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Somatic Mutations of the *ENPP2* (*Autotaxin*/*lysoPLD*) Gene in Breast Cancer

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Abstract

ENPP2, a 125 kDa secreted lysophopholipase D which originally identified as a tumor-motogen, Autotaxin, enhances cellular locomotion, cell proliferation, angiogenesis and cell survival by generating the signal molecule lysophosphatic acid or sphingosine-1-phosphate. Previous studies have suggested that expression of Autotaxin is associated with invasive phenotype in advanced breast carcinomas. Thus, to determine whether genetic alterations of *ENPP2* gene are involved in the development or progression of breast cancer, we analyzed its somatic mutation in 85 breast carcinomas by single-stranded conformational polymorphism and sequencing. Overall, six *ENPP2* mutations were found (7.0%), comprising five missense and one nonsense mutation (s). To our knowledge, this is the first report on *ENPP2* mutation in breast carcinoma, and the data indicate that *ENPP2* is occasionally mutated in breast carcinomas, and suggest that *ENPP2* mutation may contribute to the tumor development in some breast carcinomas.

Keywords: ENPP2, Lysophospholipase D, Autotaxin, Breast cancer, Somatic mutation

Ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2), a 125 kDa glycoprotein, is a member of the ecto/exo-nucleotide pyrophosphatase and phosphodiesterase, and originally purified and characterized as tumor-motogen, Autotaxin (ATX)[1-3]. Later on, this ENPP2 was revealed that a secreted-lysophopholipase D (lysoPLD) which hydrolyzes lysophospholipids to produce lysophosphatidic acid (LPA) in extracellular fluids that is identical with ENPP2/ATX[4-6]. The major substrate of ENPP2 (ATX/lysoPLD) is lysophosphatidylcholine (LPC), but also it can act on sphingosylphosphorylcholine producing sphingosine-1-phosphate, a modulator of cell motility[7]. ENPP2 has been suggested to involve in several motility-related processes such as angiogenesis, neurite outgrowth, cell proliferation, adipose tissue development and tumor invasion or metastasis[8-14].

It is interesting to know that a functionally similar enzyme, phospholipase D (PLD), which hydrolyzes phosphatidylcholine to produce phosphatidic acid, has been cloned from many species and studied extensively[15-17]. Its active site consists of duplicated HxKxxxxD sequences, commonly referred to as HKD motifs. However, in contrast to both 5′-nucleotide phosphodiesterase (PDE) and PLD, the active site for lysoPLD, which might provide insight into the enzymatic mechanism of its action, has not yet been described. In addition, the facts that ATX possesses both PDE[18] and lysoPLD enzyme activities[5] and the amino acid residue T210 is obligatory for both PDE and motogenic activities[19] suggest that an intact PDE-reactive center is necessary for motility stimulation. Recently, Koh, E. *et al*.[20] suggested that four amino acid residues (T210A, H316Q, H360Q and H475Q) are obligatory for the PDE, lysoPLD, and migration-stimulating activities of ATX by using site-directed mutagenesis methodology. This implies that PDE and lysoPLD share a common reaction mechanism and inviting design of enzymatic inhibitors as therapeutic agents for neoplastic disease. In addition, our previous study identified large-scale molecular changes responsible for aberrant expression of ATX/ENPP2 on breast cancer cells by using DNA microarrays[21]. These findings raise the possibility that genetic alteration and aberrant expression of ATX may be associated with the development of breast cancer, and provide clues for understanding the complex roles of ATX as a key regulator of lysophospholipid signaling. Thus, we analyzed somatic mutations of *ENPP2* (*ATX*/*lysoPLD*) gene by using single strand conformational polymorphism (SSCP) and sequencing in order to whether the genetic alterations of *ENPP2* (*ATX*/*lysoPLD*) gene

involved in the development and/or progression of breast cancer.

Table 1. Primer designs (GenBank Accession No. NM_006209).

Name	Primer sequence (Forward/Reverse)	Product (bp)
ATX_E7	F: 5′-GTCTCTCCTTTAAAGTCATTCCAG-3′ R: 5′-AAGTGAAGGTAACACATGTCTGAG-3′	248
ATX_E8	F: 5′-ACACAATAAATGCAAACCTTAC-3′ R: 5′-TGCTATAAAACCTTCTATGCTA-3′	240
ATX_E10	F: 5′-TTCACGCAATTCTAAACTAAGA-3′ R: 5′-TGAAAACAGATGGTGGTAAATA-3′	248
ATX_E11	F: 5′-TAGATACAAACATCCATCCAGACT-3′ R: 5′-TGTTACATGTGGTTTAGGAGAGAT-3′	228
ATX_E13	F: 5′-GGCTTCTAAGGGCAAGGATTCTTT-3′ R: 5′-CCTGTGGGGACTAACTTGCTTCTT-3′	228
ATX_E17	F: 5′-CTTTCAGTGATGACTAAGGATG-3′ R: 5′-TCTGATTTCCATTTCTATTTCA-3′	244
ATX_E18	F: 5′-CAATTCACAGTTCTGCCATCATAC-3′ R: 5′-AACTTTCAAAAGCCCCTTCTTAGA-3′	219
ATX_E21	F: 5′-TTGCCAGATAGGTATGAAAGTCAC-3′ R: 5′-ACATGAAGAACAAGTTGGATGAAC-3′	205

Mutational Analysis

Direct sequencing of aberrantly migrating band on SSCP gel led to the identification of mutation in 6 (7 %) of 85 breast cancers examined (Table 2). The mutations consisted of 5 missense mutations and 1 nonsense mutation: a GGC to GAC transition at codon 322 (G322D), a GAC to AAC transition at codon 333 (D333N), a GTG to ATG transition at codon 336

Table 2. Summary of ENPP2 (ATX/LysoPLD) mutations in breast cancer.

Exsons	No. of tissues	Nucleotide	Amino acid	
Exon 7	37T	G612A	P204P	Silent
	76T	C618T	Y206Y	Silent
Exon 10	56T	G875A	W292stop	Nonsense
Exon 11	29T	C957T	G319G	Silent
	55T	G965A	G322D	Missense
Exon 13	2T	G990A	R330R	Silent
	49T	G997A	D333N	Missense
		G1006A	V336M	Missense
	35T	G1021A	D341N	Missense
	36T	G1049A	R350Q	Missense

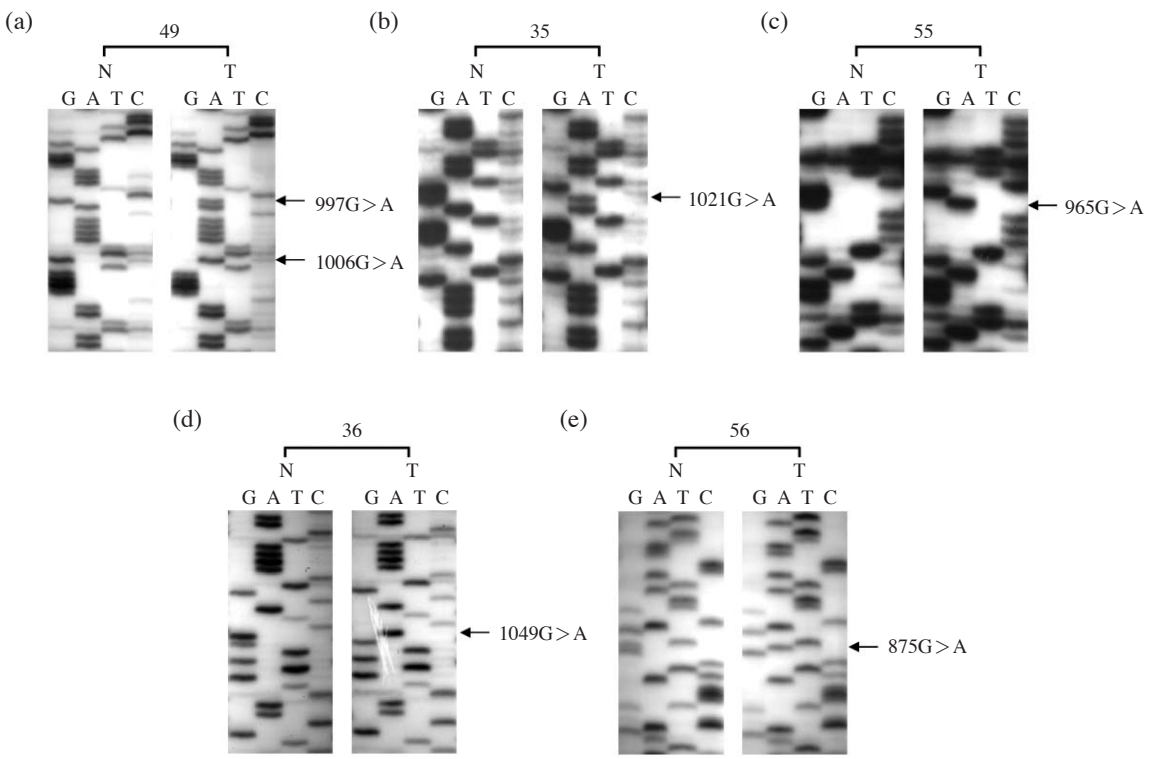

Figure 1. Mutations of the *ENPP2* gene detected in breast cancer. Representative data of DNA sequencing analysis of *ENPP2* gene from tumors (lane T) and normal tissues (lane N). Sequencing analyses from the aberrant bands in SSCP of DNA from five breast cancers showed the mutations (a) GTG to ATG (V336N) and GAC to AAC (D333N) (b) GAT to AAT (D341N) (c) GGC to GAC (G322D) (d) CGG to CAG (R350Q) (e) TGG to TAG (W292stop) in the *ENPP2* gene.

(V336N), a GAT to AAT transition at codon 341 (D341N), a CGG to CAG transition at codon 350 (R350Q) for missense mutations and a TGG to TAG transition at codon 292 (W292Stop) for a nonsense mutation (Figure 1). It is interesting to find that the case number 49 of breast cancer tissues has two missense mutations, D333N and V336N. The corresponding normal tissues showed no evidence of mutation by SSCP, indicating the mutation detected in the specimens had risen somatically. We repeated the experiments three times, including tissue microdissection, PCR, SSCP and sequencing analysis to ensure the specificity of the results, and found that the data were consistent (data not shown).

Discussion

Autotaxin, a secreted lysoPLD, was known as an ecto-nucleotide pyrophosphatase/phosphodiesterase which stimulated tumor cell (or normal cells) migration in a pertussis toxin-sensitive manner until that plasma lysoPLD was purified and found to be identical ATX[1,5]. It is now well established that ATX is unique among the ENPPs in that it primarily functions as a lysoPLD, converting LPC into the lipid mediator LPA, and this LPA acts on G protein-coupled receptors to elicit a wide range of cellular responses, ranging from cell proliferation and migration to neurite remodeling and cytokine production[24]. As biological activities of ATX in tumor progression and metastasis, there are several lines of evidences indicate a link between ATX and cancer. For example, ATX augments tumorigenic potential and angiogenesis when it introduced into *ras*-transformed cells[11,12]. ATX is also observed that it is highly overexpressed in several human cancers, including glioblastoma, lung and breast cancer, renal cell carcinoma, neuroblastoma, thyroid carcinoma and Hodgkin lymphoma[4]. Among these, ATX largely accounts for the increased motility of MDA-MB 435 human breast cancer cell line, and the expression of ATX is closely linked to invasiveness of breast cancer cells[9,25]. These findings raise the possibility that genetic alteration and aberrant expression of ATX may be associated with the development of breast cancer. For the mutational analysis of *ATX* gene, we searched somatic mutation by using SSCP and direct sequencing of aberrant bands on SSCP analysis. From the SSCP analysis, 9 aberrant bands were found, but 3 of them were confirmed as silent mutations, and finally, 5 of missense mutations and one nonsense mutation were identified.

According to ATX genomic sequence information, it consists of 25 exons and consequently, total 836 amino acid residues were translated. Protein sequence homology analysis revealed that ATX has four major conserved domains including somatomedin B, endonuclease, alkaline phosphatase and phosphodiesterase domain. Among these, the alkaline phosphatase domain include phosphodiesterase domain and display large spectrum of region in ATX sequence, we targeted this region (exon 7, 8, 10, 11, 13, 17, 18 and 21) for the somatic mutation analysis. It is very interesting to know that all five missense mutants were found in exon 11 and 13, and the nonsense mutant was in exon 10. These mutants belong to phosphodiesterase domain within alkaline phosphatase region. From the previous study for the functional domain of ATX, the phosphodiesterase domain was known to essential for its motility stimulation as well as ENPP activity[18-20]. Although functional analyses of these mutants should be tested, this fact implies that these mutants may contribute to breast cancer progression. Accumulating reports for the biological roles of ATX in physiological and pathophysiological condition have elicited that ATX is a potent motogen and mitogen with multi-enzymatic activities. However, for example, as a secreted lysoPLD, while much has recently been clarified about ATX as the major LPA-generating exoenzyme and understanding of LPA action has progressed rapidly, the exact *in vivo* functions of the ATX-LPA axis remain to be elucidated.

In conclusion, we found six mutations (five missense and one nonsense) of *ATX* genes in 85 breast cancers. We suggest that somatic mutations of the *ATX* may contribute to the development of breast cancer through the change of the enzymatic activity of ATX. Further functional analysis of the mutations identified in this study will broaden our understanding of the pathogenesis of breast caner.

Methods

Tissue Samples

Methacarn-fixed tissues of 85 breast cancer specimens (all patients are Korean) were randomly selected for study. Approval was obtained from the institutional review board of the Catholic University of Korea, College of Medicine. Informed consent was provided according to the Declaration of Helsinki. The tumor-node-metastasis (TNM) stages of the breast cancers were classified as stage I (25/85), stage II (40/85), stage III (20/85).

Microdissection and DNA Extraction

Tumor cells within tissues were selectively procured from Hematoxylin & Eosin stained slides using a

laser microdissection device (ION LMD, JungWoo International Co., Seoul, Korea). We also obtained inflammatory or surrounding normal cells for corresponding normal DNAs from the same slides in all cases. DNA extraction was performed by a modified single step DNA extraction method, as described previously[22].

Mutational Analysis

We have used published reports detailing the amino acid requirements of the ENPP associated PDE reactive center as well as the HKD motif[23] required for PLD activity[20] for the primer designing and screening mutations of *ENPP2* gene in eight separate exons. Genomic DNAs from cancer cells and corresponding non-cancerous cells were amplified with 8 sets of primers covering the addressed regions (8 exons) of *ENPP2* gene (Table 1). Numbering of DNA of *ENPP2* (*ATX*/*lsyoPLD*) was done in respect to the ATG start codon according to the genomic sequence of GenBank accession number NM_006209. Each polymerase chain reaction (PCR) reaction was performed under standard conditions in a 10 μL reaction mixture containing 20 ng of template DNA, 0.5 μM of each primer, 0.2 μM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 0.4 unit of Taq polymerase, 0.5 μCi of [^{32}P]dCTP (Amersham, Buckinghamshire, UK) and 1 μL of 10× buffer. The reaction mixture was denatured for 12 min at 95˚C and incubated for 35 cycles (denaturing for 30 s at 94˚C, annealing for 30 s at 50-54˚C, and extending for 30 s at 72˚C). The final extension was continued for 5 min at 72˚C. After amplification, PCR products were denatured 5 min at 95˚C at a 1 : 1 dilution of sample buffer containing 98% formamide/5 mmol/L NaOH and were loaded onto SSCP gel (Mutation Detection Enhancement, FMC BioProducts, Rockland, ME, USA) with 10% glycerol. After electrophoresis, the gels were transferred to 3 MM Whatman paper and dried, and autoradiography was performed with Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA). For the detection of mutations, DNAs showing mobility shifts were cut out from the dried gel, and reamplified for 30 cycles using the same primer set. Sequencing of the PCR products was carried out using the cyclic sequencing kit (Perkin-Elmer, Foster City, CA, USA) according to the manufacturer's recommendation.

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