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Influence of Neuroblastoma Stage on Serum-Based Detection of *MYCN* Amplification

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Abstract

Background—*MYCN* oncogene amplification has been defined as the most important prognostic factor for neuroblastoma, the most common solid extracranial neoplasm in children. High copy numbers are strongly associated with rapid tumor progression and poor outcome, independently of tumor stage or patient age, and this has become an important factor in treatment stratification.

Procedure—By Real Time Quantitative PCR analysis, we evaluated the clinical relevance of circulating *MYCN* DNA of 267 patients with locoregional or metastatic neuroblastoma in children less than 18 months of age.

Results—For patients in this age group with INSS stage 4 or 4S NB and stage 3 patients, serum-based determination of *MYCN* DNA sequences had good sensitivity (85%, 83% and 75% respectively) and high specificity (100%) when compared to direct tumor gene determination. In contrast, the approach showed low sensitivity patients with stage 1 and 2 disease.

Conclusion—Our results show that the sensitivity of the serum-based *MYCN* DNA sequence determination depends on the stage of the disease. However, this simple, reproducible assay may represent a reasonably sensitive and very specific tool to assess tumor *MYCN* status in cases with stage 3 and metastatic disease for whom a wait and see strategy is often recommended.

Keywords

Circulating DNA; *MYCN* amplification; neuroblastoma

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Conflict of interest statement
None declared

Introduction

The potential application of circulating DNA in the diagnosis and prognosis of cancer was first demonstrated in 1977, when high levels of circulating DNA were detected in the serum of cancer patients, and the levels of circulating DNA decreased when the patients responded to radiotherapy [1]. Subsequently, a large number of tumour-associated genetic and epigenetic changes were detected in the plasma/serum of cancer patients, including *Ras* and *p53* mutations, microsatellite alterations, aberrant promoter hypermethylation of several genes, rearranged immunoglobulin heavy chain DNA, mitochondrial DNA mutations, and tumour-related viral DNA [2–4]. Recently, we and others demonstrated that high levels of circulating *MYCN* DNA sequences, as assessed by real-time quantitative PCR, were present in the peripheral blood of patients with *MYCN*-amplified (MNA) neuroblastoma [5–7]. As the assessment of *MYCN* status is paramount to prognosis determination and therapeutic stratification in patients with neuroblastoma (NB) [8], this observation suggested that levels of circulating *MYCN* DNA sequences could serve as a sensitive, yet non-invasive, prognostic marker at the time of diagnosis. Based on these preliminary data, we have now determined the clinical relevance of circulating *MYCN* DNA by analyzing blindly serum samples from a large cohort of patients less than 18 months of age with neuroblastoma and locoregional or metastatic disease.

Patients and methods

Patients

Patients diagnosed with NB were enrolled in the study with informed consent of their parents. This study was conducted under research protocols approved by each institutional review board. Two hundred and sixty-seven serum samples (Stages 1 and 2, n=34; stage 3, n=43; stage 4, n=124; stage 4S, n= 66) were obtained from the Children's Oncology Group and from the tumor repositories of different European countries (for INSS stage 3 NB patients) at diagnosis. This retrospective study was blinded in that the sera were analyzed without knowledge of the tumor *MYCN* status determined at the time of diagnosis using standard methodology.

DNA isolation

Circulating DNA was extracted from 200 µl of frozen serum samples using the QIAmp DNA blood kit (Qiagen, Courtaboeuf, France) according to the "blood and body fluid protocol" recommended by the manufacturer.

Real-Time Quantitative PCR

Circulating *MYCN* DNA was analyzed by quantitative real-time PCR, as previously described [7]. Briefly, TaqMan PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Amplification mixtures (25 µl) consisted of template DNA (5 µl), 1× TaqMan Universal PCR MasterMix (Applied Biosystems), 200 nmol/L of each primer, and 100 nmol/L of fluorogenic probe. Simultaneously to the *MYCN* sequence, *NAGK*, located at 2p12, was measured and used as a single-copy gene reference. All PCR reactions were performed with one cycle of 95°C for 10 minutes, followed by PCR amplification with 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Standard curves were constructed in each PCR run with four-fold serial dilutions containing 100, 10, 1 and 0.1 ng/µL of a healthy donor's DNA. The dosages of the target genes in each sample were interpolated from these standard curves. The *MYCN* copy number in a given DNA sample was determined by the ratio of *MYCN* dosage to *NAGK* dosage (M/N ratio). A *MYCN*/*NAGK* cut-off ratio of 5 was considered indicative of a high

level of circulating *MYCN* DNA sequences and a surrogate for tumoral *MYCN* amplification. The MYCN copy numbers were expressed as the average of two measurements.

Results

Determination of the number of *MYCN* copies in the peripheral blood would facilitate the prediction of prognosis in patients with neuroblastoma. To assess whether this serum assay may supplant biopsy for evaluating this key biomarker, our cohort was enriched for patients in which tumor biopsy may be difficult (such as symptomatic infants with stage 4S disease) or deferred by clinical discretion (such as children with observable localized masses). Of the 124 patients with INSS stage 4 NB enrolled in the study, 83 had non-MNA tumors and 41 had MNA tumors. A high level of circulating *MYCN* DNA sequences was found in 35 of the 41 cases with MNA tumors. Of the 66 patients with INSS stage 4S disease, 6 had MNA tumors, and a high level of circulating *MYCN* DNA was found in 5 of these 6 cases. Thus, in patients less than 18 months of age with INSS stage 4 or 4S NB, serum-based determination of *MYCN* DNA sequences had good sensitivity (85% and 83%, respectively) and perfect specificity (100%, no false positives observed in either group) when compared to direct tumor *MYCN* gene determination (Table I).

Thirty-four serum samples were obtained at diagnosis from patients with INSS stages 1 and 2, and 43 from patients with INSS stage 3 disease (i.e., patients with locoregional NB). Overall, no false positive cases were observed. Twelve of the 16 INSS patients with Stage 3 disease and MNA tumors showed high levels of circulating *MYCN* DNA sequences (75% sensitivity). In contrast, significant levels of circulating *MYCN* DNA sequences were detected in the serum of only 1 of the 10 INSS stage 1–2 patients with MNA tumors, demonstrating low sensitivity of the method in this cohort (Table I).

Discussion

Our results show that the sensitivity of serum-based *MYCN* DNA sequence determination depends on the stage of NB. Whereas most patients with metastatic NB show high levels of circulating *MYCN* DNA sequences, the sensitivity of the assay appears to be lower for patients with localized NB, in particular those with a low tumor burden (INSS stages 1 and 2). This observation could be explained by the smaller quantity of dying tumor cells releasing tumor DNA in patients with favorable locoregional NB, whereas biologically aggressive disease is more frequently associated with areas of necrosis resulting in higher DNA release. However, other possibilities include a spontaneous and active release of DNA by proliferating cancer cells as observed withlymphocytes [9,10] or a stability of circulating tumoral DNA explained by a low activity of DNase I and of DNase II in patients with malignant diseases compared with the activity observed in healthy people [11]. Some of the rare false negative cases reported in patients with MNA tumors could be due to heterogeneous tumors with low *MYCN* amplification. Two patients with tumor *MYCN* amplification could only be identified using FISH analysis whereas *MYCN* detection using Real Time quantitative PCR remained negative. Beyond these particular cases, it appears that the accuracy of the assay is directly dependent on the quality of the serum samples. In the present retrospective study, false negative cases could reflect variations in the method used for serum collection and handling. We identified no false positive results using this serum assay as a surrogate for tumor MYCN amplification; the implementation of this assay is therefore unlikely to cause over treatment in patients erroneously scored as having MYCN amplified tumors.

In conclusion, the detection of *MYCN* sequences in sera appears to be a specific tool for the determination of tumor *MYCN* amplification in patients with neuroblastoma. The possibility

of determining the *MYCN* status of patients with neuroblastoma from a blood sample could potentially eliminate cases with unknown *MYCN* status (26% of all neuroblastoma patients, according to a study by Matthay et al. [12]) so that these patients can receive more appropriate treatment. Furthermore, this simple, reproducible assay may represent a safe, reasonably sensitive and very specific tool to assess tumor *MYCN* status in the cases with stage 3 and metastatic disease for whom a wait and see strategy is often recommended.

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References

1. Leon SA, Shapiro B, Sklaroff DM, et al. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res. 1997; 37:646–650. [PubMed: 837366]
2. Stroun M, Anker P, Maurice P, et al. Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology. 1989; 46:318–322. [PubMed: 2779946]
3. Anker P, Mulcahy H, Chen XQ, et al. Detection of circulating tumour DNA in the blood (plasma/serum) of cancer patients. Cancer Metastasis Rev. 1999; 18:65–73. [PubMed: 10505546]
4. Lo YM. Circulating nucleic acids in plasma and serum: an overview. Ann N Y Acad Sci. 2001; 945:1–7. [PubMed: 11708462]
5. Combaret V, Bergeron C, Noguera R, et al. Circulating MYCN DNA as a tumor-specific marker in neuroblastoma patients. Cancer Res. 2002; 62:3646–3648. [PubMed: 12097268]
6. Combaret V, Audoynaud C, Iacono I, et al. Circulating MYCN DNA predicts MYCN-amplification in neuroblastoma. J Clin Oncol. 2005; 23:8919–8920. [PubMed: 16314658]
7. Gotoh T, Hosoi H, Iehara T, et al. Prediction of MYCN amplification in neuroblastoma using serum DNA and real-time quantitative polymerase chain reaction. J Clin Oncol. 2005; 23:5205–5210. [PubMed: 16051962]
8. Rubie H, Hartmann O, Michon J, et al. N-Myc gene amplification is a major prognostic factor in localized neuroblastoma: results of the French NBL 90 study. Neuroblastoma Study Group of the Societe Francaise d'Oncologie Pediatrique. J Clin Oncol. 1997; 15:1171–1182. [PubMed: 9060561]
9. Stroun M, Anker P. Nucleic acids spontaneously released by living frog auricles. Biochem. J. 1972; 128:100–101.
10. Anker P, Stroun M, Maurice PA. Spontaneous release of DNA by human blood lymphocytes a shown in an in vitro system. Cancer Res. 1975; 9:2375–2382. [PubMed: 1149042]
11. Tamkovich SN, Cherepanova AV, Kolesnikova EV, et al. Circulating DNA and DNase activity in human blood. Ann N Y Acad Sci. 2006; 1075:191–196. [PubMed: 17108211]
12. Matthay KK, Villablanca JG, Seeger RC, et al. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. N Engl J Med. 1999; 341:1165–1173. [PubMed: 10519894]

Table I

Detection of high levels of MYCN DNA sequences in the peripheral blood and correlation with patient clinical status.

	Non Amplified Tumor		Amplified Tumor	
***MYCN* (stages 1 and 2) *n*=34**	**24**		**10**	
- No detection of circulating MYCN DNA sequences		24		9
- Detection of circulating MYCN DNA sequences		0		1
***MYCN* (stage 3) *n*=43**	**27**		**16**	
- No detection of circulating MYCN DNA sequences		27		4
- Detection of circulating MYCN DNA sequences		0		12
MYCN (stage 4 < 18 months) n =124	**83**		**41**	
- No detection of circulating MYCN DNA sequences		83		6
- Detection of circulating MYCN DNA sequences		0		35
MYCN (stage 4 S) n =66	**60**		**6**	
- No detection of circulating MYCN DNA sequences		60		1
- Detection of circulating MYCN DNA sequences		0		5