Identification of carcinoembryonic antigen-producing cells circulating in the blood of patients with colorectal carcinoma by reverse transcriptase polymerase chain reaction

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Abstract

Background—Application of the reverse transcriptase polymerase chain reaction (RT-PCR) to identification of circulating tumour cells in colorectal cancer.

Aims—To assess whether circulating malignant cells in patients with colorectal liver metastasis could be identified by RT-PCR recognition of mRNA coding for the tumour marker carcinoembryonic antigen (CEA).

Patients—A total of 31 with colorectal liver metastases and 22 no-cancer controls.

Methods—Specific cDNA primers for CEA transcripts were used to apply RT-PCR to tissue biopsy specimens, colon carcinoma cell lines, and peripheral blood samples from patients with colorectal liver metastases. A strongly CEA-expressive HT115 colorectal carcinoma cell line was used to spike blood samples from no-cancer control subjects.

Results—The limit for detection of CEA cDNA by Southern blotting using HT115 cells was 50 cells per 14 ml of spiked blood. There was a significant difference ($p=0.007$) in RT-PCR positive expression between patients with liver metastasis (26/31) compared with controls (5/22). There was no significant relation between the prevalence of CEA cDNA amplification and serum CEA level or metastasis volume in patients with liver metastasis.

Conclusions—This is the first study to suggest that identification of circulating colorectal cancer cells using RT-PCR for detection of CEA cDNA is feasible.

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been developed using various epithelial protein markers for identification of circulating melanoma,¹ prostate,² hepatocellular,³ and colon⁴ carcinoma cells.

Proteins expressing tumour associated antigens are produced in large amounts by some cancers, and increased amounts of mRNA coding for these proteins may therefore also be present within tumour cells. RNA coding for prostate specific membrane antigen and α fetoprotein has previously been identified in the blood of patients with prostate⁵ and hepatocellular⁶ cancer respectively. In this paper we have developed this approach in colorectal cancer where increased levels of the epithelial cell surface glycoprotein carcinoembryonic antigen (CEA) are released into the circulation in 80% of patients with advanced disease.⁷ CEA is not thought to be produced by cells normally found in the circulation, and identification of cDNA implies circulating cells of epithelial origin which are most likely to be tumour cells. Colorectal liver metastases are thought to develop by haematogenous spread from primary colorectal cancer.⁸ Therefore as circulating malignant cells would be most likely to be present with colorectal liver metastases, these patients were studied.

Using specific cDNA primers for CEA transcripts,⁹ we applied RT-PCR to tissue biopsy specimens, colon carcinoma cell lines, and peripheral blood samples from patients with colorectal liver metastases. The relation between CEA RT-PCR positivity and both serum CEA level and extent of liver metastases was then examined.

Methods

RNA extraction from tissues and cells

Tumour biopsy specimens from patients with colorectal liver metastases were snap frozen in liquid nitrogen at the time of operation and stored at -70°C . The biopsy specimens (50 mg) were homogenised and the RNA was extracted using RNazol B (Biogenesis) by a modified method of Chomczynski and Sacchi.¹⁰ Cells from colorectal carcinoma cell lines were counted and subjected to RNazol B treatment which extracts total RNA. All glassware used was rinsed in diethyl pyrocarbonate (DEPC)-treated water and autoclaved. Solutions were made up in DEPC-treated water.

Keywords: RT-PCR, carcinoembryonic antigen, colorectal cancer, liver metastases, circulation.

Identification of circulating viable tumour cells in the blood could be useful for cancer staging and surveillance. The reverse transcriptase polymerase chain reaction (RT-PCR) method allows small amounts of the RNA coding for a tumour cell protein to be identified. RNA identification implies that a viable cell is producing the protein coded for, because extracellular RNA is rapidly degraded if it is not within an intact cell. In the absence of a truly cancer specific protein, this approach has

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Extraction of RNA from blood

Blood was stored on ice after venepuncture and processed within two hours. The plasma was removed after centrifugation at 1000 rpm for 15 minutes. The volume was replaced by sterile phosphate buffered saline (PBS) and the blood mixed. Cell fractionation was achieved by layering the blood on to Histopaque-1077 (Sigma Chemical Co). After a 40 minute centrifugation at 1400 rpm (200 g) the plasma-Histopaque interface containing lymphocytes, and any possible epithelial or tumour cells was collected from the remaining blood cells and washed with PBS.

Cell lines and blood spiking

An aliquot of 10⁶ cells taken from each of four colorectal cancer cell lines (COLO201, HT116, HT29, and HT115, obtained from the European Collection of Animal Cell Cultures) was examined for its expression of CEA cDNA. Blood obtained from healthy volunteers was spiked with varying numbers of HT115 cells prior to blood separation, RNA extraction, and RT-PCR.

Patients

All patients had colorectal liver metastases confirmed by biopsy associated with a serum CEA increase above the upper limit of normal. The extent of increase in serum CEA and volume of liver metastases¹¹ was recorded at the time of blood sampling. A 14 ml sample of venous blood was collected, by standard transcutaneous needle venepuncture into 7 ml vacutainers containing sodium EDTA. Similarly collected blood samples from patients with no cancer history who were being treated for varicose veins or groin hernias were also analysed and used as 'no-cancer' controls. All

patients were categorised as positive if any result was positive, even if repeated sampling had also yielded negative results.

RT-PCR

Total RNA (1–2 µg) was used for reverse transcription to cDNA. cDNA was prepared using reverse transcriptase (Gibco BRL) and including RNase inhibitor (Gibco BRL) according to methods given by Clontech Laboratories and primed using random hexamer priming (Clontech Laboratories). Samples were stored at –70°C. The sequence data and full length of CEA cDNA and its related genes have been reviewed elsewhere.^{12 13} We used CEA specific primers,⁹ which give rise to a 641 bp fragment that does not include amplification of other related CEA family members. A volume of 10 µl of cDNA product was used for PCR. The conditions for PCR amplification were 94°C for four minutes, 80°C for four minutes during which time two units of *Taq* polymerase (Gibco BRL) were added. The total volume of reaction was 50 µl. This single cycle was followed by 33 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 3 minutes. The final elongation step was for 13 minutes at 72°C. Aliquots of PCR products were run on 1.5% agarose gels. Samples that had not been transcribed did not give rise to a 641 bp fragment (Fig 1). The presence of cDNA in all the samples studied was verified by RT-PCR of glyceraldehyde-3-phosphate dehydrogenase (GAP) mRNA with agarose gel electrophoresis and staining with ethidium bromide. The primer sequences are shown in Table I and the product yielded a 381 bp fragment.

Southern blot hybridisation

Blotting and hybridisation was performed according to Sambrook *et al.*¹⁴ After denaturation and neutralisation, the cDNA bands were transferred to nylon membranes (Hybond N+, Amersham) using a vacuum blotter. The membranes were probed using a CEA specific oligoprobe which lies in between two primers used (Fig 1 and Table I).⁹ The probe was 5' end labelled using [γ -³²P] ATP (Amersham International) in the presence of polynucleotide kinase (Sigma Chemical Co). Hybridisation was carried out at 48°C overnight and a final wash at 60°C in 6×SSPE/0.1% SDS was carried out as described by Churchill and Gilbert.¹⁵

The RT-PCR and blotting were carried out without knowledge of whether samples were taken from controls or patients with liver metastasis.

Results

Detection of CEA cDNA in tumour tissue

Tumour biopsy specimens from nine patients with colorectal liver metastases were studied. Amplification of the region of cDNA for which primers were selected occurred in all cases.

Figure 1: Schematic representation of human CEA showing positions of primers and probe used. Boxes represent exons as described in Schrewe *et al.*¹⁶ FPr, forward primer; RPr, reverse primer; CEAPr, CEA probe.

TABLE I Primer and probe sequences used to detect CEA and GAP cDNA

	Sequence	Product size (bp)
CEA forward primer	5'-CCATGGAGTCTCCCTCG-3'	641
CEA reverse primer	5'-GTAGCTTGCTGTGTCATTTC-3'	
CEA probe	5'-GGGATGCACCATCTGTGGG-3'	
GAP forward primer	5'-TCCCACATCACCATTCCA-3'	381
GAP reverse primer	5'-CATCAGCCACAGTTTCC-3'

Figure 2: RT-PCR for CEA cDNA with ethidium bromide staining (A) and Southern blot hybridisation (B) of liver metastases (LM) from nine patients (lanes 3 to 11), producing a 641 bp fragment run against a 100 bp ladder (lanes 1 and 13). Lane 7 is negative, but was also negative with GAP primers (results not shown) indicating an absence of any cDNA in this sample.

Although the same quantity (50 mg) of starting material was used in all these samples, the intensity of both the PCR products and subsequent Southern blot hybridisation varied, with a good correlation in intensity between ethidium bromide staining and Southern blotting (Fig 2).

Figure 3: Detection of CEA cDNA by Southern blot hybridisation of four colorectal carcinoma cell lines (HT29, HT116, COLO201, HT115) demonstrating variability in degree of CEA expression. A total of 10^6 cells of each cell line were subjected to RT-PCR and probed with a specific CEA oligoprobe.

Cell lines

All the cell lines tested were positive for CEA cDNA although the degree of expression varied (Fig 3). HT29 and HT116 were both negative by PCR amplification as assessed by ethidium bromide staining, but were weakly positive using Southern blot hybridisation. Signals from both COLO201 and HT115 were intense after Southern blot hybridisation.

Spiking of control blood with colon cancer cell lines

The cell line producing the most intense signal (HT115) was used for spiking control blood samples. This was carried out to test whether the procedure was capable of detecting CEA-producing cells in blood. RT-PCR analysis of 14 ml of blood spiked with decreasing numbers (from 10^6 down to 10) of HT115 cells, suggested a detection threshold of 10^3 cells when products were detected by ethidium bromide staining and 50 by Southern hybridisation (Fig 4). Additional weaker bands also appeared using our oligoprobe suggesting that the HT115 cell line may produce further subclones or splice variants of CEA which hybridises with the same oligoprobe.

Fifty three patients were studied. There was a significant (2×2 contingency table, $p=0.007$) in the prevalence of RT-PCR positivity in patients with colorectal liver metastasis compared with controls (Table II). Some control samples were negative when visualised by ethidium bromide staining, but were positive after Southern hybridisation (Fig 5). To establish whether epithelial cell contamination at venepuncture could produce a false positive result, the extent to which epidermal cells expressed CEA cDNA was assessed in a skin biopsy specimen from one control patient (Fig 5).

Repeat blood samples from 12 patients with liver metastasis were assessed at between one and four weeks (six patients) or more than four weeks (six patients) from the initial blood sample. Results are shown in Table III.

Effect of tumour volume and serum CEA level on RT-PCR positivity in patients with liver metastasis

There was no significant difference (Mann-Whitney U test) in serum CEA among RT-PCR positive compared with RT-PCR negative patients with liver metastasis. Similarly, there was no significant difference between liver metastasis volume in patients whose blood was RT-PCR positive compared with those who were negative (Table IV).

Discussion

This is the first reported application of RT-PCR to identifying CEA cDNA in the blood of patients with advanced colorectal cancer. Studies in CEA-producing tumour biopsy specimens confirmed that the primer and probe sequences used were capable of CEA mRNA detection. We then used an in vitro spiking method to estimate the lower detection

Figure 4: Ethidium bromide staining (A) and corresponding Southern blot hybridisation (B) of blood spiked with increasing dilutions of the HT115 cell line. The lower threshold of detection for ethidium bromide was 10^3 cells and for Southern hybridisation 50 cells in 14 ml of blood. Results for less than 50 cells are not shown here.

Figure 5: Ethidium bromide staining (A) and corresponding Southern blot hybridisation (B) of cDNA synthesised from blood of patients with colorectal liver metastasis (LM). Also included (C) are nine examples of control patient blood samples (CO) where all were negative by ethidium bromide staining (not shown) but three were positive (one weakly) by Southern blot hybridisation. The lane marked SK (below) shows the single band obtained on testing a skin biopsy specimen from a no-cancer control patient. The additional band appearing at 450 bp in lane 4 of the LM suggests the presence of a further subclone of CEA in this particular sample similar to that detected for HT115 cells.

TABLE II Prevalence of CEA RT-PCR positive cases in blood from patients with colorectal liver metastasis and no-cancer controls

	n	Positive	p*
Liver metastases	31	26 (84%)	0.007
Control	22	5 (23%)	

Venous blood samples (14 ml) from patients with colorectal liver metastasis, primary colon cancer, and controls with no known cancer were analysed by RT-PCR and subsequent Southern blotting using CEA specific primers and a CEA specific oligoprobe.

* χ^2 test compared with controls.

TABLE III Reproducibility of repeat RT-PCR cDNA assessments after less or more than four weeks

	1-4 weeks	>4 weeks
Negative to positive*	2	0
Positive to negative*	0	5
Unchanged	4	1

Blood from six patients with liver metastasis was taken twice at either one to four week intervals or after four weeks.

*Change of RT-PCR result from negative to positive or vice versa.

TABLE IV Liver metastasis volume (measured by CT) and serum CEA by RT-PCR positivity in patients with colorectal liver metastases

	n	CT volume			Serum CEA		
		Median (ml)	iqr	p*	Median (μ g/l)	iqr	p*
RT-PCR positive	26	178	18-1005.2	0.4	75	23-297	0.8
RT-PCR negative	5	481.5	0.1-1200		188.5	8-472	

CT volumes of metastases were assessed by measuring the tumour area using an image analyser. iqr, interquartile ranges; the data are not normally distributed.

*Mann-Whitney U test.

limit of our method. This suggested that CEA cDNA from HT115 colon carcinoma cells distributed in 14 ml blood could be detected down to a level of 50 HT115 cells by Southern blotting. This is within the range previously reported using RT-PCR to detect tumour associated cDNA in melanoma.^{1 17} The prevalence of RT-PCR detected CEA-producing cells in the circulation of patients with colorectal liver metastasis was more than three times that in no-cancer controls. This suggests that our method was capable of detecting circulating CEA-producing carcinoma cells in patients with colorectal liver metastases.

Of the no-cancer control group, 23% were positive by RT-PCR for CEA cDNA. One explanation for these false positive results could be that the blood sample was contaminated by CEA-producing epithelial cells cored from the skin into the venepuncture needle. In support of this our results suggested that CEA cDNA could be detected in normal

skin epithelium. Another explanation is that there may have been CEA-producing cells within the circulation of some control subjects. Although this has not to our knowledge been described, the cDNA of cytokeratins which were originally thought to be confined to epithelial tissue, have been identified in non-epithelial tissue¹⁸ and peripheral blood⁴ from healthy individuals.

Only 84% of patients with liver metastases were found to have circulating CEA cDNA (Table II), and reproducibility of the RT-PCR result in the same patient within four weeks was only 66% (Table III). Reasons for this might be that cancer cells were circulating in clumps and were not distributed homogeneously within the circulation, and that tumour shedding of malignant cells varied from day to day. Such variations could produce a negative result where a fall below the detection threshold of our assay occurred in the sampled blood. This is supported by the lower positivity rate using ethidium bromide staining compared with Southern blotting (Fig 5) in patients with colorectal liver metastasis, suggesting that cDNA levels in the clinical study were close to the lower limit of detection. Colorectal liver metastases can behave non-aggressively and roughly 10% of patients with liver metastasis are cured by liver metastasis resection.¹⁹ Thus an absence of viable circulating tumour cells might be expected in some patients with liver metastasis, although this would be unlikely to account for the negative results in our cases.

We did not find any relation between extent of liver metastases and prevalence of circulating CEA-producing cells. This is in keeping with the natural history of colorectal liver metastases.⁸ Aggressive tumour biology can produce early generalised dissemination which may not be associated with large liver metastases,²⁰ and liver metastases may grow to a large size without extrahepatic dissemination. There was also no relation between serum CEA and the prevalence of circulating CEA-producing cells. This may be because the serum level of CEA also depends on other factors such as CEA release from the cell surface²¹ and excretion by the liver.²² A similar lack of association has been reported in an RT-PCR study of CEA-producing cells in lymph nodes in gastric and breast cancer.²³

The additional bands detected with HT115 cells and in one blood sample from a patient with liver metastasis suggest the presence of smaller (by 200 bp or more) subclones or splice variants of CEA which have not previously been reported. Sequencing of the fragment or using a full length cDNA probe is needed to confirm this.

In summary, this study suggests that identification of circulating colorectal cancer cells using RT-PCR for detection of CEA cDNA is feasible. However, difficulty with the identification of non-malignant CEA mRNA-producing cells is still to be resolved. In addition it is not clear whether the reason for the low reproducibility of a positive result is a limitation of the method and could be

improved by decreasing the threshold of detection or whether it is due to the stochastic nature of metastasis. Identification of circulating cancer cells may lead to improved cancer staging and surveillance, and also to a better understanding of metastasis.

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