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Downregulation of epidermal growth factor receptor family receptors and ligands in a mutant K-ras group of patients with colorectal cancer

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Abstract. The present study investigated the expression profiles of the epidermal growth factor receptor (EGFR) family, which consists of four transmembrane tyrosine kinase receptors and their eight ligands, in 122 patients with colorectal cancer (CRC) using reverse transcription-quantitative polymerase chain reaction analysis. On comparison of the CRC primary tumor and matched adjacent normal mucosa (ANM) tissue samples, the mRNA expression levels of ErbB3, but not ErbB1, were significantly increased in CRC tissue samples, compared with those in the ANM tissues. The expression levels of the ligands exhibited opposing trends to their corresponding receptors, including EGF, BTC, AREG, EREG and HB-EGF, which were increased in the CRC tissues, whereas NRG1 and NGR2 were decreased in thee CRC tissues, compared with those in the AMN tissues. Subsequently, the present study investigated the frequency of K-ras mutations in the patients with CRC. The K-ras mutations were found to be present in 36.8% (45/122) of the cases, however, no correlation was observed between K-ras mutations and clinicopathological characteristics. In the CRC tissues, the expression levels of the EGFR family receptors and their ligands were determined in wild-type and mutant K-ras CRC cases. The expression levels of ErbB1, ErbB2, ErbB3, BTC, AREG, EREG, NRG1 and NRG2 were significantly decreased in the mutant K-ras cases, compared with those in the wild-type K-ras cases. These results suggested that the tumorigenesis of CRC with wild-type K-ras was mediated through, not only ErbB1, but also through the ErbB2 and ErbB3 pathways. Notably, although ErbB2 does not bind any ErbB ligands, ErbB2 may activate tumorigenesis via a heterodimer, rather than a homodimer. Therefore, the results of the present study suggest that the most effective strategy to target not only ErbB1, but also ErbB2 and ErbB3, is the use of monoclonal antibody treatment.

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Abbreviations: CRC, colorectal cancer; ANM, adjacent normal mucosa; EGFR, epidermal growth factor receptor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; BTC, betacellulin; AREG, amphiregulin; EREG, epiregulin; HB-EGF, heparin-binding-EGF-like growth factor; NRG, neuregulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyl transferase; ACTB, β-actin

Key words: colorectal cancer, adjacent normal mucosa, epidermal growth factor receptor, epidermal growth factor receptor ligands, reverse transcription-quantitative polymerase chain reaction, K-ras

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnose types of cancer worldwide, and accounts for ~9.4% of all malignancies (1). The most effective treatment for CRC is surgery, however, ~60% of patients who undergo curative resection experience local recurrence or distant metastasis (2). Despite advances in surgical techniques and chemotherapeutic options, the survival rate of patients with CRC has not improved substantially, and 20% of patients with CRC succumb to mortality from recurrence of the disease (3). Thus, novel and effective treatments are required to treat CRC. Among adjuvant therapies, chemotherapy, including the use of oxaliplatin, fluorouracil and leucovorin, has been an efficient strategy, however, it remains incapable of preventing recurrence in all patients (4). Therefore, targeting tumor-associated proteins and inhibiting essential processes of the tumor are being extensively investigated.

The proto-oncogene referred to as epidermal growth factor receptor (EGFR) is a well-known tyrosine kinase growth factor receptor (5). The EGFR family includes four distinct receptors: HER1/EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4, and their eight ligands: Epidermal growth factor (EGF), transforming growth factor-α (TGF-α), betacellulin (BTC), amphiregulin (AREG), epiregulin (EREG), heparin-binding-EGF-like growth factor (HB-EGF), neuregulin (NRG1) and NRG2 (5). Binding of a ligand to its receptor and the subsequent receptor hetero- and homo-dimerization leads to a phosphorylation cascade mediated via tyrosine kinases (5). Therefore, in addition to conventional treatments, EGFR family-targeted monoclonal antibodies have been used to treat patients with CRC.

Cetuximab and panitumumab, which are anti-ErbB1 monoclonal antibodies, are widely used in the treatment of CRC (6-8). However, only 20% of patients respond to anti-ErbB1 monotherapy (9).

K-ras is one of the downstream signaling molecules of the EGFR family, and is one of the most frequently mutated oncogenes, with K-ras mutations frequently found in various types of tumor (10). Furthermore, upon K-ras mutation, its downstream signaling pathway operates independently of the EGFR family and its signaling activation by ligands (11). The search for predictive markers to improve clinical outcomes has identified that the presence of a K-ras mutation predicts an adverse response, which has led to routine K-ras assessments prior to anti-ErbB1 therapy (11). However, K-ras mutations are present in only 30-40% of CRC tumors, and a significant proportion of wild-type K-ras patients (50-65%) do not respond to anti-ErbB1 therapy (9). In addition, treatments for targets other than ErbB1 are not yet available for CRC. ErbB2 is targeted by the monoclonal antibody trastuzumab (Herceptin™) in breast cancer (12,13), and therapeutic agents targeting ErbB3, including MM-121, AMG 888, TK-A3, and TK-A4 are available, however, they are not used clinically (14). A detailed evaluation, describing the synchronous modulation of expression between EGFR family receptors and their ligands, in terms of K-ras mutations in CRC tissues, has not been reported.

Therefore, in the present study, the expression profiles of the four EGFR family genes and their eight ligands were examined in CRC tissues and in adjacent normal mucosa (ANM) tissues from 122 patients using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Furthermore, K-ras gene mutations in CRC tissues were investigated, and the expression of EGFR family genes and their ligands were compared between cases of CRC exhibiting wild-type and mutant K-ras.

Materials and methods

Patients and samples. Tissue specimens were obtained from 122 patients (60 males and 62 females; 30-91 years old) who underwent resections for CRC at Sagamihara National Hospital (Sagamihara, Japan) between 2010 and 2013 (Table I). As control samples, resected specimens (5 mm^3) were obtained from the ANM at the margin of the CRC primary tumor (≥10 cm distance from the tumor). All specimens were confirmed as ANM or CRC by a pathologist. The ANM specimens were further confirmed to be free of cancer cells by the pathologist. The diameters of the primary tumors, numbers of lymph nodes with metastases and development of distant metastases were classified according to the Union for International Cancer Control TNM staging system (15). The study procedures were approved by the Research Ethical Committee of Sagamihara Hospital (Sagamihara, Japan). Informed consent for the present study was obtained from all patients and the patient's families prior to commencement.

Preparation of tissue specimens. Following surgery or biopsy, half of the ANM and CRC specimens were immediately soaked in RNAlater® RNA Stabilization Reagent (Qiagen, Hilden, Germany) and stored at -80˚C until performing RNA extraction. The other half were used for pathological examinations.

RNA extraction and cDNA synthesis. The ANM and CRC specimens were homogenized in QIAzol Lysis reagent with homogenizer beads (Qiagen) using a vortex-type homogenizer (Shakeman 2; BioMedical Science, Co., Ltd., Tokyo, Japan). Total RNA was extracted using an RNeasy® Lipid Tissue kit (Qiagen). The quality and concentration of total RNA were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). The freshly isolated total RNA from the ANM and CRC tissues was converted into cDNA using a PrimeScript™ RT Reagent kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's instructions.

RT-qPCR. RT-q-PCR was performed using a Bio-Rad CFX96 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. The thermal cycling conditions were 30 sec at 95˚C for one cycle, 50 cycles of 1 sec at 95˚C and 5 sec at 60˚C using the Bio-Rad CFX96 system (Bio-Rad Laboratories, Inc.). Results were normalized to the expression of β-actin (ACTB) and the absolute copy numbers of unknown samples were calculated by comparing the threshold cycles with the corresponding standard curve, based on the ΔΔCq method (16). Specific primers for the EGFR family members (ErbB1, ErbB2, ErbB3 and ErbB4), EGFR ligands (EGF, TGF-α, BTC, AREG, EREG, HB-EGF, NRG1 and NRG2) and housekeeping genes (HKGs), including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase (HPRT) and ACTB were purchased from Takara Bio, Inc. (Table II).

The amplified products were cloned into the pGEM®-T Easy Vector system (Promega Corporation, Madison, WI, USA). The same plasmid was linearized by enzymatic digestion at 37˚C for 2 h (Not I; Takara Bio, Inc.) and used as a quantification standard. The sequences were confirmed by DNA sequencing using a CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). The quality and concentration of the quantification standard were validated using the Agilent 2100 Bioanalyzer. The qPCR reaction mixture consisted of 5 μl SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc.), 3.5 μl RNase/DNase-free water, 0.5 μl 5 μM primer mix and 1 μl cDNA in a final volume of 10 μl. The cycle number for RT-qPCR was 50. All experiments were performed in duplicate.

Genomic DNA extraction and K-ras mutation analysis. Genomic DNA was extracted from 500 mg of the samples

Table I. Clinicopathological characteristics and percentages of K-ras mutations in patients with CRC.

Clinicopathological characteristic	Number of patients (%)	Patients with wild-type K-ras CRC (%)	Patients with mutant K-ras CRC (%)
Total cases	122 (100.0)	77 (63.2)	45 (36.8)
Gender			
Male	60 (49.2)	42 (70.0)	18 (30.0)
Female	62 (50.8)	35 (56.5)	27 (43.5)
Age (years)			
<65	35 (28.7)	22 (62.9)	13 (37.1)
≥65	87 (71.3)	55 (63.2)	32 (36.8)
Site of tumor			
Rectum	27 (22.1)	17 (63.0)	10 (37.0)
Sigmoid colon	37 (30.3)	24 (64.9)	13 (35.1)
Descending colon	8 (6.6)	6 (75.0)	2 (25.0)
Transverse colon	11 (9.0)	9 (81.8)	2 (18.2)
Ascending colon	30 (24.6)	16 (53.3)	14 (46.7)
Cecum	9 (7.4)	5 (55.6)	4 (44.4)
T stage			
pT1/T2	18 (14.8)	10 (55.6)	8 (44.4)
pT3/T4	104 (85.2)	67 (64.4)	37 (35.6)
N stage			
pN0	51 (41.8)	37 (72.5)	14 (27.5)
pN1–3	71 (58.2)	40 (56.3)	31 (43.7)
M stage			
M0	103 (84.4)	64 (62.1)	39 (37.9)
M1	19 (15.6)	13 (68.4)	6 (31.6)
Clinical stage			
I	13 (10.7)	9 (69.2)	4 (30.8)
II	38 (31.1)	28 (73.7)	10 (26.3)
III	52 (42.6)	27 (51.9)	25 (48.1)
IV	19 (15.6)	13 (68.4)	6 (31.6)

CRC, colorectal cancer; T stage, primary tumor diameter; N stage, number of lymph nodes; M stage, metastasis.

using a Quick Gene DNA tissue kit (Kurabo, Osaka, Japan). Mutations in K-ras codons 12 and 13 were detected using a multiplex PCR-Luminex method-based MEBGEN Mutation kit (Medical and Biological Laboratories, Nagoya, Japan) according to the manufacturer's instructions.

Statistical analyses. To compare expression levels in the ANM and CRC tissues, Mann-Whitney U and Wilcoxon signed-rank tests were used. All statistical analyses were performed using Prism5 for Windows (GraphPad Software, Inc, San Diego, CA, USA). Data are presented as the mean values. P<0.05 was considered to indicate a statistically significant difference.

Results

Selection of appropriate HKGs in ANM and CRC tissues. The present investigated the mRNA levels of three HKGs in the ANM and CRC tissues using RT-qPCR (Table III). The copy numbers in the experiments were compared using 1 μg total RNA. The mRNA expression levels of ACTB were similar in the ANM and CRC tissues (P=0.957). By contrast, the mRNA expression levels of GAPDH and HPRT were significantly increased in the CRC tissues, compared with those in the ANM tissues (P<0.001). Therefore, ACTB was selected as the internal standard gene in the present study. The calculated copy numbers were normalized based on the copy numbers of ACTB.

Gene expression profiles of EGFR family members and their ligands in ANM and CRC tissues. The present study determined the mRNA expression levels of EGFR family members and their ligands in the ANM and CRC tissues using RT-qPCR (Fig. 1). Among the EGFR family members, the mRNA level of ErbB2 was the highest (Fig. 1A). The mRNA expression levels of ErbB1 and ErbB3 were approximately half of the mRNA expression level of ErbB2. In addition, low expression

Table II. Primer sequences used for reverse transcription-quantitative polymerase chain reaction analysis.

Target	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT
HPRT	GGCAGTATAATCCAAAGATGGTCAA	GTCAAGGGCATATCCTACAACAAAC
ACTB	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA
ErbB1	GGTGCGAATGACAGTAGCATTATGA	AAAGGTGGGCTCCTAACTAGCTGAA
ErbB2	CAGGCACCGCAGCTCATCTA	TCCCAGGTCACCATCAAATACATC
ErbB3	CCCAGCATCTGAGCAAGGGTA	TTTAGGCGGGCATAATGGACA
ErbB4	TGATAGGCCGTTGGTTGTCTGA	CCAGGTAGACATACCCAATCCAGTG
EGF	CAACCAGTGGCTGGTGAGGA	GAGCCCTTATCACTGGATACTGGAA
TGF-α	AGATAGACAGCAGCCAACCCTGA	CTAGGGCCATTCTGCCCATC
BTC	CTTCACTGTGTGGTGGCAGATG	ATGCAGTAATGCTTGTATTGCTTGG
AREG	GTGGTGCTGTCGCTCTTGATACTC	TCAAATCCATCAGCACTGTGGTC
EREG	GTGATTCCATCATGTATCCCAGGAG	AGATGCACTGTCCATGCAAACAA
HB-EGF	GGGCATGACTAATTCCCACTGA	GCCCAATCCTAGACGGCAAC
NRG1	TCGGTGTGAAACCAGTTCTGAATA	TCTCCAGAATCAGCCAGTGATG
NRG2	ACCCTAGGCTTGGAGCTGGA	CCATTCGGGTAGCTGTGTCTTTATC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyl transferase; ACTB, β-actin; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; BTC, betacellulin; AREG, amphiregulin; EREG, epiregulin; HB-EGF, heparin-binding-EGF-like growth factor; NRG, neuregulin.

Table III. Comparisons of the expression levels of HKGs between ANM and CRC tissues.

HKG	ANM	CRC	P-value
GAPDH ($x10^6$)	11.4±3.46	21.1±4.30	<0.001
HPRT ($x10^4$)	28.7±7.48	76.7±1.91	<0.001
ACTB ($x10^6$)	19.7±8.91	20.3±8.29	0.957

Data are presented as the mean ± standard deviation. P-values were determined using the Wilcoxon matched-pairs signed rank test. HKG, housekeeping gene; ANM, adjacent normal mucosa; CRC, colorectal cancer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyl transferase; ACTB, β-actin.

levels of ErbB4 were observed in the CRC and ANM tissues. The mRNA expression levels of ErbB3 were significantly increased in the CRC tissues, compared with those in the ANM tissues (P<0.005), whereas the mRNA expression levels of ErbB1 were significantly decreased in the CRC tissues, compared with those in the ANM tissues (P<0.005). No significant differences were observed in the expression levels of ErbB2 and ErbB4 between the ANM and CRC tissues.

Among the EGFR family ligands, the mRNA expression levels of EGF, BTC, AREG, EREG and HB-EGF were significantly increased in the CRC tissues, compared with those in the ANM tissues (P<0.005, P<0.05, P<0.001, P<0.001 and P<0.001, respectively), and the mRNA expression levels of NRG1 and NRG2 were significantly decreased in the CRC tissues, compared with those in the ANM tissues (P<0.001; Fig. 1B). However, no difference was observed in the expression levels of TGF-α between the ANM and CRC tissues.

Detection of K-ras mutations in CRC tissues. The present study subsequently evaluated the genomic DNA from the tumor specimens for mutations in the K-ras gene (Table I). Mutations in K-ras codons 12 and 13 were detected in 45 of the 122 (36.8%) samples. Comparisons of the K-ras mutations and clinicopathological characteristics revealed that the mutation rate was higher in females than in males. In terms of the tumor site, K-ras mutation rates were higher in the ascending colon and cecum, and lower in the descending colon and transverse colon. The pT1/T2 T stage, pN1-3 N stage and clinical stage III groups exhibited K-ras mutation rates of >40%.

Gene expression profiles of EGFR family members and their ligands in CRC tissues with wild-type and mutant K-ras. The present study determined the mRNA expression levels of EGFR family members and their ligands in CRC tissues exhibiting wild-type or mutant K-ras using RT-qPCR (Fig. 2). The mRNA expression levels of ErbB1, ErbB2 and ErbB3, but not ErbB4, were significantly decreased in the mutant K-ras group, compared with those in the wild-type K-ras group (P<0.05; Fig. 2A). Notably, the expression levels of the ErbB1-binding ligands (BTC, AREG and EREG) and ErbB3-binding ligands (NRG1 and NRG2) were significantly decreased in the mutant K-ras group, compared with those in the wild-type K-ras group (P<0.05, P<0.005, P<0.005, P<0.005 and P<0.05, respectively; Fig. 2B). By contrast, no significant differences in the expression levels of EGF, TGF-α or HB-EGF were observed between the mutant and wild-type K-ras groups.

Discussion

In the present study, the mRNA expression levels of the four EGFR family members and their eight ligands were analyzed

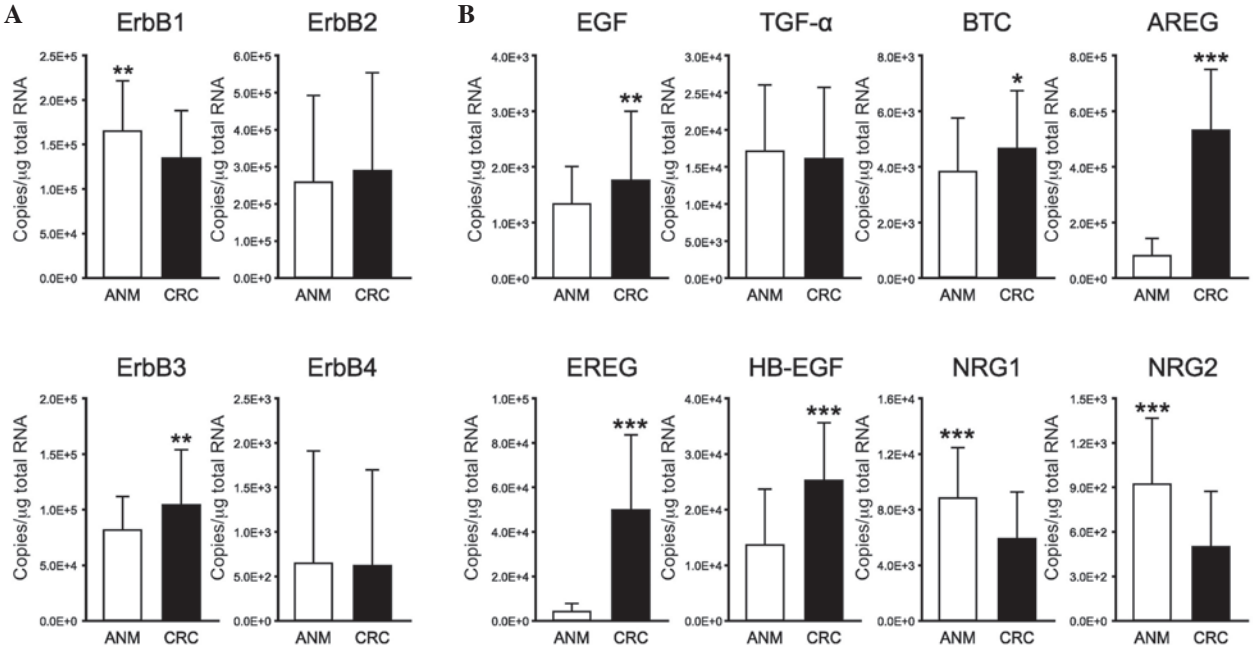

Figure 1. Gene expression profiles of EGFR family members and their ligands in ANM and CRC tissues. mRNA expression levels of (A) EGFR family members and their (B) ligands in ANM and CRC tissues were examined using reverse transcription-quantitative polymerase chain reaction. The ACTB gene was used as an internal control. All experiments were performed in duplicate. Data are presented as the mean ± standard deviation (n=122). *P<0.05, **P<0.005 and ***P<0.001 (Wilcoxon signed-rank test). (A) mRNA levels of ErbB3 were significantly increased in CRC tissues, compared with ANM tissues, whereas mRNA expression levels of ErbB1 were significantly decreased in CRC tissues, compared with those in ANM tissues. The mRNA expression level of ErbB2 exhibited the highest level of expression among the EGFR family members in the ANM and CRC tissues. (B) mRNA expression levels of EGF, BTC, AREG, EREG and HB-EGF were increased significantly in the CRC tissues, compared with those in the ANM tissues, whereas the mRNA expression levels of NRG1 and NRG2 were decreased significantly in the CRC tissues, compared with those in the ANM tissues. EGFR, epidermal growth factor receptor; ANM, adjacent normal mucosa; CRC, colorectal cancer; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; BTC, betacellulin; AREG, amphiregulin; EREG, epiregulin; HB-EGF, heparin-binding-EGF-like growth factor; NRG, neuregulin.

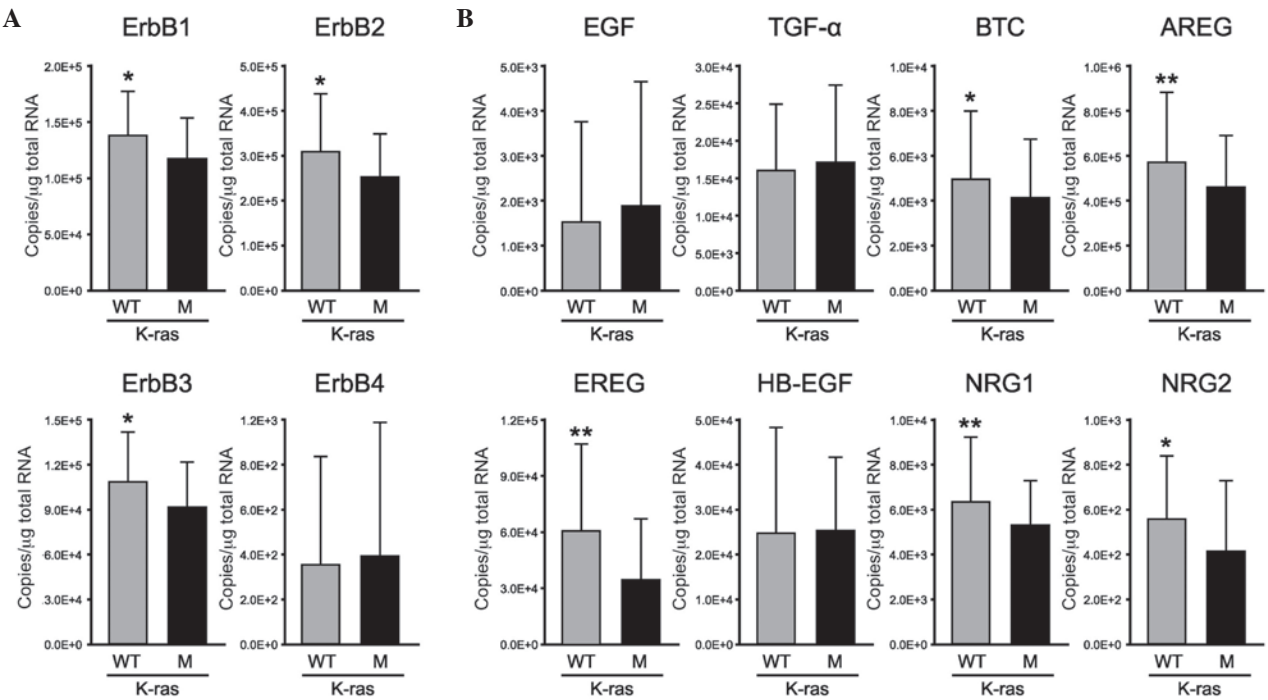

Figure 2. Gene expression profiles of EGFR family members and their ligands in CRC tissues with wild-type or mutant K-Ras. In the colorectal cancer tissues, the mRNA expression levels of (A) EGFR family members and their (B) ligands in the wild-type K-ras (gray; n=77) and mutant K-ras group (black; n=45) were measured using reverse transcription-quantitative polymerase chain reaction. The ACTB gene was used as an internal control. All experiments were performed in duplicate. Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.005 (Mann-Whitney U-test). (A) Expression levels of ErbB1, ErbB2 and ErbB3 were significantly decreased in the mutant K-ras group, compared with the wild-type K-ras group. (B) Expression levels of the ErbB1-binding ligands (BTC, AREG and EREG) and ErbB3-binding ligands (NRG1 and NRG2) were significantly decreased in the mutant K-ras group, compared with the wild-type K-ras group. EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; BTC, betacellulin; AREG, amphiregulin; EREG, epiregulin; HB-EGF, heparin-binding-EGF-like growth factor; NRG, neuregulin; WT, wild-type; M, mutant.

in tissue specimens from 122 patients with CRC using RT-qPCR. To perform accurate analyses, the present study first compared the mRNA levels of three HKGs (GAPDH, HPRT and ACTB), between the ANM and CRC tissues. Certain studies have reported that the expression levels of HKGs under hypoxic conditions vary widely (17-19). Zhong and Simons reported that mRNA expression levels of GAPDH in cell lines are increased by 21.2-75.1% under hypoxic conditions (20). In HKG comparison study of a large panel of cancer types, HPRT was suggested as the single optimal reference gene (21). Another report suggested that ACTB is the most stable gene in diabetic glomeruli and primary mesangial cells (22). In the present study, the expression levels of ACTB were almost equal in the ANM and CRC tissues. However, compared with the ANM tissues, >2-fold increases in the expression levels of GAPDH and HPRT were observed in the CRC tissues. Therefore, these results suggested that ACTB was the most suitable HKG for the analyses of patients with CRC in the presnt study.

Overexpression of EGFR family members has been associated with malignant transformation, as well as poor clinical outcomes in CRC (23). ErbB1 is overexpressed in patients with CRC (24), and high expression levels of ErbB3 have been observed in 70% of primary CRC tumors (25). In the present study, the mRNA expression levels of ErbB3 were significantly increased in CRC tissues, compared with those in ANM tissues, whereas the mRNA expression levels of ErbB1 were significantly decreased in CRC tissues, compared with those in ANM tissues. Koenders *et al* reported similar results of low expression levels of ErbB1 in CRC tissues, and concluded that the lower ErbB1 content in CRC is caused by downregulation of the receptor by a locally produced ligand (26). In the present study, the expression levels of the EGFR ligands exhibited opposing trends to their corresponding receptors, including EGF, BTC, AREG, EREG and HB-EGF, which were increased, and NRG1 and NRG2, which were decreased in the CRC tissues, compared with those in the AMN tissues. Taken together, the expression levels of EGFR family members and their ligands were altered between the ANM and CRC tissues.

To perform a detailed evaluation of the expression of EGFR in CRC, determination of K-ras gene mutations is important, as mutation of K-ras leads to the downstream signaling pathway operating independently of the EGFR family and its signaling activation by ligands. Previous studies have concluded that K-ras mutations lead to poor survival rates in patients, particularly following treatment with anti-ErbB1 antibodies and chemotherapy (27,28). Therefore, the present study evaluated genomic DNA from tumor specimens for mutations in the K-ras gene, and compared the findings with the clinicopathological characteristics of the patients to investigate the function of the EGFR family in CRC. In total, 45 mutations (36.8%) were found in the 122 samples, including common mutations in codons 12 and 13. In a previous cohort study, 37% (271/737) of patients with CRC were found to have mutations in codons 12 and 13 in the K-ras gene (29). Taback *et al* also reported that mutation of the K-ras allele at codons 12 or 13 occurred in 42% (30/72) of paraffin-embedded primary CRC tissues from a cohort of patients (30). Therefore, the incidence of K-ras mutations in the present study was concordant with those reported in previous studies of European and American patients. Comparisons of K-ras mutations with the clinicopathological characteristics revealed that the mutation rate was >10% higher at clinical stage III, compared with the total mean. Yunxia *et al* reported only tumor differentiation as potentially correlated with K-ras mutations (31), whereas another study suggested no correlation with clinicopathological parameters (32). In the present study, although no significant correlation between K-ras mutations and clinicopathological characteristics were identified, further investigations with a larger sample size are required.

Based on the K-ras mutations, the present study analyzed the expression levels of EGFR family members and their ligands in the CRC tissues. With the exception of ErbB4, the expression levels of EGFRs were decreased significantly in the mutant K-ras group, compared with those in the wild-type K-ras group, and the expression pattern of the ligands exhibited a similar trend. These results suggested enhancement of EGFR signaling without the expression of EGFR ligands in the patients with CRC and K-ras mutations. Furthermore, these results indicated that tumorigenesis of CRC with wild-type K-ras was mediated, not only ErbB1, but also by ErbB2 and ErbB3. In particular, although ErbB2 does not bind to any of the ErbB ligands, its expression levels in the present study were different between the wild-type and mutant K-ras groups. Therefore, it is possible that ErbB2 activates tumorigenesis as a heterodimer and not a homodimer. Another report suggested this possibility and found that the ErbB2-ErbB3 heterodimer is the most potent ErbB pair, with respect to the strength of the interaction, ligand-induced tyrosine phosphorylation and downstream signaling, and functions as an oncogenic unit (33,34). Using ErbB3 knockdown mice, Lee *et al* demonstrated that ErbB3 is essential in supporting intestinal tumorigenesis, and suggested that ErbB3 may be a promising target for the treatment of CRC (35). Trastuzumab is already used for the treatment of ErbB2-overexpressing breast cancer, which binds to a ErbB2 region that is not involved with receptor dimerization (36). However, clinical application has not been assessed in CRC patients due to the lack of clinical proof-of-concept (14). The results of the present study suggested that the most effective strategy to specifically target ErbB1, and also ErbB2 and ErbB3 is monoclonal antibody treatment.

In conclusion, the present study analyzed the expression profiles of the four EGFR family members and their eight ligands in ANM and CRC tissues of 122 patients using RT-qPCR. In addition, K-ras mutation analyses were performed. The results demonstrated that the expression levels of ErbB1, ErbB2, ErbB3, BTC, AREG, EREG, NRG1 and NRG2 were significantly decreased in the mutant K-ras group, compared with those in the wild-type K-ras group. Therefore, the results suggested that the enhancement of tumorigenesis was activated not only by ErbB1, but also by ErbB2 and ErbB3. The mRNA expression levels of ErbB3 were significantly increased in the CRC tissues, compared with those in the ANM tissues, whereas those of ErbB1 were significantly decreased in the CRC tissues, compared with those in the ANM tissues. Together these observations likely suggest that all ErbB2, ErbB3 and ErbB1 could be potential targets for the treatment of CRC.

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