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The performance of semi-quantitative differential PCR is similar to that of real-time PCR for the detection of the *MYCN* gene in neuroblastomas

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Abstract

Amplification of the *MYCN* gene in neuroblastomas is a potent biological marker of highly aggressive tumors, which are invariably fatal unless sound clinical management is applied. To determine the usefulness of semi-quantitative differential PCR (SQ-PCR) for accurate quantification of *MYCN* gene copy number, we evaluated the analytical performance of this method by comparing the results obtained with it for 101 tumor samples of neuroblastoma to that obtained by absolute and relative real-time PCR. Similar results were obtained for 100 (99%) samples, no significant difference was detected between the median $\log_{10}$ *MYCN* copy number (1.53 by SQ-PCR versus 1.55 by absolute real-time PCR), and the results of the two assays correlated closely (r = 0.8, Pearson correlation; $P < 0.001$). In the comparison of SQ-PCR and relative real-time PCR, SQ-PCR versus relative real-time PCR concordant results were found in 100 (99%) samples, no significant difference was found in median $\log_{10}$ *MYCN* copy number (1.53 by SQ-PCR versus 1.27 by relative real-time PCR), and the results of the two assays correlated closely (r = 0.8, Pearson correlation; $P < 0.001$). These findings indicate that the performance of SQ-PCR was comparable to that of real-time PCR for the amplification and quantification of *MYCN* copy number. Thus, SQ-PCR can be reliably used as an alternative assay in laboratories without facilities for real-time PCR.

Key words: Neuroblastoma; Semi-quantitative PCR; Real-time PCR; *MYCN*; Amplification

Introduction

Neuroblastoma is the most frequent extracranial solid cancer in infants and children younger than 5 years. It is considered to be the 4th most frequent type of cancer in children, accounting for 8-10% of all childhood cancers and being responsible for 15% of all pediatric cancer fatalities (1). The disorder is heterogeneous and prognosis and treatment can differ among children with various clinical and biological factors (2). In general, prognosis and treatment outcomes are usually favorable in infants younger than 1 year with a localized tumor (stages I, II and IVS) at diagnosis. By contrast, poor outcomes are increased among patients over the age of 1 year with advanced disease (stages III and IV), even when treated with high dose therapy followed by autologous bone marrow transplant (3).

One of the biological markers commonly used to predict neuroblastoma outcome is the *MYCN* oncogene. Amplification of this gene has a strong link with advanced stages of the disease, rapid tumor dissemination, and a poor prognosis even in the presence of favorable prognostic factors such as early stage or IVS disease (4). Earlier studies have detected amplification of the *MYCN* gene in approximately 20-25% of *de novo* neuroblastoma cases (5,6). Therefore, the determination of *MYCN* copy number together with the above mentioned factors is essential in identifying patients who need intensive treatment.

Over the past decade, several molecular biology laboratory methods have been developed and improved to assess the diagnosis of neuroblastomas. Most of these are based

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on PCR hybridization and amplification. For example, the Southern blot technique has been widely used as a standard molecular genetic method for the measurement of variations in *MYCN* copy number. However, this procedure requires a relatively large amount of genomic DNA, the use of radioactive material to label probes and it is quite costly in terms of resources, labor, and time. Early efforts to measure target sequence concentrations by standard PCR were problematic, mainly because of the inability of the method to quantify the initial concentration of the target sequence and variations in the efficiency of the reaction. These limitations were overcome by co-amplification of an internal standard in the same tube as the target molecule and by the determination of an appropriate number of cycles before PCR reaches the plateau.

An important new development over the last few years has been the introduction of real-time PCR. This method monitors the progress of the PCR at every cycle as it occurs, using fluorescence. Today, real-time PCR is the method of choice for studying changes in copy number and expression of the *MYCN* gene and DNA changes to guide treatment. Although this technique undoubtedly has a number of advantages, it is not frequently available in resource-limited settings. Where it is available, the cost of the instrumentation and reagents as well the complexity of the procedure, which requires skilled laboratory professionals, severely limit routine execution of the test. On this basis, the objective of the present study was to evaluate the performance of a previously published semi-quantitative PCR (SQ-PCR) by comparing the results of this assay to those of real-time PCR using a cohort of Brazilian samples diagnosed as neuroblastomas.

Material and Methods

We evaluated a total of 101 neuroblastoma tumor samples from surgical resections or biopsies obtained from May 2000 through April 2003, collected from various clinical sources in different locations in Brazil. Demographic and clinical data were available for 74 patients (38 boys and 36 girls). Of these 74 patients, 27% were infants below the age of 12 months at diagnosis and 73% were children over 1 year of age. The median age at diagnosis was 22 months (range: 1-114 months). Based on clinical behavior, 9 (12.3%) had stage I disease, 2 (2.7%) had stage II disease, 17 (24%) had stage III disease, and 45 (61%) had stage IV disease at the time of resection.

Tissues were frozen in liquid nitrogen and subsequently pulverized with a microdismembrator (Braun-Melsungen, Germany). Then, 3-4 mL lysing buffer (10 mM Tris-HCl, pH 8.0; 25 mM EDTA, pH 8.0; 100 mM NaCl, 0.5% SDS) containing proteinase K (0.1 mg proteinase K/mL digestion buffer) was added. After incubation for 12-16 h at 50°C, the samples were subjected to phenol-chloroform extraction and ethanol precipitation. DNA content was measured with the Quant-iT DNA Broad-range assay kit Q33130 (Molecular Probes, USA). Extracted DNA was stored at -20°C.

The resulting DNA suspension was used as template DNA in PCR amplification for *MYCN*, *TP53* and β-globin using reported primers and methods (7). Briefly, PCR was carried out in a 50-µL reaction volume containing 100 ng DNA, 5 µL 10X 200 mM Tris, pH 8.3 (Invitrogen, Brazil), 1.5 mM $MgCl_2$, 0.25 µM of each primer, 0.250 mM of each of the deoxynucleoside triphosphates, and 2 units of *Taq* polymerase (Invitrogen). Twenty-five PCR cycles were carried out as follows: initial denaturation at 94°C for 60 s, primer annealing at 60°C for 30 s, and extension at 72°C for 60 s. PCR amplification was optimized by manipulating the number of cycles and omitting the final extension step. The amplified products were analyzed by electrophoresis on 2.0% w/v agarose gel containing ethidium bromide and visualized under UV illumination. The relative intensities of the amplified PCR products were determined using the Kodak 1-D image analysis software (version 2.0.1) and expressed in arbitrary units (AU).

Real-time PCR using SYBR detection green chemistry with melting curve analysis was performed using the Rotor-Gene 2000 system (Corbett Research, Australia) with the Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen). Each 15-µL reaction contained 15 ng of the relevant genomic DNA template, 0.75 mM of each gene-specific primer as described above, 1X Platinum Quantitative PCR Supermix-UDG, 0.200 mM concentrations of each of the deoxynucleoside triphosphates, 3 mM $MgCl_2$, 1 U uracil DNA glycosylase, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, DNA polymerase antibodies, stabilizers, and 1.5 U *Taq* polymerase. The reaction consisted of 45 cycles in 3 steps: 95°C for 20 s, 55° or 60°C for 20 s, and 72°C for 30 s. Fluorescence detection was performed in each cycle at 72°C to identify the positive samples. The detection at this temperature was used to avoid the amplification signal of the primer dimer. Moreover, melting curves were measured immediately after the run by stepwise temperature increases (1°C/5 s) from 72° to 95°C. For absolute quantification, two plasmids containing a 178-bp insert of *MYCN* or a 120-bp insert of β-globin were constructed according to the instructions of the Blunt-ended PCR cloning kit (Amersham Pharmacia Biotech, UK). After replication, the recombinant plasmids were purified and carefully quantified by spectrophotometry. The copy number was calculated from the concentration, mean molecular weight of the nucleotides and plasmid length. The calibrators were then used to prepare 10-fold serial dilutions from 1 x 10^7 copies of plasmid DNA down to one starting molecule.

Since real-time PCR currently is the test most widely used, the performance of SQ-PCR was compared to that of absolute and relative real-time PCR. For absolute quantification, a standard curve of 10-fold serial dilutions of *MYCN* plasmid molecules was obtained for quantification of *MYCN* copy number in the samples. A second standard curve with the β-globin plasmid was generated to normalize sample-to-sample variation of *MYCN* values. The degree

of amplification was defined as the ratio of the number of *MYCN* molecules to the number of β-globin molecules. We also evaluated the relative quantification power of the test by calculating the *MYCN* molecules based on the observed C_T values. This was done by transforming the difference in C_T values between the tumor sample and the calibrator to a copy number ratio (8).

To obtain semi-quantitative data on *MYCN* gene, a single copy of the partial housekeeping gene β-globin and *TP53* from exon 7 were used as reference genes and co-amplified in a single tube reaction in the presence of the same DNA (Figure 1). The relative intensity of the band of the *MYCN* sequence was quantified in relation to the bands of reference genes as reported (7). The median intensity content of *MYCN* in the tumor samples was 0.39 AU without amplification (range: 0.005-0.72) and 0.86 AU with amplification (range: 0.73-0.99). Based on these values, PCR product bands showing increased intensity (>0.72 AU) were scored as amplified *MYCN* while products with intensity significantly lower (<0.36) than the references were scored as unamplified *MYCN*. After definition of the relative density, the copy number of the *MYCN* gene was calculated using the following equation: *MYCN* copy number = 1.76 x (relative intensity of the *MYCN* band) - 1.54 (7). Duplicate blind assessments of *MYCN* copy number in the 101 tumor samples were performed by two independent observers.

Data were analyzed using the SPSS software, version 16.0 (SPSS Inc., USA). Categorical data were compared by the two-tailed Fisher exact test and the two-tailed Mann-Whitney U-test was used to compare median results from different assays. Regression analysis was used to determine the correlation between the results of different assays on the samples. Statistical significance was fixed at $P < 0.05$ for all analyses.

Results

For the SQ-PCR and absolute real-time PCR, similar results were obtained for 100 (99%) samples; *MYCN* DNA was amplified in 22 samples and not amplified in 79 samples by absolute and relative real-time PCR. *MYCN* DNA was detectable by SQ-PCR alone in 21 samples (median *MYCN* copy number: 35.5; range: 1.00 to 68.0 copy numbers) and by the absolute real-time test alone in 22 samples (median *MYCN* copy number: 34; range: 4.49 to 685.0 copy numbers). No significant differences were obtained in median $\log_{10}$ *MYCN* DNA levels between samples detected by SQ-PCR and by absolute real-time PCR, with values of 1.55 (range: 0.53 to 1.83) and 1.53 (range: 0.65 to 2.82), respectively ($P = 0.325$), and the results of the two assays correlated closely ($r = 0.8$; $P < 0.001$; Figure 2C). For SQ-PCR and relative real-time PCR, similar results were obtained for 100 (99%) samples; *MYCN* DNA was amplified in 21 samples and undetectable in 79 samples by both methods. *MYCN* DNA was detectable by SQ-PCR alone in 21 samples (median *MYCN* copy

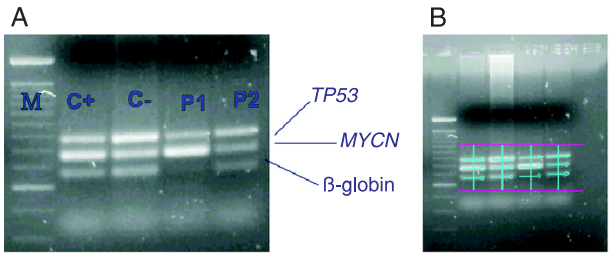

Figure 1. Gel electrophoresis showing the amplification products of the *MYCN* oncogene by differential PCR. *A*, *Lanes:* M = molecular size markers; C+ = positive control of NB19 cell lines; C- = negative control from TPH-1 cell lines; P1 = neuroblastoma patient with amplified *MYCN* gene; P2 = neuroblastoma patient with unamplified *MYCN* gene. *B*, Densitometric scanning of the PCR gel presented in Figure 1A showing *MYCN* band intensities relative to *TP53* and β-globin.

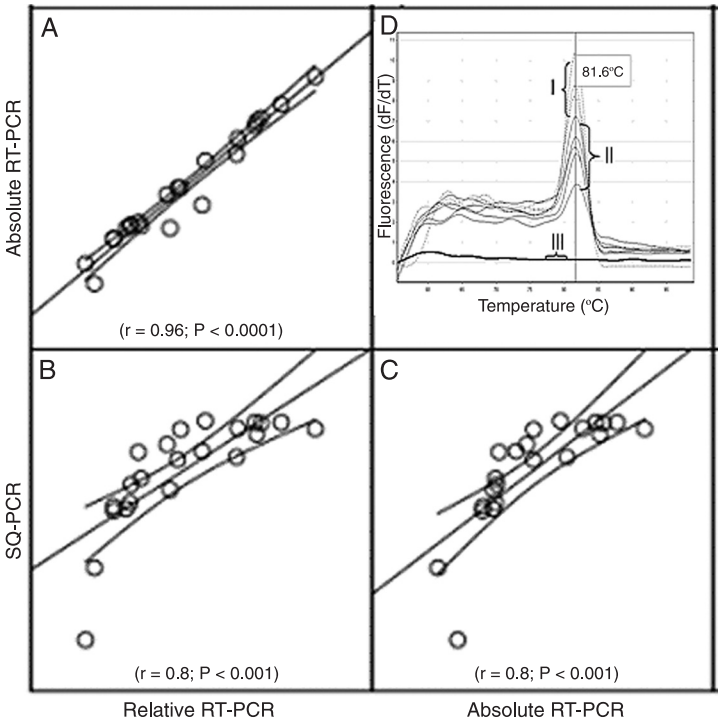

Figure 2. Detection of *MYCN* and melting curve analysis after amplification of the *MYCN* gene. *A*, Correlation of absolute and relative real-time quantitative PCR (RT-PCR). *B*, Correlation of relative RT-PCR and semi-quantitative differential PCR (SQ-PCR). *C*, Correlation of absolute RT-PCR and SQ-PCR. The results are plotted on a logarithmic scale and compared by linear regression analysis. *D*, Plasmid containing a 178-bp insert of *MYCN,* neuroblastoma patients with unamplified *MYCN* gene, and no template control indicated by the Roman numerals I, II, and III, respectively.

number: 35.5; range: 1.0 to 68.0 copy numbers) and by the relative real-time test alone in 22 samples (median *MYCN* copy number: 18.5; range: 4.00 to 282.0 copy numbers). No significant difference in median $\log_{10}$ *MYCN* DNA levels was observed between samples analyzed by SQ-PCR and by relative real-time PCR, with values of 1.55 (range: 0.53 to 1.83) and 1.27 (range: 0.53 to 2.45), respectively ($P = 0.453$), and the results of the two assays were correlated closely ($r = 0.8$; $P < 0.001$; Figure 2B).

The amplification efficiency of the standard curve in real-time PCR was 100%, with a slope value of -3.32, and displayed a good linearity of the response ($R^2 = 0.99$) (data not shown). The specificity of the PCR products was confirmed by melting curve analysis. As shown in Figure 2D, a distinct melting peak of 81.60 was obtained with real-time PCR. No melting products were detected in the no template reaction mixture (negative control) (i.e., primer dimer). The *MYCN* DNA copy numbers obtained by both methods were virtually identical, as shown in Figure 2A ($r = 0.96$; $P < 0.0001$).

Discussion

Given the poor prognosis of patients with neuroblastomas, accurate amplification of *MYCN* can be useful in *p*otentially guiding treatment decisions. The correlation of *MYCN* amplification with the progression of neuroblastomas raises concern about whether the independent amplification and expression of this gene actually contribute more significantly to the predictive power of a poor prognosis. Previous studies have demonstrated that the analysis of *MYCN* gene determined by quantitative PCR was able to effectively evaluate the degree of malignancy of neuroblastomas and to reconfirm the importance of *MYCN* amplification as a powerful predictor of a poor prognosis (9-11). In the present study, we compared the *MYCN* copy numbers obtained by two quantitative PCR methods (real-time PCR and SQ-PCR). A differential PCR for amplification of the *MYCN* gene was initially developed by Gilbert et al. (12) in 1993 and proved to be highly accurate and to have several advantages over previously described PCR-based approaches, including Southern blot. It has been shown for this method that the co-amplification of *TP53* and β-globin as internal controls together with the *MYCN* target gene in the same tube would overcome errors of under- or over-estimation of gene amplification (7). Moreover, during the evaluation process, we managed to visually discern the concentration of *MYCN*, facilitating its semi-quantification without the need for densitometry. Consistent with the results of a previous study (8), our results from the absolute and relative real-time quantification of *MYCN* from tumor samples were identical. Comparison of real-time quantitative PCR with SQ-PCR demonstrated that both assays perform equally well in determining *MYCN* molecule number and that the SQ-PCR method defined *MYCN* molecule number with an accuracy approaching that of real-time quantitative PCR. An earlier study conducted by Raggi et al. (13) measured *MYCN* molecules with *Taq* Man real-time PCR in 49 neuroblastomas, in which the presence of oncogene amplification had previously been defined by competitive PCR. The results showed a correlation ($r = 0.987$) between the two PCR assays (13). This correlation indicates that SQ-PCR can be successfully applied to the determination of *MYCN* level in neuroblastoma patients and can be used reliably as an alternative to the traditional Southern blotting method and to more expensive real-time quantitative PCR procedures. Although real-time PCR is rapid, it requires expensive amplification equipment and highly trained personnel that are only limited to high-resource laboratories. However, the alternative SQ-PCR can be easily used in any moderate resource laboratories that possess basic molecular biology equipment and that cannot afford a real-time machine.

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