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Infants and Children with Stage 4 Neuroblastoma Express Significantly Different Levels of Specific Molecular Markers

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Abstract

In neuroblastoma (NB), age at diagnosis is a strong prognostic factor. Hereby, we evaluated whether patients with metastatic NB below 1 year of age (infants) expressed lower levels of NB-specific molecular markers compared to patients over 1 year (children). Bone marrow (BM) and peripheral blood (PB) samples collected at diagnosis from 54 Italian patients with metastatic NB were analysed by RT-qPCR for *TH, PHOX2B* and *DCX* mRNA expression. Statistical analyses were then performed to evaluate the diagnostic performance and the association with other known prognostic factors.

In spite of similar levels of morphological BM infiltration, *TH, PHOX2B* and *DCX* expression levels in BM and PB samples were significantly lower in stage 4 infants than in children. Between infants with stage 4 and 4S, the former expressed significantly higher levels in BM samples but similar levels in PB samples. In children with stage 4 NB, *TH* expression levels significantly associated with the presence of BM and skeletal metastasis.

Thus, RT-qPCR analysis of BM and PB samples showed potential clinical significance that should be evaluated in future multicentre prospective studies for infants with metastatic NB in comparison with conventional morphological evaluation and other prognostic factors evaluable in the primary tumors.

Keywords: Neuroblastoma; Gene expression; Metastasis; Tyrosine hydroxylase; Doublecortin; Paired-like homeobox 2b

Abbreviations: TH, tyrosine hydroxylase; DCX, Doublecortin; PHOX2B, Paired-like homeobox 2b.

Introduction

Neuroblastoma (NB) clinical presentation is highly heterogeneous. Metastatic spread is present in 50% of cases at diagnosis and mainly involves the bone marrow (BM). The main prognostic factors are stage (International Neuroblastoma Staging System (INSS) [1]), age at diagnosis, and *MYCN* oncogene status [2]. Being a continuous variable, the effect of age on prognosis is not discrete [2-4], but an age cut off at 12 months has been used for patient risk stratification until the International Neuroblastoma Risk Group Stratification System (INRG-SS) shifted the age cut off to 18 months [2].

Prognosis of stage 4 patients over 12 months of age (children) is dismal in spite of intensive multimodal therapies [2]. Conversely, prognosis of infants (< 12 months) with either stage 4 or stage 4S (S for special [5]), is very good [6,7], unless they present with *MYCN*-amplified tumors [6,8]. However, while infants with stage 4S disease receive no or little treatment, usually targeted to life-threatening symptoms [6], stage 4 infants require low dose chemotherapy to achieve cure [7]. Thus, children and infants with metastatic NB have remarkable different outcomes, following remarkable different therapeutic regimens.

The mechanisms responsible for such profound differences in relation to age at diagnosis have been ascribed to a higher frequency of favourable histology [7], and to more favourable genetic profiles in infants than in children [9-13]. Hereby, we investigated whether infants with stage 4 disease had levels of infiltration lower than stage 4 children. To test this hypothesis we used standardized RT-quantitative(q)PCR [14] for *TH, PHOX2B* and *DCX,* in paired BM and peripheral blood (PB) samples collected at diagnosis. In these samples, tyrosine hydroxylase, the first enzyme of catecholamine synthesis; the neuronal-specific transcription factor, PHOX2B; and Doublecortin, a regulator of neuronal microtubule assembly; were all shown to be expressed only by the infiltrating neuroblastoma cells [15]. Since by INSS [1] definition infants with stage 4S NB must have lower BM infiltration than infants with stage 4 disease, these latter patients were considered as reference group.

Materials and Methods

Patients and samples

Between January 2001 and June 2008, three-hundred and twenty Italian NB patients were staged at diagnosis as 4, fifty infants as 4 and sixty-four infants as 4S [1]. Demographic, clinical and follow-up data were retrieved from the Italian NB Registry (INBR) that collects data at diagnosis, during treatment and at least yearly after treatment discontinuation [16]. All patients/guardians signed a written consent allowing the use of samples and clinical data for research purposes. The study was approved by the Ethics Committees of each Italian centre and the procedures were in accordance with the Helsinki Declaration. Diagnostic and therapeutic details are available in Document S1.

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Only 18 paired BM and PB samples were available from stage 4 infants, thus to obtain three homogeneous groups we randomly selected 18 stage 4 children and 18 stage 4S infants among those with available paired samples. Before starting the molecular analysis we checked whether the selected patients fully represented the entire cohort. As shown in Table S1, the selected patients were not significantly different from the excluded ones as for demographic, biological and clinical features, including outcome.

Detection of NB-related markers in BM and PB samples

Half mL of each BM aspirate and 2 mL of PB, taken under sedation and diluted in Acid Citric-Dextrane anticoagulant, were shipped overnight at 4°C to the Italian reference laboratory. Total RNA was then extracted as previously described [17], and reverse transcribed according to standard operating procedures [14]. Five µL of cDNA in triplicate were then separately amplified for 40 cycles with the specific primers and probe for β2-*microglobulin* (endogenous reference mRNA, forward primer: 5'-GAGTATGCCTGCCGTGTG-3'; reverse primer; 5'-AATCCAAATGCGGCATCT-3'; probe 5'-CCTCCATGATGCTGCTTACATGTCTC –3', *TH* (forward primer: 5'-ATTGCTGAGATCGCCTTCCA-3'; reverse primer; 5'-AATCTCCTCGGCGGTGTACTC-3'; probe5'-ACAGGCACGGCGACCCGATTC–3'[14], *PHOX2B* (forward primer: 5'-CAGGGACCACCAGAGCAGT-3'; reverse primer; 5- CTGCTTGCGCTTCTCGTTGA-3'; probe 5'-TACGCCGCAGTTCCTTACAAACTCTTCAC-3'[18] and *DCX* mRNA (Forward primer: 5'-CGCTATGCTCAGGATGATTTTTC-3'; reverse primer: 5'-GCTGTGGCTGATGGGTTTCC-3'; probe: 5'-CATGACTCGGCATTCATTTTCATCCAG-3') in a 7700 Sequence Detec-

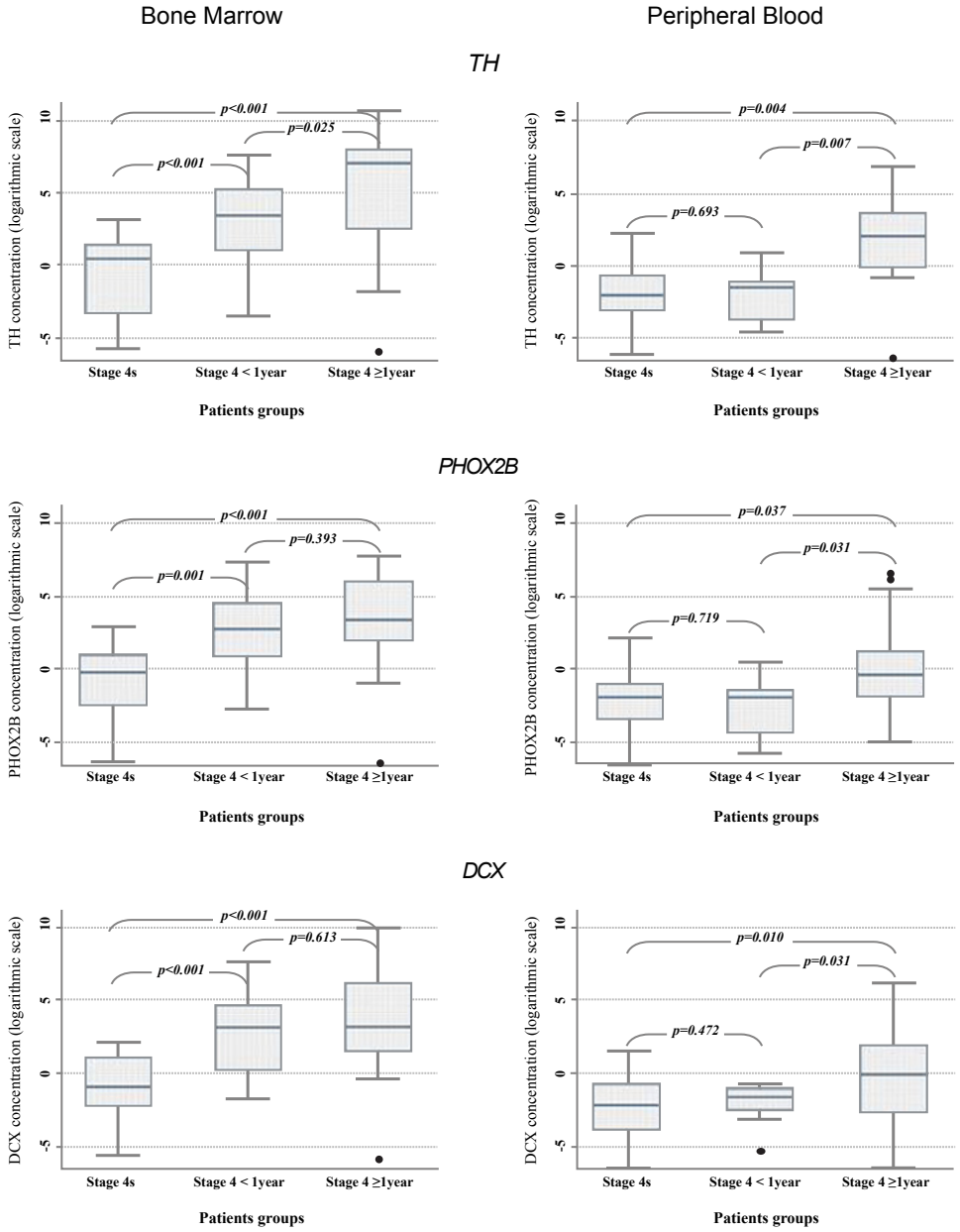

Figure 1: Level of expression of the three NB-related molecular markers in BM and PB samples from the three groups of study patients.

tion System (Applied Biosystems, Foster City, CA). Negative controls (water as template and RNA reverse-transcribed without MMLV) and a positive control (cDNA from IMR-32 NB cell line) were run in each plate.

Samples were considered positive if at least two of the three quantification cycle (Cq) values were lower than 40. Positive results of RT-qPCR analysis were expressed as ΔΔCq values [19] using β2-microglobulin as endogenous reference mRNA and the IMR-32 cell line as the exogenous reference sample, as described [14].

A checklist prepared according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) [20] is available in Doc S2.

Statistical analysis

Descriptive statistics were reported as percentages for categorical

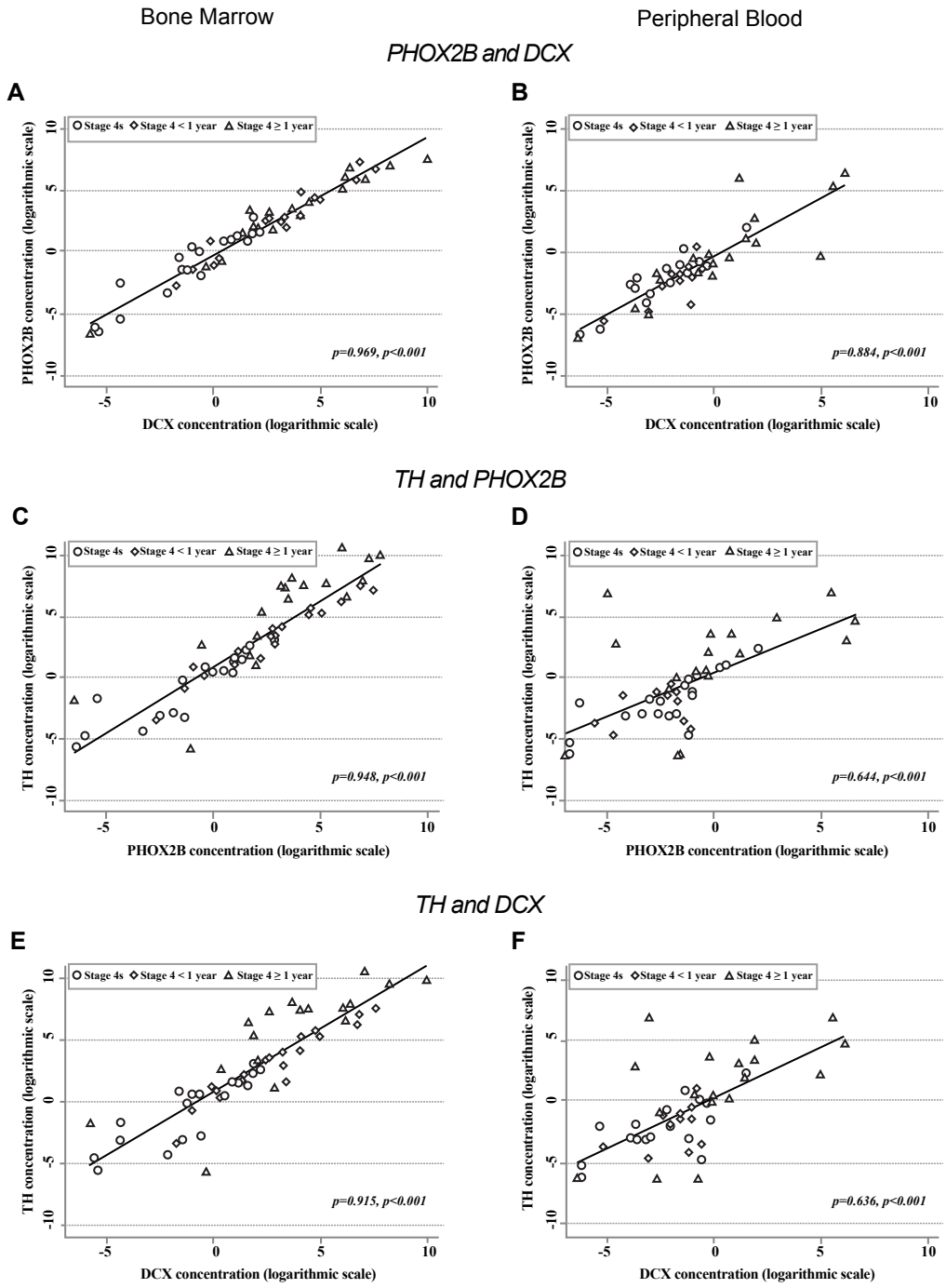

Figure 2: Correlation between ΔΔCq values measured with the three different markers in BM (panels A, C and E) and PB (panels B, D and F) samples from the study patients.

variables. For continuous data, medians with inter quartile range (IQR) were used due to the non normal distribution of the observations. Comparisons of frequency data were performed by means of the chi square test or the Fisher exact test, when appropriate, while differences in the distribution of continuous variables were analyzed by the Wilcoxon Mann-Whitney test. Correlation among concentrations of different molecular markers was assessed by the Spearman' s coefficient.

The diagnostic performance of the molecular markers was evaluated by the Receiver Operating Characteristic (ROC) analysis [21]. Analyses were made using Stata for Windows statistical package (release 11.0, Stata Corporation, College Station, TX, USA).

Results

As shown in Table 1s, the three groups of patients with metastatic NB selected for the molecular analysis of their paired BM and PB samples were similar as for gender, primary site and, most importantly, for the percentage of patients with BM involvement evaluated by MIBG scintigraphy and BM smears. As expected genetic abnormalities, such as MYCN amplification and 1p deletion were more frequent in children than in infants.

When PB and BM samples collected at diagnosis from these patients were evaluated for *TH, PHOX2B* and *DCX* mRNA expression, the levels of the three markers progressively increased, starting from stage 4S infants through stage 4 infants to stage 4 children (Figure 1), in both BM and PB samples. However, while in BM samples the levels were significantly different among the three clinical groups, in PB samples the expression levels were significantly different only between stage 4 children and infants, irrespective of stage 4 or 4S (Figure 1). Similar results were obtained when the age cut-off was shifted from 12 to 18 months (data not shown).

A perfect correlation between *PHOX2B* and *DCX* results was found both in BM and PB samples, whereas correlations between *TH* and *PHOX2B* and between *TH* and *DCX* were good in BM, but intermediate in PB samples (Figure 2). When BM and PB samples from the same patient were considered, correlation between their expression levels was good for *TH* ($\rho = 0.763$), but poor for *PHOX2B* and *DCX* ($\rho = 0.377$ and 0.393, respectively, Figure S1). By applying ROC analysis, BM *TH* $\Delta\Delta$Cq values were confirmed as the most accurate in discriminating the three groups of patients (Table 2s). A cut-off value of 19.8 allowed to allocate infants to stage 4 or 4S, and a cut-off value of 738.6 allowed to allocate a patients to the correct age group with 78% accuracy.

We next evaluated whether *TH, PHOX2B* and *DCX* expression levels significantly associated with clinical features and/or with other known prognostic markers (Table S2). No significant correlation was found in both stage 4 and 4S infants, whereas in stage 4 children BM expression values for any of the three markers, as well as PB *TH* values, significantly associated not only with the presence of BM infiltration, but also with skeletal metastasis ($p=0.008$ for BM *TH*; $p=0.032$ for PB *TH*, $p = 0.009$ for BM *PHOX2B* and $p= 0.032$ for BM *DCX*).

Discussion

Age and presence of metastatic disease are major prognostic factors in NB, thus in metastatic patients the age at diagnosis greatly impacts on both prognosis and type of therapeutic intervention. Hereby, we showed that *TH, PHOX2B* and *DCX* expression levels measured at diagnosis in BM and PB samples from infants and children with stage 4 disease were significantly different. Interestingly, the expression levels in PB samples from stage 4 infants were very close to those measured in stage 4S infants.

In general RT-qPCRs for *PHOX2B* and *DCX* gave very similar results, slightly different from those obtained for *TH* expression, that seems to give more clinical information, as demonstrated in patients with localized disease [22].

While in infants the expression levels of NB-validated markers did not associate with any clinical and biological feature, in stage 4 children *TH, PHOX2B* and *DCX* expression levels in BM samples, as well as *TH* expression levels in PB samples, associated with presence of skeletal metastasis, suggesting that elevated levels may indicate a more aggressive behaviour of metastatic cells and confirming the best performance of *TH* over the other two markers. Since the study patients were highly representative of the whole cohort of metastatic NB patients, our data suggest that the levels of expression of validated markers [15] may associate to different outcomes. Future multicentre prospective studies, however, will be necessary to assess their individual/combined clinical significance, because the number of available samples in this single-country study did not allow to perform this type of analysis.

According to both INSS [1] and INRG-SS [2], BM infiltration must be evaluated by morphological examination of smears and trephine biopsies, and a cut off of 10% of BM-infiltrating neoplastic cells allocates infants to either stage 4 or 4S. Since morphological analysis is highly dependent on the examiner's experience and skill, discrepancies in interpreting slides can not be excluded. It is interesting to note that, in spite of similar incidence of NB cases and identical frequency of all other NB stages, the percentages of stage 4 and 4S infants are remarkably different between North America and Europe/Japan [23-29]. Thus, the use of a quantitative method might also improve uniform staging across different countries.

In conclusion, RT-qPCR for validated markers should be compared with conventional morphological evaluation of BM infiltration and its potential clinical significance assessed in comparison to other prognostic markers evaluable in the primary tumors, such as favourable histology [7] and favourable genetic profiles [9-13].

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