A Sequence Variant in the Phospholipase C Epsilon C2 Domain Is Associated With Esophageal Carcinoma and Esophagitis

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A single-nucleotide polymorphism (rs2274223: A5780G:His1927Arg) in the phospholipase C epsilon gene (*PLCε*) was recently identified as a susceptibility locus for esophageal cancer in Chinese subjects. To determine the underlying mechanisms of *PLCε* and this SNP in esophageal carcinogenesis, we analyzed PLCε genotypes, expression, and their correlation in esophageal cancer cell lines, non-transformed esophageal cells, 58 esophageal squamous cell carcinomas and 10,614 non-cancer subjects from China. We found that the G allele (AG or GG) was associated with increased *PLCε* mRNA and protein expression in esophageal cancer tissues and in esophageal cancer cell lines. G allele was also associated with higher enzyme activity, which might be associated with increased protein expression. Quantitative analysis of the C2 domain sequences revealed that A:G allelic imbalance was strongly linked to esophageal malignancy. Moreover, the analysis of 10,614 non-cancer subjects demonstrated that the G allele was strongly associated with moderate to severe esophagitis in the subjects from the high-incidence areas of China (OR 6.03, 95% CI 1.59–22.9 in high-incidence area vs. OR 0.74, 95% CI 0.33–1.64 in low-incidence area; $P = 0.008$). In conclusion, the PLCε gene, particularly the 5780G allele, might play a pivotal role in esophageal carcinogenesis via upregulating PLCε mRNA, protein, and enzyme activity, and augmenting inflammatory process in esophageal epithelium. Thus, 5780G allele may constitute a promising biomarker for esophageal squamous cell carcinoma risk stratification, early detection, and progression prediction.

Key words: phospholipase C epsilon; esophageal cancer; esophagitis

Additional supporting information may be found in the online version of this article.

Abbreviations: PLCε, phospholipase C epsilon; SCC, squamous cell carcinoma; LPA, 1-Oleoyl-L-α-lysophosphatidic acid; RT-PCR, reverse transcriptional polymerase chain reaction.

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INTRODUCTION

Phospholipase C epsilon (PLCε or PLCE 1) is a phosphoinositide-specific enzyme phospholipase C that converts phosphatidylinositol 4,5-biphosphate (PtdIns (4, 5) P2) [1,2] to two intracellular second messengers, diacylglycerol and inositol 1,4,5-trisphosphate, which regulate protein kinase C activity and calcium mobilization, respectively [3,4]. These responses are involved in the regulation of cell growth, differentiation, and oncogenesis [5]. To date, six members of the PLC family (PLCβ, γ, δ, ε, η, and ζ) have been identified in mammalian cells. These members share similar catalytic properties and are characterized by distinct regulatory mechanisms [6,7], indicating that PLCs exert diverse functions in carcinogenesis. PLCε binds to and is activated by the Ras family GTPases [1,8,9]. It participates in murine skin cancer development through augmentation of inflammatory cytokine production and signaling [10–12]. A more recent report also showed that inhibition of intestinal tumorigenesis in *ApcMin/+* mice by loss of PLCε occurs via reducing cytokine-mediated inflammation in local tissue [13]. However, the involvement of PLCε in human carcinogenesis, particularly in esophageal cancer, was not been recognized until a susceptible SNP was recently identified in GWAS studies of more than 20,000 Chinese subjects [14,15]. This SNP, located within exon 26 of the *PLCε* gene at chromosome 10q23, leads to a nonsynonymous alteration from *CAC* (A allele, encoding histidine) to *CGC* (G allele, encoding arginine). These previous data showed that a G at position 5780 in the C2 domain of PLCε was associated with an increased risk of both squamous cell carcinoma (SCC) and esophagus-cardia gastric adenocarcinoma (ECA). In the current report, we demonstrate that the presence of the G allele at this locus leads to increased *PLCε* mRNA and protein expression and enzyme activity in esophageal cancer cells in vitro, and that A:G allelic imbalance is strongly associated with esophageal malignancy in primary human tissues. Interestingly, we show that the presence of the G allele is also strongly associated with moderate to severe esophagitis in non-cancer subjects from the areas of high-incidence of esophageal cancer in China.

MATERIALS AND METHODS

Human Subjects

Frozen tissues from 58 esophageal SCCs and white blood cell DNA from 10,614 non-cancer subjects were collected from an ongoing hospital-based SCC and EAC case–control study, involving multiple hospitals throughout high- and low-risk areas for esophageal SCC in China since 2007. The genotypes of *PLCE* in non-SCC patients were based on TaqMan genotyping methods, as described recently [14]. All subjects had undergone esophagogastroduodenoscopy, and non-cancer subjects with esophagitis were determined by endoscopic examination and histopathology. The diagnosis of the degree of esophagitis was made by at least three pathologists. All procedures were conducted according to Declaration of Helsinki principles and had been approved by respective institutional review boards.

DNA Extraction and PCR-Based PCLε Exon 26 Sequencing

Genomic DNA was extracted from the following materials: SCC and adjacent normal esophageal epithelia, esophageal SCC cell lines (TE-1, TE-2, TE-7, TE-8, TE-12, G5, HCE4, HCE7, EC171, EC8712, EC109, EC9706, SHEEC) and non-transformed esophageal epithelial cell lines (HET1A, HEEpic) using a DNA extraction kit (QIAGEN, Valencia, CA). PCR for *PLCε* exon 26, in which the C2 domain is located, was performed using the following primers: forward (Ex26F): 5′-TGTTCTTGGGATTCCTTTGC-3′ and reverse (Ex26R): 5′-TGCTTCTTAATTCAACTTCTTTATAGG-3′. The PCR product was directly sequenced using an ABI sequencing system (Applied Biosystems, Inc., Foster City, CA). Allelic imbalance was analyzed using Mutation Surveyor software (Softgenetics, College Station, PA).

RNA Extraction, Quantitative Real-Time PCR, Immunoblotting, and Immunohistochemical Staining

Total RNA was extracted from human esophageal cancer cell lines and tissues, samples were then subjected to reverse transcription; cDNA was used for quantitative PCR analyses of *PLCε* expression at the mRNA level. The following primers that cover the C2 domain were used for quantitative PCR analysis: forward 5′-TGTGGAACGAGCAGTTTCTG-3′ and reverse 5′-ATCGAAGAGGC TGACATGGT-3′. Cell lysate was made from esophageal cancer cell lines, followed by immunoblotting and probing with anti-PLCε antibody (provide by Dr. J. Lomasney, Northwestern University, Chicago, IL). Immunoblotting intensities were quantified using Quanty One software (Bio-Rad, Hercules, CA). PLCε immunohistochemical staining was performed as described recently [14], and immunohistochemical staining was scored by at least three pathologists using the following criteria: 0, no staining or staining area was $<10\%$; 1, positive area was between 10% and 50%; and 2, positive area was more than 50%, similar as we reported [16].

PLCε Enzyme Activity Assay

PLCε enzymatic activity was determined by measurement of total [^{3}H]inositol phosphates accumulation in esophageal cancer cells containing AG and AA alleles (TE-2, -7, -8, and -12). 1-Oleoyl-L-α-lysophosphatidic acid (LPA) was purchased from Sigma–Aldrich (St. Louis, MO) and dissolved in water containing 1.0% fatty acid-free bovine serum albumin.

Briefly, cells were seeded in a 24-well plate at a density of 200,000 cells per well. 18 h later, medium was replaced with inositol- and serum-free DMEM containing 1 μCi/well [^{3}H]myo-inositol (American Radiolabeled Chemicals, St. Louis, MO). Phospholipase C activity was quantified 16 h after labeling by incubation in inositol-free DMEM containing 10 mM LiCl, either in the absence of LPA or in the presence of 10 μM LPA. The reaction was stopped after 30 min by aspiration of the medium and addition of an ice-cold buffer containing 0.6 M perchloric acid and 0.2 mM IP_6. After neutralization with buffer containing 1 M K_2CO_3 and 40 mM EDTA, the accumulation of [^{3}H] inositol phosphates was quantified by Dowex chromatography. 0.1% Triton X-100/0.1 M NaOH was added to cells in each well to determine total lipids. Each experiment was performed in triplicate, and these triplicate experiments were also repeated independently three times.

RESULTS AND DISCUSSION

To investigate whether the G allele of the *PLCε* gene SNP at position 5780 alters *PLCε* expression, we determined PLCε genotypes and mRNA levels in 13 human esophageal squamous cancer cell lines (TE-1, TE-2, TE-7, TE-8, TE-12, G5, HCE4, HCE7, EC171, EC8712, EC109, EC9706, SHEEC). Six SCC cell lines (G5, TE-8, TE-12, HCE4, EC171, EC8712) were AA, while the remaining 7 (TE-1, TE-2, TE-7, HCE7, EC109, EC9706, SHEEC) were AG for *PLCε* at position 5780. Quantitative RT-PCR (qRT-PCR) revealed that *PLCε* mRNA levels in the seven SCC AG cell lines increased approximately 37-fold relative to the 6 AA SCC cell lines (Figure 1A; 271 ± 144 in AG cells vs. 7.3 ± 3.3 in AA cells, $P < 0.01$). Increased *PLCε* mRNA levels also correlated with higher PLCε protein levels measured by immunoblotting (Figure 1B). The overall (average) intensity of PLCE1 protein in AG cell lines were higher than those in the AA cell lines ($P < 0.02$). Interestingly, two immortalized human esophageal epithelial cell lines derived from normal cells, HET1A (AG allele) and HEEPic (AA allele) also exhibited a similar association pattern: cells with the AG allele expressed higher levels of *PLCε* mRNA (23.6 in HET1A vs. 3.6 in HEEPic) and protein (data not shown).

We then examined whether elevated PLCε mRNA and protein levels correlated with PLCε enzyme activity. For these experiments, we chose the two AG cell lines (TE-7 and TE-2) that expressed the highest levels of PLCε and the two AA cell lines (TE-8 and

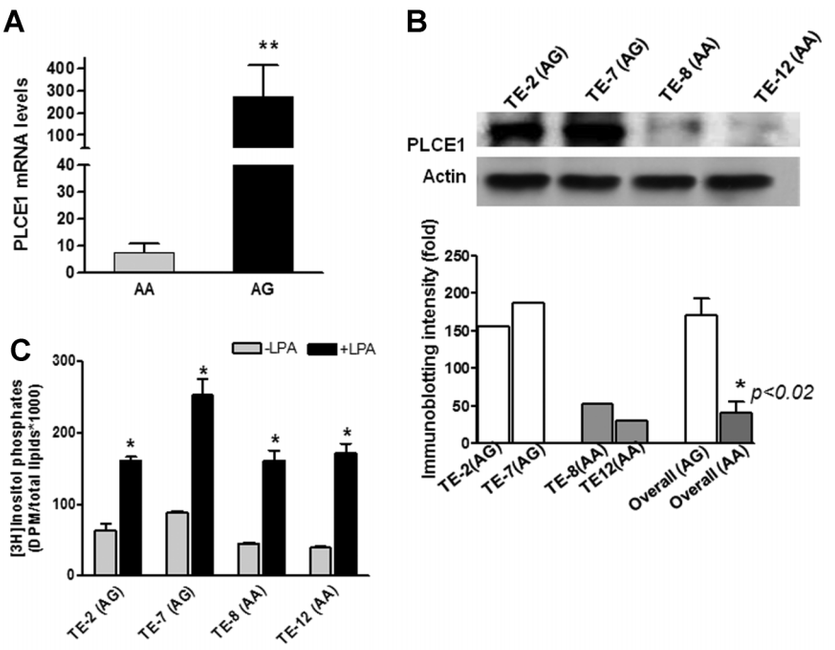

Figure 1. Correlation of A5780G genotypes with PLCε mRNA, total protein and enzyme activity levels in SCC cell lines. (A) Quantitative determination of PLCε mRNA levels in SCC cell lines ($n = 13$) by qRT-PCR. Bars represent the mean ± SD for each tumor type. $^{**}P < 0.01$. (B) A representative of immunoblotting showed that PLCE1 protein levels were higher in AG cell lines (TE-2 and TE-7) and were lower in AA cell lines (TE-8 and TE-12), using specific anti-PLCε antibody. Histograms showed immunoblotting intensities quantified by Quanty One software (Bio-Rad, Hercules, CA). $^{*}P < 0.02$, overall (average) intensity of PLCE1 protein in AG cell lines in comparison with those in AA cell lines. (C) PLCε enzymatic activity was determined by measurement of [^{3}H] inositol triphosphate in esophageal cancer cells with AA and AG alleles. Endogenous PLCε baseline activity (gray bars) was nearly twice as high in the two AG cell lines (TE-2 and TE-7) than those in the two AA cell lines (TE-8 and TE-12) (76 ± 20 vs. 42 ± 7, $P < 0.05$). LPA was used as a ligand to stimulate PLCε activity, and PLCε activities were significantly induced (black bars). Bars represent the mean ± SD for three replicates of experiments performed. $^{*}P < 0.001$, compared to individual cell line without LPA treatment.

TE-12) with the lowest levels of PLCε mRNA and protein. Consistent with findings for protein levels, endogenous PLCε baseline activity was nearly twice as high in the two AG cell lines (TE-2 and TE-7) than those in the two AA cell lines (TE-8 and TE-12) (76 ± 20 vs. 42 ± 7, $P < 0.05$). The increased enzyme activities might be linked to the increased protein expression. Previous studies have demonstrated that overexpression of activated small Rho family GTPases leads to marked elevation of intracellular inositol phosphate accumulation [17], and PLCε is a direct effector of activated Rho [18]. Similarly, the expression of G protein α-subunits Gα 12 and Gα 13 resulted in PLCε-dependent accumulation of inositol phosphates [19,20]. Therefore, agonists of G Protein coupled receptors that couple to Gα 12 and Gα 13, such as LPA (1-Oleoyl-L-α-lysophosphatidic acid), activate PLCε in a Rho-dependent manner [21]. To determine the response of PLCε activity to LPA in cells containing different alleles (AA vs. AG), we treated the two groups of ESCC cell lines with LPA and surprisingly found that AG cell lines exhibited only 2.5-fold average induction of PLCε activity, whereas AA cell lines were induced average fourfold (Figure 1C; $P < 0.05$). This finding may have been due to the failure of adapted AG allele cells to fully activate PLCε after interacting with harmful environmental factors (e.g., bile acids, bacterial infection, carcinogens, or other stressors), which reduces subsequential induction of cytokine and chemokine and development of inflammation in esophageal epithelium as a defensive response to the environmental detrimental stimulation. As a consequence, the lack of fully activated PLCε enzyme could cause epithelial cells to produce more PLCε mRNA and protein as a compensatory response through a feedback mechanism. From analysis of homology modeling of the PLCε C2 domain structure, we observed that changing His1927 to Arg in the C2 domain may affect protein–protein interaction and/or lipid recognition, but is unlikely to have an impact on ion binding by this enzyme (Supplementary Figure 1). However, further investigations using mutant allele plasmids are needed to confirm this modeling result.

PLCε-mediated cell growth promotion has been reported in various cell types [5,22,23], and the mitogenic effect of PLCε may facilitate cancer progression. We measured PLCε mRNA levels in 26 primary human esophageal SCCs and adjacent normal esophageal epithelial tissues by quantitative RT-PCR. As shown in Figure 2A, PLCε mRNA levels were significantly higher in esophageal SCCs than in adjacent normal tissues (normal, 1.0 vs. SCC, 22.0; $P < 0.01$). Consistent with these mRNA expression levels, SCC tissues with heterozygous AG expressed 1.5-fold higher protein levels than did homozygous AA tissues (1.5 ± 0.1 vs. 1.0 ± 0.3, $P < 0.05$; Figure 2B), assayed by immunohistochemical staining using an anti-PLCε antibody (Figure 2C). Our results were different from recent report that showed decreased PLCε mRNA and no changes of protein in esophageal cancer tissues compared to the adjacent normal tissues, assayed by qRT-PCR and immunohistochemical staining, respectively [24]. This discrepancy could be resulted from the patients selection: our patients were from the North Henan area, the most high incidence area of esophageal cancer in the world, the patients used in the recent report were from Shanghai area [24], the low incidence area of esophageal cancer. The different pattern of PLCε in high- and low-incidence areas was observed in esophagitis (Figure 3). We showed the results in detail later. Interestingly, allelic imbalance analysis showed an increased G allele copy number in 44% (15/34) of AG SCC tissues when compared to

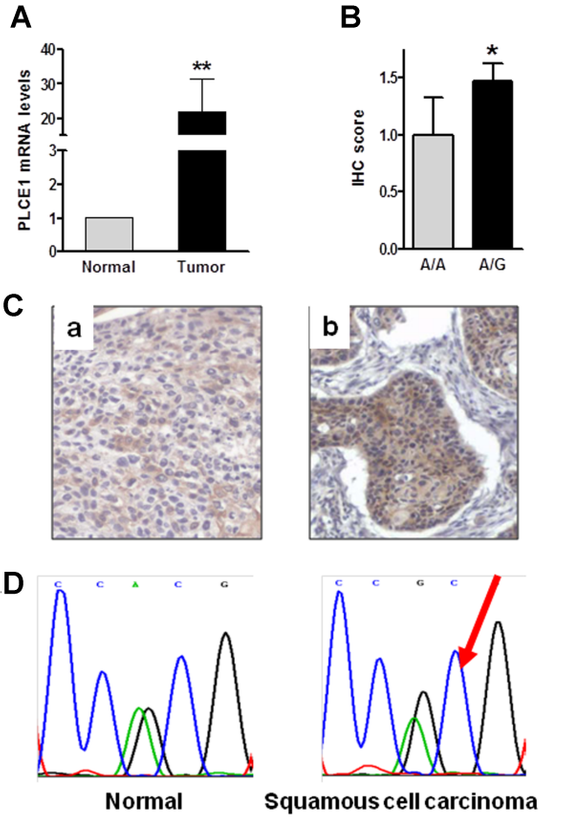

Figure 2. Genetic and expression-level alterations of PLCε in human esophageal cancer tissues. (A) Increased PLCε mRNA levels were determined by RT-PCR in SCC tissues ($n = 26$) compared to adjacent normal control tissues. Bars represent the mean ± SD of tumor and adjacent normal tissues. $^{**}P < 0.02$. (B) Immunohistochemical staining showed higher PLCε expression scores in SCC than in adjacent normal epithelia (1.5 ± 0.1 vs. 1.0 ± 0.3, $^{*}P < 0.05$). (C) Representative PLCε1 immunohistochemical staining in a AA SCC (a) and an AG SCC (b). (D) Representative sequencing results showed A5780G allelic imbalance in SCC versus normal esophageal control epithelium using Mutation Surveyor software (Softgenetics, College Station, PA). Red arrow indicates a gain of G allele in squamous cell carcinoma.

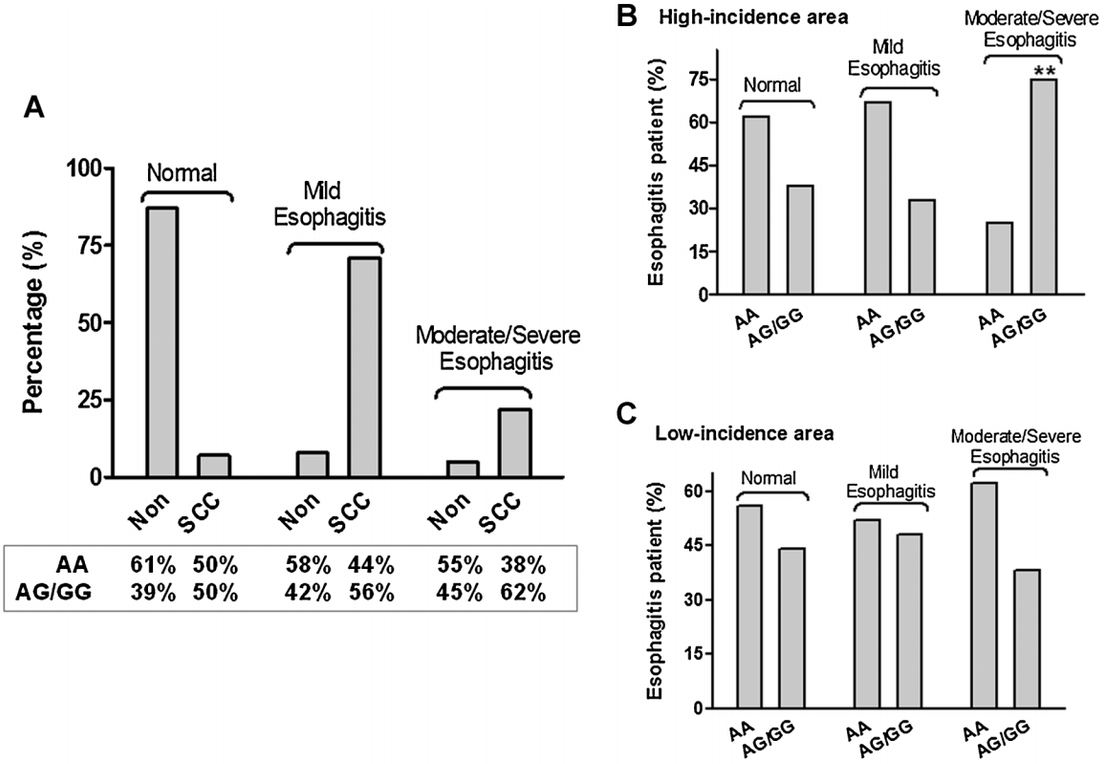

Figure 3. Association between PLCε genotypes and esophagitis in SCC and Non-SCC individuals. (A) The severity of esophagitis (mild, moderate and severe) in SCC ($n = 58$) and Non-SCC subjects ($n = 1{,}0614$) was correlated with the three PLCε genotypes. (B,C) Association between PLCε genotypes and esophagitis in Non-SCC individuals from high- and low-risk areas. The distribution and numbers in each group are provided in Table 1. $^{**}P < 0.01$.

matching normal esophageal control tissues (Figure 2D), using Mutation Surveyor software (Softgenetics, College Station, PA). These findings suggest that G allele is associated with overexpression of PLCε in primary SCC tissues.

Overexpression of PLCε protein and enhancement of PLCε enzyme activity have been reported to activate PKC and induce elevation of intracellular calcium levels [25], leading to cytokine- or chemokine-mediated inflammation in local tissues [12]. Furthermore, an association between esophagitis and the development of esophageal squamous cell cancer has been recognized and documented previously [26]. To investigate any possible association between A5780G and esophageal inflammation, we correlated SNP genotypes with presence or absence of esophagitis in individuals with cancer (SCC) and without cancer (non-SCC). 52 (89.7%) of the 58 SCC patients exhibited various degrees of esophagitis (mild, moderate, or severe), whereas only 1517 (14.3%) of the 10,614 non-SCC subjects had any esophagitis, determined by endoscopic examination and confirmed by histopathology ($P < 0.0001$). Importantly, the severity of esophagitis was associated

Table 1. Association Between PLCE1 Genotypes and Esophagitis in High- and Low-Risk Areas of China

	High-incidence area % (n)			Low-incidence area % (n)		
Genotypes	AA	AG/GG	OR (95% CI)	AA	AG/GG	OR (95% CI)
Mild esophagitis	86% (126)	80% (76)	1.00	92% (296)	91% (230)	1.00
Moderate	12% (18)	9% (9)	0.84 (0.36–1.97)	3% (10)	5% (13)	1.67 (0.72–3.89)
Severe	2% (3)	11% (10)	6.03 (1.59–22.9)*	5% (17)	4% (10)	0.73 (0.33–1.64)

OR, odds ratio; CI, confidence interval.
*$P = 0.008$.

with the AG/GG allele in these SCC patients. Eight (62%) of the 13 SCC patients with moderate or severe esophagitis were AG/GG, while only 5 (38%) of the 13 SCC patients with moderate or severe esophagitis had the AA genotype (Figure 3A). However, when non-SCC individuals with esophagitis were classified into high- or low-incidence areas for esophageal cancer, a significant association between severe esophagitis and the G allele of the PLCε gene was observed: 77% of the severe esophagitis individuals in high-incidence areas had AG/GG genotypes (Figure 3B), versus only 37% of these subjects in low-incidence areas (Figure 3C) (OR 6.03 with 95% CI 1.59–22.9 vs. OR 0.74 with 95% CI 0.33–1.64; $P = 0.008$; Table 1). These data support the hypothesis that the interaction of potential environmental factors with PLCε, particularly in the individuals with AG or GG allele, not only exists in high-incidence areas for esophageal cancer development in China, but also correlates with the severity of esophagitis. Currently, this group of individuals is under long-term follow up. Correlations between this risk allele, esophagitis, and cancer development will be clarified in future studies.

In conclusion, PLCε is likely to play a pivotal role in esophageal carcinogenesis: the presence of the 5780G allele may not only predict a high risk of future esophageal cancer development, but may also participate in esophageal cancer growth and progression by upregulating levels of PLCε mRNA, protein, and enzyme activity, ultimately leading to augmentation of the inflammatory process in esophageal epithelium. Thus, the 5780G allele in PLCε may constitute a promising biomarker for esophageal squamous carcinoma risk stratification, early detection, and progression prediction.

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