Ajou Medical Journal
Vol. 1, No. 1, 1996

Increased Expression of Phospholipase C-γ1 in Human Gastric Cancer Tissues

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Phospholipase C (PLC) plays a central role in signal transduction and is important in cellular growth, differentiation and transformation. There are currently ten known mammalian isozymes of PLC reported to date. However, their biological implication in carcinogenesis and in disease is not fully understood.

Here we examined the relative content of various PLC isozymes present in 50 paired surgical specimens from human gastric cancer patients. Western blot analysis demonstrated considerablely higher levels of PLC-γ1 protein in gastric cancer tissues from 41 of 50 specimens as compared to that of normal mucosa. On immunohistochemistry, distinct speckles or clumps of PLC-γ1 staining were observed in the cytoplasms of primary malignant lesions as well as the invading cancer cell nests, in contrast to the normal tissues which were negative. Little differences in other isozymes were found. Only PLC-δ1 was slightly increased in about two thirds of the patients, however, to a much lesser extent.

In conclusion, although the causes of increased PLC-γ1 in gastric carcinomas remain to be explored further, the elevated level of PLC-γ1 suggests an altered signal transduction pathways in gastric carcinomas and may indicate an important role played by PLC-γ1 in pathogenesis and proliferation of cancer cells.

Key Words: PLC-γ1, Over-expression, Gastric carcinoma

INTRODUCTION

Many extracellular signals such as variety of hormones, growth factors, and neurotransmitters induce a rapid hydrolysis of phosphoinositides via a receptor mediated process in transmembrane signal transduction. Hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) produces two important second messengers, namely inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 binds to the specific receptors and induces a release of calcium from intracellular storage sites such as endoplasmic reticulum[1]. Diacylglycerol activates protein kinase C which is known to be critical in cellular growth, differentiation, and transformation by phosphorylating many protein components involved in the signal transduction and transcriptional control[2].

PLC plays a key role in signal transduction by catalyzing hydrolysis of PIP_2 and generation of the second messengers[3]. To this date, there are at least ten distinct mammalian isozymes of PLCs identified, biochemically characterized from various tissues, and their respective cDNA isolated. Comparison of the deduced amino acid sequences has indicated that PLCs can be divided into three distinct types, PLC-β, PLC-γ, and PLC-δ. Each type contains more than one isozymes and these are designated by an arabic subscript following the Greek letters such as PLC-$\beta_{1\text{-}4}$, PLC-$\gamma_{1\text{-}2}$, PLC-$\delta_{1\text{-}4}$[5~9]. The sequence homology is more than 90 percent between species and all PLCs contain two distinct regions of

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amino acid similarities designated as X and Y domains which are believed to be either catalytic site or substrate binding sites[4]. Also all three subtypes of PLC contain pleckstrin homology domains[10~12]. PLC-γ_1 and -γ_2 have a unique region called *src* homology domains, SH2 and SH3[13,14], which are important in mediating protein to protein interaction and binds specifically phosphorylated tyrosine residues[15] and a proline-rich segment of the proteins[16], respectively.

As expected from their amino acid sequence differences and subcellular localization, different PLCs are activated through different mechanisms. PLC-β subfamily is activated by G-protein coupled receptors, both α and $\beta\gamma$ subunit of G-protein q family[17,18]. Activation of PLC-γ isozymes are achieved through phosphorylation at multiple tyrosine residues via receptors that has tyrosine kinase activity such as various growth factor receptors[19]. Moreover, non-receptor tyrosine kinases, most of which are cellular proto-oncogenes could activate and phosphorylate PLC-γ isozymes in vitro[20]. In addition, PLC-γ isozymes can also be activated via a tyrosine kinase independent pathway by a microtubule associated protein, tau, in bovine brain cytosol[21]. These findings suggest that the activation of various PLC isozymes could be one of the important pathways where by mitogenic signals are conveyed in both normal and pathologic states.

Information regarding the pathologic significance of the various PLCs are beginning to accummulate. The evidence of enhanced phophatidylinositol turn over in fibroblasts over-expressing PLC-γ_1 in repsonse to growth factors[22] and increased growth and oncogenic potentials[23] implies that it might have a role in carcinogenesis. Moreover, since the report of an elevated content of PLC-γ_1 in human breast carcinomas[24], increased expression of PLC-γ_1 has been also noted in colorectal carcinomas[25]. In benign diseases, such as familial adenomatous polyposis[26] or human skins in hyperproliferative conditions[27], over-expression of PLC was also reported.

Therefore, as a step toward elucidating the role played by the PLCs in the pathogenesis of stomach cancer, we have studied the relative content of 10 mammalian isozymes of PLCs in 50 paired specimens of human gastric carcinomas and report a marked increase in PLC-γ1 level in the cancer tissues and its immunohistochemical findings.

MATERIALS AND METHODS

Collection and processing of surgical specimens

Fifty paired tissues, both neoplastic and normal from the same specimens, were obtained from the material submitted to the Department of Anatomic Pathology at Ajou University Hospital, Suwon, Korea. Fresh specimens were immediately frozen in liquid nitrogen and stored at -70 °C until used. For immunohistochemical stains, samples were formalin-fixed and paraffin embedded. Duplicates were processed for the pathologic examination and the diagnosis of malignancy was verified on hematoxylin and eosin stain on the embbeded samples to ensure the results.

Preparation of the total tissue extracts

Fresh frozen neoplastic and normal gastric mucosal tissues from 50 paired surgical specimens (each weighing 1 g) was thawed in 1.5 ml of homogenization buffer containing 10 mM Tris (pH 7.4), 1% Triton X-100, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 2 μg/ml leupeptin, 2 μg/ml aprotinin, and calpain inhibitor I and II (each at 4 μg/ml). It was then ground in a Polytron homogenizer (Brinkmann) and thoroughly homogenized in a glass homogenizer with a motor-driven teflon pestle in ice. The homogenate was shaken for 2 hours at 4 °C to solubilize membrane fractions and centrifuged at 10,000 $\times$g for 30 minutes to remove the debris. The supernatant, total extract, was taken and normalized for the protein content using BCA method and used for the further studies.

Immunoblot analysis

40 μg of each samples prepared from above were treated with 4x Laemmli's sample buffer and resolved on SDS-PAGE (6% gel). Proteins were transferred to a nitrocellulose paper, incubated with various PLC isoform specific antibodies, and immunoreactive bands were visualized with horse raddish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG antibodies (Amersham) using electrochemiluminescence method. Anti-PLC isoform antibodies used in the blotting were K92-3 (anti-PLC-β1 monoclonal antibody), F7-2 (anti-PLC-γ1 monoclonal antibody), anti-γ2 monoclonal antibody, S11-2 (anti-PLC-δ1 monoclonal antibody) and others were polyclonal antibodies. The antibodies were kindly pro-

vided by Dr. Sue Goo Rhee from the Laboratory of Cell Signaling at National Institute of Health (Bethesda, Maryland, USA).

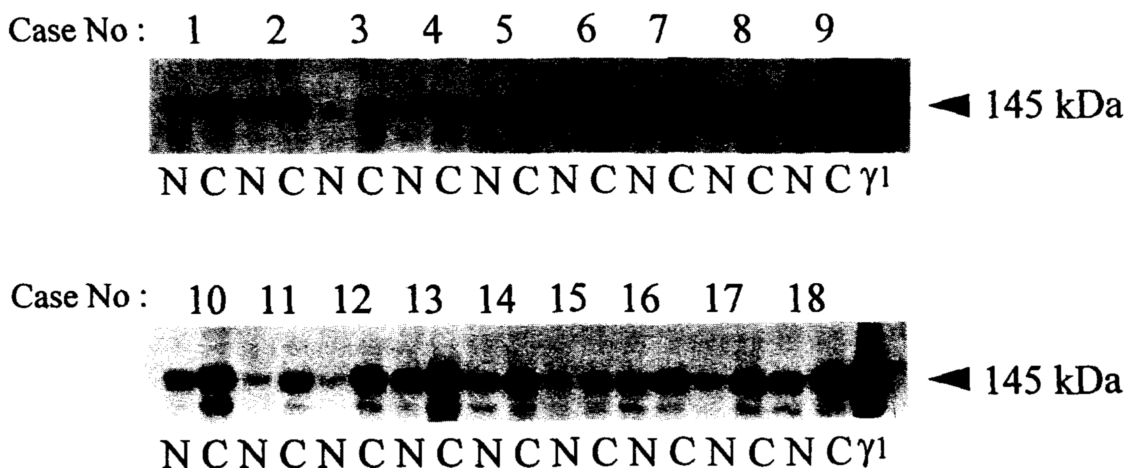
Immunohistochemistry of the gastric tissue

The immunohistochemical staining of the paraffin-embedded section of neoplastic and paired normal counter parts was performed with monoclonal antibodies against PLC-γ1 (1 μg/ml, mixture of F7-2, D7-3, and B16-5) as described elsewhere[28]. Serial 5 micrometer sections were cut from formalin-fixed and paraffin-embedded gastric tissue. After deparaffinization and hydration, sections were incubated with 10% normal goat serum to block non-specific bindings. Sections were then incubated with mixture of monclonal PLC-γ1 antibodies. Following 4 hour incubation, sections were washed three times with phosphate buffered saline and incubated with avidin-biotin reagents from LSAB kit (Dako, Denmark). For control experiment, phosphate buffered saline and anti-PLC-γ2 monoclonal antibody which were not found to be increased, were used. The immunohistochemical stains were repeated three times to ensure the relative intensity of staining and to get the representative results. The sections were reacted with AEC as chromogen and counter stained with hematoxylin. The photographs were taken under an Olympus BH2 light microscope (Olympus Optical Co., Tokyo, Japan).

RESULTS

By the use of Western blot analysis, we examined the relative level of ten known mammalian isozymes of PLC in total extracts of paired specimens. Of ten isozymes, relative content of PLC-γ1 was significantly and consistently elevated in 41 cancer tissues in comparison to their normal counterparts as judged by the intensity of immunoreactive bands. The representative results of immunoblotting from 18 pairs were demonstrated along with PLC-γ1 standard(Fig. 1). It is clear from the results that PLC-γ1 protein expression is significantly increased compared to the adjacent normal tissues in gastric cancer.

Clinical prognostic factors such as Lauren classification of the tumor, tumor size, the depth of local invasion, lymphatic vessel invasion, and lymph node metastasis were compared to the Western blot positivity of PLC-γ1. Of 50 patients



Anti-PLC-γ1 Blot

Fig. 1. Immunoblotting normal mucosa and gastric carcinoma tissue with anti-PLC-γ1 monoclonal antibody. Total extract (20 μg protein/ lane) from both normal and gastric cancer tissues were separated on 6% SDS-PAGE and transferred to nitrocellulose membrane and blotted with anti-PLC-γ1 monoclonal antibody. The bands were visualized with horse-raddish peroxidase conjugated goat anti-mouse IgG antibody using electrochemiluminescence. *N* ; normal mucosa, *C*; cancer tissue, *γ1*; purified PLC-γ1 standard. Numbers indicate each patient and the arrows indicate the molecular weight.

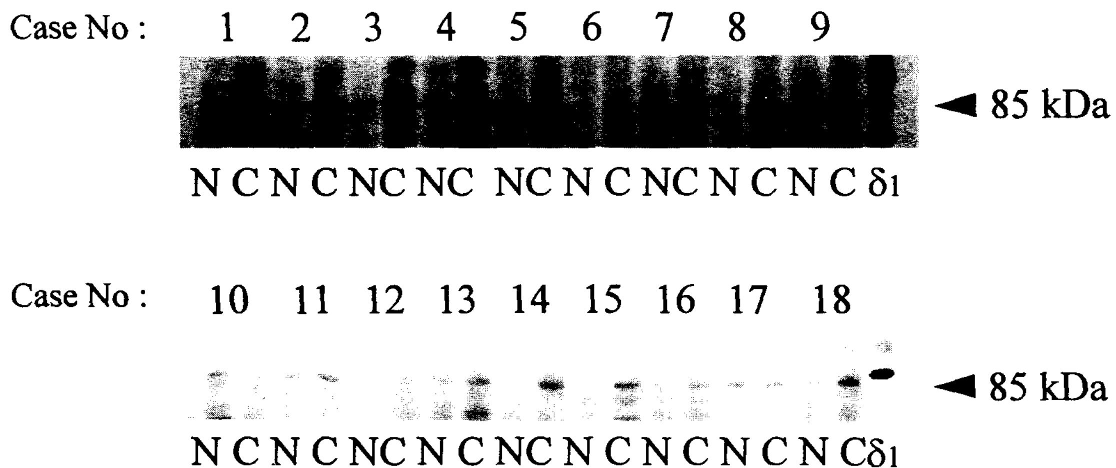
studied, 45 patients could be included for the comparison. The results were summarized in the Table 1. Although the invasion depth of the tumor seemed to correlate with the Western blot intensity of the PLC-γ1, it was not statistically significant.

Western blotting of PLC isozymes other than γ1 was also performed. However, only PLC-δ1 showed a slight increase in expression noted in about two-thirds of the cancer tissues, but the amount was small in comparison to PLC-γ1 and was much less prominent(Fig. 2).

To localize the source for the increased PLC-γ1 in cancer tissues and to rule out the possibility that epithelium-scarce normal mucosa has diluted PLC-γ1 in comparison to more densely populated malignant tissues, immunohistochemistry of 20 Western blot-positive cases were carried out. The results of Western blot agreed in 18 of 20 cases studied. PLC-γ1 immunoreactivity was intense in the neosplastic epithelium of gastric cancer (Fig. 3A and B) but staining in tumor-free normal mucosa or submucosa and adjacent connective tissues or muscle layer was not observed (Fig. 3C). The strong staining of malignant tissue against PLC-γ1 was readily

Table 1. Comparison of clinical prognostic factors of gastric carcinoma and PLC-γ1 immunoreactivity

	Case No	Phospholipase C-γ1	
		Negative (%)	Positive (%)
Lauren Classification			
Intestinal	26	3 (11.5)	23 (88.5)
Diffuse	19	5 (26.3)	14 (73.7)
Tumor size			
≤5 cm	24	5 (20.8)	19 (79.2)
〉5 cm	21	3 (14.3)	18 (85.7)
Invasion Depth			
Proper muscle	5	1 (20.0)	4 (80.0)
Subserosa	18	3 (16.7)	15 (83.3)
Serosa penetration	22	4 (18.2)	18 (81.8)
Lymphatic Invasion			
Absent	3	1 (33.3)	2 (66.7)
Present	42	7 (16.7)	35 (83.3)
Lymph Node Metatasis			
Absent	8	2 (25.0)	6 (75.0)
Present	37	6 (16.2)	31 (83.8)



Anti-PLC-δ1 Blot

Fig. 2. Immunoblotting normal mucosa and gastric carcinoma tissue with anti-PLC-δ1 monoclonal antibody. Total extract (20 μg protein/ lane) from both normal and gastric cancer tissues were separated on 6% SDS-PAGE and transferred to nitrocellulose membrane and blotted with anti-PLC-δ1 monoclonal antibody. Each bands were visualized with horse-raddish peroxidase conjugated goat anti-mouse IgG antibody using electrochemiluminescence. *N* ; normal mucosa, *C*; cancer tissue, *δ1*; purified PLC-δ1 standard. Numbers indicate each patient and the arrows indicate the molecular weight.

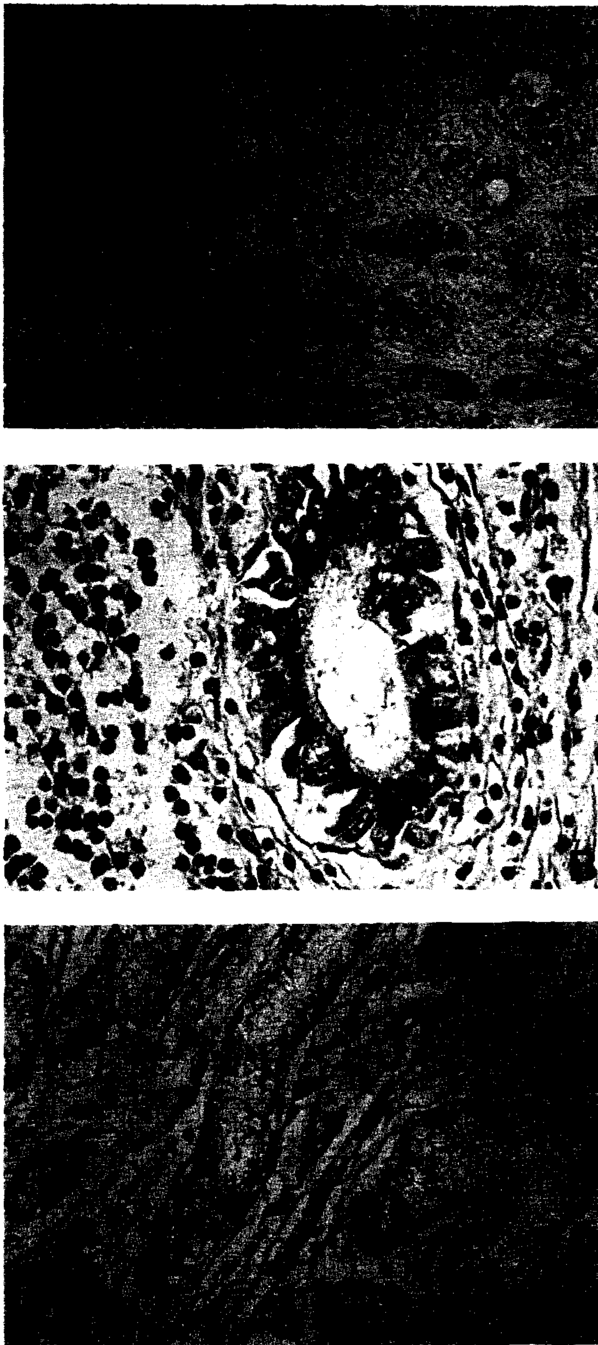


Fig. 3. Immunohistochemistry of gastric cancer and normal tissues. Immunohistochemistry of the gastric cancer and normal tissue with anti-PLC-γ1 monoclonal antibody showing clumps of PLC-γ1 positivity in cancer cells in situ and invading nests (A, ×100 and B, ×400) as compared to the normal gastric mucosa (C, ×200).

visible in primary *in situ* lesions as well as in invading cancer cell nests.

The staining in cancer tissues were characteristic. It gave a punctate appearance or revealed distinct speckles and small clumps present in the cytoplasms of malignant cells as seen in high power field. It had appearance of granules being secreted and was more prominent on the luminal side of the cytoplasms(Fig. 3B). The results were different from the previous observation that immunofluorescence against PLC-γ1 within the cells were associated with cytoskeletal compartments and was described as being linear and filamentous correlating with the actin filaments[29].

DISCUSSION

Activation of PLC by various signals and generation of second messengers, IP3 and DAG, with subsequent elevation of intracellular calcium level and activation of protein kinase C plays a crucial role in many of the biological processes such as cellular growth, differentiation, and transformation[1~3]. Thus PLC is a key player in the process of signal transduction, and at least 10 distinct mammalian isozymes of PLC have been identified, biochemically characterized from various tissues, and their respective cDNA have been isolated[4~9].

From our survey of ten known mammalian isozymes of PLC from various tissues and cell lines, it was suggested that the level of each isozyme is differentially expressed in different tissues, reflecting specialized functions of each tissue or cells to a certain degree (unpublished data). For example, PLC-γ1 and PLC-δ1 seem to be universal in many tissues. However PLC-γ2 is a predominant form in B-lymphocytes and, in T cell receptor signaling, PLC-γ1 appears to be important[30]. PLC-β2 seems to be expressed only in hematopoetic cells whereas bovine retina is rich in PLC-β4 isoform[7].

All PLCs contain two distinct regions of significant amino acid similarities designated as X and Y domains which are thought to be either the catalytic sites or the substrate binding site[4]. However, the different types of PLCs have unique features pertaining to each type in their primary structures. *src* homology domains named SH2 and SH3, resides between X and Y regions of PLC-γ type[13,14]. Pleckstrin homology domains are present in the amino terminus of PLC-β and -δ

isozymes. In contrast, split-pleckstrin homology domain is found between X and Y region of PLC-γ type[10~12]. These domains are important for protein-protein interaction and is being discovered in an ever growing number of proteins that are involved in the regulation of celluar growth and proliferation[31].

Although little is known about the events following tyrosine phosphorylation of PLC-γ1 upon growth factor receptor stimulation, it is known that after epidermal growth factor stimulation, it translocates from cytosol to cytoskeletal compartment and was found to be associated with actin filaments[29,32]. It was suggested that SH3 domain of the PLC-γ 1 directs this translocation[32] in cells and binds proline-rich segment of human dynamin through its SH3 domain[34].

Our study have demonstrated that expression of PLC-γ1 was markedly increased in majority of gastric cancer tissues suggesting a possible derangement of enhanced growth signal transduction in transformed cells. Many reports have implicated that PLC-γ1 is indeed involved in neoplastic transformation and carcinogenesis. Overexpressing PLC-γ1 in fibroblasts is known to induce transformation and increased inositol turn over[22]. A series of experiments revealed that microinjection of PLC-γ1 into quiescent NIH 3T3 cells induces DNA synthesis[35], however the mitogenic activity of PLC-γ1 is reported to be independent of its lipase activity[36]. Furthermore, recent findings clearly demonstrate that SH3 domain is essential for the induction of mitogenic activity in microinjected PLC-γ1[37]. In addition, PLC-γ1 expression is down-regulated during cell differentiation[38]. From these findings one can conclude that PLC-γ1 expression level can be used as an indicator for cellular growth and proliferation. Also there is also a recent evidence that in case of viral transformation PLC-γ1 may play a role[40].

Increased expression of PLC-γ1 has been reported in many pathologic conditions including carcinomas. In human breast carcinomas, content of PLC-γ1 as well as the state of tyrosine phosphorylation is increased compared to the normal breast tissue[24]. Also PLC-γ1 but not PLC-β1 and PLC-δ1 is increased in human colorectal carcinomas[25] and familial adenomatous polyposis[26]. Recently it has also been suggested that increased PLC-γ1 expression in colorectal cancer is associated with overexpression of factors which binds to the promoter region of PLC-γ1[38]. In hepatocellular carcinomas, increased PLC-γ1 expression and its relations with DNA ploidy has been published[39]. Our finding of increased PLC-γ1 in stomach cancer tissue contributes to this growing knowledge of PLC in carcinogenesis. But gastric cancer being the number one cause of cancer death in Korea and many Asian countries, it needs futher attention and effort to elucidate the basic mechanism underlying the overexpression. Other PLC isozymes expression was not remarkable in gastric tumor tissues. However slight increase in PLC-δ1 expression was noted in about two-thirds of the neoplastic tissues. But the amount present was low and the intensity of staining was less prominent, despite the fact that the affinity and specificity of anti-PLC-δ1 monoclonal autibody was known to be equivalent to that of the anti-PLC-γ1 antibody. In the light of the fact that the activator of PLC-δ isoforms awaits to be identified, it is difficult to draw any implication from these findings.

Immunohistochemistry of the paraffin fixed gastric cancer tissue was able to demonstrate the sources for the elevated PLC-γ1 content and identify subcellular localization of the isozymes. Cancer tissues were intensely stained with monoclonal anti-PLC-γ1 antibody whether it is present *in situ* or found in invading nests. But the adjacent normal epithelium, submucosa, endothelium of blood vessels or smooth muscle did not show the immunoreactivity. Two cases of tumor which showed positive Western blot did not stain in immunohistochemistry, however, the reason for this is not clear.

PLC-γ_1 is reported to be ubiquitous in most tissues and identified to be present in the cytosols of cells and may translocate upon stimulation, as suggested[32]. Most reports have demonstrated that the distribution of PLC-γ1 in subcellular compartment was in the form of filaments correlating with the appearance of actin filaments[29,32]. However, unlike the previous reports, we have found that the appearance of PLC-γ1 in the cytoplasms of cancer cells is in a distinct speckles or clumps, and it is worth mentioning here. Whether this is unique to gastric carcinomas or general characteristics of malignant tissues is unclear and needs further study, but it may imply the deviation from normal signal transduction and altered cellular behavior.

PLC-γ1 positivity and clinical behavior of the tumor did not correlate well, although invasion depth and Western blotting intensity seemed to relate. Thus more cases with objective methods such as densitometry is needed in the

future to link clinical significance and PLC-γ1 expression.

In conclusion, if the PLC-γ1 overexpression is a universal phenomenon in neoplastic tissues or hyperproliferative conditions, it could be considered as a prerequisite for signal amplification and enhanced mitogenesis of the tumor tissue with regard to the growth factor induced signaling pathways.

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