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Expression of CK19 mRNA and MUC-1 mRNA in the peripheral blood of patients with colorectal cancer and their clinical significances

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Abstract ***Objective:*** Using nested reverse transcription-polymerase chain reaction (Nested RT-PCR) to test the mRNA level in peripheral blood CK19 and MUC-1 in colorectal cancer patients and it's clinical significance, to discuss the feasibility of colorectal carcinoma micro-metastasis detection of molecular markers. ***Methods:*** The expression level was detected by nested RT-PCR in 20 healthy people, 20 patients with colorectal adenoma and 90 cases of patients with colorectal cancer disease peripheral blood CK19 mRNA and MUC-1 mRNA. ***Results:*** The positive expression rate of CK19 mRNA and MUC-1 mRNA were: 58.89% (53/90) and 52.22% (47/90). No CK19 mRNA healthy people 20 cases in the control group in the peripheral blood, the expression of MUC-1 mRNA in 12 cases, the expression rate of 60% (12/20). In 20 cases of colorectal adenoma diseases have the expression of CK19 mRNA in 1 cases, the expression rate of 5% (1/20), the expression of MUC-1 mRNA in 10 cases, the expression rate of 50%. Patients with colorectal cancer CK19 mRNA, MUC-1 mRNA expression rate was significantly correlated with tumor staging, the degree of differentiation of the tumor cells and tumor metastasis ($P < 0.05$). ***Conclusion:*** Marker CK19 mRNA as the detection of micro-metastasis in peripheral blood of patients with colorectal cancer has good sensitivity and specificity, but CK19 mRNA, MUC-1 mRNA can be used to judge the prognosis of patients with colorectal cancer index.

Key words colorectal cancer (CRC); peripheral blood; CK19 mRNA; MUC-1 mRNA; nested reverse transcription-polymerase chainreaction (Nested RT-PCR)

Colorectal cancer (CRC)，impacting on the quality of patients' life severely, is one of common malignant tumors which morbidity rates ranked third of malignant tumor in the world and is trending to ascend each year. Although early CRC prognosis and quality of life is good, it is often neglected because of no signs or atypia symptoms. Nested reverse transcription polymerase chain reaction (RT-PCR) is useful for detecting trace of cancer cells in peripheral blood with the sensitivity of 10^{-6}–10^{-7}. At present, there is a lack of the recognized specific molecular markers for detecting CRC micrometastasis [1]. Cytokeratin (CK) family from the epithelial tissue, is the eukaryotic cytoskeletal intermediate filament protein, while Mucin (MUC) family is a catrgary of glycoproteins on cell surface mainly exist in the mucus. As important members of the two family, CK19 and MUC-1 play an important role in occurrence and metastasis of tumor [2, 3] 2 But the level of mRNA in CRC of both aforesaid has not been reported, therefore this study adopt the method of Nested PT-PCR used to detect CK19 mRNA, MUC-1 mRNA in the peripheral blood of patients, meanwhile analysing the relationship between the mRNA level of both and the clinical pathological features of CRC. Reported the result as follows now.

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Materials and methods

General status

From April 2006 to February 2007, a total of 90 CRC patients (CRC group) admitted to the No. 252 Hospital of PLA (China) were included in this study. Female to male ratio 43:47; range 35–65 years; median age 53 years; Dukes stages: 18 cases were stage A, 20 cases were stage B, 40 cases were stage C, 12 cases were stage D; histological type: 50 cases were tubular adenocarcinoma, 40 cases were papillary adenocarcinoma; differentiation degree: 13 cases were well-differentiated, 57 cases were moderately differentiated, 20 cases were poorly differentiated; 40 cases with distant metastasis. Inclusion criteria: (1) Did not receive operation and chemotherapy; (2) Pathologically

proved. Meanwhile we selected 20 healthy volunteers (healthy group) and 20 patients with benign colorectal adenomas (benign disease group) as control. Healthy group: female to male ratio 10:10, range 28–60 years, median age 48 years; benign disease group: female to male ratio 10:10, range 30–65 years, median age 50 years.

Main reagents

Trizol was purchased from America Invitrogen; DNA polymerase, First Strand cDNA Synthesis Kit and primer were purchased from Takara Biotechnology (China) Co. Ltd., Rnase-free Dnase I, cDNA Synthesis Kit were purchased from America Fermentas, Taq PCR Master-Mix was purchased from TianGen Company (Germany), CRC cell lines A544 was provided by Xiangya Medical college, Central South University.

Primer design

According to the GenBank, designing primer of and MUC-1 by Primer Premier 5.0 primer software. CK19 outer primer: upstream 5'-AAGCTAACCATGCAGAACCTCAACGACCGC-3', downstream 5'-TTATTGGCAGGTCAGGAGAAGAGCC-3', expected proliferation product length 1069 bp; inner primer: upstream 5'-TTATTGGCAGGTCAGGAGAAGA-GCC-3', downstream 5'-CGCGACTTGATGTCCATGAGCC-GCTGGTAC-3', expected proliferation product length 745 bp. MUC-1 outer primer: upstream 5'-AGTAGCACTCACCATA-G-3', downstream 5'-CAGCCAAGGCAATGAGATAGAC-3', expected proliferation product length 510 bp; inner primer: upstream 5'-C-GTCGTGGACATTGATGGTACC-3', downstream 5'-GGTA-CCTCCTCTCACCTCCTCCAA-3', expected proliferation product length 287 bp. Reference β-actin: upstream 5'-TCATCACCATTG-GCAATGAG-3', downstream 5'-CACTGTGTTGGCGTACA-GGT-3', expected proliferation product length 154 bp.

Specimen collection and total RNA extraction and identification

Patients were treated prior to fasting venous blood about 4 mL in the EDTA anticoagulant tube, erythrocyte lysate lysed erythrocyte, nucleated cells were separated, total RNA was extracted by guanidine thiocyanate / phenol method. Add 1 mL TRIzol reagent to the sample, pipetting repeatedly to make cells fully lysed. Added 200 μL chloroform convulsed, static 2–5 min on ice，120 000 r/min centrifugal 15 min, extrcted the supernatant to EP tube, added equal volume of precool isopropanol, 120 000 r/min centrifugal 10 min, abandoned supernatant, washed and precipitated by 75% ethanol of nuclease-free, DEPC Water dissolves RNA, process available RNA solutions by RNase-free DnaseI, –80 ℃ to save. Used ultraviolet spectrophotometer to estimate the purity of RNA，ensured that the range value of absorbency ratio between 260 nm and 280 nm ($A_{260/280}$) was 1.78–1.99, 2% agarose gel electrophoresis bands was clearly visible.

Synthesis of cDNA and amplification of nested RT-PCR

Using RNA as template, followed by double PCR amplification, specific conditions were as follows. CK19 (1st round): 94 ℃ 1 min, 65 ℃ 50 s, 72 ℃ 2 min, 72 ℃ 40 s, amplification of 25 cycles; 72 ℃ extension 10 min. CK19 (2nd round): 94 ℃ 50 s, 72 ℃ 2 min, amplification of 30 cycles; 72 ℃ extension 10 min. MUC-1 (1st round): 94 ℃ 5min, 94 ℃ 45 s, 55 ℃ 45 s, 68 ℃ 1 min, amplification of 35 cycles; 68 ℃ terminal extension 7 min. MUC-1 (2nd round): 94 ℃ 2 min, 94 ℃ 45 s, 55 ℃ 45 s, 72 ℃ 1 min, 30 cycles; 72 ℃ erminal extension 7 min. Meantime, standard cDNA was a positive control, sterile water was a negative control.

Determination on PCR products

PCR amplification products was determined with 2% agarose gel electrophoresis, ethidium bromide staining, and visualized under UV light. Amplified bands appeared in 745bp and 287bp which revealed CK19-mRNA and MUC-1 mRNA positive results respectively.

Sensitivity detection

Well-grown human colon cancer cell line A544 was digested by trypsin, blending with 0.9% physiological saline 10 mL. Cell count under microscope will show the cell number per milliliter suspension. Adjust the volume of liquid to 1×10^7 cells per milliliter suspension, then diluted the suspension into cell concentration with 7 orders of magnitude as follows: 1×10^6/mL, 1×10^5/mL, 1×10^4/mL, 1×10^3/mL, 1×10^2/mL, 1×10^1/mL and 1 /mL. RNA extracted from the cells was used to make PT-PCR analysis in CK19 mRNA and MUC-1 mRNA.

Statistical analysis

Analysis was executed by SPSS 11.5 software. The χ^2 and fisher's precision test were used for data comparison. The difference was statistically significant with $P < 0.05$.

Results

Identification of total RNA

$A_{260/280}$ value of extracted RNA was 1.78–1.99, 2% agarose gel electrophoresis and gel imaging system showed two clear bands of 28S and 18S, which indicated the integrity and purity of RNA accorded with the criteria and were suitable for follow-up testing (Fig. 1).

Positive rate of CK19 mRNA and MUC-1 mRNA in different groups

Positive rate of CK19 mRNA of CRC group was higher

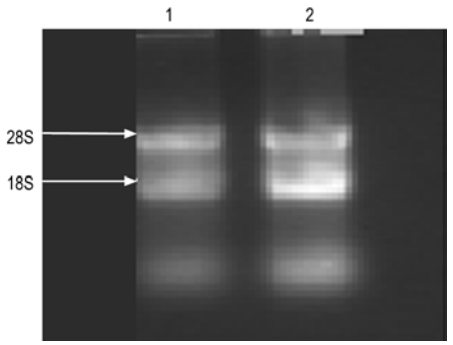

Fig. 1 Electrophoretogram of partial total RNA samples. 1 and 2: CRC patient specimens

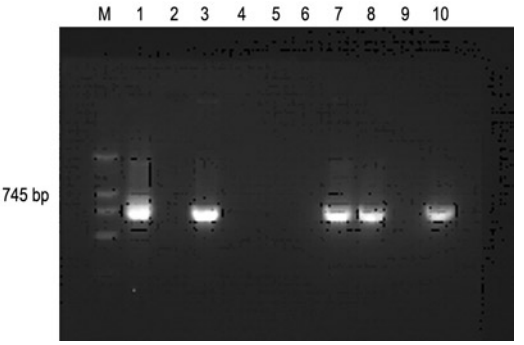

Fig. 2 CK19 mRNA expression of CRC peripheral blood Nested RT-PCR products. M: relative molecular mass reference (DNA marker); 1: positive control; 2: negative control; 3–10: CRC (3, 7–8 and 10 were positive expression)

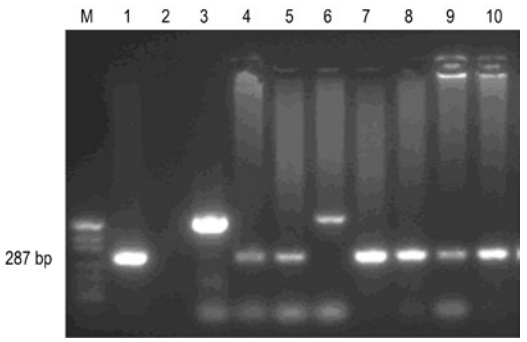

Fig. 3 MUC-1 mRNA expression of CRC peripheral blood Nested RT-PCR products. M: relative molecular mass reference (DNA marker); 1: positive control; 2: negative control; 3–10: CRC (4–5, 7–10 were positive expression)

than the other groups ($P < 0.05$), the differences of positive rate of CK19 mRNA in healthy group and benign disease group had no statistically significant ($P > 0.05$). The differences of positive rate of MUC-1 mRNA in all three groups had no statistically significant (Table 1, Fig. 2 and 3).

Relationship with patho-parameters

The positive rate of CK19 mRNA and MUC-1 mRNA in CRC was correlated with Dukes Staging, degree of differentiation and distant metastasis ($P < 0.05$), but not to the histological pattern ($P > 0.05$; Table 2).

Table 1 Comparison of positive rate of CK19 mRNA and MUC-1 mRNA in three groups [*n* (%)]

Groups	*n*	CK19 mRNA				MUC-1 mRNA			
		+		–		+		–	
		n	%	*n*	%	*n*	%	*n*	%
Healthy group	20	0	0	20	100.0	12	60.0	8	40.0
Benign disease group	20	1	5.0	19	95.0	10	50.0	10	50.0
CRC group	90	53	58.9*#	37	41.1*#	47	52.2	43	47.8

Compared with healthy group: * $P < 0.05$; compared with benign disease group: # $P < 0.05$

Table 2 The relationship between the positive rate of CK19 mRNA and MUC-1 mRNA and clinical pathological features of CRC [*n* (%)]

Clinical pathology	*n*	CK19 mRNA+	MUC-1 mRNA	*P* (CK)	*P* (MUC)
Dukes staging				0	0
A + B	38	12 (31.6)*	11 (28.9)*		
C + D	52	41 (78.8)	36 (69.2)		
Differentiated degree				0.03	0.001
Higher + medium	70	37 (52.9)*	30 (42.9)*		
Lower	20	16 (80.0)	17 (85.0)		
Distant metastasis				0	0
None	50	21 (42.0)*	17 (34.0)*		
Exist	40	32 (80.0)	30 (75.0)		
Histological pattern				0.848	0.186
Tubular adenocarcinoma	50	29 (58.0)	23 (46.0)		
Papillary adenocarcinoma	40	24 (60.0)	24 (60.0)		

Compared with the corresponding, * $P < 0.05$

Sensitivity test

The sensitivity of CK19 mRNA and MUC-1 mRNA by RT-PCR test was 10^6 (1×10^1/mL), but positive expression of mRNA was not detected in 10^7 (1 /mL) cell suspension either.

Discussion

CK19 mRNA and MUC-1 mRNA played an important role in occurrence and metastasis of tumor, which has become a popular topics of study. CK family, arised from epithelial tissue, is the eukaryotic cytoskeletal intermediate filament proteins that widely expressed on epithelial cells but lacking in mesenchymal tissue. CK19 is an important member in CK family. MUC family, mainly present in the cell surface glycoprotein of mucus, locates in human chromosome 1q21–q24 with 6 members of MUC-1, MUC-2, MUC-3, MUC-4, MUC-5a and MUC-5b [3, 4]. Some overseas studies have confirmed that MUC-1 is closely related to solid tumor, and MUC-1 was also found in the peripheral blood of healthy people, so we do not think the time is ripe for considering MUC-1 mRNA in peripheral blood as a parameter in diagnosing solid tumor micrometastasis [5, 6], meanwhile the relationship between MUC-1 mRNA and CRC clinic opathological features remains to be further studied.

Saintigny P *et al* [7] discovered that the peripheral blood CK19 mRNA positive expression rate of CRC group is higher than healthy group, and they considered that CK19 mRNA was related to the incidence of CRC. The study showed that the peripheral blood CK19 mRNA positive rate of CRC group is higher than both healthy group and benign disease group, and the difference was statistically significant ($P < 0.05$). But there was no statistical significance among the three groups in the difference of positive rate of MUC-1 mRNA ($P > 0.05$), which suggested that it still needs further study in whether can MUC-1 be used as a specific marker in CRC micro metastasis. The study also suggested that the positive rate of both CK19 mRNA and MUC-1 mRNA was related to Dukes staging and differentiation degree ($P < 0.05$). The positive rate of CK19 mRNA and MUC-1 mRNA in both "C + D" of Dukes staging and patients with poorly differentiated was higher than corresponding ($P < 0.05$), which suggested the both may play an important role in occurrence and development of CRC.

CRC, theoretically originate from epithelial tissue, remains the expression of epithelial tissue specific maker, but there is usually no epithelial tissue specific maker in mesenchymal tissue. It can be diagnosed with metastasis when pithelial tissue specific maker is tested in CRC of peripheral blood [8]. Oossterkamp HM *et al* [9] studied the condition of CK19 mRNA in cell line of human epithelial cells and non epithelial cells through RT-PCR, Northern blotting and radioimmunoprecipitation, in which different quantities of CK19 mRNA and protein were confirmed to detected, but detectable quantity in non epithelial tumor cells was lower than epithelial tumor cells. The study showed that the positive rate of CK19 mRNA and MUC-1 mRNA in CRC of peripheral blood with distant metastasis was higher than non distant metastasis, and the difference was statistically significant ($P < 0.05$). It suggested that CK19 and MUC-1 may play an important role in CRC metastasis.

Conclusions as a result, the increasing of CK-19 mRNA positive rate, even CK-19 mRNA and MUC-1 mRNA were related to Dukes staging, differentiation degree and distant metastasis, may have important significance in occurrence and development of CRC.

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