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Research Article

Identification of PI3K Isoforms in Human Prostate Cancer Cell Lines (PC3, DU145) and Human Bladder Carcinoma Cell line (5637)

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Abstract

There exists an association between PI3K pathway licentious activity and the considerable feature of high metastatic potential of the genitourinary cancer cells. Although DU 145 and 5637 have functional phosphatase and tensin homolog (PTEN) tumor suppressor gene, which antagonizes PI3K function, PC-3 is null for PTEN gene. In pursuit to explain why PTEN bearing cell lines display high metastatic behavior, we searched for any discrepancy in PI3K isoforms expression pattern between these cell lines. Gathering gene bank data files, specific primers were designed, for all the genes of 12 studied isoforms from 3 different classes of PI3K. Total RNA was extracted and examined by Real- Time PCR to compare the cells for the type and amount of the isoforms which expressed. Cα and R2 isoforms are indicative of an equal expression for PC3 and DU145, R3 transcripts revealed 80% decrease in DU145 and Cβ, R1 and C2α demonstrated an increased expression in DU145. When a comparison is made between 5637 and PC3, it can be seen that although a little decrease in the level of R3 transcripts was demonstrated, the amount of Cα, Cβ, R2, R1 and C2α increased. In conclusion in this study it is proposed that R1, R2, Cα, Cβ , C2α and R1, Cβ , C2α are candidate genes for silencing via RNAi in 5637 and DU145, respectively, to evaluate their roles in metastatic behavior of the both studied PTEN bearing cell lines.

***Keywords*:** PI3K Isoforms, Prostate, Bladder, 5637, DU145, PC3

Introduction

Phosphatidylinositol 3-kinases (PI3Ks) as lipid kinases devise pivotal roles in regulation of DNA repair, apoptosis, cell cycle, angiogenesis, metabolism and cell motility. They act as intermediate signaling molecules and transmit signals from cell surface to the cytoplasm (Akinleye, 2013). PI3K has been reported to have protein kinase activity as well. However, it remains unclear if this protein kinase activity has any role in vivo. (Vanhaesebroeck, 2012; Cantrell, 2001; Vanhaesebroeck and Waterfield, 1999).

There are at least twelve members of this family present in the human genome (Fry, 2001). There are eight mammalian PI3K enzymes that have been divided into three classes according to structural features and lipid substrate preferences (Vanhaesebroeck, 2010; Fruman, 2014; Kastan and Lim, 2000). In mammals, class I PI3Ks are the best understood and are present in all cell types (Fruman, 2014).

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Only class I PI3Ks are involved in cancer. Based on this fact it can be deducted that the three classes of PI3K have different product and substrate specificities. Only class I PI3Ks can use PIP2 to generate PIP3, class II PI3Ks produce the 3,4-bisphosphate and the 3-monophosphate of inositol lipids, and class III can only make the 3-monophosphate(Zhao , 2008).

It should be noted that Class I enzymes are able to convert PI (4, 5) P2 to PI (3, 4, 5) P3 on the inner leaflet of the plasma membrane. They are further classified to class IA and class IB (Fry, 1994; Vanhaesebroeck and Waterfield, 1999). Class IA PI3K as heterodimeric molecules consist of a P110α, β, or δ (Cα, Cβ, Cδ) catalytic subunit (Domin, 1997) and one of the five relevant p85α (R1), p55α (R1), p50α (R1), p85β (R2) or p55γ (R3) regulatory subunits (Okkenhaug and Vanhaesebroeck, 2001). P110α and P110β are widely expressed in different tissues but P110δ is primarily expressed in leukocytes(Zhao, 2008; Domin, 1997).

Although class II enzymes are structurally related to class I, unlike classes I and III, comprise no more relevant regulatory proteins. They are so called PI3K C2 as they contain a C-terminal region with

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homology to C2 domains. PI3K C2 catalyses the production of PI (3) P and PI-3, 4-P2 in vitro (Arcaro et al., 1998; Hawkins et al., 2006). Three human subclasses for class II enzymes have been reported; PI3KC2α, PI3KC2β and PI3KC2γ. While C2α and C2β are expressed in all mammalian tissues(Maria-Magdalena Georgescu, 2011; Arcaro et al., 2000; Domin et al., 1997), C2γ is mainly expressed in prostate, liver and breast(Ho et al., 1997; Rozycka et al., 1998)

Class III PI3Ks enzymes generate PI3P from PI (Volinia et al., 1995). They consist of a catalytic Vps34 (C3) subunit and a regulatory p150 (R4) subunit and seem to be primarily involved in intracellular trafficking process of proteins and vesicles (Maria-Magdalena Georgescu, 2011; Foster et al., 2003; Schu et al., 1993; Vanhaesebroeck et al., 2001).

Prostate cancer is the most common cancer diagnosed in males all over the world. It accounts for the second principal cause of cancer deaths in men (Fritz H. Schröder, 2012; Hughes et al., 2005). Metastatic prostate cancer will spread beyond the prostate gland through the body. While the primary affected organ can effectively be cured by surgery or radiation therapy, therapeutic strategies are limited for metastatic disease (Silvia Letašiová, 2012; Ross et al., 2002). Bladder cancer is the second most common cancer of the genitourinary tract, approximately 30% to 70% of superficial bladder tumors will recur after initial treatment and 10% to 30% will progress to invasive and/or metastatic disease(Lee et al., 2002). Consequently the investigation of the molecular mechanisms involved in the emergence of metastatic genitourinary cancers is of great importance.

PI3K has been shown to be involved in the secretion of matrix metalloproteinases which degrade extra cellular matrix and major components of basement membrane and which are involved in cell invasion and cell migration(Brader and Eccles, 2004). Considering the pivotal role of PI3K signaling in cancer metastasis, broad-range PI3K inhibitors have been developed. Although they have shown early signs of becoming effective anticancer drugs, isoform specific inhibitors would provide further therapeutic advantage by limiting undesirable effects of pan-inhibitor compounds (Wee et al., 2008). In this regard identifying the most relevant isoforms in cancer tissues with activated PI3K pathway will be a prerequisite. Since it is unclear which PI3K isoform(s) drive growth and survival or invasiveness feature in human genitourinary cancers containing mutations in the PTEN tumor suppressor, PI3K negative regulator; identification of the responsible downstream PI3K isoform will be important to overcome the challenge of restoring PTEN loss of function mutations in human cancers.

Materials and Methods

Cell lines and cell culture

The prostate carcinoma cell lines DU145 and PC3 and bladder carcinoma cell line 5637 (American Type Culture Collection, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran) were grown in RPMI 1640 (Gibco, Brussel, Belgium) supplemented with streptomycin (Jabberebn Hayyan, Tehran, Iran), penicillin (Sigma, Brussel, Belgium), and 10% heat-inactivated fetal bovine serum (Sigma, Brussel, Belgium) in a humidified incubator under 5% CO_2 at 37°C.

RNA isolation

Cells in subconfluent cultures were lysed in Tripure RNA-isolating reagent (Roche, Germany). All samples were extracted according to manufacturer instruction. Resulting purified intact RNA was treated with DNase I (Promega, USA) to remove genomic DNA contaminants. The RNA samples were then stored at -80°C. Experiments were performed at least three times using separate sets of cultures.

Primer for PI3K isoforms and housekeeping genes

Using criteria required for designing SYBER green assay, primers were designed using beacon designer software 6.0. To ensure the absence of secondary structure, mfold zuker program was run for the designed primers (Markham, 1995-2013). Additional criteria for a good SYBR green Real-Time assay included a relatively short amplicon (< 200 bp) for housekeeping genes and genes of interest. A blast search was performed to check the specificity of DNA sequences of primers. To amplify18SrRNA, the offered primers by Bio-rad were used (Table 1).

Table 1 : Sequences of primers and amplicon's length

Isoform	Primer	Size of product
PIK3CA (Cα)	Forward: AAAGTGTGTGGATGTGATGAATAC Reverse: GCTGTGGAAATGCGTCTGG	185 bp
PIK3CB (Cβ)	Forward: GACATCTGGGCGGTGGATTC Reverse: AAGGAGGTTGAACATTGGGTAATTG	171 bp
PIK3CD (Cδ)	Forward: TTCAGACACCATCGCCAACATC Reverse: ACAATAGCCAGCACAGGAGAGG	160 bp
PIK3CG (Cγ)	Forward: CCCGAAAGCCAAAGCGAACAG Reverse: GCCACTCTTCCTTCCTCACCTC	187 bp
PIK3C2A (C2α)	Forward: AAGTCAGCAATCTACAGGTATCTCC Reverse: TCAAGAAGAACAGCATCCCAAGG	175 bp
PIK3C2B (C2β)	Forward: CTGCGGGCTGGAGGAGTTC Reverse: GGAGATGGACGAGGTAGTTCAAGG	188 bp
PIK3C2G (C2γ)	Forward: AAGTGGCAGTTCAACAATTAGAC Reverse: CAGTAAAGACGATGGGCAACC	166 bp
PIK3R1	Forward: TGTCTGAACGGCTGAATATGAATAG Reverse: GTGTAATGTGAGGTCCCAATGC	175 bp
PIK3R2	Forward: AAGGGAGGAGGTGAACGAGAAAC Reverse: AACGGAGCAGAAGGTGAGTGG	187 bp
PIK3R3	Forward: ACAGATGCCCTCGCTTTGC Reverse: GCCTCTCCACTTCACATTCAC	160 bp
PIK3R5	Forward: GGAGGAGAGCACCAATGACATC Reverse: CAAAGCGGCAGTAGTAGAGTAGC	197 bp
PIK3C3	Forward: CCTTCAGTCTCTTCACCTCCTC Reverse: TCTTCACATTCCACTATCACATACC	158 bp
GUSB	Forward: CACGACACCCACCACCTACATC Reverse: GACGCACTTCCAACTTGAACAG	121 bp
18S rRNA	Forward: CGGCGACGACCCATTCGAAC Reverse: GAATCGAACCCTGATTCCCCGTC	99 bp
GAPDH	Forward: GCAGGGGGGAGCCAAAAGGGT Reverse: TGGGTGGCAGTGATGGCATGG	219 bp

Quantitative Real-Time RT-PCR

Real-Time PCR was performed in 25 µL of a reaction consisting of 21 µL Superscript III Platinum_R SYBR Green one-step RT supermix (Invitrogen, Belgium), 1 µM concentration of each primer and 2 µL CDNA as template. The PCR thermal profile consists of an initial incubation of 30 min at 50˚C and 10 min at 95◦C followed by 30 cycles of 95˚C for 30 s, 54˚C for 30 s, 72˚C for 30 s, and 80˚C for 10 s (data acquisition point). Amplification, detection, and data analysis were performed with MYiQ real-time detection system (Bio-rad, Milan, Italy). Each sample was run at least in duplicate. Relative quantitation of gene expression has been estimated according to the $2^{-\Delta\Delta Ct}$ method based on the threshold cycle (Ct) values(Schmittgen and Livak, 2008).

Statistical analysis

The Real-Time RT-PCR analysis was performed at least in 3 independent experiments. Each sample was run and examined in duplicate. Differences between groups were analyzed using the t-test. $P < 0.05$ was considered statistically significant.

Table 2: Location of the genes and primers

Gene type	Chromosome	Band	Primer Located Exon
PIK3C2A	11	p15.5—14	1
PIK3C2B	1	q32	6,8
PIK3C2G	12	p12	5,7
PIK3C3	18	q12.3	12,14
PIK3CA	3	q26.3	3
PIK3CB	3	q22.3	1,2
PIK3CD	1	p36.2	20,21
PIK3CG	7	q22.3	2
PIK3R1	5	q13.1	V1: 15 V2: 10
PIK3R2	19	q13.2-q13.4	7,9
PIK3R3	1	p34.1	10
PIK3R5	17	p13.1	12,13

Results

PI3K isoforms Gene Expression

Based on data obtained from Real-Time PCR, while Cα, Cβ, C2α, R1, R2 and R3 isoforms were expressed by PC3, DU145 and 5637 cell lines Cδ, Cγ , C2β, R5, C2γ and C3 isoforms were not detected in aforementioned cell lines(Table 3).

Table 3: Type of P13K isoforms demonstrated in DU145, 5637and PC3 cell lines

PK13 isoform/ cell lines	Cα	Cβ	C2α	R1	R2	R3	Cδ	Cγ	C2β	R5	C2γ	C3
DU145	+	+	+	+	+	+	-	-	-	-	-	-
PC3	+	+	+	+	+	+	-	-	-	-	-	-
5637	+	+	+	+	+	+	-	-	-	-	-	-

Ratio of the expression of PI3K isoforms in DU145 with respect to PC3

The expression level of Cα , Cβ, C2α, R1, R2 and R3 isoforms in DU145 cells were normalized against PC3 cells(null for PTEN gene). It showed that while in DU145 cells, R3 transcripts was decreased to about 80% of its amount in PC3 cells, Cα and R2 transcripts represented an equal expression for the compared cell lines. When the level of Cβ, C2α, R1 transcripts in DU145 cells were assessed, a significant increase in their relative expression was determined (Figure 1).

The expression level of PI3K isoforms in 5637 cells with respect to PC3

Looking to the result of the analysis of P13K isoforms, the data revealed increased quantity of Cα, Cβ, R2, R1 and C2α transcripts in 5637 cells. The highest increased value was detected for the R2 transcripts, while a little decrease in the level of mRNA transcripts was detected for R3 isoform (Figure 2).

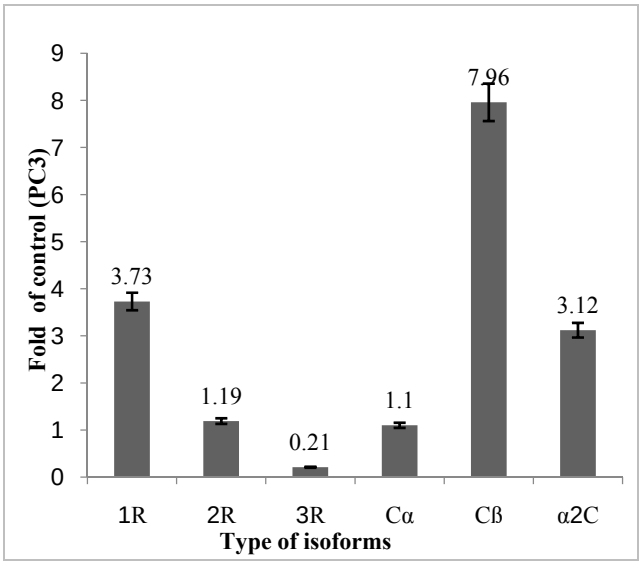

Figure1. Bar graphs showing the relative gene expression of PI3K isoforms in DU145 cell line. Total RNA was isolated and the relative gene expression of genes of interests were analyzed by Real-Time PCR. Relative quantitation of gene expression was estimated according to the $2^{-\Delta\Delta Ct}$method based on the threshold cycle (Ct) values. Each sample was assessed in at least duplicates,

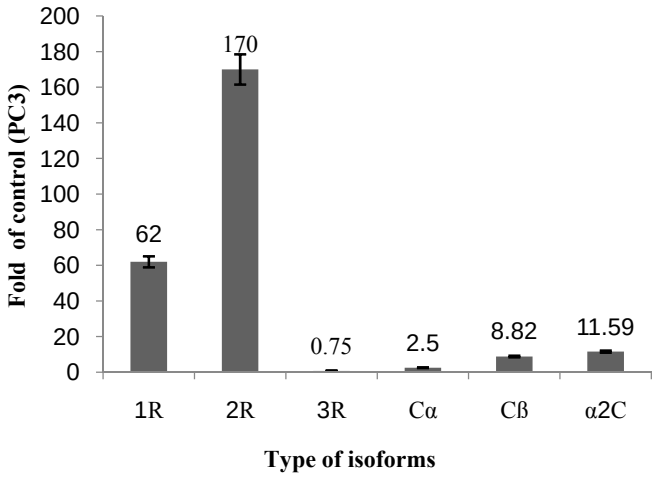

Figure 2. Bar graphs showing the relative gene expression of PI3K isoforms in 5637 cell line. Total RNA was isolated and the relative gene expression of genes of interests were analyzed by Real-Time PCR. Relative quantitation of gene expression was estimated according to the 2-ΔΔCtmethod based on the threshold cycle (Ct) values. Each sample was assessed in at least duplicates.

Relative expression level of PI3K isoforms against Cα transcripts

When we compared 5637 and DU145 cell lines against PC-3, we detected the minimum change in the amount of PI3K-Cα transcript amongst the catalytic isoforms (Figures 1 and 2). So we calculated the ratios of interested transcripts of PI3K isoforms respected to the PI3K-Cα amount in the same cell line to search for any prominent inequality in expression for regulatory subunits respected to catalytic isoforms. Data for DU145 cell line showed a significant increase in PI3K-Cβ and PI3K-C2α catalytic isoforms with respect to all the three detected regulatory subunits (R1, R2, R3) (Figure 3).

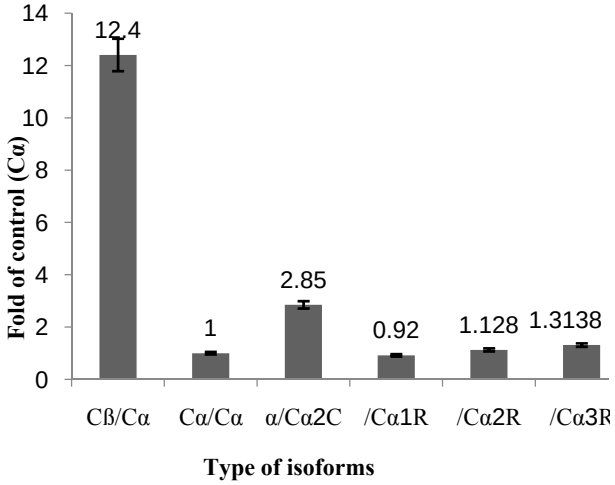

Figure 3. Bar graphs showing the relative gene expression of PI3K isoforms which were normalized against Cα transcript in Du145 cell line. Total RNA was isolated and the relative gene expression were analyzed by Real-Time PCR. Relative quantitation of gene expression was estimated according to the $2^{-\Delta\Delta Ct}$ method based on the threshold cycle (Ct) values. Each sample was assessed in at least duplicates.

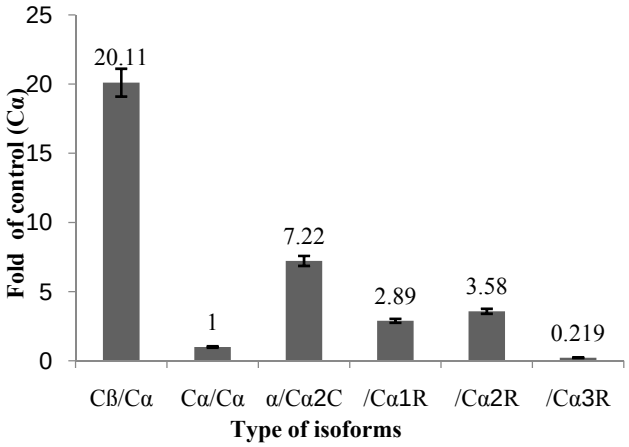

Figure 5. Bar graphs showing the relative gene expression of PI3K isoforms which were normalized against Cα transcript in PC3 cell line. Total RNA was isolated and the relative gene expression were analyzed by Real-Time PCR. Relative quantitation of gene expression was estimated according to the $2^{-\Delta\Delta Ct}$method based on the threshold cycle (Ct) values. Each sample was assessed in at least duplicates.

For 5637 cell line data showed a significant increase in R2 and a reasonable increment in PI3K-C2α, PI3K-Cβ and R1 respected to PI3K-Cα catalytic Isoform. R3 revealed neglectable increment in expression (Figures 4).

In PC3, PI3K-Cβ and PI3K-C2α relative expressions were the same as detected for DU145 and 5637. However the regulatory subunits were increased as for 5637 cell line (Figure 5).

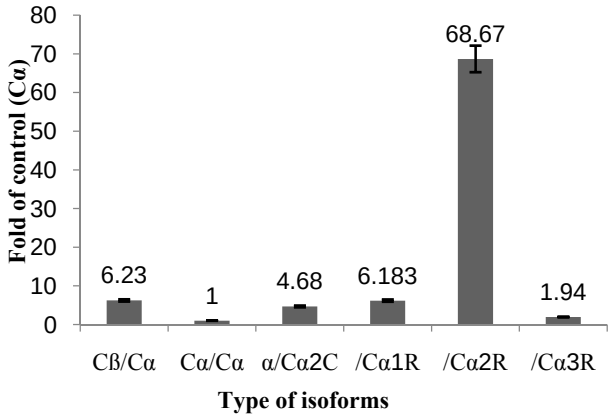

Figure 4. Bar graphs showing the relative gene expression of PI3K isoforms which were normalized against Cα transcript in 5637 cell line. Total RNA was isolated and the relative gene expression of genes of interests were analyzed by Real-Time PCR. Relative quantitation of gene expression was estimated according to the $2^{-\Delta\Delta Ct}$method based on the threshold cycle (Ct) values. Each sample was assessed in at least duplicates

Discussion

Phosphatidylinositol 3-kinase (PI3K) pathway has a central role in cancer cells proliferation, survival and migration (Osaki et al., 2004). Considering the studies indicating a component of PTEN/PI3K/Akt cassette is altered in a large number of tumors it is believed that PI3K makes a great contribution to a high percentage of cancers (Fry, 2001). Consequently PI3K family as drug targets has been under investigation (Stein and Waterfield, 2000). PTEN encodes a phosphatase against both lipid and protein substrates. The mechanism by which PTEN might act as a tumor suppressor gene may involve inhibition of the PI3K-Akt signaling pathway which is essential for cell cycle progression and survival.

PTEN is expressed in prostate normal epithelial cells. Its expression mostly reduced in prostate cancer and the loss of expression occurred in cancers with high grade or stage (Fry, 2001).

The expression of PTEN is also negatively correlated with bladder tumor grades; reduced expression of PETN might play an important role in carcinogenesis and progression of bladder cancer (Zhang, 2005).

DU-145 (prostate cell line) and 5637 (bladder cell line) have functional PTEN tumor suppressor gene, unlike this feature these PTEN bearing cell lines display highly metastatic behavior like PC-3(prostate cell line), which is null for PTEN (Bastola et al., 2002; Wang et al., 2000). In pursuit to explain why despite of bearing functional PTEN,

DU145 and 5637 are highly invasive cell lines we searched for any discrepancy in PI3K isoforms expression rate and expression pattern against DU145, PC-3 and 5637 cell lines.

We investigated the expression profile of the twelve known isoforms from PI3K family including PI3K-Cα, PI3K-Cβ, PI3K-Cγ, PI3K-Cδ, PI3K-C2α, PI3K-C2β, PI3K-C2γ, C3, R1, R2, R3, R5 isoforms. To perform precise quantitative Real-Time PCR analysis, GAPDH, 18srRNA and GUSB were included in experiments as control housekeeping genes (HKGs). Our results showed from the three studied HKGs, 18srRNA and GAPDH were the most reliable genes as reference controls and the GUSB gene revealed unstable expression in studied cell lines.

Whereas PI3K-Cα, PI3K-Cβ, PI3K-C2α, R1, R2 and R3 were the most frequently expressed isoforms in all the three studied cell lines; PI3K-Cγ, PI3K-Cδ, PI3K-C2β, PI3K-C2γ, C3 and R5 isoforms were found to be undetectable.

When PC-3 cell line was used as calibrator evaluation of quantitative gene expression demonstrated that PI3K-Cα and R2 represented an equal amount of transcripts for both DU145 and PC-3 cell lines, the expression ratio for R3 showed 80% decrease in DU145 and the ratio of gene expression for R1, PI3K-Cβ and PI3K-C2α were increased to 3.73, 7.69 and 3.12 fold respectively.

When we compared 5637 cell line against PC-3(as calibrator) the ratio of gene expression were 62.16, 170, 0.72, 2.5, 8.83 and 11.59 fold for R1, R2, R3, PI3K-Cα, PI3K-Cβ and PI3K-C2α respectively.

It is speculated that more consequences of PI3Ks in disease will be well-defined by array based PI3K gene profiling and subsequently promised that pharmaceutical intervention will develop isoform-specific PI3K inhibitors with clinical benefits to human (Vanhaesebroeck and Waterfield, 1999).

It has been shown that in cervical and ovarian cancers gene amplification of PI3K-Cα isoform culminated to its over-expression. On the other hand in ovarian and colon tumors oncogenic activating mutations were detected in R1 gene (Sawyer et al., 2003). However there exists little information about typical distribution and quantitative expression of all the family members of PI3K isoforms in normal and diseased states.

Among members of PI3K family, class I have been studied extensively. Results of gene expression analysis have suggested that both PI3K-Cα and PI3K-Cβ are widely expressed in mammalian tissues. Further experimental evidence documented the equimolar expression of catalytic and regulatory subunits in studied tissues (Geering et al., 2007).

Further compelling evidence revealed that R2 protein reactivity was higher than R1 isoform when compared for WEHI-231 and NIH 3T3 cell lines (Geering et al., 2007). This finding is completely coinciding with our data which has demonstrated high level of R2/R1 expression ratio for DU145, PC3 and 5637 cells. Other recent researches showed an increased specific activity for PI3K-Cα when compared with the other members of class IA. This could explain why its expression ratio is lower than the other catalytic subunits in our studies.

Conclusion

R1, R2, PI3K-Cα, PI3K-Cβ and PI3K-C2β isoforms in 5637 cell line and R1, PI3K-Cβ and PI3K-C2α isoforms in DU145 were increased in these both PTEN bearing cell lines respected to PC3 which is null for PTEN. To shed more light on the functional significance of isoforms over-expression we propose to silence the aforementioned isoforms by RNA interference. It should also be considered that rather than PI3K malignant activation, the extra-activation of other pathways like MAPK and PKC may bypass PTEN normal function for PI3K regulation in 5637 and DU145.

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