Overexpression of PIK3CA is associated with lymph node metastasis in esophageal squamous cell carcinoma

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Abstract. The genomic region containing *PIK3CA* was found to be amplified in esophageal squamous cell carcinoma (ESCC) tissue. *PIK3CA* at 3q26, which encodes the *p110α* catalytic subunit of phosphatidylinositol (PI) 3-kinase, is a unique intracellular signal transducer characterized by its lipid substrate specificity. In order to characterize PIK3CA in ESCC, we investigated hot-spot mutations in exons 1, 9 and 20, the copy number gain, the expression levels of mRNA and protein. Analysis in exon 9 of the *PIK3CA* gene revealed mutation in 7.7% (4 of 52) of ESCC samples. No mutation was detected in either exon 1 or exon 20. Copy number amplifications of *PIK3CA* were found in 12 of the 45 patients (26.7%). PIK3CA mRNAs were examined in 37 ESCC patients as determined by quantitative RT-PCR and the mean mRNA level of PIK3CA in ESCC tissues was 2.61-fold higher compared with that in corresponding non-tumorous esophageal epithelia ($P<0.001$). Immunohistochemically, positive immunoreaction for PIK3CA was detectable in 33 of 66 (50.0%) ESCC cases, while it was not detectable in the remaining 33 cases. Furthermore, comparing the cases with negative staining with those with positive staining for PIK3CA, the presence of node metastasis was significantly correlated with those with positive staining ($P<0.05$). This study is the first report providing comprehensive analysis of PIK3CA expression in ESCC. These results indicate that PIK3CA may play a crucial role in the development of ESCC and serve as an indicator for lymph node metastasis.

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Introduction

The genetic mechanisms underlying the development and progression of esophageal squamous cell carcinoma (ESCC) are among the most complex in human tumors and it has been reported that gene amplification is one of the essential mechanisms of oncogene activation in many cancers (1). Various chromosomal amplicons have recently been identified in ESCC by comparative genomic hybridization (CGH) (2). In particular, genomic amplification of the 3q26 region was identified to be one of the most frequent amplicons in ESCC (2-6). It would be of great interest to detect the specific gene targets within these amplicons, however, they remain largely unknown.

In search of the genes whose selection drives 3q26 amplification, a recent study has provided convincing evidence that *PIK3CA* at 3q26 (7), which encodes the *p110α* catalytic subunit of phosphatidylinositol (PI) 3-kinase (Gene Bank NM_006218), is found to be amplified and overexpressed in ovarian and cervical cancer (8-11). The increased copy number of the *PIK3CA* gene is associated with increased *PIK3CA* transcription, *p110α* protein expression and PI3K activity in ovarian cancer (9). It has been reported that aberrant PI3-kinase activation plays important roles in sustaining processes important to malignancy, including cell proliferation, adhesion, survival and motility (9,12-18). PIK3CA allows Ser/Thr kinase (Akt), which is one critical downstream target of this signaling pathway, to be phosphorylated by PDK1 and PDK2 at Thr308 and Ser473, respectively (12). After phosphorylation by Akt, the oncoprotein Mdm2 binds the transactivation domain of tumor suppressor *p53* and inhibits expression of *p53*-regulated genes involved in cell cycle arrest and apoptosis (12). In addition to Akt, PI3-kinase has been shown to regulate the activity of other cellular targets, such as the serum and glucocorticoid-inducible kinase (SGK), the small GTP-binding proteins RAC1 and CDC42 and protein kinase C (PKC), in an *Akt*-independent manner through poorly characterized mechanisms (12). The activity of these targets leads to survival, cytoskeletal rearrangement and transformation (12). More recently, somatic mutations of *PIK3CA* have been reported in carcinomas of the colon, breast, brain, liver, stomach, lung, ovary, head and neck. The

mutated *PIK3CA* protein is able to activate Akt in the absence of growth factors (19-21). A recent study has reported on the somatic mutations of *PIK3CA* in ESCC, however, the incidence is lower than in other malignancies (22). The information on both *in vitro* data and the clinicopathological relevance of PIK3CA in ESCC is, however, sparse and these findings on PIK3CA in various tumors promoted us to investigate both the genetic alterations of PIK3CA and expression patterns in ESCC which is one of the most clinically malignant neoplasms.

In order to characterize PIK3CA in ESCC, we investigated *PIK3CA* hot-spot mutations in exons 1, 9 and 20, the copy number gain, the expression of PIK3CA mRNA and protein. Finally, in addition to the International Union Against Cancer Tumor-Node-Metastasis (TNM) classification, clinicopathological factors including age, gender, vascular and lymphatic invasion, intraepithelial spread, intramural metastasis, histological grading, stage and development of recurrence were analyzed.

Materials and methods

Patients and tissues. Sixty-six representative cases with esophageal squamous cell carcinoma who underwent surgery at Nippon Medical School Main Hospital (Bunkyo-ku, Tokyo, Japan) were selected from our archives. The patients consisted of 53 males and 13 females, whose median age was 65 years (range, 45-81 years). The distribution of tumor grades was stage 0 (4 cases), stage I (19 cases), stage IIA (11 cases), stage IIB (9 cases), stage III (13 cases), stage IVA (5 cases) and stage IVB (5 cases). This study was carried out in accordance with the principles embodied in the Declaration of Helsinki, 1975 and informed consent for the usage of esophageal tissues was obtained from each patient.

DNA extraction and mutation analysis. Genomic DNAs were extracted from both tumor and non-tumorous tissues using a DNeasy tissue kit (Qiagen, Valencia, CA). The corresponding non-tumorous tissues were obtained from surgically resected tissues which were located at least 5 cm away from the tumors. Genomic DNAs from corresponding non-tumorous tissues were also analyzed to confirm whether the nucleotide substitutions detected in tumor tissues were somatic in nature or not, when nucleotide changes were detected in tumor tissues. Genomic DNAs (100 ng per sample) were amplified with primers covering the entire coding region and the exon/intron boundaries of the desired exons using the web-free software Primer3 (Table IA). Exons 1, 9 and 20 of *PIK3CA* were sequenced directly using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 3730 automated capillary sequencer. Analyses of all PCR products with sequence variants were repeated for confirmation.

PIK3CA copy number quantitation. The *PIK3CA* copy number was evaluated by real-time quantitative PCR analysis. Primers were designed for the simultaneous amplification of a 150 bp fragment of *PIK3CA* gene (3q26) and a 187 bp segment of *COL7A1* (3p21), a single copy gene taken as control (Table IB). Gene chosen as control is located on the same chromosome as the target gene to ensure that the increase of value, suggestive of an increase in copy number, is not the result of the corresponding chromosome polysomy. Reactions were performed in 25 μl volume containing 80 ng of DNA, 300 nM each primer (for both *PIK3CA* and *COL7A1*, in independent reactions) and 1X SYBR-Green PCR Master Mix according to the manufacturer's protocol. PCR universal conditions were: 10 min at 95˚C, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 31 sec. Samples were analyzed in triplicate. Each amplification reaction was checked for the absence of non-specific PCR products by running the dissociation protocol. Reactions were carried out on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems).

Sample DNA quantity (M, mean value from triplicates) was calculated by interpolation. Quantitative-PCR was applied to samples of 45 patients to validate the semiquantitave PCR approach previously described. The relative copy number (Q) of *PIK3CA* vs. *COL7A1* gene in a tumor sample (T) with respect to its normal tissue counterpart (N) was calculated using the following equation:

$$Q = \frac{qT}{qN} = \frac{M^{T}_{PIK3CA}/M^{T}_{COL7A1}}{M^{N}_{PIK3CA}/M^{N}_{COL7A1}}$$

Moreover, the results were divided into 'No amplification' for <2.0 relative copy number and 'Amplification' for ≥2.0 (23).

RNA preparation and quantitative RT-PCR. We quantified mRNA levels of *PIK3CA* using a real-time fluorescence detection method. The number of sample for quantitative RT-PCR corresponded with that for immunohistochemistry except for those samples in which tumor cells were too few to allow extraction of sufficient tumor RNA. Two micrograms of total RNA was reverse-transcribed. Taq Man® primers and probes for human *PIK3CA* (Taq Man Gene Expression Assay; Applied Biosystems) were obtained and *GAPDH* (Applied Biosystems) served as a reference, with each sample being normalized on the basis of its *GAPDH* content.

Cells and culture conditions. Two human esophageal squamous cell carcinoma cell lines: TE-series 1 and 8, were kindly provided by the Cell Resource Center for Biomedical Research, Tohoku University. All cell lines were cultured in RPMI-1640 (Sigma-Aldrich Co., St. Louis, MO), which was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco) and 10% fetal bovine serum. Cells were grown as subconfluent mono-layers in a humidified atmosphere containing 5% CO_2 at 37˚C and passaged using a treatment with 0.25% trypsin every 7 days.

Immunofluorescence analysis. TE cells grown to 70% confluence on slide glass were fixed in Zamboni solution at room temperature for 30 min. After a specific antibody against PIK3CA (Santa Cruz Biotechnology, USA) was added for 2 h at room temperature, Alexa Fluor 594-conjugated anti-goat antibody (Invitrogen Corp., Carlsbad, CA) was used as a second antibody for 60 min at room temperature. Finally, cells were stained for 10 min at room temperature with 4,6-diamidino-2-phenylindole (DAPI). Pictures were taken using

Table I. Primer sequences and experimental conditions for *PIK3CA* analysis.

A, Primer sequences of exons

Exon	Primer sequence (5' to 3')	Product length	MW	Tm (˚C)
1	F: GCCTAATCAAGTCAAACTATGGAA	24	7353.8	66
1	R: GCTATTTAAGATTACGAAGGTATTGG	26	8064.3	70
9	F: TGGTTCTTTCCTGTCTCTGAAAA	23	6995.6	64
9	R: ACATGCTGAGATCAGCCAAA	20	6119.0	58
20	F: CGAAAGCCTCTCTAATTTTGTG	22	6700.4	62
20	R: TTTTCATTCTTTTCCAATCAATGT	24	7233.8	60

B, Primer sequences of genes

Gene	Primer sequence (5' to 3')	Size (bp)	Tm (˚C)
PIK3CA	F: GAGAGGTTTCAGGAGATGTGT	150	60
	R: GGCTAGGGTCTTTCGAATGTA	187	60
COL7A1	F: GTAACAGACCTGCAAGCCAC	81	60
	R: GAGAGGGCTGGAGGTACAC	87	60

Tm, temperature.

a cooled CCD camera and MetaMorph Imaging Software (Universal Imaging Corporation, Downingtown, PA) with equal exposure times.

Immunohistochemistry for PIK3CA. For PIK3CA staining, immunohistochemical staining was performed using the standard streptavidin-biotin-peroxidase complex (SAB)-method (Histofine SAB-PO kit, Nichirei, Tokyo, Japan). The sections were incubated with commercially available goat polyclonal antibody raised against PIK3CA, diluted 1:100 at 4˚C overnight and then incubated with biotinylated rabbit anti-goat IgG+IgA+IgM for 10 min. Finally, they were incubated with streptavidin-biotin-peroxidase complex and were observed with an Olympus DP70 microscope (Olympus Optical, Tokyo, Japan). To exclude background staining by non-specific antibody binding, negative controls were included in each test.

Evaluation of PIK3CA protein expression. The prevalence of immunoreactive PIK3CA in tumors was scored semi-quantitatively and blindly by two independent investigators and confirmed by a third investigator. According to a previous study with minor modifications, the staining was scored on a scale from grade 0 to 3+ as follows: 0, no staining; 1+, <50% with weak or strong intensity; 2+, ≥50% with weak intensity; and 3+, ≥50% with strong intensity (24). For statistical analysis, we classified PIK3CA expression of score 0 or 1+ as negative and score 2+ or 3+ as positive.

Statistical analysis. All of the statistical analyses were performed with Stat-View 4.5 statistical software (SAS Institute, Inc., Cary, NC). All results were expressed as mean ± SD and $P<0.05$ was used for significance. The Mann-Whitney test was used to assess the mRNA levels between tumor samples and normal samples and either the Mann-Whitney test or Tukey-Kramer test was selected to assess the association of the mRNA levels and clinicopathological parameters. Additionally, either Fisher's exact probability test or the Chi-square test was used to examine a possible association between PIK3CA protein expression and clinicopathological parameters.

Results

Mutation analysis of PIK3CA. Most of the reported *PIK3CA* mutations cluster in small regions within the p85 binding (exon 1), helical (exon 9) and kinase (exon 20) domains, with E542K, E545K and H1047R constituting three mutational hot spots. Therefore, the analysis of the *PIK3CA* gene was restricted to these three exons and we identified *PIK3CA* sequence changes in 4 out of 52 ESCC samples (7.7%) (Fig. 1). To confirm whether these sequence changes were somatic mutations or single nucleotide polymorphisms (SNPs) of *PIK3CA*, we also examined the sequence of corresponding non-tumorous tissues. Because these sequence changes were not detected in the corresponding non-tumorous tissues, these alterations were confirmed as somatic mutations, not SNPs. With respect to exon 9, *PIK3CA* mutation A1634C was detected in 4 of 52 ESCC samples (Table II). On the other hand, no mutation was detected in either exon 1 or exon 20.

Copy number analysis of PIK3CA. Copy number amplifications of *PIK3CA* were found in 12 of the 45 patients (26.7%) for whose results in both tumorous lesion and normal lesion were available (Fig. 2 and Table III). We found no statistical

Table II. *PIK3CA* mutation in esophageal squamous cell carcinoma.

Case	Gender	Stage	Exon	Domain	Nucleotide	Amino acid
28	Male	IVA	9	Helical	A1634C	E545A
36	Male	IIB	9	Helical	A1634C	E545A
48	Male	I	9	Helical	A1634C	E545A
52	Male	IVB	9	Helical	A1634C	E545A

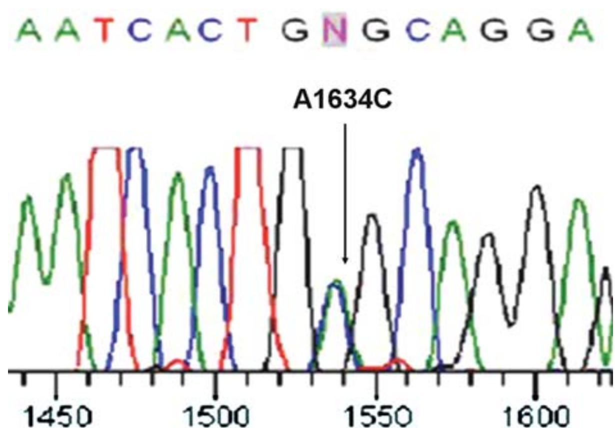


Figure 1. The analysis of the *PIK3CA* gene was restricted to exon 1, 9 and 20 and we identified *PIK3CA* sequence changes of exon 9 in 4 out of 52 (7.7%) ESCCs. The arrow indicates the location of somatic mutation. The nucleotide and amino acid alteration are indicated in the center of this figure. No mutation was detected in either exon 1 or exon 20.

significance for the correlation between *PIK3CA* amplification and its protein expression (data not shown).

Evaluation of PIK3CA mRNA status. We used quantitative RT-PCR to assess mRNA levels in ESCC specimens.

Table III. *PIK3CA* relative copy number by quantitative RT-PCR in ESCC.

Gene	No amplification (copy number <2.0)	Amplification (2.0≤ copy number)
PIK3CA (n=45)	33	12

Twenty-nine of the 37 ESCC patients (78.4%) had higher mRNA values in their tumor samples compared with corresponding normal esophageal epithelia. As a whole, the mean mRNA level of PIK3CA in ESCC samples was 2.61-fold higher compared with those in corresponding normal esophageal epithelia, as shown in Fig. 3 (P<0.001).

Immunohistochemical analysis of PIK3CA. To examine the expression and localization of PIK3CA protein in ESCC, we performed immunohistochemistry. Since the epithelial layer in some normal esophageal tissues showed weak cytoplasmic staining for PIK3CA, it was appropriate to group both no staining and 1+ staining into the negative group. Positive immunoreaction for PIK3CA was detectable in 33 of 66 (50.0%) esophageal squamous cell carcinoma specimens with >grade 2 (Fig. 4A and B), whereas the remaining 33 samples

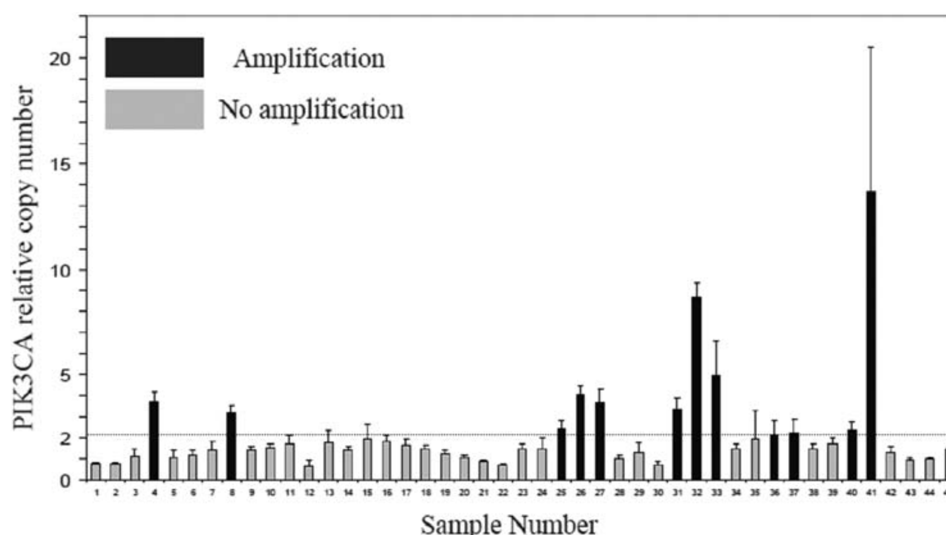


Figure 2. Histogram illustrating *PIK3CA* relative copy number in 45 samples. The case number is indicated below the panel. The *PIK3CA* copy number ratio (*PIK3CA*/*COL7A1*) in tumor samples was divided by that in normal tissue counterparts. The *PIK3CA* relative copy number (≥2.0) was considered amplified (n=12).

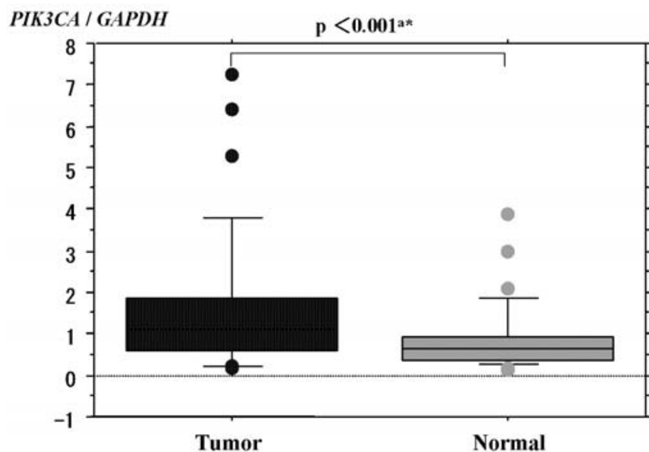


Figure 3. Quantitative real-time RT-PCR analysis of PIK3CA in 37 ESCC samples and non-tumorous esophageal epithelia. Twenty-nine of the 37 patients (78.4%) have higher mRNA values in ESCC specimens, compared with the corresponding non-tumorous esophageal epithelia. The mean mRNA expression of PIK3CA in ESCCs was 2.61-fold higher compared with non-tumorous esophageal epithelia. [a]Mann-Whitney U test.

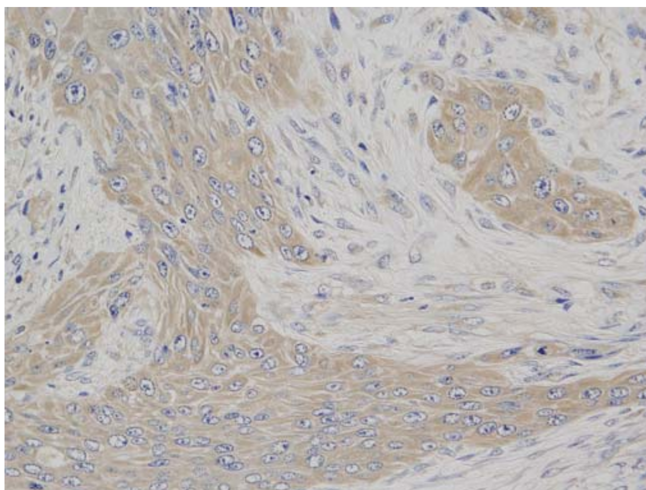
with grade 0 or 1 were negative. Immunostaining was localized mainly in the cytoplasm of the tumor cells but not in their nucleus. Furthermore, comparing negative staining with positive staining for PIK3CA, the presence of node metastasis was significantly correlated with positive staining for PIK3CA (Table IV). All other clinicopathological characteristics were statistically irrelevant to positive staining for PIK3CA.

Expression of PIK3CA in ESCC cell lines. We next used immunofluorescence techniques in established human ESCC cell lines (TE-series) to confirm whether a similar pattern of PIK3CA localization existed in culture cells. In a similar manner to that observed in tumor tissues, *PIK3CA* expression was observed in the cytoplasm (Fig. 5).

Discussion

Much of the complex fundamental biology of ESCC remains poorly understood, despite intensive study. One of the major aims of this study was to search for a molecular marker, which is associated with ESCC. Then, this study focuses on the analysis of molecular alterations associated with PIK3CA. In this study, we identified somatic mutations of *PIK3CA* in 4 out of 52 ESCC samples (7.7%), a rate which is similar to that reported for gastric cancers (4.3%) (25) and brain tumors (5%) (26) but less than that observed in colorectal (18.8-31.6%) (27) and breast (8.3-40%) tumors (28-30). Our data were partially in line with previously reported observations (22). Our analysis was limited to exons 1, 9 and 20, therefore the frequency of genomic alteration in ESCC might have been underestimated. Obviously, there are a number of factors including great variability in the types of cancers, geographical variation/influence, sample tissue preservation and methods used for DNA isolation. This is why *PIK3CA* mutations are so common among colorectal and breast cancer whereas those in ESCC are uncommon. With regard to mutational spots, *PIK3CA* amino acid substitutions at codons 542, 545 and 1047 are the most common hot spot mutations in the various cancers. In our investigation, clean sequence traces were obtained and 4 out of 52 ESCC samples (7.7%) were shown to harbor the A1634C change. The sensitivity of our sequencing method was proved by the faithful detection of the known differences between *PIK3CA* and its pseudogene (30). Along with our results, it has been reported that nearly all the mutations (21 of 24) among ovarian cancers consisted of an A1634C change in exon 9 (31). However, over 1,000 independent samples from large tumor sets identified G1624A and G1633A mutations instead of the A1634C change (27,29,32,33). These studies included 229

A



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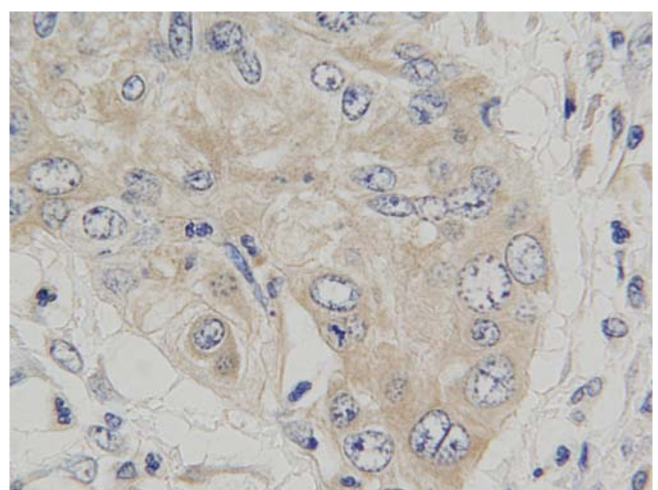

Figure 4. To examine the expression and localization of PIK3CA protein in ESCCs, we performed immunohistochemistry. Immunostaining was localized mainly in the cytoplasm of the tumor cells but not in their nucleus. The staining was scored on a scale from grade 0 to 3+ as follows: 0, no staining; 1+, <50% with weak or strong intensity; 2+, ≥50% with weak intensity; 3+, ≥50% with strong intensity. Positive immunoreactions for PIK3CA were detectable in 33 of 66 (50.0%) ESCC specimens with >grade 2 (original magnification: A, x400; B, x1000), whereas the remaining 33 samples with grade 0 or 1 were defined as negative.

Table IV. PIK3CA expression and clinicopathological factors in ESCC.

Variables	PIK3CA expression			
	n	Negative staining	Positive staining	P-value
Age				
<66 years	32	19	13	0.140[a]
≥66 years	34	14	20	
Gender				
Males	53	26	27	0.757[a]
Females	13	7	6	
T classification				
Tis and T1	30	18	12	0.138[a]
T2 and T3	36	15	21	
Lymph node metastasis				
Negative	34	21	13	0.048[a,c]
Positive	32	12	20	
Distant metastasis				
Negative	55	29	26	0.511[b]
Positive	11	4	7	
Vascular invasion				
Negative	34	18	16	0.622[a]
Positive	32	15	17	
Lymphatic invasion				
Negative	23	12	11	0.796[a]
Positive	43	21	22	
Intraepithelial spread				
Negative	43	19	24	0.103[a]
Positive	23	15	8	
Intramural metastasis				
Negative	60	31	29	0.672[b]
Positive	6	2	4	
Differentiation				
Well	30	17	13	
Mod	16	8	8	0.513[a]
Por	20	8	12	
Stage				
0	4	3	1	
I	19	9	10	
II	20	10	10	0.454[a]
III	13	4	9	
IV	10	3	7	
Recurrence				
Negative	50	28	22	0.085[a]
Positive	16	5	11	

Well, well differentiated; Mod, moderately differentiated and Por, poorly differentiated. [a]Chi-square test. [b]Fisher's exact probability test. [c]The P-value is significant.

lung cancers, 185 gastric cancers, 125 breast cancers, 109 separate ovarian cancers and 234 colon cancers. Therefore, in most *PIK3CA* mutation studies, the mutations identified in exon 9 have almost exclusively been G1624A and G1633A changes, and to date, few studies including our own have reported the A1634C mutation (31,32,34).

Genomic aberrations of *PIK3CA* encompass copy number gains and amplifications or oncogenic missense mutations

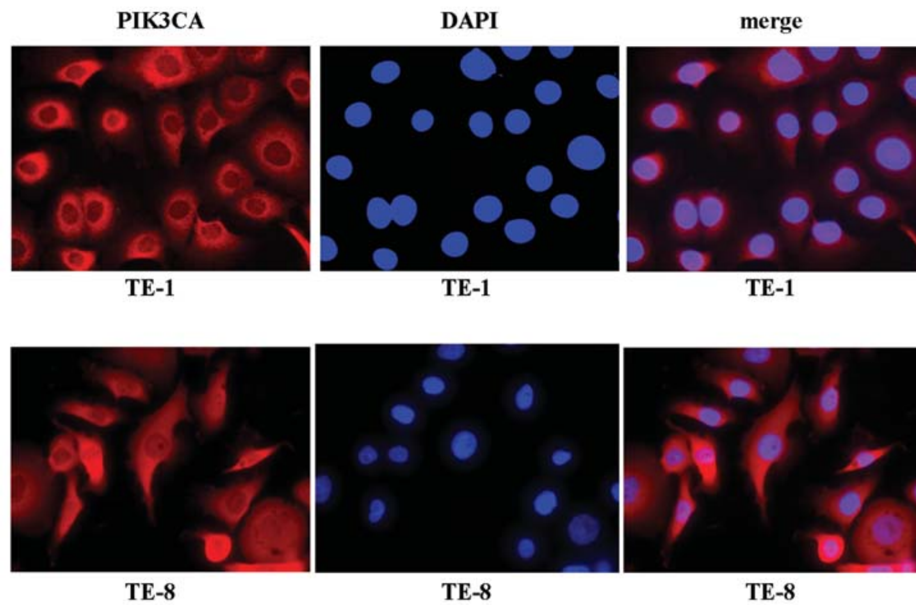

Figure 5. Immunofluorescence was assessed in established human ESCC cell lines (TE-series) to confirm whether a similar pattern of *PIK3CA* localization existed in culture cells. In a manner similar to that observed in cancer tissues, *PIK3CA* expression was observed in the cytoplasm.

(35-38). The copy number gains at 3q26 in ESCC, encompassing *PIK3CA*, have been reported previously (2). Moreover, amplification of *PIK3CA* in ESCC cell lines has been demonstrated by fluorescence *in situ* hybridization (FISH) (39). Our results showed that amplification of *PIK3CA* is not a common event in ESCC samples (26.7%). However, the copy number amplification of *PIK3CA* detected in our data is partially connected to the polyploidy of chromosome 3, or its short arm 3q, which are frequent events in ESCC (2). It is conceivable that overexpression of *PIK3CA* in tumors is not only mediated by upstream signaling pathways but is also regulated by changes in the *PIK3CA* gene itself. The evidence from other previous studies suggests that the genomic gain of *PIK3CA* and increased expression of *PIK3CA* are associated with progression of dysplasia into invasive squamous cell carcinoma in head and neck squamous cell carcinoma (HNSCC) (40). In addition, amplification of the *PIK3CA* locus was shown to be a strong predictor for an early tumor-associated death in ovarian cancer (41). However, in our study, we found no statistical significance for the correlation between *PIK3CA* amplification and its protein expression. These data are in line with previously reported observation that overexpression of PIK3CA did not correlate with its copy number in ESCC cell lines (39). These discrepancies between overexpression and amplification show that *PIK3CA* amplification is no longer specifically selected in ESCC which harbor genetic alterations, thus epigenetic events and/or other transcriptional regulation factors might influence PIK3CA expression. One possibility has been given by the inactivation of the tumor suppressor, p53, leading to enhanced PIK3CA transcription, as observed in HNSCC cell lines (42).

In PIK3CA mRNA, it has been reported that PIK3CA mRNA expression correlated with the pN status in ESCC (43). However, in this study, expression levels of mRNA were investigated only with qualitative analysis using formalin-fixed samples (43). Hence, further quantitative analyses would be considered to allow more precise outcomes. Our data using frozen samples showed that PIK3CA mRNA overexpression was highly prevalent in ESCC by quantitative RT-PCR ($P<0.001$). The vast majority of ESCC samples (78.4%) displayed an enhanced mRNA expression but no statistically significant correlations were found between PIK3CA mRNA overexpression and any clinicopathological characteristics (data not shown).

In PIK3CA protein expression, the high rate of immunoreactive PIK3CA was supposed to be identified according to the results of PIK3CA mRNA overexpression. In our study, consistent with PIK3CA mRNA overexpression in ESCC, in just half of ESCCs (50.0%) of immunoreactive PIK3CA was observed. The rate of immunoreactive PIK3CA in ESCC corresponded to that in lung squamous cell carcinoma (44). In a similar manner to that in tumor tissues, PIK3CA expression was observed in the cytoplasm of ESCC cell lines by immunofluorescence analysis.

Immunohistochemically in comparison with the negative staining group, the presence of node metastasis was significantly higher in the group staining positive for PIK3CA. It is certain that the protein expression is likely to be more reliable than the mRNA status because the *PIK3CA* protein, not mRNA, exactly affects the transcription more directly and accurately. In our study, we failed to detect the correlation between the PIK3CA mRNA status and pN status. On the other hand, a recent study highlighted a significant correlation between the PIK3CA mRNA and pN status (43). The lack of association between PIK3CA mRNA and protein could be due to transcriptional or posttranscriptional mechanisms which finely control the expression level of its protein. It is possible that PIK3CA mRNA is only transiently overexpressed in tumor cells and, as a consequence, it may be difficult to detect nearly

equivalent level of its protein. It has been reported that activation of the PI3-kinase pathway may enhance the invasion of lymph nodes by cancer cells (45) and that there is a statistically significant correlation between the presence of *PIK3CA* mutations and the presence of nodal metastasis in breast carcinoma (45). These findings indicate that *PIK3CA* is directly involved in tumor development and that the *PIK3CA* gene itself may act as an oncogene (9). According to the evidence described above, *PIK3CA* is a putative oncogene in ESCC. Taking into consideration the fact that the patients with ESCC have a poor prognosis, it is critical to identify a population of high-risk individuals who develop ESCC rapidly. Then, the high prevalence of PIK3CA expression as a biomarker may imply the presence of lymph node metastasis in ESCC.

In conclusion, to the best of our knowledge this is the first study providing comprehensive analysis of PIK3CA in ESCC. Our results indicate that PIK3CA may play a crucial role in the development of ESCC and serve as an indicator for lymph node metastasis.

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