

***KRAS* and *BRAF* Mutations in 203 Esophageal Squamous Cell Carcinomas: Pyrosequencing Technology and Literature Review**

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ABSTRACT

Background. Epidermal growth factor receptor (EGFR) signaling is one of the most promising targets for molecular-targeted therapies in esophageal squamous cell carcinoma (ESCC). Thus, the molecular diagnosis of *KRAS* and *BRAF* mutations is clinically important in therapeutic decision making. However, the frequency of *KRAS* and *BRAF* mutations in ESCCs remains inconclusive because of the limited sample sizes of previous studies (all $N \leq 80$). Pyrosequencing is a nonelectrophoretic nucleotide extension sequencing technology that can be used for mutation testing.

Methods. The frequency of *KRAS* and *BRAF* mutations was examined using a nonbiased database of 203 resected ESCCs and a high-throughput pyrosequencing assay.

Results. The validity of the *KRAS* pyrosequencing method was initially demonstrated by detection of all 4 types of *KRAS* mutations [c.35G>T (codon 12 GGT>GTT), c.35G>A (codon 12 GGT>GAT), c.34G>T (codon 12 GGT>TGT), c.38G>A mutation (codon 13 GGC>GAC)], which had been previously diagnosed using Scorpion-ARMS technology, in 9 colon cancer tissues (9 of 9; 100 %). Similar results were demonstrated for *BRAF* mutational status in 3 colon cancer cell lines (HCT116, Colo201, and HT29), which were validated by Sanger dideoxy sequencing. Subsequently, the *KRAS* mutation was found to be extremely rare (1 of 203; 0.5 %), and the *BRAF* mutation was absent (0 of 203; 0 %), in the dataset of 203 ESCCs.

Conclusions. These results suggest that *KRAS* and *BRAF* mutations play a limited role in the development of ESCC and that mutation analysis is not useful as a screening test for sensitivity to anti-EGFR therapy in ESCC.

Esophageal squamous cell carcinoma (ESCC) is the major histological type of esophageal cancer in East Asian countries and is one of the most aggressive malignant tumors.¹ Despite remarkable advances in multimodal therapies, patient prognosis remains poor, even for those whose carcinomas have been completely resected.^{2–5} The limited improvement in treatment outcomes by conventional therapies urged us to seek innovative strategies for treating ESCC, especially those that are molecularly targeted. One of the most promising targets is the inhibition of the epidermal growth factor receptor (EGFR) by monoclonal antibodies (e.g., cetuximab, panitumumab) or small molecule tyrosine kinase inhibitors (e.g., erlotinib, gefitinib).^{6–10} The EGFR signal transduction network plays a crucial role in multiple tumorigenic processes, contributing to cell-cycle progression, angiogenesis, metastasis, and protection of the cancer cell from apoptosis.¹¹ Mutations in the Kirsten Ras 1 (*KRAS*) and V-Raf Murine Sarcoma Viral Oncogene Homolog B1 (*BRAF*) genes may be predictive of response to drugs directly linked to the EGFR pathway.^{12–14} Thus, molecular diagnosis of these mutations is increasingly important in making therapeutic decisions. Several previous studies have examined the frequency of *KRAS* and *BRAF* mutations in ESCCs; however, they were all limited by small sample sizes (all $N \leq 80$) (Table 1), yielding inconclusive results.^{8,15–20}

Pyrosequencing is a nonelectrophoretic nucleotide extension sequencing technology for various applications, including mutation testing of tumors.^{21–24} This technology

TABLE 1 Studies on *KRAS* and *BRAF* mutations in ESCC

Study	Sample size	Methods	Mutation detected <i>N</i> (%)	Codons examined
Studies on <i>KRAS</i> mutations in ESCC				
Ma et al. ¹⁵	35	Pyrosequencing	2 (5.7 %)	Codons 12–13
Liu et al. ¹⁶	50	Pyrosequencing	6 (12 %)	Codons 12–13
Lorenzen et al. ⁸	37	Direct sequencing	0 (0 %)	Codons 12–13
Hollstein et al. ¹⁷	16	Direct sequencing	0 (0 %)	Codons 12–13
Victor et al. ¹⁸	27	PCR and oligomer hybridization assay	0 (0 %)	Codons 12–13
Hollstein et al. ¹⁹	25	PCR and oligomer hybridization assay	0 (0 %)	Codons 12–13
Present study	203	Pyrosequencing	1 (0.5 %)	Codons 12–13
Studies on <i>BRAF</i> mutations in ESCC				
Ma et al. ¹⁵	35	Pyrosequencing	0 (0 %)	Codon 600
Maeng et al. ²⁰	80	OncoMap	1 (1.2 %)	Codon 600
Present study	203	Pyrosequencing	0 (0 %)	Codon 600

has several advantages. First, it has higher sensitivity than classical Sanger dideoxy sequencing.²² Sanger dideoxy sequencing needs more than 20 % of tumor load in a specimen to render a reliable result, while pyrosequencing can render a reliable result with a tumor load of 5 %. Second, pyrosequencing is faster than Sanger dideoxy sequencing. Third, pyrosequencing is more cost effective. Collectively, pyrosequencing is a useful method in molecular diagnostics and large-scale epidemiological studies.^{25,26}

Therefore, in the present study, *KRAS* and *BRAF* mutations were screened using a nonbiased database of 203 ESCCs and pyrosequencing technology.

PATIENTS AND METHODS

Study Subjects

A total of 217 consecutive patients with ESCC who were undergoing curative resection at Kumamoto University Hospital between April 2005 and December 2010 were enrolled in this study. There were 13 patients excluded because of the unavailability of adequate tissue samples. We initially quantified *KRAS* and *BRAF* mutation in 204 cancer specimens and obtained valid results in 203 cases (99.5 %). Thus, 203 ESCCs were finally included in this study. Tumor staging was done by the American Joint Committee on Cancer Staging Manual (7th edition).²⁷ Written informed consent was obtained from each subject, and the institutional review board of Kumamoto University approved the study procedures.

A total of 9 patients with colon cancers harboring 4 different *KRAS* mutations [c.35G>T (codon 12 GGT>GTT;

p.Gly12Val), c.35G>A (codon 12 GGT>GAT; p.Gly12Asp), c.34G>T (codon 12 GGT>TGT; p.Gly12Cys), and c.38G>A mutation (codon 13 GGC>GAC; p.Gly13Asp)], which had been already diagnosed by Scorpion-ARMS technology, were also included in this study to validate the pyrosequencing method for the detection of *KRAS* mutations.

Genomic DNA Extraction

One pathologist marked the tumor areas on slides stained with hematoxylin-eosin. Genomic DNA was extracted from tumor lesions enriched with neoplastic cells, without adjacent normal tissue, using an FFPE kit (Qiagen, Valencia, CA). DNA was also extracted from 3 cell lines: Colo201 and HT29 with the *BRAF* mutation c.1799T>A (p.V600E), and HCT116 with wild-type *BRAF* using a QIAmp DNA mini kit (Qiagen, Valencia, CA).^{28,29} DNA was stored at -20°C before use.

Whole Genome Amplification

Whole genome amplification (WGA) is a useful technique for preserving original study material for many different assays and for future studies. In WGA, genomic DNA is amplified by polymerase chain reaction (PCR) using primers consisting of a random sequence of 15 nucleotides. Each PCR mix contained 40 pmol of the random primers, 1.0 nmol each of dNTP, 2.0 mmol/L MgCl₂, 1× PCR buffer (Applied Biosystems, Foster City, CA), 0.25 U of AmpliTaq Gold 360 (Applied Biosystems), and 5 μl of template DNA solution in a total volume of 50 μl. PCR conditions consisted of initial denaturation at

95 °C for 10 min; 50 cycles of 95 °C for 60 s, annealing (37 °C for 2 min), ramping from 37 to 55 °C (0.1 °C/s), 55 °C for 2 min, and 68 °C for 30 s; and a final extension at 72 °C for 7 min.

Pyrosequencing for KRAS and BRAF Mutations

Pyrosequencing technology has been shown to reliably detect *KRAS* mutations with 100 % analytic sensitivity and specificity, even when the proportion of mutant alleles is as low as 10 %. PCR amplification primers for pyrosequencing targeted for *KRAS* (codons 12, 13), *BRAF* (codon 600) were: *KRAS*-F, forward, 5'-NNNGCCTGCTGAAAATGACTGAA-3'; and *KRAS*-R, reverse biotinylated primer, 5'-TTA GCTGTATCGTCAAGGCACTCT-3'; and *BRAF*-F, forward biotinylated primer, 5'-CAGTAAAAATAGGTGATTTG-3'; and *BRAF*-R reverse, 5'-TCCAGACAACCTGTTCAA ACTGA-3'. Each PCR mix contained the forward and reverse primers (20 pmol each), 1.0 nmol of each dNTP with dUTP, 2 mmol/L MgCl₂, 1× PCR buffer, 1.25 U of AmpliTaq Gold 360, 0.5 U of AmpErase UNG and 5 µl of template WGA product in a total volume of 50 µl. PCR condition consisted of initial denaturation at 50 °C (10 min) for AmpErase UNG; initial denaturation at 94 °C (10 min) for AmpliTaq Gold 360; 50 cycles of 95 °C (30 s), annealing (30 s; 55 °C for *BRAF*, 57 °C for *KRAS*), and 72 °C (30 s); and a final extension at 72 °C (7 min). The PCR products were electrophoresed through an agarose gel to confirm successful amplification of the 82-bp (*KRAS*) and the 80-bp PCR (*BRAF*) product.

KRAS and *BRAF* pyrosequencing was performed using the PyroMark Q24 System (Qiagen, Valencia, CA) according to the manufacturer's instructions (Fig. 1 for *KRAS*; Fig. 2 for *BRAF*). All forward sequencing results were confirmed by reverse sequencing. In the *KRAS* pyrosequencing assay, the presence of a mutation was routinely confirmed by 3 different sequencing primers and by the creation of the frameshifted open reading frame of the mutant sequence relative to a wild-type sequence in a program. The primer *KRAS*-PF1 (5'-TGTGGTAGTTGGAGCTG-3'; pyrosequencing nucleotide dispensation order, ACTGATCG ATCGATCGATCGATCGATCG) could detect the c.35G>T (codon 12 GTT) and c.35G>A (codon 12 GAT) mutations. The primer *KRAS*-PF2 (5'-TGTGGTAGTTGGAGCT-3'; pyrosequencing nucleotide dispensation order, ATCGATCGATCGATCGATCG ATCATCG) could detect the c.34G>T (codon 12 TGT) mutation. The primer *KRAS*-PF3 (5'-TGGTAGTTGGAGC TGGT-3'; pyrosequencing nucleotide dispensation order, GATGCATGCATGCATGCATGCATGCATGC) could detect the c.34G>A (codon 13 GAC) mutation. In the *BRAF* pyrosequencing assay, the primer was 5'-CACTCCATC GAGATTTTC-3', and the pyrosequencing nucleotide dispensation order was CTGCATGCATGCTGCA.

RESULTS

Clinical Data

A summary of the clinical characteristics of the patients is given in Table 2. In this cohort, the 5-year overall survival rates of patients treated by esophagectomy were 83.9 % for stage I, 59.7 % for stage II, and 36.7 % for stage III. These rates are similar to those from the "Comprehensive Registry of Esophageal Cancer in Japan" (79.5 % for stage I, 58.9 % for stage II, and 39.8 % for stage III), which supports the absence of bias in our database.³⁰

Validation of the Pyrosequencing Assay for KRAS Mutation Detection

We first examined the validity of the pyrosequencing method using eleven colon cancer tissues harboring 4 different *KRAS* mutations [c.35G>T (codon 12 GGT>GTT; p.Gly12-Val), c.35G>A (codon 12 GGT>GAT; p.Gly12Asp), c.34G>T (codon 12 GGT>TGT; p.Gly12Cys), and c.38G>A mutation (codon 13 GGC>GAC; p.Gly13Asp)]. These mutations had already been diagnosed by the sensitive Scorpion-ARMS technology [i.e., Amplification Refractory Mutation System incorporating a unique bifunctional fluorescent primer/probe molecule (Scorpion)]. As shown in Table 3, all 4 types of mutations could be detected using the pyrosequencing technology (11 of 11, 100 %) (Fig. 1), which demonstrated that the method was reliable for the detection of *KRAS* mutations in tumors.

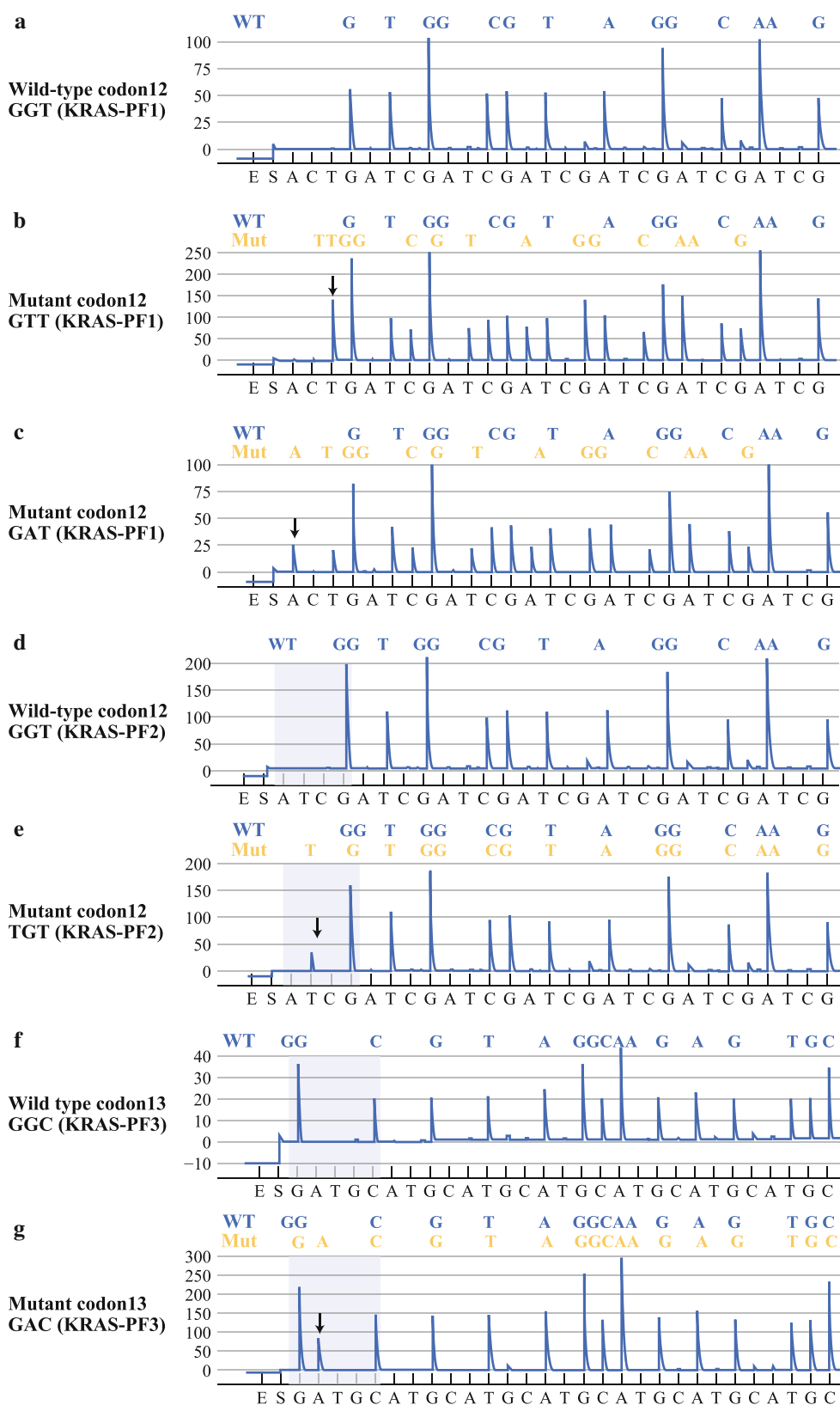
KRAS Mutation in ESCC

Pyrosequence analysis of *KRAS* codon 12 and 13 mutations was successful for 203 of 204 (99.5 %) ESCC paraffin-embedded tissues. Among the 203 ESCCs, only 1 case harbored a *KRAS* mutation [c.34G>T (codon 12 GGT>TGT; p.Gly12Cys)]. This mutation was also diagnosed by the Scorpion-ARMS technology (data not shown). This result showed that the frequency of *KRAS* mutations in ESCC was extremely low.

Validation of Pyrosequencing Assay for BRAF Mutation Detection

To validate our *BRAF* pyrosequencing assay, both pyrosequencing and Sanger dideoxy sequencing were performed on the same set of DNA samples from 3 colon cancer cell lines (HCT116, Colo201, and HT29). HCT116 possesses wild-type *BRAF*, and Colo201 and HT29 harbors a *BRAF* mutation [c.1799T>A (p.V600E)]. In Sanger

FIG. 1 Pyrograms of wild-type and mutant KRAS in colorectal carcinoma. **a** Wild-type codon 12 detected by the KRAS-PF1 primer. **b** c.35GT (codon 12 GTT) mutation detected by the KRAS-PF1 primer. **c** c.35GA (codon 12 GAT) mutation detected by the KRAS-PF1 primer. **d** Wild-type codon 12 detected by the KRAS-PF2 primer. **e** c.34GT (codon 12 TGT) mutation detected by the KRAS-PF2 primer. **f** Wild-type codon 13 detected by the KRAS-PF3 primer. **g** c.38GA (codon 13 GAC) mutation detected by the KRAS-PF3 primer. *Arrows* indicate the presence of mutant alleles



dideoxy sequencing, HCT116 showed was homozygous wild-type for the *BRAF* gene, and HT29 and COLO201 were shown to be heterozygous *BRAF* V600E mutants

(Fig. 2). Among all 3 cell lines, pyrosequencing gave the same results as Sanger sequencing for *BRAF* mutational status (Fig. 2).

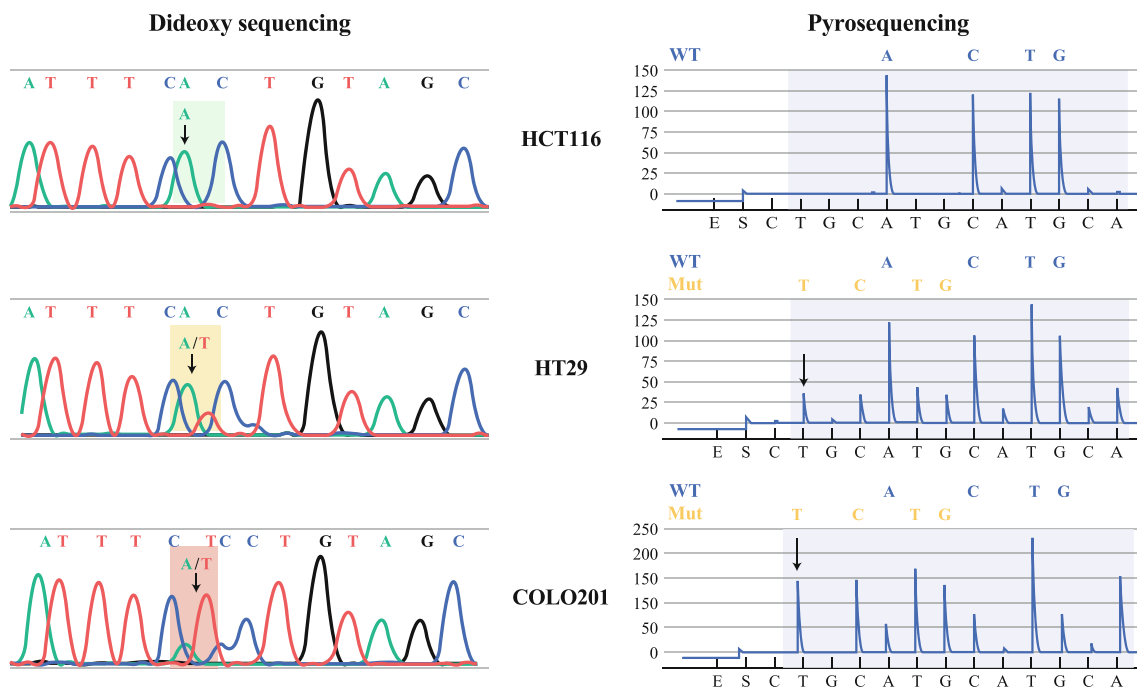


FIG. 2 Detection of BRAF V600E in colon cancer cell lines by dideoxy sequencing and pyrosequencing; detection of homozygous wild type (HCT116), heterozygous mutant (HT29, COLO201). BRAF V600E variants identified by dideoxy sequencing (left panel) and

pyrosequencing (right panel). The pyrosequencing nucleotide dispensation order is shown below each pyrogram. The numerical position for each nucleotide is indicated at the top. Arrows indicate the presence of mutant alleles

Furthermore, the *BRAF* mutation could be detected in paraffin-embedded tissues of colon cancer that had been previously diagnosed by Sanger dideoxy sequencing. Collectively, these preliminary experiments supported the validity of the pyrosequencing method for the detection of *BRAF* mutation in paraffin-embedded specimens.

BRAF Mutation in ESCC

The mutational status in *BRAF* exon 15 (V600E) was examined in 204 ESCCs, and valid results were obtained in 203 tissues (99.5%). All 203 ESCCs were wild-type at codon 600 in *BRAF* exon 15.

DISCUSSION

KRAS and *BRAF* mutational status could represent a predictive marker for anti-EGFR therapies; therefore, better understanding of the incidence of these mutations is important. However, the frequency of *KRAS* and *BRAF* mutations in ESCCs remains inconclusive because of the limited sample sizes of previous studies.^{8,15-20} The validity of the pyrosequencing method for detecting *KRAS* and *BRAF* mutations was initially demonstrated using colon cancer cell lines and colon cancer tissues harboring *KRAS* and *BRAF* mutations. Thereafter, *KRAS* mutations were shown to be extremely rare in a database of more than 200

ESCCs. In addition, the *BRAF* mutation was absent in ESCC tumors.

Pyrosequencing is a nonelectrophoretic nucleotide extension sequencing technology that can be used for mutation detection in tumors.²¹⁻²⁴ Pyrosequencing offers a higher sensitivity than classical Sanger dideoxy sequencing for the detection of *KRAS* mutations.²² In addition, because of its simplicity and cost effectiveness, pyrosequencing represents a potentially useful method in molecular diagnostics and epidemiological studies, particularly in the setting of large-scale projects and clinical assays.^{25,26}

Mutations in the *KRAS* gene occur early in the development of several types of cancers.³¹ Commonly restricted to codon 12 and 13 in exon 2, these mutations cause impaired GTPase activity and result in a continual stimulus for cellular proliferation. They have been found in more than 40% of colorectal cancers, 90% of pancreatic carcinomas, and 33% of non-small-cell lung carcinomas.³² Importantly, *KRAS* mutation status has recently become an important biomarker when identifying resistance to anti-EGFR therapy. Several previous studies have examined the frequency of *KRAS* mutations in ESCCs; only 2 studies ($N = 35$ and 50) showed the presence of *KRAS* mutations in ESCCs; however, others have demonstrated the absence of *KRAS* mutation in ESCCs.^{8,15-19} Unfortunately, all previous studies were limited by small sample sizes ($N \leq 80$). It should be noted that small studies are more

TABLE 2 Patient characteristics

Clinical characteristics	Total (N)
All cases	203
Mean age \pm SD	66.8 \pm 9.24
Sex	
Male	178 (87.7 %)
Female	25 (12.3 %)
Preoperative treatment	
Present	68 (33.5 %)
Absent	135 (66.5 %)
Cancer location	
Upper thoracic	31 (15.3 %)
Middle thoracic	105 (51.7 %)
Lower thoracic	58 (28.6 %)
Esophagogastric junction	9 (4.4 %)
Stage	
I (IA, IB)	76 (37.4 %)
II (IIA, IIB)	65 (32.0 %)
III (IIIA, IIIB, IIIC)	62 (30.5 %)
Lymph node metastasis	
Positive	94 (46.3 %)
Negative	109 (53.7 %)
Histological grade	
G1	83 (40.9 %)
G2	82 (40.4 %)
G3–4	28 (13.8 %)

TABLE 3 Comparative analysis of Scorpion-ARMS and pyrosequencing for detection of KRAS mutation in 9 colorectal carcinoma tissues

KRAS mutation type	Results of Scorpion-ARMS (n)	Results of pyrosequencing (n)	Concordance rate
Codon12 GAT	3	3	100 % (3/3)
Codon12 GTG	2	2	100 % (2/2)
Codon12 TGT	2	2	100 % (2/2)
Codon13 GAC	2	2	100 % (2/2)
Total	9	9	100 % (9/9)

prone to “publication bias” than large studies. The phenomenon of publication bias occurs because studies with null findings (e.g., absence of *KRAS* mutation in tumors) have a higher likelihood of being unwritten and unpublished compared with those with significant results (e.g., presence of *KRAS* mutation in tumors). Compared with small studies with null data, large studies with null data are

more likely to be published. As a result, large studies are less prone to publication bias than small studies. Publishing null data from well-powered studies are important because publishing significant results from small underpowered studies also leads to publication bias. In this respect, our finding that *KRAS* mutations are extremely rare in a non-biased database of more than 200 ESCCs may have considerable implications.

The *BRAF* gene encodes a serine/threonine kinase of the RAS RAF MEK MAPK signaling pathway and is mutated in a variety of cancer types.³³ The V600E point mutation in exon 15 of the *BRAF* gene has been shown to be associated with insensitivity to antigrowth signals, cell-cycle dysregulation, tumor invasion and metastasis, escape from apoptosis, unlimited replicative potential and angiogenesis, and can be used as a predictive biomarker for *BRAF*-targeted therapy. In addition, the prognostic role of the *BRAF* mutation has been emphasized in several types of cancers.^{34–37} However, the incidence of the *BRAF* mutation in ESCC remains less clear. One study showed the absence of a *BRAF* mutation in 35 ESCCs, and the other showed that only 1 tumor harbored a *BRAF* mutation among 80 ESCC tumors. In this study, the *BRAF* mutation was absent in 203 ESCCs.

In summary, *KRAS* mutations were extremely rare, and the *BRAF* mutation was absent in a nonbiased database of 203 ESCCs. This suggests that *KRAS* and *BRAF* mutations play a limited role in the development of ESCC and that mutation analysis is not useful as a predictive marker for sensitivity to anti-EGFR therapy in ESCC.

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