

Circulating *MYCN* DNA as a Tumor-specific Marker in Neuroblastoma Patients¹

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Abstract

MYCN oncogene amplification is an established indicator of the aggressiveness of neuroblastomas; it is used internationally for stratifying patients for therapy. The present study shows that high levels of *MYCN* DNA sequences are present in the peripheral blood of patients with *MYCN*-amplified neuroblastomas. Circulating *MYCN* DNA may be a powerful and noninvasive prognostic marker at the time of diagnosis. Furthermore, preliminary data strongly suggest that the release of *MYCN* sequences in the peripheral blood is an early process in disease progression, permitting us to propose this novel marker for the follow-up of patients after chemotherapy.

Introduction

Neuroblastomas are tumors derived from the sympathoadrenal lineage of the neural crest. They are the most common solid extracranial neoplasms in children, accounting for 10% of all childhood cancers and for 15% of cancer-related deaths. This tumor type is characterized by a remarkable clinical heterogeneity. In some patients, tumors will regress spontaneously or mature into benign ganglioneuromas, whereas in about half of the patients, tumors will progress despite intensive therapy. The International Neuroblastoma Staging System criteria for diagnosis requires an unequivocal pathological diagnosis and favors the identification of prognostic markers in tumor samples (1). Among the most important prognostic factors is the number of *MYCN* copies. *MYCN* amplification (from 3- to 300-fold per haploid genome) can be detected in cancer cells by Southern blot, fluorescence *in situ* hybridization, or quantitative PCR. This biological property, intrinsic to ~25% of primary tumors, is strongly associated with rapid tumor progression and a poor outcome, independently from the stage of the tumor or the age of the patient, and has become an important factor in clinical decision-making and therapy stratification (2, 3).

In several types of neoplasias, molecular techniques such as PCR have detected small amounts of free DNA in the serum and plasma of patients with melanoma, breast, head and neck, or lung cancer, suggesting that circulating DNA is a valuable target for cancer marker detection (4–11). On the basis of these observations, the present investigation aimed at determining the presence of *MYCN* DNA in the peripheral blood of neuroblastoma patients, where *MYCN* status in neuroblastoma tumors was evaluated previously by Southern blot.

Materials and Methods

Sample Preparation. Serum or plasma from 102 patients with neuroblastoma and serum or plasma from 72 volunteer controls (26 healthy volunteer donors and 46 children without malignant disease) were assayed. Initially, the samples were prepared by centrifugation at 2000 rpm for 10 min at 4°C, followed by careful aliquoting and freezing at –80°C.

DNA Isolation. Circulating DNA was extracted from 200 µl of plasma or serum using the QIAmp blood kit (Qiagen, Courtaboeuf, France) according to the blood and body fluid protocol recommended by the manufacturer. Before extraction, 5 ng of pBluescript II SK plasmid (Stratagene, La Jolla, CA) were added to each sample. After extraction, an amplification test of the plasmid was performed using 1 of the 50 µl of the elution volume of the Qiagen column to ensure the quality of the procedure.

PCR Amplification. PCR amplification of *MYCN* was performed on 1 µl of the elution volume of the Qiagen column, using specific oligonucleotide primers designed previously to amplify *MYCN* oncogene in cancer cell (12). Twenty-seven PCR cycles were performed; each one consisted of 30 s at 94°C, 60 s at 60°C, and 60 s at 72°C, followed by a final extension step at 72°C for 5 min. PCR products were separated on 2% agarose gel, and fragments were visualized after ethidium bromide staining. In parallel, we performed real-time quantitative PCR using the Taqman detection chemistry on the ABI Prism 7700 Sequence Detection System (Applied Biosystems). All primers and probes were designed with Primer Express 1.0 software System (Applied Biosystems). The sequence of the primers used for the target *MYCN* gene was: forward, 5'-CGGTCCCCCACCCTCTCTT-3'; and reverse, 5'-CGGTTTAGC-CACCAACTTCTC-3', and the sequence of the Taqman probe is FAM-CCTCAGACTGCGCTGC-TAMRA. Amplification mixtures (25 µl) consisted of template DNA (5 µl), 1× TaqMan Universal PCR MasterMix (Applied Biosystems), 300 nM of each primer, 200 nM of the probe for the *MYCN* gene, and 1× TaqMan RNase P control reagents (Applied Biosystems). The cycling conditions were 10 min of polymerase activation at 95°C, 40 cycles at 95°C for 15 s, and 60°C for 1 min. The quantification of copy number for *MYCN* was evaluated by comparison of the signals obtained for the *MYCN* gene and RNase P gene.

Results and Discussion

Using PCR amplification, we searched for the presence of *MYCN* DNA in the peripheral blood of neuroblastoma patients. The accuracy of this approach was established by comparing a large series of patients with different stages of neuroblastomas ($n = 102$, including 32 children with MNA³ tumors and 70 children with tumors exhibiting no *MYCN* amplification, as determined by Southern blot analysis) with a control group including healthy volunteer donors ($n = 26$) and children with nonmalignant diseases ($n = 46$). Using a protocol, mainly characterized by a moderate number of amplification cycles, *MYCN* sequences were detected in 31 of 32 samples from patients with MNA tumors, 1 of 70 samples from neuroblastoma patients without MNA tumors ($P < 0.00001$), and 0 of 72 samples from noncancerous individuals (Table 1 and Fig. 1). Real-time quantitative PCR confirmed all previous data and revealed that the amount of *MYCN* sequences in the peripheral blood of MNA neuroblastoma patients was 25–600-fold higher

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³ The abbreviation used is: MNA, *MYCN* amplified.

than in other individuals (neuroblastoma patients without *MYCN* amplification or controls).

Results of this study provide straightforward evidence that circulating *MYCN* is detected at diagnosis in MNA neuroblastoma patients. It is noteworthy that the presence of circulating *MYCN* is only dependent on the *MYCN* status in cancer cells. It is detectable in peripheral blood of MNA neuroblastoma patients with either localized or metastatic tumor. This observation suggests that circulating *MYCN* may be used as a prognostic marker when tumor cells are not available for molecular analysis. Considering that patients who have the most advanced disease and are at the highest risk for tumor-related death are often those from whom little or no tumor is submitted for biological studies, our data may have important clinical implications. Indeed, most protocols recommend primary radical resection only for localized tumors. In the more frequent cases, such as advanced localized neuroblastomas or metastatic neuroblastomas, up-front surgical resection is not indicated because of the associated risk of major clinical complications. Surgical or percutaneous core biopsies are carried out to provide histological diagnosis. However, these techniques do not always provide enough material for the analysis of biological markers (13). The detection of *MYCN* DNA sequences may become a crucial factor in clinical decision-making for such cases.

We also tested the potential of this novel marker in the follow-up of MNA neuroblastoma patients. For 8 of the previously studied patients, serum samples were available at the time of diagnosis, after induction chemotherapy and at the time of relapse. Whereas circulating *MYCN* was detected at diagnosis in all patients, postchemotherapy tumor response was constantly associated with an important decrease of serum *MYCN* levels (Fig. 1C). The levels of circulating *MYCN* at diagnosis and at relapse were comparable. For one patient, we were able to test a serum sample extracted 2 months before the clinical diagnosis of relapse. As shown in Fig. 1C (Lane 7), a significant increase of serum *MYCN* levels was observed, compared with the previous sample, strongly suggesting that free *MYCN* DNA in the serum is an early marker of progression.

Overall, the data provided here suggest that circulating *MYCN* DNA is a valuable prognostic marker in neuroblastoma patients and may be proposed as an early detection test, as well as a complementary, noninvasive assay in the follow-up of patients at high risk of relapse. At last, the study demonstrates that sporadic gene amplification in tumor cells leads to high levels of DNA in the peripheral blood, providing new markers in human cancers.

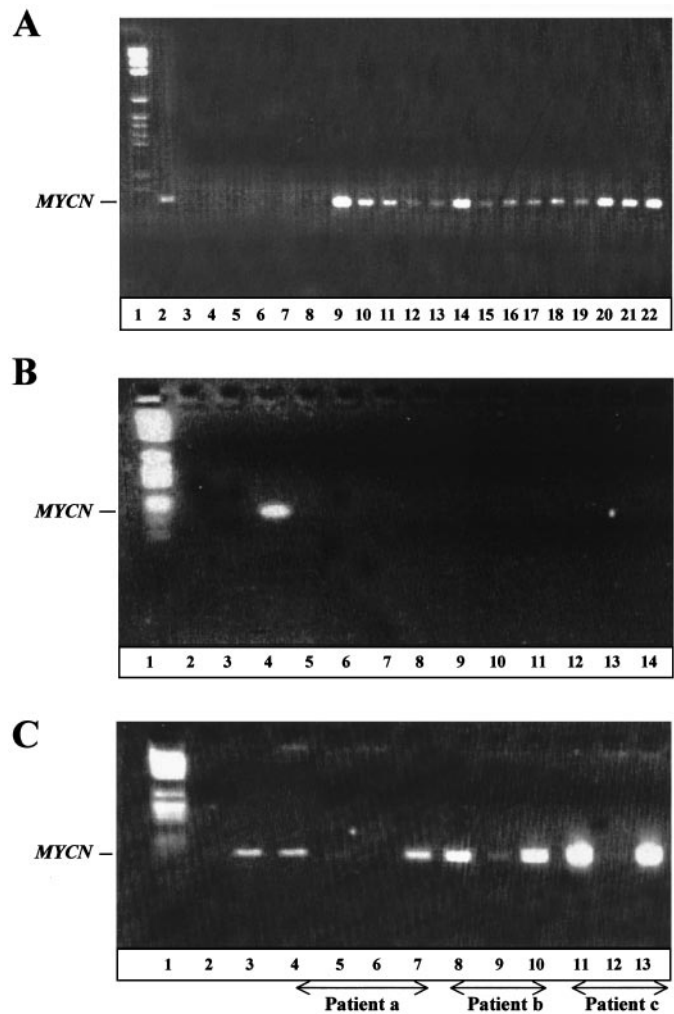


Fig. 1. Detection of *MYCN* DNA sequences in serum by PCR analysis. A, serum from neuroblastoma patients. Lane 1, molecular weight scale; Lane 2, positive control (DNA from IMR32 neuroblastoma cell line); Lanes 3–8, serum DNA from neuroblastoma patients without MNA tumors; Lanes 9–22, serum DNA from neuroblastoma patients with MNA tumors. B, serum from noncancerous individuals. Lane 1, molecular weight scale; Lane 2, control sample without DNA; Lane 3, DNA from normal lymphocytes; Lane 4, positive control (DNA from IMR32 neuroblastoma cell line); Lanes 5–9, serum DNA from healthy volunteer donors; Lanes 10–14, serum DNA from children without malignant diseases. C, follow-up of circulating *MYCN* DNA in three patients with MNA neuroblastomas. Lane 1, molecular weight scale; Lane 2, control sample without DNA; Lane 3, positive control (DNA from IMR32 neuroblastoma cell line); Lanes 4–7, serum from patient a at the time of diagnosis (Lane 4), first graft (Lane 5), second graft (Lane 6) and 2 months before relapse (Lane 7); Lanes 8–10, serum from patient b at the time of diagnosis (Lane 8), pregraft (Lane 9) and relapse (Lane 10); Lanes 11–13, serum from patient c at the time of diagnosis (Lane 11), postgraft (Lane 12) and relapse (Lane 13).

Table 1 Detection of *MYCN* DNA sequences in peripheral blood and clinical status of individuals

Individuals	n	Detection of circulating <i>MYCN</i>
Neuroblastoma patients		
<i>MYCN</i> amplification (total)	32	31
Stage 2b	1	1
Stage 3	5	4
Stage 4s	1	1
Stage 4	25	25
No <i>MYCN</i> amplification (total)	70	1
Stage 1	14	0
Stage 2	10	0
Stage 3	8	0
Stage 4s	5	0
Stage 4	33	1
Controls		
Healthy donors (adults)	26	0
Children (age <8 yr)	46	0

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