

# Expression of Phosphoinositide-specific phospholipase C enzymes in human osteosarcoma cell lines

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**Abstract** The definition of the number and nature of signal transduction pathways networking in the pathogenesis of osteosarcoma raised great interest. Intracellular calcium ions are important second messengers implicated in the control of cell death. The calcium concentration is regulated by signal transduction pathways, including the Phosphoinositides (PI) signaling. Phosphatidylinositol (4,5) bisphosphate (PIP<sub>2</sub>) is critical for many cellular activities. The levels of PIP<sub>2</sub> are regulated by means of Phosphoinositide-specific Phospholipase C (PI-PLC) family of enzymes. We delineated the panel of expression of PI-PLC enzymes in four human osteosarcoma cell lines. In MG-63 cell line, PI-PLC  $\beta$ 1,  $\beta$ 2,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\gamma$ 2,  $\delta$ 1,  $\delta$ 3 and  $\epsilon$  resulted expressed. In 143B cell line, PI-PLC  $\beta$ 1,  $\beta$ 2,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\gamma$ 2,  $\delta$ 1,  $\delta$ 3 and  $\epsilon$  were expressed. In SaOS-2 cell line, PI-PLC  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\gamma$ 2,  $\delta$ 1,  $\delta$ 3,  $\epsilon$  and  $\eta$ 1. In Hs888 cell line, PI-PLC  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\delta$ 1,  $\delta$ 3,  $\delta$ 4,  $\epsilon$  and  $\eta$ 1 the administration of U-73122 to cultures briefly modifies the levels of PI-PLC transcripts. The obtained complete expression panel of PI-PLC isoforms will be a useful tool for further functional studies about the role of the PI signal transduction pathway in osteosarcoma.

**Keywords** Signal transduction · Phospholipase C · Gene expression · Human osteosarcoma

## Abbreviations

PI	Phosphoinositides
PIP <sub>2</sub>	Phosphatidylinositol (4,5) bisphosphate
PI-PLC	Phosphoinositide-specific Phospholipase C
IP <sub>3</sub>	Inositol trisphosphate
DAG	Diacylglycerol
PKC	Protein kinase C enzymes
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
ANOVA	Analysis of variance

## Introduction

Osteosarcoma comprises less than 1 % of cancers diagnosed in the United States, occurring in less than 1000 patients per year (Mirabello et al. 2009a, b), and is the most common primary bone tumour in childhood and adolescence (Gatta et al. 2005).

The radiographic and histologic features, as well as the consistent spectrum of clinical presentations allow the diagnosis. The treatment includes surgery, radiotherapy and chemotherapy. Metastasizing tumours partially respond to current therapies and represent the primary cause of cancer related mortality (Meyers et al. 2005).

The complexity of osteosarcoma includes numerous abnormalities which were investigated (Gorlick et al. 2009). Progresses were achieved thanks to the use of epidemiologic features, genomic and molecular characterizations, and the study of osteosarcoma in animal models. A number of genetic abnormalities and environmental exposures were supposed to be involved in the development of osteosarcoma in laboratory models as well as in humans (Gorlick et al. 2009). Many efforts were made to bring multiple genetic risk factors together with epidemiologic and association data in order to identify a unifying recurrent event. However, despite the considerable improvement in the knowledge, no

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distinctive and specific dysregulation was identified and the pathogenesis of osteosarcoma is still largely unknown.

Osteosarcoma arises from a mesenchymal cell that has or can acquire the ability to produce osteoid (Gorlick et al. 2003; Marina et al. 2010). However, the cell of origin of osteosarcoma has been the subject of extensive discussions. A number of studies have also been performed as an attempt to decipher pathways associated with the pathogenesis of osteosarcoma. To define the number and the nature of the signal transduction pathways involved in the pathogenesis might improve the knowledge of the natural history of osteosarcoma. That also might help to refine the prognosis and to open the way to novel therapeutic strategies.

Intracellular calcium ions are important second messengers implicated in the control of cell death. Increases in calcium concentrations lead to necrosis or apoptosis following photodynamic therapy (Annunziato et al. 2003). Previous reports analyzed the role of calcium in osteosarcoma cells and the effect of concentration changes upon their viability. The antidepressant mirtazapine caused the death of MG-63 cells altering the cytosolic free calcium levels (Pan et al. 2006). In SaOS-2 cells, tissue factor VIIa and Xa induced transient intracellular calcium increase (Daubie et al. 2007). Calcium increase after treatment of UMR-106 cells with hematoporphyrin monomethyl ether and ultrasounds, followed by mitochondrial calcium reuptake, lead to cells death (Tian et al. 2010).

The calcium level is regulated by a complex network of signal transduction pathways, including the Phosphoinositides (PI) signaling. The regulation of PI signal transduction molecules is strictly related to a number of converting enzymes (Rhee et al. 1991). Phosphatidylinositol (4,5) bisphosphate (PIP<sub>2</sub>), one phosphorylated derivative of PI located mainly in the inner half of the plasma membrane lipid bilayer, is critical for many cellular activities, such as endo- and exocytosis, ion channel activity and cell motility. The levels of PIP<sub>2</sub> are regulated by means of Phosphoinositide-specific Phospholipase C (PI-PLC) family of enzymes (Fukami et al. 2010).

Activated PI-PLC cleaves PIP<sub>2</sub> into inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG), both crucial molecules in signal transduction (Rhee et al. 1991). IP<sub>3</sub>, a small water-soluble molecule, diffuses rapidly to the cytoplasm, where it induces calcium release from the endoplasmic reticulum by binding to IP<sub>3</sub>-gated calcium-release channels located in the reticulum membrane. The initial increase induced by IP<sub>3</sub> propagates as a wave through the cytoplasm often followed by a series of calcium spikes (Rhee et al. 1991). DAG remains bound to the membrane by its fatty acid tails and exerts two potential signalling roles. First, it can be further cleaved to release arachidonic acid, which either acts as a messenger or is used in the synthesis of eicosanoids (Tang et al. 2005). Second, DAG activates serine/threonine calcium-dependent protein kinase C enzymes (PKC). The calcium increase, induced by IP<sub>3</sub>, moves PKC to translocate from the cytoplasm

to the cytoplasmic face of the plasma membrane. The translocation activates PKC, which subsequently phosphorylates specific serine or threonine residues on further target proteins (Rhee et al. 1991; Suh et al. 2008).

The eukaryote PI-PLC family comprises a related group of complex, modular, multi-domain enzymes which cover a broad spectrum of regulatory interactions, including direct binding to G protein subunits, small GTPases from Rho and Ras families, receptor and non-receptor tyrosine kinases and lipid components of cellular membranes (Rhee et al. 1991). PI-PLC enzymes were classified on the basis of amino acid sequence, domain structure and mechanism of recruitment. Thirteen mammalian PI-PLC enzymes were identified, divided into six subfamilies:  $\beta$ (1–4),  $\gamma$ (1–2),  $\delta$ (1, 3, 4),  $\epsilon$ (1),  $\zeta$ (1), and  $\eta$ (1–2) (Suh et al. 2008).

Previous studies analyzed the expression of selected PI-PLC isoforms in human osteosarcoma. However, literature data lacked further information's about others, such as  $\epsilon$  and  $\eta$  subfamilies. The complete panel of expression of PI-PLC enzymes in tissues is critical for any kind of study. In fact, PI-PLC enzymes are strictly tissue specific (Suh et al. 2008; Bunney and Katan 2011), and our previous studies suggested that the expression panel is different in quiescent cells compared to the pathological counterpart (Lo Vasco et al. 2007, 2012). Therefore, knowledge of PI-PLC expression panel represents a preliminary tool in order to analyze the role of PI signal transduction pathway in the pathogenesis of osteosarcoma.

## Materials and methods

### Cell culture

We analyzed four cultured human osteosarcoma cell lines: MG-63, 143B, SaOS-2 and Hs888, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured as previously described (Di Cristofano et al. 2010). Briefly, cells were grown under sub-confluent or confluent conditions in medium, at 37 °C with 5 % of CO<sub>2</sub>. Cells were cultured in Dulbecco's minimum essential medium (Sigma) supplemented with 10 % fetal bovine serum (GIBCO) with penicillin (100  $\mu$ g/ml), streptomycin (100 U/ml) and sodium pyruvate. Cells were grown for 24 h, reaching a confluence of around 40–60 %. Experiments were independently repeated at least 3 times for each isoform. U-73122 (Sigma–Aldrich) was added to cultures at a concentration of 30  $\mu$ mol in dimethyl sulfoxide (DMSO) (Takenouchi et al. 2005; Lo Vasco et al. 2010). Cultures were stopped 3 and 24 h after the administration of the molecule, and suspended in TRIzol reagent for molecular biology analysis. Experiments were independently repeated at least 3 times for each isoform. Cultures were stopped 3 and 6 h after the

administration of U-73122 to be processed for western blot analyses. Experiments were independently repeated at least 2 times for each isoform.

### RT-PCR

After the confluent monolayer was obtained,  $10^6$  cells for each experiment were detached and suspended in TRIzol reagent (Invitrogen Corporation, Carlsbad, CA). Total RNA was isolated following the manufacturer's instructions. The purity of the RNA was assessed using a UV/visible spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories, Hercules, California, USA). 1  $\mu$ g total RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription (RT) kit (Applied Biosystems, Carlsbad, California, USA) according to manufacturer's instructions. Briefly, RT buffer, dNTP mix, RT random primers, multiscribe reverse transcriptase, RNase inhibitor and DEPC-treated distilled water were added in RNase-free tubes on ice. The RNA sample was added. The thermal cycler was programmed as follows: 25 °C for 10 min; 37 °C for 120 min; the reaction was stopped at 85 °C for 5 min. The final volume was 20  $\mu$ l. For PCR reactions we used the primer pairs (Bio Basic Inc, Amherst, New York, USA) listed in Table 1; for glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (Bio Basic Inc, Amherst, New York, USA)

forward 5'-CGAGATCCCTCCAAAATCAA-3' reverse 5'-GTCTTCTGGGTGGCAGTGAT-3'. The specificity of the primers was verified by searching in the NCBI database for possible homology to cDNAs of unrelated proteins. Each PCR tube contained the following reagents: 0.2  $\mu$ M of both sense and antisense primers, 1–3  $\mu$ l (about 1  $\mu$ g) template cDNA, 0.2 mM dNTP mix, 2.5 U REDTaq Genomic DNA polymerase (Sigma-Aldrich) and 1 $\times$  reaction buffer. MgCl<sub>2</sub> was added at a variable final concentration (empirical determination by setting the experiment). The final volume was 50  $\mu$ l. The amplification was started with an initial denaturation step at 94 °C for 2 min and was followed by 35 cycles consisting of denaturation (30 s) at 94 °C, annealing (30 s) at the appropriate temperature for each primer pair and extension (1 min) at 72 °C. The PCR products were analysed by 1.5 % TAE ethidium bromide-stained agarose gel electrophoresis (Agarose Gel Unit, Bio-Rad Laboratories S.r.l., Segrate, IT). A PC-assisted CCD camera UVB lamp (Vilber Lourmat, Marne-la-Vallée France) was used for gel documentation. Gel electrophoresis of the amplification products revealed single DNA bands with nucleotide lengths as expected for each primer pair. RNA samples were also amplified by PCR without RT. No band was observed, excluding DNA contamination during the procedure (data not shown). The reaction products were further quantified with the Agilent 2100

**Table 1** Primers for PI-PLC isoforms used for RT-PCR experiments

PI-PLC $\beta$ 1 (PLCB1; OMIM *607120)	forward 5'-AGCTCTCAGAACAAGCCTCCAACA-3' reverse 5'-ATCATCGTCGTCGTCACCTTCCGT-3'
PI-PLC $\beta$ 2 (PLCB2; OMIM *604114)	forward 5'-AAGGTGAAGGCCTATCTGAGCCAA-3' reverse 5'-CTTGGCAAACCTCCCAAAGCGAGT-3'
PI-PLC $\beta$ 3 (PLCB3; OMIM *600230)	forward 5'-TATCTTCTTGGACCTGCTGACCGT-3' reverse 5'-TGTGCCCTCATCTGTAGTTGGCTT-3'
PI-PLC $\beta$ 4 (PLCB4; OMIM *600810)	forward 5'-GCACAGCACACAAAGGAATGGTCA-3' reverse 5'-CGCATTTCTTGTCTTCCCTGTCA-3'
PI-PLC $\gamma$ 1 (PLCG1; OMIM *172420)	forward 5'-TCTACCTGGAGGACCCTGTGAA-3' reverse 5'-CCAGAAAGAGAG CGTGTAGTCG-3'
PI-PLC $\gamma$ 2 (PLCG2; OMIM *600220)	forward 5'-AGTACATGCAGATGAATCACGC-3' reverse 5'-ACCTGAATCCTGATTTGACTGC-3'
PI-PLC $\delta$ 1 (PLCD1; OMIM *602142)	forward 5'-CTGAGCGTGTGGTTCCAGC-3' reverse 5'-CAGGCCCTCGGACTGGT-3'
PI-PLC $\delta$ 3 (PLCD3; OMIM *608795)	forward 5'-CCAGAACCACTCTCAGCATCCA-3' reverse 5'-GCCA TTGTTGAGCACGTAGTCAG-3'
PI-PLC $\delta$ 4 (PLCD4; OMIM *605939)	forward 5'-AGACACGTCCCAGTCTGGAACC- 3' reverse 5'-CTGCTTCTTCTTCTCATATTC- 3'
PI-PLC $\epsilon$ (PLCE; OMIM *608414)	forward 5'-GGGGCCACGGTCATCCAC-3' reverse 5'-GGGCCTTCATACCGTCCATCCTC-3'
PI-PLC $\eta$ 1 (PLCH1; OMIM *612835)	forward 5'-CTTTGGTTCGGTTCCTTGTGTGG-3' reverse 5'-GGATGCTTCTGTACAGTCCTTCC-3'
PIPLC $\eta$ 2 (PLCH2; OMIM *612836)	forward 5'-GAAACTGGCCTCCAAAACACTGCCCGCCG-3' reverse 5'-GTCTTGTGGAGATGCACGTGCCCTTGC-3'

bioanalyzer using the DNA 1000 LabChip kit (Agilent Technologies, Deutschland GmbH). The PCR products of cultures treated with U-73122 were analyzed by 1.5 % TBE agarose gel electrophoresis (Submarine Agarose Gel Unit, Hoefer, San Francisco, CA). Gels were previously stained with ethidium bromide (50 ng/100 ml). A PC-assisted CCD camera (GelDoc 2000 System/Quantity One Software; Bio-Rad) was used for gel documentation and quantification. Optical densities were normalized to the mRNA content of glyceraldehydes 3 phosphate dehydrogenase (GAPDH), a typical reference constitutive transcript. Gel electrophoresis of the amplification products revealed single DNA bands with nucleotide lengths as expected for all primer pairs. To exclude possible DNA contamination during the RT-PCR, RNA samples were amplified by PCR without reverse transcription. No band was observed, suggesting also in these experiments that there was no DNA contamination in the RNA preparation procedure. Experiments were independently repeated at least 3 times for each isoform.

#### Western blot

Whole-cell lysates ( $10^6$  cells for each experiment) were prepared by lysing cells in RIPA buffer (50 mM Tris pH=7.5, NP-40, 0.1 % SDS, 100 mM NaCl, 50 mM NaF, 1 mM EDTA) supplemented with a set of protease inhibitors: 10  $\mu$ g of leupeptin per ml, 10  $\mu$ g of aprotinin per ml, 1 mM sodium benzamidine, and 1 mM phenylmethylsulfonyl fluoride. Proteins (50  $\mu$ g) were separated on 12 % polyacrylamide, 0.1 % SDS gel. Then, incubation with a monoclonal antibody specific for each PI-PLC isoform (Santa Cruz, CA) followed. Immunoreactive bands were visualized using the enhanced chemiluminescence method. Experiments were independently repeated at least 2 times for each isoform.

For PI-PLC  $\beta$ 2 and PI-PLC  $\eta$ 2, positive controls were used to test the efficacy of primers in RT-PCR analyses and of antibodies in Western Blot analyses. For PI-PLC  $\beta$ 2 human leucocytes were used as positive controls. For PI-PLC  $\eta$ 2, nervous tissue was used as positive control.

#### Statistical analysis

The concentrations obtained for each isoform were compared in the different cell lines using the ANOVA test ([http://www.physics.csbsju.edu/stats/anova\\_NGROUP\\_NMAX\\_form.html](http://www.physics.csbsju.edu/stats/anova_NGROUP_NMAX_form.html)). The panels of expression, including dosages of all PI-PLC isoforms, were compared analyzing couple of cell lineages using the *t*-test (<http://studentstest.com/>).

## Results

### RT-PCR

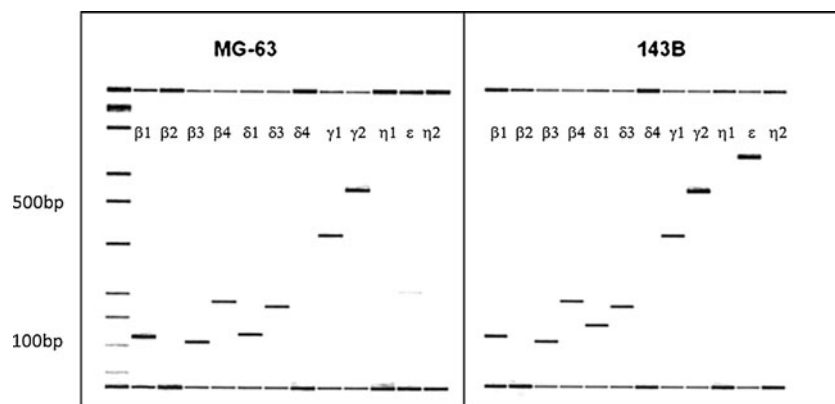
In MG-63 cell line, PI-PLC  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\gamma$ 2,  $\delta$ 1 and  $\delta$ 3 were expressed. Specific bands were obtained using gel electrophoresis analysis (Figs. 1, 2 and 3). Analysis with the bioanalyzer confirmed the data obtained with the gel electrophoresis. The concentrations obtained in 4 independent experiments measured with multiliquid analyzer are listed in Table 2. Analysis with the bioanalyzer detected a small peak for PI-PLC  $\beta$ 2, although the quantification was not possible.

In 143B cell line, PI-PLC  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\gamma$ 2,  $\delta$ 1,  $\delta$ 3 and  $\epsilon$  were expressed. Specific bands were obtained using gel electrophoresis analysis (Fig. 1). The concentrations obtained in 4 independent experiments measured with multiliquid analyzer are listed in Table 2. A weak signal for PI-PLC  $\beta$ 2 was detected with gel electrophoresis analysis. Using the bioanalyzer, a small peak was detected, although the quantification was not possible.

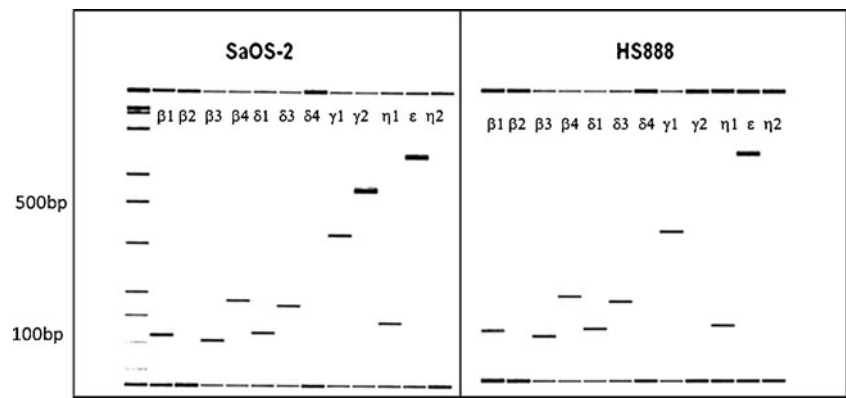
In SaOS-2 cell line, PI-PLC  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\gamma$ 2,  $\delta$ 1,  $\delta$ 3,  $\epsilon$  and  $\eta$ 1 were expressed. Specific bands were obtained using gel electrophoresis analysis (Fig. 2). Analysis with the bioanalyzer confirmed the data obtained with the gel electrophoresis. The concentrations obtained in 4 independent experiments measured with multiliquid analyzer are listed in Table 2.

In Hs888 cell line, PI-PLC  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\delta$ 1,  $\delta$ 3,  $\delta$ 4 and  $\epsilon$  were expressed. Specific bands were obtained using gel electrophoresis analysis (Fig. 2). Analysis with the

**Fig. 1** RT-PCR results in MG-63 and 143B. *Left:* MG-63 cell line. Visualization of specific bands for the transcripts of PI-PLC  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\gamma$ 2,  $\delta$ 1 and  $\delta$ 3. *Right:* 143B cell line. Visualization of specific bands for the transcripts of the genes codifying for PI-PLC  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\gamma$ 2,  $\delta$ 1,  $\delta$ 3 and  $\epsilon$



**Fig. 2** RT-PCR results in SaOS and Hs888. *Left:* SaOS-2 cell line. Visualization of specific bands for the transcripts of the genes codifying for PI-PLC  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\gamma$ 2,  $\delta$ 1,  $\delta$ 3,  $\epsilon$  and  $\eta$ 1. *Right:* Hs888 cell line. Visualization of specific bands for the transcripts of the genes codifying for PI-PLC  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\delta$ 1,  $\delta$ 3,  $\delta$ 4 and  $\epsilon$

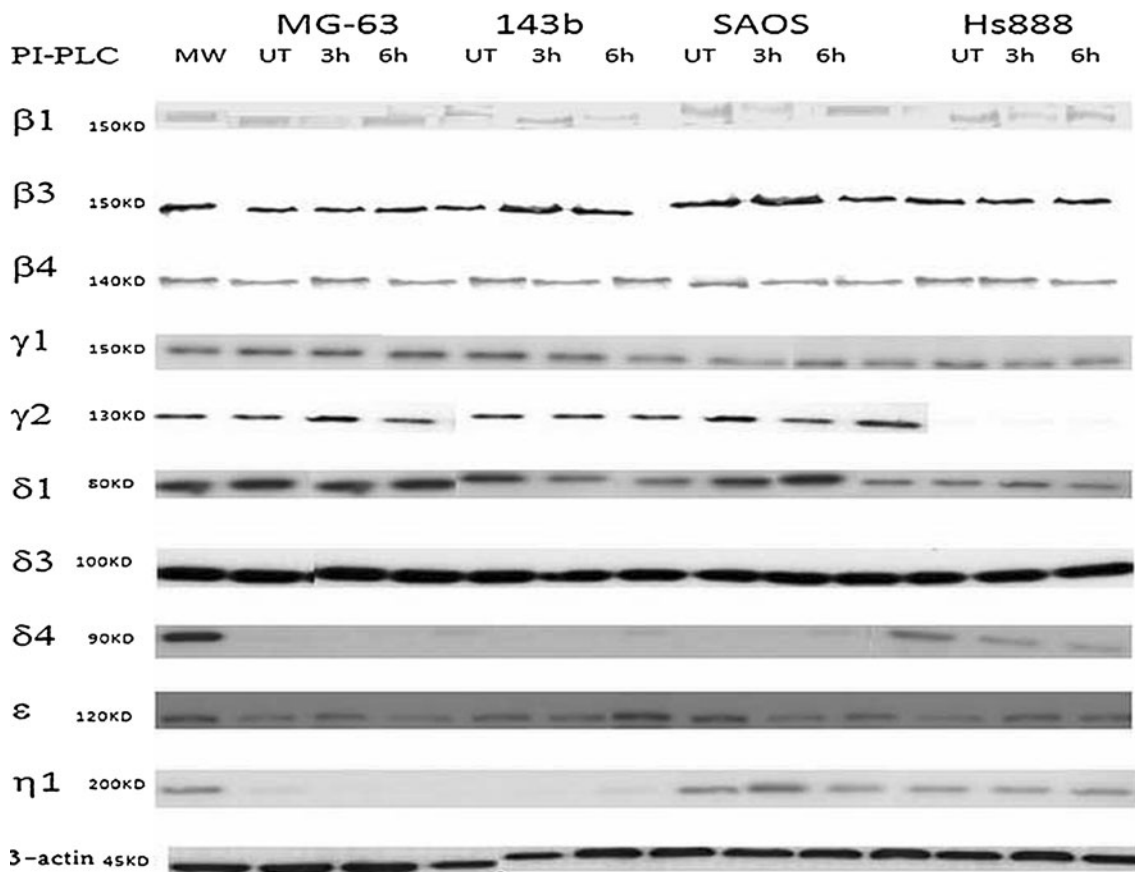


bioanalyzer confirmed the data obtained with the gel electrophoresis. The concentrations obtained in 4 independent experiments measured with multiliquid analyzer are listed in Table 2.

For PI-PLC  $\beta$ 2, in the human leucocytes used as positive controls, the corresponding specific band was obtained as expected. For PI-PLC  $\eta$ 2, in the nervous tissue used as

positive control, the corresponding specific band was obtained as expected.

After 3 h from administration of U-73122, the following results were detected: in MG-63, in 143B, in SaOS-2 and Hs888 cell lines all the tested isoforms (PI-PLC  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\gamma$ 2,  $\delta$ 1,  $\delta$ 3,  $\epsilon$ ,  $\eta$ 1 and  $\eta$ 2) resulted absent using the gel electrophoresis analysis (Tables 3, 4, 5 and 6). The GAPDH



**Fig. 3** Western Blot results of untreated cells (UT), after 3 and 6 h after U-73122 treatment in MG-63, 143B, SaOS, Hs888. Last line:  $\beta$ -actin loading controls. MW: antibody molecular weight. Visualization of specific bands indicate the presence of the PI-PLC isoforms and  $\beta$ -

actin. The gel electrophoresis results did not show differences in the presence of PI-PLC enzymes after 3 and 6 h after addition of U-73122 to the cultures with respect to untreated cells



**Table 2** Results of RT-PCR. Quantification of the expressed PI-PLC isoforms in osteosarcoma cell lines with Agilent Bio-analyzer. The concentrations were measured in 4 independent experiments, listed as EXP1, 2, 3 and 4. Concentrations are intended in nanograms per microliter

PI-PLC		MG-63	143B	SAOS	HS888	<i>p</i>
β1	EXP1	10.18	4.54	5.84	2.50	<0.01
	EXP2	10	4.44	5.88	2.5	
	EXP3	10.6	4.6	5.8	2.51	
	EXP4	10.02	4.55	5.85	2.5	
β2	EXPS1-2-3	0	0	0	0	
β3	EXP1	17.49	25.06	33.83	27.52	<0.01
	EXP2	17.58	25.06	33.9	27.51	
	EXP3	17.48	25.05	33.99	27.5	
	EXP4	17.5	25.05	33.2	27.52	
β4	EXP1	17.79	46.40	30.07	38.47	<0.01
	EXP2	17.8	46.66	30.06	38.48	
	EXP3	18.01	46.29	30.07	38.48	
	EXP4	18.01	46.49	30.07	38.46	
γ1	EXP1	8.02	10.38	11.87	11.91	<0.01
	EXP2	7.98	10.39	11.77	11.9	
	EXP3	8.2	10.38	11.9	11.9	
	EXP4	7.98	10.38	11.77	11.92	
γ2	EXP1	21.40	43.23	47.59	0	<0.01
	EXP2	22	43.02	47.55	0	
	EXP3	21.1	43.33	47.58	0	
	EXP4	21.40	43.2	47.59	0	
δ1	EXP1	15.42	9.00	12.67	54.51	<0.01
	EXP2	15.1	9	12.66	54.5	
	EXP3	15.5	9.01	12.67	54.5	
	EXP4	15.4	9.01	12.68	54.51	
δ3	EXP1	15.13	15.56	32.64	13.63	<0.01
	EXP2	15.2	15.55	32.64	13.62	
	EXP3	15.1	15.6	32.6	13.65	
	EXP4	15.13	15.56	32.65	13.64	
δ4	EXP1	0	0	0	11.59	<0.01
	EXP2	0	0	0	11.6	
	EXP3	0	0	0	11.6	
	EXP4	0	0	0	11.58	
ε	EXP1	0.77	15.55	8.0	4.43	<0.01
	EXP2	0.79	15.6	8	4.42	
	EXP3	0.77	15.46	7.9	4.41	
	EXP4	1	15.55	8	4.44	
η1	EXP1	0	0	8.18	8.29	<0.01
	EXP2	0	0	8.18	8.3	
	EXP3	0	0	8.2	8.26	
	EXP4	0	0	8.15	8.29	
η2	EXPS1-2-3	0	0	0	0	

control specific band was visualized as a positive control with gel electrophoresis analysis.

After 24 h from administration of U-73122, RT-PCR identified specific bands were obtained using the gel electrophoresis analysis. In MG-63 cell line, PI-PLC β1, β3, β4, γ1, δ1, δ3, and ε resulted expressed (Table 3). In 143B cell line, PI-PLC β1, β3, β4, γ1, γ2, δ1, δ3 and ε were expressed (Table 3). In SaOS-2 cell line, PI-PLC β1, β3,

β4, γ1, γ2, δ1, δ3, ε and η1 were expressed (Table 3). In Hs888 cell line, PI-PLC β1, β3, β4, γ1, δ1, δ3, δ4, ε and η1 were expressed (Table 3).

#### Western blot

The western blot analyses performed after administration of U-73122 did not show differences in the presence of PI-PLC

**Table 3** Statistical analysis of the results of RT-PCR in Experiment 1. Concentrations are intended in nanograms per microliter

PI-PLC isoforms	MG-63	143B	SAOS	HS888		
					SD	AADM
$\beta$ 1	10.18	4.54	5.84	2.50	3.25	2.25
$\beta$ 2	NM	NM	0	0	–	–
$\beta$ 3	17.49	25.06	33.83	27.52	6.76	4.7
$\beta$ 4	17.79	46.40	30.07	38.47	12.2	9.25
$\gamma$ 1	8.02	10.38	11.87	11.91	1.83	1.34
$\gamma$ 2	21.40	43.23	47.59	–	21.9	17.4
$\delta$ 1	15.42	9.00	12.67	54.51	21.2	12.1
$\delta$ 3	15.13	15.56	32.64	13.63	8.97	4.86
$\delta$ 4	0	0	0	11.59	5.79	2.9
$\epsilon$	0.77	15.55	8.0	4.43	6.31	4.59
$\eta$ 1	0	0	8.18	8.29	4.75	4.12
$\eta$ 2	0	0	0	0	–	–

SD standard deviation; AADM Average absolute deviation from the median

enzymes after 3 and 6 h after addition to the cultures with respect to untreated cells. For PI-PLC  $\beta$ 2, in the human leucocytes used as positive controls, the corresponding specific band was obtained as expected. For PI-PLC  $\eta$ 2, in the nervous tissue used as positive control, the corresponding specific band was obtained as expected.

#### Statistical analysis

The ANOVA test results were significant ( $p < 0.01$ ) comparing the concentrations of each isoform in the 4 cell lines (Table 3, right column).

The *t*-test used to compare the panels of expression of the cell lines did not identify statistical significant results (Table 4).

#### Discussion

The tight regulation of calcium concentration is critical to many physiological events. Unregulated elevation alters protein functions, apoptosis regulation, secretion mechanisms and cell contractile activity (Annunziato et al. 2003).

**Table 4** Statistical analysis of the results of RT-PCR. *T*-test results to compare the panels of PI-PLCs' concentration between couples of cell lines. *p* = *p*-value of statistical probability, assumed significant for  $p < 0.01$ 

Comparison of cell lines	One tailed <i>t</i> -test
MG-63/143B	$p = 0.11784$
MG-63/SAOS	$p = 0.09538$
MG-63/Hs888	$p = 0.16348$
143b/SAOS	$p = 0.39689$
143B/Hs888	$p = 0.48501$
SAOS/Hs888	$p = 0.41439$

The metabolism of calcium is regulated by a complex network of signalling pathways, including the PI transduction system (Rhee et al. 1991). Great attention was paid to PI-PLC enzymes, effector molecules in PI signalling, due to their involvement in a growing number of diseases (Rhee et al. 1991; Suh et al. 2008).

Calcium metabolism was also indicated to be critical for cells viability in human osteosarcoma cell lines (Barry 2000). Previous literature data reported the expression of PI-PLC  $\beta$ 1,  $\beta$ 3,  $\gamma$ 1,  $\gamma$ 2 and  $\delta$ 1 in a number of osteosarcoma cell lines, including MG-63 and SaOS-2 cell lines (Hoberg et al. 2005). We analyzed the expression of PI-PLC enzymes in four cultured human osteosarcoma cell lines: MG-63, 143B, SaOS-2 and Hs888.

MG-63 cells, used as experimental models for human osteoblasts, have low levels of alkaline phosphatase activity, and PTH unresponsive adenylate cyclase (Fukayama and Tashjian 1990). The present results (Tables 2 and 3; Fig. 1) accord to literature data with regard to the expression of PI-PLC  $\beta$ 1,  $\beta$ 3,  $\gamma$ 1,  $\gamma$ 2 and  $\delta$ 1. Our results partially contrast to previous literature data about PI-PLC  $\beta$ 2. Previous reports demonstrated a specific role of PI-PLC  $\beta$ 2 in the mechanotransduction and cell attachment, although the basal concentration of the enzyme in unstimulated cells was not reported (Hoberg et al. 2005). Further reports highlighted that in osteoblasts PI-PLC  $\beta$ 2 transduces signals from prostaglandin E2 and other prostanoids (Kondo and Togari 2004). In the present study, PI-PLC  $\beta$ 2 resulted weakly expressed, and the concentration was not measurable. The expression of PI-PLC  $\beta$ 2 seems usually to be correlated to specific stimuli, and probably our results differ in that we analyzed unstimulated MG-63 cells. The basal expression of PI-PLC  $\beta$ 2 in osteoblast-like cells might be low. Increase might follow to different and specific stimuli, both mechanic and biochemical. No further data were

**Table 5** Results of RT-PCR after U-73122 treatment

PI-PLC isoforms	MG-63			143B			SAOS			Hs888		
	UT	3 h	24 h	UT	3 h	24 h	UT	3 h	24 h	UT	3 h	24 h
$\beta$ 1	+	-	+	+	-	+	+	-	+	+	-	+
$\beta$ 2	-	-	-	-	-	-	-	-	-	-	-	-
$\beta$ 3	+	-	+	+	-	+	+	-	+	+	-	+
$\beta$ 4	+	-	+	+	-	+	+	-	+	+	-	+
$\gamma$ 1	+	-	+	+	-	+	+	-	+	+	-	+
$\gamma$ 2	+	-	+	+	-	+	+	-	+	-	-	-
$\delta$ 1	+	-	+	+	-	+	+	-	+	+	-	+
$\delta$ 3	+	-	+	+	-	+	+	-	+	+	-	+
$\delta$ 4	-	-	-	-	-	-	-	-	-	+	-	+
$\epsilon$	+	-	+	+	-	+	+	-	+	+	-	+
$\eta$ 1	-	-	-	-	-	-	+	-	+	+	-	+
$\eta$ 2	-	-	-	-	-	-	-	-	-	-	-	-

UT untreated cells. + mRNA expressed; - mRNA unexpressed

available about expression of the remaining enzymes in MG-63 line. In the present study, PI-PLC  $\beta$ 4 and PI-PLC  $\delta$ 3 were expressed. PI-PLC  $\epsilon$  known to be a downstream effector of Ras superfamily GTPases and an upstream activator of small GTPases, both Ras and Rap (Seifert et al. 2008), was weakly expressed. The expression of PI-PLC  $\beta$ 1 is significantly higher. The expression PI-PLC  $\beta$ 3,  $\beta$ 4,  $\gamma$ 2 and  $\epsilon$  were significantly lower with respect to the other cell lines. These observations require further studies in order to evaluate the role of PI-PLC  $\beta$ 2 and PI-PLC  $\epsilon$  in MG-63 cells.

143B, thymidine kinase negative cells, are known to develop osteolytic tumours (Kaminski et al. 2003). No literature data were available for PI-PLC expression in 143B cell line. In the present study, PI-PLC  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\gamma$ 2,

$\delta$ 1,  $\delta$ 3 and  $\epsilon$  were expressed (Table 2; Fig. 1). PI-PLC  $\beta$ 2 was weakly expressed and the concentration not measurable. The expression of PI-PLC  $\epsilon$  is significantly higher with respect to the other cell lines. Further studies are required to evaluate the role of PI-PLC  $\beta$ 2 and PI-PLC  $\epsilon$  in 143B cells.

SaOS-2 is an established epithelial-like human osteosarcoma cell line (Fogh et al. 1975) used as experimental model for human osteoblasts (McQuillan et al. 1995; Rodan et al. 1987). The present results (Table 2; Fig. 1) accord to previous literature data with regard to the expression of PI-PLC  $\beta$ 1,  $\beta$ 3,  $\gamma$ 1,  $\gamma$ 2 and  $\delta$ 1. No further data were available about the expression of the remaining isoforms. In the present study, PI-PLC  $\beta$ 4,  $\delta$ 3,  $\epsilon$  and  $\eta$ 1 were also expressed. Moreover, the expression of PI-PLC  $\delta$ 3 was

**Table 6** Results of western blot after U-73122 treatment

PI-PLC isoforms	MG-63			143B			SAOS			Hs888		
	UT	3 h	6 h	UT	3 h	6 h	UT	3 h	6 h	UT	3 h	6 h
$\beta$ 1	+	+	+	+	+	+	+	+	+	+	+	+
$\beta$ 2	-	-	-	-	-	-	-	-	-	-	-	-
$\beta$ 3	+	+	+	+	+	+	+	+	+	+	+	+
$\beta$ 4	+	+	+	+	+	+	+	+	+	+	+	+
$\gamma$ 1	+	+	+	+	+	+	+	+	+	+	+	+
$\gamma$ 2	+	+	+	+	+	+	+	+	+	-	-	-
$\delta$ 1	+	+	+	+	+	+	+	+	+	+	+	+
$\delta$ 3	+	+	+	+	+	+	+	+	+	+	+	+
$\delta$ 4	-	-	-	-	-	-	-	-	-	+	+	+
$\epsilon$	+	+	+	+	+	+	+	+	+	+	+	+
$\eta$ 1	-	-	-	-	-	-	+	+	+	+	+	+
$\eta$ 2	-	-	-	-	-	-	-	-	-	-	-	-

UT untreated cells. + enzyme present; - enzyme absent



significantly higher with respect to the other cell lines. These data suggest a possible role of the PI-PLC induced calcium osteosynthesis even in osteosarcoma.

Hs888 cells were derived from lung metastasis of osteosarcoma. No literature data were currently available for PI-PLC expression in Hs888 cell line. In the present study, PI-PLC  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\delta$ 1,  $\delta$ 3,  $\delta$ 4 and  $\epsilon$  were expressed. Surprisingly, PI-PLC  $\gamma$ 2 resulted unexpressed, whereas it was detected in the other three cell lines we analyzed. Therefore, in the present study, PI-PLC  $\gamma$ 2 resulted absent exclusively in the metastatic counterpart of the human osteosarcoma. Previous literature data suggested that PI-PLC  $\gamma$ 2 isoform is required for the early phase in osteoclast differentiation (Kertész et al. 2012). PI-PLC  $\delta$ 4 was expressed exclusively in Hs888. Noteworthy, studies of breast cancer MCF-7 cells suggested that the abnormal expression of PI-PLC  $\delta$ 4 contributes to oncogenesis through up-regulation of ErbB expression and activation of ERK pathway (Leung et al. 2004). The expression of PI-PLC  $\beta$  and PI-PLC  $\delta$ 1 was significantly higher with respect to the other cell lines.

Remarkably, PI-PLC  $\eta$ 1 was detected exclusively in SaOS-2 and Hs888 cells. PI-PLC  $\eta$ 1, the most calcium sensitive isoform, is essential for downstream ERK1/2 phosphorylation (Kim et al. 2011). Although the role of PI-PLC  $\eta$ 1 is not fully elucidated, the activation of PI-PLC  $\eta$ 1 is known to enhance the GPCR mediated calcium pathway (Kim et al. 2011).

Further investigations, performed using RT-PCR, demonstrated that the transcription of PLC genes was absent after 3 h from the administration of U-73122, a specific inhibitor for PI-PLC isoforms. These observations accord to previous literature data described in other tumours (Lo Vasco et al. 2011) indicating that U-73122 also acts upon the genes' expression. After 24 h from the administration of U-73122, the expression returns similar to untreated cells, accordingly to previous data (Lo Vasco et al. 2011).

Given the tissue specificity of PI-PLC enzymes, the panel of expression presently delineated slightly differed in the MG-63, 143B, SaOS and Hs888 osteosarcoma cell lines. Selected enzymes, such as PI-PLC  $\beta$ 1,  $\beta$ 3,  $\beta$ 4,  $\gamma$ 1,  $\delta$ 1 and  $\delta$ 3, were commonly expressed, although at variable concentrations, in all four cell lines. PI-PLC  $\eta$ 2 was commonly unexpressed in all the analyzed cell lines. Beside the results regarding PI-PLC  $\beta$ 2 and PI-PLC  $\epsilon$ , already commented, the remaining enzymes were differently expressed. In our previous report, we had recently analyzed the expression of PI-PLC isoforms in cultured human fibroblasts (Lo Vasco et al. 2013). PI-PLC  $\beta$ 2,  $\eta$ 1 and  $\eta$ 2 had resulted unexpressed and PI-PLC  $\delta$ 4 inconstantly expressed (Lo Vasco et al. 2013). Interestingly, also in the osteosarcoma cell lines we presently analyzed, PI-PLC  $\beta$ 2 and  $\eta$ 2 were not expressed and PI-PLC  $\delta$ 4 inconstantly expressed. The present results suggest that human cultured fibroblasts and human osteosarcoma cell

lines share similar expression panel for PI-PLC isoforms. This observation will require further studies in order to investigate the possible relationship.

Moreover, although it is not possible to formulate conclusions about these findings, the differences in the panel of expression of PI-PLC enzymes might be due to the different commitment of the cell lines. In fact, PI-PLC  $\gamma$ 2 was expressed in MG-63, 143B and SaOS-2, whereas it was absent in Hs888 cells. The concentration measured in 143B and SaOS-2 cells was about 50 % higher than in MG-63 cells. Notably, recent literature data indicated that PI-PLC  $\gamma$ 2 is involved in osteoclast differentiation and bone resorption (Kertész et al. 2012). Therefore, unique among the analyzed cell lines, Hs888 did not express PI-PLC  $\gamma$ 2 and expressed PI-PLC  $\delta$ 4. The peculiar expression of these two isoforms in Hs888 cells might be related to the metastasizing ability of the lineage.

PI-PLC  $\eta$ 1 was expressed in SaOS-2 epithelial-like lineage and in Hs888 cells. PI-PLC  $\eta$ 1 is considered a sensor activated by very small calcium increases, and, therefore, involved in the finest regulation of signalling.

The statistical comparison of the results resulted significant. However, further studies are required in order to confirm these observations and the possible use of this finding as diagnostic or prognostic tool. Moreover, the peculiar expression of specific enzymes in distinct cell lines deserves further studies in order to analyze the specific role and crosstalk of each expressed enzyme in specific osteosarcoma lineages.

Little is known about the events leading to osteosarcoma, with special regard to the complex network of molecular pathways which regulate the cells survival and metabolism. The present work offers a first contribute to further studies addressed to analyze the role of PI signal transduction pathway in osteosarcoma cells, representing a tool to improve our knowledge of the metabolic activity during the disease.

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