# Circulating Tumor Cell (CTC) Count and Epithelial Growth Factor Receptor Expression on CTCs as Biomarkers for Cetuximab Efficacy in Advanced Colorectal Cancer

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**Abstract.** *Background/Aim: The purpose of this study was to establish whether CTC count and epidermal growth factor receptor (EGFR) expression in CTCs predicted outcome in patients with advance colorectal cancer (ACC) receiving cetuximab as third-line treatment. Patients and Methods: Between October 2008 and March 2011, 63 patients with KRAS wild-type ACC were treated with cetuximab-containing chemotherapy at the Cancer Institute Hospital. We measured the CTC count and EGFR expression on CTCs using the CellSearch System (Veridex LLC, NJ, USA). Results: Nineteen patients (30%) with a high number of CTCs had a significantly lower overall survival compared with 44 patients with a low number of CTCs. No significant difference was observed in progression-free survival between the two groups. Out of the 33 patients positive for CTCs (one or more CTC), seven patients (21%) were positive for EGFR expression. No statistically significant difference was observed in clinical outcome between EGFR-positive and EGFR-negative patients. Conclusion: A high CTC count predicted reduced overall survival in patients with ACC treated with cetuximab-combination chemotherapy as third-line treatment. These results suggest that the assessment of CTCs might provide with important prognostic information for such patients.*

The number of circulating tumor cells (CTCs) as measured by the CellSearch system has been shown to have prognostic significance in patients with breast (1, 2), lung (3), prostate (4, 5), colorectal (6-8), and gastric cancer (9), so recent efforts have concentrated on detecting CTCs in the peripheral blood of patients with cancers.

Cohen *et al*. reported that the number of CTCs before and during treatment was an independent predictor of progression-free survival (PFS) and overall survival (OS) in patients with advanced colorectal cancer (ACC) (6). Detection of three or more CTCs *versus* fewer than three CTCs before and after initiation of a new systemic treatment regimen was associated with shorter median PFS and OS. Our previous study supported the clinical utility of CTC enumeration in improving the ability to accurately assess first-line treatment in individual Japanese patients (7).

Cetuximab is a monoclonal antibody that specifically blocks epidermal growth factor receptor (EGFR), which participates in signaling pathways that are deregulated in cancer cells, including colorectal cancer. Cunningham *et al*. reported that cetuximab alone or in combination with irinotecan had significant efficacy in patients with irinotecan-refractory ACC (10).

Tol *et al*. assessed the prognostic and predictive role of CTCs in patients with ACC treated in a randomized phase III trial with first-line chemotherapy and targeted agents (capecitabine, oxaliplatin, and bevacizumab, or the same schedule with addition of weekly cetuximab). According to their report, the CTC count before the commencement of first-line treatment independently predicted PFS and OS in these patients (8). The relationship between CTC count and response to third-line treatment in patients with ACC refractory to second-line treatment, however, is unclear. Moreover, the correlation between CTC levels and clinical outcome in patients treated with cetuximab as third-line treatment remains to be clarified. The purpose of this study was to use the CellSearch system to investigate the potential of CTC count and EGFR expression on CTCs as a surrogate marker of clinical outcome in patients treated with cetuximab with or without irinotecan, as third-line treatment.



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*Key Words:* mCRC, cetuximab, circulating tumor cells, colorectal cancer.

## Patients and Methods

*Patients*. All the patients in this study were enrolled in accordance with the protocols established by the Institutional Review Board of the Cancer Institute Hospital of the Japanese Foundation for Cancer Research. Between October 2008 and March 2011, 63 patients with *KRAS* wild-type ACC were treated with cetuximab, with or without irinotecan as third-line therapy. Written informed consent for collection of CTCs was obtained from each patient. Eligible patients had been previously treated with fluoropyrimidine, irinotecan, and oxaliplatin (with or without bevacizumab).

*Sample preparation for isolation of CTCs from blood*. For isolation of CTCs from patients with ACC, 10-ml samples of blood were drawn into a Cell Save Preservative Tube (Veridex LLC, Raritan, NJ, USA). The CellSearch system (Veridex LLC) consists of the CellPrep system, the CellSearch Epithelial Cell Kit (for measurement of CTCs) and the CellSpotter Analyzer. The CellPrep system is a semi-automated sample preparation system, and the CellSearch Epithelial Cell Kit consists of ferrofluids coated with epithelial cell-specific EpCAM antibodies to immunomagnetically enrich epithelial cells; a mixture of two phycoerythrin-conjugated antibodies that bind to cytokeratin 8, 18 and 19; an antibody to CD45 conjugated to allophycocyanin; nuclear dye 4',6-diamidino-2-phenylindole (DAPI) to fluorescently label the cell; and buffers against wash, permeabilize and resuspend the cells (8). In addition, the surface EGFR expression level in the CTC subset was assessed using CellSearch® Tumor Phenotyping Reagent EGFR.

Briefly, 7.5 ml blood was mixed with 6 ml buffer, centrifuged at 800 ×*g* for 10 min and then placed in the CellPrep system. After aspiration of the plasma and buffer layer by the instrument, ferrofluids were added. After incubation and subsequent magnetic separation, unbound cells and remaining plasma were aspirated. The staining reagents were then added in conjunction with a permeabilization buffer to fluorescently label the immunomagnetically labeled cells. After incubation in the system, magnetic separation was repeated and excess staining reagents aspirated. As the final step in the procedure, the cells were resuspended in a MagNest Cell Presentation Device (Veridex LLC). This device consists of a chamber and two magnets that orient the immunomagnetically labeled cells for analysis using the CellSpotter Analyzer.

*Sample analysis*. The MagNest is placed on the CellSpotter Analyzer, a 4-color, semi-automated fluorescence microscope. Image frames covering the entire surface of the cartridge are captured. Captured images containing objects that meet predetermined criteria are automatically presented in a web-enabled browser; an operator makes the final selection of cells. The criteria for an object to be defined as a CTC include a round-to-oval morphology, a visible nucleus (DAPI-positive), positive staining for cytokeratin and negative staining for CD45. Results of cell enumeration and EGFR expression in the CTCs are always expressed as the number of cells per 7.5 ml blood (Figure 1).

*Evaluation of efficacy*. Tumor response was assessed using computed tomography (CT) approximately every eight weeks according to the Response Evaluation Criteria in Solid Tumor (RECIST) criteria (11). Treatment outcome was determined by OS and PFS.

Table I. *Patients' characteristics*.

| | Number of patients | % |
|---|---|---|
| Median age, years (range) | 61 (33-81) | |
| Gender: Male/female | 34/29 | 54/46 |
| Treatment arm | | |
| Irinotecan (+/−) | 55/8 | 87/13 |
| Prior bevacizumab chemotherapy (+/−) | 39/24 | 62/38 |
| Primary site | | |
| Colon/Rectum | 41/22 | 65/35 |
| Number of affected organs | | |
| 1 | 19 | 30 |
| >1 | 44 | 70 |
| Metastasis site | | |
| LLD/non-LLD | 10/53 | 16/84 |
| Number of CTCs | | |
| <3 | 44 | 70 |
| ≥3 | 19 | 30 |
| PS | | |
| 0/1/2 | 50/11/2 | 79/18/3 |

LLD, Liver-limited disease; CTCs, circulating tumor cells; PS, performance status.

*Statistical analysis*. The primary objective was to assess the prognostic and predictive value of CTCs in patients with ACC treated with cetuximab. Patients were prospectively divided into two subgroups: a low CTC count group, defined as these having fewer than three CTCs per 7.5 ml; and a high CTC count group, defined as these having three or more CTCs per 7.5 ml. This cut-off level of three CTCs was chosen on the basis of the results of previous studies (7, 8, 12). Survival curves were estimated using the Kaplan–Meier method and compared using log-rank testing. Univariate and multivariate Cox proportional hazards models were built using sex (male *versus* female), age (65 years *versus* more than 65 years), performance status (PS) (0 *versus* 1, 2), primary site (colon *versus* rectum), liver-limited disease (LLD) (LLD *versus* non-LLD), the number of affected organs (one *versus* more than one), treatment arm (cetuximab with *versus* without irinotecan) and CTC count at baseline as covariates for PFS and OS.

## Results

*Patients' characteristics*. A total of 63 patients were enrolled between October 2008 and March 2011. All patients were treated with a fluoropyrimidine, irinotecan, and oxaliplatin. Thirty-nine patients (62%) received bevacizumab. Fifty-five patients (87%) received cetuximab with irinotecan, and the remaining eight patients (13%) received cetuximab alone. The baseline characteristics of the patients in the CTC study subset are shown in Table I. The median duration of follow-up at the time of this analysis was 8.7 months.

*CTC level and imaging to assess response to therapy*. A total of 61 patients (97%) were evaluated. Two patients were not evaluated by CT due to their death before the first evaluation.

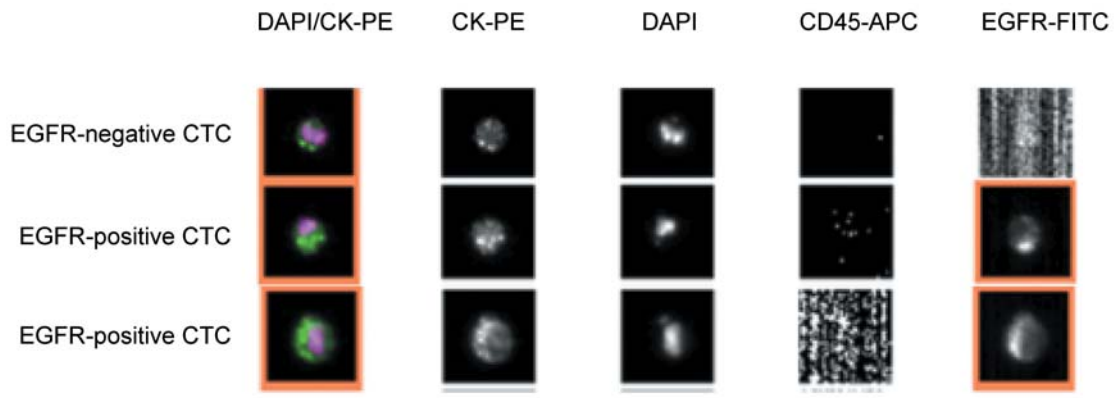


Figure 1. *Image galleries after CellSearch processing. Epidermal growth factor receptor (EGFR)-positive CTCs were cytokeratin (CK)- and DAPI-positive, but CD45-negative.*

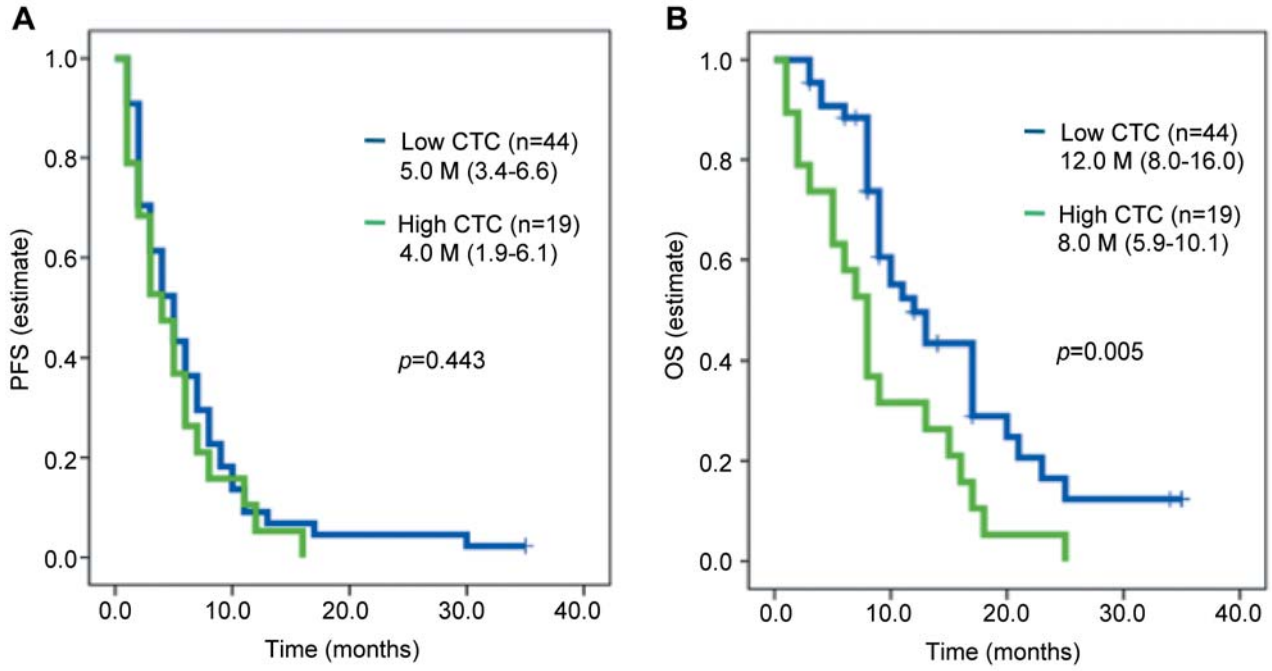


Figure 2. *Kaplan-Meier plots of progression-free survival (PFS) (A) and overall survival (OS) (B) in patients with advanced colorectal cancer high or low circulatuing tumor cells (CTCs) count at baseline.*

Eighteen patients (29.5%) had a high number of CTCs at baseline. The best objective responses were achieved as follows: a response [partial response (PR) or complete response (CR)] was observed in 13 patients (21.3%); stable disease (SD) in 19 patients (31.1%); and progressive disease in 29 patients (47.5%). The disease control rate (CR + PR + SD) was 52.4%. No significant difference was observed in efficacy (response rate/disease control rate) between the high and low CTC groups ($p$=0.498, 27.7% *versus* 18.6%; $p$=1.0, 50.0% *versus* 53.4%).

*Analysis of clinical outcome and CTC count*. Out of the 63 patients with evaluable baseline CTC results, 19 patients (30%) had a high number of CTCs. The median number of CTCs was one (range=0-220, mean=7.3) at baseline. No statistically significant difference was observed in patient characteristics between the high- and low-CTC groups. The median PFS was 4.0 months in the high CTC and 5.0 months in the low CTC group [log-rank $p$=0.443; hazard ratio (HR)=1.2, 95% Confidence interval (CI)=0.7-2.1] (Figure

Table II. *Independent predictive factors by univariate Cox regression analysis for progression-free survival and overall survival.*

| Factor | Hazard ratio | 95% CI | *p*-Value |
|---|---|---|---|
| Progression-free survival | | | |
| CTCs (≥3) | 1.218 | 0.706-2.100 | 0.479 |
| Gender (female) | 0.490 | 0.288-0.835 | 0.009 |
| Age (<65) | 0.643 | 0.383-1.078 | 0.094 |
| PS (1/2) | 1.029 | 0.556-1.907 | 0.926 |
| Primary site (rectum) | 1.078 | 0.576-1.906 | 0.879 |
| Non-LLD | 0.570 | 0.636-1.825 | 0.781 |
| Number of affected organs (>1) | 1.090 | 0.619-1.918 | 0.766 |
| Treatment arm (without Irinotecan) | 1.055 | 0.500-2.227 | 0.889 |
| Overall survival | | | |
| CTCs (>=3) | 2.169 | 1.217-3.865 | 0.007 |
| Sex (female) | 0.770 | 0.439-1.349 | 0.361 |
| Age (<65) | 0.818 | 0.456-1.468 | 0.500 |
| PS (1/2) | 1.256 | 0.640-2.465 | 0.507 |
| Primary site (rectum) | 1.048 | 0.576-1.906 | 0.879 |
| Non-LLD | 0.570 | 0.291-1.120 | 0.103 |
| Number of affected organs (>1) | 1.219 | 0.633-2.349 | 0.554 |
| Treatment arm(without Irinotecan) | 1.263 | 0.591-2.698 | 0.546 |

CTC, Circulating tumor cells; PS, performance status; LLD, Liver-limited disease.

Table III. *Independent predictive factors by multivariate Cox regression analysis for progression-free survival and overall survival.*

| Factor | Hazard ratio | 95% CI | *p*-Value |
|---|---|---|---|
| Progression-free survival | | | |
| Age (<65) | 0.726 | 0.429-1.231 | 0.373 |
| Sex (female) | 0.525 | 0.306-0.904 | 0.020 |
| Overall survival | | | |
| CTCs (≥3) | 2.081 | 1.162-3.727 | 0.014 |
| Non-LLD | 0.627 | 0.318-1.237 | 0.178 |

CTCs, Circulating tumor cells; LLD, liver-limited disease.

2A). No statistically significant difference was observed between the two groups. The median OS was 8.0 months in the high-CTC and 12.0 months in the low-CTC group (log-rank $p<0.005$; HR=2.2, 95% CI=1.2-3.9) (Figure 2B). The median OS was a statistically significantly lower in patients in the high-CTC group in comparison with in those in the low-CTC group.

*Univariate and multivariate analyses of predictors of clinical outcome*. Univariate Cox regression analysis was used to assess the ability of sex, age, PS, primary site, liver limited disease, the number of affected organs, treatment arm and CTC count to predict PFS and OS. (Table II) Only the CTC count was significantly associated with OS. The HR (95% CI) of death for the CTC count was 2.2 (1.2–3.8). Additionally, sex was associated with PFS. In the multivariate Cox regression analysis, the CTC count was the strongest predictor of OS ($p=0.014$; HR=4.1) (Table III).

*Analysis of the relationship between clinical outcome and EGFR expression on CTCs*. Out of the 33 patients positive for CTCs (one or more CTC), seven patients (21%) were positive for EGFR expression. No statistically significant difference was observed in response between EGFR-positive and EGFR-negative patients. The median PFS was 3.6 months in the EGFR-positive and 4.8 months in EGFR-negative patients ($p=0.367$; HR=1.5, 95% CI=0.6-3.5). The median OS was 8.0 months in EGFR-positive and 8.0 months in the EGFR-negative patients ($p=0.973$; HR=0.98, 95% CI=0.4-2.4). No statistically significant difference was observed in clinical outcome between EGFR-positive and EGFR-negative patients.

## Discussion

We investigated the prognostic and predictive potential of CTCs in patients with ACC receiving cetuximab, with or without irinotecan. The CTC count before third-line treatment was shown to be an independent prognostic factor for OS but not for PFS. Earlier studies reported that the CTC count before first-line treatment was a strong independent prognostic factor for PFS and OS (8). However, it should be noted that these studies included patients who had received first- or other lines of treatment, not just third-line. On the other hand, to our knowledge, the present study is the first to only include patients with ACC receiving a cetuximab-containing regimen as third-line treatment. The *KRAS* mutational characterization of ACC tumors is now determined as a matter of routine before any treatment decision is made. The presence of *KRAS* mutation is a specific predictive biomarker for lack of efficacy of cetuximab. However, the prognostic factors for cetuximab in patients with wild-type KRAS remain to be clarified. Targeted therapies such as cetuximab are likely to increase the economic burden associated with the management of ACC. Therefore, the evaluation of the cost-effectiveness and cost-utility of targeted therapies is of substantial interest to healthcare providers and policy makers. Mittmann *et al*. reported that the mean incremental cost-effectiveness ratio of cetuximab compared with best supportive care for patients with wild-

type *KRAS* tumors was lower than that for the entire CO 17 study population, even though the incremental cost-effectiveness remained high (12). Analysis of cost-effectiveness is increasingly being used as a measure to direct treatment allocation in an environment of shrinking healthcare resources. It is therefore highly questionable whether cetuximab-based therapy in third-line treatment should be selected for patients with wild-type *KRAS* tumors. The present findings allow us to propose a new strategy for selection of patients for cetuximab therapy as third-line treatment. In short, we propose that patients with a high CTC count should receive best supportive care rather than third-line treatment.

To our knowledge, this is the first study to assess the predictive potential of EGFR expression in CTCs. Analysis of EGFR expression by immunohistochemical techniques or gene amplification, however, has been suggested to have no predictive value for response to cetuximab in colorectal cancer (13). One possible reason for this is suggested to be a difference in EGFR expression between the primary tumor and distant metastases, but there seems to be little agreement on this among earlier reports (13, 14). The results of this study indicate that EGFR expression in CTCs does not predict response to cetuximab. Expression of EGFR was detected in 21% of CTC-positive blood samples. Similarly, earlier reports showed that EGFR expression was detected in 18% of CTC-positive blood samples and there was no direct correlation between detection of EGFR expression in CTCs and EGFR expression in metastases (15). Further study is needed to investigate this discrepancy in EGFR expression between primary tumors, metastatic sites, and CTCs.

In conclusion, a high CTC count predicted a decrease in OS in patients with ACC receiving cetuximab in combination with chemotherapy as third-line treatment. These results suggest that the assessment of CTCs might provide with important prognostic information for such patients.

## Disclosure Statement

None.

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## References

1 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*: 781-791, 2004.

2 Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, Matera J, Allard WJ, Doyle GV and Terstappen LW: Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res *12*: 4218-4224, 2006.

3 Tanaka F, Yoneda K, Kondo N, Hashimoto M, Takuwa T, Matsumoto S, Okumura Y, Rahman S, Tsubota N, Tsujimura T, Kuribayashi K, Fukuoka K, Nakano T and Hasegawa S: Circulating tumor cell as a diagnostic marker in primary lung cancer. Clin Cancer Res *15(22)*: 6980-6986, 2009.

4 Shaffer DR, Leversha MA, Danila DC, Lin O, Gonzalez-Espinoza R, Gu B, Anand A, Smith K, Maslak P, Doyle GV, Terstappen LW, Lilja H, Heller G, Fleisher M and Scher HI: Circulating tumor cell analysis in patients with progressive castration-resistant prostate cancer. Clin Cancer Res *13*: 2023-2029, 2007.

5 Okegawa T, Nutahara K and Higashihara E: Prognostic significance of circulating tumor cells in patients with hormone refractory prostate cancer. J Urol *181(3)*: 1091-1097, 2009.

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 *31*: 3213-3221, 2008.

7 Matsusaka S, Suenaga M, Mishima Y, Kuniyoshi R, Takagi K, Terui Y, Mizunuma N and Hatake K: Circulating tumor cells as a surrogate marker for determining response to chemotherapy in Japanese patients with metastatic colorectal cancer. Cancer Sci *102(6)*: 1188-1192, 2011.

8 Tol J, Koopman M, Miller MC, Tibbe A, Cats A, Creemers GJ, Vos AH, Nagtegaal ID, Terstappen LW and Punt CJ: Circulating tumour cells early predict progression-free and overall survival in advanced colorectal cancer patients treated with chemotherapy and targeted agents. Ann Oncol *21*: 1006-1012, 2010.

9 Matsusaka S, Chìn K, Ogura M, Suenaga M, Shinozaki E, Mishima Y, Terui Y, Mizunuma N and Hatake K: Circulating tumor cells as a surrogate marker for determining response to chemotherapy in patients with advanced gastric cancer. Cancer Sci *101*: 1067-1071, 2010.

10 Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I and Van Cutsem E: Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med *351*: 337-345, 2004.

10 Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC and Gwyther S: New guidelines to evaluate the response to treatment in solid tumors. J Natl Cancer Inst *92*: 205-216, 2000.

11 Mittmann N, Au HJ, Tu D, O'Callaghan CJ, Isogai PK, Karapetis CS, Zalcberg JR, Evans WK, Moore MJ, Siddiqui J, Findlay B, Colwell B, Simes J, Gibbs P, Links M, Tebbutt NC and Jonker DJ; Working Group on Economic Analysis of National Cancer Institute of Canada Clinical Trials Group; Australasian Gastrointestinal Interest Group: Prospective cost-effectiveness analysis of cetuximab in metastatic colorectal cancer: Evaluation of National Cancer Institute of Canada Clinical Trials Group CO.17 Trial. J Netl Cancer Inst *101*: 1182-1192, 2009.

12 Chung KY, Shia J, Kemeny NE, Shah M, Schwartz GK, Tse A, Hamilton A, Pan D, Schrag D, Schwartz L, Klimstra DS, Fridman D, Kelsen DP and Saltz LB: Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. J Clin Oncol *20*: 1803-1810, 2005.

13 Scartozzi M, Bearzi I, Mandolesi A, Galizia E, Pierantoni C, Loupakis F, Berardi R, Zaniboni A, Quadri A, Zorzi F, Biagetti S, Loretelli C, Biscotti T, Labianca R, Masi G, Falcone A and Cascinu S: Epidermal growth factor receptor (EGFR) status in primary colorectal tumors does not correlate with EGFR expression in related metastatic sites: Implications for treatment with EGFR-targeted monoclonal antibodies. J Clin Oncol *23*: 4772-4788, 2004.

14 Scartozzi M, Bearzi I, Mandolesi A, Galizia E, Pierantoni C, Loupakis F, Berardi R, Zaniboni A, Quadri A, Zorzi F, Biagetti S, Loretelli C, Biscotti T, Labianca R, Masi G, Falcone A and Cascinu S: Epidermal growth factor receptor (EGFR) status in primary colorectal tumors correlates with EGFR expression in related metastatic sites: Biological and clinical implications. Ann Oncol *16*: 1503-1507, 2005.

15 Lankiewicz S, Rother E, Zimmermann S, Hollmann C, Korangy F and Greten TF: Tumour-associated transcripts and *EGFR* deletion variants in colorectal cancer in primary tumour, metastases and circulating tumour cells. Cell Oncol *30*: 463-71, 2008.

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