PERGAMON

European Journal of Cancer 39 (2003) 1234–1241

European Journal of Cancer

www.ejconline.com

Intrinsic variability in the detection of micrometastases in lymph nodes for re-staging of colorectal cancer: effect of individual markers and tissue samples

M. Salto-Tellez[a,b,1], S. L. Kong[a,1], A.P.K. Leong[c], E.S.C. Koay[a,b,*]

[a]*Department of Pathology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore*[2]
[b]*Molecular Diagnosis Centre, Department of Laboratory Medicine, National University Hospital, 5 Kent Ridge Road, Singapore 119074, Singapore*
[c]*Department of Surgery, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore*

Received 10 July 2002; accepted 6 January 2003

Abstract

In this study, we investigated whether (a) carcinoembryonic antigen (CEA), cytokeratin-20 (CK-20) and guanylyl cyclase C (GCC) are clinically useful markers for the molecular detection of submicroscopic metastases in colorectal cancer (CRC) and (b) whether overexpression of CEA, CK-20 and GCC can be reliably detected in formalin-fixed, paraffin-embedded tissues as well as frozen lymph nodes. We studied 175 frozen lymph nodes and 158 formalin-fixed, paraffin-embedded lymph nodes from 28 cases of CRC. CEA or CK-20 or GCC-specific polymerase chain reaction (PCR) was carried out on mRNA transcripts extracted from the nodal tissues. Ten out of 11 Dukes' B CRC cases had detectable CEA and CK-20 while 6 out of 11 Dukes' B CRC cases had detectable GCC. In general, the difference of re-staged cases when comparing frozen and paraffin-embedded samples was marked; the only statistically significant correlation between frozen and paraffin tissue was for the CEA marker. Our results indicated a high incidence (>50%) of detecting micrometastases in histologically-negative lymph nodes at the molecular level.

Keywords: Micrometastases; Colorectal cancer; Lymph nodes; CEA; CK-20; GCC

1. Introduction

Five-year survival rates drop from approximately 80% in colorectal cancer (CRC) patients with stage II (Dukes' B) disease (no lymph node metastases or "node-negative") to less than 50% in those with stage III (Dukes' C) disease (presence of metastases in locoregional lymph nodes or "node-positive") [1]. Examination of lymph nodes for the staging of CRC by conventional histology is thus the most important prognostic factor, but it is suboptimal to predict the outcome of Dukes' B stage [2], presumably due to submicroscopic metastases. The presence of these "micrometastases" may well account for the significant proportion (approximately 30%) of patients with morphologically documented lymph node-negative tumours who still succumb to their disease after surgery [3].

The main problems with current lymph node evaluation are sampling error and poor sensitivity for detecting individual tumour cells or small tumour foci. Histological examination only samples a very small percentage of each lymph node and it has been calculated that a pathologist has only a 1% chance of detecting a micrometastatic focus of a three-tumour cell diameter [1]. Immunohistochemistry adds a higher rate of detection, but with no obvious clinical implications [4,5].

Technical advances now permit the detection of micrometastases in locoregional lymph nodes at the molecular level. Mori and colleagues [6] showed the applicability of detection of carcinoembryonic antigen (CEA) by reverse transcriptase-polymerase chain reaction (RT-PCR), and since then several reports have indicated the value of RT-PCR methods coupled to gel electrophoresis for micrometastases detection and dis-

* Corresponding author. Tel.: +65-6772-4564; fax: 65-6778-0671.
E-mail address: patkoaye@nus.edu.sg (E.S.C. Koay).
[1] Both these authors contributed equally to this paper.
[2] Address where work was done.

0959-8049/03/$ - see front matter
doi:10.1016/S0959-8049(03)00231-4

crimination between groups within Dukes' B with different prognoses [1,7–9]. However, there are variations regarding the type of tissue (fresh [1,7–9] versus formalin-fixed, paraffin-embedded [8]), and the specific biomarker used (CEA [1,9], guanylyl cyclase C (GCC) [7,8] or cytokeratin-20 (CK-20) [9,10]).

Recent results of large-scale, randomised trials have confirmed that patients with locally advanced (node-positive) CRC benefit from the administration of adjuvant chemotherapy following definitive surgical resection [11]. However, because of the potential toxicity of the compounds used, adjuvant therapy cannot be applied preemptively to all cancer patients and is standard treatment only for Dukes' C, but not for those with Dukes' B disease. Thus, a potential discriminator of those Dukes' B CRC requiring adjuvant therapy would be useful.

Several molecular markers have been shown to discriminate between prognostic groups. CEA is accepted as a useful tumour marker for the surveillance of gastrointestinal carcinoma patients, especially for those with CRC, and has been widely used postoperatively to detect early relapse [12]. Cytokeratins are major cellular proteins with many subgroups in some species [13,14], such as CK-8, 18 and 19. In general, their specificity is suspect because they often show false-positive bands, probably derived from pseudogenes. However, CK-20 appears to be relatively specific for gastrointestinal adenocarcinomas [15–17]. GCC is expressed in normal intestinal mucosal cells, adenomatous polyps, primary and metastatic colorectal tumours, but is absent in extra-intestinal tissues or tumours. Expression of GCC has been detected by RT-PCR in all of the histologically-confirmed colorectal tumours and CRC cell lines that have been examined, and may be a specific biomarker for metastatic CRC [7,8].

The aims of this study were (a) to analyse if overexpression of CEA, CK-20 and GCC can be reliably detected in formalin-fixed, paraffin-embedded tissue when compared with frozen material in the same lymph nodes and (b) to establish if CEA, CK-20 and GCC are clinically useful markers for the detection of submicroscopic metastases in CRC at the molecular level.

2. Patients and methods

2.1. Cell lines

The DLD-1 cell line, derived from human colorectal adenocarcinoma with epithelial morphology, was purchased from the American Type Culture Collection (ATCC, VA, USA). Growth requirements for the cell line were as follows: Roswell Park Memorial Institute (RPMI) 1640 medium with 2 mmol/l L-glutamine, 10 mmol/l N-(2-Hydroxethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), 1 mmol/l sodium pyruvate, 1.5 g/l sodium bicarbonate, 10% fetal bovine serum and 4.5 g/l glucose (growth medium and fetal bovine serum from Gibco-BRL, MD, USA).

2.2. Patients and tissues

A total of 193 lymph nodes from 28 consecutive CRC cases (2 Dukes' A; 11 Dukes' B; 15 Dukes' C) diagnosed at the Pathology Department of the National University of Singapore/National University Hospital in 1999, an average of approximately 8 lymph nodes per patient, were studied (the specific details of these patients are indicated in Table 1). The CRC specimens were sampled in the fresh state immediately after surgery, allowing

Table 1
Clinicopathological characteristics for all the CRC patients

Gender	
Male	15 (54%)
Female	13 (46%)
Age (years)	
Median (min–max)	63 (42–83)
Stage	
A	2 (7%)
B	11 (39%)
C/C1/C2	15 (54%)
Tumour size (cm)	
Median (min–max)	3.5 (2.0–6.0)
Tumour type	
Colon	21 (75%)
Rectum	7 (25%)
Differentiation	
Well	6 (21%)
Moderate	18 (64%)
Poor	4 (14%)
Invasive Margin	
Infiltrative	20 (71%)
Expanding	8 (29%)
Lymphoid response	
Yes	9 (32%)
No	19 (68%)
Crohn's-like lymphocytic response	
Yes	13 (46%)
No	15 (54%)
Intravascular/lymphatic invasion	
Yes	17 (61%)
No	11 (39%)
Perineural invasion	
Yes	14 (50%)
No	14 (50%)
Jass prognostic group	
I	3 (11%)
II	5 (18%)
III	6 (21%)
IV	14 (50%)

CRC, colorectal cancer.

less than 20 min between tumour excision and the last resected lymph node. Half of each resected lymph node was snap-frozen at −152 °C, while the other half was fixed for 24 h in 10% formalin and embedded in paraffin (each lymph node individually). After the first search for lymph nodes in the fresh state, further lymph nodes were identified and retrieved from the fixed CRC specimens. However, in no instance did the extra lymph nodes retrieved change the staging based on histological examination of the fresh lymph nodes alone.

There were 35 extra frozen lymph nodes without corresponding paraffin-embedded material from 13 cases because the paraffin-embedded tissue had been fully used for diagnostic purposes before the molecular study was carried out. In 6 other CRC cases, there was insufficient frozen material for successful PCR amplification and thus 18 extra paraffinised nodes were available in these cases. After histological sections were taken for routine histopathological diagnosis, further sections of the paraffinised tissue were used for total RNA isolation and cDNA synthesis, as was a portion of the complementary frozen half.

For negative control samples, 9 lymph nodes from specimens diagnosed with diverticulitis and no history of cancer were taken. The histologically-positive lymph nodes in Dukes' C CRC and the aforementioned cell line served as positive controls.

2.3. RNA extraction

Two 10 μm sections of each block were pooled and the formalin-fixed, paraffin-embedded tissue was deparaffinised with xylene. The Paraffin Block RNA Isolation Kit (Ambion Inc., TX, USA) was used to extract RNA from paraffin-embedded lymph nodes. Total RNA was extracted from frozen lymph nodes with Tri Reagent® (Molecular Research Center, OH, USA), following the recommended protocol of the manufacturer. Total RNA from the DLD-1 CRC cell line was isolated as well, to serve as a positive control. Elimination of genomic DNA contamination of the RNA extract by DNase treatment was incorporated into both procedures. To prevent RNA degradation, extracted RNA was kept at −80 °C until further investigation steps were taken.

2.4. RT-PCR

RT-PCR was performed using the Advantage RT-PCR kit (Clontech Laboratories, CA, USA). The optimal RT-PCR was carried out in a reaction consisting of 8 units of Moloney-murine leukaemia virus (MMLV) reverse transcriptase, MMLV buffer, 4 pmol/μl of random hexamers, 0.5 mmol/l of deoxynucleotide triphosphates (dNTPs), 10 nmol/l of dithiothreitol (DTT), 30 Units of RNase inhibitor and 1 μg of total RNA, made up to 25 μl with diethyl pyrocarbonate (DEPC)-treated water. Reaction tubes were incubated at 70 °C for 5 min, 42 °C for 60 min and 95 °C for 5 min.

2.5. PCR

PCR was carried out with the Gene Amp 9600 (Applied Biosystems, CA, USA) thermal cycler. The sequences of primer pairs used for synthesis and amplification of CEA, CK-20 and GCC with the appropriate thermal cycling conditions are shown in Table 2. All the primer pairs used were designed to bridge two adjacent exons and generate intron-spanning amplicons, so as to remove background interference of genomic DNA. The CEA-specific PCR was carried out with 2.5 mmol/l of $MgCl_2$, 1.25 Units of AmpliTaq (Perkin Elmer, CA, USA), 0.2 mmol/l of dNTP mix (Amersham Pharmacia Biotech UK Ltd., England) and 0.2 μmol/l of each CEA-specific primer, in 1× PCR buffer (Perkin Elmer, CA, USA). Nested CK-20-specific PCR was performed with 1.5 mmol/l of $MgCl_2$, 1.25 Units of AmpliTaq, 0.3 mmol/l of dNTP mix and 0.3 μmol/l of each CK-20-specific primer, in 1° PCR buffer. The hemi-nested GCC-specific PCR was carried out with 2 mmol/L of $MgCl_2$, 1.25 Units of AmpliTaq, 0.4 mmol/l of dNTP mix and 0.2 μmol/l of each GCC-specific primer, in 1×PCR buffer. Amplified products were separated by electrophoresis on a 3% agarose gel (Bio-Rad, CA, USA) and visualised by ethidium bromide staining. Amplicon identity was confirmed by sequence analysis on the ABI PRISM™ 377 DNA sequencer (Applied Biosystems, CA, USA).

A separate PCR run for the housekeeping *abl* gene, which serves as a control for the RNA extraction and cDNA synthesis steps, was carried out. The amplification was performed with 1.5 mmol/l of $MgCl_2$, 0.2 mmol/l of dNTP mix, 1.0 Unit of AmpliTaq and 0.4 μmol/l of each *abl* gene-specific primer, in a 1×PCR buffer under the conditions shown in Table 2.

2.6. Statistical analysis

Fisher's exact test was performed to compare the proportion of positive results among the different stage groups. We also used the McNemar test and Kappa statistics to evaluate the numbers of CEA, CK-20 and GCC-positive results in frozen and fixed tissues.

3. Results

A total of 28 tumours from 28 patients were studied (2 Dukes' A, 11 Dukes' B and 15 Dukes' C). Fifty-three lymph nodes (11 frozen and 42 paraffin-embedded) showing no amplification from the control *abl* gene PCR were excluded from the database, to eliminate

Table 2
Oligonucleotide sequences and PCR conditions used for synthesis of amplicons

Genes	Primers (5′–3′)	Denaturing temperature (°C) and time (min)	Annealing temperature (°C) and time (min)	Extension temperature (°C) and time (min)	Number of cycles	Size of PCR products (bp)
CEA [12]	F: GGGCCACTGTCGGCATCATGATTGG R: TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC	95 (1)	69 (0.5)	72 (1)	30	131
CK-20 [10]	(Outer) F: CAGACACACGGTGAACTATGG R: GATCAGCTTCCACTGTTAGACG	95 (1)	63 (1)	72 (1)	35	370
	(Inner) F: CTGTTTGTTGGCAATGAGAAAATGG R: GTATTCCTCTCTCAGTCTCATACT	95 (1)	61 (1)	72 (1)	35	303
GCC [24]	(Outer) F: GGGCACAAGGAGTATGGTTCTAAC R: CGAGTTCGTGGGAGAAATAGGA	94 (1)	69 (1)	72 (1)	35	695
	(Inner) F: GGGCACAAGGAGTATGGTTCTAAC R: GTAGCGTTCACAGTCACATTTAGG	94 (1)	66 (1)	72 (1)	35	277
abl [25]	F: TGTTGACTGGCGTGATGTAGTTGCTTGG R: TTCAGCGGCCAGTAGCATCTGACTT	94 (1)	55 (1)	72 (1)	35	275

PCR, polymerase chain reaction; CEA, carcinoembryonic antigen; CK-20, cytokeratin-20; F, forward; R, reverse; GCC, guanylyl cyclase C.

false-negative results. This study was thus based on materials from the remaining 164 frozen lymph node samples and 116 fixed lymph node samples derived from a total of 175 frozen lymph nodes and 158 paraffinised lymph nodes from the 28 cases, an average of 7–8 lymph nodes per case, which represented 94% of the original frozen lymph nodes and 73% of the original fixed lymph nodes. The average number of lymph nodes per case analysed for molecular studies in our study is slightly below the number of recommended lymph nodes ($n=9$) for maximum sensitivity suggested in the literature for histological examination [18].

The results for CEA, CK-20 and GCC amplification both in frozen and paraffin-embedded lymph nodes for all the 28 cases are shown in Table 3. None of the lymph nodes (frozen or fixed) from the two patients with Dukes' A disease showed any amplification of the CEA, CK-20 and GCC gene fragments, an indirect indicator of the specificity of the assay. Of the morphological Dukes' B, 34 of 65 (52%) frozen lymph nodes and 18 of 52 (35%) fixed lymph nodes yielded CEA-positive results. CEA-positivity was also detected in 34 (22 frozen and 12 fixed) histologically-negative lymph nodes from the 15 Dukes' C cases. Presence of CK-20 was detected in 25 of 65 (38%) Dukes' B frozen nodes and 10 of 52 (19%) Dukes' B fixed nodes, as well as 63 of 93 (68%) Dukes' C frozen nodes and 12 of 55 (22%) Dukes' C fixed nodes. There were 22 histologically-negative Dukes' C (16 frozen and 6 fixed) lymph nodes with CK-20 positive results. For the GCC marker, 13 of 65 (20%) Dukes' B frozen nodes, 7 of 52 (14%) Dukes' B fixed nodes, 31 of 92 (34%) Dukes' C frozen nodes, and 11 of 57 (19%) Dukes' C fixed nodes yielded positive results. Among the Dukes' C cases, there were 13 histologically-negative frozen lymph nodes and 7 histologically-negative fixed lymph nodes with demonstrable bands of the correct GCC amplicon size. The collective results are tabulated in Tables 3 and 4. Fig. 1 is an illustration of the electrophoretic separation.

Our results for the Dukes' B cases indicated that 10 of 11 (91%) morphological Dukes' B had detectable overexpression of CEA and CK-20, while 6 of 11 (55%) morphological Dukes' B cases were re-staged as molecular Dukes' C by GCC. Molecular upstaging occurred in the cDNA synthesised from *both* frozen and fixed tissue sources for 6 of 10 cases expressing CEA, 3 of 10 cases expressing CK-20, and 4 of 6 cases expressing GCC, respectively. CEA was detected only in frozen tissues for 3 cases (patient numbers 7, 9 and 12 in Table 3) and only in fixed tissue for 1 case (patient number 6). The presence of CK-20 was detected only in frozen tissues for 5 cases (patient numbers 3, 10, 11, 12 and 13) and only in fixed tissues for the remaining 2 cases (patient numbers 5 and 6). GCC overexpression was found only in the frozen tissue of patient number 7 and only in the fixed tissue in patient 11.

Table 3
Detection of micrometastases in lymph nodes of colorectal cancer: comparison of the different tumour markers

Patient No.	Stage	No. of lymph nodes		CEA		CK-20		GCC	
		Frozen tissue	PET	Frozen tissue	PET	Frozen tissue	PET	Frozen tissue	PET
1	A	5	6	0/5	0/6	0/5	0/6	0/5	0/6
2	A	2	0	0/2	0	0/2	0	0/2	0
3	B	8	7	4/8	2/7	2/8	0/7	1/8	2/7
4	B	11	8	7/11	1/8	7/11	3/8	3/11	1/8
5	B	0	1	0	0/1	0	1/1	0	0/1
6	B	0	4	0	3/4	0	3/4	0	0/4
7	B	7	7	4/7	0/7	2/7	2/7	2/7	0/7
8	B	6	5	6/6	5/5	0/6	0/5	0/6	0/5
9	B	4	2	3/4	0/2	3/4	1/2	4/4	1/2
10	B	5	5	3/5	1/5	1/5	0/5	0/5	0/5
11	B	4	5	2/4	5/5	1/4	0/5	0/4	1/5
12	B	6	3	2/6	0/3	2/6	0/3	0/6	0/3
13	B	14	5	3/14	1/5	7/14	0/5	3/14	2/5
14	C	2	2	2/2	2/2	2/2	0/2	1/2	0/2
15	C	5	5	5/5	4/5	4/5	0/5	3/5	0/5
16	C	2	2	2/2	1/2	2/2	1/2	2/2	1/2
17	C	6	6	4/6	4/6	4/6	2/6	2/6	0/6
18	C	12	5	11/12	3/5	11/12	1/5	8/12	3/5
19	C	0	1	0	1/1	0	1/1	0	0/1
20	C	8	7	5/8	2/7	6/8	6/7	6/8	0/7
21	C	10	4	5/10	2/4	8/10	0/4	2/10	0/4
22	C	0	6	0	3/6	0	0/6	0	1/6
23	C	12	9	8/12	4/9	6/12	0/9	1/12	5/9
24	C	3	1	1/3	1/1	3/3	1/1	1/3	0/1
25	C	10	1	9/10	1/1	1/10	0/1	2/10	0/1
26	C	5	2	3/5	2/2	4/5	0/2	2/5	0/2
27	C	2	0	1/2	0	2/2	0	1/2	0
28	C	15	7	12/15	5/7	9/15	0/7	0/15	1/7

PET, paraffin-embedded tissue.

Table 4
Cross-tabulation of stage and different micrometastases[a]

	Stage		
	A	B	C/C1/C2
CEA (PET)			
+	–	7	14
–	2	4	1
CK-20 (PET)			
+	–	5	6
–	2	6	9
GCC (PET)			
+	–	5	5
–	2	6	10
CEA (FROZEN)			
+	–	9	13
–	2	–	–
CK-20 (FROZEN)			
+	–	8	13
–	2	1	–
GCC (FROZEN)			
+	–	5	12
–	2	4	1

[a] Numbers refer to patient numbers. For some analyses, data are missing.

Statistical analysis showed that for formalin-fixed tissues there was a significant difference in the proportion of positive CEA among the different stage groups (Fisher's exact test, $P=0.008$), but there was no evidence that the proportions of positive CK-20 and positive GCC differed among the different stage groups. In contrast, there was a significant difference among the stage groups in the frozen tissues that were positive for CEA (Fisher's exact test, $P=0.004$), positive for CK-20 (Fisher's exact test, $P=0.004$) and positive for GCC (Fisher's exact test, $P=0.013$).

Application of the McNemar test to evaluate whether the fixed and frozen tissues can be used interchangeably to measure CEA, CK-20 and GCC expression to detect micrometastasis showed that there was no significant difference between the two sampling methods for measuring CEA, but there were significant differences for CK-20 ($P<0.0005$) and GCC ($P=0.039$).

Likewise, Kappa statistics for CEA was 0.429 (95% Confidence Interval (CI) from 0.0091 to 0.8481), which indicates moderate agreement, while those for CK-20 and GCC were 0.133 (95% CI from −0.0258 to 0.2925) and 0.094 (95% CI from −0.2153 to 0.4040) respec-

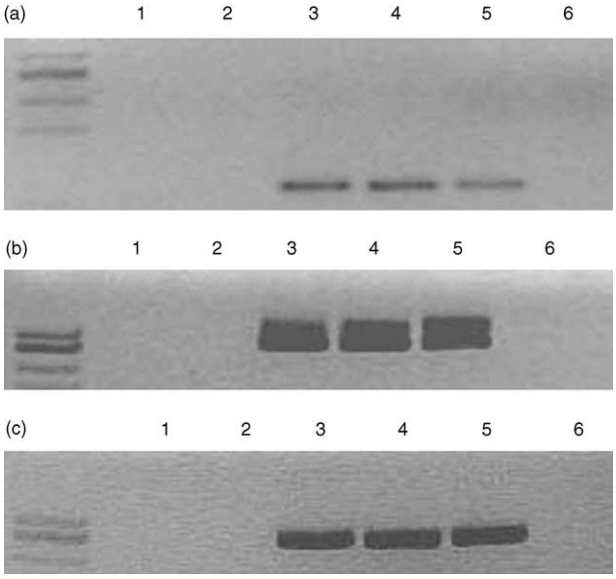

Fig. 1. Representative results. (a) CEA results: 1—lymph node patient 1 (fixed); 2—lymph node patient 7 (fixed); 3—lymph node patient 11 (fixed); 4—lymph node patient 15 (fixed); 5—positive control (CRC cell line); 6—negative control. (b) CK-20 results: 1—lymph node patient 1 (frozen); 2—lymph node patient 8 (frozen); 3—lymph node patient 13 (frozen); 4—lymph node patient 23 (frozen); 5—positive control (CRC cell line); 6—negative control. (c) GCC results: 1—lymph node patient 2 (frozen); 2—lymph node patient 10 (frozen); 3—lymph node patient 9 (frozen); 4—lymph node patient 18 (frozen); 5—positive control (CRC cell line); 6—negative control.

tively, both indicating slight agreement. However, this was a study with a relatively small sample size, which may partially explain the wide ranges of Confidence Intervals for all of the estimates.

4. Discussion

The prognosis of CRC is related to the degree of penetration of the tumour through the bowel wall and the presence or absence of lymph node involvement or distant organ metastasis, and some evidence has been shown in this and some previous studies that the presence of micrometastases in regional lymph nodes detected by a molecular method is a prognostic factor in stage II CRC.

Many investigators now believe that to precisely determine the nature of any given primary cancer and predict its metastatic potential, multiple tumour markers applied to the same tissue may be required [19]. At clinical diagnosis, solid cancers contain a wide spectrum of genetically and epigenetically heterogeneous cells. This results in numerous functional abnormalities that influence cell structure and function and accounts for the wide range of phenotypes and genotypes identified within tumours. This was the rationale for our PCR-based molecular assessment of tumour stage of CRC using multiple tumour markers such as CEA, GCC and CK-20, to obtain a more complete picture. These three markers have been used in the past because of their presumed specificity for the detection of malignant CRC cells in locoregional lymph nodes.

Our study showed CEA, CK-20 and GCC amplification in histologically-negative lymph nodes from stage II and stage III CRC. It is, to our knowledge, the only attempt to date addressing the comparison of several molecular markers and different tissue sampling protocols in the same study. However, our cases were too recent to allow a reliable correlation with significant follow-up of the long-term survival rate among the prognostically heterogeneous Dukes' B patients.

Our main finding is that the amount of lymph nodes with detectable expression of markers, presumably specific for malignant cells, varies widely with the type of tissue preservation and the type of molecular marker used. As a result, the number of cases that would have been re-staged is not constant. We refer to the CK-20 analysis in the paraffinised tissue as an example: patient 5 could only have been re-staged with this marker, while patient 3 was re-staged with all markers, except CK-20 in the paraffinised tissue.

Our study shows a significantly higher proportion of cases re-staged with CEA and CK-20 than previously reported in the literature, probably due to the efficiency of our PCR protocol. GCC is the only marker in which the percentage of re-staged cases was similar to previously reported numbers [7,8]. However, with this marker, a few results were still controversial: case 7 would have been re-staged only following the results in the frozen tissue, while case 11 would only have been up-staged following the results in the paraffin-embedded tissue.

Several studies have shown that the RT-PCR assay has a high sensitivity for detecting disseminated micrometastases of CRC to regional lymph nodes. According to a report by Liefers and colleagues [1], micrometastases by detection of CEA were found in 14 of the 26 (54%) stage II patients. In a study by Mori and colleagues [6], 30 out of 117 (26%) examined lymph nodes obtained from 13 carcinoma patients showed positive lymph node metastases by routine histological examination. They were able to demonstrate an increased number of positive lymph nodes to 77 (66%) by the RT-PCR method. Dorubi and colleagues [20] showed the presence of CK-20 in frozen lymph nodes from 4 out of 15 (27%) samples from stage II patients. Futamura and colleagues [9] showed a total of 102 among 202 (50.5%) lymph nodes were positive for either CEA or CK-20, or both (47.0, 40.1 and 36.6%, respectively). Waldman and colleagues [7] reported that GCC mRNA was detected by RT-PCR in lymph nodes from one third of patients with Dukes' B CRC and from all patients with Dukes' C CRC. Similarly, in a study by Cagir and colleagues [8], GCC was detected in the lymph nodes of all 10 patients with stage II disease who developed recurrent

disease up to 3 years after diagnosis. Our study highlights the idea that these findings must be reviewed in the context of a multi-marker, multi-tissue approach in a long-term, multi-centre study before the technique is used in routine clinical practice.

Merrie and colleagues [21] addressed one of the major methodological limitations of earlier studies based on the RT-PCR gel electrophoresis approach, namely, the lack of appropriate controls. In our study, apart from external controls of colorectal lymph nodes from surgical resections with no malignancy and the use of a CRC cell line, we had positive results in all histologically-positive lymph nodes (with amplifiable RNA) from Dukes' C tumours and negative results in all lymph nodes from Dukes' A tumours, confirming the high specificity of our method.

The comments of Ghosseini [22] on one of the cited studies probably underlines one of the main reasons accounting for the variability of markers and tissue samples, namely, that because of the power of PCR, there may be a cut-off level at which the detected signal represents baseline ('physiological'') expression of constitutional genes rather than overexpression by a small number of malignant cells. This is the central idea of those that advocate a quantitative (real-time PCR-based) assay [23], rather than a qualitative approach to the detection of micrometastases. Although this approach may represent an improvement in the standardisation of the technique, we feel that some of the precautionary measures highlighted in our study should apply equally to a quantitative analysis, particularly the choice of an in-house gene with no fluctuation in overexpression *at any time* to allow consistent comparison with the signal arising from a few bona fide malignant cells, and the incorporation of a DNAse step during transcript extraction, to eliminate any contamination of the mRNA extract by intrinsic genomic DNA.

A larger study, involving a greater number of cases, and based on a quantitative real-time PCR assay using hybridisation probes and SYBR Green chemistry is currently being pursued in our laboratory, to obtain a better correlation of biomarker detection with other clinicopathological characteristics in CRC.

Acknowledgements

This work was supported by grants from the National Medical Research Council, Singapore (NMRC-R-179-000-013-214 and NMRC/0383/1999) and the Singapore Cancer Registry (SCS-R-179-000-014-593).

References

1. Liefers GJ, Cleton-Jansen AM, van de Velde CJ, *et al*. Micrometastases and survival in stage II colorectal cancer. *N Engl J Med* 1998, **339**, 223–228.
2. Sloane JP. Molecules and micrometastases. *Lancet* 1995, **345**, 1255–1256.
3. Bustin SA, Dorudi S. Molecular assessment of tumor stage and disease recurrence using PCR-based assays. *Mol Med Today* 1998, **4**, 389–396.
4. Cutait R, Alves VAF, Lopes LC, *et al*. Restaging of colorectal cancer based on the identification of lymph node micrometastases through immunoperoxidase staining of CEA and cytokeratins. *Dis Colon Rectum* 1991, **34**, 917–920.
5. Greenson JK, Isenhart CE, Rice R, Mojzisik C, Houchens D, Martin Jr EW. Identification of occult micrometastases in pericolic lymph nodes of Dukes' B colorectal cancer patients using monoclonal antibodies against cytokeratin and CC49. Correlation with long-term survival. *Cancer* 1994, **73**, 563–569.
6. Mori M, Mimori K, Inoue H, *et al*. Detection of cancer micrometastases in lymph nodes by reverse transcriptase-polymerase chain reaction. *Cancer Res* 1995, **55**, 3417–3420.
7. Waldman SA, Cagir B, Rakinic J, *et al*. Use of guanylyl cyclase C for detecting micrometastases in lymph nodes of patients with colon cancer. *Dis Colon Rectum* 1998, **41**, 310–315.
8. Cagir B, Gelmann A, Park J, *et al*. Guanylyl cyclase C messenger RNA is a biomarker for recurrent stage II colorectal cancer. *Ann Intern Med* 1999, **131**, 805–812.
9. Futamura M, Takagi Y, Koumura H, *et al*. Spread of colorectal cancer micrometastases in regional lymph nodes by reverse transcriptase-polymerase chain reactions for carcinoembryonic antigen and cytokeratin 20. *J Surg Oncol* 1998, **68**, 34–40.
10. Funaki NO, Tanaka J, Ohshio G, Onodera H, Maetani S, Imamura M. Cytokeratin 20 mRNA in peripheral venous blood of colorectal carcinoma patients. *Br J Cancer* 1998, **77**, 1327–1332.
11. Markman M. *Basic Cancer Medicine*. Philadelphia, WB Saunders, 1997 [pp. 78–79].
12. Gerhard M, Juhl H, Kalthoff H, Schreiber HW, Wagener C, Neumaier M. Specific detection of carcinoembryonic antigen-expressing tumor cells in bone marrow aspirates by polymerase chain reaction. *J Clin Oncol* 1994, **12**, 725–729.
13. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalog of human cytokeratins: Patterns of expression in normal epithelial, tumor and cultured cells. *Cell* 1982, **31**, 11–24.
14. Franke WW, Moll R. Cytoskeletal components of lymphoid organs. *Differentiation* 1987, **36**, 145–163.
15. Moll R, Schiller DL, Franke WW. Identification of protein IT of the intestinal cytoskeleton as a novel type I cytokeratin with unusual properties and expression patterns. *J Cell Biol* 1990, **111**, 567–580.
16. Moll R, Lowe A, Laufer J, Franke WW. Cytokeratin 20 in human carcinomas. A new histodiagnostic marker detected by monoclonal antibodies. *Am J Pathol* 1992, **140**, 427–447.
17. Moll R, Zimbelmann R, Goldschmidt MD, *et al*. The human gene encoding cytokeratin 20 and its expression during fetal development and in gastrointestinal carcinomas. *Differentiation* 1993, **53**, 75–93.
18. Cianchi F, Palomba A, Boddi V, *et al*. Lymph node recovery from colorectal tumor specimens: recommendation for a minimum number of lymph nodes to be examined. *World J Surg* 2002, **26**, 384–389.
19. Guan RJ, Van Dam J. Molecular detection of micrometastases: science on stage [Review]. *Am J Gastroenterol* 1999, **94**, 3062–3064.
20. Dorubi S, Kinrade E, Marshall NC, Feakins R, William NS, Bustin SA. Genetic detection of lymph node micrometastases in patients with colorectal cancer. *Br J Surg* 1998, **85**, 98–100.
21. Merrie AEH, Yun K, McCall JL. Detection of carcinoembryonic antigen messenger RNA in lymph nodes from patients with colorectal cancer [Discussion]. *N Engl J Med* 1998, **339**, 1642.
22. Ghossein RA. [Discussion]. *N Engl J Med* 1998, **339**, 1642.

23. Mitas M, Mikhitarian K, Walters C, *et al*. Quantitative real-time RT-PCR detection of breast cancer micrometastasis using a multigene marker panel. *Int J Cancer* 2001, **93**, 162–171.
24. Carrithers SL, Barber MT, Biswas S, *et al*. Guanylyl cyclase C is a selective marker for metastatic colorectal tumors in human extra-intestinal tissues. *Proc Natl Acad Sci USA* 1996, **93**, 14827–14832.
25. Evans H, Sillibourne J. Detection of the Bcr-Abl gene in CML/ALL patients. *Promega Notes Magazine* 1996, **57**, 21–23.