)
Healthy individuals	30	1.252±0.31	64.2±7.4
Chronic cholecystitis	90	1.078±0.21[a]	55.1±5.9[a]
Cholecystolithiasis			
Gallbladder carcinoma	33	0.738±0.23[bc]	45.9±5.7[bd]

[a]P<0.05, [b]P<0.01 *vs* normal control; [c]P<0.05, [d]P<0.01 *vs* chronic cholecystitis and cholecystolithiasis.

Table 2 The number and adhering activity of CR1 in different groups before operation (mean±SE)

Group	Cases	CR1(A_{405})	Adhering activity(%)
Healthy individuals	30	1.252±0.31	64.2±7.4
cholecystolithiasis	46	1.027±0.27[a]	54.4±5.9[a]
Gallbladder carcinoma with cholecystolithiasis	18	0.731±0.26[bc]	45.1±6.1[bd]
Simple gallbladder carcinoma	15	0.728±0.23[bc]	44.8±6.4[bd]

[a]P<0.05, [b]P<0.01 *vs* normal control; [c]P<0.05, [d]P<0.01 *vs* chronic cholecystitis.

Table 3 The number and adhering activity of CR1 in different groups before operation (mean±SE)

Type	Cases	CR1(A_{405})	Adhering activity(%)
Simple hyperplasia	59	1.175±0.22	61.1±6.2
Atypical hyperplasia	21	0.906±0.24[b]	50.8±5.7[b]
Gallbladder carcinoma (I-III stage)	23	0.761±0.23[b]	41.6±6.1[b]
Gallbladder carcinoma (IV-V stage)	10	0.602±0.31[b]	30.4±6.5[b]
F		6.4	11.5
P		<0.01	<0.01

[b]P<0.01 *vs* each group.

Changes of genomic density polymorphism of CR1 in patients with gallbladder carcinoma

The distribution of genomic density polymorphism of CR1 in patients was 67.8% in high expression genomic type, 24.8% in moderate expression genomic type, 7.4% in low expression genomic type. Compared with healthy controls, no difference was observed between the patients with gallbladder carcinoma and healthy in the spot mutation rate of CR1 density gene (χ^2 = 0.521, P>0.05). The number and adhering activity of CR1high expression genomic type gallbladder carcinomas (0.749±0.22, 42.1±6.2) were significantly lower than those in healthy individuals (1.240±0.29, 63.9±7.2). The number and adhering activity of CR1 moderate expression genomic type gallbladder carcinomas (0.641±0.19, 34.2±5.1) were also significantly lower than those in healthy individuals (0.921±0.23, 54.8±7.1), but no difference was observed between the number and adhering activity of CR1 lower expression genomic type gallbladder carcinomas (0.582±0.18, 44.3±5.5) and those of healthy individuals (0.610±0.20, 45.8±5.7) (P>0.05).

DISCUSSION

The immune functions of erythrocytes have been studied at gene levels[5-8]. RBCs are the major cellular component of the peripheral blood and occupy 50% of the total blood volume[9]. Normal RBC cytosol contains the majority of natural killer enhancing factors and other factors, such as PIF. Damaged RBC could produce tumor necrosis factor inducing factor. RBCs appear to have an important regulating role in immune function, and RBC activity involves in regulating multiple cytokines such as IL-2, IL-3, CSF, IL-6, TNF-r, TNF. CD44 and CD58 expressed by RBC could serve as the center in controlling immune status, which can directly affect the development and progression of tumors[8-11]. The changes of CR1 quantitative expression and adhering activity are consanguineously related to the development and metastasis of liver carcinoma.

In our study, the number and adhering activity of CR1 high expression genomic type gallbladder carcinomas were significantly lower than those in healthy individuals, indicating that defective expression of CR1 in gallbladder carcinoma is mostly acquired through central peripheral mechanisms.

Gallbladder carcinoma is often associated with cholecystolithiasis and cholecystitis in 40-100% of cases[19-28]. Although no carcinogenic substance has so far been isolated from the bile or the stones in patients with cholecystolithiasis and cholecystitis, many scholars suggested that gallstone might play a role as a chronic injury factor to induce a series of epithelial pathologic changes[29-38]. Gong *et al.*[17] reported that in 150 consecutive cholecystectomy specimens for detection of cholelithiasis or cholecystitis, 76.68% exhibited epithelial hyperplasia, 16.89%

Table 4 The number and adhering activity of CR1 on preoperative and postoperative day (mean±SE)

Group	Preoperative	3rd d	1st wk	2nd wk	3rd wk
CR1 (A_{405})	0.738±0.23	0.310±0.25[b]	0.480±0.25[b]	0.740±0.24	0.85±0.32[a]
Adhering activity (%)	45.4±4.9	31.8±5.1[b]	38.9±5.2[b]	46.8±5.9	52.7±5.8[a]

[a]P<0.05, [b]P<0.01 *vs* normal control.

Table 5 The number and adhering activity of CR1 in gallbladder carcinoma with adjacent lymph nodes and metastasis (mean±SE)

Group	Healthy individu als	Infiltrating (-)	Infiltrating (+)	Adjacent lymph node (-)	Adjacent lymph node (+)	Metastasis (-)	Metastasis (+)
Cases	30	15	18	18	15	23	10
CR1 (A_{405})	1.252±0.31	0.95±0.22[a]	0.81±0.29[ab]	0.88±0.27[a]	0.73±0.25[ab]	0.78±0.26[a]	0.44±0.29[ad]
Adhering activity (%)	64.2±7.4	52.8±6.7[a]	48.1±7.2[ab]	47.6±7.0[a]	41.1±7.1[ab]	46.1±6.1[a]	38.9±6.2[ad]

[b]P<0.01 *vs* normal control; [a]P<0.05, [d]P<0.01 *vs* negative.

atypical hyperplasia, 1.32% carcinoma *in situ* and 2.11% invasive carcinoma. Simple epithelial hyperplasia was found in the mucus adjacent to invasive carcinoma.With the passage of time, a significant number of atypical hyperplasiae presumably would progress to a higher grade lesion, becoming carcinoma. Toyonaga *et al*.[39] reported that in 200 consecutive cholecystectomy specimens for detection of cholelithiasis or cholecystitis, 83% simple epithelial hyperplasiae, 13.5% atypical hyperplasiae and 3.5% carcinomas *in situ*. In general, a significant number of atypical hyperplasiae presumably would progress to a higher grade lesion, 80% atypical hyperplasiae could become precancer lesions. Albores-Saavedra *et al*.[11] results showed that cholelithiasis or cholecystitis produced a series of epithelial pathologic changes, such as simple epithelial hyperplasia, atypical hyperplasia and carcinoma *in situ*, which represented the precancer lesion of gallbladder carcinoma. The probable sequence of events appear to be as follows. Hyperplasia has been found to have atypical hyperplasia which in turn may progress to neoplasia. With the passage of time, a significant number of atypical hyperplasiae presumably would progress to carcinoma *in situ* and invasive carcinoma[24]. So almost all scholars have suggested that a small number of hyperplasiae of the gallbladder would evolve toward *in situ* carcinoma which finally becomes invasive carcinoma[40]. In our study, the number and adhering activity of CR1 in patients with gallbladder carcinoma were significantly lower than those in patients with chronic cholecystitis and cholelithiasis and healthy individuals ($P<0.01$). The number and adhering activity of CR1 in patients with chronic cholecystitis and cholelithiasis were significantly lower than those in healthy individuals. There was a positive linear correlation between quantitative expression and the adhering activity of ECR1 ($r = 0.79$, $P<0.01$). Compared with data the on preoperative day, the number and adhering activity of CR1 in patients with gallbladder carcinoma decreased greatly on the third postoperative day and in the first postoperative week, indicting that surgery plays an injurious role in the disorder of RBC and cytokine functions[12,41,42]. The number and adhering activity of CR1 recovered in the second postoperative week, and increased greatly in the third postoperative week.

The number and the adhering activity of CR1 in gallbladder carcinoma patients with infiltrating, adjacent lymph node and distant organ metastases were significantly lower than those in patients without metastasis. This study demonstrates CR1 can be used as an immune therapy for gallbladder carcinoma.

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