Featured Article

The Tumor-Associated Antigen *PRAME* Is Universally Expressed in High-Stage Neuroblastoma and Associated with Poor Outcome

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ABSTRACT

Purpose: **The tumor-associated antigen *PRAME*, a potential candidate for immunotherapeutic targeting, is frequently expressed in a variety of cancers. However, no information about its presence in neuroblastoma is available to date. We therefore evaluated and quantified *PRAME* expression in a considerable number of neuroblastoma tumors and assessed its impact on the outcome of patients.**

Experimental Design: **Qualitative analysis of *PRAME* expression was assessed by reverse transcription (RT)-PCR screening of 94 patients with primary neuroblastoma. The same cohort was used for semiquantitative determination of transcript levels by Northern blotting, comparing the signal intensities of patients with those of testis total RNA. For more precise quantification of *PRAME* expression, real-time RT-PCR was performed in 88 patients of the above cohort and 7 additional patients, thus leaving a total of 101 patients that were analyzed with either method. Furthermore, association with tumor stage, age of patients at diagnosis, and *MYCN* amplification was determined as well as the prognostic impact of *PRAME* expression.**

Results: **RT-PCR screening detected *PRAME* expression in 93% of primary neuroblastoma and 100% of patients with advanced disease. Furthermore, RT-PCR and Northern blot analysis showed a highly significant association of *PRAME* expression with both higher tumor stage ($P < 0.01$) and the age of patients at diagnosis ($P < 0.01$). Finally, precise quantification of *PRAME* expression by quantitative real-time reverse transcription-PCR displayed significant impact on the outcome of patients.**

Conclusions: PRAME **expression in neuroblastoma is extraordinarily common and was universally seen in patients with advanced-stage disease in our study. Furthermore, significant impact of *PRAME* expression on the outcome of patients was shown. Thus, *PRAME* may present a particularly attractive target for immunotherapeutic strategies in neuroblastoma.**

INTRODUCTION

Treatment of cancer by inducing a specific immune response has been the focus of recent studies and might provide an efficient therapeutic concept in the near future (1–3). However, suitable antigens for specific targeting are still rare, and their identification remains a major task for promoting further immunotherapeutic strategies. Cancer-testis antigen(s) (CTA) belong to the group of tumor-associated antigens and represent possible target proteins for such approaches. Their expression is encountered in a variety of malignancies of different histological origin but is negligibly low in healthy tissues, with male germinal cells usually being the sole exception (4). Because of this exclusive expression pattern, CTAs are considered to be promising candidates for tumor immunotherapy, because they restrict the effects of an induced immune response to the malignant tissue.

In neuroblastoma, a pediatric tumor arising from the neural crest that represents the most common solid malignancy in patients under the age of 15, established CTA such as members of the MAGE family or NY-ESO-1 that can only be detected in a fraction of cases (5–8). Possible benefits of an induced immune response against these antigens would therefore be limited to a few patients. With overall survival being poor in advanced neuroblastoma stages despite intensive treatment regimes, identification of candidates for immunotherapeutic targeting that show a more common expression pattern in this entity is of crucial clinical relevance. *PRAME* (preferentially expressed antigen in melanoma), a CTA that was first discovered in a patient with melanoma (9), has since been found to be expressed in a large variety of cancer cells including squamous cell lung carcinoma, medulloblastoma (10), renal cell carcinoma (11), and acute leukemia (12–14). Furthermore, *PRAME*-specific lysis of multiple tumor cell lines by CTL in an HLA-restricted manner has been demonstrated recently (15). Hence, *PRAME* is considered to be an attractive potential candidate for future immunotherapeutical approaches.

To date, no information is available about *PRAME* expression in neuroblastoma, except for the observation of *PRAME*-specific transcripts in two of three cases of neuroblastoma in reverse-transcription (RT)-PCR screening for *PRAME* in a panel of multiple tumors (9). Therefore, we here present the first analysis of *PRAME* expression in a considerable number of primary neuroblastomas and evaluate the impact of *PRAME*-transcript levels on the outcome of patients.

PATIENTS AND METHODS

Patients. The study comprised 101 patients enrolled in the German Neuroblastoma Trials NB90, NB95S, and NB97, who were diagnosed between 1991 and 2002. Stage was

Received 12/28/03; revised 3/16/04; accepted 4/13/04.

Grant support: This work was supported by the Fördergesellschaft Kinderkrebs-Neuroblastom-Forschung e.V.

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classified according to the International Neuroblastoma Staging System (INSS), as follows: stage 1, 25% ($n = 26$); stage 2, 8% ($n = 8$); stage 3, 17% ($n = 17$); stage 4, 29% ($n = 29$); and stage 4S, 21% ($n = 21$). The age of patients at diagnosis ranged from 0 to 179.8 months, with a median age at diagnosis of 7.5 months. Median follow-up for patients without fatal events was 32.9 months (range 0.2 to 109.6). Tumor cell content was determined by a pathologist, and only samples with a minimum of 80% tumor cells were processed for analysis.

Statistical Analysis. To compare variables of interest, Fisher's exact test, χ^2 test, Kruskal-Wallis test or Mann-Whitney *U* test were used where appropriate. Kaplan-Meier estimates for event-free survival (EFS) and overall survival were calculated and compared by log-rank test. Recurrence, progression of disease, and death from disease were counted as events. Death resulting from therapy complications was not counted as an event but censored for EFS analysis.

Analysis of Chromosomal Alterations and N-*myc* proto-oncogene (MYCN) Amplification. Determination of the MYCN status was assessed by fluorescent *in situ* hybridization. We used the locus-specific DNA-probes n-myc + *D2Z*. Probes were obtained from ONCOR (Gaithersburg, MD). Fluorescent *in situ* hybridization was performed in a dual color procedure following the manufacturer's protocol with one exception: DNA-probes were applied on the slides and denatured on a hot plate (75°C, 4 min). According to the European Network for Quality Assurance in Higher Education guidelines (16), MYCN amplification was defined as over 4-fold increase of MYCN signal number in relation to the number of chromosomes 2.

Sample Preparation. Thirty to sixty mg of snap frozen primary tumor specimen were cryosliced to 20 μm slices and homogenized in TRIzol reagent (Invitrogen, Karlsruhe, Germany) using the FastPrep FP120 cell disrupter (Qbiogene, Inc., Carlsbad, CA) according to the manufacturer's protocol. Total RNA was isolated following the TRIzol protocol. Integrity of total RNA was assured by RNA Nano Chip assays on the 2100 Bioanalyzer (Agilent, Waldbronn, Germany) according to the manufacturer's protocol.

Serial Analysis of Gene Expression (SAGE). Gene expression profiles of five primary neuroblastomas were generated using SAGE (17). Construction of SAGE libraries was performed using the I-SAGE kit (Invitrogen Life Technologies, Karlsruhe, Germany) according to the manufacturer's protocol. A total of 131,920 sequence tags were cataloged, and genes corresponding to tags were identified by comparison to the human UniGene reference sequence database.[1]

Reverse Transcriptase (RT)-PCR. First-strand cDNA synthesis was performed using 2 μg of total RNA and oligodeoxythymidine acid-primers (Sigma-Aldrich, Taufkirchen, Germany) in a total volume of 20 μl according to the SUPERSCRIPT II protocol (Invitrogen, Karlsruhe, Germany). PCR was carried out in a total volume of 50 μl containing 1 μl first-strand cDNA, 2 units *Taq*DNA Polymerase (Invitrogen), 125 nM sense and antisense primer each, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 nM deoxynucleoside triphosphates each (Amersham Biosciences), and 1.5 mM $MgCl_2$. Oligonucleotide sequences used for amplification are available upon request. Cycling conditions consisted of a single denaturation step at 95°C for 3 min, followed by 95°C for 45 s, 60°C for 45 s, 72°C for 1 min, and a final extension step at 72°C for 5 min. Thirty-five cycles were performed for amplification of both *PRAME* and β-actin (positive control). No template control PCR was performed to exclude unspecific amplification. PCR products were visualized on a 2% agarose gel, and correct identity of amplificates was confirmed by direct sequencing of PCR products using the BigDye terminator sequencing kit, version 3.1 (Applied Biosystems, Foster City, CA).

Northern Blotting. Ten μg of total RNA from primary tumor samples were size-fractioned on a 1% denaturating formaldehyde-agarose gel and transferred to positively charged nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany) using NorthernMax One-Hour Transfer Buffer (Ambion Europe, Cambridgeshire, United Kingdom). Templates for probes for *PRAME* and β-*actin* were generated by RT-PCR (as described above) and were checked by direct sequencing using the BigDye terminator sequencing kit, version 3.1 (Applied Biosystems). Blots were prehybridized for 1 h and hybridized overnight at 42°C in UltraHyb Hybridization Buffer (Ambion) with high sensitivity strippable DNA probes labeled with [α^{32}P]dATP generated using the Strip-EZ DNA kit (Ambion) following the manufacturer's guidelines. After hybridization, membranes were washed 2 times for 5 min at room temperature in 2× SSC, 0.1% SDS and 2 times for 15 min at 42°C in 0.1× SSC, 0.1× SDS and exposed at −80°C to Kodak BioMax MR-1 films (Amersham) amplifying signals by use of BioMax MS Intensifying Screens (Amersham).

Quantitative Real-Time RT-PCR. Quantitative real-time RT-PCR was performed using the SYBR Green I reagent on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). PCR reactions were carried out in a total volume of 30 μl containing 26.8 μl of SYBR-Green I PCR master mix (Applied Biosystems), 0.4 μl of undiluted first-strand cDNA, and 1.4 μl of forward and reverse primers (Eurogentec, Seraing, Belgium) each. Oligonucleotide sequences were selected in successive exons (intro-spanning) to avoid amplification of contaminating genomic DNA (sequences available on request). To enable calculation of relative expression levels, serial dilutions (undiluted, 1:3, 1:9, and 1:27) of cDNA of the cell line IMR-32 were used for the generation of standard curves. All PCR reactions were performed in 96-well optical reaction plates with optical caps (Applied Biosystems) as duplicates and triplicates for the determination of standard curves, respectively. Cycling conditions comprised a single step at 50°C for 2 min, subsequent 10 min polymerase activation at 95°C, followed by 95°C for 15 s and 60°C for 1 min (annealing and elongation). Forty cycles were run for amplification of templates. Data evaluation of real-time RT-PCR experiments was performed by normalizing relative expression levels of target genes to the geometrical mean of relative expression levels of the three housekeeping genes cyclophilin A (*PPIA*), phosphoglycerate kinase (*PGK1*), and porphobilinogen-deaminase (*PBGD*).

[1] http://www.ncbi.nih.gov/SAGE/.

RESULTS

Comprehensive gene expression profiles of three stage 4S and two stage 4 neuroblastomas were constructed by SAGE (17, 18). A total of 131,920 tags was sequenced with about 20,000–30,000 tags per library.[2] *PRAME* displayed significant differential expression according to the criteria of Audic and Claverie (19) as it lacked specific tag counts in all libraries from stage 4S neuroblastoma and was represented by 10 tags in each profile from stage 4 tumors. A similar expression pattern was observed by microarray analysis of the same tumor samples with Agilent Human 1A and Human 1B arrays (data not shown). These data suggested that *PRAME* might be linked to advanced neuroblastoma stages.

We therefore evaluated *PRAME* expression in a larger number of samples by both RT-PCR and Northern blot analysis. The panel of tumors used for this survey consisted of 94 primary neuroblastomas and comprised 46 samples of localized disease (stage 1–3), 28 samples of stage 4 disease, and 20 samples of stage 4S disease. Qualitative analysis of *PRAME* expression by RT-PCR detected specific amplification of *PRAME* in 93% (87 of 94) of all tumor samples, leaving 7% (7 of 94) of patients without detectable transcript levels. Amplification of β-*actin* as a positive control was seen in all patients, and unspecific amplifications for both *PRAME* and β-*actin* were excluded by no template controls. Absence of specific amplificates for *PRAME* was restricted to patients with favorable disease, with five samples of stage 4S and a single case of stage 1 and stage 2a, respectively, contributing to this group. Consequently, *PRAME* expression was universally observed in patients with advanced disease.

For quantitative information on *PRAME* expression by Northern blot analysis, commercially available testis total RNA and spleen total RNA were used as positive and negative control, respectively, because Northern blot analysis of various healthy tissues exclusively revealed *PRAME*-specific signals in testis (9). Considerable variation of *PRAME* signal intensities was observed, and results were therefore evaluated in a semiquantitative manner by summarizing patients in four groups according to the relation of their *PRAME* signal intensities to *PRAME* signal intensities of testis RNA. Scores from 0 to 3 were given to each tumor sample, with 0 being no detectable *PRAME* expression, 1 referring to *PRAME* signals in tumor RNA being weaker than signals in testis RNA, 2 accounting for equal signal intensities in both RNA, and 3 referring to samples with higher *PRAME* signal intensities than testis RNA (Fig. 1). Results of semiquantitative interpretation of Northern blot analysis are listed in Table 1. Application of this discrimination revealed highly significant association of higher levels of *PRAME* expression with advanced tumor stages ($P < 0.01$) and higher age of patients at diagnosis ($P < 0.01$). Because both are established valuable markers for the prognosis of patients, we assessed impact of *PRAME* expression on the outcome of patients by Kaplan-Meier analysis of both EFS and overall survival. Three-year EFS of patients with no detectable signal (score 0) was 0.89 (±0.07), compared with 0.72 (±0.09; score 1), 0.62 (±0.13; score 2), and 0.57 (± 0.10; score 3), respectively, although this was not significant ($P = 0.31$), probably because of the small number of patients per group. In contrast, distinguishing patients with a score of 0 from a combined group with scores of 1–3 pointed to an impact on the prognosis of patients. Whereas 3-year EFS was 0.89 (±0.07) for patients with a score of 0, it was 0.62 (±0.07) in the other group ($P = 0.07$; Fig. 2*A*). Similar results were observed for RT-PCR analysis, where no events occurred in patients in which no *PRAME* expression was detected by RT-PCR, whereas 3-year EFS was 0.66 (±0.06) for patients with amplification of *PRAME*-specific transcripts ($P = 0.13$; Fig. 2*B*). Comparing data from both techniques, it was suggested that lack of statistical significance was in part influenced by both the small number of patients without *PRAME* amplificates in RT-PCR analysis ($n = 7$) and the classification of patients according to semiquantitative analysis. We therefore reasoned that a more precise quantification of expression levels might reveal significant influence of *PRAME* expression on patients' outcome and applied quantitative real-time RT-PCR (QPCR).

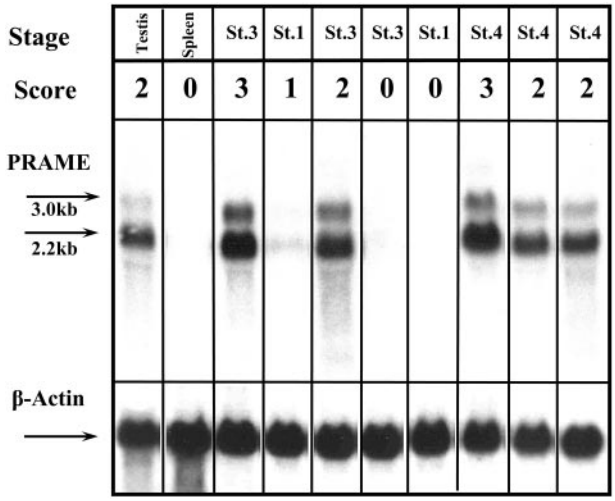

Fig. 1 Northern blot analysis of *PRAM*E expression in primary neuroblastoma. Tumor stages are listed in the *top row* with scores depicted below. Scores were determined by relating *PRAME*-specific signal intensities of tumor RNA (*Lanes 3–10*) to intensities of testis RNA (*Lane 1*; score 0, no signal; score 1, patient's signal < testis signal; score 2, patient's signal = testis signal; score 3, patient's signal > testis signal). Spleen total RNA served as negative control (*Lane 2*). Loading of near equal amounts of RNA was assured by stripping and rehybridizing blots with a probe for β-*actin*.

QPCR amplification of *PRAME* was performed in a panel of 95 primary tumor samples using SYBR green dye detection 1. Samples from 88 patients were analyzed previously by both RT-PCR and northern blotting. In addition, RNA from seven tumor samples without sufficient material for Northern analysis were used for this survey. Thus, the panel of tumors used for QPCR covered 27 samples of stage 4, 20 samples of stage 4S, and 48 samples of localized (stage 1–3) primary neuroblastoma. Specific amplification of *PRAME* by QPCR was observed in all patients, including six of seven patients with negative results in the conventional RT-PCR. QPCR data of the seventh patient

[2] M. Fischer, unpublished observations.

Table 1 Semiquantitative interpretation of *PRAME* expression by Northern blotting

Scores were determined by relating the *PRAME* signal intensities of patients to those of testis RNA. Both absolute numbers of cases (bold numbers) and percentage of patients within each stage (numbers in italics) are listed.

		Stage 1	Stage 2	Stage 3	Stage 4	All patients
Score	Stage 4S	$n = 24$	$n = 7$	$n = 15$	$n = 28$	$n = 94$
0	**9**	**8**	**1**	**2**	**0**	**20**
	45%	*33%*	*14%*	*13%*	*0%*	*21%*
1	**8**	**10**	**3**	**7**	**6**	**34**
	40%	*42%*	*43%*	*47%*	*21%*	*36%*
2	**1**	**2**	**2**	**1**	**8**	**14**
	5%	*8%*	*29%*	*7%*	*29%*	*15%*
3	**2**	**4**	**1**	**5**	**14**	**26**
	10%	*17%*	*14%*	*33%*	*50%*	*28%*

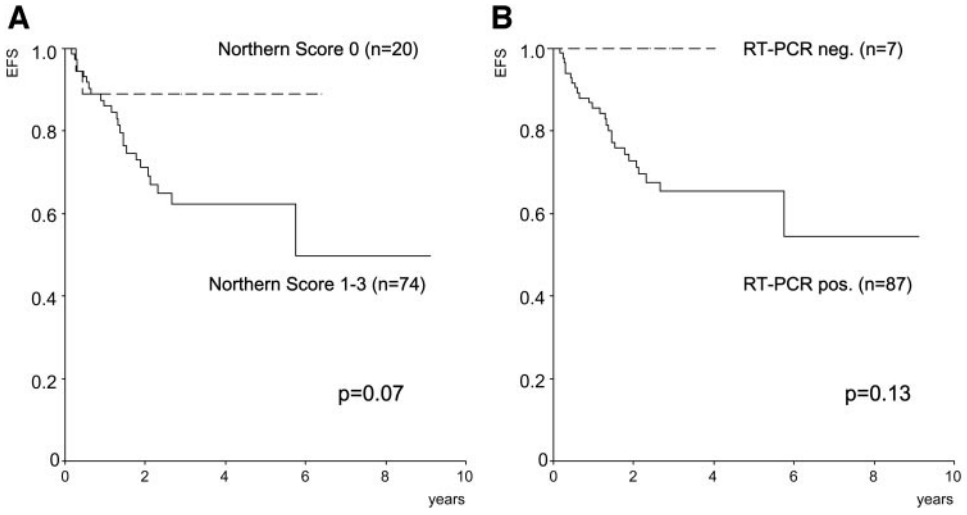

Fig. 2 Univariate Kaplan-Meier analysis for event-free survival according to Northern blot scores (*A*) and reverse transcription (RT)-PCR amplification of *PRAME* (*B*)

could not be generated because of the lack of further material from this patient. For quantification of *PRAME* transcript levels, relative QPCR values for *PRAME* were normalized to the geometrical mean of the three control genes *PBGD*, *PPIA*, and *PGK1*. We observed that reliability of transcript quantification by QPCR in primary neuroblastoma is enhanced considerably by this normalization procedure.[3] Subsequently, normalized expression levels were related to the minimum value within the cohort, thus describing values as the *n*-fold multiple of the lowest level within the panel of tumors. By using this calculation, 130,000-fold oscillation of *PRAME* expression between minimum and maximum values was observed. Results of QPCR were highly concordant with both results from Northern blotting (Fig. 3*A*) and conventional RT-PCR (data not shown). Moreover, highly significant association of higher QPCR values with both tumor stage (Fig. 3*B*) and age of patients at diagnosis (>1 year *versus* <1 year, each $P < 0.01$, data not shown) was observed. For assessment of prognostic impact of *PRAME* expression levels, different thresholds of QPCR values were set, with cutoffs of 15,000 and 20,000 displaying significant influence on EFS ($P = 0.02$ and $P = 0.01$, respectively). Whereas EFS was 0.76 (±0.06) for 63 patients with values <20.000, it was 0.45 (±0.10) for the 32 patients with values ≥20,000 ($P = 0.013$). The threshold of 20,000 also exerted significant impact on overall survival (0.89 ± 0.05 *versus* 0.77 ± 0.08; $P < 0.05$). Moreover, a trend toward significant prognostic influence was found for thresholds of low QPCR values. EFS was 0.92 (±0.07) for 13 patients with QPCR values <100 *versus* 0.61 (±0.06) for 82 patients with values ≥100 ($P = 0.085$). No fatal events were observed in patients with QPCR values <100, whereas 3-year overall survival for patients with values >100 was 0.83 (±0.05; $P = 0.16$).

Given these data, we combined both thresholds creating groups of patients with QPCR values <100, 100–20,000, and >20.000. Three-year EFS was 0.92 (±0.07) for 13 patients with QPCR values <100, 0.71 (±0.08) for 50 patients with values from 100–20,000 and 0.45 (±0.10) for 32 patients with values >20,000 ($P = 0.03$). Thus, *PRAME* expression levels functioned to significantly discriminate the outcome of patients. Three-year overall survival for these groups was 1.00, 0.86 (±0.06), and 0.77 (±0.08), respectively, although not statistically significant ($P = 0.10$; Fig. 4). Despite the obvious influence of *PRAME* expression on the prognosis of patients, no

[3] M. Fischer. Data submitted for publication.

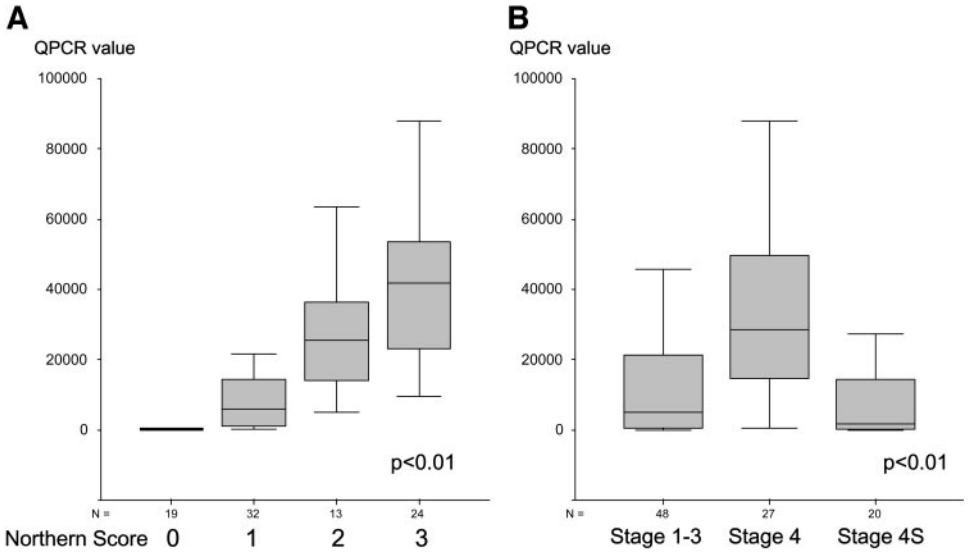

Fig. 3 Correlation of QPCR values with scores of Northern blot analysis (*A*) and with tumor stages (*B*). Median QPCR values for Northern scores were 75 (score 0), 5,800 (score 1), 25,515 (score 2), and 41,750 (score 3), respectively. For tumor stages median QPCR values were as follows: localized stages, 5,000; stage 4, 28,689; and stage 4S, 1,824. *QPCR*, quantitative real-time RT-PCR.

significant association with amplification of the *MYCN* oncogene, another important marker for prognosis, was detected in this study. However, *MYCN* oncogene was restricted to patients who showed amplification of *PRAME* by RT-PCR as well as QPCR values >100. Thus, the lack of significant correlation with *MYCN* oncogene might be biased by the limited number of patients with *MYCN* oncogene ($n = 10$) in this cohort.

DISCUSSION

Although the necessity for treating patients with favorable stage 1 or stage 4S neuroblastoma was successfully reduced over the last decades, most patients with higher stage disease still follow a fatal course despite intensive treatment regimes. Therefore, identification of target antigens for novel therapeutic strategies is of crucial clinical importance. Because *PRAME*, a potential target for future immunotherapeutic treatment, displayed significant differential expression in SAGE libraries of three-stage 4S and two-stage 4 primary neuroblastoma, its abundance was evaluated in a considerable number of patients.

Our data show that *PRAME*-specific transcripts can be found in 93% of patients with primary neuroblastoma, as determined by RT-PCR. To date, such remarkable frequency of *PRAME* expression has only been observed in melanoma (9–11, 14, 20). Moreover, to our knowledge, this incidence exceeds that of any other CTA in primary neuroblastoma (5–8, 21, 22). In addition, *PRAME* expression was detected in all specimens from advanced tumor stages, thus comprising all high-risk patients who are potential candidates for immunotherapeutic treatment options. However, although the number of patients in whom RT-PCR successfully detected *PRAME* transcripts is remarkable, benefits of immunotherapeutical strategies also depend on the percentage of neuroblastoma tumor cells positive for this CTA. It is assumed that targeting of antigens expressed in small portions of tumor cells are probably of lesser therapeu-

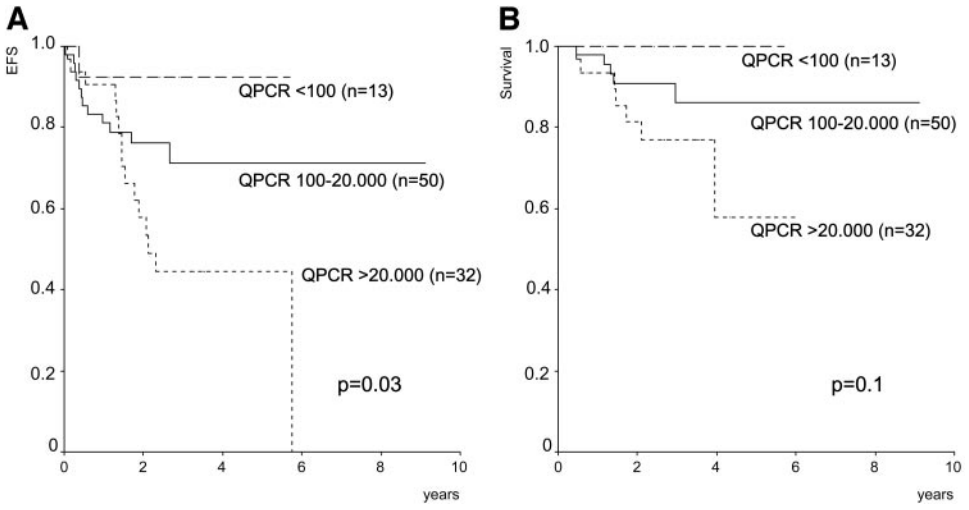

Fig. 4 Univariate Kaplan-Meier analysis for event-free survival (*A*) and overall survival (*B*) for three groups of patients with QPCR values <100, 100–20,000, and >20,000. *QPCR*, quantitative real-time RT-PCR.

tical value (4). Unfortunately, efforts to quantify the number of tumor cells expressing *PRAME* with commercially available antibodies were neither successful for immunohistochemical staining of cryosliced neuroblastoma specimen nor for Western blotting or fluorescence activated cell sorting analysis of cultured neuroblastoma cells (data not shown). Thus, we focused on measuring gene transcript levels, which are usually closely related to the amount of translated protein and also provide information about a gene's biological function. In our study, we performed both Northern blotting and QPCR in a total of 101 patients with primary neuroblastoma. Data from both techniques were highly concordant ($P < 0.01$) as shown in Fig. 3*A* and also matched excellently with qualitative information from conventional RT-PCR. Comparing all three techniques, we found that QPCR was the most sensitive method for detecting gene-specific transcripts. Specific amplification of *PRAME* by QPCR was successful in all patients, including those cases in which conventional RT-PCR did not detect *PRAME* transcripts. This effect might be explained by the higher number of cycles in the QPCR protocol and by the higher sensitivity of SYBR green dye detection in comparison with ethidium bromid visualization of PCR products. Given the notion that conventional RT-PCR showed specific amplification in 13 of 20 Northern blot negative patients, it can be derived that sensitivity of QPCR is superior to that of Northern Blotting. More precisely, we would estimate that in our study, QPCR was approximately 75-fold more sensitive than Northern blotting, as this was the median QPCR value for patients with a score of 0 in Northern blotting. Although transcript levels of these patients should be considered rather low, it is nonetheless noteworthy that sensitive detection methods reveal measurable amounts of *PRAME* transcripts in all samples of neuroblastoma in our survey. In contrast, high abundance of *PRAME* transcripts can be assumed in patients with Northern scores ≥ 2, as was noted in 40% of all patients and 80% of patients with stage 4 disease (Table 1). Regarding data from Ikeda *et al.* (9) who observed higher *PRAME* expression levels in testis than in melanoma cells from the patient in whom *PRAME* originally was discovered, transcript levels of *PRAME* in neuroblastoma should be considered to be very high in the majority of patients with higher tumor stages. Although reflections on the question of *PRAME*'s immunogenicity allow for different conclusions (23), lysis of various tumor cell lines by CTL in an HLA-restricted manner has been demonstrated by Kessler *et al.* (15). Taking these results into account, our observation of universal expression of *PRAME* in advanced stages of neuroblastoma and high expression levels in the majority of these patients qualify *PRAME* as a particularly attractive target for immunotherapy in neuroblastoma.

Given the fact that significant association of higher levels of *PRAME* expression with both advanced tumor stages and patient's age at diagnosis was shown by all techniques, we furthermore assessed the impact of *PRAME* on the prognosis of patients. Regarding RT-PCR data, we noted that the outcome of patients in the subset of samples that did not show amplification of *PRAME* transcripts was excellent. Although no events occurred in this group, data for EFS and overall survival lacked statistical significance, probably because of the small numbers of patients. Discriminating groups according to their scores in Northern blotting resulted in 3-year EFS of 0.89 (± 0.07) for patients with a score of 0 and 0.62 (± 0.07) for the combined group of patients with scores 1–3 ($P = 0.08$), thus pointing to a possible influence of *PRAME* expression on the outcome of patients but still without statistical significance. A closer look at the clinical data revealed that one of the two patients who had an event in the group with a Northern score of 0 was classified as stage 4S and displayed deletion of the chromosomal region of 11q, as assessed by fluorescent *in situ* hybridization. This cytogenetic aberration has been shown to be associated with poor prognosis in both localized and stage 4S disease in a highly significant manner (24). Because the patient described showed *PRAME* expression by RT-PCR, we reasoned that the more sensitive and precise determination of *PRAME* transcript levels by QPCR might disclose significant influence on the outcome of patients. Therefore Kaplan-Meier analysis on EFS with different QPCR scores as cutoff were performed, and significant impact on the prognosis of patients for various thresholds between 10,000 and 20,000 was observed, whereas a low threshold of 100 showed only a trend toward significant influence on the outcome of patients. With regard to the still elusive function of *PRAME*, one can speculate that high abundance of PRAME transcripts is necessary for inducing unfavourable biological effects in patients with neuroblastoma. However, when assigning patients to three groups with QPCR values <100, 100–20,000, and $>20{,}000$, *PRAME* expression levels functioned for significant discrimination of the outcome of patients. Thus, lower levels of *PRAME* also appear to be of biological importance.

In general, data that highlight clear association of expression of tumor-associated antigens with the outcome of patients are still rare, although association of tumor-associated antigens with higher tumor stages or advanced disease were shown in a couple of studies (25–27). Similar to our observation, Steinbach *et al.* (28) showed that *PRAME* exerted significant influence on the outcome of patients in a panel of 50 children with acute myeloid leukemia and that *PRAME* expression correlated significantly with WBC count at diagnosis or t (8, 21). Maybe, the fact that *PRAME* seems to be of prognostic value for different malignancies signifies that *PRAME* expression is somehow linked to the transformation of tumor cells to a more aggressive phenotype.

Taken together, our data demonstrate that *PRAME* expression is an extraordinarily common phenomenon in primary neuroblastoma that includes all patients with advanced disease. Moreover, remarkably high levels of *PRAME* transcript were found in higher stages of neuroblastoma. Finally, *PRAME* expression in neuroblastoma specimen also exerts significant influence on the outcome of patients. We therefore conclude that patients with neuroblastoma might benefit from any successful immunotherapeutic approach targeting *PRAME*, although the question whether patients will be able to mount an immunological response to this antigen has yet to be clarified.

ACKNOWLEDGMENTS

We thank Yvonne Kahlert for excellent technical assistance, Dr. Karen Ernestus for assessment of tumor cell content, and Gabriele Benz-Bohm for excellent cooperation.

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