Original Article (Imaging, Diagnosis, Prognosis)

**PIK3CA** mutation is associated with a favorable prognosis among patients with curatively resected esophageal squamous cell carcinoma

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TRANSLATIONAL RELAVANCE

*PIK3CA* mutations and subsequent activation of the AKT pathway play a crucial role in human neoplasms. *PIK3CA* mutations have been associated with poor prognosis in patients with colorectal or lung cancer. In contrast, the relationship between *PIK3CA* mutations and favorable prognoses has been shown in breast cancer. However, no large-scale study has examined the prognostic impact of *PIK3CA* mutations in esophageal squamous cell carcinoma (ESCC). In this study, we quantified the *PIK3CA* mutations in exons 9 and 20 using a non-biased database of 219 curatively resected ESCCs and pyrosequencing technology. This is, by far, the largest study on the prognostic role of *PIK3CA* mutations in ESCC to date, and it shows that *PIK3CA* mutations in ESCC are associated with favorable prognoses. Our data suggest that *PIK3CA* mutational status can have a potential role as a prognostic biomarker.
ABSTRACT

Purpose: PIK3CA encodes the catalytic subunit of PI3K, p110α. Mutant PIK3CA stimulates the AKT pathway and promotes cancer cell proliferation. PIK3CA mutations have been associated with poor prognosis in patients with colorectal or lung cancer. In contrast, the relationship between PIK3CA mutations and favorable prognoses has been shown in breast cancer. However, the influence of PIK3CA mutations on the prognosis of esophageal squamous cell carcinoma (ESCC) patients remains unclear.

Experimental Design: Using a non-biased database of 219 curatively resected ESCCs and eight esophageal cancer cell lines, we evaluated PIK3CA mutational status by pyrosequencing. The expression of p53 and phosphorylated AKT (i.e., AKT activation) was evaluated by immunohistochemistry.

Results: PIK3CA mutations in exon 9 and/or 20 were detected in 46 cases (21%). No ESCC cell line harbored PIK3CA mutations. PIK3CA mutations were significantly associated with phosphorylated AKT expression, but not with p53 expression, sex, age at surgery, tobacco use, alcohol use, or histological grade. Compared with wild-type PIK3CA cases, patients with PIK3CA mutations in exons 9 and/or 20 experienced significantly better disease-free survival [log-rank \( P=0.0089; \) univariate HR= 0.37, 95% confidence interval (CI) 0.15–0.75, \( P=0.0042; \) multivariate HR=0.34, 95% CI 0.10–0.86, \( P=0.021 \)] and overall survival (log-rank \( P=0.012; \) univariate HR=0.38, 95% CI 0.16–0.78, \( P=0.0060; \) multivariate HR=0.35, 95% CI 0.10–0.90, \( P=0.028 \)).

Conclusion: PIK3CA mutations in ESCC are associated with longer survival, suggesting its role as a prognostic biomarker. Future studies are needed to confirm this association.
and to elucidate the exact mechanisms by which \textit{PIK3CA} mutations affect tumor behavior.
INTRODUCTION

Esophageal squamous cell carcinoma (ESCC), the major histological type of esophageal cancer in East Asian countries, is one of the most aggressive malignant tumors (1). Despite the development of multimodality therapies, the prognosis of patients remains poor, even for those who undergo complete resection of their carcinomas (2-4). The limited improvement in treatment outcome provided by conventional therapies has prompted us to seek innovative strategies for treating ESCCs, especially those that are molecularly-targeted. The identification of new prognostic or predictive molecular markers for ESCC may improve the risk-adapted therapeutic strategies and help stratify patients in future clinical trials for treatment with drugs targeting these molecular changes (5, 6).

Activation of the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway plays an important role in the development of a variety of human carcinomas, and several efforts are underway to define therapeutic inhibitors of this signaling pathway (7, 8). PI3K interacts with phosphatidylinositol-3-phosphate at the membrane and catalyzes the phosphorylation of AKT, which activates the downstream signaling pathway (8). Mutations in the \textit{PIK3CA} gene, which encodes the p110α catalytic subunit of PI3K (7, 8), have been identified in many human cancers (9). The majority of activating \textit{PIK3CA} mutations map to 3 sites (codons 542 and 545 in exon 9 and codon 1,047 in exon 20) (10). In studies of ESCC, \textit{PIK3CA} mutation has been detected in 2.2% to 11.8% of analyzed cases (11-14), and its prognostic role has been still unknown. Previous studies in large numbers of cases (more than 200) have shown that \textit{PIK3CA} mutations are associated with unfavorable prognosis in patients with colorectal (15-19) or lung cancer...
(20). On the other hand, a study utilizing 590 patients with breast cancer demonstrated the relationship between PIK3CA mutation and a favorable prognosis, although it was not statistically significant in multivariate analysis (21) (**Table1**). Given accumulating evidence on vital roles of the PIK3/AKT pathway in cancer cells, we hypothesized that PIK3CA-Wild type ESCCs and PIK3CA-mutation ESCCs might behave differently.

To test this hypothesis, we quantified the PIK3CA mutations in 219 samples of curatively resected ESCCs using pyrosequencing, and examined the prognostic significance of PIK3CA mutations in ESCC. In addition, we also evaluated the relationship between PIK3CA mutations, phosphorylated AKT (p-AKT) expression, which was the most successful biomarker for evaluating the status of AKT activation, and p53 expression, which was one of the important molecular alterations in ESCC.

**METHODS**

**Study subjects**

A total of 235 patients with ESCC who underwent curative resection at Kumamoto University Hospital between April 2005 and December 2011 were enrolled in this study. Fifteen patients were excluded because of the unavailability of adequate tissue samples. We initially quantified PIK3CA mutations in 220 cancer specimens, and obtained valid results in 219 (99.5%) of the cases. Thus, a total of 219 ESCCs were ultimately included in this study. Patients were observed at 1- to 3-month intervals until death or 31 March 2012, whichever came first. Tumor staging was performed by the American Joint Committee on Cancer Staging Manual (7th edition) (22). Eighty-two patients received
preoperative treatment [50 patients; chemotherapy (cisplatin, 5-FU either with or without docetaxel), five patients; radiation therapy, 27 patients; chemoradiotherapy].

Disease-free survival was defined as the length of time after surgical treatment of the cancer during which the patient survived with no sign of cancer recurrence. Overall survival was defined as the time between the date of the operation and the date of death. Cancer-specific survival was defined as the time between the date of operation and the date of death, which was confirmed to be attributable to ESCC. In our 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 were comparable with 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), certainly supporting the absence of bias in our database. Written informed consent was obtained from each subject, and the study procedures were approved by the institutional review board. The term “prognostic marker” is used throughout this article according to the REMARK Guidelines (23).

**Cell Lines**

The TE-1, TE-4, TE-8, TE-9, TE-11, TE-14, TE-15 (obtained from the Institute of Development, Aging, and Cancer, Tohoku University) and KYSE30 (obtained from Japanese Collection Research Bioresources, Japan) human esophageal cancer cell lines were cultured in a medium supplemented with 10% fetal bovine serum in 5% CO₂ atmosphere at 37°C.

**Genomic DNA Extraction**
Genomic DNA was extracted from 220 paraffin-embedded tissue specimens of surgically resected esophageal cancers. Tumors areas were marked on hematoxylin–eosin stained slides by one pathologist (Y.B). 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 ESCC cell lines using a QIAmp DNA mini kit (Qiagen).

**Pyrosequencing for PIK3CA mutations**

The exon 9 PCR primers were as follows:

\[ \text{PIK3CA} \text{ 9-F, 5'}\text{-biotin-AACAGCTCAAAGCAATTTCTACACG-3';} \]
\[ \text{PIK3CA} \text{ 9-R, 5'}\text{-ACCTGTGACTCCATAGAAAAATCTTT-3'.} \]

Each PCR mix contained the forward and reverse primers (10 μM each), 0.2 mM each of dNTP Mix with dUTP, 0.2 U of AmpErase (Uracil-N glycosylase; Applied Biosystems), 2 mM of MgCl2, 1× PCR buffer, 0.9 U of AmpliTaq Gold 360 (Applied Biosystems), and 3.5 μl of template WGA product in a total volume of 35 μl. The exon 20 PCR primers were:

\[ \text{PIK3CA} \text{ 20-F, 5'}\text{-biotin-CAAGAGGCTTTGGAGTATTTCA-3';} \]
\[ \text{PIK3CA} \text{ 20-R, 5'}\text{-CAATCCATTTTTGTGTTGCCA-3'.} \]

Each PCR mix contained the same components described for the exon 9 PCR reactions except 2.5 μl of template WGA product was used in a total volume of 25 μl.

PCR conditions consisted of initial denaturing at 50°C (10 min) for AmpErase UNG; initial denaturing at 94°C (10 min) for AmpliTaq Gold 360; 50 cycles of 95°C (30 sec), annealing (30 sec; 60°C for PIK3CA exon 9, 57°C for PIK3CA exon 20), and 72°C (30 sec); and final extension at 72°C (7 min). The PCR products were electrophoresed on
agarose gels to confirm successful amplification of the 81-bp (exon 9) and 74-bp (exon 20) products.

*PIK3CA* pyrosequencing was performed using the PyroMark Q24 System (Qiagen) according to the manufacturer’s instructions. All forward sequencing results were confirmed by reverse sequencing. In the *PIK3CA* exon 9 pyrosequencing assay, we routinely confirmed the presence of a mutation by three different sequencing primers and by the creation of frameshifted reading of a mutant sequence relative to a wild-type sequence in a program. Representative pyrograms of wild-type and mutant exon 9 and 20 are shown in Figure 1. The primer *PIK3CA* 9-RS1 (5’-CCATAGAAAATCTTTTCTCCT-3’; nucleotide dispensation order, ATCGACTACACTGACTGACTGACTGACTGACTGACTG) could detect the c.1634A>G mutation. The primer *PIK3CA* 9-RS2 (5’-TTCTCCTTGCCTTCAGTGATTT-3’; nucleotide dispensation order, ATACACATGTCAGACTAGCTAGCTAGCTAGCTAG) was particularly sensitive to detect c.1624G>A mutation. The primer *PIK3CA* 9-RS3 (5’-TAGAAAATCTTTTCTCCTG-3’; nucleotide dispensation order, ATAGCACTGACTGACTGACTGACTGACTGACTGACTG) could detect the most common mutation (c.1624G>A). For *PIK3CA* exon 20, we used the primer *PIK3CA* 20-RS (5’-GTTGTCCAGCCACCA-3’; nucleotide dispensation order, CTGACGATACTGTCATCATATGCACTGACTGACTG) to detect various exon 20 mutations (c.3140A>G, c.3139C>T). Nucleotide dispensation orders were designed so that if any of the common mutations were present it caused a shift in the reading frame and resulted in additional new peak(s) (indicated by arrowheads in Figure 1) following the mutated nucleotide.
**Immunohistochemistry**

For p53 staining, we used the anti-p53 antibody [Mouse monoclonal anti-p53 (PAb1801 Ab-2), Santa Cruz Biotechnology, CA]. The second antibody used was a ready-for-use anti-mouse EnVision-Peroxidase system (Dako Japan Inc., Tokyo, Japan). For p-AKT staining, we utilized the pan-AKT antibody [Rabbit monoclonal anti-Phospho-Akt (Ser473) (736E11), Cell Signaling Technology, Boston, MA] and a subsequent reaction was performed with the Vectastain ABC Elite avidin/biotin/peroxidase kit (Vector Laboratories Inc., Burlingame, CA, USA). In each case, we recorded nuclear p53 expression and cytoplasmic p-AKT expression as no expression, weak expression, moderate expression, or strong expression compared to normal esophageal epithelial cells. The positivity of p53 and p-AKT was defined as the presence of weak to strong expression. Among 219 esophageal cancers, we observed p53 expression in 117 tumors (53%) and p-AKT expression in 79 tumors (36%) (Supplemental figure 1).

**Statistical methods**

For the statistical analyses, we used the JMP (Version 9, SAS Institute, Cary, NC) and the SAS software programs (Version 9.1, SAS Institute). All P values were two-sided. For the survival analysis, the Kaplan-Meier method was used to assess the survival time distribution, and the log-rank test was used. To assess the independent effect of the PIK3CA mutations on mortality, the tumor stage (IA+IB, IIA+IIB, IIIA+IIIB+IIIC) was used as a stratifying (matching) variable in Cox models using the “strata” option in the SAS “proc phreg” command to avoid residual confounding and overfitting. We constructed a multivariate, stage-stratified Cox proportional hazard model to compute a hazard ratio.
(HR) according to PIK3CA mutation status, sex (male vs. female), age at surgery (continuous variable), tobacco use (yes vs. no), alcohol use (yes vs. no), year of diagnosis (2005–2008 vs. 2009–2011), preoperative treatment (yes vs. no) and histological grade (G1 vs. G2-4). A backward stepwise elimination with a threshold of $P=0.2$ was used to select variables in the final model. For cases with missing information in any of the categorical variables [tobacco use (1.4%) and alcohol use (2.3%)], we included those cases in a majority category of that variable in the initial model. After the selection was complete, we assigned separate missing indicator variables to those cases with missing information in the final model. An interaction was assessed by including the cross product of the PIK3CA variable and another variable of interest (without data-missing cases) in a multivariate Cox model, and thereafter the Wald test was performed.

RESULTS

PIK3CA mutational status in ESCC

Among 219 patients who had undergone curative resection of stage I to III ESCC, we examined PIK3CA exon 9 and 20 mutations by pyrosequencing technology (Figure 1). PIK3CA mutations were detected in 46 (21%) of 219 cases; 11 cases in exon 9 only, 21 cases in exon 20 only, and 14 cases in both exon 9 and 20. The most common mutation was the c.3140A>G (p.H1047R) mutation, which was present in 35 tumors, followed by c.1633G>A (p.E545K) in 16 tumors (Supplementary Table 1). All ESCC cell lines (TE-1,
TE-4, TE-8, TE-9, TE-11, TE-14, TE-15 and KYSE30) showed wild-type statuses in both exon 9 and 20.

**PIK3CA mutations and patients characteristics**

*PIK3CA* mutations were significantly associated with p-AKT expression (*P*=0.0043). In contrast, there was no significant relationship between *PIK3CA* mutations and p53 expression. *PIK3CA* mutations were not significantly associated with any clinical, epidemiological or pathological characteristics (Table 2).

**PIK3CA mutations and patient survival**

We assessed the influence of *PIK3CA* mutations on clinical outcome in patients with curatively resected ESCC. During the follow-up of the 219 patients, there were a total of 71 esophageal cancer recurrences, 70 deaths, and 52 deaths confirmed to be attributable to esophageal cancer. The median follow-up time for censored patients was 2.2 years.

In the Kaplan-Meier analysis, patients with *PIK3CA* mutations experienced significantly longer disease-free survival (log rank *P*=0.0089), cancer-specific survival (log rank *P*=0.021), and overall survival (log rank *P*=0.012) than those with wild-type *PIK3CA* (Figure 2). In the univariate Cox regression analysis, compared with patients with wild-type *PIK3CA* tumors, those patients with *PIK3CA*-mutated tumors experienced significant improvement in the disease-recurrence rate [hazard ratio (HR): 0.37; 95% CI: 0.15–0.75; *P*=0.0042], in cancer-specific mortality (HR: 0.35; 95% CI: 0.12–0.81; *P*=0.011) and in overall mortality (HR: 0.38; 95% CI: 0.16–0.78; *P*=0.0060) (Table 3). In_
the multivariate Cox model adjusted for the clinical, epidemiological and pathological features, *PIK3CA* mutations were found to be associated with an improvement in the disease-recurrence rate (multivariate HR: 0.34; 95% CI: 0.10–0.86; *P*=0.021), cancer-specific survival (multivariate HR: 0.33; 95% CI: 0.075–1.02; *P*=0.054) and overall survival (multivariate HR: 0.35; 95% CI: 0.10–0.90; *P*=0.028) (Table 3). Other features (sex, age at surgery, tobacco use, alcohol use, year of diagnosis, and histological grade) were not significantly associated with patient survival in the multivariate analyses.

We also assessed *PIK3CA* exon 9 mutations and exon 20 mutations separately. However, there was no significant difference in mortality between exon 9 and exon 20 mutations (log rank *P*=0.59). In addition, patients with concomitant *PIK3CA* mutations in exons 9 and 20 experienced a similar prognosis with those with *PIK3CA* mutation in either exon 9 or 20 alone (log rank *P*=0.98) (Supplementary figure 2).

**Survival analyses of the interaction between *PIK3CA* mutations and other variables**

We also examined whether the influence of *PIK3CA* mutations on cancer-specific survival was modified by any of the clinical, pathological and epidemiological variables evaluated. The effect of *PIK3CA* mutations was not significantly modified by tobacco use, alcohol use, year of diagnosis, tumor grade, or tumor stage (all *P*>0.29; Figure 3).

**Survival analyses for patients with or without preoperative therapy**
The relationship between preoperative chemotherapy and/or radiotherapy and \textit{PIK3CA} mutation is not known. Therefore, we performed the additional survival analyses for two subgroups; one is a cohort without preoperative therapy (n=137) and another is a cohort with preoperative treatment (n=82). Patients with \textit{PIK3CA} mutations experienced better disease-free survival compared with wild-type \textit{PIK3CA} cases both in cohort without preoperative treatment (log rank \( P=0.080 \), univariate HR=0.41, 95% CI 0.12-1.03, \( P=0.058 \)) and cohort with preoperative treatment (log rank \( P=0.064 \), univariate HR=0.34, 95% CI 0.083-0.96, \( P=0.041 \)) (Supplementary figure 3). Notably, we did not observe a modifying effect of the preoperative treatment on the relationship between \textit{PIK3CA} mutation and the recurrence rate (\( P \) for interaction = 0.76, Figure 3).

**DISCUSSION**

\textit{PIK3CA} mutations and subsequent activation of the AKT pathway are considered to play a crucial role in human neoplasms (24-26). The PI3K/AKT pathway has emerged as a central node in cancer cell signaling pathways downstream of growth factors, cytokines, and other cellular stimuli (7, 8). We conducted this study to examine the prognostic impact of \textit{PIK3CA} mutations among 219 patients with curatively resected esophageal squamous cell carcinomas (ESCCs). We found that \textit{PIK3CA} mutations in ESCCs are associated with a good prognosis, suggesting that \textit{PIK3CA} mutational status may be a biomarker that can be used to identify patients who will experience a favorable clinical outcome.
Examining biomarkers or prognostic factors is important in cancer research (27-31). Previous studies examining the relationship between PIK3CA mutations and prognosis in human cancers have yielded variable results (Table 1; all n ≥ 200). In addition, the relationship between PIK3CA mutations and a better prognosis has been demonstrated in several small-sized studies (all n < 200) of breast cancer and ovarian cancer (32-35). This discrepancy might be due to differences in the tumor histological type. A study of ESCC showed that PIK3CA mutational status is not associated with patient survival; however, this study was limited by its small sample size (n=87) (11). The importance of large-scale studies cannot be emphasized enough, because small studies with null results have much higher likelihood of being unpublished than small studies with “significant” results, leading to publication bias. In contrast to the previous study, our study examined PIK3CA mutational status in a much larger non-biased cohort of ESCCs. Nonetheless, our finding of the correlation between PIK3CA mutations and favorable prognosis in esophageal cancer needs to be confirmed by independent studies in the future.

We also found the significant relationship between PIK3CA mutation and p-AKT expression (i.e., AKT activation) in ESCCs. This finding is in agreement with previous results on colorectal cancer, bladder cancer, and pancreas neoplasms (36-38). There was no perfect correlation between PIK3CA mutation and p-AKT, in part because there were other mechanisms of AKT activation (i.e., PTEN loss and/or methylation, PIK3CA amplification, and PIK3RI mutation). Given the well-known roles of the AKT pathway in tumor proliferation, invasion, and survival (8, 39-41), one could expect that PIK3CA mutations would imply poor clinical outcome. It is very common to assume that the
presence of oncogene activation or tumor suppressor inactivation implies aggressive tumor behavior. However, this presumption does not always hold true. Esophageal cancers develop through the accumulation of multiple genetic and/or epigenetic alterations, and each tumor has its own unique combination of these molecular aberrations. Tumors harboring mutant \textit{PIK3CA} may activate the AKT pathway, while tumors harboring wild-type \textit{PIK3CA} may not. In order to acquire malignant characteristics, wild-type \textit{PIK3CA} tumors require alternative molecular aberrations to the AKT pathway. These alternative aberrations may lead to more aggressive phenotypes that the AKT pathway actually does.

Another possible explanation for the relationship between \textit{PIK3CA} mutations and good prognosis may be due to “tumor suppressive” roles of the AKT pathway (42). In an experimental study utilizing primary esophageal epithelial cells, inducible activation of AKT results in growth arrest and a senescent phenotype in normal epithelial cells (43). That AKT activation induces growth arrest is reminiscent of the effects of oncogenic \textit{Ras} in primary human cells. Indeed, as with oncogenic \textit{Ras}, activation of AKT in primary cells may represent an anti-tumorigenic effect (44, 45). In addition, one study on colorectal cancer reported that p-AKT expression is associated with low stage cancer and good prognosis (36). Future studies are necessary to elucidate the biological mechanisms by which \textit{PIK3CA} mutation activation affects esophageal cancer behavior.

Interestingly, we detected \textit{PIK3CA} mutations in 21% of ESCCs. This frequency is slightly higher compared with those of previous studies on ESCCs. This difference might be due to a difference in the patient cohorts or the methods used to assess \textit{PIK3CA} mutation. In the current study, we used pyrosequencing, which has been shown to be
more sensitive than regular Sanger sequencing in *KRAS* mutation analysis (46).

Pyrosequencing is a non-electrophoretic nucleotide extension sequencing technology that can be used for mutation detection in tumors. Pyrosequencing assay for *PIK3CA* mutation detection is certainly useful, because most activating *PIK3CA* mutations cluster in the hotspots of exons 9 and 20, affecting the functionally important helical and kinase domains.

In summary, our large cohort study suggests that *PIK3CA* mutations are associated with a favorable clinical outcome in stage I to III esophageal cancer, supporting its role as a prognostic biomarker. Future studies are needed to confirm this association, and to elucidate the exact mechanisms by which *PIK3CA* mutation affects tumor behavior.

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REFERENCES


an official journal of the American Association for Cancer Research.


Table 1. Studies on prognostic significance of PIK3CA mutations in several types of cancers

<table>
<thead>
<tr>
<th>Author (year)</th>
<th>Type of cancer</th>
<th>Prognostic effect</th>
<th>Sample size</th>
<th>HR (95%CI)</th>
<th>Method</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.68 (1.24–5.77; OS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farina et al. (2011)</td>
<td>Colon Cancer</td>
<td>unfavorable</td>
<td>685</td>
<td>N/A</td>
<td>Single nucleotide primer extension, real time PCR</td>
<td>(16)</td>
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<tr>
<td>De Roock et al. (2010)</td>
<td>Colon Cancer</td>
<td>unfavorable</td>
<td>743</td>
<td>2.27 (1.10–4.66; PFS)</td>
<td>MassARRAY system</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.30 (1.46–7.45; OS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>He et al. (2009)</td>
<td>Rectum Cancer</td>
<td>unfavorable</td>
<td>240</td>
<td>3.4 (1.2-9.2; LR)</td>
<td>Direct sequencing</td>
<td>(18)</td>
</tr>
<tr>
<td>Kalinsky et al. (2009)</td>
<td>Breast Cancer</td>
<td>favorable</td>
<td>590</td>
<td>0.7 (0.4–1.2; CS)</td>
<td>MassARRAY system</td>
<td>(21)</td>
</tr>
<tr>
<td>Ogino et al. (2009)</td>
<td>Colon Cancer</td>
<td>unfavorable</td>
<td>450</td>
<td>2.23 (1.21–4.11; CS)</td>
<td>Pyrosequencing</td>
<td>(19)</td>
</tr>
<tr>
<td>Kawano et al. (2006)</td>
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<td>235</td>
<td>N/A</td>
<td>Direct sequencing</td>
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<tr>
<td>Current study</td>
<td>ESCC</td>
<td>favorable</td>
<td>219</td>
<td>N/A</td>
<td>Pyrosequencing</td>
<td></td>
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</table>

Abbreviations: CS, cancer-specific survival; DFS, disease-free survival; LR, local recurrence; N/A, not available; OS, overall survival; PFS, progression-free survival; RFS, relapse-free survival.
Table 2: *PIK3CA* mutational status in esophageal cancers, and clinical and tumor features

<table>
<thead>
<tr>
<th>Clinical, epidemiological or pathological feature</th>
<th>Total N</th>
<th><em>PIK3CA</em></th>
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<td></td>
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<td>Mutant</td>
<td>Wild-type</td>
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<tr>
<td>All cases</td>
<td>219</td>
<td>46</td>
<td>173</td>
<td>0.10</td>
<td>0.44</td>
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<td>Mean age ± SD</td>
<td>66.9 ± 8.8</td>
<td>64.7 ± 9.2</td>
<td>67.5 ± 8.7</td>
<td>0.10</td>
<td>0.44</td>
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<td>Sex</td>
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</tr>
<tr>
<td>Male</td>
<td>193 (88%)</td>
<td>39 (85%)</td>
<td>154 (89%)</td>
<td></td>
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<tr>
<td>Female</td>
<td>26 (12%)</td>
<td>7 (15%)</td>
<td>19 (11%)</td>
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<td>Tobacco use</td>
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<td>160 (74%)</td>
<td>35 (76%)</td>
<td>35 (74%)</td>
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<td>11 (24%)</td>
<td>11 (26%)</td>
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<tr>
<td>Alcohol use</td>
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<td></td>
<td>0.65</td>
<td></td>
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<tr>
<td>Yes</td>
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<td>37 (80%)</td>
<td>140 (84%)</td>
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<tr>
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<td>35 (17%)</td>
<td>9 (20%)</td>
<td>26 (16%)</td>
<td>0.65</td>
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<td>0.83</td>
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<tr>
<td>2005 to 2008</td>
<td>116 (53%)</td>
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<td>91 (53%)</td>
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<tr>
<td>2009 to 2011</td>
<td>103 (47%)</td>
<td>21 (46%)</td>
<td>82 (47%)</td>
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<tr>
<td>Preoperative treatment</td>
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<tr>
<td>Present</td>
<td>82 (37%)</td>
<td>15 (33%)</td>
<td>67 (39%)</td>
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<tr>
<td>Absent</td>
<td>137 (63%)</td>
<td>31 (67%)</td>
<td>106 (61%)</td>
<td>0.44</td>
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<tr>
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<td></td>
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<tr>
<td>IA</td>
<td>29 (13%)</td>
<td>9 (20%)</td>
<td>20 (12%)</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>IB</td>
<td>46 (21%)</td>
<td>11 (24%)</td>
<td>35 (16%)</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>IIA</td>
<td>18 (8.2%)</td>
<td>6 (13%)</td>
<td>12 (6.9%)</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>IIB</td>
<td>45 (21%)</td>
<td>7 (15%)</td>
<td>38 (22%)</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>IIIA</td>
<td>33 (15%)</td>
<td>7 (15%)</td>
<td>26 (15%)</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>IIIB</td>
<td>19 (8.7%)</td>
<td>1 (2.2%)</td>
<td>18 (10%)</td>
<td>0.22</td>
<td></td>
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<tr>
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<td>5 (11%)</td>
<td>24 (14%)</td>
<td>0.22</td>
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</tr>
<tr>
<td>Histologic grade</td>
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<td></td>
<td>0.58</td>
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<tr>
<td>G1</td>
<td>80 (37%)</td>
<td>16 (35%)</td>
<td>64 (37%)</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>109 (50%)</td>
<td>26 (57%)</td>
<td>83 (48%)</td>
<td>0.58</td>
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<tr>
<td>G3</td>
<td>25 (11%)</td>
<td>3 (6.5%)</td>
<td>22 (13%)</td>
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<tr>
<td>G4</td>
<td>5 (2.3%)</td>
<td>1 (2.2%)</td>
<td>4 (2.3%)</td>
<td>0.58</td>
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</table>

(Table 2, continued)
(Table 2, continuing from the previous page)

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<th>P value</th>
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<td>Wild-type</td>
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<td>19 (41%)</td>
<td>98 (57%)</td>
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<tr>
<td>negative</td>
<td>102 (47%)</td>
<td>27 (59%)</td>
<td>75 (43%)</td>
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<td>p-AKT expression</td>
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<td>25 (54%)</td>
<td>54 (31%)</td>
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<tr>
<td>negative</td>
<td>140 (64%)</td>
<td>21 (46%)</td>
<td>119 (69%)</td>
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</table>

(%) indicates the proportion of cases with a specific clinical, pathological, epidemiological or molecular feature among each group (wild type or mutant).
Table 3: **PIK3CA** status in esophageal cancer and patient mortality

<table>
<thead>
<tr>
<th>PIK3CA</th>
<th>Total N</th>
<th>Disease-free survival</th>
<th>Cancer-specific survival</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Univariate HR (95% CI)</td>
<td>Multivariate stage-matched HR (95% CI)</td>
<td>Univariate HR (95% CI)</td>
</tr>
<tr>
<td>Wild type</td>
<td>173</td>
<td>1 (referent)</td>
<td>1 (referent)</td>
<td>1 (referent)</td>
</tr>
<tr>
<td>Mutant</td>
<td>46</td>
<td>0.37 (0.15-0.75)</td>
<td>0.34 (0.10-0.86)</td>
<td>0.35 (0.12-0.81)</td>
</tr>
<tr>
<td>P value</td>
<td>0.0042</td>
<td>0.021</td>
<td>0.011</td>
<td>0.054</td>
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</table>
**Figure Legends**

**Figure 1:** *PIK3CA* exon 9 and exon 20 Pyrograms (antisense strand). (A) Wild-type exon 9 sequenced with the 9-RS1 primer. (B) The c.1634A>G mutation (arrow) causes a shift in the reading frame and results in a new peak at A (arrowhead), which serves as a quality assurance. (C) Wild-type exon 9 sequenced with the 9-RS2 primer. (D) The c.1624G>A mutation (arrow) causes a shift in the reading frame and results in a new peak at A (arrowhead). (E) Wild-type exon 9 sequenced with the 9-RS3 primer. (F) The c.1633G>A mutation (arrow) causes a shift in the reading frame and results in new peaks (arrowheads). (G) Wild-type exon 20. (H) The c.3139C>T mutation (arrow) causes a shift in the reading frame and results in a new peak at T (arrowhead). (I) The c.3140A>G mutation (arrow) causes a shift in the reading frame and results in a new peak at G (arrowhead). Mut indicates mutant; WT, wild type.

**Figure 2:** Kaplan-Meier curves for disease-free survival (left panel) and cancer-specific survival (right panel) in stage I to III ESCC according to *PIK3CA* mutational status.

**Figure 3:** Stratified analysis of *PIK3CA* mutation and cancer-specific mortality. Loge (adjusted HR) and 95% CI for *PIK3CA*-mutated tumors (vs. *PIK3CA* wild-type tumors) in various strata are shown.
A. Wild-type (Exon9-RS-1)

B. PIK3CA mutation c.1634A>G (Exon9-RS-1)

C. Wild-type (Exon9-RS-2)

D. PIK3CA mutation c.1624G>A (Exon9-RS-2)

E. Wild-type (Exon9-RS-3)

F. PIK3CA mutation c.1633G>A (Exon9-RS-3)

G. Wild-type (Exon20-RS)

H. PIK3CA mutation c.3139C>T (Exon20-RS)

I. PIK3CA mutation c.3140A>G (Exon20-RS)

Figure 1
Figure 2

Disease free survival

- PIK3CA mutant
- PIK3CA wild type

Survival rate vs. Disease free survival

Cancer-specific survival

- PIK3CA mutant
- PIK3CA wild type

Survival rate vs. Cancer-specific survival

Overall survival

- PIK3CA mutant
- PIK3CA wild type

Survival rate vs. Overall survival

p=0.0089

p=0.021

p=0.012
Figure 3
Clinical Cancer Research

PIK3CA mutation is associated with a favorable prognosis among patients with curatively resected esophageal squamous cell carcinoma

Hironobu Shigaki, Yoshifumi Baba, Masayuki Watanabe, et al.

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