Identification of DEIN, a Novel Gene with High Expression Levels in Stage IVS Neuroblastoma

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Abstract

Neuroblastoma at stage IVS, defined by dissemination to specific tissues and age <1 year at diagnosis, regularly follows spontaneous regression without cytotoxic treatment. To uncover the molecular characteristics of this subtype, Serial Analysis of Gene Expression (SAGE) profiles from stage IVS and fatal stage IV tumors were compared. A SAGE tag (GCAACTTTAAC) was detected that was overrepresented in stage IVS disease and that had no reliable match in current National Center for Biotechnology Information databases of SAGE profiles, thus pointing to a novel gene. The corresponding gene, which maps to chromosome 4q33-34, was identified using a modified 3'- and 5'-rapid amplification of cDNA ends PCR and was designated as DEIN (differentially expressed in neuroblastoma). The gene comprises five transcript variants and its sequence overlaps with expressed sequences of the yet uncharacterized UniGene cluster Hs.61435. DEIN exhibits nucleotide sequence conservation over a broad range of species with an overall homology of 65% between human and mouse. As none of the predicted amino acid sequences is homologous to known proteins, it remains to be determined whether DEIN represents a coding or noncoding RNA. Northern blot analysis and semiquantitative reverse transcription-PCR showed high DEIN expression in neuroblastoma, whereas expression was absent or weak in most normal adult tissues. Analysis of 121 primary neuroblastomas by real-time reverse transcription-PCR revealed a strong association with age at diagnosis <1 year and particularly with stage IVS disease (both \( P < 0.001 \)). The characteristic expression pattern of DEIN suggests a specific role of this gene in the unique biology of stage IVS tumors and may help to molecularly define this special subtype of neuroblastoma. (Mol Cancer Res 2007;5(12):1276–84)

Introduction

Neuroblastoma is a malignant pediatric tumor originating from migrating neural crest cells, which accounts for ~8% of all childhood cancers (1). Remarkably, the tumor may follow contrasting patterns of biological behavior, which are most strikingly reflected by the clinically recognizable syndromes defined as stage IVS and stage IV, which both present with disseminated disease although having completely different courses: whereas stage IVS tumors are characterized by spontaneous regression of the primary tumor and metastases with little or no therapy, patients with stage IV disease present with rapidly progressing metastatic disease that is difficult to treat and has a poor prognosis. The molecular mechanisms leading to the diverse clinical courses of disseminated neuroblastoma are largely unknown and remain one of the most intriguing questions in neuroblastoma research. Whereas delayed onset of programmed cell death of neuroblasts has been proposed to account for the phenomenon of spontaneous regression of stage IVS disease and tumors in children diagnosed before the age of 1 year (2), the reasons for dissemination of disease before regression in stage IVS patients remain elusive. In recent years, gene expression profiling using microarrays has been used to identify the molecular characteristics of neuroblastoma (3-8); however, whereas several gene expression patterns associated with favorable patients’ outcome were described, none of these did contribute to the elucidation of the peculiar biology of stage IVS neuroblastoma or reported on a specific molecular marker defining this subtype of the disease.

Serial Analysis of Gene Expression (SAGE) has been shown to be a powerful tool to identify differentially expressed genes in cancer and to effectively discover novel cancer targets (9, 10). Using this technique, we have generated gene expression profiles of five favorable stage IVS and three unfavorable stage IV neuroblastomas to identify transcripts that represent specific markers of these two subtypes and that may account for the characteristic biology of either phenotype (11). In this study, we describe a novel gene designated as DEIN, the expression of which is strongly associated with stage IVS disease and which may contribute to the molecular definition of this particular subtype of neuroblastoma.
Results
Identification of the Full-length cDNA Sequences of the Novel Gene DEIN

The SAGE tag GCAACTTAAC was found to be abundantly represented in libraries generated from five stage IVS and three stage IV neuroblastomas [absolute tag count: 144; tag count normalized to 10^6 tags: 809 corresponding to 0.08% of the total tag population; SAGE data have been deposited in National Center for Biotechnology Information Gene Expression Omnibus (GEO)1 and are accessible through GEO Series accession no. GSE4991]. The normalized expression value was 2.5-fold higher in stage IVS as compared with stage IV profiles, which was a significant difference according to Fisher’s exact test (P < 0.001). Tag-to-gene alignment using the SAGEmap database2 assigned this tag to UniGene cluster Hs.177532, which has recently been retired. Transcribed sequences of this cluster have been incorporated into UniGene cluster Hs.61435; however, all of them are designated “suboptimal members of this cluster” because they do not match the model reference sequence of this cluster (XM_496724). In line with this, tag-to-gene alignment using the SAGEGenie database3 did not assign this tag to any known gene. According to SAGEmap, tag GCAACTTAAC was rarely detected in published human SAGE libraries, with only the following, of 327 profiles, showing more than one copy: medulloblastoma (GEO accession no. GSM14779; absolute tag count: 5; tag count normalized to 10^6 tags: 69), adrenal cortex affected by primary adrenocortical disease (GSM37212; 4; 127), mammary gland carcinoma (GSM14745; 3; 36), normal heart (GSM1499; 2; 27), and normal liver (GSM785; 2; 29).

To convert the tag GCAACTTAAC back into its corresponding full-length transcript, we carried out Generation of Longer cDNA fragments from SAGE tags for gene identification (GLGI; ref. 12), 5’-rapid amplification of cDNA ends (RACE)-PCR, and reverse transcription-PCR (RT-PCR). Using GLGI, two 3’-cDNA fragments measuring 57 and 158 bp were detected, the latter of which comprised the 57-bp fragment and an additional 101 bp at the 3’ end. Both fragments contain a polyadenylation signal (AATAAA) within the last 50 bp 5’ of the polyadenylate tail. Considering this additional sequence information, the cDNA was mapped to chromosome 4q33-34. Consistent with our results, several 3’ reads of transcribed sequences assigned to cluster Hs.61435 match the experimentally generated 3’ ends (e.g., CR623023; Fig. 1A).

To determine the full-length sequence of the identified transcript, a combined 5’-RACE and RT-PCR approach was used. In total, five major transcript variants were detected by these experiments (Fig. 1A), all of which comprise the exons 1, 2, and 4 at their 5’ end but differ in their 3’ regions. Variant A covers 4,186 bp and exhibits the same splicing sites between exons 4 and 5 as XM_496724, the computationally predicted model sequence of UniGene cluster Hs.61435. Variant B, which is 5,278 bp in length, consists of exons 1 and 2 as well as the complete genomic nucleotide sequence from the 5’ part of exon 4 to the 3’ part of exon 5 (designated as exon 4’). Variants C and E are identical to variant A in their 5’ part and their 3’ tail but display enlarged introns between exons 4 and 5. Both variants exhibit variable splicing sites at the 5’ end of exon 5, which comprises 153- and 356-bp isoforms in variant C and 2,734-, 2,787-, 2,864-, and 2,999-bp isoforms in variant E. Because various transcribed sequences of UniGene cluster Hs.61435 (Fig. 1A) differ considerably in their 3’ ends from our experimentally determined variants, we carried out additional 3’-RACE experiments. Two alternative 3’ ends were identified, one of which is identical to the 3’ end of XM_496724 and the other extends 32 bp into 3’ direction (variant D). A SAGE tag (ATTGTGTAC) corresponding to these shorter transcripts was detected in our SAGE profiles; however, the total tag count for this transcript was low with only a weak indication of differential expression (total absolute tag count in all profiles: 4; 1 and 3 tags in stage IVS profiles neuroblastoma 1 and neuroblastoma 2, respectively) as opposed to tag GCAACTTAAC.

Typical intronic splicing sites (GT/AG) were identified in all isoforms. The 5’ ends of all variants extend 21 bp into 5’ direction of the 5’ end of the model sequence XM_496724. In a few cDNA clones of variant C, an additional exon of 209 bp (exon 3) was identified.

To determine whether the variants identified by RACE and RT-PCR represent full-length transcripts, Northern blot analysis of primary neuroblastoma samples was done with five specific probes (Fig. 1B). In stage IVS tumors, two intense signals of about 4 and 5 kb were detected with probes I to IV, whereas probe V only revealed a 5-kb transcript (Fig. 1A and B, results with probes I, III, and V are shown). The two signals at 4 and 5 kb correspond well to variants A and B, respectively. Because no additional signals were observed in the Northern blot analysis with probe III, which was designed to detect the four variants A, B, D, and E, one can suggest that the latter two variants represent minor isoforms of this gene. The finding of a single signal at 5 kb using probe V is in line with the proposed structure of variant B (Fig. 1A) because this region is not covered by any other exon of this gene. Transcript variant C was not detected in an additional Northern blot analysis using a probe specific to exons 1, 2, and 4 (data not shown). Because of the strong and differential expression of both variants A and B in primary neuroblastoma, the gene was designated as DEIN (differentially expressed in neuroblastoma). A search in the human UCSC BLAT database4 disclosed numerous transcribed sequences with the splicing sites of variant A (e.g., BC101437 and BC101981) as well as sequences that match a fragment specific for variant B (BC009184, AU118035, and AL522836). In contrast, only one expressed sequence tag (EST) showed a splicing site identical to variant C (EST R24426) and no transcribed sequence with the splice sites of variant E or D was found. These findings support our experimental results suggesting that the isoforms A and B represent the major transcript variants of DEIN.

None of the cDNAs encoding the variants A to E of DEIN contain repetitive sequence elements. Transcript variant A harbors an open reading frame encoding a putative protein of 134 amino acids, which is identical to the putative protein XP_496724

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3 http://cgap.nci.nih.gov/SAGE/AnatomieViewer
4 http://genome.ucsc.edu/cgi-bin/hgBlat
The transcript variants B to E comprise open reading frames ranging from 38 to 143 amino acids in length. Strong or adequate contexts of start codons according to Kozak's rule, together with conservation of start codons and the following amino acid sequences in other species, have been regarded as minimal prerequisites for attributing the status of a coding transcript to the sequence (13, 14). The only putative start codon of DEIN in a strong context is located in exon 4 and corresponds to the coding sequence of XM_496724. However, because this start codon is not conserved in other species, and the predicted proteins do not show significant homology to known proteins or conserved domains as determined by a protein-protein-BLAST-alignment, it remains to be determined whether these sequences represent coding transcripts or noncoding RNAs.

The nucleotide sequences of DEIN variants A and B are conserved among various mammals such as dog, mouse, and rat, with similarities ranging from 62% to 71% (Fig. 2). A 250-bp region located between two CpG islands in the last exon of these variants shows the highest degree of conservation, particularly in a 43-bp segment exhibiting 88.4% identity between human and zebrafish (Figs. 1A and 2). Various conserved transcription factor binding sites were detected in this sequence by in silico prediction. The highest homology was found for transcription factor binding sites of Nkx-2.5, Pbx-1, GATA-1, and CCAAT/enhancer binding protein-β, which were conserved in all species with only 1-bp variation in zebrafish and/or chicken (data not shown). In other species, numerous transcribed sequences that match the genomic region of DEIN were found in a BLAT search. In mouse, most of these ESTs correspond to exons 2 and 4 and originate almost exclusively from heart (numerous ESTs) as well as fetal and embryonic tissues, including trophoblast (BQ032256 and BU755815), hematopoietic stem cells (DT915657), and a sympathetic ganglion of a 2-day neonate.

5 http://www.ncbi.nlm.nih.gov/_BLAST
Expression of the Novel Gene DEIN in Stage IVS Neuroblastoma

Northern blot analysis revealed strong DEIN expression in five stage IVS tumors and weak DEIN expression in six stage IV tumors, which supports the SAGE results suggesting differential expression of DEIN between these two stages (Fig. 1B). In contrast, no hybridization signal to any of the polyadenylate RNAs from a commercially available Northern blot containing RNAs of 10 different human adult tissues (brain, liver, placenta, small intestine, colon, pancreas, spleen, prostate, testis, and ovary) was observed, indicating that expression in these tissues is very low or absent (data not shown). To further evaluate DEIN expression, a panel of 16 different human adult and embryonic as well as 6 neuroblastoma cDNAs were analyzed by semiquantitative RT-PCR with primers selectively detecting the major variants A and B as well as the minor variant E. A 140-fold oscillation between minimum and maximum relative mRNA levels with a median value of 22.4 was detected. Stage IVS tumors displayed a 3-fold higher median expression value for DEIN as compared with localized stage I to III or stage IV tumors (P < 0.001; Fig. 4A). Transcript levels of localized tumors were also significantly higher than those of stage IV tumors (P = 0.033). Moreover, patients diagnosed before the age of 1 year had significantly higher expression values of DEIN as compared with those diagnosed later (P < 0.001; Fig. 4B). When stage IVS patients (who are <1 year by definition) were excluded from this analysis, DEIN expression still was significantly higher in patients diagnosed before the age of 1 year (P < 0.001; Fig. 4C), indicating that the strong correlation between expression and age is not simply due to the high number of stage IVS patients in this cohort. Significantly higher mRNA levels were also found in younger patients after applying an age cutoff at 18 months (P = 0.005). In contrast, no significant association between DEIN expression and the cytogenetic aberrations MYCN amplification (P = 0.43), 1p deletion (P = 0.13), 11q deletion (P = 0.09), 3p deletion (P = 0.32), or 17q gain (P = 0.43) was observed.

To evaluate whether DEIN mRNA levels are associated with neuroblastoma patients’ outcome, Kaplan-Meier curves for event-free survival and overall survival were calculated. Choosing the median expression value (22.4) as a threshold, DEIN expression was found to be significantly correlated with event-free survival: whereas 3-year event-free survival was 0.76 ± 0.06 for patients with expression levels >22.4, it was 0.59 ± 0.06 for patients with lower transcript levels (P = 0.014; Fig. 4D). Overall survival for these two groups was 0.93 ± 0.03 and 0.78

* GNF Expression Atlas 2 Data from U133A and GNF1H Chips: http://genome.ucsc.edu/cgi-bin/hgGene.
DEIN subgroup (3-year event-free survival: 0.70 ± 0.05, respectively (*P* = 0.093). Considering only patients with disseminated neuroblastoma (stages IV and IVS), DEIN expression again distinguished a favorable and an unfavorable subgroup (3-year event-free survival: 0.70 ± 0.08 versus 0.44 ± 0.08, *P* = 0.006; Fig. 4E). In the analysis of the prognostic relevance of DEIN expression within the three subgroups of localized stages, stage IVS and stage IV patients revealed that DEIN expression was associated with patients’ outcome only in the cohort of stage IVS disease (3-year event-free survival: 0.90 ± 0.07 versus 0.57 ± 0.19, *P* = 0.021; Fig. 4F). To test whether DEIN expression is an independent prognostic marker for neuroblastoma patients, a multivariate analysis according to Collett (15) was done considering the variables age at diagnosis (continuous), 11q status (aberration versus normal), MYCN amplification (amplified versus nonamplified), stage (localized versus stage IV versus stage IVS), 1p status (aberration versus normal), and DEIN expression level according to quantitative real-time RT-PCR (relative expression <22.4 versus >22.4). In this analysis, only age at diagnosis (*P* < 0.001; hazard ratio, 1.14; 95% confidence interval, 1.08-1.20), loss of 11q (*P* = 0.005; hazard ratio, 2.50; 95% confidence interval, 1.34-4.67), and *MYCN* amplification (*P* = 0.006; hazard ratio, 3.34; 95% confidence interval, 1.54-7.21) were independent prognostic markers, whereas stage (*P* = 0.311), loss of 1p (*P* = 0.222), and DEIN expression (*P* = 0.157) were not.

In contrast to these findings, examination of variant C transcript levels by quantitative real-time RT-PCR in a cohort of 76 primary neuroblastoma specimens (stage IV, *n* = 49; stage IVS, *n* = 27) with primers specific for this isoform did not reveal a significant association of this splice variant with stage or age, whereas an inverse correlation with *MYCN* amplification was observed (*P* = 0.001). In addition, relative expression levels of the major transcript variants and variant C were not correlated (Pearson’s correlation coefficient, 0.18).

**Discussion**

We report on the identification and genomic characterization of DEIN, a novel gene that exhibits a characteristic expression pattern in primary neuroblastoma. With a combined RACE and RT-PCR approach, the full-length sequences of the main transcript variants of this gene were characterized. According to SAGE, Northern blot, and quantitative real-time RT-PCR, variants A and B represent the major transcripts of DEIN in primary neuroblastoma. These isoforms are strongly expressed in stage IVS tumors, whereas lower expression levels were observed in stage IV tumors. In contrast, expression of transcript variant C did not differ in these stages, as determined by quantitative real-time RT-PCR, and seemed to be expressed at lower levels in neuroblastoma because it could not be detected by Northern blot hybridization and C1 values of quantitative real-time RT-PCR analysis were high (data not shown). In addition, low numbers of SAGE tag ATTTGTTACA corresponding to transcript D suggested that this isoform is a minor splice variant in neuroblastoma as well. Because there is a significant sequence overlap between DEIN and expressed sequences of UniGene Cluster Hs.61435, we conclude that these transcripts originate from a single gene. Whereas DEIN variants A and B are mainly expressed in neuroblastoma, the remaining isoforms may represent splice variants expressed at lower levels in neuroblastoma or variants that are preferentially expressed in other tissues.

The genomic sequence of DEIN was found to be conserved among various species with the highest degree of conservation in a 250-bp region in the last exon of variants A and B. This region may represent a *cis-regulatory* element for DEIN transcription (Fig. 2), which is supported by the finding of several conserved putative transcription factor binding sites within this sequence. Interestingly, the transcription factor binding site CCAAT/enhancer binding protein-β has been shown to have a regulatory function in differentiation and apoptosis of neuroblastoma cells (16), the latter of which is suggested to account for spontaneous regression in stage IVS neuroblastoma. Alternatively, this DNA segment could also represent a currently unknown *cis-regulatory* element for the control of expression of *HAND2*, a gene that is located closely upstream and in antisense orientation to DEIN and functions as a key regulator of neurogenesis in the developing sympathetic nervous system. This suggestion is supported by the high degree of homology of *HAND2*, its enhancer element for expression in branchial arches (located between exon 3 and 4 of DEIN; ref. 17), and the present putative regulatory region between human and zebrafish (Fig. 2), whereas the remaining sequences of DEIN variants A and B are not conserved in zebrafish.

![FIGURE 3. Analysis of DEIN and β-actin (control) expression levels in 22 human tissues after 25 and 35 cycles of amplification by RT-PCR. The primers used for amplification were F1 and R3 (Table 1).](image-url)
Only one of the putative open reading frames of the DEIN transcripts has a start codon in a strong context according to Kozak’s rule, but this codon is not conserved in other species and the resulting proteins do not have known functional domains or match known proteins. Because comparative genome analysis (i.e., detection of significant conservation of open reading frames in related species) is suggested to be a reliable method to determine whether a transcript is protein coding or not (18), one may suggest that DEIN comprises noncoding RNAs. In line with this, SAGE tags not matching a known gene have been reported to frequently correspond to noncoding RNAs that are expressed in antisense orientation to known genes or from loci outside of currently annotated exons (i.e., intergenic and intronic regions; ref. 19). The high degree of conservation of certain nucleotide segments of DEIN in different species could be due to specific consensus secondary structures that many noncoding RNAs evolutionary conserve and that are related to their function (18).

RT-PCR and Northern blot analyses revealed strong expression of transcripts A and B in primary neuroblastoma and neuroblastoma cell lines, whereas lower expression levels were observed in heart, uterus, placenta, adrenal gland, cerebellum, testis, and fetal liver (Fig. 3). These findings are consistent with the rare occurrence of the corresponding SAGE tag in expression profiles of human tissues currently published in the SAGEmap database and with tissue sources of ESTs of UniGene cluster Hs.61435. Interestingly, the tissue expression pattern of DEIN strongly parallels that of HAND2. According to the UniGene database, cluster Hs.61435 shares common tissue sources for ESTs with HAND2, such as neuroblastoma, heart, uterus, placenta, and brain, and a substantial number of ESTs assigned to these clusters originate from embryonic tissues or those of children <3 years of age. Together, these findings might suggest that HAND2 and DEIN are regulated by common transcriptional mechanisms in a restricted range of tissues during early developmental stages.

This hypothesis is further supported by the observation of high DEIN transcript abundances in neuroblastoma of patients <1 year old, whereas older patients showed lower levels of expression. In addition, the description of two ESTs in mouse with sequence homology to variant A that originated from a sympathetic ganglion of a 2-day mouse neonate, together with the observation of high DEIN expression in neuroblastoma of infants, might suggest that DEIN may particularly play a role in the development of the sympathetic nervous system of mammals.

FIGURE 4. Relative expression levels of DEIN in neuroblastoma subsets according to stage (A), age at diagnosis (B), and both (C) as determined by quantitative real-time RT-PCR. Kaplan-Meier curves for event-free survival of neuroblastoma patients of all stages (D), disseminated stages IV and IVS (E), and stage IVS only (F) according to high and low DEIN expression levels (see text).
Within primary neuroblastoma subtypes, highest expression levels of the main variants A and B of DEIN were found in stage IVS tumors. Whereas numerous genes such as TrkA (2), CD44 (20), ECEL1 (21), and ICE (22) have been shown to be strongly expressed in favorable neuroblastosoma comprising both locoregional and stage IVS disease, this is the first report of a gene that exhibits high transcript abundances selectively in stage IVS neuroblastoma. One could therefore speculate that DEIN might be involved in biological processes related to the unique stage IVS phenotype, which is characterized by dissemination to specific tissues followed by subsequent spontaneous regression. In addition, whereas multivariate analyses suggested that the clinical value of DEIN expression levels for patients’ outcome prediction seems to be limited as compared with established prognostic markers, the characteristic expression pattern could contribute to the definition of this stage as a separate biological subtype of neuroblastoma. Moreover, high expression levels of DEIN were found to be associated with age at diagnosis <1 year even after exclusion of stage IVS tumors. In light of the beneficial outcome of infants in comparison with patients >12 to 18 months (23, 24), expression of DEIN may represent a molecular correlate of the distinct biology of these tumors in comparison with neuroblastoma developing in older children. However, the biological role of DEIN in neuroblastoma and normal development has yet to be determined by functional analyses.

Materials and Methods
Generation of SAGE Profiles and Selection of Tags for Gene Identification

SAGE libraries were constructed from eight primary neuroblastomas using the I-SAGE Kit (Invitrogen) as described elsewhere (11). Tag-to-gene alignment was done by comparing SAGE tags to the human UniGene reference database, build #166.7 Differentially expressed tags that could not be assigned to known genes, tags linked to UniGene clusters consisting only of ESTs, and those assigned to multiple genes were selected for further characterization by GLGI.

Generation of cDNA, Cloning, and Sequencing of PCR Products

Generation of cDNA used for GLGI, 5’-RACE-PCR, and RT-PCR was done with 0.4 to 2 μg of polyadenylate RNA of a primary stage IVS tumor that had a high count for tag GCAACTTAAC (GEO accession no. GSM112808). Synthesis of cDNA for semiquantitative RT-PCR and real-time RT-PCR was carried out using 2 μg of total RNA of normal human tissues (Clontech), neuroblastoma cell lines, and primary neuroblastoma samples. For RT-PCR, cDNA synthesis was done with the Qiagen LongRange 2Step RTPCR kit according to the manufacturer’s protocol. For real-time RT-PCR, first-strand cDNA synthesis was done with oligo(dT) primers (Sigma-Aldrich) in a total volume of 20 μL according to the SUPERSCRIPT II protocol (Invitrogen). Cloning of PCR products was carried out using the TOPO TA-Vector (Invitrogen). Sequencing was done with the BigDye terminator sequencing kit, version 3.1 (Applied Biosystems).

Generation of Longer cDNA Fragments from SAGE Tags for Gene Identification (GLGI) 5’- and 3’-RACE-PCR

To identify tags that did not match a known gene, GLGI was done as described previously (12). GLGI is a modified 3’-RACE-PCR and converts SAGE tags back into their corresponding 3’-cDNA fragments. 5’- and 3’-RACE-PCR was done with the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer’s protocol. Primers used for amplification are available on request.

Northern Blot Analysis

Ten micrograms of total RNA from primary neuroblastoma samples were size fractionated on a 1% denaturing formaldehyde agarose gel and transferred onto a nylon membrane (Roche Diagnostics) using Northern Max One-Hour Transfer Buffer (Ambion). Expression in normal adult tissues was assessed using the FirstChoice Human Blot II (Ambion) containing 2 μg of polyadenylate RNA from 10 adult human tissues. Five cDNA probes for DEIN and one probe for β-actin were generated by RT-PCR. Primers used for amplification of probes I to V are shown in Table 1 (F1 + R1 for probe I, F2 + R4 for probe II, F3 + R5 for probe III, F5 + R6 for probe IV, and F6 + R7 for probe V). Blots were hybridized overnight at 42°C in UltraHyb Hybridization Buffer (Ambion) with high-sensitivity strippable DNA probes labeled with [α-32P]dATP (Strip-EZ DNA kit, Ambion). After hybridization, membranes were washed, air-dried, and exposed to Kodak BioMax MR-1 films (Amersham).

RT-PCR

PCR was carried out in a total volume of 50 μL containing 1-μL first-strand cDNA, 2 units HiFi Platinum-Taq DNA polymerase (Invitrogen), 125 mmol/L sense and antisense primers each, 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 200 mmol/L deoxynucleotide triphosphates each, and 1.5 mmol/L MgSO4 (Invitrogen). Cycling conditions consisted of a single denaturation step at 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 58°C for 1 min, and a final extension step at 72°C for 15 min. For amplification of overlapping subfragments of variants A and B, the primer combinations F7 + R6, F5 + R5, and F3 + R1 were used (Table 1). For amplification of variant E, the primers F7 to F9 together with R4 were used. PCR products were visualized on a 2% agarose gel. To exclude amplification of contaminating DNA, RT-PCR was done with negative controls without reverse transcriptase in first-strand cDNA reactions.

Semiquantitative RT-PCR

PCR was carried out in a total volume of 30 μL containing 1-μL first-strand cDNA, 1.2 units Platinum-Taq DNA polymerase (Invitrogen), 75 mmol/L sense and antisense primers (F1 + R3) each, 12 mmol/L Tris-HCl (pH 8.4), 30 mmol/L KCl, 120 mmol/L deoxynucleotide triphosphates each (Invitrogen), and 1 mmol/L MgCl2. Cycling conditions consisted of a single denaturation step at 95°C for 3 min, followed by various cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. To exclude amplification of contaminating DNA, RT-PCR was done with negative controls without reverse transcriptase in first-strand cDNA reactions.
Expression of the Novel Gene DEIN in Stage IVS Neuroblastoma

Table 1. Oligonucleotides Used as Primers in This Study

<table>
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<tr>
<th>Method</th>
<th>Name</th>
<th>Nucleotide Sequence</th>
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<td>GLGI</td>
<td>GF</td>
<td>5’-GGATCCTCGATCCCGAAGTCTAAC-3’</td>
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<td>GR</td>
<td>5’-CTACTCTAGAGCGCGCCCTT-3’</td>
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Note: Forward primers are denoted by F; reverse primers are denoted by R. GF and GR indicate the sense primer based on the SAGE tag sequence for GLGI and the universal antisense primer for GLGI, respectively. GSP1 was used as the gene-specific primer used for the detection of variant D by 3’-RACE. Primers F1-9 and R1-7 were used for RT-PCR, semi-quantitative RT-PCR, quantitative real-time RT-PCR, and generation of Northern blot probes as indicated in the main text.

Acknowledgments
We thank Dr. Barbara Hero for critical reading of the manuscript.

References
5. Yamanaka Y, Hamazaki Y, Sato Y, et al. Maturational sequence of differentiation and growth arrest path from the putative promoter region of DEIN were detected using in silico prediction based on the TRANSFAC algorithm. CpG islands within the nucleotide sequence of DEIN were detected with CpGplot.

Statistical Analysis
Variables of interest (stage, age at diagnosis, and cytogenetic aberrations) and relative expression levels of DEIN variants A/B/E or C were compared by \( \chi^2 \) test, Kruskal-Wallis test, or Mann-Whitney U test where appropriate. Expression levels of variants A/B/E and C were correlated with Pearson’s correlation coefficient. For DEIN expression, Kaplan-Meier estimates for 3-year event-free survival and overall survival were calculated and compared by log-rank test. Relapse, progression, and death were regarded as events. Death resulting from therapy complications was censored for event-free survival and overall survival analyses. Multivariate Cox regression was applied to analyze the prognostic value of DEIN expression in comparison with other markers for event-free survival. Models were built using a stepwise variable selection procedure recommended by Collett (15). In the first step, all variables were tested in a univariate Cox regression calculation one at a time. In the second step, all variables that seemed to be important in step 1 were fitted together in a multivariate Cox regression model.

In the third step, all variables that were not important in step 1 were added to the variables that were important in step 2, one at a time. The likelihood-ratio test \( P \) value for inclusion was <0.05; for exclusion, the \( P \) value was >0.10.


d a global alignment between two sequences. Potential transcription factor binding sites within the putative promoter region were detected using in silico prediction based on the TRANSFAC algorithm. CpG islands within the nucleotide sequence of DEIN were detected with CpGplot.
Identification of DEIN, a Novel Gene with High Expression Levels in Stage IVS Neuroblastoma

Harald Voth, André Oberthuer, Thorsten Simon, et al.


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