Clinical Significance of Cytokeratin 20-Positive Circulating Tumor Cells Detected by a Refined Immunomagnetic Enrichment Assay in Colorectal Cancer Patients

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Abstract

Purpose: Current immunomagnetic enrichment method can only detect general epithelial antigens of circulating tumor cells (CTC). Further characterization of the CTCs to provide specific information on the tumor type is not possible. We attempted to overcome this drawback by developing the methodology for using a gastrointestinal-specific anti-cytokeratin (CK) 20 antibody to detect CTCs in colorectal cancer patients' blood.

Experimental Design: The protocol was validated using a colorectal cancer SW480 cell line. The clinical significance of findings in colorectal cancer was investigated by detecting CK20-positive CTCs (pCTC) in patients with colorectal cancer, other common cancers, colorectal adenoma, benign colorectal diseases, and normal subjects. Moreover, the malignant nature of CK20 pCTCs was examined by comparing chromosome 17 aberration patterns with those from the corresponding primary tumors.

Results: The assay successfully showed CK20-positive SW480 cells. When applied in patient samples, the detection rates were 62% (132 colorectal cancer patients; median number = 11 CTCs), 0% (120 patients with other common cancers), 6% (50 colorectal adenoma patients), 0% (120 patients with benign colorectal diseases), and 0% (40 normal subjects). Furthermore, statistical analysis showed that CK20 pCTC numbers were associated with tumor-node-metastasis stage and lymph node status. Using the median CK20 pCTC numbers as the cutoff points, stratified groups of colorectal cancer patients had significant differences in their recurrence, metastasis, and survival. Finally, chromosome 17 aneusomy in 90% of colorectal cancer patients with CK20 pCTCs matched with those from the primary tumors.

Conclusions: Detection of CK20 pCTCs using the new protocol could generate clinically important information for colorectal cancer patients.

The identification of circulating tumor cells (CTC) can be used to detect malignancy, predict metastasis, evaluate prognosis, assist in the management of cancer patients, and monitor recurrence and metastasis after primary therapy (1–4). Approaches to detect CTCs can be classified into molecular-based methods, which detect target mRNA expression, and cytometric methods, which isolate and quantify individual cells (1). Although researchers have reached an important consensus that cytopathologic examination of CTCs after immunomagnetic enrichment with further characterization of their malignant potential represents a promising approach (5–7), the current immunomagnetic enrichment method uses either a broad-spectrum anti-cytokeratin (CK) antibody (Miltenyi Biotec), combined anti-CK8, anti-CK18, and anti-CK19 antibodies (CellSearch System; Veridex), or an anti-$BerEP_4$ antibody (Dynal Biotech, Invitrogen) against general epithelial antigens of various tumor and normal cells. Therefore, specific information on the primary tumor type is not available. We hypothesize that one would overcome this limitation by blocking the Fc region of the anti-$BerEP_4$ antibody with a goat anti-mouse antibody during immunomagnetic enrichment, so that an anti-CK20 antibody can be used to show the gastrointestinal origin of the $BerEP_4$-positive cells in the blood of colorectal cancer patients. This modification can improve immunomagnetic CTC detection by allowing tumor- or tissue-specific antibodies to bind to their respective antigens, so that an accurate diagnosis of the tumor type can be made. CK20 is a more specific marker than CK, CK8, CK18, CK19, and $BerEP_4$

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Translational Relevance

Our work has shown that the number of preoperation cytokeratin (CK) 20-positive circulating tumor cells (pCTC) is correlated with tumor-node-metastasis stage and lymph node status in colorectal cancer patients. Besides, using the median CK20 pCTC numbers in the selective patient groups as the cutoff points, preoperation CK20 pCTCs correlated with recurrence, metastatic disease, and overall survival in colorectal cancer patients. Therefore, detection of preoperation CK20 pCTCs may be useful as a noninvasive prognostic factor to predict the biological behavior of colorectal cancer. Moreover, preoperation CK20 pCTC levels may be used to stratify the colorectal cancer patients into poor and good prognosis groups, which may in turn affect the patients' treatment strategies. In general, the discoveries of CK20 pCTCs in patients with colorectal cancer and colorectal adenoma but not in normal subjects show that this noninvasive test has high discriminatory value for colorectal cancer detection. Finally, the success of this refined immunomagnetic enrichment assay has opened up new possibilities in CTC detection because CTC shed from various cancers may be further characterized after immunomagnetic enrichment with their respective tumor markers using immunocytochemical staining, *in situ* hybridization, or even molecular profiling using quantum dot technology.

because the former antigen is expressed mainly in tumor and normal cells from the gastrointestinal tract, whereas the latter antigens are expressed in virtually all carcinomas and all nonneoplastic epithelial cells (8, 9). Therefore, we expect that CK20-positive CTCs (pCTC) may more accurately reflect the colorectal cancer patients' micrometastatic condition. In this study, the concept of this refinement was validated in a model using the human colorectal cancer SW480 cell line. Furthermore, this refined assay was used to evaluate the clinical significance of CK20 pCTCs in colorectal cancer by detecting such cells in patients with colorectal cancer, other common cancers, colorectal adenoma, benign colorectal diseases. We further correlated CK20 pCTC numbers to the clinicohistopathologic conditions in colorectal cancer patients. In addition, the malignant nature of CK20 pCTCs in colorectal cancer patients was investigated by comparing the pattern of chromosome 17 aberrations with those in the corresponding primary tumors using fluorescence *in situ* hybridization (FISH) because chromosome 17 gain is common in colorectal cancer (10, 11). Finally, the potential of CK20 pCTCs in predicting the effectiveness of 5-fluorouracil (5-FU)-based chemotherapy was explored by comparing the CK20 pCTC numbers in colorectal cancer patients before 5-FU-based therapy and 24 months after treatment. The information obtained in this study would be very useful for understanding the prognostic and diagnostic potential of CK20 pCTCs in colorectal cancer patients.

Materials and Methods

Validation of the refined immunomagnetic enrichment assay and recovery experiments in SW480 cells. The refined protocol was compared with the conventional protocol for SW480 cells capture and detection. In brief, three cell suspensions each of 3,000 SW480 cells in 1 mL PBS were first incubated with Dynabeads (CELLection Epithelial Enrich, Dynal Biotech, Dynal) for 1 h at 4°C. One cell suspension captured by the conventional protocol and a cytospin spot was prepared for immunocytochemical staining according to the manufacturer's instructions (CELLection Epithelial Enrich, Dynal Biotech, Dynal). For those captured by the refined protocol, a modification was done by incubating the cell suspensions with a goat anti-mouse antibody (1:50; DakoCytomation) for another hour at 4°C after incubation with Dynabeads. All other procedures were the same as those in the conventional protocol. The aim of using the goat anti-mouse antibody was to block the immunomagnetic bead-linked mouse anti-$BerEP_4$ antibody at the Fc region to prevent the mouse anti-$BerEP_4$ antibody from being linked to the secondary rabbit anti-mouse antibody during immunocytochemical staining. To verify that the blocking of the mouse anti-$BerEP_4$ antibody was successful in this refined protocol, one captured cytospin spot was prepared for immunocytochemical staining as in the conventional protocol, whereas another cytospin spot was incubated with an anti-CK20 antibody (1:10; clone $KB_{sB}20.8$; DakoCytomation) before immunocytochemical staining to detect CK20-positive $BerEP_4$ captured cells (Fig. 1). All immunocytochemical stainings were done inside an automatic Ventana Benchmark XT immunostainer using an ultraView universal diaminobenzidine tetrahydrochloride detection kit (Ventana Medical Systems).

SW480 cells were serially diluted 10-fold into blood from healthy volunteers. The final concentrations of SW480 cells were 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 1/10 mL blood, respectively, and they were captured using the same refined protocol as above in triplicates for CK20 immunocytochemical staining. The number of CK20-positive SW480 cells for each concentration was counted under the microscope.

Patients' blood samples. Between October 2003 and February 2007, blood samples were taken from five cohorts of patients. In the first cohort, blood samples from 132 colorectal cancer patients and 50 colorectal adenoma patients were taken at two time points: (*a*) before operation [stages I-III colorectal cancer patients based on tumor-node-metastasis (TNM) classification], before therapeutic intervention (TNM stage IV colorectal cancer patients), and before endoscopy (colorectal adenoma patients) and (*b*) on first follow-up (5 days after operation for colorectal cancer patients and 7 days after endoscopy for colorectal adenoma patients). Moreover, 62 of 101 TNM stage I to III colorectal cancer patients were followed up for 24 months from their respective diagnosis for recurrent or metastatic colorectal cancer as the remaining 39 patients did not have sufficient follow-up period. Finally, overall survival curve of 83 TNM stage I to IV colorectal cancer patients were plotted using the median number of preoperation CK20 pCTC as the cutoff point. In the second cohort, blood samples from 30 patients each with colitis, hemorrhoids, colorectal ulcers, and hyperplastic polyps were taken before surgical treatment. In the third cohort, blood samples from 30 patients each with breast cancer, prostate cancer, liver cancer, and lung cancer were taken before operation or treatment. In the fourth cohort, blood samples from another 20 colorectal cancer patients were taken before operation to study both the preoperation CK20 pCTCs and the pattern of chromosome 17 aberrations, which were compared with those in the corresponding primary tumors using FISH. In the fifth cohort, blood samples were collected from 15 TNM stage III colorectal cancer patients before 5-FU-based chemotherapy and 24 months after treatment. Finally, 40 normal subjects were also recruited for comparison. Although the normal subjects did not undergo colonoscopy to confirm their status, their plasma samples had been tested for carcinoembryonic antigen protein and all were within the normal range. Informed consent was obtained from all patients and healthy individuals. The clinicopathologic characteristics of the studied subjects were shown in Supplementary Table S1. The study was approved by the Clinical Research Ethnics Committee of the Prince of Wales Hospital and Queen Elizabeth Hospital.

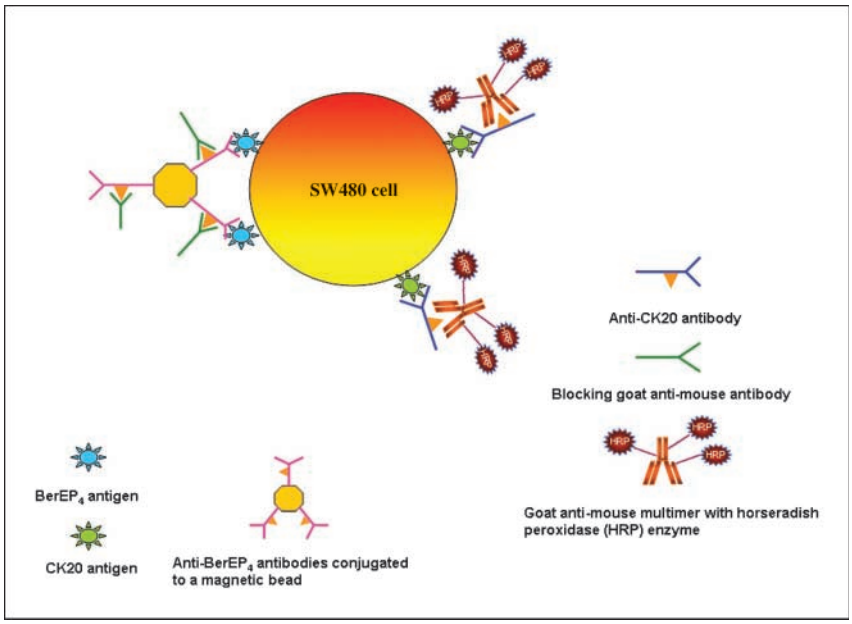

Fig. 1. Principle of the refined immunomagnetic enrichment assay for the capture and detection of CK20-positive SW480 cells.

Refined immunomagnetic enrichment assay, immunocytochemical staining, and examination of CK20 pCTCs in blood samples. Ten milliliters of blood from each sample were collected in EDTA tubes. The mononuclear cells were collected by centrifugation through a Ficoll density gradient (GE Healthcare) according to the manufacturer's instructions. The CTCs were isolated from the mononuclear cells using the refined protocol as in the SW480 cells, and CK20 immunocytochemical staining was done for each patient sample. The criteria used to identify a CK20 pCTCs in a blood sample were (*a*) positive CK20 staining, (*b*) the cell should have a round-to-oval morphology, and (*c*) the cell size was at least double that of a lymphocyte. The CK20 pCTCs were examined and quantified by two independent assessors (S.C.C. Wong and A.K.C. Chan), and an average cell number was calculated for each patient sample.

FISH for chromosome 17 aneusomy. The cytospin slides prepared from blood samples were fixed in 10% formalin in 95% alcohol for 15 min at room temperature before retrieval in spotlight cell treatment solution (Zymed, Invitrogen) for 13 min at 37°C. A chromosome 17 spectrum green DNA probe (CEP 17; Vysis, Abbott Molecular) was applied to each cytospin slide and incubated for 8 min at 75°C for codenaturation of probe and chromosomal DNA. Hybridization was carried out for 48 h in a humidified chamber at 37°C. Posthybridization wash was done at 73°C for 2 min with 2× SSC followed by a second wash at room temperature for 2 min. Slides were then counterstained and mounted with 4′,6-diamidino-2-phenylindole before being examined for chromosome 17-positive signals using a fluorescence microscope (×100 oil immersion objective; Nikon Eclipse E600). Paraffin-embedded sections were done with the same procedures as above, except that they were retrieved with the paraffin pretreatment reagent kit II (Vysis, Abbott Molecular). The interpretative criteria were (*a*) 2 signals indicated that the cell was disomic, (*b*) >2 signals indicated that the cell showed chromosome gain, and (*c*) <2 signals were not considered as aneusomy in an individual cell because it might be caused by hybridization inefficiency or miscounting and the percentage of well-preserved normal cells with <2 signals should be <10%. The stained slides (all areas for blood samples and 10 areas for paraffin-embedded sections under ×1,000 magnification) were evaluated by two independent observers (S.C.C. Wong and E.S.F. Lo) and an average value for the number of CTCs with aneusomy was calculated for each patient sample. Leukocytes from normal blood donors served as hybridization controls, whereas those from patients served as an internal control for each sample. In paraffin sections, adjacent normal epithelial cells served as an internal control.

Statistical analysis. χ^2 test was used to examine the association between preoperation CK20 pCTCs and recurrent or metastatic colorectal cancer. Besides, multivariate regression was used to analyze whether preoperation CK20 pCTC was correlated with the clinicohistopathologic factors of the patients and Cox proportional hazards model was applied to detect the independent prognostic factors of survival. (Statistical Package for the Social Sciences version 12.0 software; SPSS). Finally, log-rank test was used to examine whether the overall survival of selected patient groups stratified by the median of preoperation CK20 pCTCs had significant difference (GraphPad Prism software version 4.0; GraphPad Software). $P < 0.05$ was considered to be statistically significant.

Results

Validation of the refined immunomagnetic enrichment assay and recovery experiments. $BerEP_4$-positive SW480 cells were detected using the conventional protocol (Fig. 2A). However, $BerEP_4$-positive staining in SW480 cells was no longer observed after using the refined protocol (Fig. 2B). Addition of anti-CK20 antibody on cells captured by the refined protocol showed CK20-positive SW480 cells (Fig. 2C). In recovery experiments, proportional amount of CK20-positive SW480 cells was found in each concentration of spiked cells for every trial, except in those with 1 SW480 cell/10 mL blood, which the cell was detected once in triplicate tests. The mean CK20-positive SW480 cell recovery was 51% with a range of 35% to 68%.

CK20 pCTCs in patients with colorectal cancer, colorectal adenoma, and normal subjects. In general, CK20 pCTC cannot be found in any of the 40 normal subjects (Fig. 3A); therefore, the baseline was set at 0 CTC and detection of ≥1 CTC/10 mL blood was considered to be positive. Detailed analysis showed that the overall detection rates in colorectal cancer patients, colorectal adenoma patients, and normal subjects were 62% (82 of 132; range, 0-306), 6% (3 of 50; range, 0-10), and 0% (0 of 40), respectively (Fig. 3A). When we divided the colorectal

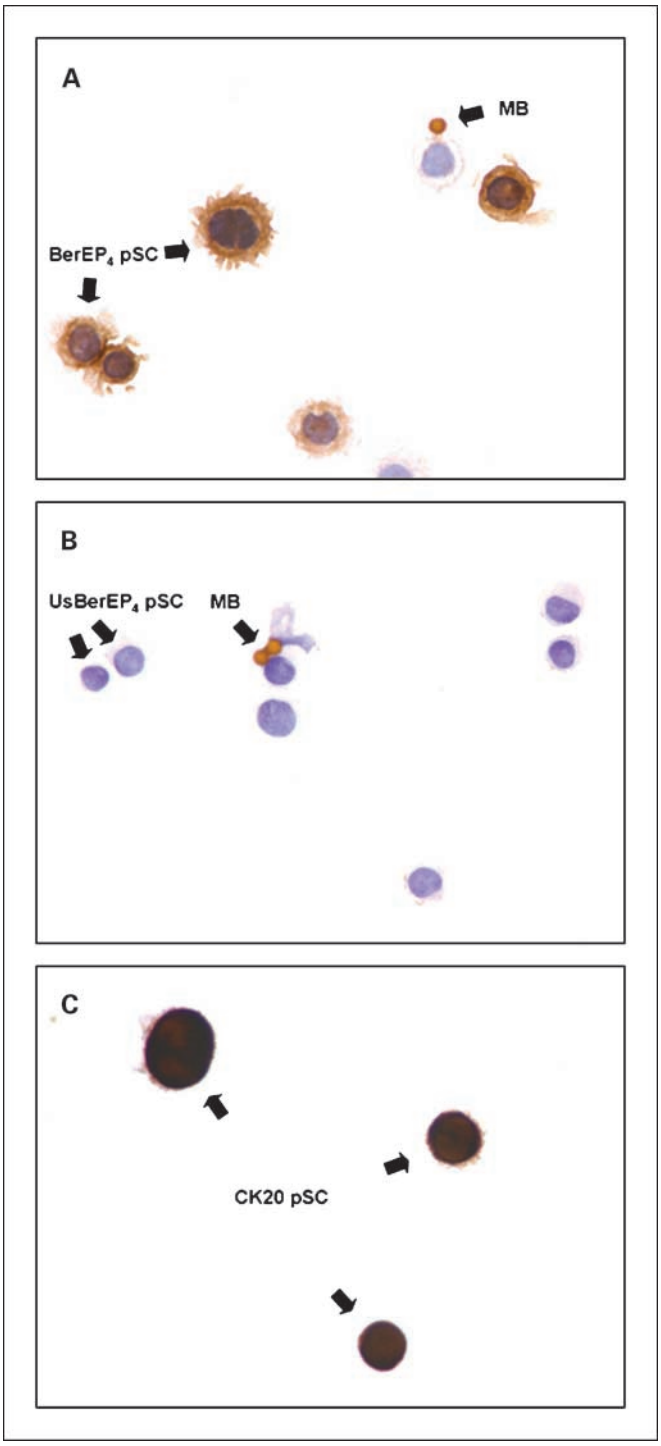

Fig. 2. Validation of the refined immunomagnetic enrichment assay. *A,* positive $BerEP_4$ staining in SW480 cells using the conventional immunomagnetic enrichment assay. *B,* negative $BerEP_4$ staining in SW480 cells using the refined immunomagnetic enrichment assay. *C,* positive CK20 staining in SW480 cells using the refined immunomagnetic enrichment assay. *$BerEP_4$ pSC,* $BerEP_4$-positive SW480 cells; *MB,* magnetic bead(s); *Us$BerEP_4$ pSC,* unstained $BerEP_4$-positive SW480 cells; *CK20 pSC,* CK20-positive SW480 cells. Original magnification, ×400.

cancer patients into different TNM stages, the detection rates were 39% (11 of 28; stage I), 55% (18 of 33; stage II), 73% (29 of 40; stage III), and 77% (24 of 31; stage IV; Fig. 3B). On their first follow-up, only 101 colorectal cancer patients (stage I-III) were recruited because 31 patients with stage IV did not have operation. Among them, 58 (57%) of 101 patients had detectable preoperation CK20 pCTCs and 51 (88%) of 58 patients were found to have a decreased number of postoperation CK20 pCTCs, whereas 7 (12%) of 58 patients had slightly increased number of postoperation CK20 pCTCs (Fig. 3C). In contrast, only 3 colorectal adenoma patients had a detectable pre-endoscopy CK20 pCTCs and all patients did not have any CK20 pCTC found after endoscopy (Fig. 3D). A typical CK20 pCTC from a colorectal cancer patient was shown in Fig. 4.

Multivariate regression analysis. Multivariate regression analysis was applied to examine whether preoperation CK20 pCTCs was correlated with the clinicohistopathologic factors of the patients. Significant associations were found with TNM stage ($P < 0.001$) and lymph node status ($P < 0.01$) but not for age ($P = 0.836$), sex ($P = 0.759$), tumor stage ($P = 0.471$), and degree of differentiation ($P = 0.284$).

Recurrent or metastatic colorectal cancer. The median number of preoperation CK20 pCTCs from the 101 TNM stage I to III colorectal cancer patients was 8. Using this median number as the cutoff point, 33 and 29 of the colorectal cancer patients had preoperation CK20 pCTCs >8 and ≤8, respectively. Among them, 17 patients with preoperation CK20 pCTCs >8 and only 5 patients with preoperation CK20 pCTCs ≤8 had recurrent or metastatic colorectal cancer after follow-up for 24 months and the association between preoperation CK20 pCTCs and recurrent or metastatic colorectal cancer was highly significant ($\chi^2 = 7.95$; $P < 0.001$).

Survival of colorectal cancer patients. Kaplan-Meier overall survival curves were plotted for 40 patients with preoperation CK20 pCTCs >11 and 43 patients with preoperation CK20 pCTCs ≤11, where 11 is the median number of preoperation CK20 pCTCs from the first cohort of 132 colorectal cancer patients. Our results showed that the survival for these two groups of patients was significantly different ($P < 0.0001$, log-rank test; Fig. 5). Moreover, the independent prognostic factors of survival identified by the Cox proportional hazards regression model were found to be preoperation CK20 pCTCs ($P = 0.005$) and lymph node status ($P = 0.041$; Supplementary Table S2).

CK20 pCTCs in patients with benign colorectal diseases and other common cancers. No pretreatment CK20 pCTC can be detected in patients with benign colorectal diseases (colitis, hemorrhoids, colorectal ulcers, and hyperplastic polyps) and other common cancers (breast cancer, prostate cancer, liver cancer, and lung cancer).

Chromosome 17 aneusomy in CK20 pCTC colorectal cancer patients. Twelve (60%) of 20 patients with detectable preoperation CK20 pCTCs had chromosome 17 gains, whereas the remaining 8 (40%) of 20 patients had a chromosome 17 diploid pattern. Moreover, the chromosome 17 FISH signals in CK20 pCTCs were consistent with those from the primary tumors in 18 (90%) patients. Discordance was observed in 2 patients in whom chromosome 17 gains were found in 8% and 12% of the tumor cells, whereas the blood samples had only a disomic pattern (Supplementary Table S3). Typical chromosome 17 gains detected by FISH in the blood and primary tumor samples from the same patient were shown in Fig. 6A and B. Leukocytes in the blood samples and normal colorectal epithelium in the paraffin-embedded tumor tissues showed disomic pattern.

Prediction for the effectiveness of 5-FU-based chemotherapy in 15 TNM stage III colorectal cancer patients. Three definite trends were observed based on examining CK20 pCTC numbers before 5-FU-based chemotherapy and 24 months after treatment: 8 patients showed an increase in CK20 pCTC numbers with 5 (62.5%) patients experienced relapse; 3 patients showed an insignificant change in CK20 pCTC numbers with 1 (33.3%) patient experienced relapse; and 4 patients showed a decrease in CK20 pCTC numbers with 1 (25%) patient experienced relapse.

Discussion

Although the clinical significance of CTCs from patients with tumors is still debatable (12, 13), the technology platform has improved rapidly. Over the last few years, CTC detection has become more standardized and reliable (14, 15). A typical example is the detection of CTCs with the CellSearch System, which allows the defined stratification of the risk of death in metastatic breast cancer patients (6). However, the anti-CK antibody panel (CK8, CK18, and CK19) in this system is not specific in tumor typing. Therefore, we hypothesized that the detection of a specific marker in CTCs with quantification might be helpful in the prognosis and diagnosis of colorectal cancer patients.

The success of the refined immunomagnetic enrichment assay was confirmed in the SW480 cell line model, which has opened up a new scenario in CTC detection. Although the detection of 1 spiked SW480 cell/10 mL blood in the recovery experiment was not consistent, that finding is expected as the refined assay would be more reliable in detecting a higher amount than a smaller amount of target cells. This limitation may lead to false-negative results when the number of CK20 pCTCs/10 mL blood sample is only 1. Although the clinical significance of 1 CTC is currently not known, the proportion of false-negative findings from this refined assay would be explored in future. On the other hand, the main advantage of this refined assay is that it allows the enumeration and analysis

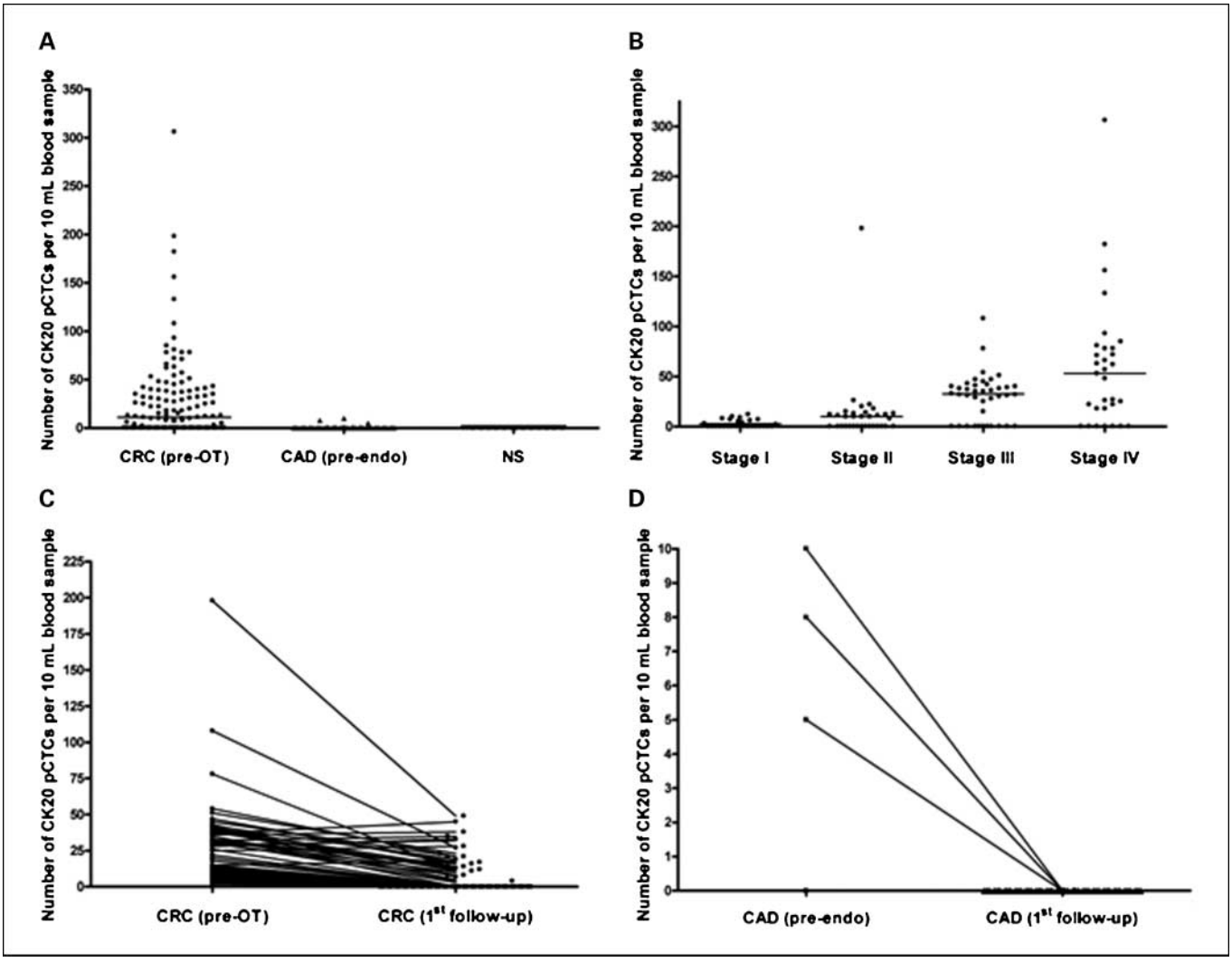

Fig. 3. CK20 pCTCs in blood samples. *A,* number of CK20 pCTCs/10 mL blood sample in 132 colorectal cancer patients (*pre-OT*), 50 colorectal adenoma patients (*pre-endo*), and 40 normal subjects. *B,* number of CK20 pCTCs/10 mL blood sample in 28 stage I, 33 stage II, 40 stage III, and 31 stage IV colorectal cancer patients. *C,* number of CK20 pCTCs/10 mL blood sample in 101 colorectal cancer patients (pre-OT and first follow-up) with interconnecting lines between the two time points. *D,* number of CK20 pCTCs/10 mL blood sample in 50 colorectal adenoma patients (pre-endoscopy and first follow-up) with interconnecting lines between the two time points. *OT,* operation; *pre-endo,* pre-endoscopy. *Black horizontal line,* median in each group of subjects (*A* and *B*).

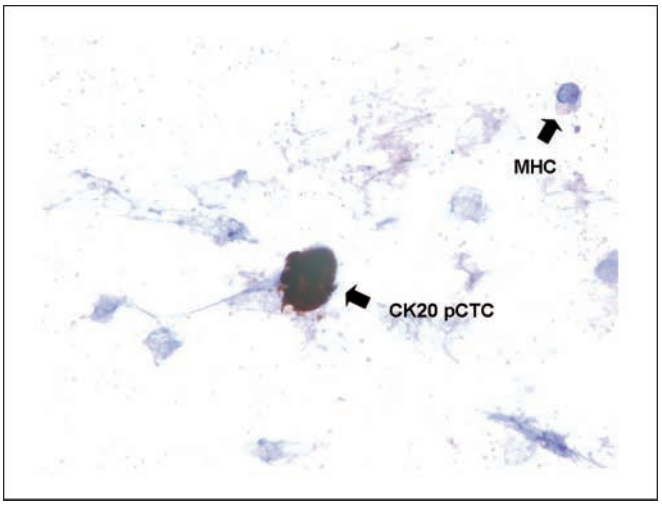

Fig. 4. Typical CK20 pCTC from a colorectal cancer patient sample. *MHC,* mononuclear hematopoietic cell. Original magnification, ×400.

of gastrointestinal-specific CK20 pCTCs that can offer several advantages over a general epithelial antigen $BerEP_4$ pCTCs. First, a detailed understanding of the micrometastatic process in colorectal cancer patients can be provided. Second, a thorough investigation to the patients especially in the gastrointestinal tract can be implemented. Third, false-positive results due to various kinds of benign epithelial cells can be greatly reduced. All these advantages have justified an intensive investigation in the significance of CK20 pCTCs in colorectal cancer patients with four objectives.

The first objective is to evaluate (*a*) the sensitivity of preoperation CK20 pCTCs in colorectal cancer and colorectal adenoma detection, (*b*) the origin of those CK20 pCTCs, and (*c*) the prognostic potential of preoperation CK20 pCTCs. The results showed that the overall detection rate in colorectal cancer is only 62% and detailed analysis indicated that the detection rates are higher for stages III and IV colorectal cancer, whereas those are lower for stages I and II colorectal cancer. This is logical because the dissemination of tumor cells into blood is a micrometastatic process, which has a higher metastatic potential in stages III and IV tumors than stages I and II tumors (16). Moreover, our observation, which is supported by a previous report, showed that the CTCs are the metastatic precursors with an increased malignant potential when compared with the parental cells in the primary tumor (17). On the other hand, pre-endoscopy CK20 pCTCs can only be found in 3 colorectal adenoma patients with severe dysplasia and this low percentage (6%) is reasonable because colorectal adenoma is a premalignant lesion. In summary, our results suggest that the presence of CK20 pCTCs may be a late event in colorectal carcinogenesis. Five days after operation, CK20 pCTC numbers were found to be decreased in 76% (22 of 29) stage III, 100% (18 of 18) stage II, and 100% (11 of 11) stage I colorectal cancer patients. This evidence can verify that the origin of those preoperation CK20 pCTCs is the primary tumor. Another conclusion that can be drawn from these results is that early detection and operation would reduce those CK20 pCTCs and might diminish the risk of metastasis to other distant organs. On the other hand, 7 stage III patients who had a slightly increased CK20 pCTC numbers after operation would be monitored to examine whether the postoperation CK20 pCTCs would lead to relapse in future. The detection rate of CK20 pCTCs in various TNM stages of colorectal cancer patients using this refined assay (62%) was higher than that using CellSearch System in colorectal cancer patients by Sastre et al. (36.2%; ref. 18). This discrepancy can be explained by the fact that Sastre et al. performed blood collection after operation in TNM stage I to III colorectal cancer patients that most of them had partial or complete clearance of CTCs (18). In fact, the percentage of CK20 pCTC detection in our cohort of colorectal cancer patients at their first follow-up after operation was only 28.7% (29 of 101). Another reason that can explain this difference in all patients is that Sastre et al. used ≥2 CTCs (18) rather than ≥1 CTC in this study as the cutoff point for positivity. In colorectal adenoma, 3 severe dysplasia patients who had 5, 8, and 10 CK20 pCTCs, respectively, before endoscopy did not have any CK20 pCTC found after endoscopy suggest that the origin of those CK20 pCTCs is the adenoma lesion. Our results support previous reports that some colorectal adenoma tissue specimens already have cancer cell clones with unfavorable histology (19–21). Therefore, early removal can prevent them from changing into a malignant lesion later. Finally, the fact that CK20 pCTC cannot be found

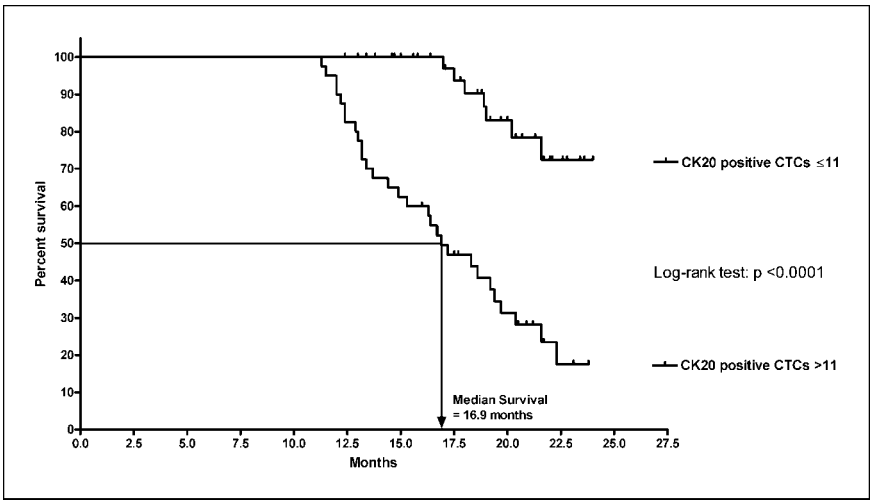

Fig. 5. Kaplan-Meier overall survival curve for 43 colorectal cancer patients with CK20 pCTCs ≤11 and 40 colorectal cancer patients with CK20 pCTCs >11.

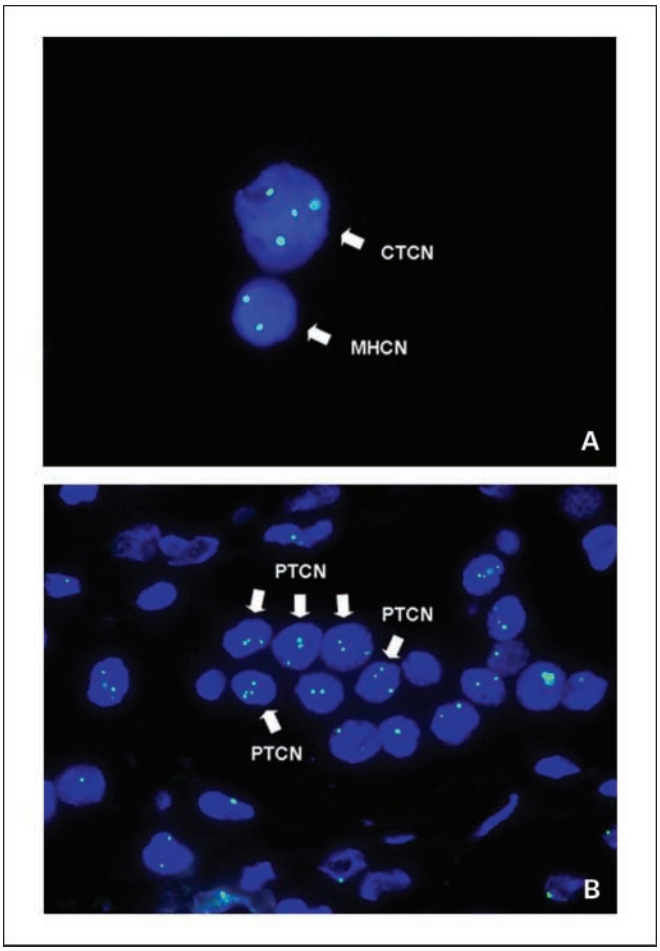

Fig. 6. Chromosome 17 gains detected by FISH in (*A*) a CTC from a blood sample and (*B*) a primary tumor sample from the same colorectal cancer patient. *CTCN,* CTC nucleus; *MHCN,* mononuclear hematopoietic cell nucleus; *PTCN,* primary tumor cell nuclei. Original magnification, ×1,000.

in any of the 40 healthy subjects suggests that this assay has a low false-positive rate. Another important finding from this study is that preoperation CK20 pCTC number has prognostic potential as shown by its significant correlation to TNM stage and lymph node status in multivariate analysis. Furthermore, using the median CK20 pCTC numbers in selective patient groups as the cutoff points, preoperation CK20 pCTCs correlated with recurrence, metastatic disease, and overall survival in colorectal cancer patients. Actually, the patients with CK20 pCTCs >11 were mainly of stages III and IV patients, whereas those with CK20 pCTCs ≤11 were mostly of stages I and II patients. This may be one explanation why stages III and IV patients have a greater risk of recurrence, metastasis, and shorter survival than stages I and II patients.

In the second objective, we did not observe any pretreatment CK20 pCTC in patients with various kinds of benign colorectal diseases. This finding can imply that those benign colorectal diseases do not have micrometastatic potential. In fact, the significance of pretreatment CK20 pCTC in colorectal cancer detection would be greatly reduced if it is found in patients with benign colorectal diseases. Therefore, we propose that patients with benign diseases should be included in all tumor marker evaluation studies to have a comprehensive assessment of the markers' potential in prognosis and diagnosis.

In the third objective, we explored whether CK20 pCTCs can be found in other cancers because currently one major limitation in immunomagnetic enrichment CTC detection is that only broad-spectrum antibodies are used; therefore, detection is not specific to any kind of tumor or tissue system. Previous studies indicated that immunomagnetic enrichment CTC detection using broad-spectrum antibody is very promising only in metastatic breast cancer (5–7), whereas there are still very scanty reports in colorectal cancer (18, 22, 23). Using this refined immunomagnetic enrichment assay with standardized immunocytochemical staining and stringent assessment criteria, no CK20 pCTC was found in all blood samples from patients with breast cancer, prostate cancer, liver cancer, and lung cancer. These results are encouraging because it can prove that this refined immunomagnetic enrichment assay in CK20 pCTC detection is specific to colorectal cancer among the types of cancers that have been tested.

The fourth objective is to investigate the malignant nature of those CK20 pCTCs because CK20 is an antigen that is present in both tumor and normal colorectal epithelial cells. We investigated whether our assessment criteria during slide examination could really select CK20 pCTCs rather than CK20-positive circulating epithelial cells. Although we have already shown that CK20 pCTCs were decreased after operation in a majority of colorectal cancer and colorectal adenoma patients that the origin of them should be from the primary tumor, more solid evidence is necessary for definitive interpretation of our data before further investigation of its value in both clinical monitoring of colorectal cancer and chemotherapeutic treatment as the level of CTCs has been shown to reflect the disease status in prostate and breast cancer patients (24, 25). Chromosome 17 gain is a frequent event in colorectal tumor (10, 11). As aneusomy is an early event in cancer and hematopoietic cells are normally disomic, changes of chromosome 17 copy numbers in CK20 pCTCs can be used to infer the malignant nature of epithelial cells (26). Our results provide evidence that our assessment criteria are capable to pick up those CK20 pCTCs. There are several possibilities for the discrepancy in chromosome 17 aberration pattern between the blood and their respective tumor samples in 2 patients: (*a*) we may miss a CTC with chromosome 17 gain in each blood sample, (*b*) there may have overlapping signals if >2 copies are present in each blood sample, and (*c*) the number of tumor cells shed into the circulation from those 2 samples may not be high enough to be detectable by this refined immunomagnetic enrichment assay in the blood because chromosome 17 gain was only found in 8% and 12% of their respective primary tumor cells.

The effect of 5-FU-based chemotherapy on CK20 pCTCs was explored to examine whether the CK20 pCTC numbers can be used to predict the effectiveness of this treatment. Although the sample size is too small to perform a valid χ^2 test, the results can still be classified into three groups. The first group includes patients with increasing number of CK20 pCTCs detected 24 months after 5-FU-based treatment and they should be the poor response group because 62.5% of the patients had relapse. The second group of patients, with insignificant change of CK20 pCTCs found, is insensitive to this treatment. We hypothesize that this group of patients may consist of CK20

pCTCs with good prognosis as only 33.3% of the patients had relapse. The last group of patients with decreasing number of CK20 pCTCs found should be the good response group because only 25% of the patients had relapse. Based on these results, CK20 pCTCs may be a potential marker to indicate the chemotherapeutic effectiveness of 5-FU-based treatment in TNM stage III colorectal cancer patients and a larger-scale study would be carried out in the future to validate our findings.

In conclusion, this study is the first to detect CK20 pCTCs in colorectal cancer patients using a gastrointestinal-specific anti-CK20 antibody. Quantification of CK20 pCTCs as detected by this refined immunomagnetic enrichment assay has high potential for the differential diagnosis of colorectal cancer and their serial measurements may be clinically useful to monitor disease progression. Finally, the success of this refined immunomagnetic enrichment assay has opened up new possibilities in CTC detection as CTC shedding from various cancers may be further characterized after immunomagnetic enrichment with their respective specific tumor markers using immunocytochemical staining (27), *in situ* hybridization (28), or even molecular profiling using quantum dot technology (29, 30).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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