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Technical note

Optimal reference genes for qPCR in resting and activated human NK cells—Flow cytometric data correspond to qPCR gene expression analysis

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ABSTRACT

Natural killer cells (NK cells) are cytotoxic lymphocytes critical to the innate immune system engaged in rapid response against tumor or virus infected cells. After activation NK cells acquire enhanced cytotoxicity and are capable of producing cytokines to stimulate other immune cells. Quantitative PCR (qPCR) is a method of choice for gene expression analysis but the usage of reliable reference genes for the normalization process is critical. Commonly used reference genes may vary in expression level between different experimental conditions providing wrong quantitative results of the studied genes' expression levels. Fourteen potential endogenous control genes were analyzed by qPCR method in NK-92 cell line that shows characteristics of human natural killer cells and is often used in studies on biology of NK lymphocytes. NK-92 cells were stimulated with IL-2 or TNF for 2, 24 or 72 h. Results were analyzed with RefFinder, a program which enables evaluation and screening of reference genes and integrates the currently available major computational programs (Genorm, Normfinder, BestKeeper and Delta Ct). The most stable gene in activated and non-activated NK cells was *B2M*, followed by *IPO-8* and *GAPDH* and the least stable were *HPRT1*, *PPIA* and *RPL32*. The normalization process was performed on *SOD2* gene and the results of qPCR experiments were confirmed by flow cytometry. The flow cytometric data corresponded to the results of qPCR gene expression analysis performed for the reference genes qualified by RefFinder as the most stable.

1. Introduction

Natural killer cells (NK cells) are effector lymphocytes of the innate immune system involved in antitumor and antiviral responses. NK-92 cell line shows characteristics of human natural killer cells and is frequently used as a suitable model to study NK lymphocytes. Similarly to NK cells, NK-92 cells are $CD3^{-}56^{+}$. They can bind to cancer cells or virus infected cells and secrete perforin and granzymes. After activation they are cytotoxic for K562 cells and produce cytokines (i.e. IFNγ, TNF, IL-10, GM-CSF) to stimulate other cells of the immune system (Reefman et al., 2010). Cytokines, such as IL-2 and tumor necrosis factor (TNF) are important modulators of NK cells' effector functions. IL-2 is a pleiotropic cytokine required for proliferation and activation of NK cells (Dybkaer et al., 2007). TNF is a proinflammatory cytokine engaged in the regulation of the activity of most immune cells including NK lymphocytes. NK cells express on their surface several TNF family receptors (TNFRs), by which they interact with dendritic cells in the reciprocal activating crosstalk via cell-to-cell contacts and transmembrane TNF (Munich et al., 2012). The activation of lymphocytes changes the cellular expression of many genes. Dybkaer and coworkers demonstrated that IL-2 exerted a broad range of effects on NK cells ranging from the regulation of cell cycle, cell survival, cytotoxicity and secretion of many cytokines in a sequential manner. They found that IL-2-induced activation of NK cells results in the upregulation of many members of the TNF and TNFR superfamily. IL-2 triggers also many other signaling pathways in NK cells: PI3K/AKT, NF-κB and calcineurin/NFAT (Dybkaer et al., 2007). Similarly, binding of TNF to its receptors activates NF-κB, apoptosis, extracellular signal regulated kinase (ERK), p38 mitogen-activated protein kinase (p38MAPK) and c-Jun N-terminal kinase (JNK) (Aggarwal et al., 2012). Manganese superoxide dismutase (SOD2) is a mitochondrial enzyme that belongs to the antioxidant enzyme system of oxygen-respiring organisms. It catalyzes the dismutation of highly reactive superoxide radicals into hydrogen peroxide and molecular oxygen. It is controlled by NF-κB transcription factor activated under conditions of oxidative stress and in response to treatment by toxic stimuli, such as TNF, IL-1, ionizing radiation, etc. (Xu et al., 2008).

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Quantitative PCR is a powerful technique to determine changes in gene expression by measuring cellular level of respective mRNA. The accuracy of results depends, however, on precise data normalization process. The most common method involves the application of reference genes which are supposed to be constitutively expressed in specific cells/tissues and theoretically should display stable expression. However, it appeared that expression of some commonly used reference genes can be altered in different biological samples and experimental conditions (Dheda et al., 2004). A possible approach to overcome this problem is the assessment of the accuracy of candidate reference genes for specific experimental treatments (Stephens et al., 2011).

The study aimed to establish stable reference genes upon activation of NK cells, assess gene expression analysis with the use of the most stable genes identified by RefFinder and compare the qPCR results to flow cytometry data to verify the obtained results.

2. Material and methods

2.1. Cell cultures

Human NK-92 cell line (obtained from ATCC, CRL-2407, USA) was maintained in α-MEM medium (1×10^6/ml) with 12.5% fetal bovine serum, 12.5% horse serum, 2 mM L-glutamine, 0.2 mM myo-inositol, 0.1 mM β-mercaptoethanol, 0.02 mM folic acid, 100 U/ml penicillin, and 100 μg/ml streptomycin (SigmaAldrich, St. Louis, MO, USA). Cells were stimulated with IL-2 or TNF (100 U/ml; BD Biosciences, San Jose, CA, USA) for 2 h, 24 h or 72 h. Non-activated cells were used as control cells in all experiments.

2.2. RNA extraction

Total cellular RNA was isolated using the Total RNA Mini kit (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's instruction. Concentration and quality of RNA samples were determined spectrophotometrically with NanoDrop ND-1000 (Thermo Fisher Scientific, Fitchburg, WI, USA). Samples with a 260/280 ratio of 1.9 or greater were used for preparation of cDNA. The quality of the isolated total RNA was analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using Agilent RNA 6000 Nano Kit according to the manufacturer's instruction. RNA integrity was assessed by RNA Integrity Number (RIN). The RIN software algorithm allows for the classification of eukaryotic total RNA, based on a scoring system from 1 to 10, with 1 denoted degraded and 10 intact RNA.

2.3. Reverse transcription (RT) reaction

Complementary DNA (cDNA) was synthesized from 1 μg of total RNA with the use of 0.5 μg oligo$(dT)_{18}$ primers (Sigma-Aldrich, Munich, Germany), 200 U RevertAid Reverse Transcriptase, 1 mM dNTP mix and 2 U Ribo-Lock (Fermentas-Thermo Fischer Scientific, Fitchburg, WI, USA) in total volume of 20 μl. RT reaction was performed according to the manufacturer's protocol and the resulting cDNA was stored at −20 °C until use in qPCR reaction.

2.4. Real time qPCR

The qPCR reaction contained 1.5 μl of cDNA (5 × diluted), 200 nM of each forward and reverse primer, 6 μl of SensiFAST SYBR No-ROX Kit (Bioline, London, UK) and was performed in total volume of 12 μl in StepOnePlus Real-Time PCR System (Life Technologies-Applied Biosystems, Grand Island, NY, USA). The mRNA expression levels of fourteen putative reference genes were analyzed (Table 1). *SOD2* gene utilized for validation with the use of reference genes' data was amplified in the same conditions. Primers specific for the selected genes were designed using Primer-BLAST software and are listed in Table 1 (Wierzbicki et al., 2014). The calibration curves for all gene-specific qPCR assays were performed. All PCRs displayed efficiency between 91.2% and 104.8% (Table 1). Each PCR was performed in triplicate and contained negative (no-template) control (water instead of cDNA) and 5 × diluted pooled cDNA as a run-to-run precision control.

2.5. Data analysis

Geometric Ct (the threshold cycle) mean values from triplicate reactions were calculated for further analysis and used to determine the relative gene expression stability. Results were analyzed with RefFinder, a web-based software tool (http://www.leonxie.com/referencegene.php?type=reference) that integrates four major computational programs (Genorm, Normfinder, BestKeeper and Delta Ct) and enables evaluation of reference genes. A gene expression normalization factor (M value) was calculated for each sample. The gene with the lowest M value was the most stable.

2.6. Flow cytometry

NK-92 cells (1×10^6 cells) were stimulated with 100 U/ml of IL-2 (BD Biosciences, San Jose, CA, USA) for 2 h, 24 h and 72 h. Simultaneously control, unstimulated cells were cultured. Then cells were collected and stained for surface antigens with anti-CD56-APC antibodies (clone NCAM16.2; BD Biosciences, San Jose, CA, USA). Subsequently cells were fixed and permeabilized with Cytofix/Cytoperm Buffer (BD Biosciences, San Jose, CA, USA) and stained with anti-MnSOD-FITC antibodies (clone MnS-1; eBioscience, San Diego, CA, USA) according to the manufacturer's protocol. Samples were run on a BD FACSCalibur flow cytometer equipped with argon-ion laser (488 nm) and data were analyzed with BD CellQuest Pro software (BD Biosciences, San Jose, CA, USA). Isotype controls for intracellular staining were also prepared.

2.7. Statistics

Normality of qPCR and flow cytometric data distribution was analyzed by Shapiro–Wilk test. Student's t test for normal distribution of qPCR data and Wilcoxon test for flow cytometric samples were used to compare experimental data (Statistica, version 10; Statsoft, Tulsa, OK, USA).

3. Results

3.1. RNA quality control

The average concentration of RNA isolated from NK-92 cells stimulated with IL-2 (100 U/ml) or TNF (100 U/ml) and from unstimulated cells (all samples were prepared in triplicates) was comparable between the analyzed groups and amounted respectively: 278.26 ± 141.93 ng/μl (range 131.9–415.3 ng/μl), 282.68 ± 167.19 ng/μl (range 107.99–441.2 ng/μl) and 226.3 ± 146.19 ng/μl (range 67.1–354.5 ng/μl). RNA samples presented high RNA quality and integrity; mean $A_{260/280}$ ratio was 1.99 ± 0.07 (range 1.84–2.05); average RIN value was 9.22 ± 1.47 (range 5.7–10.0).

3.2. Identification of the optimal reference genes

RefFinder program ranked the tested genes according to the determined gene stability from the most stable (the lowest M value) to the least stable (the highest M value), as shown in Fig. 1. The most stable reference gene for both activated (stimulated with IL-2 100 U/ml or TNF 100 U/ml) and non-activated NK-cells by this ranking was *B2M*, followed by *IPO8* and *GAPDH*. The least stable gene was *HPRT1*, then *PPIA* and *RPL32* (Fig. 1).