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ORIGINAL ARTICLE

Epidermal growth factor receptor (EGFR) mRNA levels and protein expression levels in primary colorectal cancer and corresponding liver metastases

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Abstract
Purpose High expression levels of EGFR mRNA are reported to be associated with a higher response probability in epidermal growth factor receptor (EGFR) targeted drugs. Our aim was to determine how EGFR gene expression levels in primary colorectal cancer (CRC) were related to those in liver metastases.
Methods 31 pairs of primary CRC and corresponding liver metastases were analyzed. Gene expression level was measured using real-time RT-PCR.
Results No significant difference was observed between median mRNA expression levels of EGFR in primary cancer and those in corresponding liver metastases ($P = 0.99$). When matched tissue sets were compared on an individual basis, there was a significant correlation for EGFR mRNA expression between primary cancer and corresponding liver metastases ($r_s = 0.78$, $P < 0.0001$).
Conclusions A good prediction of EGFR mRNA levels in liver metastases can be obtained by measuring those in the primary CRC.

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Introduction

Epidermal growth factor receptor (EGFR) is known to be involved in signaling pathways affecting cellular growth, differentiation, and proliferation [1]. In order to block the activation of this receptor, EGFR-targeted therapies have been developed. Gefitinib and Erlotinib are small-molecule EGFR tyrosine-kinase inhibitors which compete with ATP for binding sites in the intracellular catalytic domain of the EGFR tyrosine-kinase and prevent EGFR autophosphorylation and downstream signaling. Cetuximab and Panitumumab are anti-EGFR monoclonal antibodies, representing promising results for metastatic colorectal cancer (CRC) [2–4].

Biomarkers for predicting the efficacy of these EGFR-targeted drugs have been investigated. Recent data show that the level of EGFR expression as measured immunohistochemically (IHC) did not predict clinical benefits when patients were treated with an EGFR-targeted drug [5, 6]. On the other hand, it is reported that the CRC patients with lower EGFR mRNA levels had a longer overall survival when treated with Cetuximab [7]. Another report indicated that EGFR mRNA expression was higher in responders to gefitinib than in non-responders in patients with non-small cell lung cancer [8]. Regarding epiregulin and amphiregulin, which are both EGFR ligands, high mRNA expression levels of those genes are reported to be related to longer progression-free survival in patients with CRC treated with Cetuximab [9]. These recent data demonstrate that mRNA expression is a potentially useful biomarkers for predicting the efficacy of anti-EGFR drugs.

Colorectal cancer (CRC) is the fourth most common malignancy and the second leading cause of cancer death in the United States [10]. For most patients with CRC, liver metastases are the main cause of death. As controlling liver metastases are considered essential in the treatment of CRC, it is reasonable to expect that measurement of the EGFR mRNA expression level in liver metastases would provide the best prediction of therapy benefit. However, in many—if not most—cases, only biopsies of the patient's primary tumor are readily available for analysis. Therefore, it is important to know the relationship between the levels of response determinants in the primary tumor and the corresponding liver metastases in order to determine whether or not analysis of tissue biopsies of the primary tumor is useful for tumor response prediction.

In this study, we investigated the relationship between EGFR mRNA expressions in primary CRC and corresponding liver metastatic tumors, using laser-capture microdissection and real-time RT-PCR. We also performed IHC staining of EGFR using the same samples, and determined whether they were compatible with mRNA expression levels.

Materials and methods

Patients and samples

Thirty-one pairs of primary colorectal cancer and corresponding liver metastases were analyzed in this study (18 males and 13 females; median age, 66 (range, 45–85). These patients had undergone surgical resection of primary colorectal adenocarcinoma and liver metastasis between 1988 and 1999 at the Department of Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan. All of the patients were Japanese, and all gave their written informed consent according to the institutional regulations. The characteristics of the 31 patients are shown in Table 1. Seventeen patients had solitary liver metastases and 14 had multiple (two or more) metastases. The metastases in sixteen patients were synchronous, and in 15, metachronous. Of the patients with metachronous liver metastases, four had received 5-FU based chemotherapy as adjuvant therapy after the primary resection. Resections of the liver metastases in these patients were performed at least 2 months after completion of the adjuvant chemotherapy. Adjacent normal colorectal mucosa and adjacent normal liver tissue were also evaluated as a control in each patient. The EGFR mRNA expression and protein expression were evaluated in these 31 patients using real-time RT-PCR and IHC staining.

This study has been approved by the institutional ethics committee and has been performed in accordance with the Declaration of Helsinki.

Table 1 Demographic and clinical parameters of patients with metastatic CRC

Characteristics	Frequency	%
Age		
Mean (range)	66 (45–85)	
Gender		
Male	18	58
Female	13	42
Anatomical Site		
Right colon	5	16
Transverse colon	3	10
Left colon	13	42
Rectum	10	32
Histology		
Well differentiated	16	52
Moderately differentiated	13	42
Mucinous	2	6
Dukes grade		
A	1	3
B	10	32
C	20	65
Number of liver metastases		
Solitary	17	55
Multiple	14	45
Liver synchronicity		
Synchronous	16	52
Metachronous	15	48

Microdissection

Formalin-fixed, paraffin-embedded tumor specimens, and adjacent normal tissues were cut into serial sections with a thickness of 10 μm. For pathological diagnosis, one slide was stained with H&E and evaluated by a pathologist. Other sections were stained with nuclear fast red (NFR, American MasterTech Scientific Inc., Lodi, CA) to enable clear examination of the histology. Laser-capture microdissection (P.A.L.M. Microlaser Technologies AG, Munich, Germany) was performed in all the tumor samples to ensure that only tumor cells were dissected [11]. Adjacent normal colorectal mucosa and liver tumor tissues were dissected from the slide using a scalpel.

RNA isolation and cDNA synthesis

RNA isolation from formalin-fixed paraffin-embedded specimens was done as previously described [12]. In brief, tissue samples were heated at 92ºC for 30 min in 4 M dithiothreitol (DTT)-GITC/sarc (4 M guanidinium isothiocyanate,

50 mM Tris-HCl, pH 7.5, 25 mM EDTA) (Invitrogen; #15577-018). To the tissue suspensions were added 50 µl of 2 M sodium acetate, pH 4.0, followed by 600 µl of freshly prepared phenol/chloroform/isoamyl alcohol (250:50:1). The suspensions were centrifuged at 13,000 rpm for 8 min in a chilled (8°C) centrifuge. The upper aqueous phase was removed and combined with glycogen (10 µl) and 300–400 µl of isopropanol. The tubes were placed at −20ºC for 30–45 min to precipitate the RNA. After centrifugation at 13,000 rpm for 7 min in a chilled (8°C) centrifuge, the supernatant was carefully poured off and the pellet was re-suspended in 50 µl of 5 mM Tris.

After RNA isolation, cDNA was prepared from each sample as described previously [13].

Reverse transcription-PCR

Quantitation of EGFR and an internal reference gene (β-actin) was done using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System (Taqman); Applied Biosystems, Foster City, CA) as described previously [14, 15]. The sequences of the primers and probe used were: for EGFR, Forward 5′- TGCGTCT CTTGCCGGAAT -3′, Reverse 5′- GGCTCACCCTCCAGA AGCTT -3′, Probe 3′- (FAM) ACGCATTCCCTGCCTCG GCTG (TAMRA)-3′; and for β-actin, Forward 5′- TGAG CGCGGCTACAGCTT-3′, Reverse 5′-TCCTTAATGTC ACGCACGATTT-3′, Probe 5′-(FAM) ACCACC ACGGCCGAGCGG(TAMRA)-3′. The PCR reaction mixture consisted of each primer at a concentration of 1,200 nM; 200 nM probe; 0.4 U of AmpliTaq Gold Polymerase; dATP, dCTP, dGTP, and dTTP, each at 200 nM; 3.5 mM MgCl2; and 1× Taqman Buffer A containing a reference dye; to a final volume of 20 µl (all reagents from PE Applied Biosystems, Foster City, CA, USA). Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 46 cycles at 95°C for 15 s and 60°C for 1 min. Gene expression values (relative mRNA levels) are expressed as ratios (differences between the Ct values) between the genes of interest and an internal reference gene (β-actin) that provides a normalization factor for the amount of RNA isolated from a specimen.

Immunohistochemistry

IHC staining for EGFR was done using the EGFR pharmDx kit (DakoCytomation, Carpinteria, CA), according to the manufacturer's instructions. EGFR expression was defined as any membrane staining above background level. Both the primary and metastatic neoplasms were considered positive when >1% of the tumor cells had membranous staining. Cytoplasmic staining without associated membrane staining was reported as negative.

Statistical analysis

The comparisons between median mRNA levels of the primary CRC and the corresponding liver metastases, and between these median mRNA levels and those of the corresponding adjacent normal tissues were assessed using the Wilcoxon signed-rank test. The correlation between the mRNA levels of primary cancers and of liver metastases was assessed using Spearman's rank correlation. Statistical significance was recognized at *P*-values less than 0.05. All values were two-sided.

Results

Median gene expression levels of primary tumor and liver metastases

Median EGFR gene expression levels in primary CRC, corresponding liver metastases, corresponding adjacent normal colon, and liver tissue are shown in Table 2. There were no significant differences in median EGFR mRNA levels between primary CRC and liver metastases (median value: 1.35 vs. 1.24, $P = 0.99$). The median value of EGFR mRNA from normal liver tissue is significantly higher than that from normal colon mucosa (median value: 5.06 vs. 1.39, $P < 0.0001$). EGFR mRNA levels in primary CRC did not significantly differ from that in adjacent normal colon mucosa (1.35 vs. 1.39: $P = 0.37$). On the other hand, EGFR mRNA levels in liver metastases were significantly lower than those in normal adjacent liver tissue (1.24 vs. 5.06: $P < 0.0001$).

Table 2 Median EGFR gene expression levels in primary CRC, corresponding liver metastases, corresponding adjacent normal colon and liver tissues

	Primary site		Metastatic liver site	
	Cancer	Normal	Cancer	Normal
EGFR	1.35 (0.21–4.56) $P = 0.37$	1.39 (0.01–4.19)	1.24 (0.02–4.59) $P < 0.0001$	5.06 (1.64–9.54)

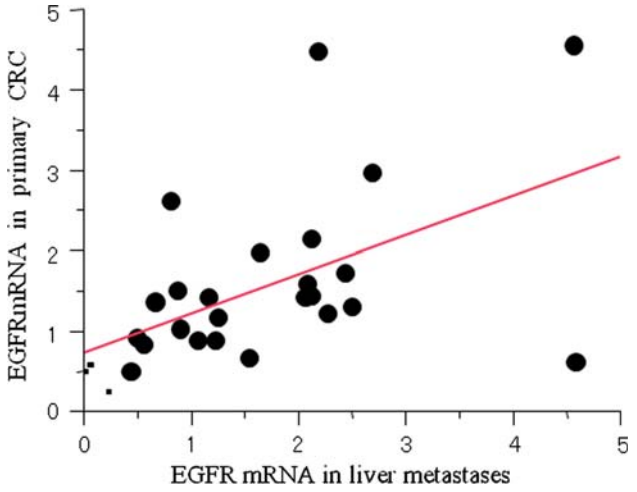

Fig. 1 Correlation of EGFR mRNA levels between primary colorectal tumor and liver metastases. Significant correlation was seen between primary tumor and liver metastases ($r_s = 0.59$, $P = 0.0012$). Gene expression values are expressed as ratios between the EGFR and an internal reference gene (β-actin)

Correlation between primary CRC and liver metastases mRNA level

When matched tissue sets were compared on an individual basis, there was a strong significant correlation for EGFR mRNA expression between primary CRC and corresponding liver metastases ($r_s = 0.59$, $P = 0.0012$) (Fig. 1). The correlation coefficient of EGFR expression between primary tumor and liver metastases was $r_s = 0.65$, $P = 0.017$ in the synchronous metastasis group and $r_s = 0.54$, $P = 0.046$ in the metachronous metastasis group.

Table 3 EGFR immunohistochemical status in primary colorectal cancer and corresponding liver metastases

		Liver metastases	
Primary CRC		Positive	Negative
	Positive	4	3
	Negative	3	21

IHC analysis

Of the 31 primary lesions, 7 (22.6%) were evaluated as EGFR-positive. Of the 31 liver metastatic lesions, 7 (22.6%) were EGFR-positive (Table 3).

Four of the seven primary positive patients showed EGFR-positive reactions in their liver metastases (57.1%). Twenty-one patients were negative in both the primary and the metastatic tumors. Overall, the EGFR status in the primary tumor matched that in paired liver metastases in 25 out of 31 patients (80.6%).

Correlation between mRNA expression and IHC expression

The median mRNA levels in primary CRC in the patients with positive IHC expression did not differ statistically from those in the patients with negative IHC expression (1.44 vs. 1.23: $P = 0.18$). However, in liver metastases, the patients with positive IHC expression showed significantly higher mRNA levels than those with negative IHC expression (2.17 vs. 1.07: $P = 0.013$) (Fig. 2).

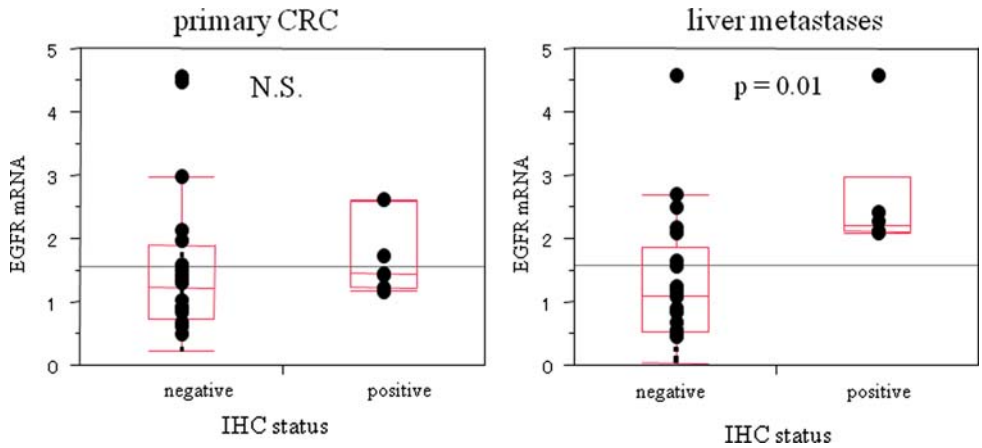

Fig. 2 Comparison between mRNA expression and protein expression in primary CRC and liver metastases. In primary CRC, no difference of mRNA levels was observed between positive and negative protein expression on immunohistochemical evaluation (1.44 vs. 1.23: $P = 0.18$). However, in liver metastases, the patients with positive protein expression showed significantly higher mRNA levels than those with negative expression (2.17 vs. 1.07: $P = 0.013$). Boxes indicate the first and third quartiles (median inside); and bars represent the range of values falling within 1.5 times the interquartile range. Gene expression values are expressed as ratios between VEGF and an internal reference gene (β-actin)

Discussion

Since, there have been few reports demonstrating mRNA expression of EGFR, there is as yet no consensus on the importance of measuring mRNA as a biomarker. Recently, the significance of K-ras status in CRC patients as a biomarker for Cetuximab and Panitumumab, which are monoclonal antibodies against EGFR, has frequently been reported, and showed a marked difference of efficacy between the patients with K-ras wild-type and those with the mutated type [16–20]. The frequency of K-ras mutation in CRC patients was reported to be 20–50% [21–23], but not in the rest of the patients, who have a wild-type K-ras and who show sensitivity to EGFR antibody. Thus, there is a slight possibility that using other biomarkers along with the K-ras status would provide more benefit to the patients treated with Cetuximab or Panitumumab. One of the other candidates for biomarkers is the EGFR mRNA expression level. Vallbohmer et al. reported that CRC patients with a lower EGFR mRNA amount had a longer overall survival than patients with a higher mRNA amount when treated with Cetuximab [7]. Dziadziuszko et al. demonstrated that EGFR mRNA expression was higher in responders to gefitinib, a small-molecule inhibitor of EGFR, than in non-responders in non-small-cell lung cancer patients [8].

In this study, there is a strong correlation in EGFR mRNA levels between primary CRC and corresponding liver metastases. This correlation was marked in synchronous metastases ($P = 0.017$), but was preserved even in metachronous metastases ($P = 0.046$). We have reported similar data in VEGF mRNA expression [24]. However, mRNA expression is not always the same in a primary lesion and its metastases. We previously measured four different gene expressions related to 5-FU metabolism, and showed only a weak correlation in one gene and no correlation in the other three [12]. In a clinical setting, cases of liver metastases that have recurred during the follow-up periods after surgery for primary CRC are often encountered. If mRNA expression from a primary tumor previously operated on could help in determining whether an anti-EGFR drug should be used against liver metastases, it would be benefit of patients. Therefore, RNA extraction from paraffin-embedded samples is useful because it does not require special techniques of sample preservation.

Our data showed that the EGFR mRNA expression in cancer tissue of primary site was relatively lower than in adjacent normal mucosa, although EGFR is considered to be over-expressed in colorectal cancer. Maurer et al. measured EGFR expression in both IHC and northern blot analysis, reported that no difference in EGFR immunostaining was evident between normal colon and cancer, and on mRNA level, it was decreased in cancer [25]. Piazzi et al. measured EGFR mRNA by real-time PCR, reported that mean EGFR mRNA content was significantly higher in normal mucosa than in neoplastic tissue [26]. They also measured activated EGFR by ELISA, showed that activated EGFR was higher in neoplastic tissue than in normal mucosa, and concluded that the EGFR content is frequently lower but more activated in cancer tissue than in paired normal mucosa. These reports support our data of low EGFR mRNA expression in cancer tissue.

To the best of our knowledge, there was no report compared EGFR mRNA expression between primary tumor and corresponding metastatic tumor. The comparison of EGFR immunohistochemistry between primary tumor and metastases were reported several times [27–30]. However, in most of those manuscripts, the expression in primary tumor did not match with that in metastases. Scartozzi et al. evaluated EGFR immunohistochemistry from primary tumors and related metastatic sites in 99 CRC patients, reported that EGFR status in primary CRC was not concordant with that in related metastatic sites, concluded that the detection of the EGFR in primary colorectal cancer could be inadequate for planning therapy with EGFR-targeted monoclonal antibodies [28]. One possible explanation for elucidating the discrepancy between our mRNA data and these IHC data is that the lack of objectiveness in immunohistochemistry. For example, even though the same antibody and the same experimental kit were used, the frequency of EGFR-positive expressions in CRC by IHC varied from 12.3 [31], 53 [28], to 84% [29]. Our data showed EGFR-positive staining in 22.6% of cases, when the same Dako PharmDx kit was used. This discrepancy is probably due to the laboratory conditions, sample conditions, and the personal opinion of each pathologist. In this study, we found a certain correlation between IHC results and mRNA expression levels in liver metastases, but the relationship did not reach statistical significance in the primary tumors. This failure to reach statistical significance is estimated to be due to underpowered sample size, but possibly it may also be due to the instability of IHC. Spindler et al. evaluated the mRNA expression, protein expression by IHC, and the gene copy number using the same samples, and reported a poor correlation between them [32]. Real-time RT-PCR is able to quantify mRNA expression objectively, and if laser-capture microdissection is used, it should enable us to show the precise mRNA expression data from the tumor tissues alone [33]. Our report is of importance because it not only represents the first publication of data comparing EGFR mRNA expression between primary and metastatic tumors but also shows a strong correlation between them.

In summary, we found that, in this set of tumor specimens, there is a positive correlation between EGFR mRNA expression in liver metastases and that in primary tumors, indicating that regulation of this gene is not altered

appreciably during the metastatic process. Further clinical trials are warranted to confirm both this correlation and the significance of EGFR mRNA as a predictive marker for EGFR-targeted drugs.

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