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

KAZUNORI OTSUKA[1*], HIROO IMAI[1*], HIROSHI SOEDA[1], KEIGO KOMINE[1], CHIKASHI ISHIOKA[2] and HIROYUKI SHIBATA[1]

[1]*Department of Clinical Oncology, Graduate School of Medicine, Akita University, Akita, Japan;*
[2]*Department of Clinical Oncology, Institute for Development, Aging and Cancer, Tohoku University, Sendai, Japan*

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, NL, 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).

*These Authors contributed equally to this work.

Correspondence to: Hiroyuki Shibata, Department of Clinical Oncology, Graduate School of Medicine, Akita University, Akita, Japan. E-mail: hiroyuki@med.akita-u.ac.jp

Key Words: Colorectal cancer, circulating tumour cells, biomarkers, chemotherapy.

Table I. *Patients' background*.

Case	Age (years)/Gender	Primary	Metastases	Stage	CTC (n/7.5 ml)
1	76/F	Ce/tub1	(Li), Lym	IV	0, 0
2	68/M	A/tub1	Li, Lu	IV	2
3	60/F	T/tub1	Li	IV	4, 28, 73, 18, 12, 16, 6
4	65/M	R/tub1	Lu	IV	0, 0
5	57/F	A/tub2	Li	IV	1
6	78/F	Ce/tub1	Li	IV	0
7	77/M	A/tub1	PC	IV	0
8	68/F	R/tub1	(–)	III	0
9	52/M	R/tub2	Li	IV	0
10	66/M	R/tub1	Li, Lu	IV	0
11	80/M	R/tub1	Lu	IV	0, 0
12	68/M	T/tub2	Li, PC	IV	1
13	70/M	A/tub1	Lu, Li, PC	IV	1, 0
14	54/F	Ce/MAC	PC	IV	0

M, Male; F, female; Ce, cecum; A, ascending; T, transverse; R, rectum; Li, liver; Lym, lymph nodes; Lu, lung; PC, peritonitis carcinomatosa; tub1, well-differentiated tubular adenocarcinoma; tub2, moderately-differentiated; MAC, mucinous adenocarcinoma.

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 Cell Tracks 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 and TTF are shown in Table II. Chemotherapeutic agents included an oxaliplatin-based regimen with or without bevacizumab (BV) (n=5), an irinotecan-based regimen (n=5), 5-fluorouracil (5-FU) plus leucovorin (n=1), and no therapy (n=1). In the latter case, time-to-progression (TTP) was applied. The Pearson product-moment correlation coefficient was calculated. A negative correlation was observed between the number of CTCs and the therapeutic outcome, but this relationship was not significant ($y=4.71-0.0076x$; correlation coefficient=–0.3897; $p=0.21$) (Figure 1).

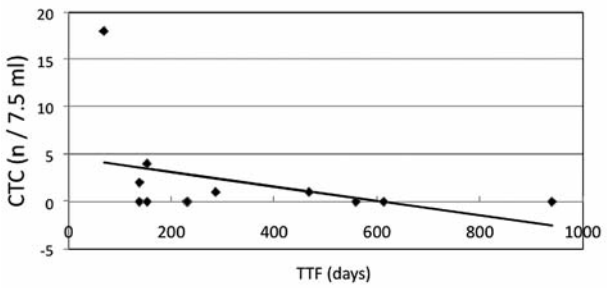

Figure 1. *Correlation between the number of circulating tumor cells (CTCs) during therapy and time-to-treatment failure (TTF).*

Table II. *Number of circulating tumor cells detected during therapy and treatment outcome.*

Case	CTC (n/7.5 ml)	TTF (days)
1	0	940 (FOLFOX+BV)
2	2	139 (Xelox)
3	4	153 (Xelox+BV)
4	18	69 (IRIS+BV)
5	0	232 (CPT-11+Pmab)
6	1	468 (FOLFIRI+Pmab)
7	0	139 (SOX)
8	0	230 (SOX)
9	0	613 (FOLFIRI+BV)
10	0	153 (CTP-11+Cmab)
11	0	559 (FL)
12	0	287 (no therapy, TTP)

TTF, Time-to-treatment failure; FOLFOX, 5-fluorouracil + leucovorin + oxaliplatin; BV, bevacizumab; Xelox, capecitabine + oxaliplatin; IRIS, irinotecan + S1; Pmab, panitumumab; FOLFIRI, 5-fluorouracil + leucovorin + irinotecan; SOX, S1 + oxaliplatin; CPT-11, irinotecan ; FL, 5-fluorouracil + leucovorin; TTP, time-to-progression.

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 tumour 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 (IRIS) 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;

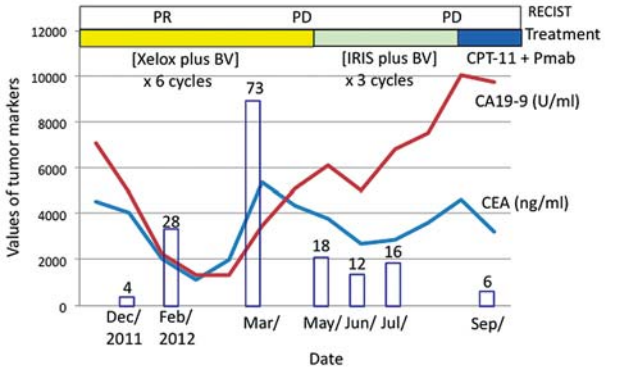

Figure 2. *Change in number of circulating tumor cells (CTCs) during the treatment of case 3. The number of CTCs is indicated by white bars. Carcinoembryonic antigen (CEA) is indicated in blue and carbohydrate antigen 19-9 (CA19-9) in red. The result of response evaluation criteria in solid tumors (RECIST) in each timing is indicated at the top. Xelox, Capecitabine + oxaliplatin; BV, bevacizumab; IRIS, irinotecan + S1; CPT-11, irinotecan; Pmab, panitumumab; PR, partial response; PD, progress disease.*

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 III). 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 III). The CTC DNA obtained from our examination seemed to be inadequate for

Table III. *Molecular analysis of circulating tumor cells (CTCs).*

Case	CTC (n/7.5 ml)	*KRAS*		RNAS extraction
		In CTC	In tissue sample	
2	2	Wild-type*	○ Wild-type*	nd
3	4	NA		nd
	28	Wild-type*		nd
	73	nd	× (G13D)	NO
	18	nd		NO
	12	nd		NO
5	1	Wild-type*	○ Wild-type	nd
12	1	NA	(G12V)	nd
13	1	Wild-type*	× (G13D)	nd

NA, Not amplified; nd, not done; NO, not extracted; ○, match; X, no match.

KRAS Scorpion-ARMS analysis. We also made three attempts to obtain RNA from the CTCs captured in case 3, where the number of CTCs was 12, 18, and 73, but all failed (Table III).

Discussion

CTCs have been recently detected in various types of cancers, including colonic, breast, and prostatic cancer (11, 12). The importance of CTC analysis has been proposed, including its use as a prognostic or predictive biomarker. In this study, we examined the practical availability of CTC analysis using the CellSearch system, which involves outsourcing the analysis to a commercial laboratory. The detection rate and the number of cells identified were rather low, even for stage IV CRC. Previous studies reported detection rates of over two CTCs per 7.5 ml of blood in 30-40% of patients with metastatic CRC (4-6); in patients with metastatic breast and prostate cancer, the same rate was observed in 60% of the patients (11, 12). Our observations are similar to the former.

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.

Acknowledgements

This study was partially supported by a grant from the Project for Development of Innovative Research on Cancer Therapeutics, the Ministry of Education, Culture, Sports, Science, and Technology, Japan (for CI and HS).

References

1 Roock WD, Claes B, Bernasconi D, Schutter JD, Biesmans B, Fountzilas G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G, Cappuzzo F, Frattini M, Molinari F, Saletti P, Dosso SD, Martini M, Bardelli A, Siena S, Sartore-Bianchi A, Tabernero J, Macarulla T, Di Fiore F, Gangloff AO, Ciardiello F, Pfeiffer P, Qvortrup C, Hansen TP, Van Cutsem E, Piessevaux H, Lambrechts D, Delorenzi M and Tejpar S: Effects of *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol *11(8)*: 753-762, 2010.

2 Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, Hayes DF, McAllister PK, Morton RF and Schilsky RL: American Society of Clinical Oncology provisional clinical opinion: Testing for *KRAS* gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. J Clin Oncol *27(12)*: 2091-2096, 2009.
3 Salazar R, Roepman P, Capella G, Moreno V, Simon I, Dreezen C, Lopez-Doriga A, Santos C, Marijnen C, Westerga J, Bruin S, Kerr D, Kuppen P, van de Velde C, Morreau H, Van Velthuysen L, Glas AM, Van't Veer LJ and Tollenaar R: Gene expression signature to improve prognosis prediction of stage II and III colorectal cancer. J Clin Oncol *29(1)*: 17-24, 2011.
4 Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe AG, Uhr JW and Terstappen LW: Tumour cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res *10(20)*: 6897-6904, 2004.
5 Cohen SJ, Alpaugh RK, Gross S, O'Hara SM, Smirnov DA, Terstappen LW, Allard WJ, Bilbee M, Cheng JD, Hoffman JP, Lewis NL, Pellegrino A, Rogatko A, Sigurdson E, Wang H, Watson JC, Weiner LM and Meropol NJ: Isolation and characterization of circulating tumor cells in patients with metastatic colorectal cancer. Clin Colorectal Cancer *6(2)*: 125-132, 2006.
6 Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, Picus J, Morse M, Mitchell E, Miller MC, Doyle GV, Tissing H, Terstappen LW and Meropol NJ: Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol *26(19)*: 3213-3221, 2008; erratum in: J Clin Oncol *27(11)*: 1923, 2009.
7 Hoeppener AE, Swennenhuis JF and Terstappen LW: Immunomagnetic separation technologies. Recent Results Cancer Res *195*: 43-58, 2012.
8 Hiraiwa K, Takeuchi H, Hasegawa H, Saikawa Y, Suda K, Ando T, Kumagai K, Irino T, Yoshikawa T, Matsuda S, Kitajima M and Kitagawa Y: Clinical significance of circulating tumor cells in blood from patients with gastrointestinal cancers. Ann Surg Oncol *15(11)*: 3092-3100, 2008.
9 Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, Picus J, Morse MA, Mitchell E, Miller MC, Doyle GV, Tissing H, Terstappen LW and Meropol NJ: Prognostic significance of circulating tumor cells in patients with metastatic colorectal cancer. Ann Oncol *20(7)*: 1223-1229, 2009.
10 Thelwell N, Millington S, Solinas A, Booth J and Brown T: Mode of action and application of Scorpion primers to mutation detection. Nucleic Acids Res *28(19)*: 3752-3761, 2000.
11 Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW and Hayes DF: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med *351(8)*: 781-971, 2004.
12 Moreno JG, O'Hara SM, Gross S, Doyle G, Fritsche H, Gomella LG and Terstappen LW: Changes in circulating carcinoma cells in patients with metastatic prostate cancer correlate with disease status. Urology *58(3)*: 386-92, 2001.
13 Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, Smith MR, Kwak EL, Digumarthy S, Muzikansky A, Ryan P, Balis UJ, Tompkins RG, Haber DA and Toner M: Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature *450(7173)*: 1235-1239, 2007.
14 Stott SL, Hsu CH, Tsukrov DI, Yu M, Miyamoto DT, Waltman BA, Rothenberg SM, Shah AM, Smas ME, Korir GK, Floyd FP Jr., Gilman AJ, Lord JB, Winokur D, Springer S, Irimia D, Nagrath S, Sequist LV, Lee RJ, Isselbacher KJ, Maheswaran S, Haber DA and Toner M: Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. Proc Natl Acad Sci USA *107(43)*: 18392-18397, 2010.
15 Maheswaran S, Sequist LV, Nagrath S, Ulkus L, Brannigan B, Collura CV, Inserra E, Diederichs S, Iafrate AJ, Bell DW, Digumarthy S, Muzikansky A, Irimia D, Settleman J, Tompkins RG, Lynch TJ, Toner M and Haber DA: Detection of mutations in EGFR in circulating lung-cancer cells. N Engl J Med *359(4)*: 366-377, 2008.
16 Diaz LA Jr., Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, Allen B, Bozic I, Reiter JG, Nowak MA, Kinzler KW, Oliner KS and Vogelstein B: The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature *486(7404)*: 537-540, 2012.

Received November 27, 2012
Revised December 22, 2012
Accepted January 3, 2013