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DETECTION OF OCCULT TUMOUR CELLS IN LYMPH NODES OF COLORECTAL CANCER PATIENTS USING REAL-TIME QUANTITATIVE RT-PCR FOR CEA AND CK20 mRNAS

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The purpose of our study was to develop specific, sensitive, objective assays for early detection of disseminated tumour cells in patients with colorectal cancer (CRC). Carcinoembryonic antigen (CEA) and cytokeratin 20 (CK20) were chosen as markers because they are selectively expressed in epithelial cells with maintained expression in CRC. Real-time quantitative RT-PCR assays with RNA copy standards were constructed. Regional lymph nodes were collected from patients with CRC (n = 51) and benign intestinal disease (n = 10). Results were compared to routine histopathology and anti-CEA immunohistochemistry. Lymph node levels of CEA and CK20 mRNA correlated strongly ($p < 0.0001$, $r = 0.8$). Lymph nodes from non-CRC patients had <0.01 CEA and <0.001 CK20 mRNA copies/18S rRNA unit. Lymph nodes from 3/6 Dukes' A, 17/26 Dukes' B, 10/10 Dukes' C and 7/9 Dukes' D patients had CEA mRNA levels above cut-off. Corresponding figures for CK20 mRNA were 3/6, 10/26, 9/10 and 5/9, respectively. CEA mRNA levels varied from 0.001 to 100 copies/18S rRNA unit in Dukes' A and B, and 50% of the Dukes' B patients had CEA mRNA levels within the range of Dukes' C patients. Three Dukes' B patients have died from CRC or developed distant metastases. All 3 had high CEA and CK20 mRNA levels. Determination of mRNA was superior to immunohistochemistry in showing CEA expression in lymph nodes. The present qRT-PCR assay for CEA mRNA seems to be a superior tool to identify individuals with disseminated tumour cells. Future extended studies will establish the clinically most relevant cut-off level.

Key words: *colorectal neoplasm; carcinoembryonic antigen; cytokeratin 20; real-time quantitative RT-PCR; lymph node metastases; minimal residual cancer*

Surgery is the only curative treatment for colorectal cancer (CRC). A substantial number of tumour recurrences occur, however, after potentially curative surgery. The tumour stage based on the histopathologic examination of the resected specimen combined with the perioperative findings is the best prognostic predictor in CRC.[1–3] Adjuvant chemotherapy has proven positive to patients operated for colon cancer in Dukes' Stage C (anyTN1-2M0, Stage III) indicating high risk of minimal residual cancer, thereby reducing the relative mortality rate by one-third.[2–4] There are also a number of patients who will die from tumour recurrences among patients with no revealed lymph node metastases (5–15% in Dukes' Stage A [T1-2N0M0, Stage I] and 20–50% in Stage B [T3-4N0M0, Stage II]). These patients do not routinely receive adjuvant chemotherapy. It has been argued that early detection of residual cancer will improve the success rate of adjuvant chemotherapy initiated when the tumour burden is low[5,6] but this remains to be proven clinically. It is of vital importance to improve the selection criteria in identifying patients who may benefit from adjuvant chemotherapy. Moreover, such improved selection criteria may be of large interest when selecting patients to intense follow-up protocols after potentially curative surgery.[7]

Various techniques have been applied for detection of tumour cells disseminated to regional lymph nodes, bone marrow, and blood of patients with CRC.[6] Immunocytochemistry and immunohistochemistry (IHC) have been used for detection of cells expressing tumour markers, and the more sensitive RT-PCR has been utilized to detect mRNA for proteins expressed in tumour cells. Early detection of disseminated tumour cells by these methods has been hampered partly by the fact that none of them are sufficiently potent in combining specificity with both sensitivity and objectivity. Most investigators have used cytokeratins 8/18 (CK8/18), CK19, CK20 or carcinoembryonic antigen (CEA) as markers for tumour cells. These proteins are selectively expressed in epithelial cells and are indicators of disseminated tumour cells when detected at locations outside epithelial compartments.[8–10] CEA is a well-established tumour marker in adenocarcinoma and is used primarily for postoperative follow-up.[7,11,12] Moreover, CEA has been suggested as a prognostic factor and a parameter for staging of CRC.[13,14] Carcinoembryonic antigen has been used by several groups as a target antigen for radioimmunoimaging, radioimmunoguided surgery and radioimmunotherapy in CRC,[15–19] and is a highly glycosylated protein expressed normally on the surface of epithelial cells along the alimentary tract and particularly on the columnar epithelial cells in the colon. Both CEA and CK20 are expressed in epithelial cells of the large bowel and their expression is retained in CRC.[9,10,20] Specific detection of CEA at both the protein and mRNA levels has been complicated by the existence of a number of closely related genes (*e.g.*, 4 are expressed in immune cells).[10]

We report on the construction of real-time quantitative RT-PCR assays for CEA mRNA and CK20 mRNA and compare their utility for detection of disseminated tumour cells in regional lymph nodes of patients with CRC. Lymph node mRNA analysis is compared to other indicators of disseminated tumour cells.

Abbreviations: CEA, carcinoembryonic antigen; CK20, cytokeratin 20; CRC, colorectal cancer; ELISA, enzyme linked immunosorbent assay; IBD, inflammatory bowel disease; IHC, immunohistochemistry; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction.

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MATERIAL AND METHODS

Patients

Between September 1998 and November 2001 surgery for CRC was carried out in 51 patients (32 men, 19 women; median age = 69 years, range = 52–90). Thirty-five tumours were located in the colon and 16 in the rectum. Seven patients with rectal cancer received preoperative irradiation with 25 Gy. Radical excision of the tumours with wide lymph node dissection was carried out in 42 patients. Nine patients had distant metastases. Five patients received adjuvant chemotherapy. According to Dukes' classification there were 6 tumours in Stage A (T1-2N0M0, Stage I), 26 in Stage B (T3-4N0M0, Stage II), 10 in Stage C (anyTN1-2 M0, Stage III) and 9 in Stage D (anyTanyNM1, Stage IV). At follow-up in May 2003, 32 patients were alive (median = 41 months, range = 22–53). Two were alive with tumour recurrence. Fourteen patients had died from CRC and 5 patients had died from non-cancer disease. Controls included 7 men and 3 women (median age = 30 years, range = 18–61) undergoing colorectal surgery for ulcerative colitis ($n = 6$), Crohn's disease ($n = 3$) or rectal prolapse ($n = 1$). Informed consent was obtained from the patients. The local Research Ethics Committee of the Medical Faculty, Umeå University, Sweden, approved our study.

Lymph nodes

Lymph nodes (1–4) were dissected from surgically removed specimens. Lymph nodes with overt metastases and apparently normal nodes were cut into halves with separate knives under sterile conditions to prevent RNA cross-contamination. One half of each node was fixed in 10% buffered formalin and embedded in paraffin for routine H&E staining and anti-CEA IHC. The other half was snap frozen in liquid nitrogen and stored at −70°C until RNA extraction. Ninety-eight lymph nodes were collected from CRC patients. Eighty-two lymph nodes were from 42 potentially cured patients with tumours in Dukes' Stages A–C (11, 55 and 16, respectively), and 16 lymph nodes were from 9 patients with tumours in Dukes' Stage D. Thirty-four lymph nodes were collected from the 10 control patients.

CRC tissue

An approximately 0.5 × 0.5 × 0.5 cm piece was collected from the outer rim of the tumour specimens immediately after resection, snap-frozen and kept at −70°C until RNA extraction.

Blood cells

Peripheral blood mononuclear cells (PBMC) and granulocytes were isolated from peripheral blood of healthy adults as described.[21] *In vitro* activated PBMC were obtained by incubation in HEPES buffered RPMI1640 supplemented with 0.4% human serum albumin with addition of either 50 ng anti-CD3 mAb OKT3/ml, 3 μg Concanavalin A/ml or a combination of phorbolmyrestate acetate (5 μg/ml) and Ionomycin (1 mg/ml) as activators.[22] PBMC were incubated with these stimulants in parallel cultures for 4, 7, 20, 48 and 72 hr, washed, snap frozen and stored at −70°C until RNA extraction.

Tumour cell lines

The human cell lines LS174T (well-differentiated colon carcinoma), HT29 (moderately differentiated colon carcinoma) and HL-60 (promyelocytic leukaemia) were grown in Parker199 medium (SBL Vaccin, Stockholm, Sweden) supplemented with 8% FCS and antibiotics.

RNA preparation and real-time qRT-PCR

Total RNA was extracted by the acid guanidine-phenol-chloroform method and precipitated RNA dissolved in RNase free water containing 1,000 U/ml of the RNase inhibitor rRNasin (Promega, Madison, WI) as described.[23] Total amount of RNA was determined by OD_{260} measurements in selected PBMC and lymph node samples before the addition of rRNasin. Purity was ascertained by an OD_{260}/OD_{280} ratio >1.8.

Real-time qRT-PCR assays for quantitative determination of CEA and CK20 mRNAs were constructed using the TaqMan EZ technology (Applied Biosystems, Foster City, CA). Specific primer pairs placed in different exons were used with a reporter dye-labeled probe hybridizing over the boundary between 2 exons in the amplicon. In the CEA mRNA assay the primers and probe were placed at and around the M/3′ and 3′-untranslated region (3′-UTR) exon boundary in the mRNA sequence. In the CK20 assay the primers and probe were placed at and around the boundary between exons 1 and 2 (see Table I for sequences of primers and probes used in the 2 assays). Emission from released reporter dye was monitored by the ABI Prism 7700 Sequence Detection System (Perkin-Elmer, Wellesley, MA). The RT-PCR profile was: 50°C for 2 min, 60°C for 30 min, 95°C for 5 min followed by 45 cycles of 94°C for 20 sec and 62°C for 1 min. Total RNA extracted from LS174T and HT29 cells were used for optimization of the CEA and CK20 assay, respectively. Specific RNA copy standards were prepared for both assays (see below). Determinations were carried out in triplicates and expressed as copies of mRNA/μl as determined from parallel RT-PCR of serial dilutions of the RNA copy standard. The concentration of 18S rRNA was determined in each sample by real-time qRT-PCR according to the manufacturer's protocol (Applied Biosystems). No copy standard is available for the 18S rRNA assay. Therefore the 18S rRNA content was expressed as arbitrary units defined as the amount of 18S rRNA in 1 pg total RNA extracted from PBMC. Results are expressed as mRNA copies per unit of 18S rRNA. Quantitative PCR assays for determination of possible DNA signals were carried out using the same primers, probe and PCR profile without the RT step and using AmpliTaqGOLD DNA polymerase (Applied Biosystems) instead of recombinant thermostable *Thermus thermophilus* (rtTh) polymerase.

All samples of RNA extracted from lymph nodes were checked for presence of immune cell RNA by RT-PCR for CD45 to certify the correct tissue origin (see Lundqvist *et al.* for primer sequences).[23]

Cloning and sequencing

RT-PCR products were subjected to cloning and sequencing as described.[24]

RNA copy standard preparation

Total RNA from LS174T and HT29 were used as starting material for CEA and CK20 RNA copy standard preparation, respectively. The copy standards were prepared from cloned and

TABLE I – PROPERTIES OF PRIMERS AND PROBES USED IN THE CEA AND CK20 mRNA REAL TIME QUANTITATIVE RT-PCR ASSAYS

Property	CEA	CK 20
Forward primer sequence	5′-CTGATATAGCAGCCCTGGTGTAGT-3′	5′-CGACTACAGTGCATATTACAGACAAA-3′
Reverse primer sequence	5′-TGTTGCAAATGCTTTAAGGAAGA-3′	5′-GACACACCGAGCATTTTGC-3′
Probe sequence[1]	5′-TTCATTTCAGGAAGACTGACAGTTGTTTTGCTT-3′	5′-CTGCGAAGTCAGATTAAGGATGCTCAACT-3′
Amplicon length	82 bases	82 bases
NCBI accession number	M29540	X73502
Amplicon position	2215–2296	90–171

[1]Reporter dye at the 5′-end is FAM. Quencher dye at the 3′-end is TAMRA.

sequenced products of real-time qRT-PCR reactions as described in Fahlgren *et al.*[24] The copy standards were checked by RT-PCR and PCR to certify that the DNA content was <0.1%. Concentration of RNA copies was calculated on the basis of the OD_{260} value, the molecular weight of the transcript and Avogadro's number.

Because the sensitivity of the assay is influenced by the proportion of the mRNA species of interest compared to unrelated mRNA, standard curves were set up by diluting the copy standard in buffer containing RNA extracted from PBMC. The concentration of RNA added to the standard (200 ng/μl) was chosen from determinations of RNA concentration by OD_{260} measurements in lymph node RNA ($n = 5$).

Histopathology

Sections of formalin-fixed, paraffin-embedded lymph nodes were stained with H&E and subjected to routine histopathologic examination at the Department of Pathology, Umeå University Hospital.

IHC

Four-micrometer sections of formalin-fixed, paraffin-embedded lymph nodes were subjected to 2 cycles of antigen retrieval in a microwave oven in 10 mM citric acid buffer (pH 6.0). Endogenous peroxidase was quenched in 3% H_2O_2 in methanol. Non-specific binding was blocked using 5% horse serum. CEA staining was carried out using the CEA specific mAb II-7 (IgG1, Dakopatts, Glostrup, Denmark),[21] and the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA). Sections were counterstained with Mayer's hematoxylin and mounted. One anti-CEA stained section/lymph node was inspected. Sections on which the primary or secondary antibody was omitted served as negative controls.

Determination of CEA concentration in serum

Serum was prepared from blood collected in the morning of the day surgery was carried out and stored at −70°C. CEA concentrations were determined in duplicate samples by ELISA according to the manufacturer's protocol (ICN Biomedical Research Products, Costa Mesa, CA). All samples were analyzed on the same occasion.

Statistical analysis

Differences between patient groups in levels of CEA mRNA in lymph nodes were analyzed using Mann-Whitney's rank sum test. Correlation between CEA mRNA levels in lymph nodes and CK20 mRNA levels in lymph nodes, CEA mRNA levels in tumour tissue and serum concentrations of CEA were analyzed using Spearman correlation test. The statistical significance of differences in frequencies between patient groups was determined by Fisher's exact test. Two-tailed analyses were used throughout. A p-value < 0.05 was considered statistically significant.

RESULTS

Sensitivity, specificity and reproducibility of the CEA and CK20 mRNA real-time qRT-PCR assays

We used the 3′-primer for specific reverse transcription, so-called TaqMan EZ technology. This method was about 10 times more sensitive compared to a qRT-PCR assay in which cDNA was prepared by random hexamers followed by specific PCR amplification with AmpliTaqGOLD DNA polymerase (data not shown). The detection limit of the CEA mRNA assay was 10 mRNA copies and there was a linear relationship between log concentration of RNA and PCR cycles up to 10^8 copies. The CK20 assay was slightly less sensitive (about 30 copies with a similar range).

The CEA assay was highly specific. CEA mRNA levels were below the detection limit of the assay in 7/10 PBMC samples and extremely low ($2–5 \times 10^{-4}$ copies/18S rRNA unit) in 3/10 PBMC samples. None of the PBMC samples gave a detectable signal in the CK20 mRNA assay ($<9 \times 10^{-5}$ copies/18S rRNA unit). Pooled RNA from polyclonally activated PBMC was negative in both assays. No CEA mRNA signal was obtained in RNA extracted from purified granulocytes and the granulocytic HL-60 cell line ($<7 \times 10^{-5}$ copies/18S rRNA unit). The latter cell line is known to express several closely related CEA gene family members, notably CEACAM1 (the new nomenclature for the CEA family as described in Beauchemin *et al.*[25] was used), CEACAM3, CEACAM6 and CEACAM8. Granulocytes and HL-60 cells gave weak signals (6×10^{-3} and 2×10^{-4} copies /18S rRNA unit, respectively) in the CK20 assay. Thus, for PBMC there was a difference in sensitivity of at least 5 orders of magnitude compared to colorectal cancer cells for both assays. The same was true for granulocytes and cancer cells in the CEA mRNA assay. In the CK20 mRNA assay, however, the difference between granulocytes and cancer cells was only in the order of 2,000 times. Randomly selected real-time qRT-PCR products from the CEA and CK20 mRNA assays were sequenced and gave the expected sequences. When purified DNA was used as template a very weak signal was seen in the CEA mRNA assay whereas no signal was seen in the CK20 mRNA assay. To ascertain that signals from contaminating genomic DNA were not falsely interpreted as positive, all RNA samples were also subjected to PCR without reverse transcription. No DNA signal was seen in any of the lymph node RNA samples in our study ($n = 132$).

The reproducibility of the assays was high at concentrations above 100 copies/μl RNA (mean C.V. = 1.7%, range = 1.1–2.8%, $n = 11$) as demonstrated by analysis of RNA from 12 lymph nodes in 6 independent experiments with new dilutions of the copy standard in each experiment. Figure 1*a* shows the results for the CEA mRNA assay.

To allow comparison between lymph nodes, the CEA and CK20, mRNA contents were normalized to the content of 18S rRNA of the same sample. Figure 1*b* shows the results of experiments carried out to give an estimate of what a given value of CEA mRNA copies/18S rRNA unit represents in terms of proportion of tumour cells among the immune cells. LS174T cells were added to PBMC at different ratios, thereafter RNA was extracted and the CEA mRNA and 18S rRNA content determined. There was an almost linear relationship between CEA mRNA copies/18S rRNA unit and tumour cell: PBMC ratio from 1:10,000 up to 1:1. One CEA mRNA copy/18S rRNA unit corresponded to approximately 2,000 LS174T cells/10^6 PBMC (Fig. 1*b*). For HT29 cells one CEA mRNA copy/18S rRNA unit corresponded to approximately 80,000 cells/10^6 PBMC (data not shown). CK20 mRNA levels were determined in the same experiments. One CK20 mRNA copy/18S rRNA unit corresponded to 700 HT29 cells/10^6 PBMC.

Most lymph nodes from non-cancer patients contain low but significant levels of CEA and CK20 mRNAs

Thirty-four lymph nodes from 9 inflammatory bowel disease patients (IBD) and one patient with rectal prolapse (= controls) were analyzed in the 2 mRNA assays. The results are shown in Figure 2*b*. As can be seen values for CEA mRNA varied from undetectable to 7×10^{-3} copies/18S rRNA unit and for CK20 mRNA from undetectable to 9×10^{-4} copies/18S rRNA unit. There was a good correlation between CEA and CK20 mRNA values ($p = 0.003$, $r = 0.6$). Comparing different lymph nodes from the same individual, we found that nodes could vary considerably both in their CEA mRNA and in their CK20 mRNA values (Fig. 3*a*). Based on the levels of these control nodes we established tentative cut-off values for CEA mRNA to 10^{-2} copies/18S rRNA unit and for CK20 mRNA to 10^{-3} copies/18S rRNA unit.

CEA mRNA and CK20 mRNA levels in regional lymph nodes of CRC patients span over several logs and are significantly above those in lymph nodes of non-cancer patients

Ninety-eight regional lymph nodes from 51 CRC patients representing all 4 Dukes' stages were analyzed in both mRNA assays (Fig. 2). The CEA and CK20 mRNA levels in all lymph nodes (CRC + controls) were strongly correlated with each other ($p <$

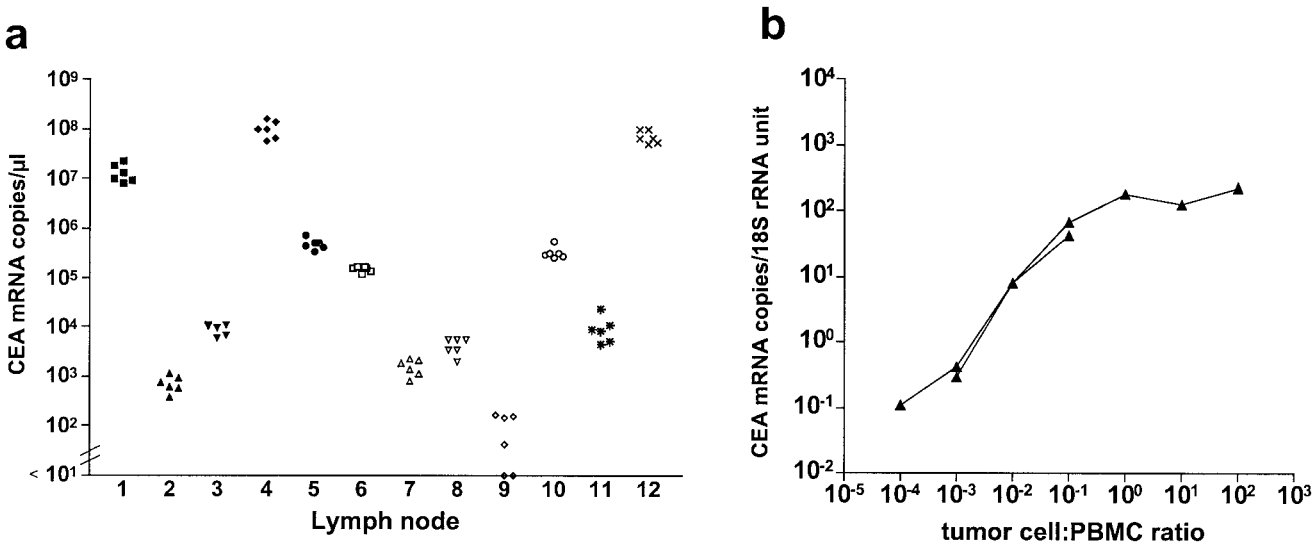

FIGURE 1 – (*a*) Results from 6 independent determinations of the CEA mRNA concentration in extracts of 12 individual lymph nodes. Each test was carried out with parallel external standard. (*b*) Results from 2 independent experiments in which LS174T colon carcinoma cells were admixed with PBMC, RNA extracted from the mixture and the concentrations of CEA mRNA copies and 18S rRNA units in the extracts determined by real-time qRT-PCR.

0.0001, $r = 0.79$; Fig. 2*a*). CEA mRNA levels were approximately 10 times higher than CK20 mRNA levels. Both CEA mRNA and CK20 mRNA values in lymph nodes from CRC patients varied by several order of magnitude between patients, Dukes' C and D patients displaying the highest values. Interestingly, a wide variation in CEA and CK20 values was also seen within each Dukes' stage, especially in Dukes' B. This was partly due to the fact that individual nodes from the same patient sometimes showed highly different mRNA levels (Fig. 3*a*, data for CEA mRNA). One reason for the large variation in mRNA values between different regional lymph nodes from the same CRC patient is that tumour cells do not naturally spread evenly to all local nodes. To investigate whether there was a relationship between the mRNA level in the original tumour and the lymph nodes, CEA mRNA levels were also determined in RNA extracted from the original tumours of 10 patients with tumours in Dukes' Stage B. CEA mRNA levels in the original tumours varied between 13 and 260 mRNA copies/18S rRNA unit (Fig. 3*b*). There was no correlation between the CEA mRNA levels in the lymph nodes and the original tumour ($p > 0.05$; Fig. 3*b*). Very similar results were obtained for CK20 mRNA (data not shown). Thus, the variation in CEA and CK20 mRNA expression level is likely to reflect different degree of tumour cell infiltration rather than variation in CEA and/or CK20 mRNA expression level/cell in the original tumour.

CEA mRNA and CK20 mRNA levels in regional lymph nodes of CRC patients: correlation to Dukes' stage and other clinical parameters

We argued that because detection of a single lymph node with overt metastasis is sufficient for classification of a tumour as Dukes' Stage C, it is correct to use the lymph node with the highest CEA mRNA level as the indicator for tumour cell dissemination. Figure 4 shows the result when only the lymph node with the highest CEA mRNA level is taken into consideration for each patient. On average, all 4 CRC patient groups had significantly higher CEA mRNA levels in lymph nodes than controls (Fig. 4) with a median CEA mRNA copies/18S rRNA unit of 0.0003 in controls and median values of 0.02, 0.04, 39.5 and 0.08 for CRC patients with tumours in Dukes' Stage A, B, C and D, respectively. Further comparisons between CRC patient groups showed that patients with Dukes' Stage C tumours had significantly higher CEA mRNA levels in their lymph nodes than patients with tumours in Dukes' Stage A or B ($p = 0.005$ and 0.0002 for Dukes' C compared to Dukes' A and B, respectively).

The CK20 mRNA values in lymph nodes from CRC patients were also analyzed as described above. Using the cut-off values determined above lymph nodes from 3/6 Dukes' A patients were elevated in both assays. For Dukes' B patients 17/26 and 10/26 were elevated in the CEA mRNA assay and the CK20 mRNA assay, respectively. For Dukes' C elevated values were seen in 10/10 and 9/10 patients, respectively, and for Dukes' D in 7/9 and 5/9, respectively (Table II). Thus, lymph nodes with CEA mRNA but not CK20 mRNA levels above cut-off were frequent in Dukes' B-D CRC patients (Fig. 2*c–f*). Two lymph nodes from patients with Dukes' B tumours even had significant CEA mRNA levels without detectable CK20 mRNA (Fig. 2*d*). Conversely, 2 lymph nodes, one from a patient with a Dukes' A tumour and one from a patient with a Dukes' B tumour, had CK20 mRNA but not CEA mRNA above cut-off. Both lymph nodes, however, were only marginally above the CK20 mRNA cut-off (Fig. 2*c*,*d*). These 2 patients did not have other lymph nodes positive for CK20 or CEA mRNA.

Two of the patients with tumours classified as Dukes' Stage B have died from CRC (Fig. 4). Both had elevated CEA and CK20 mRNA values in their lymph nodes. A third Dukes' B patient with highly elevated CEA and CK20 mRNA levels has developed distant metastases since operation. Lymph nodes from patients with cancer in Dukes' Stage D showed a dispersed pattern with regard to CEA and CK20 mRNA contents (Figs. 2,4). CEA mRNA levels were above 100 CEA mRNA copies/18S rRNA unit in cases where the primary tumour was classified as Dukes' Stage C locally. Primary tumours of those with lower CEA mRNA values in the highest lymph node were classified as either Dukes' Stage A or B locally, *i.e.*, with lymph nodes judged as normal in routine histopathology.

Seven patients with rectal tumours classified as Dukes' Stage A, B or C received preoperative irradiation. The CEA mRNA content in their lymph nodes with highest level ranged from 4×10^{-3} to 3 copies/18S rRNA unit. In this limited material we find no effect of preoperative irradiation on CEA mRNA levels in regional lymph nodes.

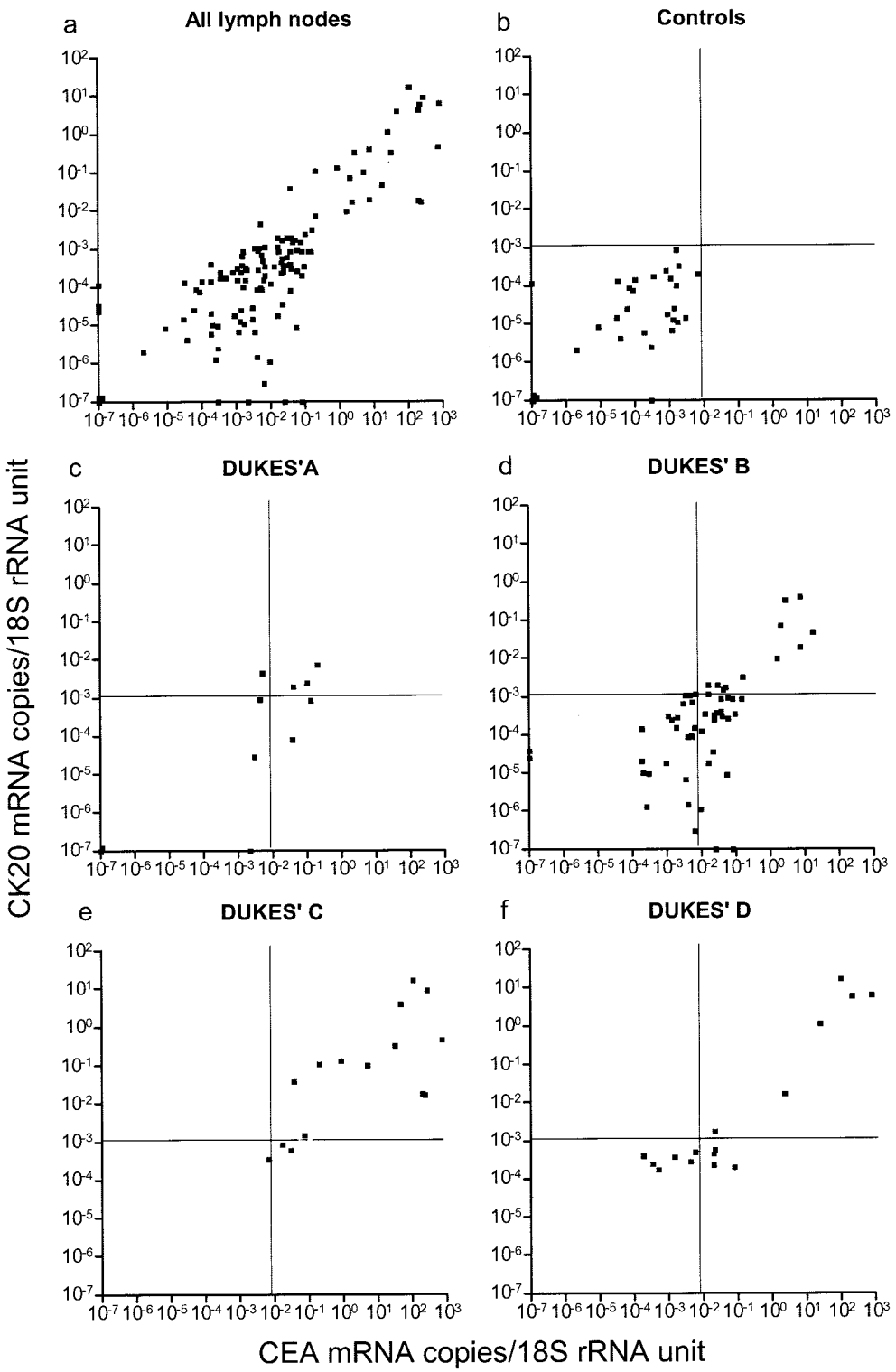

FIGURE 2 – Correlation between CEA and CK20 mRNA levels in lymph nodes collected from specimens removed by colorectal surgery from non-cancer patients (*b*), and CRC patients with tumours in Dukes' Stages A (*c*), B (*d*), C (*e*) and D (*f*), respectively. (*a*) The collected results from all lymph nodes analyzed. RNA was extracted and the concentrations of CEA and CK20 mRNA copies and of18S rRNA in the extract determined by real-time qRT-PCR. Values are normalized by calculating the CEA mRNA copies/18S rRNA unit and CK20 mRNA copies/18S rRNA unit ratios. Each dot represents one lymph node.

Real-time qRT-PCR is more sensitive than IHC for detection of CEA expressing cells in lymph nodes

A total of 96 lymph nodes from 42 CRC patients with tumours in Dukes' Stages A–D ($n = 75$) and 7 non-cancer patients ($n = 21$) were analyzed both for CEA mRNA levels and presence of cells expressing the CEA protein by IHC. Nine lymph nodes had overt metastases. All were positive in IHC (>25 CEA^+ cells/section) and had CEA mRNA levels above 5 copies/18S rRNA unit (Fig. 5*a*). Sixty-six lymph nodes from CRC patients were judged as negative for tumour infiltration by routine histopathology. Twenty-three of these nodes showed positive staining with anti-CEA mAb. All had low frequency of positively stained cells (1–25 CEA^+ cells/section; Fig. 5*a*). Interestingly, a large number of lymph nodes from CRC patients that were negative for infiltrating tumour cells both by routine histopathology and anti-CEA IHC had CEA mRNA levels above the cut-off level 10^{-2} copies/18S rRNA unit (Fig. 5*a*).

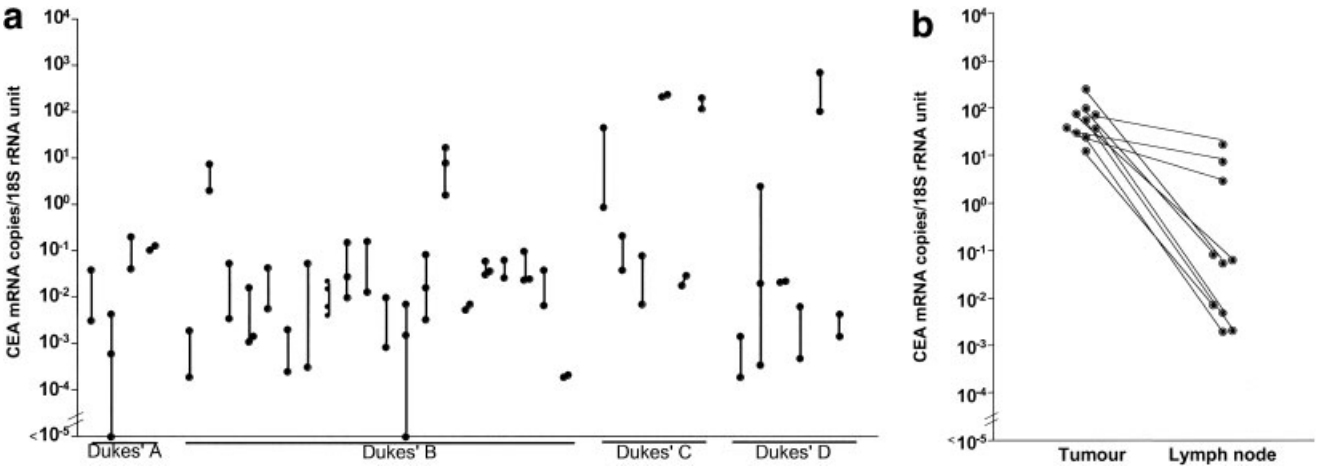

FIGURE 3 – (*a*) Comparison between CEA mRNA levels in different lymph nodes from the same patient. Results from patients from whom more than one lymph node was collected are shown. Individual lymph nodes are indicated by dots and lymph nodes from the same patient connected by vertical solid lines. Dukes' stage of the tumour is indicated along the X-axis. (*b*) Comparison between CEA mRNA levels in the original tumour tissue (tumour) and the lymph node with the highest CEA mRNA level (lymph node) of 10 CRC patients with tumours in Dukes' Stage B. Solid lines connect the corresponding tumour tissue and lymph node values in each individual.

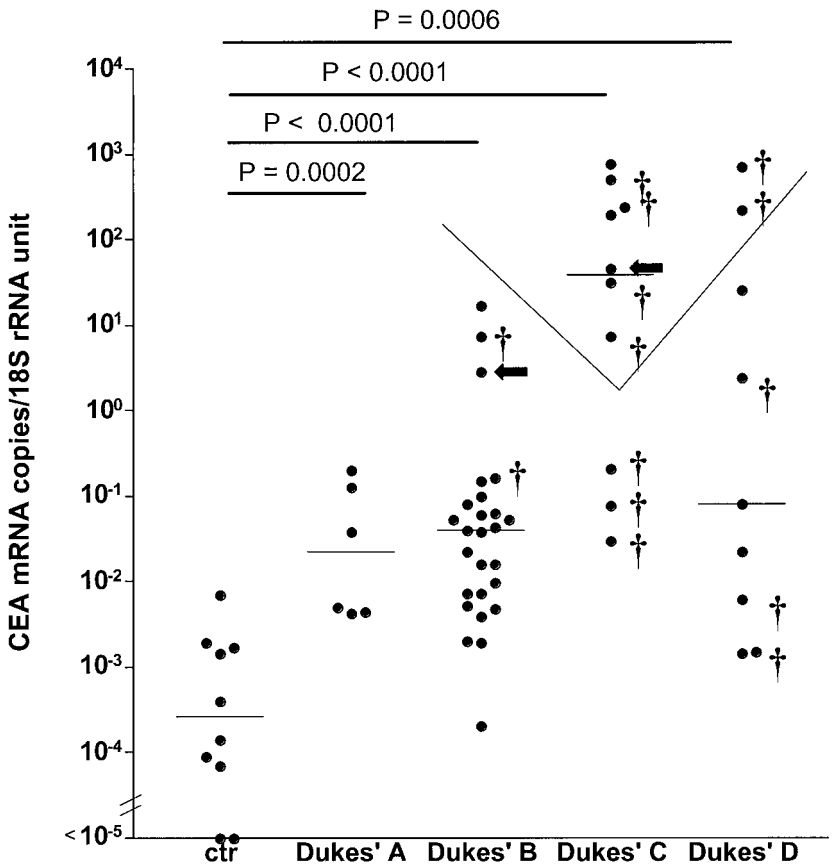

FIGURE 4 – Results of CEA mRNA levels in lymph nodes when each patient is represented by the lymph node with highest CEA mRNA level only. †indicates a patient who died from CRC. A fat arrow indicates a patient with tumour recurrence. Lymph nodes above the v-shaped line had tumour cells identified by routine H&E staining. Horizontal bars indicate medians; *p*-values from comparisons between CEA mRNA levels in lymph nodes from non-cancer patients and CEA mRNA levels in lymph nodes of CRC patient groups with tumours in Dukes' Stage A, B, C and D, respectively, are indicated.

CEA^+ cells were only detected in one of the lymph nodes from controls. This positive node had one CEA^+ cell (Group 1–5 CEA^+ cells in Fig. 5*a*) and was from the control patient, who had the highest CEA mRNA levels in lymph nodes. More than 5 CEA^+ cells/section was used as cut-off for positivity in anti-CEA IHC because all non-cancer patients were below this value. The frequency of CRC patients with lymph nodes positive for CEA expressing cells in IHC was lower than that of patients with lymph nodes positive for CEA mRNA by qRT-PCR (Table II). This difference was most pronounced in patients with tumours in Dukes' Stage B. Only 20% of the patients had lymph nodes positive in anti-CEA IHC and 65% had lymph nodes with CEA mRNA levels above cut-off ($p = 0.003$).

Serum CEA levels do not correlate to CEA mRNA levels in lymph nodes

CEA concentration was determined in preoperative serum collected from 36 CRC patients. No correlation was seen between the

TABLE II – COMPARISON BETWEEN DETECTION RATES OF CEA AND CK20 IN PATIENTS WITH CRC

Patient group	Regional lymph nodes[1]				Serum[2]
	CEA mRNA	CK20 mRNA	CEA and/or CK20 mRNA	CEA^+ cells	CEA protein
Dukes' A	3/6[3]	3/6[3]	4/6	0/4[4]	0/5[5]
Dukes' B	17/26	10/26	18/26	4/21	6/17
Dukes' C	10/10	9/10	10/10	7/10	5/7
Dukes' D	7/9	5/9	7/9	5/7	4/7

[1]One to four regional lymph nodes were collected at the time of surgery. RNA was extracted from half of each lymph node and levels of CEA mRNA, CK20 mRNA and 18S rRNA determined by real-time qRT-PCR. In most cases, the second half of the lymph node was assayed for CEA expressing cells by IHC.–[2]Serum was prepared from peripheral blood collected on the morning of the day for surgery and CEA concentrations determined by ELISA.–[3]Number of patients/total number of patients analyzed who had at least one lymph node with CEA mRNA or CK20 mRNA levels above cut-off. Cut-offs were set at 0.01 copies/18S rRNA unit and 0.001 copies/18S rRNA unit for CEA mRNA and CK20 mRNA, respectively.–[4]Number of patients/total number of patients analyzed who had at least one lymph node with > 5 CEA^+ cells/section.–[5]Number of patients/total number of patients analyzed who had CEA serum concentrations > 2.5 ng/ml.

serum CEA concentration and the CEA mRNA level in the lymph node with the highest level in the individual patient (Fig. 5*b*). Using a cut-off point of >2.5 CEA ng/ml serum[26] one-third of the patients with tumours in Dukes' Stage B were positive (Table II). This was intermediate to the frequency of patients with Dukes' B tumours that had CEA mRNA levels above cut-off in lymph nodes and those who had lymph nodes with CEA^+ cells in IHC (Table II). The 2 patients with Dukes' B tumours who died from CRC had slightly elevated CEA serum concentrations (4 and 6 ng/ml) and high CEA mRNA levels in their lymph nodes (compare Fig. 4).

DISCUSSION

The purpose of our study was to develop an objective, quantitative, specific and reproducible assay for the determination of disseminated tumor cells in lymph nodes from patients with CRC. This is an important clinical goal because success in treatment of CRC is dependent on correct classification of tumor stage. A number of studies have addressed the question of finding tumor cells in regional lymph nodes using various techniques such as increasing the number of lymph nodes for classical histologic examination[27] combined with serial sectioning,[28] IHC with tumor marker specific mAbs, extraction of tumor marker proteins and qualitative RT-PCR.[6,29–32] Although of value, each method has its particular limitation including quantitative aspects and objectivity. The first assay we developed was a real-time quantitative RT-PCR assay for CEA mRNA. We chose CEA as the tumor marker because it is a well established marker for CRC and the gene family is known in detail allowing the construction of a specific mRNA assay in which related mRNAs do not interfere notably with mRNAs expressed by immune cells. These mRNAs include CEACAM1, CEACAM3, CEACAM6 and CEACAM8.[33,34] Specificity studies with PBMC and granulocytes demonstrated that there was no interference. We believe the fact that we found very low level expression in a few PBMC samples reflects the presence of CEA mRNA presumably from occasional epithelial cells.[35] We also established a CK20 mRNA assay. Although no interference by PBMC was seen, we detected a weak but significant interference by granulocytes in line with the results of Neumaier *et al.*[36] This interference is probably negligible in the analysis of lymph nodes but may be a problem when searching for tumor cells in blood. There were two reasons for choosing to analyze at the mRNA level instead of at the protein level. First, mRNA is more likely to reflect the presence of living tumor cells than protein[37,38] and it has been shown that dendritic cells/macrophages can transport protein components from apoptotic epithelial cells to lymph nodes.[39,40] Second, the sensitivity of mRNA assays are much higher than protein assays. Real-time quantitative RT-PCR was chosen because the assay is objective, quantitative, reproducible and has a large measuring range. In the qRT-PCR assay used detection of the amplicon is achieved by a probe that specifically hybridizes with the amplicon. This is superior to assays in which the read-out is binding of a fluorescent dye to double stranded DNA (SYBR®-Green) or a primer that displays fluorescence after binding (Amplifluor fast gene system). The importance of using a fluorochrome-labeled internal probe that hybridizes to a sequence in the amplicon corresponding to the continuous sequence over an exon boundary in the mRNA instead of binding of a fluorescent primer was revealed by a recent study.[41] In that study, a qRT-PCR assay that lacks this specificity control and consequently does not allow discrimination between correct and illegitimate PCR products was used. Indeed, the reported sequence of the amplicon in PBMC[41] shows complete identity to an intron sequence 1.4 Mb distal to the CEA gene, strongly suggesting amplification of genomic DNA contaminating the RNA in the PBMC samples used by the authors (our unpublished results).

To appropriately deal with the sampling problem we have chosen to extract RNA from one half of the lymph node. The other half was used for routine histopathology. In the future, if qRT-PCR assays are adopted in clinical practice, the entire gland should be extracted. Several lymph nodes with high CEA mRNA levels were negative for CEA in IHC. The explanation for this discrepancy is most likely related to sampling. The probability of finding tumor cells in a tissue section is low (<1% of the lymph node volume) compared to detecting CEA mRNA in RNA extracted from half the lymph node volume.

In practice, there might sometimes be a problem of distinguishing lymph nodes from certain fat deposits. To make sure that lymph nodes are analyzed we check for presence of immune cell RNA by RT-PCR for CD45, a ubiquitous leukocyte antigen.

Another aspect of the sampling problem that is not addressed in our study is the problem of selecting the primary draining lymph nodes, where metastatic cells are most likely to be found. As seen in our study, occasionally there are large differences in mRNA levels of both markers between different nodes from the same patient. The evolving technique using the sentinel node concept can be utilized in identification of the appropriate lymph nodes thereby reducing the total workload.[42] Even though the risk of false negative sentinel nodes (skip lymph nodes)[43,44] is a major concern, the method is likely to improve the sampling procedure.

Using the qRT-PCR assay for CEA mRNA we find that lymph nodes from patients with IBD also gave positive signals. The mRNA levels were low, however, compared to those of CRC patients. The average CEA mRNA level in lymph nodes of control patients (median $= 8 \times 10^{-5}$ copies/18S rRNA unit) would correspond to ~1 tumour cell/10^7 immune cells as judged from experiments in which LS174T cells were admixed to PBMC. It was evident that we were most likely dealing with dislodged epithelial cells and not cross-reactivity with mRNA from immune cells when the samples were tested for the epithelial cell selective cytokeratin CK20 mRNA. CEA and CK20 mRNA correlated significantly, and all but one of the CEA mRNA positive lymph nodes contained detectable amounts of CK20 mRNA. Probably, there is a minor release of epithelial cells to the lymphatics also in benign conditions, particularly in ulcerative colitis, where changes in the colonic epithelium are prominent. Although CRC was not observed in the resected UC specimens at histopathologic examination, we cannot entirely exclude the detected dislodged cells in lymph nodes of UC patients. These cells may actually be cancer cells because there is an increased CRC risk in UC estimated to ~8% accumulated risk 20 years after diagnosis.[45,46] Using qRT-PCR it is possible to set a cut-off point for significant CEA and CK20 mRNA levels. Therefore, occasional low CEA and CK20 mRNA levels in controls is not an obstacle in estimating tumour cell dissemination.

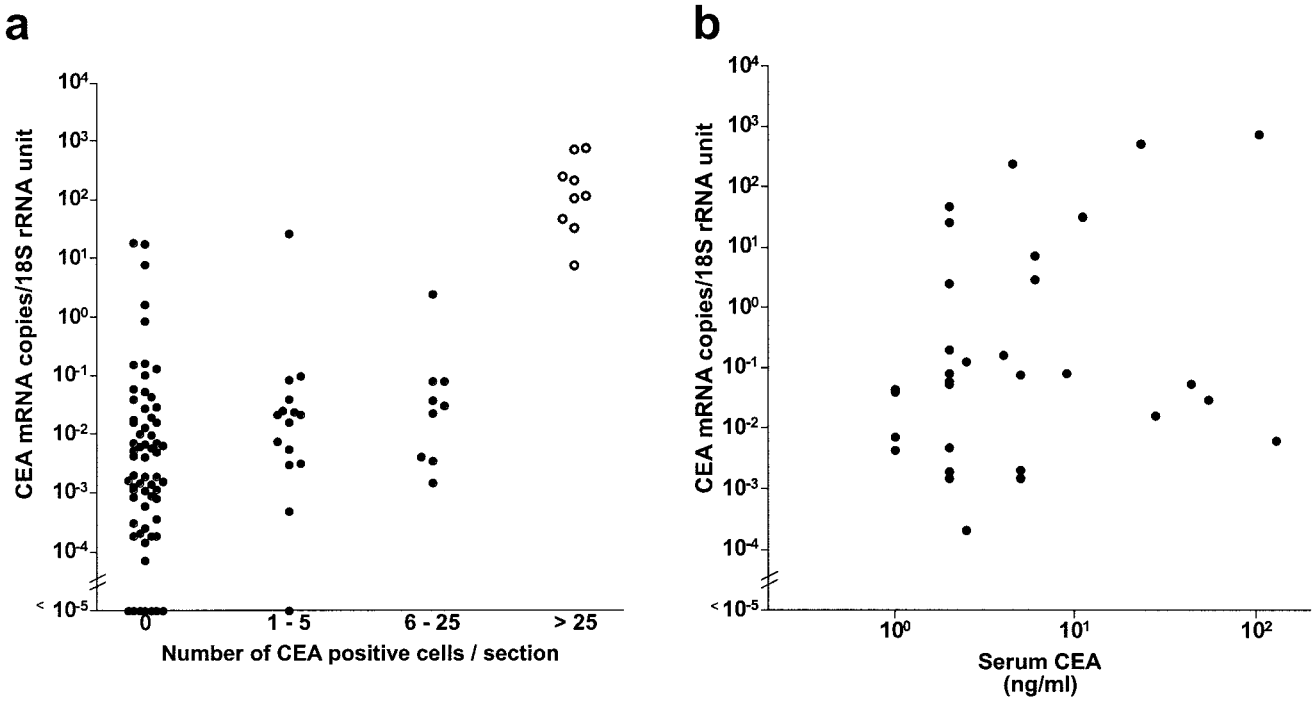

FIGURE 5 – (*a*) CEA mRNA levels and frequencies of cells expressing the CEA protein in lymph nodes of CRC patients and controls including results from analyses of both CRC and non-cancer patients. CEA mRNA levels were determined in half of the lymph node. One section from the other half of the lymph node was stained with the CEA specific mAb II-7 in IHC and inspected for positively stained cells. Sections with positively stained cells were divided into 3 groups: sections with single or few scattered CEA^+ cells (1–5); sections with one or several small aggregates of CEA^+ cells (6–25); and sections with CEA^+ cells in larger aggregates (>25). Open circles indicate lymph nodes with tumour cells identified by routine H&E staining and filled circles lymph nodes in which no tumour cells were detected by this method. (*b*) Comparison between CEA mRNA levels in lymph nodes and concentration of CEA protein in preoperative serum of CRC patients with tumours in Dukes' Stage A–D. Serum was collected in the morning the same day as the surgical procedure and CEA concentration was determined by ELISA. The CEA serum concentration is plotted against the value of the lymph node with the highest CEA mRNA level.

Regardless of the tumour stage, the majority of patients with CRC had at least one lymph node with higher CEA mRNA level than controls. The CEA levels in the group of patients with Dukes' Stage B tumours did not show a Gaussian distribution, suggesting heterogeneity in lymph node involvement. Thus, patients with tumours in Dukes' Stages A and B showing the highest levels of CEA mRNA may constitute the patients at risk of tumour recurrences. Two of the patients with high CEA mRNA level and Dukes' B tumours have died from cancer and a third patient with high levels has developed distant metastases after operation. All 3 patients also had elevated levels of CK20 mRNA.

The picture was somewhat different for CK20 mRNA. Although all but one CRC patient with tumours in Dukes' Stage C had lymph nodes with CK20 mRNA levels above controls, only 40–50% of the patients with tumours in Dukes' Stage A, B, and D had elevated levels. The majority of lymph nodes from CRC patients had more than a 10-fold lower level of CK20 mRNA compared to CEA mRNA suggesting a lower expression level of CK20 mRNA in the tumour cells. This could partly explain the lower sensitivity using CK20 mRNA as a marker for disseminated tumour cells. Lymph nodes containing elevated CK20 mRNA levels, but not CEA mRNA levels, were only detected in 2 patients. The CK20 mRNA levels were only marginally above those of lymph nodes in controls. The 2 patients did not have other lymph nodes with CEA mRNA levels above cut-off. There is a possibility that they have unusual tumours with low CEA levels and normal CK20 levels. Blood and lymph node samples from CRC patients giving signals for CK20 but not CEA mRNA have been reported[47,48] indicating the existence of such tumours. The atypical colon carcinoma cell line HT29 was found to have higher CK20 than CEA mRNA levels. Furthermore, the CEA mRNA levels were very low compared to the cell line LS174T.[49] These results are compatible with the possibility that HT29 originates from such a tumour. A larger patient material has to be analyzed to evaluate whether analysis also of CK20 mRNA would be useful to increase sensitivity for disseminated tumour cells by allowing detection of cells originating from tumours with low CEA expression.

The large differences in CEA mRNA and CK20 mRNA values between nodes from patients of the same Dukes' stage (particularly Dukes' B and D) deserve some comments. Apart from the problem of finding the draining nodes, it is known that CEA and CK20 expression levels are related to the degree of cellular differentiation.[37,50] For CEA mRNA the levels differed approximately 20 times between individual primary tumours. For CK20 mRNA this difference was actually larger (~50 times) mainly due to an exceptionally low value in one primary tumour. The explanation for the low values found in lymph nodes from some Dukes' D patients is puzzling. Possibly these tumours have spread through other pathways, either directly to the blood or have local advanced disease without liver or lymph node involvement.[51]

Using qualitative RT-RCR for CEA mRNA Liefers *et al.*[52] analyzed lymph nodes from 26 Stage II CRC patients. The cancer-specific survival rate was 91% in node negative patients compared to 50% for patients positive for CEA mRNA in their lymph nodes. Similarly, presence of CK20 mRNA in regional lymph nodes was reported as a prognostic factor for overall survival in colorectal cancer patients.[53,54] Our finding of 3 Dukes' B patients who had high CEA mRNA and CK20 mRNA levels and who later developed metastatic CRC are in line with these findings.

Having established 2 reliable quantitative and specific assays for disseminated tumour cells of colonic origin it will now be of

utmost importance to determine the clinically most relevant cut-off levels. It is likely that a certain minimum level of tumour cell dissemination to the lymph node is needed before a poor prognosis due to spread of disease prevails. A large prospective study with long follow-up time is needed to settle this question.

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