Science in China Series B: Chemistry

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Dissemination profile of perioperative tumor cells in peripheral blood of colorectal cancer patients detected by multiple marker genes

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This work proposes a method to assess the molecular profile of perioperative circulating tumor cells in peripheral blood (PB) of colorectal cancer patients for differentiating the dissemination process of tumor cells. Two-point quantification of multiple marker genes was designed for describing the profile. The expression levels of cytokeratin 20 (CK20), carcino-embryonic antigen (CEA) and survivin mRNA in PB and tumor tissue samples in 37 colorectal cancer patients from 1 d pre-operation to 2 h post-operation were detected with real-time quantitative reverse transcription-polymerase chain reaction. β-Actin mRNA was used as internal control to standardize the results of different mRNA expression levels. The data analysis using Stata statistical packages, Chi-Square test and Mann-Whitney test indicated the expression level of CEA mRNA in PB increased significantly, while those of CK20 and survivin mRNA decreased significantly. Quantitative comparison with tumor tissues indicated that the increase of CEA mRNA level in PB coincided with the decrease of CK20 and survivin mRNA levels in different tumor cells. These results showed surgical manipulation caused tumor cells shedding into blood from primary tumor tissue and significant increase of CEA mRNA level, while occult tumor cells with high expression levels of CK20 and survivin mRNA before surgery decreased after surgery.

real-time quantitative RT-PCR, multimarker genes, circulating tumor cells, colorectal cancer

1 Introduction

A major cause of death in colorectal cancer patients results from distant metastases after resection[1,2]. This phenomenon suggests that the occult tumor cells might exist at an early stage in tumor progression of colorectal cancer patients. Many publications in animal studies have demonstrated that surgical manipulation would cause tumor cells shedding into blood from primary tumor tissues[3,4], and the study in humans showed that the enhanced cancer cell dissemination is related to metastatic relapse[5−7]. However, the evidence in humans is not sufficient[8]. Therefore, it is urgent to investigate the dissemination and molecular profile of tumor cells for understanding the micrometastatic process.

Both our previous work[5,9,10] and some studies[11,12] have demonstrated that carcino-embryonic antigen (CEA) mRNA expressing cells in peripheral blood (PB) significantly increase following surgical manipulation in esophageal and colorectal cancer patients, and the increased tumor cells during surgery are speculated to be released from primary tumor tissues. Some researchers reported that survivin mRNA expression level significantly decreased following surgical manipulation[13], which is contrary to the change of CEA mRNA in the same kind of cancer patients. Obviously, exploring the

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Received September 11, 2009; accepted September 21, 2009
doi: 10.1007/s11426-009-0267-9

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Supported by the Outstanding Medical Talents Program (Grant No. RC2007069) from Department of Health of Jiangsu Province, the National Science Funds for Creative Research Groups (Grant No. 20821063), the Major Research Plan (Grant No. 90713015) and General Programs (Grant Nos. 20845005 & 20875044) from the National Natural Science Foundation of China

underlying reason for the contrary change of expression levels in different marker genes will provide valuable information for understanding the metastatic process of this cancer. This work designed a two-point quantification of multiple marker genes with real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) technology for seeking the reasonable explanation.

RT-PCR has been widely used to detect occult tumor cells in the PB of colorectal cancer patients by single marker gene[14−17]. However, no marker gene has been found to be consistently and specifically expressed in all of the primary tumors for a particular malignancy[18]. Moreover, tumor cell dissemination is non-linear, and marker gene expression may vary between a primary tumor and its metastasis[19,20]. Therefore, multiple marker genes have been recently applied to improve the detection sensitivity in this cancer patients[21−23], and single gene quantification at multiple time points has also been proposed for exploring the micrometastatic characteristic[24,25]. However, in colorectal cancer patients this method has not been used due to lack of effective marker genes combination applicable in PB.

To provide detailed information of metastatic characteristic and process, this work developed a strategy by combining multiple marker genes with two time points for describing the molecular profile of perioperative circulating tumor cells in perioperative colorectal cancer patients. Using three genes, cytokeratin 20 (CK20), CEA and survivin mRNA, as markers, we analyzed their expression levels (ΔC_t values, the change of cycle threshold) in PB collected at one day preoperative and two hours postoperative and primary tumor tissues. The results obtained provided valuable information for understanding the process of tumor cell dissemination in colorectal cancer.

2 Experimental

2.1 Patients

According to the rules of the local ethical committee, 74 fresh PB samples and 37 primary tissue samples were collected from 37 consecutive patients with pathologically colorectal carcinoma from January 2007 to July 2008 in the Tumor Hospital of Jiangsu Province, China. PB samples were collected from peripheral vein one day before surgery and two hours after surgery, separately. 36 PB samples were collected from 18 nonmalignant patients who underwent thoracotomy over the same period to compose a control group. Total RNA was extracted immediately after PB sample collection and red cell lysis without any kind of enrichment. Tissue samples were obtained from these patients during surgery and shock-frozen in liquid nitrogen for later RNA extraction.

2.2 Cell lines

The human colorectal carcinoma cell line HcT 116 and esophageal squamous carcinoma cell line TE-10 were used to evaluate the sensitivity of various mRNAs by serial dilutions (1, 10, 10^2, 10^3, 10^4, 10^5 and 10^6 cancer cells in 5×10^6 leucocytes). Leucocytes were separated from the PB of healthy volunteers.

2.3 RNA preparation and cDNA synthesis

Total RNA was extracted from tissue or PB samples using TRI REAGENT (Molecular Research Center Inc, USA) according to the manufacturer’s instructions, and its purity and quality were assessed by UV-Vis spectrophotometer (BIO-TEK, USA) and 1% agarose gel-electrophoresis for the integrity assay. cDNA was derived from 4 μg of total RNA by random primed reverse using commercially available RevertAid™ First Strand cDNA Synthesis kit k1622 (Fermentas, America) according to the recommended protocol by the manufacturer.

2.4 Real-time quantitative PCR

Icycler iQ™ Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories Inc., USA) was used for real time PCR. The sequences of primers and Taqman probes are listed in Table 1. PCR was performed with 2.0 μL cDNA (corresponding to 400 ng of total RNA), 1× reaction buffer, $MgCl_2$ (2.0 mM for β-actin, CK20 and survivin, 3.0 mM for CEA), 0.2 mM dNTPs, 200 nM forward and reverse primers, 200 nM probe, 2.5 U Taq DNA polymerase in a final reaction volume of 50 μL. The reaction program began with an initial denaturation step of 2 min at 94℃, followed by 45 cycles at 94℃ for 30 s and 52℃ for 60 s for β-actin, CK20 and survivin or 60℃ for 60 s for CEA.

2.5 Standardization of quantitative results and cut-off values

To standardize the results of different mRNA expression levels in PB and tissue samples, β-actin mRNA was used as internal control to determine the ΔC_t values according to $\Delta C_t = C_t^{\text{marker gene}} - C_t^{\beta\text{-actin}}$. The results obtained showed that this choice was valid. The cut-off values of ΔC_t for marker genes were used to exclude the effect of non-cancerous cells, which were obtained according to the following procedure: (i) the correlations between expression levels (ΔC_t) of three marker genes and number of cancer cells were firstly determined; (ii) the ΔC_t values corresponding to the lowest numbers of cancer cells detectable were used as detection limits of marker mRNAs; (iii) the upper limits of 95% confidence interval (CI) of ΔC_t values of marker genes in control group were used as the expression levels in non-cancerous cells; (iv) the smaller ΔC_t values between results (ii) and results (iii) were considered as the cut-off values of three marker mRNAs at the corresponding time points, respectively. If the ΔC_t value in PB sample was smaller than the cut-off value, the marker mRNA was defined to be positive at the corresponding time point.

Table 1 Used oligonucleotide sequences

Marker gene	Primers and probes sequences (5′ to 3′)	Amplicon size (bp)
CK20	F: CTGAATAAAGACCTAGCTCTCCTCAAA	77
	R: GTGTTGCCCAGATGCTTGTG	
	P: AGGAGCATCAGGAGGAAGTCGATGGC	
CEA	F: AATAATTCCATAGTCAAGAGCATCACA	125
	R: CAGGGCTGCTATATCAGAGCAAC	
	P: CTGGAACTTCTCCTGGTCTCTCAGCTGG	
Survivin	F: CCTGGCAGCCCTTTCTCA	121
	R: TCAGTGGGGCAGTGGATG	
	P: CCGCATCTCTACATTCAAGAACTGGC	
B-actin	F: AGCACAGAGCCTCGCCTTT	76
	R: GGCGATATCATCATCCATG GT	
	P: CCACACCCGCCGCCAGCT	

F, Forward primer; R, reverse primer; P, TaqMan probe. The probes were labeled at the 5′-end with reporter dye molecule 6-carboxyfluorescein (FAM) and the 3′-end with quencher dye 6-carboxy-*N*,*N*,*N*,*N*- tetramethyl rhodamine (TAMRA). The primers were designed to extend across at least one intron so that eventual DNA contamination would not pose a significant problem.

2.6 Statistical analysis

Data analysis was carried out using Stata statistical packages. Chi-Square test was used to analyze the correlations between the positive detection rates of marker genes at two sampling time points and between positive detection rates and clinicopathological parameters. Mann-Whitney test was used to compare the expression levels of marker genes between two time points, in which the result of the marker gene in PB sample was abandoned if it was negative at both two time points. The *p* value with less than 0.05 was considered as statistically significant difference.

3 Results and discussion

3.1 Cut-off values

RT-PCR has been commonly used to detect the expression levels of mRNA markers. The presence of mRNA markers in blood suggests active expression by circulating tumor cells[26]. However, possible degradation of RNA and unstability of reverse transcription and PCR process require standardizing the RT-PCR results. This study used β-actin mRNA as internal control to normalize the results, which made the expression levels of marker genes in different PB samples comparable.

The calibration curves of CK20 and survivin mRNA expressing cells were established with HcT 116 cell line, while the calibration curve of CEA mRNA expressing cells was established with TE-10 cell line since the former did not express CEA mRNA. The slopes and intercepts of calibration curves were −3.93 and 28 for CK20, −3.31 and 24.5 for survivin and −3.69 and 27 for CEA with the R^2 values of 0.983, 0.988 and 0.988, respectively. All *p* values were less than 0.0001. Ten cells expressing CK20 mRNA could be detected in each 5×10^6 leucocytes, and 1 cell expressing CEA or survivin mRNA in each 5×10^6 leucocytes could be detected. According to the calibration curves, the detection limit of ΔC_t values were 24.2 for CK20, 27 for CEA and 24.5 for survivin mRNA.

Although this work used cell lines to establish the calibration curves, it was impossible to transform their ΔC_t values in tissue samples into relative numbers of cancer cells. Furthermore, the same cell might express different marker genes. Thus the ΔC_t values, not the numbers of cancer cells, were considered to show the expression levels of marker genes. The smaller the ΔC_t value of marker gene was, the higher the expression level was.

The upper limits for 95% CI of ΔC_t values of all markers in control group were found to be more than the detection limits of cell lines. Therefore, 24.2, 27 and 24.5 were used as cut-off values for CK20, CEA and survivin mRNA, respectively.

3.2 Expressions of marker genes in primary tissue samples

When the ΔC_t value of one sample was smaller than the cut-off value, the corresponding marker mRNA in the samples was defined to be positive. The positivity detection rates of these marker genes in primary tumor tissues were 97.3% (36/37) for CK20 and CEA, and 89.2% (33/37) for survivin mRNA, and their ΔC_t values are shown in Figure 1. The medians of ΔC_t values for CEA, CK20 and survivin mRNA were 2.3, 4.2 and 9.6, respectively, indicating significant difference. In 4 patients with survivin negative of tumor tissues, 3 patients were found to be survivin positive in the blood before surgery and their expression levels decreased after surgery. One patient with CK20 negative of tumor tissue was found to be CK20 positive in the blood before surgery and its expression level decreased after surgery.

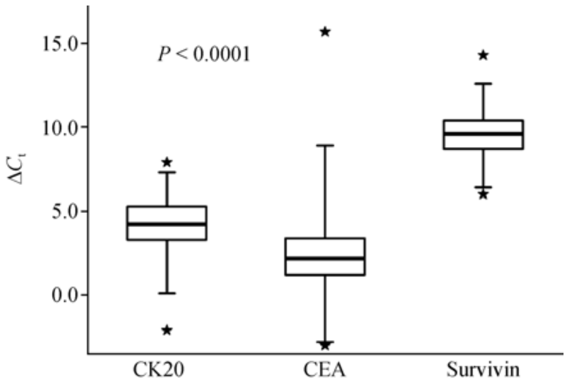

Figure 1 Box-whisker comparative plots of ΔC_t values of marker genes in primary tumor tissues in 37 colorectal cancer patients.

3.3 Positivity detection rates in PB samples

According to the cut-off values, the positivity detection rates of CK20, CEA and survivin mRNA in PB before and after surgery are listed in Table 2. 19 of 37 PB samples (51.4%) collected from colorectal cancer patients before surgery showed CEA mRNA negative, but after surgery these patients showed positive results of CEA mRNA. 6 patients (6/37, 16.2%) showed CEA mRNA positive both before and after surgery. In whole, the positivity detection rate increased following surgical manipulation ($p < 0.05$). These results were consistent with previous works in esophageal cancer[10,12] and colorectal cancer[11].

Different from the changes of CEA mRNA, after surgery the positivity detection rates of CK20 and survivin mRNA significantly decreased. In 37 colorectal cancer patients, 10 patients with CK20 mRNA positive before surgery became CK20 mRNA negative after surgery, and 20 patients remained the positive results, only 1 patient with CK20 mRNA negative became positive after surgery. The whole positivity detection rate of CK20 mRNA significantly decreased from 81.8% to 56.6% following surgical manipulation, consistent with that reported previously[7]. At the same time, 16 patients with survivin mRNA positive before surgery became survivin mRNA negative after surgery, 10 patients showed survivin mRNA positive both before and after surgery, and all negative patients kept negative expression of survivin mRNA in PB after surgery. The whole positivity detection rate of survivin mRNA changed from 70.3% to 27.3%.

Table 2 Positivity detection rates of marker genes

Marker genes	One day before surgery	Two hours after surgery	*P* values
CK20	30/37 (81.8%)	21/37 (56.6%)	0.024
CEA	7/37 (18.9%)	26/37 (70.3%)	< 0.0001
Survivin	26/37 (70.3%)	10/37 (27.3%)	< 0.0001

After surgery the distribution of the positivity detection rates of survivin mRNA was correlated with clinical pathological stage (Figure 2). No other correlation between the positivity detection rate and clinical pathological stage was observed.

3.4 Expression levels of three marker genes in PB samples

The expression levels of CK20, CEA and survivin mRNA before and after surgery are shown in Figure 3.

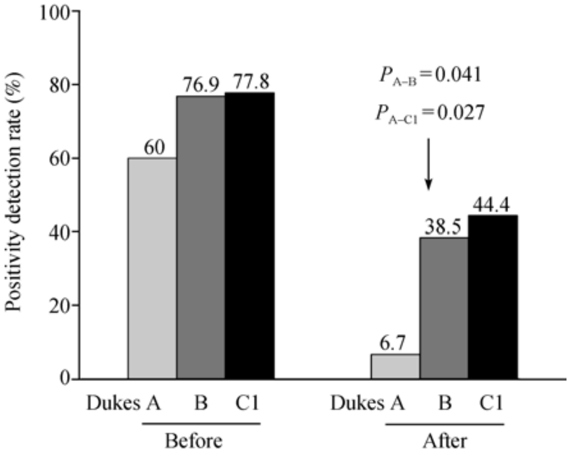

Figure 2 Positivity detection rates of survivin mRNA in peripheral blood after surgery for two hours according to clinical pathological stages in 37 colorectal cancer patients.

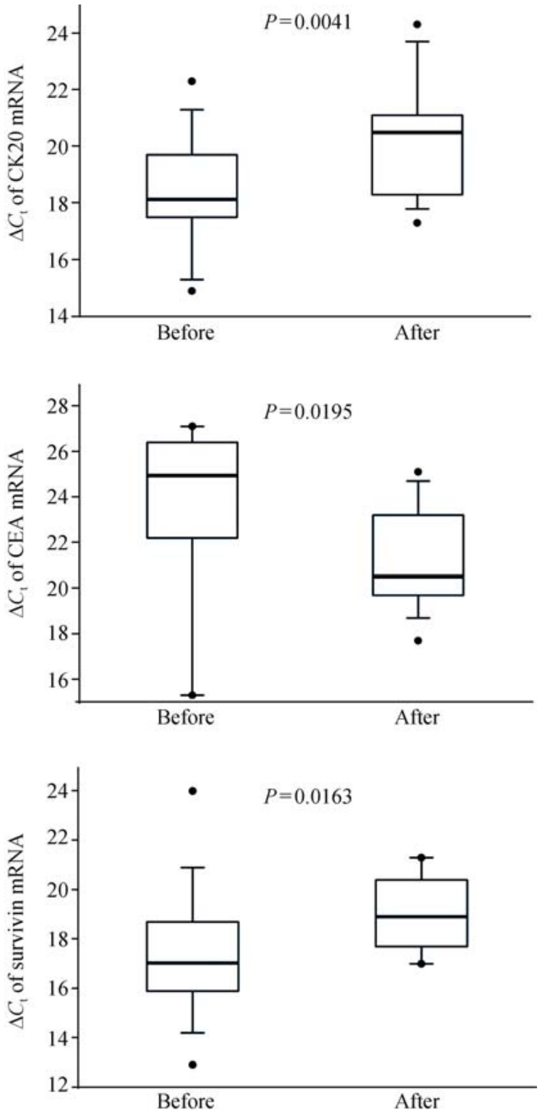

Figure 3 Box-whisker comparative plots of ΔC_t values of marker genes in peripheral blood of 37 colorectal cancer patients one day before and two hours after surgery.

Both the medians of ΔC_t values of CK20 and survivin mRNA before surgery (18.2 and 17.2, respectively) were significantly lower than those after surgery (20.5 and 19.1, respectively). Moreover, the median of ΔC_t values of CK20 mRNA for 20 positive patients at both two time points significantly increased from 18.5 (95% CI, 17.8—19.4) to 20.5 (95% CI, 19.4—21.3) ($p = 0.0138$), indicating the decrease of expression levels. Similarly, the median of ΔC_t values of survivin mRNA for 10 positive patients at two time points also increased significantly from 16.2 (95% CI, 14.8—17.7) before surgery to 19.1 (95% CI, 18.0—20.0) after surgery ($p = 0.0051$). This result showed the decrease of expression levels and was also consistent with some reports in esophageal[13] and gastrointestinal cancer[27].

Different from the expression levels of CK20 and survivin mRNA, the median of ΔC_t values of CEA mRNA before surgery (24.9) was significantly more than that after surgery (20.7). The expression level of CEA mRNA significantly increased following surgical manipulation ($p < 0.05$).

3.5 Relativity of expression levels of three marker genes between PB and primary tissue samples

The above results indicated an independently increasing process on the surface for the expression level of CEA mRNA. According to the relative quantification equation, the expression level of CEA mRNA in primary tumor tissues was nearly 10 times more than that of CK20 mRNA and 100 times more than that of survivin mRNA (Figure 1). It suggested that the detectable possibility would be CEA > CK20 > survivin mRNA, once tumor cells shed from primary tumor tissues into blood, which could also be testified from these results on the positivity detection rates of these three markers in the blood after surgery (Table 2). Thus, the shedding of primary tumor tissues or cells with much higher expression level of CEA mRNA into PB during surgical manipulation caused the significant increase of expression level of CEA mRNA.

On the other hand, the above results suggested that circulating tumor cells or occult tumor cells before surgery significantly decreased in the blood after surgical manipulation. As shown in Figure 1 and Table 2, the positivity detection rates of the three marker genes in PB before surgery showed significantly different sequence from large to small from their expression levels in primary tumor tissues. It has been reported that circulating tumor cells or occult tumor cells that existed in blood before surgery were genetically heterogeneous to cancer cells from primary tumor tissues[28,29]. From the above results, the high expression of CK20 or survivin mRNA before surgery was found in the blood in four patients with the two markers negative in their primary tumor tissues and the expression levels decreased after surgery, which suggested a second process of tumor cell disseminating during surgery: occult tumor cells, with high expression levels of CK20 and survivin mRNA before surgery, decreased after surgery. The reason may be due to the lack of their origin and their apoptosis led to the decrease of CK20 and survivin mRNA expression levels, which still needed further investigation.

Some previous publications indicated that the anti-apoptosis of survivin increased with the tumor progression in cancer patients[30－32]. In the above results, the positivity detection rate of survivin mRNA after surgery was correlated with pathological stages, while it was unrelated to pathological stages before surgery. The patients with Dukes B and C1 showed significantly higher positivity detection rates than patients with Dukes A (Figure 2). As well known, patients with worse stage had worse outcome. Therefore, in colorectal cancer patients, the expression levels of survivin mRNA after primary tumor tissues are resected in a short time may suggest the "true face" of the growing status of occult tumor cells, which is correlated with the outcome of the patients. Our previous work showed the rapid increase of the expression level of survivin mRNA in the blood after surgery for three days and its correlation with the recurrence of esophageal cancer patients[9]. This correlation in colorectal cancer patients will be investigated in our further work.

4 Conclusions

In despite of the relatively small number of patients, this is the first work to assess the changes of expression levels in different marker genes and differentiate the dissemination process of tumor cells during surgery. The results suggest that surgical manipulation of colorectal cancer patients causes tumor cells shedding into blood from primary tumor tissues, which is in good agreement with that reported in a review[5], and the dissemination of tumor cells leads to significant increase of expression level of CEA mRNA, which does not increase the expression levels of survivin and CK20 mRNA in PB. Occult tumor cells, existing in blood before surgery with high expression of CK20 and survivin mRNA, decrease after surgery, which may be due to the lack of origin and apoptosis of occult tumor cells.

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