Expression of B-myb in Neuroblastoma Tumors Is a Poor Prognostic Factor Independent from MYCN Amplification

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Although these clinical and molecular features have been associated with disease outcome, their interrelationship and their impact in the context of known risk factors is not always clear. For instance, the deletion of the short arm of chromosome 1 is a risk factor if associated with the amplification of MYCN but, when present alone, does not correlate with an adverse prognosis (10). On the contrary, MYCN amplification has been demonstrated to contribute significantly to risk estimation and constitutes by far the most useful genetic indicator (11). Nevertheless, the predictive value of MYCN amplification is also incomplete because this genetic trait is present only in a fraction of patients with poor outcome. Thus, the identification of new independent prognostic indicators that may broaden the predictive value, thus far provided by a well-recognized marker such as MYCN amplification, is strongly needed in human NB.

The myb genes are crucial for the control of proliferation and differentiation in a number of cell types including NB cells (12–15). We and others have assessed the functional significance of myb genes expression in NB cells in vitro (14, 15). The expression of c-myb, a transcriptional regulator essential for the proliferation of hematopoietic cells (16), is down-regulated in NB cell lines during differentiation (15). RNA and DNA antisense experiments have demonstrated that inhibition of c-myb mRNA impairs the proliferation (17), and increases the susceptibility, of NB cells to apoptosis in vitro (18). B-myb is also expressed in NB cells and, like c-myb, is down-regulated during differentiation (19). In addition, when constitutively expressed, it inhibits differentiation of NB cell lines (19). A-myb expression was also detected in NB cells (20), but much less is known about its regulation and functional significance. Together, these findings prompted us to investigate whether the expression of myb genes in NB tumors correlates with disease stage and prognosis.

Materials and Methods

Patients. The study was carried out in a group of 64 children with pathological diagnosis of NB. Clinical staging was according to Brodeur et al. (4); 10 patients were at stage 1, 5 at stage 2A, 2 at stage 2B, 10 at stage 3, 33 at stage 4, and 4 at stage 4S. Survival was calculated from diagnosis to last follow-up (n = 46; median = 37 months; range, 11–84 months) or until death (n = 18; median = 18.5 months; range, 1–53 months).

RT-PCR on NB Cell Lines and Tumors. Total RNA (500 ng; Ref. 21) from human NB cell line LAN-5 was reverse-transcribed for 45 min at 40°C in the presence of 2 μM random hexamers (Amersham Life Science LTD, Buckinghamshire, United Kingdom), 0.8 mM dNTPs in 1× RT buffer (Amersham) and 200 units of Moloney Murine Leukemia Virus reverse transcriptase (Amersham) in a total volume of 50 μL. PCR mixes were prepared using 5 μL of the reverse transcriptase mix in 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, 0.4 μM upstream primer, 0.4 μM downstream primer, 0.2 mM dNTPs, and 2 units Ex Taq polymerase (TaKaRa Shuzo Co., LTD, Otsu, Shiga, Japan) in a total volume of 50 μL. PCR conditions were: 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C for 20, 25, 30, and 35 cycles. A final extension step of 7 min at 72°C was carried out in each case. Fifteen μL of each PCR product were run on a 1% agarose gel in 1×

Abstract

The transcription factors of the Myb family are expressed in several tissues and play an important role in cell proliferation, differentiation, and survival. In this study, the expression of A-myb, B-myb, and c-myb was investigated in a group of 64 neuroblastomas at different clinical stages by a sensitive reverse transcription-PCR technique and correlated with patients’ survival. All of the myb genes were frequently expressed in neuroblastoma tumors. Interestingly, the expression of B-myb, which was detected in 33 cases, was associated with an increased risk of death (P = 0.027 in a univariate analysis), whereas there was no correlation with A-myb and c-myb expression. In addition, in a multivariate Cox regression analysis that included myb gene expression, MYCN status, age at diagnosis, and tumor staging, MYCN amplification and B-myb expression were independently associated to an increased risk (P < 0.01 and P = 0.015, respectively). In overall survival curves obtained by stratifying the neuroblastoma cases on the basis of MYCN status and B-myb expression, the group of patients without MYCN amplification and positive for B-myb expression had worse survival probability than that without MYCN amplification and nonexpressing B-myb (P < 0.01). In summary, these findings provide the first demonstration that B-myb expression can be a useful prognostic marker in human neuroblastoma. Moreover, B-myb expression has a prognostic value complementary to MYCN amplification and can identify a group of high-risk patients that would not be predicted on the basis of the MYCN status only.

Introduction

NB, a solid tumor of early childhood, derives from the embryonic neural crest. In vitro (1) and in vivo (2) NB cells frequently maintain the ability to differentiate along pathways reminiscent of their embryonic origin (1). The differentiation potential of the tumor cells is probably related to the clinical outcome, which varies from aggressive and generally fatal to spontaneous regression (3). Several clinical, biological, and genetic parameters have been used to assess the prognosis and to help the clinician in devising the most effective therapies for NB patients. Age (1), tumor staging (4), and ferritin (5) and lactate dehydrogenase (6) serum levels are presently used for these purposes. Among the genetic characteristics, MYCN amplification (7) as well as the deletion of the short arm of chromosome 1, where one or more tumor suppressor genes are thought to be located (8), define a group of patients at high risk. The expression of trkA and low-affinity nerve growth factor receptor have been associated with a benign outcome. Accordingly, the absence of these receptors was linked to a more aggressive disease (9).

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TBE and transferred onto a nylon membrane (Amersham) according to the manufacturer’s instructions. The filter was hybridized to a B-myb-specific 5’ end-labeled oligonucleotide probe (specific activity: 5 × 10^6 cpm/μg, 1 × 10^6 cpm/ml in the hybridization mix), representing an internal portion of the amplified product at 45°C in 5× SSC and 100 μg/ml sonicated salmon sperm DNA for 16 h. Washings were carried out in 2× SSC and 0.1% SDS for 15 min at room temperature and for 30 min in the same buffer at 50°C. The filter was exposed to an X-ray film (Eastman Kodak Company, Rochester, NY) in the presence of an intensifier screen for 20 h at ~80°C. RT-PCR on total RNA extracted from NB samples was carried out as described above. Thirty PCR cycles were used for detection of B-myb, A-myb, and c-myb transcripts, and 25 cycles were used to detect β-actin. RT-PCR, blotting, and hybridization conditions were as above. B-myb and A-myb blots were exposed for 20 h, c-myb for 72 h, and β-actin for 4 h. Densitometric analysis was carried out using Image Tool Version 2.00 software (The University of Texas, Health Sciences Center, San Antonio, TX). The expression level of each myb gene in each sample was normalized against the correspondent β-actin expression and referred in arbitrary densitometric units, after setting the level of expression in LAN-5 as 1. The complete analysis was repeated twice, and the results from each experiment were averaged. Primers and probes are as follows: B-myb, upstream primer 5’-AAAAACAGTTGAGGAGAAG-3’, downstream primer: 5’-CAGGGGAGTTCAATTATAC-3’, probe: 5’-ATCGGTACACATCTGGAAGC-3’, A-myb, upstream primer 5’-GCTGAGAATGAGTTAAGG-3’, downstream primer: 5’-GCTGATTITCTCATCACTG-3’, probe: 5’-AGCCTGTGAGACACTAGT-3’, c-myb, upstream primer 5’-CCATGTGACATTTAATCG-3’, downstream primer 5’-CTAGACAGATGTCTACAGGC-3’, probe: 5’-GCTCATTGTATGTTAATGAC-3’, β-actin, upstream primer 5’-TCATCCATTGTGCAATGAG-3’, downstream primer 5’- ACTTGTTGGCGTACAGGT-3’, probe: 5’-TGGAGTCCTGTGGCATCCAGGA-3’.

Statistical Analyses. Univariate and multivariate regression analyses according to the Cox proportional hazard model (22), Kaplan and Mayer survival curves (23), and log-rank significance tests were carried out using the software package SPSS 7.0 for Windows (SPSS Inc., Chicago, IL).

Results and Discussion

To analyze the scarce biological material often obtained from tumor biopsies, a sensitive semiquantitative RT-PCR method was devised to detect B-myb, A-myb, and c-myb specific transcripts in NB specimens. The human NB cell line LAN-5, which expresses detectable amounts of A-myb, B-myb, and c-myb (Ref. 14 and G. Raschella et al.), was used as control of the appropriate experimental conditions. Total RNA from LAN-5 cells was reverse-transcribed using random hexamers and amplified by PCR for 20, 25, 30, and 35 cycles in the presence of A-myb, B-myb, or c-myb specific primers. After electrophoretic separation and transfer onto nylon membranes, the amplified products were hybridized under stringent conditions with a 32P-oligonucleotide complementary to an internal portion of the amplified sequences. The increase in the specific signal after autoradiography remained in the linear range up to 35 cycles for all of the myb genes (see Fig. 1A for the linearity of B-myb amplification). Thus, in the experiments with RNA from tumor samples, we subjected the reverse-transcribed cDNAs to 30 amplification cycles. Similarly, the linearity of β-actin amplification was tested, and 25 cycles of PCR were chosen (not shown).

Total RNA from NB samples was analyzed for the expression of A-myb, B-myb, and c-myb by the RT-PCR technique described above. Complete details of the procedure are given in “Materials and Methods.” Fig. 1B shows the autoradiograms derived from the hybridization of some of the samples to B-myb, A-myb, and c-myb specific probes, and to β-actin. The latter probe was used to monitor the integrity of the starting material from each sample and to normalize the amount of B-myb, A-myb, and c-myb mRNAs. For each sample, the level of A-myb, B-myb, and c-myb mRNA was calculated taking the corresponding level in the LAN-5 cell line as 1 after normalization against the amount of β-actin mRNA. The cutoff level to define a myb-positive sample was ≥0.25 in both analyses. The experiment was repeated twice by two investigators (V. C. and B. T.), and negative and positive samples were the same in both analyses. Expression of myb genes is frequent in human NB tumors. A-myb and c-myb transcripts were detected in 42 and 21 patients, respectively. B-myb expression was detectable in 33 patients and ranged from 0.25 to 6.9 (data not shown). These data and other clinical and biological parameters are summarized in Table 1.

First, we carried out a univariate regression analysis based on the Cox proportional hazard model (22) to assess the prognostic value of the
expression of myb genes. A-myb, B-myb, and c-myb expression, MYCN status, deletion of chromosome 1p, clinical stage, and age at diagnosis were analyzed for their relationship to overall survival. In this analysis, MYCN amplification (P = 0.001), age at diagnosis (P = 0.04), and deletion of chromosome 1p (P = 0.0001) were associated with an increased risk of death (Table 2). Interestingly, the expression of B-myb was also associated with an increased risk (P = 0.027; Table 2). On the contrary, neither A-myb (P = 0.47), nor c-myb (P = 0.10), nor clinical stage (P = 0.11) reached statistical significance.

Next, we used a multivariate regression analysis based on the Cox proportional hazard model to test the independent value of each parameter in predicting overall survival. The chromosome 1p deletion was not tested because of the high number of noninformative samples, which would have lowered the total number of testable cases and the statistical power of the analysis. MYCN amplification (P < 0.01) and B-myb expression (P = 0.015) resulted in independent prognostic indicators associated with an increased risk (Table 3). Also in this multivariate analysis, A-myb and c-myb expression and tumor stage did not associate with an increased risk (P = 0.43, 0.64, and 0.81, respectively), whereas age at diagnosis >12 months was close to statistical significance (P = 0.07) in predicting an increased risk (Table 3). It should be pointed out that the statistical power of the analysis could be insufficient to detect weaker prognostic factors given the relatively small number of patients. Thus, we cannot rule out the possibility that some of the excluded parameters may have some independent prognostic significance in a bigger cohort of patients. This is likely for the age at diagnosis, which has been commonly used in assessing the prognosis of NB (1), although the biological basis of this correlation is unknown.

NB patients were then grouped based on the basis of B-myb expression and MYCN amplification, the two parameters with independent prognostic value in the Cox analysis described above, and the overall survival curves were calculated according to the method of Kaplan and Meier (23). NB patients were divided in the following four groups: (a) group 1: B-myb+; MYCN (n = 25); (b) group 2: B-myb+, MYCN (n = 26); (c) group 3: B-myb+, MYCN (n = 5); and (d) group 4: B-myb+, MYCN (n = 7). Noteworthy, group 1 and group 2 define two patient populations at different risk (Fig. 2A); group 1 has a good survival probability (80% in our set of patients), whereas group 2 has a lower survival probability (50%) and differs significantly from group 1 (P < 0.01). Groups 3 and 4 (Fig. 2B) define patients at high risk with a low survival probability (20 and 24%, respectively) and do not differ significantly from each other (P = 0.66), regardless of the expression of B-myb.

Together, these data indicate that, independently from MYCN status, the expression of B-myb can define a group of NB patients at intermediate risk who may benefit from an aggressive therapy similar to that tested in patients with MYCN amplification (24).

B-myb expression in NB cells has been linked to enhanced survival and reduced differentiation potential (19). For example, constitutive expression of B-myb impairs the ability of NB cells to complete the differentiation program when stimulated with chemical inducers (19), consistent with similar effects in other cellular systems (25). Furthermore, B-myb is a cell cycle-regulated gene with a peak of expression at the G1-S boundary (26) necessary for progression through the S phase (27). Together, these data provide a rationale for the potential use of B-myb expression as a prognostic factor in NB tumors. The expression of B-myb in a large number of NB patients may reflect the proliferative state of the tumor cell population and/or its reduced propensity to differentiate. In vitro, the expression of B-myb prevents NB cells from terminal differentiation maintaining them in an immature proliferative state (19). Thus, it might be expected that B-myb-expressing tumors have a worse prognosis compared with those that do not express B-myb. Indeed, the subset of B-myb-positive patients was clearly a group at higher risk compared with that in which B-myb expression was not detectable. This remains to be determined whether the consequences of B-myb expression in NB were reflected in an increased proliferative potential or in a reduced differentiation propensity.

Our data also fit nicely with a recent report in which, by serial analysis of gene expression (SAGE), B-myb has been identified as one of the genes overexpressed in primary non-small cell lung carcinomas, compared with normal epithelial tissue (28).

Our study also suggests that the role of B-myb does not overlap with that of A-myb and c-myb; these latter genes were coexpressed with B-myb in a cohort of NB patients, but there was no obvious correlation with survival. The lack of prognostic value of c-myb expression was somehow surprising; an explanation may rest in the short half-life of c-myb mRNA (~40 min; Ref. 15), compared with that of B-myb mRNA, which is instead long-lived (~16 h; Ref. 29). Moreover, unlike c-myb, B-myb expression is tightly associated with the S phase. Thus, B-myb expression may reflect the pool of proliferating cells more accurately than c-myb. Nevertheless, because c-myb has been shown to regulate proliferation of NB cells in vitro (17) and to act as a survival factor in NB (18) and in other cell types (30–32), the...
The two groups did not differ significantly (log-rank test, \( P_{1} = 0.66 \)). Survival probability was 20% in the first group and 24% in the second. The two groups did not differ significantly (log-rank test, \( P_{2} = 0.01 \)). Survival probability for patients \( B-myb \) without MYCN amplification (\( B-myb \)) and expression of \( c-myb \) in each group.

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