

Identification of KRAS and PIK3CA but not BRAF mutations in patients with gastric cancer

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Received June 12, 2014; Accepted February 26, 2015

DOI: 10.3892/mmr.2015.3530

Abstract. Cetuximab, an immunoglobulin G1 chimeric monoclonal antibody directed against the epidermal growth factor receptor, is currently considered to be the strategy with the most potential for the treatment of gastric cancer due to the low frequency of KRAS mutations in patients with gastric cancer. However, the therapeutic success of cetuximab in colorectal cancer (CRC) has demonstrated that the clinical effect of cetuximab is closely dependent not only on KRAS mutations, but also BRAF and phosphoinositide-3-kinase, catalytic, α polypeptide (PIK3CA) mutations. In the present study, the status of KRAS, BRAF and PIK3CA mutations in gastric cancer were investigated concomitantly in order to aid the selection of patients eligible for treatment with cetuximab. Mutations in KRAS (exon 2), BRAF (exon 15) and PIK3CA (exon 9 and exon 20) were retrospectively evaluated by high resolution melting analysis and DNA direct sequencing in samples from 156 patients with gastric cancer. Mutations in either KRAS or PIK3CA were identified in 13 samples (8.3%), 7 samples with KRAS mutations and 6 samples with PIK3CA mutations. No mutations in the BRAF gene were identified. The frequency of mutations in either KRAS or PIK3CA were significantly higher in patients without lymph node metastasis than those with. Furthermore, KRAS and PIK3CA mutations were mutually exclusive. The present study, therefore, suggested that it may be necessary to evaluate KRAS and PIK3CA mutations concomitantly for the selection of patients eligible for treatment with cetuximab.

Introduction

Gastric cancer is one of the most common types of cancer globally, with a particularly high incidence in Northeast Asian countries, including China, Japan and Korea (1). The traditional therapeutic strategies for gastric cancer have failed to effectively treat the condition (2). In previous years, targeted therapies against the molecular variants specific to the carcinogenesis of various types of human cancer have been developed. However, the development of targeted therapies for gastric cancer lags behind other types of cancer. Trastuzumab, a monoclonal antibody against human epidermal growth factor receptor 2 (HER2) widely used for the treatment of breast cancer, has been approved for the treatment of patients with gastric cancer expressing HER2 based on the encouraging results of Trastuzumab for the treatment of gastric cancer (3). Gefitinib, an epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) successfully used for the treatment of non-small cell lung cancer with an EGFR mutation in exon 19 or 21, appears to be effective for certain patients with gastric cancer (4). Furthermore, EGFR mutations in exon 21 have been identified in patients with gastric cancer by a Portuguese research group and in our previous study (5,6). These findings indicate that certain patients with gastric cancer would benefit from TKIs. However, only a specific subpopulation of patients are able to benefit from treatment with trastuzumab or EGFR-TKIs.

Cetuximab, an immunoglobulin G1 chimeric monoclonal antibody directed against EGFR, exhibits anticancer effects by binding to the extracellular domain of EGFR with a higher affinity than the natural ligands of the receptor, inhibiting its dimerization and phosphorylation, and then blocking the downstream signaling transduction pathways, mainly KRAS/BRAF/mitogen activated protein kinase and phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (7). Cetuximab was initially approved for the treatment of patients with colorectal cancer (CRC), particularly those with EGFR-positive tissue samples. Notably, it was reported that over half of all patients with gastric cancer expressed EGFR, suggesting that certain patients with gastric cancer may benefit from cetuximab treatment (8). Furthermore, cetuximab, either alone or in combination with various chemotherapeutic drugs, has been evaluated in clinical trials for the

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Key words: KRAS, phosphoinositide-3-kinase, catalytic, α polypeptide, BRAF, mutation, cetuximab, gastric cancer

treatment of patients with gastric cancer (9-11). However, the majority of patients, including those with EGFR-positive tissue samples were not sensitive to cetuximab. The previous results appear to be conflicting. Therefore, it is hypothesized that certain factors may impair the sensitivity of patients with gastric cancer to cetuximab. Currently, cetuximab has been well demonstrated to be effective in patients with CRC without KRAS mutations as mutant KRAS with an elevated kinase activity would result in a continuously activated state, leading to a continuous and self-sufficient signaling transduction pathway, thus rendering cetuximab ineffective (12). Furthermore, it has been clearly demonstrated that mutations of other downstream effectors of EGFR, such as BRAF and PIK3CA, are also able to render cetuximab ineffective (13,14). Therefore, the evaluation of KRAS, BRAF and PIK3CA mutations in gastric cancer may aid the selection of patients eligible for treatment with cetuximab.

In previous years, KRAS mutations have been reported in gastric cancer with a frequency, ranging between 0 and 21% (15-18). However, the exact frequency of mutations in the KRAS gene and their association with clinicopathological features of the patients remains to be elucidated. Although previous studies had considered BRAF mutations to be rare in gastric cancer, further studies are required to confirm this hypothesis as no more than ten studies have investigated the status of the BRAF mutation to date, to the best of our knowledge (18-25). In contrast to KRAS and BRAF mutations, a mutation in PIK3CA has been identified in gastric cancer with a relatively higher frequency between 4.3 and 25.0% (18,26-28). However, no definitive conclusion can be drawn regarding whether the PIK3CA mutation coexists with the KRAS mutation in gastric cancer.

Therefore, the previously mentioned data revealed that mutations in KRAS, BRAF and PIK3CA may be involved in gastric cancer. In addition, these variants were usually evaluated by different groups from different countries and data regarding comprehensive analysis of KRAS, BRAF and PIK3CA mutations in gastric cancer were limited. The aim of the present study was to evaluate concomitantly the status of KRAS, BRAF and PIK3CA mutations in 156 gastric cancer samples in order to aid the selection of patients eligible for treatment with cetuximab.

Patients and methods

Study population. A total of 156 gastric cancer samples [53 fresh-frozen and 103 formalin-fixed, paraffin-embedded (FFPE)] were collected from patients who underwent surgical resection at The First Affiliated Hospital of Dalian Medical University and The Third People's Hospital of Dalian (Dalian, China). The present study was initiated with the approval of the ethics committee of The Second Affiliated Hospital of Dalian Medical University. Informed consent was obtained from patients prior to the collection of the samples. None of patients in the present study had received preoperative radio- or chemotherapy. The clinicopathological features of all patients were verified by two experienced pathologists (Table I).

DNA extraction. The DNA extraction of the 53 fresh-frozen samples has been previously completed by our group (17).

Therefore, in the present study, DNA was only extracted from the 103 FFPE samples. An $\sim 5\text{-}\mu\text{m}$ thick single section from each sample was selected for hematoxylin and eosin (H&E) staining. An experienced pathologist analyzed the H&E sections and marked the area containing at least 70% tumor cells. Subsequently, according to the marked area in the H&E section, the tumor tissue was dissected from eight sections of each FFPE sample and then genomic DNA was extracted using an FFPE DNA extraction kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions.

Mutation analysis of KRAS (exon 2), BRAF (exon 15) and PIK3CA (exon 9 and 20). The status of the KRAS (exon 2) mutation in the 53 fresh-frozen samples has been previously analyzed and reported by Liu *et al* (17). In the present study, the mutation status of KRAS (exon 2) in 103 FFPE tumor samples, and BRAF (exon 15) and PIK3CA (exon 9 and 20) in 156 tumor samples was screened using polymerase chain reaction-high resolution melting (PCR-HRM) analysis. PCR primers for KRAS, BRAF and PIK3CA are shown in Table II. The final PCR reaction mixture (10 μl) contained: i) KRAS exon 2: 1X PCR buffer, 200 μM dNTPs, 2.5 mM MgCl_2 , 1 μM each primer, 0.25 units HotStart Taq (Takara Bio Inc., Dalian, China), 5 ng genomic DNA and 1X LC Green Plus (Biofire Diagnostics, Salt Lake City, UT, USA); ii) BRAF exon 15 and PIK3CA exon 9: 1X PCR Buffer, 200 μM dNTPs, 2 mM MgCl_2 , 1 μM each primer, 0.25 units HotStart Taq, 5 ng genomic DNA and 1X LC Green Plus; iii) PIK3CA exon 20: 1X PCR Buffer, 200 μM dNTPs, 2.5 mM MgCl_2 , 0.5 μM each primer, 0.25 units HotStart Taq, 5 ng genomic DNA and 1X LC Green Plus. The PCR amplification conditions were as follows: i) KRAS exon 2: 95 °C for 10 min; 45 cycles of 95°C for 30 sec, 54°C for 10 sec and 72°C for 1 min; ii) BRAF exon 15 and PIK3CA exon 20: 95°C for 10 min; 45 cycles of 95°C for 30 sec, 56°C for 10 sec and 72°C for 30 sec; and iii) PIK3CA exon 9: 95°C for 10 min; 45 cycles of 95°C for 30 sec, 60°C for 10 sec and 72°C for 30 sec.

DNA sequencing. The PCR product was purified using the GeneJET™ gel extraction kit (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions and then sequenced on an ABI Prism 3730 sequence detection system (Takara Bio, Inc.). A 173 bp amplicon was generated by sequencing primers of KRAS exon 2 following the same method as the PCR-HRM primers above. A 124 bp amplicon was generated by sequencing primers of PIK3CA exon 9, forward: 5'-GTAACAGACTAGCTAGAG-3' and reverse: 5'-CTGTGACTCCATAGAAAATC-3'. A 158 bp amplicon was generated by sequencing primers of PIK3CA exon 20, forward: 5'-GAATGCCAGAACTACAATC-3' and reverse: 5'-TGTGTGGAAGATCCAATC-3'.

The sequencing conditions for KRAS and PIK3CA were as follows: 95°C for 10 min; 45 cycles of 95°C for 30 sec, 54°C for 10 sec and 72°C for 1 min.

Statistical analysis. All statistical analyses were conducted using the χ^2 test and Fisher's exact test with SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Table I. Associations between gene mutations and clinicopathological features of patients.

Clinicopathological feature	No.	KRAS		PIK3CA		KRAS/PIK3CA	
		Mutation	P-value	Mutation	P-value	Mutation	P-value
Gender			1		1		0.956
Male	115	5		4		9	
Female	41	2		2		4	
Age (years)			0.439		1		0.385
≤65	78	2		3		5	
>65	78	5		3		8	
Lymph node metastasis			0.131		0.359		0.036
Present	116	3		3		6	
Absent	40	4		3		7	
Differentiation grade			0.742 ^a		0.53 ^a		0.317 ^a
Well	5	1		1		2	
Moderate	42	2		2		4	
Poor	92	4		3		7	
MAC	17	0		0		0	
Depth of invasion			0.433		1		0.296
T1	4	1		0		1	
T2-T4	152	6		6		12	

^aWell and Moderate vs. Poor and MAC. MAC, mucinous adenocarcinoma. PIK3CA, phosphoinositide-3-kinase, catalytic, α polypeptide.

Table II. Primers for mutation analysis using polymerase chain reaction-high resolution melting.

Primer	Sequence (from 5' to 3')
KRAS exon 2	Forward: AGGCCTGCTGAAAATGACTG Reverse: TCAAAGAATGGTCCTGCACC
BRAF exon 15	Forward: CTCTTCATAATGCTTGCTCTGATAGG Reverse: TAGTAACTCAGCAGCATCTCAGG
PIK3CA exon 9	Forward: CTAGCTAGAGACAATGAATTAAGGGAAA Reverse: CATTITAGCACTTACCTGTGACTCCA
PIK3CA exon 20	Forward: TGAGCAAGAGGCTTTGGAGT Reverse: TCATTTTCTCAGTTATCTTTTCAGTTCAAT

PIK3CA, phosphoinositide-3-kinase, catalytic, α polypeptide.

Results

A total of 156 gastric cancer samples were screened for mutations in KRAS (exon 2), BRAF (exon 15) and PIK3CA (exon 9 and 20). As a whole, 8.3% (13/156) of the analyzed samples harbored a mutation in either KRAS or PIK3CA. No mutations in the BRAF gene were identified. Notably, the frequency of mutations in either KRAS or PIK3CA was significantly higher in patients without lymph node metastasis than those with (17.5 vs. 5.2%, $P=0.036$). No significant association was observed between mutations in either KRAS or PIK3CA and other clinicopathological features (Table I).

KRAS mutations. Mutations in the KRAS gene were identified in 7 out of 156 gastric cancer samples (4.5%). In addition to the five mutations (two G13D, one G12V and two G12D) previously described by Liu *et al* (17), a further two mutations were found to occur at codon 12, leading to the substitutions of glycine for aspartic acid (G12D) and alanine (G12A), respectively (Fig. 1). In addition, the two mutations occurred exclusively in female patients aged over 65 years whose tumors were histologically classified into poorly-differentiated adenocarcinoma without lymph node metastasis. The mutation types and the clinicopathological features of the patients are summarized in Table III. The results demonstrated that no significant associa-

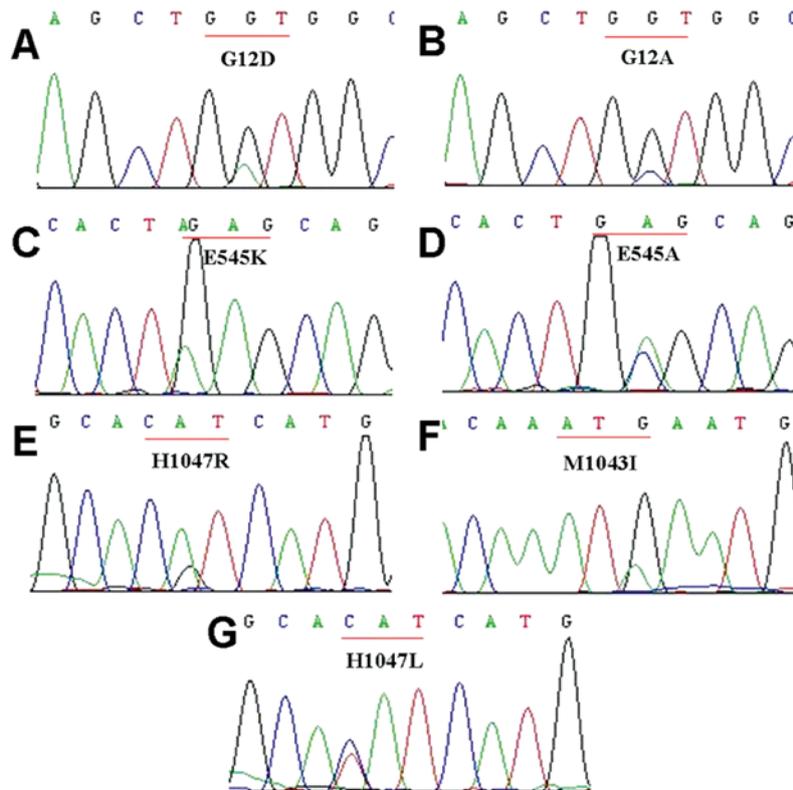


Figure 1. Sequencing chromatograms of (A and B) KRAS exon 2, (C and D) PIK3CA exon 9, and (E, F and G) PIK3CA exon 20 mutations. PIK3CA, phosphoinositide-3-kinase, catalytic, α polypeptide.

tion was found between mutations in the KRAS gene and the clinicopathological features of the patients (Table I).

BRAF mutations. Mutations in the BRAF gene were not detected in the present study, which is consistent with the results of previous studies.

PIK3CA mutations. Mutations in the PIK3CA gene were identified in 6 out of 156 gastric cancer samples (3.8%), including three mutations in exon 9 (two E545K and one E545A) and four mutations in exon 20 (two H1047R, one M1043I and one H1047L) (Fig. 1). Furthermore, two different types of PIK3CA mutations, namely E545A in exon 9 and H1047L in exon 20, occurred simultaneously in one patient with gastric cancer. By contrast, mutations in the PIK3CA gene were found to occur in a mutually exclusive manner with mutations in the KRAS gene. The mutation types and the clinicopathological features of the patients are summarized in Table III. In addition, no significant association was found between mutations in the PIK3CA gene and the clinicopathological features of the patients (Table I).

Discussion

In the previous decade, molecular targeted therapy has been an important area of investigation for the treatment of human cancer. However, contemporary targeted therapy for gastric cancer is less than satisfactory, with few encouraging therapeutic effects. In addition to trastuzumab, which has been approved for the treatment of patients with gastric cancer,

gefitinib appears to be useful clinically. It is worth noting that trastuzumab and gefitinib are applicable to only a small fraction of patients with gastric cancer. Cetuximab, which was initially implemented for the treatment of patients with CRC, is currently undergoing clinical trials for the treatment of patients with gastric cancer. However, clinical outcomes from certain trials have been unsuccessful (9,10). In CRC, cetuximab has been well demonstrated to be effective in patients without mutations in the KRAS gene (12). However, certain patients without mutations in the KRAS gene do not respond to cetuximab in clinical practice, suggesting that other molecular variants may be associated with the inefficacy of cetuximab. Various studies have demonstrated that patients with CRC carrying mutations of other downstream effectors of EGFR, such as BRAF and PIK3CA, also exhibit resistance to treatment with cetuximab (13,14). Therefore, a previous study suggested that patients with CRC without KRAS, BRAF and PIK3CA mutations are more likely to respond to cetuximab (29). Based on the therapeutic success of cetuximab in CRC, it was hypothesized that it is necessary to evaluate the status of KRAS, BRAF and PIK3CA mutations in gastric cancer for selecting patients eligible for treatment with cetuximab.

KRAS, a molecular switch of intracellular signaling transduction pathways, is essential in transferring extracellular growth signals into the nucleus. Mutant KRAS protein exhibits elevated kinase activity and is able to constitutively activate downstream signaling transduction pathways independent of stimuli from activated EGFRs. A review conducted by Kiaris and Spandidos (30) demonstrated that

Table III. Mutation types and clinicopathological features of the patients.

Patient no.	KRAS	PIK3CA		Gender	Age (years)	Differentiation grade	Lymph node metastasis
		Exon 9	Exon 20				
1	G13D			Male	61	Well	Absent
9	G12D			Male	72	Moderate	Present
27	G12V			Male	64	Poor	Present
29	G12D			Male	70	Poor	Present
49	G13D			Male	73	Moderate	Absent
68	G12D			Female	69	Poor	Absent
152	G12A			Female	75	Poor	Absent
39			H1047R	Female	81	Moderate	Absent
45		E545K		Male	65	Moderate	Present
70			H1047R	Female	73	Well	Absent
83			M1043I	Male	45	Poor	Absent
108		E545K		Male	61	Poor	Present
122		E545A	H1047L	Male	67	Poor	Present

PIK3CA, phosphoinositide-3-kinase, catalytic, α polypeptide.

a mutation in the KRAS gene was associated with numerous types of human cancer, including pancreatic cancer, CRC and lung cancer (30). To date, a wide variety of studies have investigated the status of mutations in the KRAS gene in gastric adenocarcinoma with the frequency ranging between 0 and 21% (15-18). The frequency of mutations in the KRAS gene in different studies has varied markedly. In order to improve understanding of the status of mutations in the KRAS gene in gastric cancer, a large international multi-center study, including 712 patients with gastric cancer was conducted in 2013 (31). In the study, which is the largest to date, the overall frequency of mutations in the KRAS gene was 4% and KRAS mutation was not associated with clinicopathological features, including ethnicity, gender and stage of tumor differentiation. The discrepancy between previous studies and the multi-center study may be predominantly due to the small sample size. Thus far, the majority of the studies included ≤ 100 patients with gastric cancer, rendering the interpretation of any conclusion difficult. In our previous study (17), the frequency of mutations in the KRAS gene was 9.6%. Furthermore, the patients with KRAS mutations were exclusively males although no significant difference was found, due to the small sample size. However, in the current study with a larger sample size, it was identified that the frequency of mutations in the KRAS gene was 4.5%. In addition, the patients with mutations in the KRAS gene were not only males but also females. No significant association was identified between KRAS mutation and the clinicopathological features of the patient, including gender, age, differentiation grade and depth of invasion. Thus, mutations in the KRAS gene may occur in all patients with gastric cancer, rendering its evaluation necessary for the identification of patients suitable for cetuximab therapy.

BRAF, a member of the RAF kinase family, is an essential downstream effector of KRAS. BRAF is commonly activated by somatic mutation and mutated BRAF is able to constitu-

tively activate downstream signaling transduction pathways regardless of stimulus from EGFR or KRAS. To date, BRAF mutations have been identified in numerous types of human cancer, including melanoma, papillary thyroid cancer and CRC (32-34). However, data regarding the BRAF mutation in gastric cancer are limited. To date, a small number of studies involving 943 patients with gastric cancer have investigated the status of the BRAF mutation and found that the average frequency of mutations in the BRAF gene was only 2.5% (ranging between 0 and 11%) (18-25). Therefore, it is not noteworthy that no mutations in the BRAF gene were found in the present study. Mutations in the BRAF gene were absent or occurred only occasionally in Chinese patients with gastric cancer. Therefore, it is not necessary to evaluate the status of the BRAF mutation in order to select patients eligible for treatment with cetuximab.

PI3K, another downstream effector of EGFR, usually interacts with KRAS in the regulation of cellular functions. PIK3CA, encoding the catalytic subunit of PI3K, was initially demonstrated to be mutated in human cancer by Samuels *et al* (27). Subsequently, PIK3CA mutations have been identified in various types of human cancer, including breast cancer, CRC, liver cancer and ovarian cancer (28,35,36). In gastric cancer, the frequency of PIK3CA mutations varied between 4.3 and 25% (18,26-28). In the present study, the frequency of PIK3CA mutations was 3.8%, slightly lower than that observed in previous studies. The discrepancy among these studies may be due to a number of reasons, including sample size, mutation detection method used and the ethnicity of the patients. In CRC, it has been reported that PIK3CA mutations usually coexist with KRAS mutations (28,37). Notably, the PIK3CA mutation was also observed in a concomitant manner with KRAS mutation in gastric cancer (26,28). However, in the present study, it was identified that PIK3CA and KRAS mutations were mutually exclusive. Therefore, a

definitive conclusion regarding the association of the PIK3CA mutation with the KRAS mutation in gastric cancer requires confirmation in further studies. By contrast, it was identified that mutations in either KRAS or PIK3CA were more likely to occur in patients without lymph node metastasis, which indicated that mutations of downstream effectors of EGFR occurred earlier in gastric carcinogenesis. In any case, KRAS and PIK3CA mutations should be evaluated prior to patients receiving cetuximab.

In conclusion, in the present study ~8.3% of Chinese patients with gastric cancer harbored a mutation in either KRAS or PIK3CA, suggesting that these patients would not benefit from cetuximab. Therefore, in the future, KRAS and PIK3CA, but not BRAF mutations should be evaluated in order to select patients eligible for treatment with cetuximab.

Acknowledgements

The authors would like to thank the doctors of the Second Affiliated Hospital of Dalian Medical University for the collection of the samples. This study was supported by the National Natural Science Foundation of China (grant no. 81071805).

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