Absolute Quantification of Four Isoforms of the Class I Phosphoinositide-3-kinase Catalytic Subunit by Real-Time RT-PCR

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Class I phosphoinositide-3-kinase (PI3K) consists of four isoforms of the catalytic subunit, p110α, -β, -δ and -γ, generated from the genes PIK3CA, -B, -D and -G, respectively. These isoforms show different tissue distribution and some specific and indispensable functions in various biological pathways such as development, inflammation, autoimmunity and malignancy. In human cancers, frequent genomic amplification and gain-of-function mutations of PIK3CA were reported, which suggests an oncogenic potential. However, the role played by the other three isoforms in human cancer remains to be determined. We wanted to investigate the relationship between all the isoforms in human cancers. Here, we have established a system for the simultaneous absolute-quantification of all four isoforms by real-time reverse transcription polymerase chain reaction (RT-PCR). The reliability of this system was confirmed using three main criteria: (i) good correlation of each standard curve, (ii) high specificity of the PCR reactions and (iii) excellent reproducibility. Using this system, we investigated human monocytic specificity of the PCR reactions and (iii) excellent reproducibility. To date, however, there are no reports of mutation in the other isoforms15,41) and little is known about the precise roles played by each of the four isoforms. To better understand class I PI3K signaling in cancer or other diseases, quantitative data is required to evaluate the relative expression levels of the individual isoforms of the catalytic subunit. However, only limited information on the expression patterns of these isoforms is currently available. Here, we have developed a system for the absolute quantification of all four isoforms of class I PI3K catalytic subunits. We believe our technique will help to understand the role played by each isoform in human cancer.

MATERIALS AND METHODS

Cell Lines and Cell Culture A panel of 39 human cancer cell lines (termed JFCR39) was previously described.42,43) All cell lines in JFCR39 and U937 were maintained in RPMI 1640 supplemented with 5% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 mg/ml) at 37 °C in humidified air containing 5% CO2.

Total RNA Preparation Total RNA was extracted with RNeasy Mini kit (Qiagen, Chatsworth, CA, U.S.A.) according to the manufacturer’s recommendations. The purity of total RNA was assessed by the NanoDrop® ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.) and the quality by the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, U.S.A.) (data not shown).

cDNA Preparation One microgram of total RNA was used in the first-strand cDNA synthesis with random hexamers (Applied Biosystems Inc., Foster City, CA, U.S.A.) and Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturers’ instructions.

Plasmid Construction PIK3CA and -B were isolated by a PCR technique using cDNAs reverse-transcribed from total RNA isolated from 24 normal human tissues (Premium Total RNA; Clontech, Mountain View, CA, U.S.A.). PIK3CD was isolated from the cDNA reverse-transcribed from the total RNA of U937. Primer sequences are shown in Table 1. Amplified DNA fragments were cloned into pcDNA3.1/myc-His A (Invitrogen) and all constructs were verified by double-strand sequencing (data not shown). PIK3CG was cloned into pGEM5zf (+) (Stratagene, La Jolla, CA, U.S.A.).

Quantitative Real-Time RT-PCR Analysis Real-time PCR was performed on the ABI PRISM 7000 Sequence Detection System by using TaqMan® Universal PCR Master Mix (Applied Biosystems) and TaqMan® Gene Expression Assays probe and primer mix (Applied Biosystems) according to the manufacturer’s specifications. The Assay Identifi-

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cation numbers for each gene are as follows: PIK3CA, Hs00180679_m1; PIK3CB, Hs00178872_m1; PIK3CD, Hs00192399_m1 and PIK3CG, Hs00176916_m1. Gene-specific probes were labeled by using reporter dye FAM at the 5’ end. A non-fluorescent quencher and the minor groove binder were linked at the 3’ end of probe. The thermal cycler conditions were as follows: hold for 10 min at 95 °C, followed by two-step PCR for 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min. All samples were performed in duplicate and all experiments were performed twice. Amplification data were analyzed with an ABI Prism Sequence Detection Software 2.1 (Applied Biosystems).

RESULTS AND DISCUSSION

Real-Time RT-PCR Absolute Quantification System of PIK3CA, -B, -D and -G

Absolute quantification by real-time PCR requires a cloned cDNA construct of the target gene of known concentration. Full-length cDNA constructs of all four isoform genes were prepared as described in Materials and Methods. To generate a standard curve for quantification of templates in the reaction mixture, cloned cDNA constructs were serially diluted 5 times at a ratio of 1:10. Thus, the dynamic range for each gene was from 10 to 100000. Pearson’s correlation coefficient of standard curve was >0.99 (Fig. 1A). The reproducibility between the two experiments for each gene were confirmed as shown in Fig. 1B. Agarose-gel electrophoresis was used to assess the size of transcript obtained by RT-PCR of total RNA from JFCR39 using isoform-specific primers (Fig. 2). A single band of the expected size was detected for each isoform in most of the cell lines tested. However, the transcript corresponding to PIK3CG could not be detected in several cell lines, suggesting low levels of expression in cancer cells, which were derived from ectodermal origin. Reliability of the established isoform-specific absolute-quantification system using real-time RT-PCR was confirmed by an excellent correlation coefficient of each standard curve (Fig. 1A), reproducibility (Fig. 1B) and specificity of the PCR reactions (Fig. 2). This method can be applied by intercalator assay using isoform-specifically-designed primers and fluorescent DNA intercalator such as SYBR Green I. However, the specificity of the reaction of intercalator assay is lower than TaqMan-probe assay which we selected to develop the system stated above.

Gene Expression Analysis in U937

We used the real-time PCR analysis to investigate PI3K expression in U937. This cell-line was derived from hematopoietic origin. Therefore the cells were thought to have the expression of all four isoforms and that is why we chose this cell-line to analyze

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<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’)*</th>
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<tr>
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<tr>
<td>PIK3CA_reverse 1</td>
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* Sequences written with bold characters contain restriction-enzyme sequence for cloning.

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![Fig. 1. Evaluation of the Absolute Quantitative Real-Time RT-PCR System for Four Isoforms of Class I PI3K Catalytic Subunit](image)

(A) Standard curves of each gene (representative data are shown). Pearson’s correlation coefficient was >0.99 for each gene. Dynamic ranges of each curve were 10 to 100000 in all cases. (B) Reproducibility of each gene. Pearson’s correlation coefficient was 0.99 for PIK3CA, 0.99 for -B, 0.94 for -D and 0.99 for -G, respectively.
first by the system we have developed. Transcripts corresponding to all four isoforms were detected (Fig. 3A). The relative expressions of PIK3CA, PIK3CB, PIK3CD and PIK3CG were 10.4%, 18.2%, 61.1% and 10.3%, respectively. The higher level of expression of PIK3CD relative to the other isoforms is consistent with the fact that PIK3CD is predominantly expressed in leukocytes.22,25) This absolute-quantification system also enabled us to integrate the expression data of each isoform. Our analysis revealed the quantitative expression of the whole class I or class IA (PIK3CA, -B and -D) PI3K catalytic subunit as shown in Fig. 3B. There are numerous reports of isoform-specific inhibitors of PI3K (listed in1,26)). These chemicals are useful tools for understanding the non-redundant function of these isoforms as well as gene-targeting studies in mice. However, classical PI3K inhibitors that contributed to the early studies of PI3K, such as...
wortmannin or LY294002, are not PI3K-specific. Furthermore, the recently developed PI3K-specific small-molecule inhibitor ZSTK474 does not possess isoform-specificity. Thus, the biological role of these isoforms remains unclear.

In the present study, we have established an absolute quantification system for the four isoforms of class I PI3K catalytic subunit. Quantitative expression analysis in vitro and in vivo will also shed light to the isoform-specific roles related to cancer and other human diseases (e.g. autoimmune disease such rheumatoid arthritis).

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REFERENCES

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