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Tissue-specific selection of optimal reference genes for expression analysis of anti-cancer drug-related genes in tumor samples using quantitative real-time RT-PCR

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ABSTRACT

Gene transcription analysis in clinical tumor samples can help with diagnosis, prognosis, and treatment of cancers. We aimed to identify the optimal reference genes for reliable expression analysis in various tumor samples by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Using a one-step TaqMan-based qRT-PCR, 5 commonly used reference genes (ACTB, GAPDH, RPLPO, GUSB, and TFRC) and 10 anticancer drug-related genes (TYMS, RRM1, TUBB3, STMN1, TOP2A, EGFR, VEGFR2, HER2, ERCC1, and BRCA1) were analyzed in 327 tissue samples from lung, rectal, colon, gastric, esophageal, and breast tumors. According to the expression stability assessments obtained by using three programs (geNorm, NormFinder, and BestKeeper) and a comprehensive ranking method, the optimal reference genes for lung, gastric, esophageal, and breast tumors were RPLPO, GAPDH, ACTB, and ACTB, respectively. For rectal tumors, a combination of the 3 most stable genes (GUSB, ACTB, and RPLPO) was suitable for qRT-PCR, whereas for colon tumors, a combination of the 4 most stable genes (GAPDH, ACTB, GUSB, and RPLPO) was optimal for qRT-PCR. Based on the expression data of target genes normalized against selected reference genes, the principal component analysis revealed 4 expression patterns in 6 different tissues. One pattern was observed in gastric, rectal, and colon tumor tissues, which are gastrointestinal tumors. Expressions in the breast, lung, and esophageal tissues were separately represented as one pattern. Our results could facilitate the practice of personalized cancer medicine based on the gene expression profile of the patients.

1. Introduction

Anti-cancer drug therapy is the main method for cancer treatment, but common drug resistance and severe adverse drug reactions are major impediments to the successful treatment of cancer patients. For chemotherapeutic drugs, only less than 30% of patients show responsiveness. Personalized medicine revolution is the trend for cancer treatment. Biomarkers are becoming increasingly capable of distinguishing genotypic or phenotypic traits of individual tumors and are being used to select optimal treatment protocols (Lee et al., 2005; Moen et al., 2012; Watters and McLeod, 2003). Gene transcription level alteration is an important genotypic biomarker category and is related to anti-cancer drug sensitivity or resistance. A number of genes (ERCC1, BRCA1, TUBB3, STMN1, TOP2A, RRM1, and TYMS) have been identified as potential markers for predicting drug efficacy, particularly chemotherapeutic drugs (Table A.1). For example, the expression level of excision repair cross-complementing 1 (ERCC1) gene, which is crucial in the repair of platinum-DNA adducts, was reported to negatively affect the effectiveness of platinum-based therapy and could be used as a major predictor of disease response to platinum-based chemotherapy (Bepler et al., 2006; Ceppi et al., 2006; Lord et al., 2002; Olaussen et al., 2006; Shirota et al., 2001; Ueda et al., 2011). Furthermore, several randomized prospective clinical studies confirmed that customized cisplatin chemotherapy based on quantitative ERCC1 mRNA expression improved the survival of patients with non-small-cell lung cancer (Bepler et al., 2013; Cobo et al., 2007), thereby indicating that ERCC1 mRNA expression assessment is feasible in a clinical setting and can predict the response to cisplatin-based treatment. Therefore, the expression level assessment of drug-related genes in patient tumor tissue before treatment is useful for therapeutic decision making.

Real-time reverse transcription polymerase chain reaction (RT-PCR) has been recognized as the most sensitive and accurate method for mRNA detection and quantitative gene expression analysis. This method allows the simultaneous measurement of gene expression in many different samples for a limited number of genes and is the method of choice for the accurate determination of gene expression when the quality and/or quantity of RNA are low. The critical step for quantitative RT-PCR (qRT-PCR) application is the selection of reference genes for normalization; such genes are thought to be stably expressed across different samples, treatments, and physiological states (Kozera and

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Rapacz, 2013; Radonic et al., 2004). Traditionally, housekeeping genes (HKGs), such as GAPDH and ACTB, are used as reference genes. However, the expression levels of the HKGs or reference genes are regulated and often vary in different tissues or under different experimental conditions (de Kok et al., 2005; Rubie et al., 2005). Furthermore, because of the low yield of mRNA in formalin-fixed paraffin-embedded (FFPE) tissues, the selection of proper reference genes or HKGs has become increasingly stringent to obtain reliable qRT-PCR data; more than one reference gene maybe required (Soes et al., 2013). As of this writing, few studies have investigated the expression stability of reference genes in different FFPE tumor tissues. Studies performing reliable transcriptional analysis based on tissue-specific reference genes are also few.

Therefore, to select the optimal reference genes for expression analysis of anticancer drug-related genes in various tumor samples by qRT-PCR, we conducted an extensive evaluation of 5 of the most commonly used reference genes, ACTB, GAPDH, RPLPO, TFRC, and GUSB, which are the reference genes used in breast 21 gene oncotype DX (Paik et al., 2004), in 6 different types of tumor tissue samples (the lung, colon, rectal, gastric, breast, and esophageal). The expression profiles of 10 drug-related genes (ERCC1, BRCA1, TUBB3, STMN1, TOP2A, RRM1, TYMS, HER2, EGFR, and VEGFR2) in 6 different types of tissue samples were determined based on the normalization of the selected tissue-specific reference genes.

2. Materials and methods

2.1. Tissue samples and cells

A total of 327 tissue specimens from breast (42), gastric (80), esophageal (25), colon (54), rectum (55), and lung carcinomas (71) were obtained from Taizhou Hospital of the Taizhou University School of Medicine (Taizhou, China). All samples were FFPE and were prepared after surgical resection. Two independent expert pathologists histologically reviewed these samples. No patient received neoadjuvant treatment prior to the primary surgery. All patients gave informed consents for sample retention and analysis for research purposes according to institutional guidelines. The research ethics committee of Northwest University, China approved the present study.

Cells from the HeLa cell line were grown in Dulbecco's Modified Eagle's Medium and supplemented with 10% FBS, antibiotics (penicillin 100 U/mL and streptomycin 100 mg/mL) at 37 °C and 5% CO_2, with passages every 3 d.

2.2. RNA extraction

Total RNAs from FFPE tissues and HeLa cell cultures were separately extracted by using AllPrep® DNA/RNA FFPE Kit (Qiagen, Hilden, Germany) and TRIzol reagents (Invitrogen, USA) according to the instructions of the manufacturers. The total RNA was quantified by optical density with NanoDrop 2000 (Thermo Fisher Scientific Inc., Rockford, IL, USA), and gel electrophoresis was used to evaluated the total RNA quality. Intact rRNA sub-units of 28S and 18S were observed on the gel, thereby indicating minimal degradation of the RNA.

2.3. PCR primer and probe design

All primers and probes were designed by Primer 3.0 (http://primer3.ut.ee/) and Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA) based on the reference mRNA sequences of target genes (*TYMS*, *RRM1*, *TUBB3*, *STMN1*, *TOP2A*, *EGFR*, *VEGFR2*, *HER2*, *ERCC1*, and *BRCA1*) and reference genes (*ACTB*, *GAPDH*, *GUSB*, *RPLPO*, and *TFRC*), which were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Table 1 presents the primer and probe sequence data. At least one primer sequence or corresponding probe spanning two exons was chosen to minimize the inaccuracies from genomic DNA contamination. Sequences of all primers, probes, and amplicons were analyzed using NCBI/primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to check the corresponding specificity to the target template.

2.4. One-step TaqMan qRT-PCR

TaqMan probe-based one-step real-time quantitative PCR was performed in the Roche LightCycler 480 real-time PCR System (Roche, Germany) by using One Step PrimeScript™ RT-PCR Kit (TAKARA, Dalian, China). Reactions were conducted in a 20 μL reaction system, each containing 1 μL of Primer and Probe Mix (20 μM), 1 μL total RNA (50 ng/μL), 10 μL 2× One Step RT-PCR Buffer III, 0.4 μL *TaKaRa Ex Taq* HS (5 U/μL), and 0.4 μL PrimeScript RT enzyme Mix II. Thermo cycling conditions were set as the initial reverse transcription step for 5 min at 42 °C and the polymerase activation step for 10 s at 95 °C, followed by 45 cycles of 5 s at 95 °C for template denaturation, and 34 s at 60 °C for annealing and extension and fluorescence detection. All samples were amplified in technical triplicates. Negative controls without templates were included in each run. Both agarose electrophoresis profiles of the qRT-PCR products and dissociation curve analysis were used to check the specificity of the qRT-PCR. The average threshold cycle (Ct) values for targeted and reference genes were obtained from each reaction to determine the occurrence of gene expression in the tested samples.

2.5. Standard curve construction and amplification efficiency optimization

Before the mRNA expression quantification in tumor samples, standard curves using standard RNAs were established to ensure that the amplification efficiency of both target and reference genes was at 100% or was approaching 100%. Total RNAs from HeLa cell lines were prepared as the standard RNA. The standard curve was constructed under the abovementioned reaction conditions using 5 different concentrations of RNA templates, as follows: 5, 10, 25, 50, and 100 ng/μL.

2.6. Data analysis and statistics

For each primer set, standard curves made from serial dilutions of standard RNA were used to estimate PCR reaction efficiency (E) using the formula: $E\ (\%) = (10^{[1/\text{slope}]} - 1) \times 100$. The Ct values used in the analysis represented the mean of three values (three technical triplicates). Three different statistical tools, geNorm v3.5 (http://medgen.ugent.be/genorm) (Vandesompele et al., 2002), NormFinder v0.953 (http://moma.dk/normfinder-software) (Andersen et al., 2004), and BestKeeper v1 (http://www.genequantification.de/bestkeeper.html) (Pfaffl et al., 2004) were used according to the recommendations of the developer to assess expression stability and the ranking of the candidate reference genes. The relative mRNA expression level of each targeted gene was normalized against selected reference genes as calculated by the $2^{-\Delta Ct}$ method, where $\Delta Ct = Ct_{\text{targeted gene}} - Ct_{\text{reference gene.}}$

Statistical analyses were conducted with GraphPad Prism software (Version 5.0, San Diego, CA, USA) and SPSS 16.0 (SPSS Inc., Chicago, IL, USA). To evaluate the differential expression of certain target genes in different tumor types, one-way analysis of variance (ANOVA) with Dunnett's test was used, with the statistical significance set at $p < 0.05$. To investigate the overall trend governing the differences in expression profile of different tumor tissues, the average mRNA expression value of each target gene in each type of tumor was globally analyzed by a multivariate analysis with principal component analysis (PCA) (Palma et al., 2010).

3. Results

3.1. Expression profile of the candidate reference genes

Under the same reaction system, the expressions of the 5 candidate reference genes in the tested samples were detected. In the total samples, the Ct values ranged from 16.45 to 35.15. When referring to a

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