

Telomerase activity in circulating colorectal tumour cells

Research Article

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Summary

The detection of viable circulating tumour cells (CTC) in colorectal cancer (CRC) patients may be useful in devising new prognostic / diagnostic strategies and in understanding the metastatic process. This study used telomerase as a marker for CTC which has the advantage over most previous CTC studies in that it is both highly cancer-specific and only detectable in viable cells. Blood samples were taken from 35 CRC patients pre-operatively and 7 days post-operatively and from 10 healthy normal controls. Peripheral blood mononuclear cells were isolated using density gradient centrifugation and epithelial cells separated using BerEP4-conjugated magnetic beads. Telomerase activity was assessed using the TeloTAGGG γ PCR-ELISA assay. CTC were detected in 11/35 pre-operative, 19/35 post-operative and 0/10 control samples. 11/35 patients who were negative pre-operatively showed CTC post-operatively. CTC did not correlate with any clinical markers, however gender was a significant factor in CTC status with females most likely to be CTC positive pre-operatively ($p < 0.01$). The current study describes novel methodology to detect viable CTC in CRC patients. The methodology may be valuable in conjunction with established methods in the diagnosis of symptomatic patients. Interesting differences in the biology of colorectal cancer between genders has also been described.

Colorectal cancer is the second most common cancer in the UK and accounts for more than 18,000 deaths annually. Surgical resection is the mainstay of treatment for colorectal cancer but nearly half of all patients who undergo a potentially curative resection will relapse, principally because of undetected metastases at the time of surgery (Midgley and Kerr, 2000). This indicates that the metastatic process is already underway prior to surgical resection.

The detection of tumour cells in the circulation of cancer patients is not new. As early as 1869 Ashworth described a cancer case in which cells similar to those in the tumour were found in the blood after death (Ashworth, 1869; Ghossein and Bhattacharya, 2000). However inadequate detection strategies and conflicting reports on the significance of such cells hindered development in this field. In recent years many studies have used RT-PCR directed against epithelial specific antigens to detect circulating tumour cells (CTC). The field with respect to breast cancer has recently been reviewed by Ring et al,

(2004) with the potential clinical value being highlighted. Cytokeratin 20 detected by RT-PCR is one of the most common approaches in colorectal cancer (Wyld et al, 1998; Wharton et al, 1999; Weitz et al, 1999; Hardingham et al, 2000); however the detection of this marker in some samples from healthy individuals questions the specificity (Wyld et al, 1998). Other authors have used RT-PCR against carcinoembryonic antigen (CEA) mRNA (Castells et al, 1998). This group also detected CEA mRNA in patients with inflammatory bowel disease suggesting the presence of circulating, non-neoplastic, colonic epithelial cells. Zippelius et al, (1997) state that the limiting factors in the detection of micrometastatic tumour cells by RT-PCR are 'the illegitimate transcription of tumour associated or epithelial specific genes in haematopoietic cells and the deficient expression of the marker gene in micrometastatic tumour cells'. Furthermore, there are problems with such PCR-based studies in that they do not necessarily prove the epithelial cells are either viable or malignant.

Recently many authors have developed alternative methods for the detection of CTC. The method that has gained most popularity is the use of immunomagnetic separation technology. The epithelial cell specific antibody, BerEP4, is the most frequently chosen reagent to be coupled to magnetic beads (Soria et al, 1999; Hardingham et al, 2000; Gauthier et al, 2001). The cells collected after incubation with the antibody-conjugated magnetic beads after positive or negative selection strategies are removed for further analysis; in this case telomerase activity. Measuring telomerase activity has two principal advantages: firstly, with few exceptions, telomerase is a highly cancer-specific marker and secondly, only viable cells are detected as the assay requires active telomerase. Previous studies using magnetic beads have shown telomerase being detected in 15/17 (88%) of hepatocellular carcinoma patients (Tatsuma et al, 2000), 21/25 (84%) of metastatic breast cancer patients (Soria et al, 1999), 11/15 (73%) of stage IIIB or IV non-small cell lung cancer and 8/11 (72%) of Dukes stage C or D colon cancer patients (Gauthier et al, 2001). All of these studies have relied on a single blood sample. To date telomerase has not been detected by this method in any healthy normal controls.

The aim of this study therefore was to assess colorectal cancer patients for telomerase activity in CTC, both pre-operatively to assess possible use of this method as a prognostic tool, and post-operatively to identify how surgery affects the release of CTC.

II. Materials and Methods

A. Blood samples

Blood samples (10ml) were collected in Potassium/EDTA vacutainers from 35 patients undergoing surgery for primary colorectal cancer at Castle Hill Hospital, Hull, UK and 10 healthy age-matched normal controls. Samples were obtained from patients 1 day pre-operatively and 7 days post-operatively and all were processed within 2 hr of collection. Local Research Ethics Committee approval was granted and written consent obtained from all subjects.

B. Isolation of epithelial cells

Blood samples were diluted with 10ml PBS, mixed gently and peripheral blood mononuclear cells (PBMC) obtained by standard Hypaque (Sigma, Poole, UK) differential centrifugation. The PBMC were resuspended in 500µl PBS-1% v/v Bovine Serum Albumin (BSA) and 5×10^6 pre-washed immunomagnetic beads (Dynal, Merseyside, UK) covalently coated with the epithelial specific antibody BerEP4 (Dako, Cambridgeshire, UK) were added. The mixture was rotated at 4°C for 30min and the beads plus epithelial cells harvested using a magnet (Dynal). The cells were washed 3 times in PBS-0.1% BSA, resuspended in FBS with 10% v/v Dimethyl Sulphoxide and stored at -80°C overnight before transfer to liquid nitrogen for storage until assay.

C. Telomerase PCR ELISA

Telomerase activity was assessed using the *TeloTAGGG* Telomerase PCR ELISA PLUSi kit (Roche, Sussex, UK), all reagents were supplied in the kit unless otherwise stated. The manufacturer's protocol was followed throughout.

1. Preparation of cell lysates

Cells were thawed on ice, resuspended in 100µl Lysis buffer and incubated on ice for 30min. Cells were then centrifuged (13000g, 20min, 4°C), supernatants removed, and aliquoted prior to storage at -80°C. The protein concentration of lysates was determined using the Bio-Rad Protein Assay (Bio Rad Labs, Hemel Hempstead, UK) and all assays standardised to 0.2µg/µl.

2. PCR conditions

As telomerase is an RNA dependent enzyme, negative controls were prepared by incubating 5µl lysate (1 µg protein) with 1µl RNase (Sigma) for 20min at 37°C, then 10min at 65°C. For all samples a PCR master mix was prepared consisting of reaction mixture (25µl) and internal standard (IS; 5µl) per tube. Cell lysate, RNase-treated lysate or control template (3µl) was added to the relevant tubes and these were subjected to thermal cycling (Techne Progene, SLS, Nottingham, UK) according to the following protocol: Primer elongation 30min 25°C, telomerase inactivation 5min 94°C, amplification (30sec 94°C, 30sec 50°C, 90sec 72°C) x 30 cycles, 10min 72°C.

D. Hybridisation and ELISA

Following PCR, two aliquots of amplification product (2.5µl) were denatured at room temperature for 10min with denaturation reagent (10µl). The denatured hybridisation products were then hybridised separately to one of two digoxigenin labelled detection probes either specific for telomeric repeats (hybridisation buffer T) or the internal standard (IS buffer), mixed briefly and then added to a streptavidin coated microtitre plate. The plate was covered and incubated at 37°C on a shaker (300rpm) for 2 hr. Hybridisation solutions were removed and the wells washed three times with Washing buffer. Anti-DIG-HRP working solution (100µl) was added and incubated with shaking at room temperature for 30min. The solution was then removed and wells washed five times. TMB substrate (100µl) was added and incubated with shaking (300 rpm) at room temperature for approximately 10min (until colour development). Stop reagent (100µl) was added and the absorbance of samples measured (450nm - 690nm) on an Anthos plate reader (Lab Tech International, East Sussex, UK). The mean of the absorbance readings of the negative controls were subtracted from the absorbance readings of the samples. Samples were regarded as telomerase positive if the difference in absorbance was higher than the two-fold background activity as recommended by the manufacturer's protocol.

In initial optimisation experiments analysis of a subset of both normal (n=5) and tumour (n=7) lysates were repeated 6 times to ensure the reproducibility and reliability of the assay, in subsequent experiments the analysis of all cell lysates was performed twice. PCR amplification and subsequent analysis of RNase pre-treated negative control lysates were performed at each analysis alongside experimental lysates, again to ensure the specificity of the assay.

E. Statistical analysis

All statistical analyses were carried out using Fisher's exact test (for differences between 2 variables) or Chi squared test (for 3 or more variables) utilising ArcusTM PRO-11.

III. Results

Patients were considered to have CTC if a positive telomerase result was obtained. The CTC status for each individual patient and their clinicopathological data are shown in **Table 1**. Telomerase was not detected in circulating epithelial cells from any of the healthy normal

Table 1: Detection of circulating tumour cells (CTC) in 35 colorectal cancer patients

Patient	Age (years)	Sex	Dukes' stage	Tumour site	Recurrence	Follow-up (months)	CTC status	
							Pre	Post
1	30	F	A	Rectum	N	29	+	+
2	76	F	A	Rectum	N	27	-	-
3	62	M	A	Rectum	Y	26	+	-
4	66	M	A	Sigmoid	N	21	-	+
5	64	M	A	Rectum	N	21	-	+
6	69	F	B	Rectum	N	29	+	+
7	68	F	B	Transverse	N	28	-	-
8	63	F	B	Rectum	Y	9	+	+
9	73	F	B	Sigmoid	Y	24	+	-
10	66	F	B	Descending	N	21	+	+
11	61	M	B	Sigmoid	Y	26	-	+
12	66	M	B	Rectum	N	24	-	-
13	68	M	B	Sigmoid	N	20	-	-
14	65	M	B	Sigmoid	N	16	-	+
15	68	M	B	Sigmoid	N	25	-	-
16	61	M	B	Sigmoid	N	19	-	-
17	70	M	B	Sigmoid	N	25	+	+
18	80	F	C	Rectum	N	27	+	+
19	71	F	C	Sigmoid	N	25	-	+
20	64	F	C	Rectum	N	26	+	+
21	55	F	C	Rectum	N	25	-	-
22	78	F	C	Sigmoid	Y	18	-	-
23	50	F	C	Sigmoid	Y	27	+	-
24	70	M	C	Sigmoid	N	25	-	+
25	55	M	C	Rectum	Y	26	-	+
26	81	M	C	Rectum	Y	28	-	+
27	47	M	C	Rectum	Y	26	-	-
28	55	M	C	Rectum	N	23	-	+
29	57	M	D	Rectum	Y	6	-	+
30	54	M	D	Rectum	Y	25	-	-
31	72	M	D	Sigmoid	Y	20	-	-
32	61	M	D	Sigmoid	Y	20	-	-
33	49	M	D	Rectum	Y	5	-	-
34	80	M	D	Transverse	Y	7	-	+
35	80	M	D	Sigmoid	Y	20	+	+

^a (+/- denotes positive/negative status)

Table 2: Patients categorised according to CTC status

Category	Patients (n)	CTC status	
		Pre-operatively	Post-operatively
1	13	Negative	Negative
2	11	Negative	Positive
3	8	Positive	Positive
4	3	Positive	Negative

controls. These were an age matched population and their consistent negativity demonstrates the reliability and reproducibility of the assay, as did the analysis in duplicate of all samples.

Each patient was placed into 1 of 4 categories (**Table 2**): category 1 patients were CTC negative at both samplings (n=13), category 2 patients were CTC negative pre-operatively but CTC positive post-operatively (n=11), category 3 patients were positive at both samplings (n=8) and category 4 patients were CTC positive pre-operatively

but negative post-operatively (n=3).

CTC status was not found to correlate with Dukes' stage, recurrence, tumour location or survival. Gender was identified to be a significant factor in CTC status (**Table 3**). In comparing each CTC category between males and females, males were significantly more likely than females to be in category 2 (p=0.03) and females were significantly more likely to be in category 3 than males (p=0.03). Further analysis grouping categories 1 and 2 together, i.e. negative pre-operative categories) and

Table 3: CTC status and gender

Category	Female	Male	Total	P= ²
1	4 (31% ¹)	9 (69%)	13	NS
2	1 (9%)	10 (91%)	11	0.03
3	6 (75%)	2 (25%)	8	0.03
4	2 (67%)	1 (33%)	3	NS
Total	13	22	35	
p= ³	NS	0.001		

The distribution of patients according to gender for each category is shown.

¹ Indicates % of total patients in category which were female

² Statistical analyses were performed between gender within each category

³ Statistical analyses were performed for each gender across category

NS: Not Significant

categories 3 and 4 (positive pre-operative categories) revealed that females were significantly more likely to be CTC positive pre-operatively than males ($p=0.007$). However, when the distribution in females alone was compared between each category there was no significant difference. In contrast when the distribution of males alone were compared between categories they were significantly more likely to be in category 1 or 2, i.e. CTC negative pre-operatively, than category 3 or 4 ($p=0.001$).

Further analysis to investigate whether the gender of this cohort of patients was associated with age, Dukes stage, recurrence, tumour location or survival did not identify any correlation.

IV. Discussion

This study has described the highly specific detection of viable tumour cells in the peripheral blood of colorectal cancer patients in a technique demonstrated to be both reproducible and reliable. CTC were detected pre-operatively in 11/35 (31%) of the patients. For 8 of these patients the CTC remain detectable at 7 days, however the remaining 3 patients were CTC negative indicating that live CTC were no longer present. 13/35 (37%) of the patients fell into Category 1 with CTC not being detected either pre or post-operatively. It may be expected that lower Dukes stage tumours would be less likely to have CTC. It is a reasonable assumption that the presence of CTC would be an indicative factor for the presence of metastases and consequently CTC would be more common in higher Dukes stage tumours. However, the present study demonstrates that the metastatic process is not this simple as the category 1 patients consisted of 1 Dukes A, 5 Dukes B, 3 Dukes C and 4 Dukes D patients. The cancer biology of this category of tumours may intrinsically differ from tumours that are disseminated into the circulation. It is important to remember that this study only addresses the phenomenon of blood borne metastases as other studies have shown that lymphogenic tumour cell dissemination may be equally as important. Weitz et al, (1998) using Cytokeratin 20 mRNA RT-PCR studied 279 lymph node, blood and bone marrow samples from 20 colorectal cancer patients. A high proportion of patients with histopathologically tumour-free lymph nodes were found to have tumour cells in these nodes and/or the

mesentery vessels by this technique but only 2 patients had tumour cells in the blood. These authors suggest that lymphogenic tumour cell dissemination is a very common and early event in colorectal cancer preceding hematogenous tumour cell dissemination, however it must be noted that RT-PCR is not specific for viable cells.

An interesting recent study by Nozawa et al (2003) assessed telomerase activity in blood samples from mesenteric (tumor-drainage) vein and peripheral vessels of 41 colon cancer patients in relation to liver metastases. The authors identified high telomerase activity of mesenteric samples reflecting the existence of liver metastasis of colorectal cancer.

Many studies have shown that surgical manipulation can provoke cell dissemination (Van der Pompe et al, 1998; Weitz et al, 1998; Crisan et al, 2000) and this is supported by the 11 Category 2 patients who were CTC negative pre-operatively and CTC positive post-operatively. However, as statistical analysis of the results did not identify any significance between the presence of CTC and clinicopathological factors it indicates that CTC may not be an important prognostic factor in overall patient survival. This conclusion is supported by Bessa et al, (2003) who, using RT-PCR based methodology, concluded that postoperative detection of CTC had no prognostic significance in patients with colorectal cancer undergoing surgical resection with curative intent.

The observation that gender was a highly significant factor in relation to the presence of CTC with females generally being more likely to have CTC pre-operatively than males is an extremely interesting finding. This supports the idea that there may be differences in the biology of the same cancer between genders. Such an observation has been made by a number of other studies in colorectal and other cancers, and a number of hormones and other factors implicated to behave differently between genders. Aberrant hypermethylation of promoter CpG islands is an important mechanism for the inactivation of tumour suppressor genes and in gastric cancer, Kang et al, (2003) identified that male patients showed higher numbers of methylated genes than females. In colorectal cancer, a large study ($n=867$) revealed loss of hMLH1 expression is more likely ($p<0.0001$) to occur in females than males (Kakar et al, 2003). Similarly, studies of Apolipoprotein E gene polymorphism in 206 colorectal cancer patients and 353 healthy controls revealed a strong

association with both colorectal cancer risk and prognosis in a gender dependent manner (Watson et al, 2002).

In patient treatment, gender has also been implicated to be a predictive factor in response to treatment with 5-Fluorouracil (Yamashita et al, 2002). The authors studied the expression of Dihydropyrimidine dehydrogenase (DPD), the initial rate limiting enzyme in the catabolism of 5-fluorouracil. DPD expression levels are believed to correlate with the 5-FU sensitivity of malignant tumours. DPD expression was quantitated in 97 tumour specimens and 92 adjacent normal tissue specimens from 97 patients. The DPD expression in the tumour tissues was significantly lower in females than males although in the normal tissues there was no significant difference between the genders. The authors conclude that CRC patients who will benefit most, because of lowered DPD expression, must be given priority and female gender is a predictive factor for a better response to chemotherapy with 5-FU.

From this study it would appear that there is a factor(s) specific to males which means they are less likely to have CTC pre-operatively than females. Not only is the incidence of pre-operative CTC significantly less for males than females, but also when considering the male group alone, males are significantly more likely to be in a CTC negative pre-operative group. However it is important to remember that the group sizes are small and the study therefore needs to be expanded to fully elucidate such differences.

It is a very interesting phenomenon that the biology of cancer differs between genders and as evidence for such a difference accumulates it is becoming increasingly important to consider gender in future cancer studies. This is supported by a recent study by McArdle et al, (2003) who showed that following apparent curative resection for colorectal cancer and after adjusting for case-mix, male gender adversely affected 5-year survival. The data presented in our study supports the fact that gender differences must be considered when designing an individual patient's treatment.

In conclusion, this study has demonstrated methodology to determine the presence of viable CTC in colorectal cancer patients. The ability to study the presence of viable CTC is of paramount importance in further understanding the metastatic process and this study has described a highly reproducible and reliable assay which may be applied to a wide range of cancer studies. This methodology has previously been found valuable in studies of metastatic breast cancer patients (Soria et al, 1999), hepatocellular carcinoma patients (Tatsuma et al, 2000), non small cell lung carcinoma patients and Dukes stage C or D colon cancer patients (Gauthier et al, 2001). For colorectal cancer patients the technique could have an important role to play, in conjunction with faecal occult blood testing and lower GI endoscopy, in the diagnosis of symptomatic patients. The technique may also be very valuable in monitoring patients during chemo- or radiotherapy.

The study has indicated that although the presence of CTC is not clinically significant in this cohort size they are strongly associated with gender. This association, implicating differences in the biology of colorectal cancer

between gender, should be assessed in other cancers and indicates an important new avenue for cancer research.

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