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Analysis of Epidermal Growth Factor Receptor Expression and *KRAS* Mutations in Gastric Cancer : A Single-institution Retrospective Analysis

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ABSTRACT

Background : Epidermal growth factor receptor (EGFR) is expressed in many solid cancers and is a potential target for therapeutic agents. Mutations of Kirsten rat sarcoma viral oncogene homolog (*KRAS)* are associated with resistance to EGFR inhibitors in solid tumors. However, the clinical relevance of EGFR expression and *KRAS* mutations in gastric cancer is unknown. We used standard methods to examine the clinical significance of these biomarkers in gastric cancer.

Methods : The EGFR status and *KRAS* mutations in gastric cancer tissues from 98 patients were evaluated, and their relation to clinicopathological characteristics was examined.

Results : The expression of EGFR was found in 78 cases (79.6%), and *KRAS* mutations were detected in 5 cases (5.1%). The expression of EGFR was significantly correlated with Borrmann type ($P = 0.004$). Mutations of *KRAS* were associated with tumor classification ($P = 0.013$). EGFR expression was not significantly associated with overall survival. Although expression of wild-type *KRAS* tended to be associated with a poor prognosis, this association was not statistically significant ($P = 0.095$).

Conclusions : Among a group of patients with gastric cancer the frequency of EGFR expression was high but that of *KRAS* mutations was low. These biomarkers are not associated with prognosis.

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Key words : epidermal growth factor receptor, *KRAS*, gastric cancer

INTRODUCTION

Gastric cancer, as the fourth most common cancer and the second most common cause of cancer death worldwide, remains a major health problem[1]. The main therapy is surgical treatment, yet, for advanced cases, 5-year survival rates remain poor, as do prognoses for unresectable or metastatic cases. Although combination chemotherapy regimens including fluorouracil or platinum have greater survival benefits than does optimal supportive care for patients with advanced gastric cancer, outcomes remain poor[2]. Therefore, these patients require more effective agents are treatment.

Epidermal growth factor receptor (EGFR), a member of the ErbB protein family, is a transmembrane receptor-type tyrosine kinase involved in tumor cell proliferation, survival, adhesion, migration, differentiation, and angiogenesis[3]. Upon ligand stimulation, EGFR forms either homodimers or heterodimers, resulting in activation of the cytoplasmic domain[4]. Stimulation of EGFR with epidermal growth factor (EGF) or transforming growth factor-α promotes cell proliferation in various systems, including the

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gastrointestinal tract[5].

Expression of EGFR has been thought to be associated with patient survival in various types of cancer. For example, in non-small cell lung cancer and colorectal cancer, increased EGFR expression is associated with a more advanced stage and a poor prognosis[6,7]. Importantly, molecularly targeted therapies against EGFR and vascular endothelial growth factor receptor have improved treatment outcomes of patients with colorectal cancer. However, other than trastuzumab, molecularly targeted agents are rarely used to treat gastric cancer. Molecularly based approaches are expected to be critical for predicting clinical outcomes and guiding treatment strategies in patients with gastric cancer[8-11]. Although the expression of EGFR in gastric cancer has been well studied, the results have been variable[12-14]. Therefore, further studies of EGFR expression in gastric cancer and subsequent development of specific EGFR-targeted agents are needed to advance treatment strategies for gastric cancer.

The Kirsten rat sarcoma viral oncogene homolog gene (*KRAS*), a member of the *RAS* family, plays an important role as a molecular switch in the EGFR-RAS-RAF–mitogen-activated protein kinase pathway. Recently, *KRAS* mutations have been shown to predict the ineffectiveness of molecularly targeted therapy for solid tumors. Indeed, the *KRAS* mutation status is associated with resistance to EGFR inhibitors. Although several reports have described the presence of *KRAS* mutations in gastric cancer, the clinical significance of these mutations has not been clarified[15-17].

These findings suggest that determining EGFR expression and *KRAS* mutations could be important for assessing prognosis and identifying patients who might be treated with EGFR-targeted therapies. Therefore, in the present study, we used standard methods to examine the frequency of EGFR expression and *KRAS* mutations in advanced gastric cancer. We also examined the relationships among EGFR expression, *KRAS* mutations, clinicopathological characteristics, and survival.

METHODS

Patient characteristics

The subjects were 98 patients with advanced gastric cancer who underwent gastrectomy at The Jikei University Kashiwa Hospital,Chiba, Japan, from January 2006 through December 2010. The following clinicopathological variables were evaluated by reviewing medical and pathological records : age, sex, histological subtype, lymphatic invasion, vascular invasion, invasion depth, lymph node metastasis, and pathological stage. Cancer was staged according to the Union for International Cancer Control Staging System, 7th edition. Clinical outcomes were determined from the date of surgery until death or July 2, 2013, with a follow-up period ranging from 1 to 89 months (mean, 33.9 months). Only 1 case was lost to follow up and regarded as censored data ; this case was included in the survival analysis. This study was approved by the institutional review board of The Jikei University Hospital. Written informed consent was obtained from all patients enrolled in this study.

EGFR expression analysis

Fresh tissues were fixed in neutral buffer formalin for 24 hours, followed by infiltration with melted paraffin wax. Following histologic evaluation to identify and exclude necrotic and hemorrhagic areas, 4-μm-thick tissue sections were cut from the paraffin blocks and used for immunohistochemistry staining to detect EGFR with an EGFR PharmDx kit (Dako Japan, Tokyo, Japan) according to the manufacturer's instructions. Reactivity was scored as 0 when there was no membranous reactivity within the tumor or as positive when there was reactivity above background signals within the tumor cell membranes. Evaluation of all immunohistochemistry slides was performed by 2 pathologists of our hospital. Positive staining was classified by intensity as 1+ if weak (faint brown membranous staining), 2+ if moderate (brown membranous staining of intermediate darkness producing a complete or incomplete circular outline of the neoplastic cell), or 3+ if strong (dark brown or black membranous staining, producing a thick complete or incomplete circular outline of the neoplastic cell). We defined score 0 as negative expression and score 1+, 2+, or 3+ as positive expression, as has been performed in colorectal cancer[18].

The percentage of cells exhibiting each level of staining intensity (1+, 2+, or 3+) was determined when the intensity of EGFR staining was heterogeneous[19]. Typical staining intensities for EGFR are shown in Figure 1.

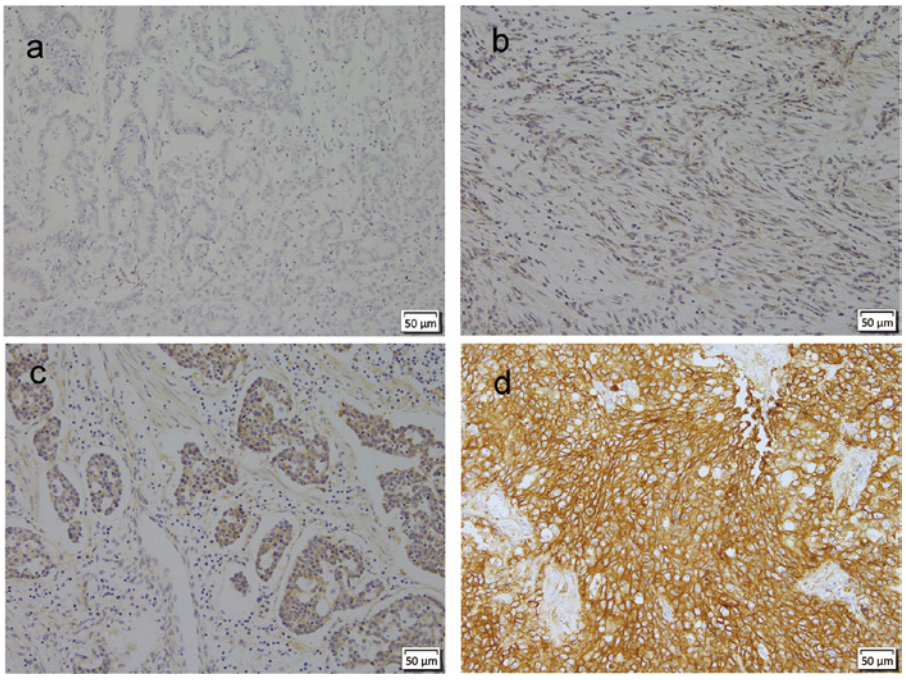

Fig. 1. Typical examples of immunohistochemical staining for EGFR. a: score 0; b: score 1+; c: score 2+; d: score 3+. Original magnification, 20×.

KRAS mutation analysis

Samples of DNA were extracted from formalin-fixed tumor tissue sections. Tumor cell-rich areas in hematoxylin and eosin section were marked under a microscope, and tissue was scratched from the area of another deparaffinized unstained section. DNA from pieces of the scratched tissue sample was isolated using the QIAamp DNA FFPE Tissue Kit (QIAGEN KK, Tokyo, Japan). Per sample, FFPE sections were used for three things that have been sliced into 10 μm. Isolation and purification process of DNA, according to the instructions that came with the kit. The final elution from the spin column was performed with 100 μL of a Tris-ethylenediaminetetraacetic acid buffer. Screening of tumor DNA for *KRAS* mutations in codon 12 or 13 was performed with direct sequencing. The 107-bp region in exon 2 of *KRAS* that encompasses the mutation hotspots in codons 12 and 13 was amplified with the polymerase chain reaction (PCR) using the c-Ki-*ras*/12 primer set (c-Ki-*ras*/12 forward, 5′-GACTGAATATAAACTTGTGG-3′; c-Ki-*ras*/12 reverse, 5′-CTATTGTTGGATCATATTCG-3′; Takara Bio Inc., Otsu, Japan) and *Taq* polymerase with 3′-exonuclease activity (TaKaRa *Ex Taq*; Takara Bio Inc.). The PCR conditions, before and after denaturation for 10 minutes at 95°C, conducted 40 cycles 20 seconds at 94°C, 20 seconds at 60°C, for 30 seconds at 72°C as 1 cycle, and final extension of 10 minutes at 72°C. The reverse primer was used for cycle sequencing reactions. Sequencing analysis was performed with a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

The association between clinicopathological characteristics and EGFR expression or *KRAS* mutations were assessed with the Wilcoxon signed-rank test, chi-square test, or Fisher's exact test when appropriate. The survival rate was calculated with the Kaplan-Meier method, and statistical analysis was performed with the log-rank test. Differences or associations with a p value of less than 0.05 were considered significant.

Results

The mean age of the patients was 68.5 years (range, 34-89 years) (Table 1). The 98 adenocarcinomas consisted of 18 well-differentiated tumors, 30 moderately differentiated tumors, 38 poorly differentiated tumors, and 12 signet ring cell tumors. The expression of EGFR was positive in 78 cases (79.6%) and negative in 20 cases (20.4%) (Table 2).

Table 1. Patient characteristics

		Patients (N = 98) Number	%
Age (years)		68.5 ± 11.1	
Sex	Female	39	39.8
	Male	59	60.2
Tumor classification	T2	5	5.1
	T3	41	41.8
	T4	52	53.1
Borrmann's type	1	3	3.1
	2	24	24.5
	3	54	55.1
	4	16	16.3
	5	1	1.0
Node classification	N0	17	17.3
	N1	21	21.4
	N2	29	29.6
	N3	31	31.6
Histological grade	Well-differentiated	18	18.4
	Moderately differentiated	30	30.6
	Poorly differentiated	38	38.8
	Signet ring cell	12	12.2
Lymphatic invasion	0	8	8.2
	1+	30	30.6
	2+	34	34.7
	3+	26	26.5
Vascular invasion	0	20	20.4
	1+	43	43.9
	2+	27	27.6
	3+	8	8.2
TNM	I B	3	3.1
	II A	6	6.1
	II B	20	20.4
	III A	14	14.3
	III B	11	11.2
	III C	14	14.3
	IV	30	30.6
Postoperative chemotherapy	Yes	58	59.2
	No	40	40.8

The positive staining for EGFR was classified as 1+ in 42 patients, 2+ in 11 patients, and 3+ in 25 patients. The expression of EGFR was significantly correlated only with Borrmann type cancer, and the percentage of patients with positive staining for EGFR was significantly lower in patients with type 4 cancer (P = 0.004). Other clinicopathological characteristics, including age, sex, tumor classification, node classification, histological grade, lymphatic invasion, vascular invasion, and TNM classification, were not significantly correlated with EGFR expression.

The expression of EGFR (negative or positive) was not related to overall survival (Fig. 2a), and no associations were observed between overall survival and any subgroup of EGFR expression (i.e., score 0, 1+, 2+, or 3+ ; Fig. 2b).

Mutations of KRAS were found in 5 patients (5.1%) (Table 2). Interestingly, the frequency of *KRAS* mutations was significantly higher in patients with T3 cancers (P = 0.013) than in patients with other cancers (Table 2). No other associations were found between clinicopathological characteristics and *KRAS* mutational status. While patients with wild-type *KRAS* tended to have worse overall survival (Fig. 2c), this association was not statistically significant (P = 0.095). The clinicopathological features of the 5 cases with *KRAS* mutations are listed in Table 3.

All 5 cases with *KRAS* mutations were found in patients with lymphatic invasion and vascular invasion ; however, these associations were not statistically significant (Table 3). We also found no association between *KRAS* status and EGFR expression.

All detected *KRAS* mutations occurred at codons 12 or 13 of exon 2. Of the 5 mutations, 3 occurred at codon 13, including 2 G-to-A transversion mutations in the second base and one G-to-T transversion mutation in the first base. The 2 mutations occurring at codon 12 were G-to-A transversion mutations in the second base.

DISCUSSION

The present study found that a high percentage of patients had gastric cancer tumors expressing EGFR but that a low percentage of patients had tumors with mutated *KRAS*. These data provide insights into the potential use of EGFR-targeted therapies for the treatment of gastric cancer.

The frequency of EGFR expression in gastric cancer

Table 2. Comparison of clinicopathological parameters of EGFR expression status and KRAS mutation status

Clinicopathological feature		EGFR expression					*KRAS* status		
		Negative	Positive			*P*-value	Wild-type	Mutated	*P*-value
			1+	2+	3+				
Number of patients		20	42	11	25		93	5	
Age (years)		67.2 ± 9.9	68.9 ± 11.3			0.422	68 ± 11.0	78 ± 7.0	0.050
Sex	Female	7	16	4	12	0.623	37	2	1.000
	Male	13	26	7	13		56	3	
Tumor classification	T2	1	1	1	2	0.981	5	0	0.013
	T3	4	19	5	13	T2 vs T3+T4	36	5	T2+T4 vs T3
	T4	15	22	5	10		52	0	
Borrmann's type	1	0	1	0	2	0.004	2	1	0.804
	2	3	10	2	9	1-3 + 5 vs 4	23	1	1-3 + 5 vs 4
	3	9	22	9	14		51	3	
	4	8	8	0	0		16	0	
	5	0	1	0	0		1	0	
Node classification	N0	5	7	1	4	0.495	16	1	1.000
	N1	4	8	3	6	N0 vs N1-N3	20	1	N0 vs N1-N3
	N2	3	12	4	10		27	2	
	N3	8	15	3	5		30	1	
Histological grade	well	4	5	2	7	0.161	17	1	0.230
	mod	3	11	7	9	well+mod vs poor+sig	27	3	well+mod vs poor+sig
	poor	8	21	1	8		37	1	
	sig	5	5	1	1		12	0	
Lymphatic invasion	0	1	2	1	4	0.718	8	0	1.000
	1+	5	17	2	6	0 vs 1+-3+	29	1	0 vs 1+-3+
	2+	10	14	5	5		31	3	
	3+	4	9	3	10		25	1	
Vascular invasion	0	3	6	3	8	0.718	20	0	0.553
	1+	8	22	3	10	0 vs 1+-3+	38	5	0 vs 1+-3+
	2+	7	12	4	4		27	0	
	3+	2	2	1	3		8	0	
TNM	I B	1	0	1	1	0.964	3	0	0.660
	II A	1	2	0	3	I+II vs III+IV	5	1	I+II vs III+IV
	II B	4	11	2	3		19	1	
	III A	1	5	2	6		12	2	
	III B	1	4	3	3		10	1	
	III C	4	7	1	2		14	0	
	IV	8	13	2	7		30	0	
Postoperative chemotherapy	Yes	12	26	6	14	0.934	54	4	0.715
	No	8	16	5	11	Yes vs No	39	1	Yes vs No

Abbreviations : well, well-differentiated ; mod, moderately differentiated ; poor, poorly differentiated ; sig, signet ring cell

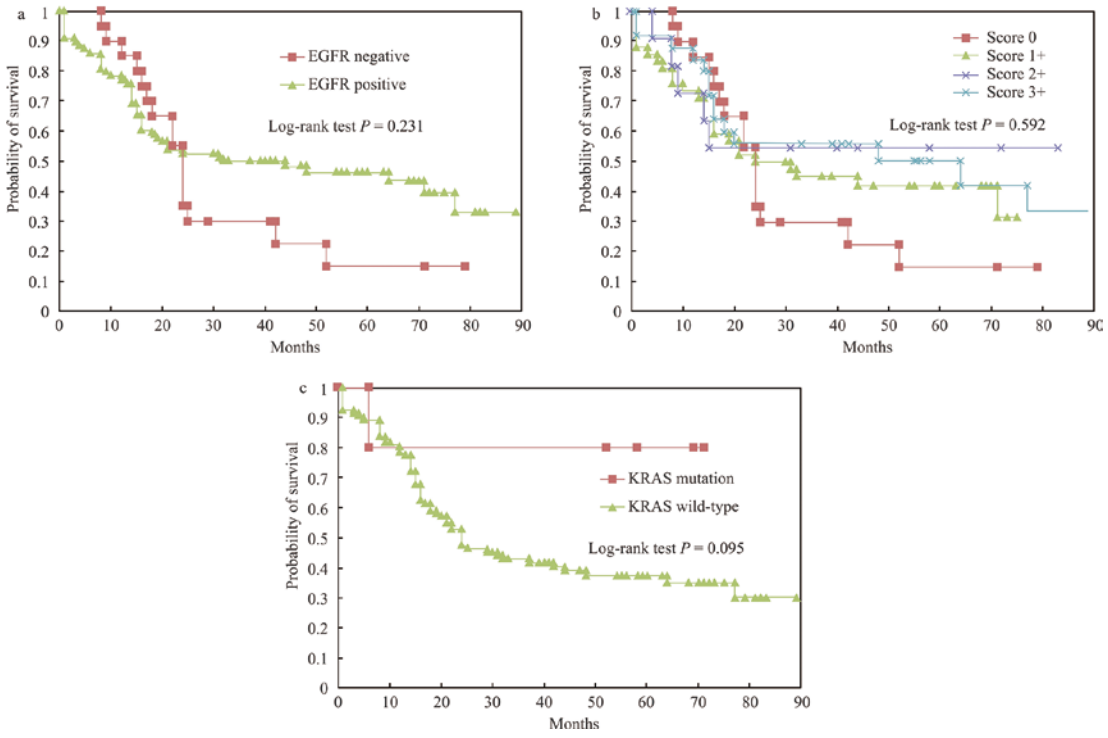

Fig. 2. Survival curves according to EGFR expression status and *KRAS* mutation status and calculated with the Kaplan–Meier method. (a) EGFR expression in the EGFR-negative and EGFR-positive groups and resulting survival curves. (b) EGFR expression in four subgroup according to immunohistochemical staining results (score 0, 1+, 2+, and 3+) and resulting survival curves. (c) *KRAS* mutation status in *KRAS*-mutant and wild-type groups and resulting survival curves.

Table 3. *KRAS* mutation type and patient characteristics

Case number	Sex	Age	*KRAS* mutation			Differentiation grade	Wall penetration depth	Lynphatic invasion	Vascular invasion	Node classification	Tumor stage	Postoperative chemotherapy	EGFR expression
			Codon	Nucleotide change	Borrmann type								
1	M	82	13	GGC–GAC	Type3	mod	T3	3	1	3	III B	Yes	1+
14	M	81	13	GGC–TGC	Type3	mod	T3	2	1	2	II A	Yes	2+
16	M	64	13	GGC–GAC	Type2	mod	T3	2	1	0	II A	Yes	0
24	F	82	12	GGT–GAT	Type1	well	T3	1	1	1	II B	Yes	1+
33	F	81	12	GGT–GAT	Type3	poor	T3	2	1	2	III A	No	1+

Abbreviations : well, well-differentiated ; mod, moderately differentiated ; poor, poorly differentiated

has often been examined during the last 2 decades. However, the reported frequencies of EGFR expression have varied greatly[12-14], possibly because of the use of different antibodies, the subjectivity of the pathologic interpretation, and the use of different scoring systems. In the present study, we used the Dako EGFR pharmDx kit, which is commonly used to identify patients who have colorectal cancer that can be treated with anti-EGFR antibodies. The frequency of EGFR expression was higher in our study than in previous studies that evaluated gastric cancer by means of immunohistochemical techniques. However, we found no associated between EGFR expression and overall survival. This finding is contradictory to those of previous studies in which high levels of EGFR in gastric cancer were associated with a poor prognosis for overall survival[20,21]. However, a high level of EGFR has also been reported as a positive prognostic factor predicting the efficacy of chemotherapy in patients with advanced gastric cancer[22-24]. On

the other hand, several studies have found that EGFR expression is not correlated with treatment outcome[25,26]. These discrepancies are likely due to differences in immunohistochemical scoring systems for classifying EGFR expression. Furthermore, immunohistochemical examinations can be affected by such variables as tissue fixation, choice of primary antibodies, and scoring system, potentially leading to conflicting results among studies. Therefore, to reach a consensus regarding the clinical significance of EGFR in gastric cancer, standardized methods should be developed for analyzing EGFR expression in cancer tissue.

The protein KRAS is a signal transducer downstream of tyrosine kinase receptors, including EGFR, and is an important element within complex signaling cascades involved in the development and progression of cancer. The mutually exclusive relationship between *KRAS* mutations and EGFR mutations has been suggested to determine the resistance of cancers with *KRAS* mutations to EGFR inhibitors ; therefore, the detection of *KRAS* mutations might be important for identifying patients who have cancer resistant to EGFR inhibitors[15,16]. In the present study *KRAS* mutations were found in only 5 cases (5.1%) ; this low incidence was consistent with the findings of previous reports[15-17]. Interestingly, we found that *KRAS* mutations were associated with tumor stage, a relationship that has not been previously reported in gastric cancers[16]. This discrepancy may be due to the small sample size of our study and the low rate of *KRAS* mutations. Although associations between tumor stage and KRAS mutations have been found in a previous study with small sample sizes for other cancers, no such associations were found in another study with a large sample size[27,28]. To investigate these concepts more fully, further studies should have larger sample sizes.

In gastric cancer, trastuzumab has recently been introduced as a potential human EGFR 2 (HER2) antagonist ; however, trastuzumab is only indicated for patients with HER2-positive cancer, as defined by immunohistochemistry scores of 2+ or 3+ and positive results for fluorescence in situ hybridization of HER2 amplification[29]. Therefore, to significantly improve outcomes perhaps only patients with immunohistochemistry scores of 3+ should be selected for treatment with EGFR-targeted agents. On the other hand, *KRAS* mutations have been shown to be associated with the resistance of colorectal cancer to anti-EGFR therapy. Mutations of *KRAS* occur in up to 40% of patients with colorectal cancer. However, as we have found in the present study, the rate of *KRAS* mutations in gastric cancer was low but the rate of EGFR expression was high. Therefore, anti-EGFR therapy might be effective and improve the prognosis of patients with gastric cancer.

In chemotherapy for patients with colorectal cancer, the relationship between EGFR expression score and the response rate to treatment with cetuximab, which specifically targets EGFR, is unclear. Response rates to monotherapy with cetuximab have been as low as 3%[30]. However, several trials have examined the activity of cetuximab in combination with chemotherapy in patients with advanced esophageal or gastric cancer[31-33] ; objective response rates have varied from 40% to 65%. Thus, the addition of cetuximab appeared to increase the response rate to chemotherapy, and standard chemotherapy combined with EGFR-targeting agents may be more effective in the treatment of gastric cancer. Unfortunately, in the phase III EXPAND trial[34], which assessed the efficacy of cetuximab in combination with cisplatin and capecitabine as first-line chemotherapy for patients with gastric cancer, the addition of cetuximab did not improve the survival of progression-free patients with advanced gastric cancer. However, the development of new anti-EGFR drugs is expected to improve the sensitivity and responsiveness to treatment of EGFR-expressing tumors. Indeed, a recent study shows that treatment with nimotuzumab, a new anti-EGFR monoclonal antibody, significantly improves progression-free and overall survival in patients who have gastric cancer with moderate and high levels of EGFR (i.e., 2+ and 3+)[35]. Although the small sample size and various biases associated with the retrospective design of the present study preclude in-depth statistical analysis, our results indicate that the genetic heterogeneity of patients with gastric cancer, with respect to KRAS mutations, might define a treatment-resistant group of patients that would require an alternative therapeutic approach.

Conclusions

Molecularly targeted therapy for gastric cancer is still in its infancy. Our study in patients with gastric cancer found that the frequency of EGFR expression was extremely high but that of *KRAS* mutations was extremely low. Therefore, EGFR-targeted therapy may be effective for

treating gastric cancer. Additional multicenter phase III trials with larger sample sizes are needed to assess the potential use of EGFR inhibitors in the treatment of gastric cancer.

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