BRIEF METHODS

Detection of MYCN Amplification in Neuroblastoma using Competitive PCR Quantitation

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Amplification of the MYCN oncogene in the pediatric tumor neuroblastoma is almost always associated with rapid tumor progression and poor outcome irrespective of tumor stage (Brodeur et al, 1992). The use of MYCN amplification as a prognostic indicator is of particular relevance in view of the clinical heterogeneity that is a characteristic feature of neuroblastoma: tumor phenotype may vary from a benign tumor requiring little or no treatment to a very aggressive malignancy that results in high mortality. Thus assessment of MYCN amplification is now an essential component of the routine diagnostic evaluation of new neuroblastoma patients (Favrot et al, 1996), and it is therefore important to be able to assess MYCN amplification status quickly and reliably. The techniques most frequently used are fluorescence in situ hybridization (FISH) and Southern blotting. The latter method is not ideal for routine diagnostic use because it is time-consuming and expensive, a relatively large amount of tumor material is needed and normal stromal cell infiltration of the tumor may mask amplification. The use of FISH allows a result to be obtained much more quickly (2–3 days), it can be performed on tumor imprints and allows analysis at the single cell level. However, this technique is costly and very sensitive to the quality of the tumor imprint preparations. Polymerase chain reaction (PCR) provides an attractive alternative to Southern blotting and FISH for the detection of MYCN amplification. It is extremely rapid, requires minimal amounts of material, and is straightforward to carry out. There is a requirement however for the development of a well validated, reliable method of quantitation of the PCR product.

Previously reported methods have relied on direct comparison of the level of MYCN amplification with that of a co-amplified single copy control gene sequence (Norris et al, 1994). Drawbacks of this method include variation in amplification efficiency of MYCN and control genes and the need to analyze the PCR reaction before the plateau phase is reached. More reliable quantitation may be achieved by using a single primer pair to amplify both the target sequence and a competitor DNA fragment that is of slightly different size or that has an internal sequence modification (eg, a single nucleotide change resulting in gain/loss of a restriction enzyme sequence) (Cross, 1995). This allows equal efficiency of amplification of test and competitor sequences, thus maintaining a standard ratio of test/competitor product at all stages of the PCR reaction, including the plateau phase. Consequently we have used this approach to develop a competitive, quantitative PCR assay for the determination of MYCN amplification status. We show that this assay distinguishes very clearly between neuroblastoma cell lines containing single copy MYCN (SH-EP1) and approximately 120-fold amplified MYCN (BE(2)-C). In addition the sensitivity of the assay is sufficient to detect as little as a 5-fold increase in MYCN copy number, a factor that is important in the detection of amplification in tumor tissue, which is infiltrated with stromal cells. We also show that results from 3 neuroblastoma tumor samples and a bone marrow sample obtained using quantitative PCR are concordant with those obtained using FISH.

Construction of a MYCN competitor fragment for quantitative PCR was carried out by first amplifying a 340 bp fragment from exon 3 of the MYCN oncogene (bases 7101–7440 Genbank Acc. No. Y00664), using primers MYCN3F: 5' CAAAGGCTAAGAGCTTGAGC and MYCN3R: 5' GTGCAAAGTGGCAGTGAGTG (30 PCR cycles: 5 seconds at 94° C, 20 seconds at 60° C, and 30 seconds at 72° C). The MYCN PCR product

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was digested with *Hae*III yielding fragments of 156, 27, 27, and 130 bp. The two largest restriction fragments were ligated together after gel purification thereby creating a 286 bp Δ MYCN fragment. This fragment was gel-purified and cloned into the *Sma*I site of pUC18. Recombinant plasmid p Δ MYCN was introduced into DH5 α cells, and after replication plasmid DNA was isolated for use as a competitor template in PCR reactions. A full length MYCN fragment of 340 bp was cloned in the same way (pMYCN). DNA concentrations were determined using Hoechst 33258-based fluorometry.

Verification of equal amplification efficiency for full length target and p Δ MYCN competitor DNA was obtained by co-amplifying a standard amount of pMYCN with increasing amounts of p Δ MYCN and separating the PCR products on agarose gel. Visual inspection of gel images showed that equal band intensities were obtained when equimolar amounts of input DNA were used.

Confirmation of the linearity of amplification was demonstrated by titration of normal human genomic DNA against increasing amounts of p Δ MYCN. PCR fragments were separated on agarose gels. A curve was obtained by plotting the log of the number of competitor molecules added to each reaction versus the log of the ratio of the resultant band intensities (full length MYCN/competitor p Δ MYCN) as determined by densitometric analysis of gel images (UVP Enhanced Analysis System, Ultra-Violet Products, LTD., Cambridge, United Kingdom). The data obtained (Fig. 1) showed a linear relationship between the ratio of the

Figure 1.

A linear relationship between the ratio of the resultant band intensities (full length MYCN/competitor p Δ MYCN) and the number of competitor molecules added per reaction was established in control single copy MYCN samples. The initial ratio of test DNA to competitor DNA is maintained during PCR and hence provides a reliable estimate of MYCN amplification status in the test DNA sample.

A

B

C

D

Figure 2.

Agarose gel electrophoresis of competitive PCR products shows an upper band of 340 bp amplified from tumor or cell-line DNA and a lower band of 286 bp amplified from the p Δ MYCN plasmid competitor. A constant amount of tumor/cell line DNA template is included in each PCR reaction, so that the level of amplification from this template is determined by competition from increasing, known amounts of plasmid competitor. The point at which amplification from both templates is equal determines approximate MYCN copy number in the test sample. **A**, Neuroblastoma cell-lines, SH-EP1 and SK-N-AS with single copy MYCN and BE(2)-C and Kelly with amplified MYCN. Lanes 1–5 on each gel contained 0.25, 1.4, 4.4, 79, and 250 pg of plasmid template in the PCR reaction, equivalent to approximately 1, 2, 5, 100, and 300 copies of MYCN/haploid genome. **B**, A dilution series of BE(2)-C DNA demonstrating gel patterns characteristic of low, moderate, and high levels of MYCN amplification. **C**, MYCN single copy and amplified tumor identified by competitive PCR. **D**, FISH analysis of the same tumors showing single copy and amplified MYCN. The results obtained for the amplified sample show the variation in amplification levels between cells.

resultant band intensities and the number of competitor molecules added per reaction, confirming that the initial ratio of target to competitor is maintained during the PCR reaction and thus providing a reliable estimate of MYCN amplification status in the test DNA sample. Small amounts of heteroduplex product, generated during the PCR reaction, did not affect the results. The mean equivalence point (ie, the number of p Δ MYCN molecules equivalent to the number of copies of MYCN in 200 ng of normal genomic DNA), determined using DNA from 3 different individuals, was $(4.98 \pm 0.682) \times 10^5$. This figure allows calcula-

tion of the amounts of competitor DNA required to allow a direct visual estimation of MYCN copy number following agarose gel electrophoresis (without densitometric analysis) in nonamplified and amplified tumor tissue and cell lines.

A standard protocol for the detection of MYCN amplification, using the information generated in the experiments described above was produced. An important feature of this protocol was to minimize the amount of template DNA and the number of PCR reactions required for MYCN quantitation in each sample because tumor tissue is often limited. Thus a total of 5 reactions (each with 100 ng test DNA and 0.25, 1.4, 4.4, 79, and 250 pg competitor DNA was used to cover the range of amplification levels from single copy to approximately 300-fold amplification. It was noted during initial investigations that DNA in dilute solution (<1 ng/ μ l) is extremely sensitive to degradation, and thus Δ MYCN dilutions were freshly prepared for each experiment. In addition, a parallel experiment using genomic DNA with single copy MYCN was carried out in all cases to allow for variation between experiments.

Investigation of MYCN amplification level in a number of neuroblastoma cell-lines confirmed that this protocol was capable of differentiating clearly between the non-MYCN amplified cell-lines SH-EP1 and SK-N-AS, and the MYCN amplified cell-lines BE(2)-C and Kelly (Fig. 2a). The level of amplification in the latter 2 cell-lines was approximately 120-fold and 240-fold, respectively. The sensitivity of the assay was further demonstrated using a dilution series of BE(2)-C DNA. In this case dilutions equivalent to 1, 5, 30, and 240-fold amplification of MYCN were clearly distinguishable on direct visual inspection of the PCR products on agarose gels (Fig. 2b). This is an important finding because a significant level of normal stromal cell infiltration in the tumor may mask high level amplification (>100 -fold) in tumor cells and produce an apparent low to moderate level of amplification in the tissue as a whole.

The final step in the validation of the MYCN competitive PCR assay was to demonstrate amplification in clinical material. Investigation of three solid neuroblastoma tumors indicated that one tumor was nonamplified and two tumors showed a moderate increase in MYCN copy number (Fig. 2c). Analysis of the same tumors using FISH confirmed the absence of

amplification in the first tumor and showed significant amplification in the others, although the level of amplification was very variable from cell to cell (Fig. 2d). In another patient MYCN amplification was demonstrated by FISH in approximately 40% of cells in the tumor tissue. However, PCR results showed amplification in a metastatic bone-marrow aspirate, but not in the solid tumor tissue. Hematoxylin and eosin (HE) staining of a section of this tumor revealed a significant level of necrosis, suggesting that the quality and/or quantity of the DNA was insufficient to allow detection of the expected level of amplification.

In conclusion, we describe a very quick and straightforward competitive PCR assay for the determination of MYCN amplification in tumor tissue, which could be easily carried out in a diagnostic laboratory using only basic equipment. We suggest that, when used in parallel with routine histological examination to assess the proportion of viable tumor cells in a tissue sample, the assay will allow the accurate identification of clinically significant levels of amplification in the majority of neuroblastoma tumors. The assay could be used as a primary screen for the rapid identification of MYCN-amplified tumors, with a requirement for additional FISH analysis in apparently nonamplified tumors to verify single copy MYCN and in tumors containing very high levels of stromal infiltration.

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