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Original Article

Copy number loss of variation_91720 in PIK3CA predicts risk of esophageal squamous cell carcinoma

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Abstract: Purpose: Esophageal squamous cell carcinoma (ESCC) is one of the most fatal cancers worldwide. However, the etiology is complex and unclear. 3q26 harboring abundant oncogenes have been identified as the loci of ESCC susceptibility. In the present study, we examined whether CNVs on 3q26 would be associated with the risk, TNM stage and prognosis of ESCC. Methods: Variation_91720 in phosphatidylinositol 3-kinase catalytic subunit (PIK3CA) and Variation_91733 in sex-determining region Y-box 2 overlapping transcript (SOX2OT) were selected for investigation. The study included 204 ESCC patients and 208 healthy controls. The copy number of the selected sites and mRNA was detected by real-time fluorescence quantitative polymerase chain reaction and calculated using the CopyCaller v2.0 software program. Results: The copy number distribution of Variation_91720 was significantly different in ESCC cases and matched controls (p<0.001). Copy number loss of Variation_91720 may increase the risk of ESCC (OR=6.217, 95% CI=3.117-12.400; adjusted OR =6.251, 95% CI=3.130-12.428). PIK3CA mRNA expression was higher in tumor tissue (P=0.0003) and increased with the copy number gain of Variation_91720. Conclusion: Our findings suggest that copy number loss of Variation_91720 in PIK3CA predicts risk of ESCC, which might serve as a biomarker that for early diagnosis of ESCC.

Keywords: Copy number variation, esophageal squamous cell carcinoma, phosphatidylinositol 3-kinase catalytic subunit, sex determining region Y-box 2 overlapping transcript, susceptibility

Introduction

Esophageal cancer (EC) is one of the most common cancers and is the seventh most frequent causes of cancer-related mortality in the world. Esophageal squamous cell carcinoma (ESCC) is the predominant pathologic type of EC in China [1]. The prognosis of ESCC is extremely poor due to difficulties in early diagnosis and thus biomarkers of ESCC are urgently needed.

Copy number variation (CNV), a major source of genetic variation, refers to the copy number change of a particular fragment which is at least 1-kilobase (kb) in size [2]. Overlapping wider nucleotides, CNVs usually encompass functional DNA sequences and affect gene expression variability [3, 4]. It has been reported that CNVs may contribute to the predisposition of many cancers, such as colorectal cancer, pancreatic cancer, breast cancer, ovarian cancer, lung cancer and lymphoma [5-7]. However, few studies have focused on the association between CNVs and ESCC.

Increased copy number involving chromosome 3q26 is a frequent and early event in ESCC and other epithelial cancers [8, 9]. A number of oncogenes including PRKCI [8], MDS1-EVI1 [9], SOX2 [10], located on 3q26 have been considered to be potential target genes in ESCC. Moreover, numerous genome-wide association studies (GWAS) have identified that genetic polymorphisms on 3q26 are associated with the risk of cancers, such as bladder cancer, lung cancer and so on [11, 12].

In the present study, we examined whether CNVs on 3q26 would be associated with the risk, TNM stage and prognosis of ESCC and found that copy number loss of Variation_91720 in phosphatidylinositol 3-kinase catalytic sub-

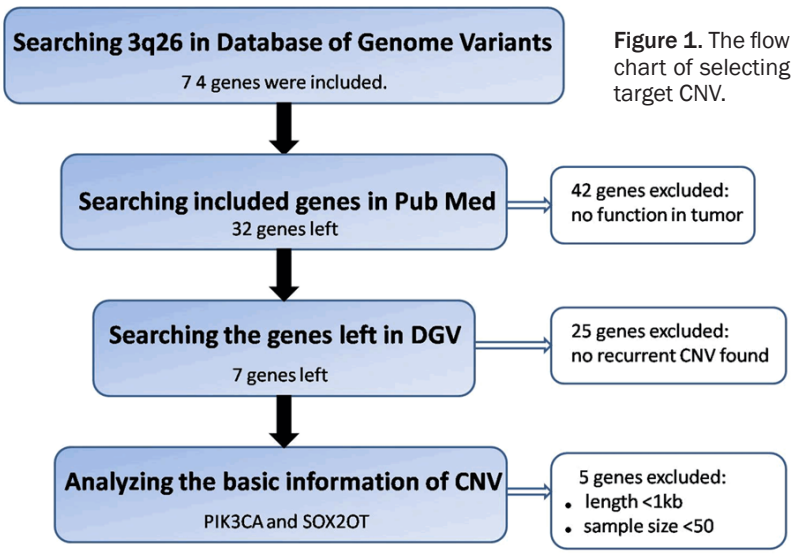

Figure 1. The flow chart of selecting target CNV.

unit (PIK3CA) increases the risk of esophageal squamous cell carcinoma.

Materials and methods

Study population and tissue samples

A total of 204 patients with ESCC and 208 cancer-free controls were enrolled in this study. All subjects were genetically unrelated Chinese Han people from southwestern China and treated at Southwest Hospital, Third Military Medical University, from December 2006 to September 2011. All ESCC patients were diagnosed by gastroscopy biopsy at the outpatient clinic of Southwest Hospital. Exclusion criteria for ESCC patients were as follows: previous malignancy, radiotherapy, or chemotherapy. The cancer-free controls were carefully matched to the ESCC cases based on age, sex, residential area, and had no previous malignancy. Tumor and adjacent non-tumor tissues (5 cm away from tumor tissues) were obtained from 48 ESCC patients who underwent radical esophagectomy at the Department of Cardiothoracic Surgery, Southwest Hospital. The pathologist confirmed the histopathologic nature of the samples. The study was approved by the ethics committee of the Third Military Medical University. Written informed consent was obtained from all participants.

Selection of CNV sites

By searching the Database of Genomic Variants (DGV) and PubMed, we found tumor-associated genes on 3q26 chromosome. Then we searched these genes in DGV to exam whether these genes overlapped with recurrent CNVs which were reported by two or more research teams. At last, we evaluated the basic information of CNVs including length, sample size, frequency and position. The inclusion criteria were as follow: i) longer than 1 kb, ii) sample size bigger than 50, iii) frequency higher than 0.05%, and iv) better to contain regulatory element. Only CNV fulfilled all the criteria above would be selected for subsequent experiments.

DNA extraction and CNV genotyping

Genomic DNA was extracted from the peripheral blood using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's protocol. The quantity and quality of DNA was determined using NanoDrop 1000 (Thermo Scientific, Waltham, MA).

CNVs were determined by TaqMan Copy Number Assays. Probes were designed and synthesized by the manufacturer (Applied Biosystems, Foster City, CA). RNase P (Applied Biosystems) was selected as a reference gene assay. Copy number was calculated by Applied Biosystems Copy Caller™ Software v2.0.

RNA extraction and RT-qPCR

Total RNA was extracted from ESCC patients' tumor and adjacent tissues using a Trizol Reagent kit (Invitrogen Life Technologies, Carlsbad, CA). The quality of the RNA was determined by spectrophotometry and gel electrophoresis, and then reverse-transcribed into complementary DNA (cDNA). cDNA was determined by real-time fluorescence quantitative PCR using SYBR Premix Ex Taq (Takara, Japan). The qPCR primer sequences were as follows: PIK3CA-forward 5'-TTGCTGTTCGGTGCTTGG-3' and PIK3CA-reverse 5'-GACTTGCCTATTCAGGTGCTTC-3'. Glyceraldehyde-3-phosphate dehy-

Table 1. Demographic characteristics of ESCC patients and controls

	Case (n=204) N (%)	Control (n=208) N (%)	*P*-value[a]
Age (years)			
<55	58 (28.43)	65 (31.25)	0.532
≥55	146 (71.57)	143 (68.75)	
Sex			
Male	119 (58.33)	112 (53.85)	0.359
Female	85 (41.67)	96 (46.15)	
Smoking			
Ever	101 (49.51)		
Never	103 (50.49)		
Drinking			
Ever	107 (52.45)		
Never	97 (47.55)		
TNM stage			
I	51 (25.00)		
II	110 (53.92)		
III	39 (19.12)		
IV	4 (1.96)		

[a]*P* value was calculated using the chi-square test.

drogenase (GAPDH) was used as the reference gene.

Statistical method for analysis

Differences in distributions of demographic characteristics and the CNVs genotype between ESCC and controls were evaluated by the chi-square test. The OR and 95% CI were used to estimate the association between CNVs and the risk and TNM stage of ESCC. Survival analysis was performed using Kaplan-Meier survival curves. Difference in the expression of PIK3CA mRNA in tumor and adjacent tissues was evaluated with the Paired-Samples T test. The One-way ANOVA test was used to compare PIK3CA mRNA expression among patients with different copy number of Variation_91720. For all analyses, differences were considered significant when the *p*-value was less than 0.05. SPSS v13.0 software was used for the statistical analysis.

Results

Selection of CNVs on 3q26

Searching 3q26 in the DGV, we found 74 genes. Only 32 genes of them were reported to have a role in the tumor biology. We searched these 32 genes in the DGV and excluded 25 genes which didn't overlap with recurrent CNVs. Then 7 genes were left. CNVs in TNIK, PRKCI, PLD1, FNDC3B and NCEH1 were either shorter than 1 kb or sample size smaller than 50. Only CNVs in PIK3CA and sex-determining region Y-box 2 overlapping transcript (SOX2OT) reached the criteria (**Figure 1**). Among all the CNVs in PIK3CA and SOX2OT, Variation_91720 was the only CNV spanning a regulatory sequence of PIK3CA and Variation_91733 was nearest to SOX2 in SOX2OT. So Variation_91720 and Variation_91733 were finally selected.

Association between CNVs and ESCC risk

All included subjects reached qualifying criteria for the study. Sex and age did not significantly differ between ESCC cases and controls (**Table 1**). The copy number of Variation_91720 in PIK3CA and Variation_91733 in SOX2OT was determined in 204 ESCC patients and 208 matched controls. The distribution of copy number of Variation_91720 in ESCC patients was significantly different from that in controls ($P<0.001$), whereas there was no statistic difference in Variation_91733 copy number distribution between two groups ($P=0.583$) (**Table 2**). Copy number loss of Variation_91720 may increase the risk of ESCC (OR=6.217, 95% CI=3.117-12.400; adjusted OR=6.251, 95% CI=3.130-12.428). Neither copy number gain of variation_91720 nor Variation_91733 genotype was significantly associated with the susceptibility of ESCC (**Table 2**).

Effect of CNVs on TNM stage and prognosis of ESCC

Copy number of Variation_91720 and Variation_91733 were not associated with the TNM stage of ESCC patients ($P=0.991$ and $P=0.545$ respectively) (**Table 3**) Moreover, Kaplan-Meier survival curves showed that Variation_91720 and Variation_91733 didn't influence the prognosis of ESCC patients (**Figure 2**).

PIK3CA mRNA expression in tissues

PIK3CA mRNA expression in tumor tissue was significantly higher than that in adjacent tissues ($P=0.0003$) (**Figure 3**). We further explore whether copy number of Variation_91720 would influence the mRNA expression of

Table 2. Associations between CNV and ESCC risk

CNV	Patients N (%)	Controls N (%)	*P*-value	Crude OR (95%)	Adjusted OR (95%)*
Variation_91720 in PIK3CA					
CN<2	53 (25.98)	11 (5.29)	**<0.001**	**6.217 (3.117-12.400)**	**6.251 (3.130-12.482)**
CN=2	124 (60.78)	160 (76.92)		1 (Reference)	1 (Reference)
CN>2	27 (13.24)	37 (17.79)		0.942 (0.544-1.630)	0.946 (0.542-1.653)
Variation_91733 in SOX2OT					
CN<2	28 (13.73)	30 (14.42)	0.583	0.910 (0.520-1.593)	0.921 (0.525-1.613)
CN=2	161 (78.92)	157 (75.48)		1 (Reference)	1 (Reference)
CN>2	15 (7.35)	21 (10.10)		0.697 (0.347-1.400)	0.697 (0.347-1.401)

Bold type: statistically significant, P<0.05. *Adjusted for age and sex.

Table 3. Associations between CNV and TNM stage of ESCC patients

CNV	TNM stage		*P*-value	Crude OR (95%)	Adjusted OR (95%)*
	I	II+III+IV			
Variation_91720 in PIK3CA					
CN<2	13 (25.49)	40 (26.14)	0.991	1.026 (0.486-2.163)	0.756 (0.342-1.674)
CN=2	31 (60.78)	93 (60.78)		1 (Reference)	1 (Reference)
CN>2	7 (13.72)	20 (13.07)		0.952 (0.368-2.467)	0.852 (0.306-2.224)
Variation_91733 in SOX2OT					
CN<2	5 (9.80)	23 (15.03)	0.545	1.676 (0.600-4.687)	1.702 (0.587-4.935)
CN=2	43 (84.31)	118 (77.12)		1 (Reference)	1 (Reference)
CN>2	3 (5.88)	12 (7.84)		1.458 (-/392-5.415)	1.435 (0.376-5.474)

*Adjusted for age, sex, smoking and drinking.

PIK3CA. We found PIK3CA mRNA expression in tumor tissues increased with the copy number gain of Variation_91720, whereas no significant difference was detected in adjacent tissues (**Figure 4**).

Discussion

CNV has recently attracted more attention than single nucleotide polymorphism (SNP) for its potentially important role in phenotypic diversity and evolution. Although SNPs on 3q26 have been identified as candidate loci in various cancers, it is the first study to indentify a functional CNV on 3q26 associated with ESCC risk.

PIK3CA encodes the p110alpha catalytic subunit of phosphatidylinositol 3-kinase (PI3K) that generates second messengers involved in a variety of cellular functions, including proliferation, survival, and invasion [13, 14]. The PI3K signaling pathway is described as one of the most frequently deregulated pathways in various cancers [14-16], including ESCC [13]. In the present study, we found PIK3CA was significantly overexpressed in esophageal tumor tissues indicating PIK3CA was an important oncogene in ESCC. PIK3CA mRNA expression in tumor tissues increased with the copy number gain of Variation_91720, but copy number loss of Variation_91720 increases the risk of ESCC, which suggested the carcinogenic potential of PIK3CA did not increase along with its up-regulated expression. It may involve the negative feedback regulation of PIK3CA. Further study was needed to find out the mechanism

SOX2OT, located on 3q26, overlaps with SOX2 and has the same transcription direction with SOX2. SOX2OT is reported to promote cell proliferation of lung cancer and reduce cell proliferation of breast cancer [17, 18], so it is hard to tell whether SOX2OT function as an oncogene or not. But the SOX2OT expression level is elevated and is closely related to SOX2 in ESCC [19], indicating that SOX2OT might play an important role in the oncogenesis of ESCC. In

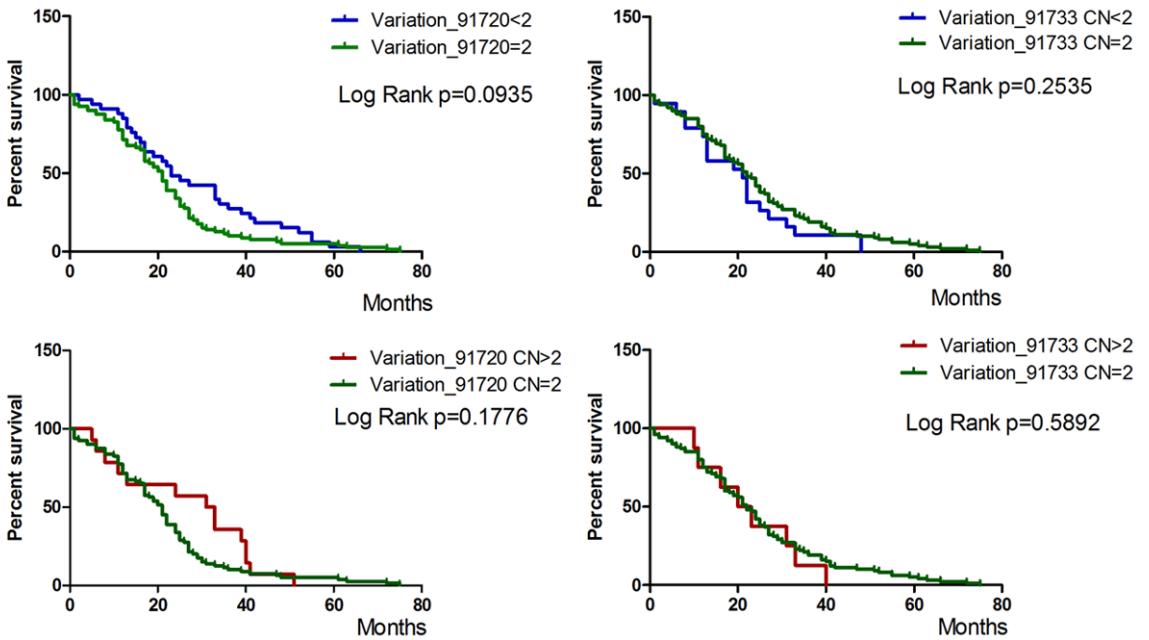

Figure 2. Survival curve for ESCC patients with different CNV genotype.

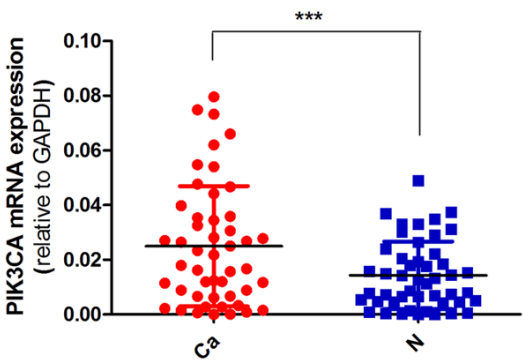

Figure 3. PIK3CA mRNA expression in tissues. PIK-3CA mRNA expression is significantly higher in tumor tissues. ***; p<0.001.

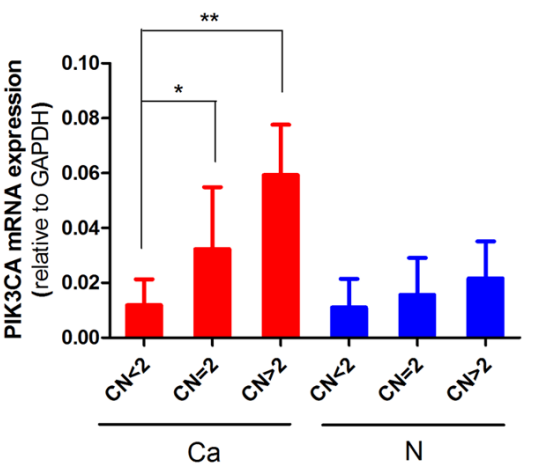

Figure 4. The association between Variation_91720 and PIK3CA expression. PIK3CA mRNA expression in tumor tissues increased with the copy number gain of Variation_91720. *; p<0.05, **; p<0.01.

the present, we didn't found the association between Variation_91733 and the risk, TNM stage and prognosis of ESCC.

This may be related to our study limitations. On one hand, the general information of the subjects included is insufficient, especially the history of alcohol and tobacco use, which may influence our results. We obtained detailed information of the ESCC cases and only lack the information of some of the healthy controls due to unsustained follow-up. We excluded one ESCC patient who had a history of heavy smoking or/and drinking to avoid the bias from alcohol and tobacco. In addition, the sample size needs to be larger to strengthen the conclusion.

To conclude, this study explored the association between CNV and ESCC, and identified Variation_91720 in PIK3CA is associated with the susceptibility of ESCC in a Chinese Han population. Thus, Variation_91720 in PIK3CA is a potentially useful biomarker for early diagnosis and timely treatment of ESCC. Future studies with a larger sample size and more patient

risk factors are needed to confirm the association between CNV and ESCC susceptibility.

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Disclosure of conflict of interest

None.

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