Reliable Transcript Quantification by Real-Time Reverse Transcriptase-Polymerase Chain Reaction in Primary Neuroblastoma Using Normalization to Averaged Expression Levels of the Control Genes *HPRT1* and *SDHA*

Matthias Fischer, Matthias Skowron, and Frank Berthold

From the Department of Pediatric Oncology, University Children's Hospital Cologne, Cologne, Germany

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) represents a sensitive and efficient technique to determine expression levels of target genes in multiple samples and is increasingly used in clinical oncology to evaluate the patient's outcome or to detect minimal residual disease. Normalization of raw data are required to obtain comparable results between different specimens and is usually achieved by correlating transcript abundances of target genes with those of a single control gene with putatively stable expression levels. In this study, expression stability of six supposed control genes was evaluated in 64 samples of primary neuroblastoma and *HPRT1* and *SDHA* mRNA levels were shown to exhibit the least expression variability among the samples. Because application of more than one control gene may enhance reliability of real-time RT-PCR results, various normalization factors consisting of the geometrical mean of multiple control gene expression values were calculated and evaluated by mRNA quantification of 14 target genes. Comparison with transcript levels determined by oligonucleotide-array expression analysis revealed that target gene mRNA quantification became most consistent after normalization to averaged expression levels of *HPRT1* and *SDHA*. This normalization factor was in addition demonstrated to be not associated with stage of disease or *MYCN* amplification status of the tumor. Thus, these data indicate that the geometrical mean of *HPRT1* and *SDHA* transcript levels represents a suitable internal control for biological and clinical studies investigating differential gene expression in primary neuroblastoma by real-time RT-PCR. (*J Mol Diagn* 2005, 7:89–96)

Quantification of target gene expression levels by real-time reverse transcriptase-polymerase chain reaction (RT-PCR)^{1,2} is becoming increasingly significant in neuroblastoma as in other human malignancies to gain insight into the molecular pathology of the tumor, to identify novel prognostic markers, or to evaluate minimal residual disease response.^{3–9} A common feature of most transcript quantification techniques is the requirement for normalization, because a number of variables may influence the results.^{2,10} Ideally, abundances of mRNA levels are determined as transcript copies per cell.^{2,11} However, this approach is not feasible in many studies, in particular if samples of solid tissues are analyzed. As a surrogate, the amount of total RNA is often used to receive comparable results of expression levels between different samples, assuming that the cells under investigation contain approximately equal amounts of total RNA and mRNA. This assumption, however, does not hold true in many cases because it has been shown that even a particular cell type may contain different quantities of total RNA and/or mRNA under various physiological conditions.^{12,13} In addition, determination of the cell count or total RNA amount does not take varying RNA quality or enzymatic efficiencies into account, which may have a significant impact on the results in RT-PCR reactions.^{10,14} Thus, expression levels of target genes are usually normalized to expression levels of internal control genes that are supposed to show stable expression in the tissues of interest. However, it has been demonstrated by a number of studies that frequently used control genes such as *β-actin* and *GAPDH* exhibit considerably varying transcript levels in cells of different histological origin and under various physiological or experimental conditions.^{2,11,13,15–17} The finding that a universal control gene

Supported by the Fördergesellschaft Kinderkrebs-Neuroblastom-Forschung e.V. and Kind-Philipp-Stiftung (to M.S.).

M.F. and M.S. contributed equally to this work.

Accepted for publication July 16, 2004.

Address reprint requests to Dr. Matthias Fischer, University Children's Hospital Cologne, Department of Pediatric Oncology, Joseph-Stelzmann-Str. 9, 50924 Cologne, Germany. E-mail: matthias.fischer@medizin.uni-koeln.de.

Table 1. Control Genes Evaluated in This Study and Oligonucleotide Sequences Used as Primers for Amplification

Symbol	Gene name	Sequence	Accession no.
LMNB1 for	Lamin B1	5'-GCTGCTCCTCAACTATGCTAAGAA-3'	NM_005573
LMNB1 rev		5'-TCTTTTCGAATTCAGTGCTGCTTC-3'	
PBGD for	Porphobilinogen-deaminase	5'-CTACTTTCCAAGCGGAGCCAT-3'	NM_000190
PBGD rev	(Hydroxymethylbilane synthase)	5'-CCACGCGAATCACTCTCATCT-3'	
PPIA for	Peptidylprolyl isomerase A	5'-GCTCGTGCCGTTTGGCA-3'	NM_021130
PPIA rev		5'-GCAAACAGCTCAAAGGAGACG-3'	
PGK1 for	Phosphoglycerate kinase 1	5'-AGGGAAAAGATGCTTCTGGG-3'	NM_000291
PGK1 rev		5'-AAGTGAAGCTCGGAAAGCTTCTAT-3'	
SDHA for	Succinate dehydrogenase complex,	5'-TGGAACAAGAGGGCATCTG-3'	NM_004168
SDHA rev	subunit A, flavoprotein (Fp)	5'-CCACCACTGCATCAAATTCATG-3'	
HPRT1 for	Hypoxanthine	5'-TGACACTGGCAAAACAATGCA-3'	NM_000194
HPRT1 rev	phosphoribosyltransferase 1	5'-GGTCCTTTTCACCAGCAAGCT-3'	

Forward and reverse primers are indicated by "for" and "rev", respectively, following the gene symbol.

does apparently not exist led to the conclusion that putative reference genes have to be evaluated carefully for every individual cell type separately.^{2,10,11,16,18}

The present study evaluates the stability of expression levels of six putative control genes in primary tissue samples of the pediatric tumor neuroblastoma and establishes a hierarchy of the control genes according to their degree of regulation among the samples. It furthermore shows that normalization to more than one control gene considerably reduces the influence of varying expression levels of single control genes on target gene mRNA quantification. Finally, it reveals that a normalization factor consisting of the geometrical mean of *HPRT1* and *SDHA* expression levels is not associated with stage or *MYCN* amplification status of primary neuroblastoma.

Materials and Methods

Sample Preparation and cDNA Synthesis

Neuroblastoma cell lines IMR-5, IMR-32, and CHP-134 were grown to subconfluency in RPMI 1640 medium (Biocrom, Berlin, Germany) supplemented with 10% fetal calf serum and 0.1% Plasmocin (InvivoGen, San Diego, CA). Cells were harvested, washed once with phosphate-buffered saline, and subsequently used for RNA isolation. Approximately 40 mg of snap-frozen tissue samples of 64 primary neuroblastomas were cryosliced into 20- μ m-thick tissue sections and transferred into 2-ml reaction tubes. After samples were homogenized by the FastPrep FP120 cell disrupter (Qbiogene Inc., Carlsbad, CA), total RNA was prepared using the TRIzol reagent following the instructions of the supplier (Invitrogen, Karlsruhe, Germany). RNA concentrations were quantified by spectrophotometric OD₂₆₀ measurement and integrity of RNA was checked by agarose gel electrophoresis. First strand cDNA was synthesized in a total volume of 21 μ l using 2 μ g of total RNA each, 500 ng of oligo-(dT₁₂₋₁₈)-primers (Invitrogen) and SuperScript II reverse transcriptase according to the manufacturer's protocol (Invitrogen).

Real-Time RT-PCR

Real-time RT-PCR was performed using the SYBR Green I reagent on the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). PCR reactions were performed in a total volume of 30 μ l containing 26.8 μ l of 1 $\times$ SYBR Green PCR master mix (Applied Biosystems), 0.4 μ l undiluted first strand cDNA, and 1.4 μ l of 2.5 μ mol/L forward and reverse primer (Eurogentec, Seraing, Belgium) each. To enable calculation of relative expression levels, serial dilutions (undiluted, 1:3, 1:9, and 1:27) of cDNA of the cell lines IMR-32 or CHP-134 (for assessment of interassay variance and comparison of real-time RT-PCR results to oligonucleotide array data) were used for the generation of standard curves of each gene separately. Oligonucleotides hybridizing specifically to corresponding sequences of control genes *PBGD*, *PPIA*, *PGK1*, *HPRT1*, *SDHA*, and *LMNB1* as well as target genes *PCBP4*, *SNAP91*, *BASP1*, *DBH*, *IGFBP7*, *HSPA5*, *STMN4*, *TUBA3*, *IFI27*, *PRAME*, *ROBO1*, *CLSTN3*, *CADPS*, and *EVL* served as primers in PCR reactions (Tables 1 and 2). Oligonucleotides were selected in successive exons (intron-spanning) for each gene except for *TUBA3* to avoid amplification of contaminating genomic DNA. PCR reactions were performed in duplicates using 96-well optical reaction plates with optical caps (Applied Biosystems). Cycling conditions consisted of a single incubation step at 50°C for 2 minutes and a subsequent heating to 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. To evaluate amplification of genomic DNA or nonspecific products, aliquots of each reaction mixture were analyzed by agarose gel electrophoresis.

Oligonucleotide Array Analysis

Gene expression profiles of seven primary neuroblastoma samples (three tumors of stage 4 and four tumors of stage 4S) were generated using Agilent Human 1A and 1B oligonucleotide microarrays (Agilent Technologies, Palo Alto, CA). For each tumor sample, 2 μ g of total RNA

Table 2. Target Genes Evaluated in This Study and Oligonucleotide Sequences Used as Primers for Amplification

Symbol	Gene name	Sequence	Accession no.
TUBA3 for	Tubulin, alpha 3	5'-CATTGAAAAGTTGTGGTCTGATCA-3'	NM_006009
TUBA3 rev		5'-GCTTGGGTCTGTAAACAAAGCAT-3'	
STMN4 for	Stathmin-like 4	5'-GGCCCAGAAGATGGAATCCAA-3'	NM_030795
STMN4 rev		5'-GCACCTCCTCGGCGTGCTT-3'	
IGFBP7 for	Insulin-like growth factor binding	5'-CCAGGTCAGCAAGGGCACCT-3'	NM_001553
IGFBP7 rev	protein 7	5'-CAGTGACATTCCAGATGTCCTT-3'	
HSPA5 for	Heat shock 70-kd protein 5	5'-CTACAGCTTCTGATAATCAACCA-3'	NM_005347
HSPA5 rev		5'-CCACGAGGAGCAGGAGGAAT-3'	
DBH for	Dopamine beta-hydroxylase	5'-CAGAAGTACTTCCACCTCATCA-3'	NM_000787
DBH rev		5'-GTCGCGGTTGAAGGAGTTCCA-3'	
BASP1 for	Brain abundant, membrane attached	5'-GAAAGCCAAGGAGAAAGACAAGA-3'	NM_006317
BASP1 rev	signal protein 1	5'-CGGCCTTGCCCTCGGCGT-3'	
EVL for	Enah/Vasp-like	5'-CCTGCTGGGAGCGTGAATGA-3'	NM_016337
EVL rev		5'-CCTCCTTCACCTTGTGGAGC-3'	
PCBP4 for	Poly(rC) binding protein 4	5'-CCTGAACGCATCACCACCAT-3'	NM_020418
PCBP4 rev		5'-CTGACTGGCAGGATGACAA-3'	
IFI27 for	Interferon, alpha-inducible protein 27	5'-GAGTTGCCTCGGGCAGCCT-3'	NM_005532
IFI27 rev		5'-GCCCAGGATGAACCTTGGTCAA-3'	
SNAP91 for	Synaptosomal-associated protein,	5'-GCGGATCTTAACATCAAGGATT-3'	BC060818
SNAP91 rev	91-kd homolog (mouse)	5'-GTACTGGTGGCTCCCTTTGA-3'	
PRAME for	Preferentially expressed antigen in	5'-TGCAGGCTCTCTATGTGGACT-3'	NM_006115
PRAME rev	melanoma	5'-ATGCACATGCCCTTCCATTCCGA-3'	
ROBO1 for	Roundabout, axon guidance	5'-GCGTGCAGTACTAAGGGAACA-3'	NM_002941
ROBO1 rev	receptor, homolog 1 (Drosophila)	5'-GGCTTCTTACATGAACATAATGAA-3'	
CADPS for	Ca2+-dependent activator protein for	5'-CAGCTTCCAAATATGTGGATGTA-3'	NM_003716
CADPS rev	secretion	5'-CATCTCCTCATTGACCTTATCA-3'	
CLSTN3 for	Calsyntenin 3	5'-GTTATCGGCTGCGACACGGA-3'	NM_014718
CLSTN3 rev		5'-GGTTCATGCTGTGCAGGACATT-3'	

Forward and reverse primers are indicated by "for" and "rev," respectively, following the gene symbol.

were linear amplified and aliquots were labeled with either Cy5 or Cy3 dyes (Perkin Elmer, Rodgau, Germany) using Agilent's Low RNA Input Fluor Linear Amp kit according to the manufacturer's protocol. One μg of Cy-labeled cRNA samples of each tumor specimen was hybridized along with the same amount of reverse-color Cy-labeled product from a pool consisting of equal amounts of cRNA from all tumor samples analyzed. After dye correction, target gene expression levels were normalized to the global mean array intensity of the respective sample. Data analysis was performed using the Luminator Gene Expression Data Analysis System, version 2.0 (Rosetta Inpharmatics, Seattle, WA).

Data Analysis

After averaging duplicate C_t measurements, relative expression levels were calculated according to the standard curve method as described by the user bulletin 2 of the ABI Prism 7700 sequence detection system (Applied Biosystems). Calculation of the control gene stability measure M was performed according to Vandesompele and colleagues.¹¹ In brief, real-time RT-PCR expression levels a_{ij} of n internal control genes are determined in m tissue samples. An array A_{jk} of m elements is calculated for every combination of two internal control genes j and k , consisting of the $\log_2$ -transformed expression ratios a_{ij}/a_{ik} (Equation 1). The pairwise variation V_{jk} for the control genes j and k represents the SD of A_{jk} elements (Equation 2). The gene stability measure M_j for control

gene j is the arithmetic mean of all pairwise variations V_{jk} (Equation 3).

($\forall j, k \in [1, n]$ and $j \neq k$):

$$A_{jk} = \left\{ \log_2 \left(\frac{a_{ij}}{a_{ik}} \right) \right\}_{i=1 \rightarrow m} \quad (1)$$

$$V_{jk} = \text{st.dev.}(A_{jk}) \quad (2)$$

$$M_j = \frac{\sum_{k=1}^n V_{jk}}{n-1} \quad (3)$$

Normalization factors NF_2 to NF_6 were determined for each sample by calculating the geometrical mean of expression levels of the two best performing control genes (NF_2) and stepwise inclusion of additional control genes in the order of their expression stability (NF_3 to NF_6).¹¹ To analyze independence of the proposed normalization factor of stage and *MYCN* status of the tumor, distributions of normalization factor values within each subgroup were compared to one another and statistically evaluated using the Kruskal-Wallis and Mann-Whitney U -tests. Comparison of target gene expression levels determined by real-time RT-PCR and oligonucleotide array was enabled by calibrating transcript abundances measured by either method to the minimal expression value of the respective gene within the set of tumors analyzed.

Table 3. Determination of the Interassay Variance of Real-Time RT-PCR Experiments

	PBGD	LMNB1	PPIA	PGK1
Max/min IMR-5	1.7	1.8	2.2	2.1
Coefficient of variation (%)	15.9	17.6	22.2	17.8
Max/min patient 587	2.0	1.3	2.4	2.0
Coefficient of variation (%)	22.5	8.9	25.0	20.2

Control gene expression levels of two samples (neuroblastoma cell line IMR-5 and patient 587) were analyzed in 10 independent PCR runs. Indicated are the fold-differences between maximum and minimum values and the coefficient of variation of each control gene.

Results

Relative expression levels of the putative control genes porphobilinogen-deaminase (*PBGD*),¹⁹ cyclophilin A (*PPIA*),^{20,21} phosphoglycerate kinase (*PGK1*),²⁰ lamin B1 (*LMNB1*),²⁰ hypoxanthine phosphoribosyltransferase 1 (*HPRT1*),¹¹ and succinate dehydrogenase complex subunit A (*SDHA*)¹¹ were determined in 64 primary neuroblastoma samples by real-time RT-PCR using SYBR Green 1 dye detection. Aliquots of the reactions were analyzed by agarose gel electrophoresis to evaluate PCR specificity. Detection of a single specific amplification product of the expected length and absence of visible primer dimers demonstrated a high PCR specificity in all cases. Amplification efficiencies of real-time RT-PCR reactions were compared by plotting the ΔC_t values of different primer combinations of serial dilutions against the LOG of starting template concentrations. Resulting slopes ranged from -0.84 to 1.18 for different reactions (data not shown), which indicates significantly varying amplification efficiencies.¹⁸ Thus, amplification results of the various genes investigated in this project are not directly comparable to one another. Relative expression levels were therefore determined by adjusting C_t values of the samples to standard curves derived from serial cDNA dilutions of neuroblastoma cell lines IMR-32 or CHP-134, which were generated for each gene in every run separately.

To determine the interassay variation of the real-time polymerase-chain reaction, expression levels of four selected genes (*PBGD*, *PPIA*, *PGK1*, and *LMNB1*) were analyzed in two samples (patient 587 and neuroblastoma cell line IMR-5) in 10 independent PCR runs. After adjustment of the C_t values of each gene to their respective standard curve, the resulting relative expression levels varied from 1.3-fold to 2.4-fold between minimum and maximum values with coefficients of variation ranging from 9 to 25% (Table 3), which is in the range of results that have been reported previously.^{16,18,22} These data, however, indicate that expression values of distinct samples differing less than twofold cannot reliably be assigned to distinct transcript abundances, because they might result from variations of the experimental procedure.

Expression stability of the six control genes was assessed in primary neuroblastoma using a calculation termed as "internal control gene-stability measure M " that was recently published by Vandesompele and colleagues.¹¹ In this calculation, the pairwise variation of

Figure 1. Average expression stability values M of remaining control genes during stepwise exclusion of the least stable reference genes. Calculations were performed according to Vandesompele and colleagues¹¹ (see text).

every control gene with all other control genes is determined as the SD of the logarithmically transformed expression ratios. The internal control gene stability measure M is defined as the average pairwise variation of a particular gene with all other genes (see Material and Methods). Low M values correspond to stable expression levels and vice versa. Stepwise exclusion of the gene with the highest M value thus results in a ranking of the genes in order of their expression stability, which ends up with the two housekeeping genes showing the most stable expression among the samples. Calculation of M values for the control genes examined in this project identified *HPRT1* and *SDHA* as the most stably expressed control genes, followed by *PPIA*, *PBGD*, and *PGK1*, whereas *LMNB1* turned out to be the strongest regulated gene (Figure 1 and Table 4).

Table 4. Order of Expression Stability of Control Genes in Primary Neuroblastoma

Gene ranking	Distance (bp)*
<i>LMNB1</i>	2077
<i>PGK1</i>	1807
<i>PBGD</i>	1315
<i>PPIA</i>	1503
<i>HPRT1/SDHA</i>	1331/2054

Control genes are ranked in order of increasing variability from bottom to the top. The two most stable control genes *HPRT1* and *SDHA* cannot be ranked because of the requirement of gene ratios for gene stability measure M calculation.

*Nucleotide distances of the PCR amplicons to the 3' end of the respective transcript.

Table 5. Correlation of Expression Data Determined by Real-Time RT-PCR after Normalization to Various Normalization Factors and Oligonucleotide-Array Analysis

Gene	NF ₆	NF ₅	NF ₄	NF ₃	NF ₂	SDHA	HPRT1
<i>BASP1</i>	0.81	0.73	0.72	0.67	0.80	0.89	0.64
<i>DBH</i>	0.82	0.85	0.84	0.87	0.90	0.67	0.80
<i>SNAP91</i>	0.81	0.84	0.82	0.83	0.87	0.68	0.94
<i>PCBP4</i>	0.79	0.78	0.77	0.77	0.85	0.78	0.77
<i>EVL</i>	0.00	-0.02	0.05	0.00	-0.14	-0.12	-0.20
<i>HSPA5</i>	0.83	0.89	0.89	0.95	0.94	0.88	0.60
<i>IGFBP7</i>	0.92	0.94	0.92	0.94	0.97	0.99	0.83
<i>STMN4</i>	0.57	0.60	0.55	0.43	0.69	0.75	0.34
<i>TUBA3</i>	0.15	0.28	0.21	0.27	0.41	0.32	0.49
<i>IFI27</i>	0.93	0.93	0.92	0.94	0.94	0.89	0.93
<i>PRAME</i>	0.99	0.99	0.99	0.97	0.98	0.98	0.95
<i>ROBO1</i>	0.53	0.60	0.60	0.63	0.65	0.69	0.55
<i>CLSTN3</i>	0.02	-0.15	-0.13	-0.24	-0.01	0.26	-0.38
<i>CADPS</i>	0.93	0.96	0.95	0.94	0.90	0.89	0.80

Correlation of target gene transcript levels measured by the two methods are indicated by Pearson's correlation coefficient *r* for each normalization factor.

Although RNA quality of each sample had been carefully assessed, it cannot be ruled out that small amounts of RNA degradation might have contributed to sample-to-sample variability of measured expression values. Because oligo(dT) primers were used for mRNA conversion into cDNA, RNA degradation is expected to result in increased variations of RT-PCR results corresponding to the distance of the PCR amplicon to the poly(A) tail of the transcript. Thus, the proximity of PCR amplicons to the poly(A) tail of control gene mRNAs were compared to the *M* values determined for the respective gene. Table 4 shows that distances of PCR amplicons to the poly(A) tail of the transcripts did not correlate with measured expression stabilities, which suggests that inconsistencies of RNA integrity did not contribute considerably to sample-to-sample variations of control gene mRNA levels.

The use of averaged expression levels of multiple control genes that are not co-regulated has been suggested to enhance reliability of normalization of real-time RT-PCR data,¹¹ because individual outlying values of single control genes might be compensated by expression levels of additional genes. To test whether normalization to averaged expression levels of multiple control genes results in more accurate determination of target gene transcript abundances, the geometrical mean of the two best-performing control genes *SDHA* and *HPRT1* was calculated for each sample and denoted as normalization factor 2 (NF₂). Likewise, normalization factors NF₃ to NF₆ were calculated by stepwise inclusion of additional control genes in the order of their expression stability. Expression levels of 14 target genes (*PCBP4*, *DBH*, *SNAP91*, *HSPA5*, *BASP1*, *IGFBP7*, *STMN4*, *TUBA3*, *IFI27*, *PRAME*, *ROBO1*, *CLSTN3*, *CADPS*, and *EVL*) were measured by real-time RT-PCR in seven primary neuroblastoma samples and normalized to *SDHA*, *HPRT1*, and NF₂ to NF₆ separately, and were then compared to their transcript abundances determined by oligonucleotide array analysis of the same samples. Correlation of relative expression levels determined by the two methods was assessed by calculating Pearson's correlation coefficient *r* for each gene. In three cases (*EVL*, *TUBA3*, and *CLSTN3*), no or very poor correlation (*r* < 0.5) of real-time RT-PCR and

array data were observed irrespective of the normalization strategy applied (Table 5). The remaining 11 genes correlated best after normalization to NF₂ (Table 5), with nine genes showing a high correlation (*r* ≥ 0.8) of relative expression levels. Inclusion of additional genes did not further improve but rather resulted in a slightly impaired overall correlation suggesting that normalization of target gene mRNA levels to the geometrical mean of *HPRT1* and *SDHA* transcript abundances yields the most reliable results.

Because identification of differentially expressed genes in the various subtypes of neuroblastoma is a major goal of current neuroblastoma research, a normalization factor serving as an internal control should be not associated with particular biological phenotypes of this malignancy. The panel of tumors analyzed in this study comprised 26 samples derived from localized disease (stage 1 to 3), 19 samples of stage 4S disease that usually shows spontaneous regression and 19 samples of stage 4 neuroblastoma. Nine samples derived from tumors of various stages contained an amplification of the oncogene *MYCN*, which is associated with an unfavorable prognosis. To ascertain independence of the normalization factor from stage or *MYCN* amplification status of the tumor, geometrical means of *HPRT1* and *SDHA* expression levels were calculated for each sample and distributions of the resulting values were compared between the groups. No correlation was observed for either stage (*P* = 0.985) or *MYCN* amplification (*P* = 0.664), indicating that the proposed normalization factor is not associated with the major biological phenotypes of this malignancy (Figure 2).

Discussion

Expression levels of target genes measured by real-time RT-PCR are usually normalized by comparison with transcript abundances of reference genes. However, evaluation of expression stability of well established and frequently used control genes such as *β-actin* and *GAPDH* revealed that expression levels of control genes may vary

Figure 2. Distribution of normalization factor NF_2 values among the samples with regard to stage (A) and *MYCN* amplification status (B) of the tumor. The number of tumor samples of each category is indicated by (n). NF_2 values are depicted in arbitrary units.

considerably in the samples of interest,^{2,11,13,15–17} which may lead to a serious distortion of experimental results. It has been shown by gene expression profiling using serial analysis of gene expression,²³ for example, that induction of expression of the oncogene *MYCN* in a neuroblastoma cell line results in up-regulation of the putative control gene *GAPDH*,¹² which has been used in many studies for normalization.^{4–6,8,9} If *GAPDH* is used as an internal control for experiments investigating differential

gene expression between *MYCN*-amplified tumors that usually show enhanced *MYCN* expression²⁴ and nonamplified neuroblastomas, which is a major topic of current neuroblastoma research,^{12,25} genes that are up-regulated in *MYCN*-amplified tumors might be missed and genes that are stably expressed might be classified as down-regulated by mistake because of the co-regulation of *MYCN* and *GAPDH* expression levels. This example illustrates the necessity for validation of control genes for every single cell type. Accordingly, evaluation of expression stability of control genes for each individual experiment has been claimed in a number of previous publications.^{2,10,11,16,18} In the present study, the six putative control genes *PPIA*, *PGK1*, *LMNB1*, *HPRT1*, *SDHA*, and *PBGD* were evaluated regarding their expression stability in various subtypes of primary neuroblastoma. Calculation of the internal control gene stability measure M^{11} revealed that *SDHA* and *HPRT1* exhibit the most constant expression levels among the samples, followed by *PPIA*, *PBGD*, *PGK1*, and *LMNB1*.

It has been suggested previously, that normalization to the geometrical mean of more than one control gene compensates for outlying values of single reference genes in individual samples and may therefore more accurately reflect transcript abundances of target genes.¹¹ In the present study, this hypothesis was tested by comparison of expression values of 14 target genes determined by real-time RT-PCR and by oligonucleotide array expression profiling in seven primary tumor samples. Normalization to the geometrical mean of mRNA levels of the two best-performing control genes *SDHA* and *HPRT1* improved correlation of results as compared to normalization to expression values of each of these genes alone. Inclusion of additional genes into the normalization factor did not further enhance correlation of transcript abundances, indicating that normalization to averaged expression levels of *SDHA* and *HPRT1* reveals reliable results. Expression levels of three target genes did not correlate irrespective of the normalization factor used, which might in part be because of distinct splice variants measured by either method. This suggestion is supported by the observation that expression levels of *EVL* and *CLSTN3* determined by real-time RT-PCR correlated well with transcript abundances obtained by serial analysis of gene expression ($r = 0.77$ and 0.82 , respectively, data not shown).

The pediatric tumor neuroblastoma shows remarkable heterogeneity in its biological and clinical behavior.²⁶ Some tumors regress spontaneously, in particular those of children that have been classified as stage 4S disease. In contrast, many other patients show an adverse outcome despite intensive therapy. Tumors of these children usually present as stage 4 disease and frequently harbor a genomic amplification of the oncogene *MYCN*. The underlying molecular mechanisms of these completely different biological subtypes are still to be characterized. A major goal of current neuroblastoma research is the identification of genes that are responsible for either spontaneous regression or fatal progression of the disease,^{12,25,27,28} and quantitative PCR is increasingly used

to evaluate correlation of marker gene expression levels with the biological phenotype of the tumor or outcome of disease.^{3-7,9} Comparison of expression levels of candidate genes by real-time RT-PCR experiments therefore requires a normalization factor that is supposed to show no considerable variation between the different subtypes. In the present study, no significant difference was observed for distributions of normalization factor values with regard to stage and *MYCN* amplification status (Figure 2), which suggests that the normalization factor is not associated with the major biological and clinical subtypes of neuroblastoma and that it therefore represents a suitable control for real-time RT-PCR experiments in this malignancy.

In a recent study by Vandesompele and colleagues,¹¹ expression stability of 10 putative control genes including *PBGD* (denoted as *HMBS* in that study), *SDHA*, and *HPRT1* was investigated in 34 neuroblastoma cell lines using real-time RT-PCR. Although overall expression stability of reference genes appeared to be slightly lower in the cell lines in comparison to primary tumors analyzed in the present work as indicated by higher *M* values, both *SDHA* and *HPRT1* were shown to exhibit rather constant expression levels and were ranked among the three most stable control genes. Thus, normalization of target gene expression levels to a normalization factor consisting of *HPRT1* and *SDHA* might be suitable for both primary neuroblastoma and neuroblastoma cell lines.

In conclusion, this study provides additional evidence to previous suggestions that control genes in real-time RT-PCR experiments analyzing transcript abundances have to be evaluated for each individual cell type. Furthermore, it is demonstrated that normalization to the geometrical mean of more than one control gene may reduce the impact of varying control gene expression levels by compensating single outlying values. Finally, we propose that mRNA levels of target genes in primary neuroblastoma determined by real-time RT-PCR are appropriately normalized using the geometrical mean of expression levels of the control genes *HPRT1* and *SDHA*, which may result in an enhanced reliability of studies investigating correlation of marker gene expression levels with clinical and biological characteristics of the tumor.

Acknowledgments

We thank Yvonne Kahlert for excellent technical assistance; Dr. André Oberthür for providing expression data determined by oligonucleotide array analysis; Dr. Barbara Hero, Tom Christiansen, and Dr. Christoph Fischer for lessons in statistical analysis; Hichem Gallala for helpful discussions; Dr. Rüdiger Spitz and Dr. Ralf Küppers for critical reading of the manuscript; and the Competence Network Pediatric Oncology and Hematology (KPOH, supported by the Bundesministerium für Bildung und Forschung) for providing tumor samples.

References

1. Heid CA, Stevens J, Livak KJ, Williams PM: Real time quantitative PCR. *Genome Res* 1996, 6:986-994
2. Bustin SA: Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000, 25:169-193
3. Krams M, Parwaresch R, Sipos B, Heidorn K, Harms D, Rudolph P: Expression of the c-kit receptor characterizes a subset of neuroblastomas with favorable prognosis. *Oncogene* 2004, 23:588-595
4. Tajiri T, Liu X, Thompson PM, Tanaka S, Suita S, Zhao H, Maris JM, Prendergast GC, Hogarty MD: Expression of a MYCN-interacting isoform of the tumor suppressor BIN1 is reduced in neuroblastomas with unfavorable biological features. *Clin Cancer Res* 2003, 9:3345-3355
5. Poremba C, Hero B, Heine B, Scheel C, Schaefer KL, Christiansen H, Berthold F, Kneif S, Stein H, Juergens H, Boecker W, Dockhorn-Dworniczak B: Telomerase is a strong indicator for assessing the proneness to progression in neuroblastomas. *Med Pediatr Oncol* 2000, 35:651-655
6. Cheung IY, Lo Piccolo MS, Kushner BH, Kramer K, Cheung NK: Quantitation of GD2 synthase mRNA by real-time reverse transcriptase polymerase chain reaction: clinical utility in evaluating adjuvant therapy in neuroblastoma. *J Clin Oncol* 2003, 21:1087-1093
7. Cohn SL, London WB, Huang D, Katzenstein HM, Salwen HR, Reinhardt T, Madafoglio J, Marshall GM, Norris MD, Haber M: MYCN expression is not prognostic of adverse outcome in advanced-stage neuroblastoma with nonamplified MYCN. *J Clin Oncol* 2000, 18:3604-3613
8. Lambooy LH, Gidding CE, van den Heuvel LP, Hulsbergen-van de Kaa CA, Ligtenberg M, Bokkerink JP, De Abreu RA: Real-time analysis of tyrosine hydroxylase gene expression: a sensitive and semi-quantitative marker for minimal residual disease detection of neuroblastoma. *Clin Cancer Res* 2003, 9:812-819
9. Dotsch J, Harmjan A, Christiansen H, Hanze J, Lampert F, Rascher W: Gene expression of neuronal nitric oxide synthase and adrenomedullin in human neuroblastoma using real-time PCR. *Int J Cancer* 2000, 88:172-175
10. Lion T: Current recommendations for positive controls in RT-PCR assays. *Leukemia* 2001, 15:1033-1037
11. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002, 3:0034.1-0034.12
12. Boon K, Caron HN, van Asperen R, Valentijn L, Hermus MC, van Sluis P, Roobeek I, Weis I, Voute PA, Schwab M, Versteeg R: N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. *EMBO J* 2001, 20:1383-1393
13. Schmittgen TD, Zakrajsek BA: Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *J Biochem Biophys Methods* 2000, 46:69-81
14. Bishop GA, Rokahr KL, Lowes M, McGuinness PH, Napoli J, DeCruz DJ, Wong WY, McCaughan GW: Quantitative reverse transcriptase-PCR amplification of cytokine mRNA in liver biopsy specimens using a non-competitive method. *Immunol Cell Biol* 1997, 75:142-147
15. Suzuki T, Higgins PJ, Crawford DR: Control selection for RNA quantitation. *Biotechniques* 2000, 29:332-337
16. Hamalainen HK, Tubman JC, Vikman S, Kyrola T, Ylikoski E, Warrington JA, Lahesmaa R: Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. *Anal Biochem* 2001, 299:63-70
17. Tricarico C, Pinzani P, Bianchi S, Paglierani M, Distanti V, Pazzagli M, Bustin SA, Orlando C: Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. *Anal Biochem* 2002, 309:293-300
18. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* 2001, 25:402-408
19. Westerman BA, Neijenhuis S, Poutsma A, Steenbergen RD, Breuer RH, Egging M, van Wijk IJ, Oudejans CB: Quantitative reverse transcription-polymerase chain reaction measurement of HASH1 (ASCL1), a marker for small cell lung carcinomas with neuroendocrine features. *Clin Cancer Res* 2002, 8:1082-1086

20. Korz C, Pscherer A, Benner A, Mertens D, Schaffner C, Leupolt E, Dohner H, Stilgenbauer S, Lichter P: Evidence for distinct pathomechanisms in B-cell chronic lymphocytic leukemia and mantle cell lymphoma by quantitative expression analysis of cell cycle and apoptosis-associated genes. *Blood* 2002, 99:4554–4561
21. Feroze-Merzoug F, Berquin IM, Dey J, Chen YQ: Peptidylprolyl isomerase A (PPIA) as a preferred internal control over GAPDH and beta-actin in quantitative RNA analyses. *Biotechniques* 2002, 32:776–782
22. Schmittgen TD, Zakrajsek BA, Mills AG, Gorn V, Singer MJ, Reed MW: Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal Biochem* 2000, 285:194–204
23. Velculescu VE, Zhang L, Vogelstein B, Kinzler KW: Serial analysis of gene expression. *Science* 1995, 270:484–487
24. Nisen PD, Waber PG, Rich MA, Pierce S, Garvin Jr JR, Gilbert F, Lanzkowsky P: N-myc oncogene RNA expression in neuroblastoma. *J Natl Cancer Inst* 1988, 80:1633–1637
25. Alaminos M, Mora J, Cheung NK, Smith A, Qin J, Chen L, Gerald WL: Genome-wide analysis of gene expression associated with MYCN in human neuroblastoma. *Cancer Res* 2003, 63:4538–4546
26. Schwab M, Westermann F, Hero B, Berthold F: Neuroblastoma: biology and molecular and chromosomal pathology. *Lancet Oncol* 2003, 4:472–480
27. Berwanger B, Hartmann O, Bergmann E, Bernard S, Nielsen D, Krause M, Kartal A, Flynn D, Wiedemeyer R, Schwab M, Schafer H, Christiansen H, Eilers M: Loss of a FYN-regulated differentiation and growth arrest pathway in advanced stage neuroblastoma. *Cancer Cell* 2002, 2:377–386
28. Ohira M, Morohashi A, Inuzuka H, Shishikura T, Kawamoto T, Kageyama H, Nakamura Y, Isogai E, Takayasu H, Sakiyama S, Suzuki Y, Sugano S, Goto T, Sato S, Nakagawara A: Expression profiling and characterization of 4200 genes cloned from primary neuroblastomas: identification of 305 genes differentially expressed between favorable and unfavorable subsets. *Oncogene* 2003, 22:5525–5536