Practical Utility of Circulating Tumour Cells as Biomarkers in Cancer Chemotherapy for Advanced Colorectal Cancer

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Abstract. Molecular-targeted therapies require the assessment of targets and their related molecules. Circulating tumour cells (CTCs) are considered a very good source of samples for these purposes. In this study, we applied a practical method for examining CTCs to evaluate the effects of chemotherapy on advanced colorectal cancer (CRC). Even in stage IV CRC, CTCs were detected in only 38.5% (n=5/13) of the cases. However, in cases where CTCs were detected, the change in the number of CTCs compared before and after chemotherapy appeared to be associated with the therapeutic outcome. Changes in the number of CTCs may be a good predictive biomarker. Problems with this method are yet to be resolved, including the detection rate and the stability of the sample source for subsequent molecular analysis.

Recent advances in chemotherapy have been mainly due to the development of molecular-targeted agents. The use of these therapies depends on the molecular diagnosis related to the target molecules themselves or other molecules located in their signalling pathways. For the treatment of colorectal cancer (CRC), administration of antibodies against epidermal growth factor receptor (EGFR) is effective for patients with the wild-type Kirsten rat sarcoma viral oncogene homolog (KRAS) phenotype (1, 2). Genotyping of v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) and phosphoinositide 3-kinase catalytic subunit (PI3CA) should also be considered (1). In addition, overall expression profiling using products such as the 18-gene signature ColoPrint is under consideration for the molecular diagnosis of metastatic CRC (3). In any case, molecular diagnosis requires the use of DNA or RNA derived from resected specimens. Such samples are archival and thus do not represent the real-time status of the disease and its potential molecular targets. Furthermore, because almost all targets of chemotherapy for advanced-stage cancer are metastatic lesions, it is often difficult to obtain samples.

Analysis of circulating tumour cells (CTCs) from patients with cancer has recently become possible (4-6). CTCs are attractive sources for tumour analysis, as they can be obtained safely and are real-time tumour samples. The CellSearch system (Veridex LLC, Raritan, NJ, USA), an immunomagnetic enrichment method, has been approved by the US Food and Drug Administration (7). In this method, ferrofluid coated with antibodies against epithelial cell adhesion molecule (EpCAM) is employed for the selection of epithelial cells. Antibodies against cytokeratin 8, 18, and 19 are also used for positive selection, and an antibody against CD-45 is used for negative selection to eliminate leukocytes. Diamidino-2-phenylindole (DAPI), a marker of cell nuclei, is used in the negative selection of red blood cells and debris. In a present study, no healthy volunteer was found to have more than one CTC (4). CTC analyses have been included in several clinical trials (8, 9). Some of the results are promising, but further confirmation is needed.

In this study, we counted CTCs in blood from patient with stage IV CRC and analyzed the clinical importance and utility of samples for molecular diagnosis. We demonstrated the potential usefulness of CTC analysis and noted that further modification of the methodology is needed.

Patients and Methods

Fourteen patients with CRC stage III and IV, treated at the Department of Clinical Oncology at the Akita University Hospital from January 2012 to October 2012 were enrolled after obtaining their informed consent. This study was scientifically and ethically approved by the Committee of the School of Medicine of Akita University (#828).
Collection of CTCs. CTCs were obtained from 20 ml of peripheral venous blood drawn from each patient. CTCs were collected using the CellSearch kit (Veridex LLC, Raritan, NL, USA) and the CellTracks autoprep machine (Veridex LLC, Raritan, NL, USA). Identification of CTCs was confirmed using the Cell Tracks analyzer. In brief, CTCs were selected using anti-EpCAM and anti-cytokeratin antibodies (positive selection) and the anti-CD-45 antibody (negative selection).

Mutation analysis of KRAS. DNA was extracted from CTCs and mutational analysis of KRAS was conducted using the Scorpion-ARMS real-time PCR method (10). The mutations analysed included Gly12Ala, Gly12Asp, Gly12Arg, Gly12Cys, Gly12Ser, Gly12Val, and Gly13Asp.

RNA extraction. RNA was extracted from CTCs using the NucleoSpin RNA XS kit (Takara Bio, Tokyo, Japan). CTCs are lysed by incubation in the lysis buffer. Residual genomic DNA is removed by on-column digestion with DNase, and total RNA was eluted.

Statistical analysis. The Pearson product-moment correlation coefficient between CTC number and therapeutic outcome was determined using STAT III mate (ATMS, Tokyo, Japan).

Results

Detection rate of CTCs in patients with stage IV CRC. The demographic information on the CRC cohort is presented in Table I. The age of the patients ranged from 52 to 80 years. Thirteen patients with stage IV CRC and one with stage III CRC were included. Primary sites of stage IV CRC were as follows: cecum (n=3), ascending colon (n=4), transverse colon (n=2), and rectum (n=5). Nine patients had liver metastases, five had lung metastasis, and four had cancerous peritonitis. The overall rate of CTC detection was 38.5% (n=5/13). In patients with liver metastases, the detection rate was particularly high (55.6%, 5/9), whereas CTCs were not detected in patients with stage IV CRC without liver metastasis. The number of CTCs was less than 2 cells per 7.5 ml of whole blood in 80% (4/5) of the CTC-positive cases. In only one case were CTCs detected repeatedly; the median number of CTCs was 16 per 7.5 ml of whole blood (range, 2–73). In cases 1, 4, and 11, CTCs were re-analyzed immediately after the disease was judged as progressive; no CTCs were detected in any of these cases.

Correlation between CTC number and therapeutic outcome. As stage IV CRC is a systemic disease, we considered that CTCs may be more prevalent in this stage. However, CTCs were not always detected, even in stage IV cancer. In order to determine whether the presence of CTCs is related to the therapeutic outcome, we analyzed the relationship between the number of CTCs and the time-to-therapeutic failure (TTF) of chemotherapy administered when CTCs were counted. The number of CTCs was 16 per 7.5 ml of whole blood (range, 2–73). In cases 1, 4, and 11, CTCs were re-analyzed immediately after the disease was judged as progressive; no CTCs were detected in any of these cases.
Potential use of CTCs as a predictive biomarker for outcome of chemotherapy for CRC. Comparison of the number of CTCs before and after chemotherapy could predict the treatment outcome. In case 3, we detected CTCs several times. We compared the change in CTC number with other evaluative methods, such as Response Evaluation Criteria in Solid Tumors (RECIST) and the tumor markers carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9). As shown in Figure 2, an increase in the number of CTCs was observed during Xelox plus BV treatment, three months prior to RECIST evaluation, and one month prior to the increase in tumour markers. The same trend was observed for treatment with irinotecan plus S1 plus BV. In case 13, the number of CTCs declined from 1 to 0 during capecitabine plus oxaliplatin (XELOX) therapy. A decrease in CTCs was associated with partial response (PR; RECIST) evaluated at two-month intervals beginning with the initiation of therapy and was also associated with a decrease in tumour markers between the baseline measurement and during therapy of CEA (from 1636.1 to 187.5 ng/ml) and CA19-9 (from 2137.0 to 411.8 U/ml). The number of CTCs did not increase for four months, and the disease kept within stable disease (SD; RECIST) criteria during this period. These observations demonstrate that if CTCs are detectable, changes in the number present after treatment may be useful for predicting therapeutic outcomes much earlier than that with the current methods.

In cases where CTCs were not present initially, they were not detected even after disease progression (cases 1, 4, and 11; Table I). In the CTC-negative cases, we did not obtain any predictive values.

**Utility of CTCs as a sample source for molecular analysis.** We attempted to analyse KRAS in the DNA derived from CTCs collected in cases 2, 3 (twice), 5, 12, and 13, using the Scorpion-ARMS method. No DNA was amplified in case 3 or case 12, where the number of CTCs was 4 and 1 per 7.5 ml of whole blood, respectively (Table II). In the other four cases, where the number of CTCs ranged from 1 to 28 per 7.5 ml of whole blood, the DNA was insufficiently amplified, and no KRAS mutants were amplified. For cases 3 and 13, we compared the results of Scorpion-ARMS analysis from surgically removed tissue samples and CTCs. While analysis of the tissue samples identified both cases as having the KRAS G13D mutation, analysis of CTC DNA from the same cases did not yield any result (Table II). The CTC DNA obtained from our examination seemed to be inadequate for
In general, the number of CTCs in patients with metastatic CRC seems to be lower than that observed in patients with metastatic breast cancer. The cell surface markers used in the CellSearch system (i.e. cytokeratin and adhesion-related EpCAM) may be less abundant in patients with metastatic CRC compared with those with metastatic breast cancer. Another possibility is that a fraction of the CTCs may transform to mesenchymal cells through epithelial-mesenchymal transition (EMT). This EMT may be more frequent in CTCs from metastatic CRC than from those in metastatic breast cancer. The method used to collect CTCs may require modification according to the type of cancer. Immunomagnetic separation has been reported to improve CTC detection rates. For example, cytokeratin 20 was positive in CTCs in 92.9% of patients with metastatic CRC after column immunomagnetic separation (5).

Many reports describe a relationship between therapeutic outcomes and baseline number of CTCs or number of CTCs during therapy (5, 6, 9). However, in this study, there was no correlation between the number of CTCs during therapy and the outcomes. This observation may be due to the low detection rate of CTCs in metastatic CRC. Once CTCs are detected, the change in the number of CTCs could be a good predictive marker of ongoing treatment, as shown in our cases. In contrast to single measurements of CTC number (either baseline or during therapy), changes in CTC counts during therapy could be used to determine whether to continue or change the therapy. Prospective studies should be conducted in the future in order to clarify these points.

CTCs are viewed as a good source of DNA and RNA for analyses (13-15). However, the DNA obtained using the CellSearch system was not suitable for KRAS Scorpion-ARMS analysis in this study. The PCR conditions, such as primer sequences, composition of reaction buffer, and annealing temperature, may require modification. RNA was not recovered from CTCs using the CellSearch system.

Recently, circulating DNA was shown to be useful for identifying acquired resistance to antibodies against EGFR in metastatic CRC (16). This method seems to be much more potent than CTC analysis for KRAS mutation detection. However, a next generation sequencer is necessary to use this method, and the balance between cost and effectiveness should be discussed before choosing this method for daily clinical use. Furthermore, CTCs may be rich in molecular information derived from RNAs or proteins rather than DNA. Analysis of these molecules may be advantageous over that of circulating DNA.

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**References**


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