Aus dem Gerhard Domagk-Institut für Pathologie

der Westfälischen Wilhelms-Universität Münster

Direktor: Univ.-Prof. Dr. med. W. Böcker

und

dem Institut für Pathologie

der Heinrich-Heine Universität Düsseldorf

Direktor: Univ.-Prof. Dr. med. H.E. Gabbert

Assessment of Protein 4.1 in Neuroblastoma Tumors

DISSERTATION

zur Erlangung des Grades eines Doktors der

Medizin

Der Medizinischen Fakultät der Heinrich-Heine-Universität

Düsseldorf

vorgelegt von

Urs Lichtenauer

2004

Aus dem Gerhard Domagk-Institut für Pathologie

der Westfälischen Wilhelms-Universität Münster

Direktor: Univ.-Prof. Dr. med. W. Böcker

und

dem Institut für Pathologie

der Heinrich-Heine Universität Düsseldorf

Direktor: Univ.-Prof. Dr. med. H.E. Gabbert

Assessment of Protein 4.1 in Neuroblastoma Tumors

DISSERTATION

zur Erlangung des Grades eines Doktors der

Medizin

Der Medizinischen Fakultät der Heinrich-Heine-Universität

Düsseldorf

vorgelegt von

Urs Lichtenauer

2004

Als Inaugural-Dissertation gedruckt

mit Genehmigung der Medizinischen Fakultät

der Heinrich-Heine-Universität Düsseldorf

gez.: Prof. Dr. med. Wolfgang H. M. Raab

Dekan

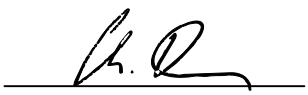
Referent: Prof. Dr. med. Christopher Poremba

Korreferent: Priv.-Doz. Dr. med. Dominik Schneider

Meiner Familie in Dankbarkeit gewidmet

ABSTRACT

Protein 4.1 (p4.1) ist ein multifunktionelles Potein, das als wichtiges Strukturelement des erythrozytären Zytoskeletts bekannt geworden ist. In den letzten Jahren konnten zudem in nicht-erythrozytärem Gewebe verschiedene Isoformen mit unterschiedlichen und teilweise ungeklärten Funktionen identifiziert werden. P4.1 gehört zu einer schnell wachsenden Proteinfamilie, zu der auch der NF2 Tumor Suppressor Merlin/Schwannomin gezählt wird.
Das p4.1 Gen – EPB4.1 – wurde auf dem Chromosomenlokus 1p33-1p34.2 vermutet. Mit Fluoreszenz in Situ Hybridisierung und Radiation Hybrid Mapping wurde der Genort von EPB4.1 jetzt präzisiert und auf Genlokus 1p36 relokalisiert. Interessanterweise gehören Deletionen der Telomerischen Enden von 1p36 zu den häufigsten Aberrationen beim Neuroblastom. Pathophysiologisch wird der Verlust eines Tumorsuppressor-Gens angenommen. Dieser konnte jedoch trotz intensiver Bemühungen bisher nicht identifiziert werden.
In dieser Dissertation wurde untersucht, ob es sich bei EPB4.1 um das gesuchte Tumorsuppressor-Gen bei Neuroblastomen handelt. Hierfür wurde EPB4.1 auf Mutationen und Variationen in der Genexpression in 78 verschiedenen Neuroblastom-Primärtumoren und 5 Neuroblastom-Zelllinien untersucht. P4.1 Transkripte konnte in fast allen Tumorproben nachgewiesen werden. Erwartungsgemäß fehlte das Stopcodon-reiche Exon 3, so dass die nicht-erythrozytäre, 135 kdDa große p4.1 Isoform vorherrschend war. Single-strand-conformational-polymorphism (SSCP) und Sequenzierungs-Analysen ergaben Missense Mutationen in Exon 4 (vier Tumoren) – mit Verlust der Wildtyp-Expression – und Exon 8 (ein Tumor); Silent Mutationen konnten in Exon 21 (sechs Tumoren) und Exon 16 (ein Tumor), und intronische Aberrationen in den Introns 9, 10, 17, 18 und 21 (sieben Tumoren) nachgewiesen werden. Jedoch konnten die zunächst vielversprechenden Alterationen in Exon 4 und Exon 21 später auch im Blut gesunder Probanden nachgewiesen werden und müssen insofern als Polymorphismus interpretiert werden. Die anderen Mutationen waren selten, mit zweifelhaften Einfluss auf das Genprodukt und daher nicht signifikant. In einem weiteren Schritt wurde das Probenmaterial entblindet und die gefundenen Alterationen mit den klinischen Tumorstadien sowie den teilweise vorhandenen 1p36 Loss of Heterozygosity (LOH) - Daten der Tumoren verglichen. Es konnte keine Korrelation festgestellt werden.
Um funktionelle Untersuchungen durchzuführen, wurde Tumor-p4.1 geklont und in eine humane Zelllinie transfiziert. Dieses mutierte p4.1 konnte ungewöhnlicherweise ausschließlich im Bereich der Plasmamembran der Zellen nachgewiesen werden. Die biologische Signifikanz dieses Ergebnisses ist unklar.
Zusammenfassend ist festzustellen, dass p4.1, das durch seine Relokalisation als sehr attraktiver Tumorsuppressor-Kandidat bei Neuroblastom Tumoren galt, durch die Ergebnisse dieser Studie als solcher in dem hier untersuchten Kollektiv ausgeschlossen werden konnte.



Unterschrift Betreuer

TABLE OF CONTENTS

INTRODUCTION

The Tumor Suppressor Protein 4.1

Protein 4.1 is the product of the gene EPB4.1, was originally identified in the red blood cell cytoskeleton and is believed to be a tumor suppressor. It is a prototypical member of a superfamily that includes ERMs (ezrin, radixin, moesin), p4.1 homologues, the NF2 tumor suppressor gene merlin/schwannomin, and protein tyrosine phosphatases (Chishti et al., 1998; Peters et al., 1998). Generally, it is believed, that the products of tumor-suppressor genes inhibit or suppress cell proliferation. The term tumor-suppressor gene is misleading because the physiologic function of these genes is to regulate cell growth, not to prevent tumor formation. However, the loss of these genes is a key event in many, possibly all, human tumors.

Many tumors can be classified as sporadic or familial. To explain the familial and sporadic occurrence of the apparently identical tumor, Knudson proposed his now famous “two-hit” hypothesis of oncogenesis (Knudson, Jr. and Strong, 1972). He suggested that in hereditary cases, one genetic change – the first hit – is inherited from the affected parent and is therefore present in all somatic cells of the body, whereas a second mutation – the second hit - occurs later on in life in one of these affected cells, which subsequently develops into tumor.

In sporadic cases, however, both mutations (hits) occur somatically within a single cell, whose progeny then form the tumor. A person carrying an inherited mutant allele in all somatic cells is therefore - except for the increased risk of developing cancer - perfectly normal. Because such a person is heterozygous at the particular gene locus, it implies that heterozygosity for the gene does not affect cell behavior. Cancer develops when the cell becomes homozygous

for the mutant allele or, put another way, looses heterozygosity for the normal gene. Since the gene is associated with cancer when both normal copies are lost, it is sometimes referred to as a recessive cancer gene. Tumor-suppressor genes seem to encode various components of this growth inhibitory pathway (Cotran et al., 1999c).

Taken the above into account, it becomes clear, why germ line mutations in the NF-2 gene, located on human chromosome 22q12, for example, predispose to the development of neurofibromatosis type 2. Interestingly, somatic mutations affecting both alleles of NF-2 have also been found in sporadic tumors of the central nervous system such as schwannomas, meningiomas and ependymomas (Rouleau et al., 1993; Ruttledge et al., 1994). The product of the NF-2 gene, called merlin, or schwannomin, shows a great deal of homology with the red cell membrane protein 4.1, in a way that both are crucial for cell structure integrity. Merlin binds, on one hand to actin, and, on the other to CD44, a transmembrane protein that is involved in matrix interactions. How the loss of merlin eventually leads to tumor formation is not known (Belliveau et al., 1995; Lutchman and Rouleau, 1995).

Protein 4.1's membrane stabilizing abilities are founded on its interactions with spectrin, which is the major protein of the membrane cytoskeleton. The individual spectrin dimers are like segments of an extensive cable network that are linked to each other head to head to form tetramers. Lateral connections between spectrin tetramers are established through actin. The two-dimensional spectrin cable meshwork so formed is tethered to the inner surface of the cell membrane by ankyrin and protein 4.1. Ankyrin forms a bridge between spectrin and the transmembrane anion transporter, called band 3, whereas protein 4.1 connects spectrin to glycophorin A. Together these proteins are responsible for maintenance of the normal shape, strength, and flexibility of the red cell membrane (Cotran et al., 1999b). Purified Protein 4.1 has also been shown to specifically interact with tubulin (Correas and Avila, 1988) and myosin (Pasternack and Racusen, 1989).

Besides its homology to merlin, further evidence regarding the tumor suppressor capabilities of Protein 4.1 was given, when hDlg, the human homologue of the Drosophila discs-large (Dlg) tumor suppressor protein, and a prototypical member of the MAGUK superfamily (membrane-associated guanylate kinase homologues), was found to binds to the FERM domain of Protein 4.1. The HOOK domain located between the SH3 and guanylate kinase-like domain of hDlg (Lue et al., 1994; Marfatia et al., 1994; Marfatia et al., 1996) mediates this association. Germline mutations of the Drosophila Dlg gene result in imaginal disc neoplasia (Woods and Bryant, 1989). Interestingly, while examining the expression of hDlg transcripts in neuroblastoma cell lines, Mori et al. (Mori et al., 1998) have recently reported an altered hDlg splicing pattern as compared to the hDlg splicing pattern seen in normal brain tissue. These observations suggest, that hDlg and protein 4.1 may participate in the formation of a protein complex that plays an important role in the suppression of growth signals.

In summery, Protein 4.1 is a multifunctional protein originally identified in red blood cells, that has tumor suppressor homologies and properties, and mediates interactions between the erythrocyte cytoskeleton and the overlying plasma membrane (Arpin et al., 1994; Conboy, 1993).

Protein 4.1 contains four functional domains

Four functional domains have been characterized so far: First, there is a 30 kDa basic N-terminal domain which mediates membrane-cytoskeleton (transmembranal) interactions via protein p55, glycophorin C and D (Alloisio et al., 1993; Hemming et al., 1995; Marfatia et al., 1994; Reid et al., 1989), and a minor band 3 binding site (Anderson and Marchesi, 1985; Hemming et al., 1995; Pasternack et al., 1985).

P55 is a peripheral membrane protein, which shows the presence of a *src* homology motif 3 (SH3) and has a potentially active guanylate kinase domain (Alloisio et al., 1993). Band 3 serves as the major anion channel of the erythrocyte (Jons and Drenckhahn, 1992; Rothstein et al., 1979) and is also a binding site for hemoglobin, protein 4.1 and several glycolytic enzymes (Cassoly, 1983; Haest, 1982; Murthy et al., 1981; Yu and Steck, 1975). This domain is typical for the p4.1 superfamily.

Secondly, a 16 kDa hydrophilic domain is existent, which serves as a protein kinase C phosphorylation site (Horne et al., 1985; Horne et al., 1990).

A third domain, which is 10 kDa in size, is a cAMP-dependent phosphorylation site, which has been shown to be critical for the interaction with spectrin and actin (Correas et al., 1986; Horne et al., 1993; Lorenzo et al., 1994; Morris and Lux, 1995; Schischmanoff et al., 1995). A binding site for the fodrin/actin complex in nonerythroid cells (Kontrogianni-Konstantopoulos et al., 2001) has also been found in this area.

Finally there is a 22/24 kDa C-terminal domain, which is quite acidic (Leto and Marchesi, 1984). No specific binding or regulatory function has yet been assigned to this domain.

Recently, other EPB4.1-like genes have been identified. They share strong homology with the domains mentioned above, and are believed to function accordingly.

Nonerythroid Protein 4.1

In contrast to the strict peripheral localization of p4.1 in mature red blood cells, classified p4.1R, protein 4.1 in nucleated cells is more diversely distributed and comes in several different isoforms (Tang et al., 1990). They seem to be concentrated at points of cell-cell or cell-matrix contact, along stress fibers, on cytoplasmic matrixlike structures (Cohen et al., 1982), but unlike erythroid p4.1 particularly at perinuclear regions such as centrosomal and Golgi structures (Chasis et al., 1993; Leto et al., 1986). Additionally, p4.1 isoforms of nucleated cells have been observed to be present in centrioles, the nucleus, and the nuclear matrix of mammalian cells (De Carcer et al., 1995; Gascard et al., 1999; Krauss et al., 1997b; Krauss et al., 1997a; Lallena and Correas, 1997; Luque et al., 1998), possibly interacting with the nuclear mitotic apparatus protein (Mattagajasingh et al., 1999). Therefore they seem to play an important role in organizing the nuclear architecture and serve as structural elements (Krauss et al., 1997b). Moreover, during interphase, p4.1 is located in the nucleus and it rapidly redistributes to the developing spindle poles when the nuclear envelope dissembles in prometaphase, a characteristic of nuclear matrix proteins that leave the nucleus during interphase/mitosis transition to become structural components of the mitotic apparatus, seen both in mammalian cells (He et al., 1995) and Drosophila (Saunders et al., 1997). The variety of p4.1 isoforms combined with the different possible protein-protein interactions leads to the suggestion, that each of those isoforms may have specific and discrete functions (Granger and Lazarides, 1984; Granger and Lazarides, 1985). Nonerythroid p4.1 subsequently should play an important role in the regulation of cell turnover rate, cell growth, and - to a certain extend - in cell to cell contact (Krauss et al., 2002; Tang and Tang, 1998).

Nevertheless, little is yet known about the specific cytochemistry, biochemical functions, or physiologic importance of nonerythroid p4.1.

EPB4.1 encodes Protein 4.1

The gene encoding protein 4.1 (EPB4.1) contains at least 23 exons (Huang et al., 1993), of which 20 exons are translated. Two first methionine sites are present, the first within exon 2, and the second within exon 4. Stopcodons can be found in exon 3 and exon 21. Multiple p4.1 isoforms (e.g. protein 4.1 homologues 4.1B, 4.1G, p4.1H, p4.1I, 4.1N) are originated with this constellation by alternative pre-mRNA splicing, differential use of two translation initiation sites, and posttranslational modification. They are heterogeneous with regard to molecular mass, abundance, and cellular localization (Anderson et al., 1988; Baklouti et al., 1997; Gascard et al., 1998; Granger and Lazarides, 1985; Parra et al., 2000). This complexity of alternative splicing events results in protein products, which range in size from 30 up to 210 kDa (Anderson et al., 1988).

Erythroid Protein 4.1 is in fact a 80 kDa polypeptide translated from the second translation initiation ATG located in exon 4. It is this 80 kDa protein that is reduced or missing in patients with severe hereditary diseases, such as 4.1(-) hereditary elliptocytosis (HE) (Conboy et al., 1986).

Nonerythroid p4.1 polypeptides result from tissue-specific alternative splicing translated from the upstream ATG located in exon 2 (Conboy et al., 1991). The predominant form of nonerythroid p4.1 is represented by the ~135 kDa polypeptide that is expressed in a variety of nonerythroid tissues and is localized in the nucleus of nucleated cells (Baklouti et al., 1996; Chasis et al., 1993; Conboy et al., 1991; Parra et al., 2003) (Figure 1).

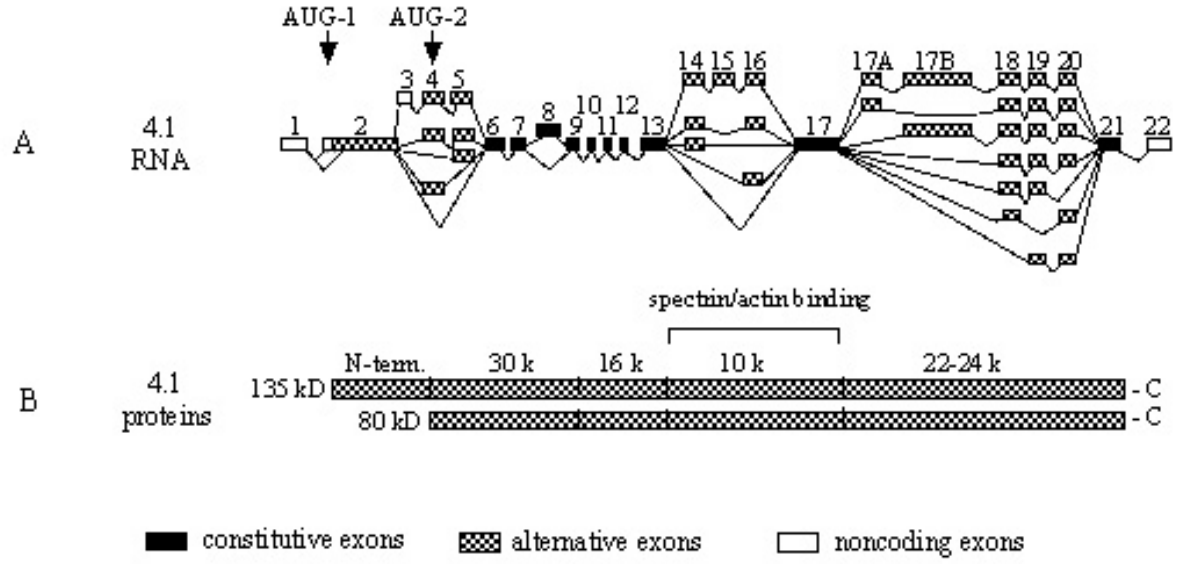


Figure 1: Protein 4.1 map
(A) A schematic diagram of p4.1 RNAs displaying multiple combinations of splicing pathways possible among p4.1 alternative exons. In this format, exons are coded as follows: *solid bars*, constitutive; *shaded bars*, alternative; *open bars*, noncoding. The arrows on top indicate the position of alternative translation initiation sites, AUG-1 and AUG-2.
(B) Examples of p4.1 isoforms derived from different translation initiation sites. The 80 kD prototypical red cell isoform is produced is produced from AUG-2 and can be present in nucleated and nonnucleated cells. Chymotryptic fragments of this isoform include a 30-kD membrane binding domain, a 16-kD domain, a 10-kD spectrin/actin binding domain, and the 22 – 24-kD domain (Leto and Marchesi 1984). Higher molecular mass p4.1 isoforms, present in nucleated cells, use AUG-1 to generate an additional 209-amino acid "NH_2-terminal extension".

Reassignment of EPB4.1 to 1p36

The EPB4.1gene has been originally mapped to human chromosome 1p32---1pter (Conboy et al., 1986). In 1991 Tang and Tang (Tang and Tang, 1991) subsequently refined the localization, which thereafter has been cited extensively in the literature, to human chromosome 1p33-34.2, by using fractional length (Flpter). In this method, chromosomal locations are calculated by determining the fractional length of the hybridization signal relative to the total length of the chromosome.

While examining the 4.1 gene in normal and neuroblastoma cell lines by fluorescence in situ hybridization (FISH) analysis, evidence for a more distal location of EPB4.1, namely at 1p36.1, was found. Radiation hybrid mapping further confirmed and further defined this new location. Both PCR primer pairs used for RH mapping produced identical raw scoring data. Analysis of the raw data, performed by RHMAPPER, linked EPB4.1 to chromosome 1p36, located 4.5 cR (~1.2 Mb) downstream of the Whitehead framework marker D1S511 (LOD > 3). The discrepancy in p4.1 map position can be explained by the limitations in using Flpter values to accurately determine chromosome location. It is known that Flpter values are affected by heteromorphic variation in chromosome size, and chromosome 1 is especially variable in size (Mascio et al., 1995).

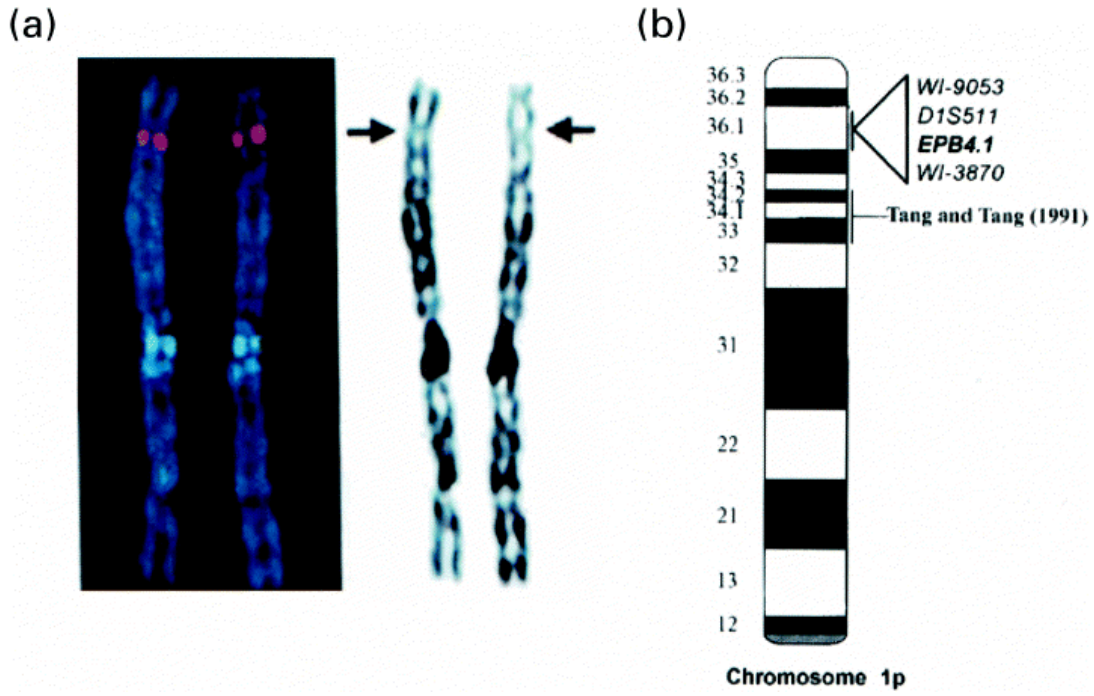


Figure 2: Reassignment of EPB4.1.
(a) Fluorescence *in situ* hybridization (FISH). Subchromosomal location of EPB4.1 to the region 1p36.1 using PAC probe (Rhodamine signal) hybridized to normal human chromosomes. FITC signals depict a (peri-) centromeric region of chromosome 1. DAPI-inverted images of chromosome 1 (right) simulate G-banding patterns.
(b) Ideogram of human chromosome 1p and location of EPB4.1 relative to markers mapped in the Whitehead RH framework map. EPB4.1 is located at 1p36, distally to the previously reported localization.

The new distal position of EPB4.1 is important in the elucidation of genes mapped to chromosome 1p36 in the search for diseases, which show abnormalities at that site, such as neuroblastomas, meningiomas, and other human cancers.

In fact, the deletion of the distal short arm of chromosome 1 in the region of band p36 - determined either karyotypically (Biegel et al., 1993) or by loss of heterozygosity (LOH) (Amler et al., 1995; Caron et al., 1993; Maris et al., 1995; Muresu et al., 1995; White et al., 1997) - is the most characteristic cytogenetic abnormality in neuroblastomas and has been demonstrated in several studies in 70 – 80 % of tumors with a diploid or near-diploid content of DNA (Cotran et al., 1999a), making it a consensus deletion for this disease (Martinsson et al., 1997; Spieker et al., 2001).

Neuroblastomas

Neuroblastoma is the third most common tumor of the peripheral nervous system and of neural crest origin (Brodeur, 1994), and is responsible for approximately 15% of all childhood cancer deaths. It is an embryonal cancer of the postganglionic sympathetic nervous system, which most commonly arises in the adrenal gland. Neuroblastoma is the most common malignant disease of infancy (Brodeur, 2003; Gurney et al., 1997), and 96% of cases occur before the age of 10 years (Grovas et al., 1997). Despite the fact that neuroblastoma is sometimes diagnosed in the perinatal period (Knudson, Jr. and Strong, 1972), no environmental influences or parental exposures that impact on disease occurrence have been identified consistently (Bunin et al., 1990). Thus, the etiology of neuroblastoma remains obscure.

The clinical hallmark of neuroblastoma is heterogeneity, with the likelihood of tumor progression varying widely according to anatomic stage (Figure 2a) and age of diagnosis. In general, children diagnosed before 1 year of age and/or with localized disease are curable with surgery and little or no adjuvant therapy. Particularly in infants the tumor may regress spontaneously in some patients, whereas in other patients, the tumor may mature into a benign ganglioneuroma (Maris, 1999). D'Angio et al (D'Angio et al., 1971) first recognized a distinct subset of infant patients who present with a unique pattern of extensive disseminated disease, but who reliably have spontaneous disease regression (stage 4s). In contrast, older children often have extensive hematogenous metastases – most commonly to the bone – at diagnosis, and in about 50 to 70% of the cases the tumor will progress despite intensive therapy (Brodeur, 2003; Grovas et al., 1997).

a)

Stage	Description
1	Localized tumor confined to the area of origin; complete gross excision, with or without microscopic residual disease; identifiable ipsilateral and contralateral lymph nodes negative microscopically
2A	Unilateral tumor with incomplete gross excision; identifiable ipsilateral nonadherent lymph nodes negative microscopically
2B	Unilateral tumor with complete or incomplete gross excision; positive ipsilateral nonadherent lymph nodes; identifiable contralateral lymph nodes negative microscopically
3	Tumor infiltrating across the midline (vertebral column) with or without regional lymph node involvement; or unilateral tumor with contralateral regional lymph node involvement; or midline tumor with bilateral regional lymph node involvement or extension by infiltration
4	Dissemination of tumor to distant lymph nodes, bone, bone marrow, liver, or other organs (except as defined in stage 4S)
4S	Localized primary tumor as defined for stage 1 or 2 with dissemination limited to liver, skin and/or bone marrow (<10% tumor) in infants younger than 1 y

b) **c)**

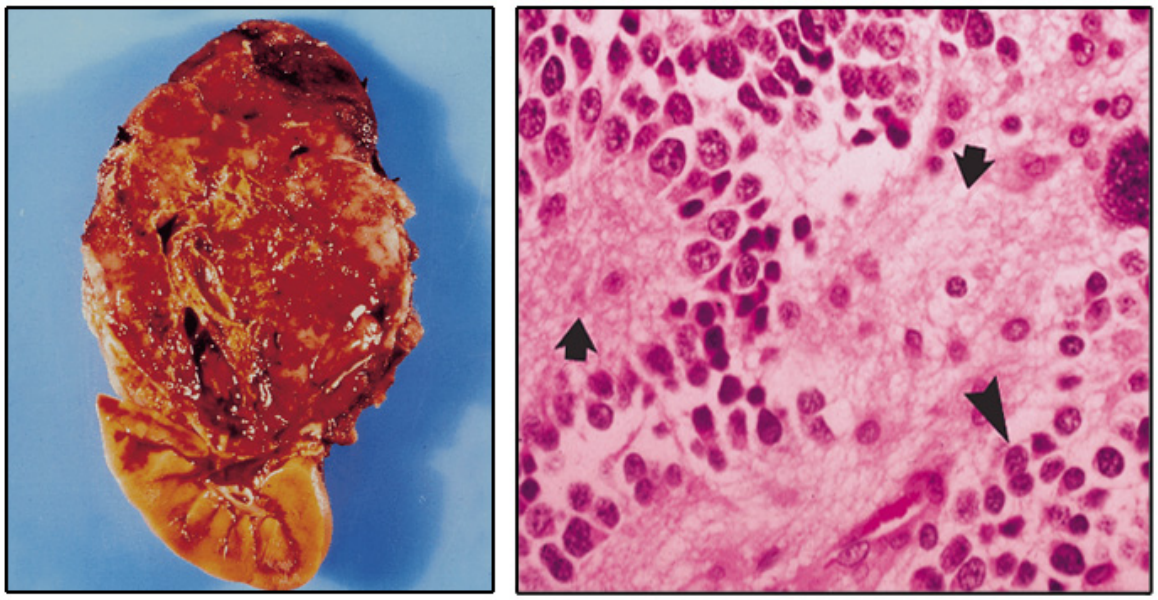


Figure 2: Neuroblastoma:
a) Clinical staging of NB
b) Adrenal NB. Photograph of a bisected kidney reveals a heterogeneous, hemorrhagic adrenal NB arising above the kidney.
c) Immature neuroblastic tissue. High-power photomicrograph (original magnification, x300; hematoxylin-eosin stain) of NB demonstrates neuroblasts, which are the dark purple cells with scant cytoplasm (arrowhead), and neurophil, which is the fine, fibrillary light pink-staining material (arrows).
(Schwab M., et al. *Lancet Oncol.* 2003 Aug;4(8):472-80)

Histologically, neuroblastoma is a small round cell tumor. It is possible for the neoplasm to be composed of undifferentiated neuroblasts or neuroblasts, which are associated with ganglion

and Schwann-like cells (Figure 2b + c). The refractivity to the treatment and the development of clinical unresponsiveness suggest that biologically neuroblastoma is a heterogeneous tumor. It is primarily characterized by amplification of the N-myc oncogene and other factors and, as mentioned above, chromosome 1p deletions, strongly suggesting the presence of a tumor suppressor gene.

Several attempts to identify this tumor suppressor gene, however, failed.

Protein 4.1 – a candidate tumor suppressor for neuroblastomas

So, is p4.1, located at the site commonly deleted in neuroblastomas, the responsible tumor suppressor gene? Although an attractive candidate tumor suppressor, protein 4.1 may have been disregarded as such for neuroblastoma tumors by virtue of its mislocalized chromosomal position. The refinement of this locus, however, makes further investigations of p4.1 and its involvement especially in neuroblastomas, but also other human cancers linked to chromosome 1p36 - like meningiomas – plausible and very attractive.

Further motivation and support is given by a recent experiment in which protein 4.1 deficient mice have been generated and exhibit erythroid membrane and neurobehavioral abnormalities (Shi et al., 1999). In addition, another study demonstrated that protein 4.1R is also localized in granule cells in the cerebellum and in the dentate gyrus. Interestingly, 4.1R null mice suffer from deficits in movement, coordination, balance, and learning (Walensky et al., 1998). Whether or not these mice develop neuronal proliferation defects with aging remains to be examined. However, numerous studies showed, that p53 null mice are often susceptible to induced tumor development (Jacks et al., 1994; Williams et al., 1994). It is noteworthy here that the p53 tumor suppressor knockout mice – in contrast to p4.1R deficient mice - display normal development, and develop tumors only late in life (Donehower et al., 1992; Williams et al., 1994). These observations suggest that a similar paradigm for p4.1, being a tumor

suppressor itself with various critical functions affecting the cell structure and cell proliferation, may determine a tumor susceptibility of 4.1R null mice.

Excitingly – using FISH analysis in a first attempt to address this issue – the loss of one allele of the p4.1 gene on both copies of the large derivative of Chromosome 1 in the neuroblastoma cell line Lan-5 could be detected (Figure 3). Furthermore, the 1p36 region in the SK-N-AS neuroblastoma cell line was deleted in a Comparative Genomic Hybridization (CGH) study.

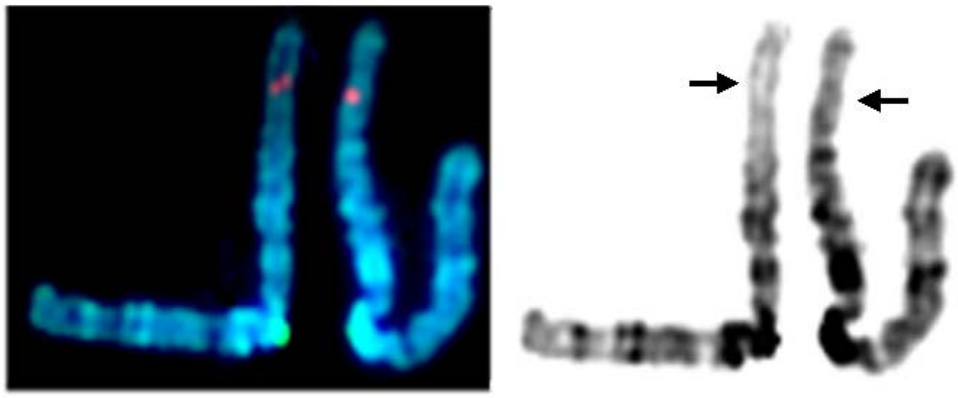


Figure 3: Protein 4.1 LOH in Lan-5
FISH analysis of the neuroblastoma cell line Lan-5 revealed the loss of one of the alleles encoding Protein 4.1 at the short arm of chromosome 1.

Assessing the involvement of EPB4.1 – now relocated to 1p36.1 – in neuroblastoma tumors, which most commonly show chromosomal abnormalities in this region, was therefore the primary objective in this doctorial thesis work. I addressed this issue by screening 78 neuroblastoma tumors and 5 neuroblastoma cell lines for alterations on the DNA as well as on the RNA level. Also, a p4.1 transcript was cloned and cultured followed by an expression study to gain further knowledge of the alternative use of the EPB4.1 gene. Clinical data was evaluated and protein studies finally were used to get more insights regarding the localization and function of p4.1 in neuroblastoma tumors.

MATERIAL & METHODS

Presence and alternative splicing pattern of EPB4.1 transcripts

Plan:

Using a complex alternative splicing machinery, EPB4.1 produces several isoforms depending on the inclusion or exclusion of various exons. For example, the inclusion of a stop codon-rich exon 3 would generate an 80 kDa isofom by utilizing a start codon in exon 4, whereas the exclusion of exon 3 results in the synthesis of a 135 kDa isofom by using a start codon in exon 2. The expression of the 135 kDa isoform of p4.1 is the predominant event in non-erythroid cells.

The project was started making sure that non-erythroid p4.1 is expressed in neuroblastoma tumors at all, and if so, to also find out, what splicing pattern is being used. Therefore, RT-PCR was performed on all 78 neuroblastoma primary tumors using primers flanking exon 3.

Briefly, this method requires only very small amounts of RNA, much less than is necessary for a Northern blot or even a nuclease protection assay. The RNA is reverse transcribed by Reverse Transcriptase - an enzyme present in retroviruses to convert their RNA genomes into DNA - using six-base oligonucleotides with random sequence ('random oligos'), that anneal all over the RNA and act as primers to produce a cDNA strand, complementary to RNA, which is in a second step amplified by regular PCR. This method is explained in the chapter ‘PCR and SSCP-analysis’.

RT-PCR results here should hence demonstrate that all p4.1 transcripts from neuroblastomas lack exon 3 and consequently permit assembling of the expected full length 135 kDa nonerythroid isoform.

Procedure:

RT-PCR/First strand synthesis

For c-DNA synthesis, the SuperScript system (Invitrogen) was used according to the manufacturers protocol: 2 µl of Random Hexamers, 5 µl of tumor mRNA, and 3 µl of DEPC-treated water were added to a sterile 1.5-ml microcentrifuge tube. This mixture was heated to 70 °C for 10 min and quick-chilled on ice. The content was collected by brief centrifugation. 4 µl of 5X First Strand Buffer, 2 µl of 0.1 M DTT, 1 µl of 10 mM dNTP Mix were added, and the mixture was gently vortexed and then collected by brief centrifugation. The tube was placed at 37 °C for 2 min to equilibrate the temperature. 3 µl of SuperScript™ II RT were added to a total reaction volume of 20 µl, which was mix gently and finally incubate at 37 °C for 1 h. The reaction was terminated thereafter by placing the tube on ice.

Amplification

Exon 2 forward primer P4full.F (5'GGA TCT TCA TTC ATT AAG CAG TG), and exon 4 reverse primer P4full.R (CAC TCT TCA AGT CCT TCT C) were used to perform PCR on neuroblastoma cDNA. PCR components for each reaction included 1 µM primers, 1 µl of template, 1X reaction buffer, 5 % of DMSO, 200 µM dNTPs, and 50 U/ml Taq polymerase (Perkin Elmer). PCR conditions were: denaturation at 94 °C for 10 min, 35 cycles at 94 °C for 60 sec, 55 °C for 60 sec (annealing), and 72 °C for 60 sec, with final extension at 72 °C for 10 min. Products were analyzed in a UV-light box after they were electrophoresed on a

1% agarose gel as described earlier. The expected size for the PCR product from the transcript with exon 3 was 182 bp. A 102 bp product was expected from the transcript excluding exon 3.

PCR and single-stranded conformational polymorphism (SSCP) analysis

Plan:

Genomic DNA from 78 neuroblastoma tumors and 5 neuroblastoma cell lines (AS, CHP-134B, NGP, Lan-5, and SK-N-DZ) were subjected to SSCP analysis to screen EBP4.1 for aberrations or mutated alleles. Regions of interest – EBP4.1's 22 exons – were therefore first amplified by regular Polymerase Chain Reaction (PCR) using 22 sets of primers.

Amplification

Description of method:

PCR can be used to produce vast quantities of DNA of a particular gene or stretch of DNA of interest.Two oligonucleotide primers of approximately 20 base-pairs in length, complementary to opposite DNA strands flanking the particular DNA sequence of interest, are used to amplify that sequence by means of repeated cycles of denaturation of the double-stranded DNA, primer annealing to the single-stranded DNA and primer extension of the single-stranded DNA with DNA polymerase to produce double-stranded copies of the DNA sequence of interest.

The successive cycles of DNA synthesis result in an exponential amplification of the DNA target sequence and can result in an increase as great as 105-fold in the sequence of interest. This is sufficient DNA to be directly visualized by ultraviolet fluorescence after staining with ethidium bromide.

Great care has to be taken with PCR to avoid DNA from an extraneous source to be amplified, giving false positive or negative results. Necessary controls to detect this are built in. Thermocycler machines carry out the required temperature changes necessary to repeat the 20-25 cycles of amplification. The original problem that the high temperatures required to denature the double-stranded DNA resulted in the inactivation of DNA polymerase I used for primer extension, so that further aliquots of that enzyme had to be added in each cycle of the amplification process, was overcome by the use of DNA polymerase isolated from the bacterium Thermophilus aquaticus, which grows naturally in hot springs and is heat-stable! Kary B. Mullis won the Noble Price for this discovery in 1993 (30).

Table 1: Primer-Sequences
Sequence of the primers used for SSCP analysis. The size of the PCR product and the annealing temperature of each primer set are indicated

Primers	Sequence	Size (bp)	Temp (°C)
P4-1F P4-1R	GGC AGT TGC CTG CGC CAA TCA G ACG GTC CGA GCC TTG GGG GTA G	258	60
P4-2NF P4-2NR	TAG GGT TTA GGG TTT TGC CAA CAG CAC CTG AGA TTT GGG CCT TTT GAG	363	60
P4-2CF P4-2CR	AAG GAG CGG ACA TCA GAA AGC AGA AAG TGG TGG AAT TAC AGG CAT GAG	375	60
P4-3F P4-3R	GTT GTT AAA CTA AAC ACG ACT TGT TAT GGA GTC CAA ATC TCA GAC CT	273	55
P4-4F P4-4R	GTG TTT ATT ACT GAC TTG GCG ATG TCA CCA CTT ACG CAA CTC TAG ATG	323	60
P4-5F P4-5R	CAG AGT GAA TCC CCA TCT CTT TTG GCT TAG AGA AGC AGG TAT AGA TAC	281	60
P4-6F P4-6R	GAT AGT CAG TGA TCT TGC TTC CAG AAA GTG ACT GAC TCA CAG CC	211	60
P4-7F P4-7R	CTT TGT GTT TAT TTT ATA G TTT TTC TTC AGT TCT GAA TA	207	55
P4-8F P4-8R	TTT TTC TCT TTT GGC TCT AG AAC CCT GGA GAC ATA TTC AC	256	60
P4-9F P4-9R	ATG TTT TTA TTC TAT CAA AG CTT TTG AAT GTT TTA AAA TAC	239	60
P4-10F P4-10R	TGG TTG TTA TCT TCA GAT TAT C TCT CAG ATG ACT TAA TAA GG	332	55
P4-11F P4-11R	GTG GCA CCA TAT TTC TCT GGT AC AGA GTG CCA GTC ATA CAA TGA AAG G	287	55
P4-12F P4-12R	TGG AAA CCC TGA GAA TTG TGA AAC GCC CAA CCC TGA GCT TCT TTA ATT CGG	403	55
P4-13F P4-13R	CTT TTC CCT TTC TCA CGT AG GGT ATT TGG ACT GAT GAC ATA C	251	55
P4-14F P4-14R	CTA CTT TTA TTT CTT AAA TG AAG CTT CTG TTT TGC CAT AAT T	248	60
P4-15F P4-15R	TTT GTG CCA ATA GGC CAC TT GAA AGT GAA AAA TGG CTA AC	226	55
P4-16F P4-16R	GCC AAT TTT CAG TTT TTT CCC GCA AG AGC ACA GAA TGA AGA AAA GGG GAT C	228	55
P4-17F P4-17R	ACG GGT TGC CCT TGA TTA TGT TC AGC TCT AAG CTT TCT CTC AGA TAC AG	340	55
P4-18F P4-18R	ACA CTT TGA TCA GAT CAG TGT CAG AAG ATG ACT AGA AAC TCT GTG AAG AAA GG	292	55
P4-19F P4-19R	TGT GCA GCT GAA GAG CAA AGC TGC ACT AGG GTC CTT CTT GCA GAG AGG	271	55
P4-20F P4-20R	ATG ACA TCT TCT CTT TGG TCT CTT CAA CAT TCC CAC	123	55
P4-21F P4-21R	GCC CTT GTT TCT GTC TTT ATC CAG AGC CCT CTC A	188	55

Procedure:

22 sets of primers – summarized in Table 1 – were designed and tested prior to use. PCR components for each reaction included 1 µM primers, 1 µl of template, 1X reaction buffer, 5 % of DMSO, 200 µM dNTPs, 0.1 µCi/µl of [α-^{32}P] dCTP (Dupont), and 50 U/ml Taq polymerase (Perkin Elmer). PCR conditions were: denaturation at 94 °C for 10 min, 35 cycles at 94 °C for 60 sec, 55 °C for 60 sec (annealing), and 72 °C for 60 sec, with final extension at 72 °C for 10 min.

After amplification, the product was analyzed by SSCP electrophoresis.

SSCP Analysis

Description of method:

Single stranded DNA tends to refold and to build complex structures, which are stabilized by intramolecular effects, in particular hydrogen-bonds on the base pair level. The electrophoretic mobility of those structures on a nondenaturating gel depends not only on the length of the molecule, but also on the conformation, which, in return, depends on the DNA-sequence. After denaturation, the reaction products partially renaturate into double stranded DNA, which travels faster on the Gel (bands further down on the gel). Single stranded DNA travels slower (upper part). For detection, the primers are marked using radioactive [α-^{32}P], so that the reaction products are visualized by the darkening of a photo film, which is called autoradiography. Usually there will be 2 bands of different mobility showing, representing the two complementary single strands. In samples containing mutations, there will be two extra bands in the upper part of the gel resulting from the mutant allele. There also will be additional bands in the lower part of the gel right above the normal, heteroduplex bands. If the wild-type allele is deleted and only a mutant allele is present, only two bands will show. This

is the reason why, to determine the difference between wild-type and mutant alleles, a normal control is mandatory (Figure 4).

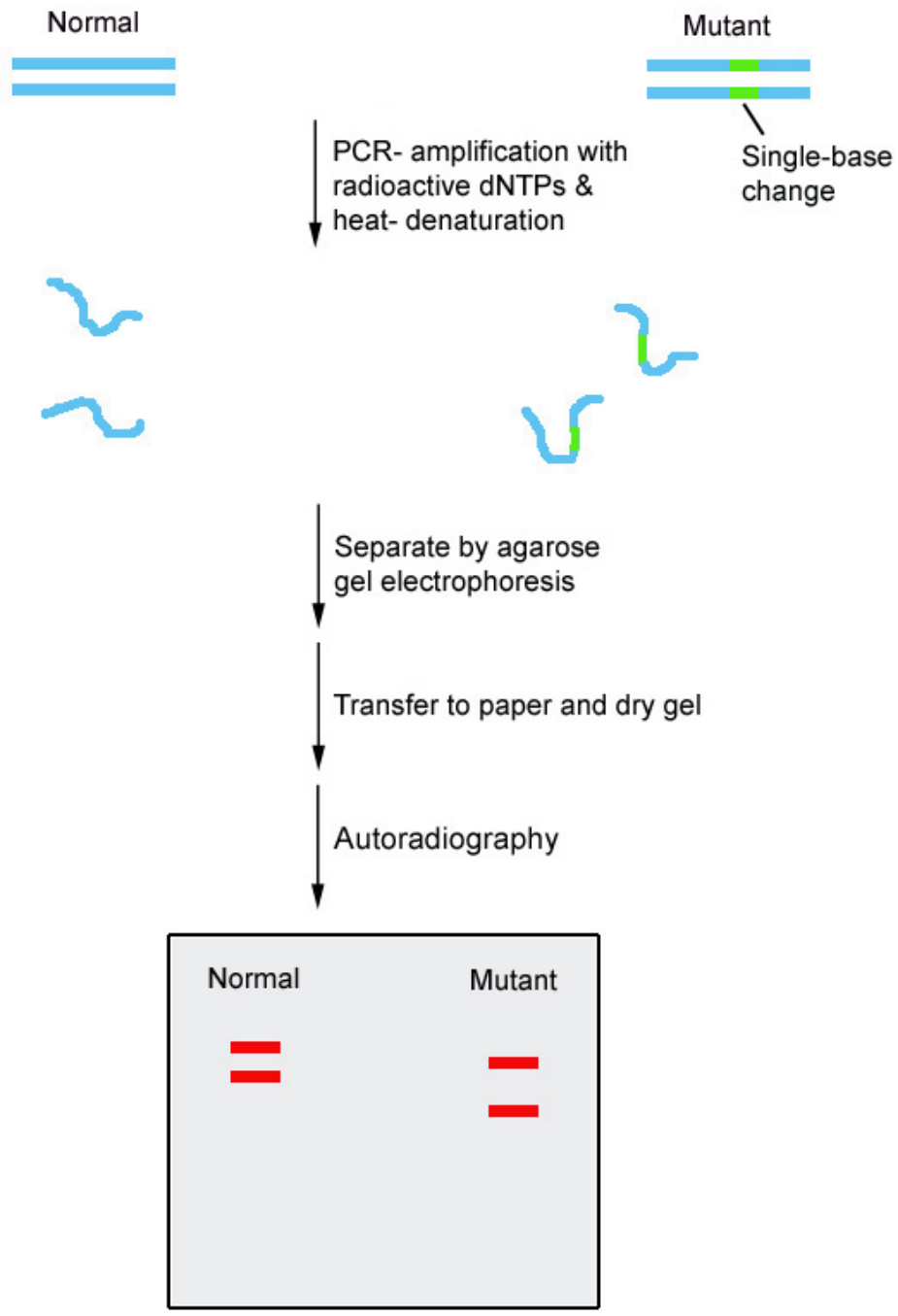


Figure 4: SSCP Analysis
Single-stranded conformation polymorphisms (SSCPs) can be used to detect single-base changes 'dark green'. Single stranded DNA molecules that differ by only one base frequently show different electrophoretic mobilities in nondenaturing gels. Differences between normal and mutant DNA are revealed by autoradiography.

SSCP analysis is a quite simple and very sensitive tool (~ 90% sensitivity), but is not suitable for fragments greater than 200 bp. In addition, it cannot identify the type, or the position of the detected mutations. Sequencing of suspicious bands is therefore necessary to answer these questions (Sheffield et al., 1993).

Procedure:

For SSCP analysis, PCR amplified DNA samples were mixed with loading buffer and denaturated by heating them to 95 °C for 5 min and chilling them instantly by placing them on ice-water for 10 min. For the SSCP-gel, the small glass plate was wiped with binding buffer. After a 5 min wait, the plate was gently wiped two times with ethanol 95%. Thereafter the large glass plate was cleaned with ethanol 70% and Sigmacote (Sigma) was applied to both glass plates' inner side, in order to avoid the gel to stick to either of the glass plates after completion of the electrophoresis. Spacers were set corresponding to the desired thickness of the gel, and the plates were clamped together using four metal clamps.

The nondenaturating polyacylamid gel was mixed using 0.25X of MDE gel solution (FMC BioProducts), 0.6X TBE, 10% glycerol, 0.05% (w/v) of APS, and 0.05% of Temed.

Immediately after the addition of the polymerizing agents, the gel solution was carefully poured between the two glass plates, taped together, taking care to avoid and eliminate air bubbles.

After pouring, the gel immediately was laid horizontally and a well forming comb was inserted into the gel and held in place by metal clamps. The polyacrylamide gels were allowed to polymerize for at least 30 minutes prior to use. After polymerization, the comb and the tape at the bottom of the gel were removed. The vertical electrophoresis apparatus was assembled by clamping the top and bottom buffer wells onto the gel, and adding running buffer to the

buffer chambers. The wells were cleaned by circulating buffer into the wells with a syringe and, immediately prior to the loading of each sample, the excessive gel in each well was suctioned out with a mouth pipette.

PCR products were loaded into the gel slots, and were electrophoresed at 8 watts for 16 hours. After electrophoresis, the glass plates were separated and the gel was blotted to Whatman paper, covered with plastic wrap, dried by heating on a Hoefer vacuum gel drier, and exposed to X-ray film (Kodak). Depending on the intensity of the signal, exposure times varied from 4 hours to several days. After exposure, the films were developed by automated processing. The autoradiogram then was finally placed on a light-box and the sequence was manually read and analyzed.

To further investigate detected mutations, aberrant bands were excised from the gel and placed in 50 µl of DEPT-water overnight to dissolve the DNA. The latter was reamplified using regular PCR under the same conditions mentioned above. The product was confirmed and purified by adding 3 µl of dye to each PCR-product and loading 4 µl of this mixture on a 1% agarose gel, which was composed of 100 ml of TBS-buffer, ph 7.5, 0.7 g agarose plus 2.5 µl of ethidium bromide. Electrophoresis was performed for 15 min with 100 Volts. Bands were identified under UV-light, cut out and transferred to sterile 1.5 ml vials. DNA was extracted from the agarose gel by applying 300 µl of QG-buffer (Quiagen) into each tube and placing them in a 50 °C water bath for approximately 10 min, before 100 µl of isopropanol were added. Vials were mixed gently and the content transferred to purple columns (Quiagen Mini Kit), which thereafter were centrifuged for 15 sec at 14000 rpm. The flow through was discarded and each column was filled with 700 µl of PE-buffer and centrifuged 2 times at 14000 rpm for 1 min to wash the DNA product. Columns were then placed on 1.5 ml collection tubes. DNA could now be collected by adding 15 µl of clean water directly onto the

filter of each column and centrifuging them at full speed for 30 sec. Successful extraction was confirmed under a UV-light box after 1 µl of DNA from each collection tube had been mixed with 3 µl of dye and loaded on a 1% agarose gel, which subsequently was electrophoresed for 15 min at 100 Volt as described above. The DNA was now ready to be subjected to DNA sequencing for identifying the type and location of the expected mutations.

Sequencing

Description of Method:

Sequencing is a method to determine the order of the nucleotides. As in a regular PCR with radioactivity described above, thermal-stable DNA-Polymerase is used and the DNA repeatedly anneals, extends and melts. The crucial difference is, that in four different reactions dideoxynucleotides (ddNTPs) are added, which are base-specific and differ from normal deoxynucleotides (dNTPs) in that they cannot be extended by DNA-Polymerase, leading to a termination of the chain reactions and to DNA fragments. The result is a heterogeneous population of molecules that end at one of the four nucleotides in each of the four separate reactions. These populations of molecules are then resolved on a denaturing polyacrylamide gel, one lane per base-specific reaction. The order of the bands across the lanes represents the DNA sequence, which can now be compared to the sequence of the normal – wild type – DNA to identify type and location of aberrations (Figure 5).

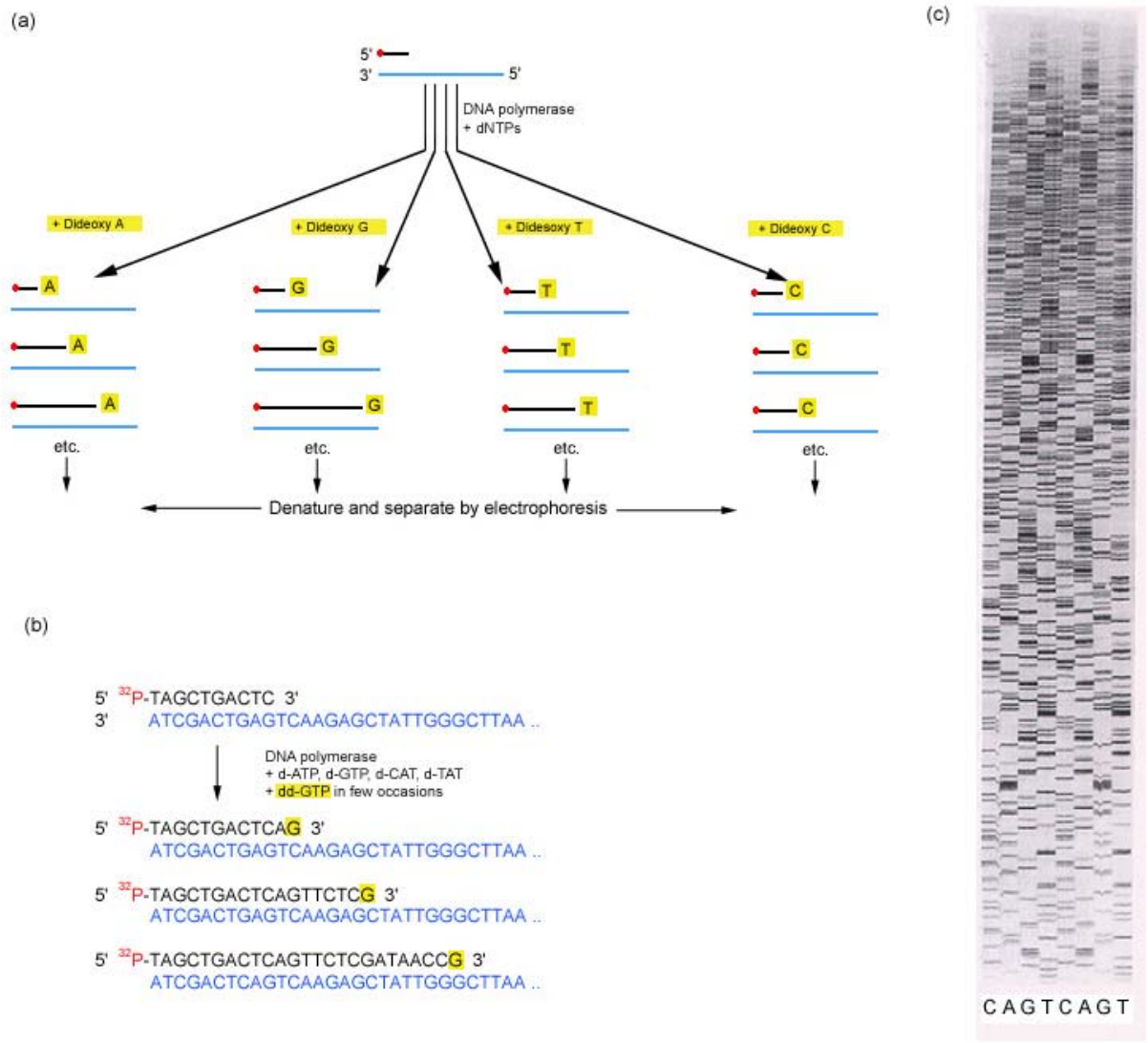


Figure 5: Sanger (dideoxy) method for sequencing DNA fragments
(a) A single strand of the DNA to be sequenced (blue line) is hybridized to a 5'-end-labeled synthetic deoxynucleotide primer. The primer is elongated in four separate reaction mixtures containing the four normal deoxynucleoside triphosphates (dNTPs) plus one of the four dideoxynucleoside triphosphates (ddNTPs) in a ratio of 100 to 1. A ddNTP molecule can add at the position of the corresponding normal dNTP, but when this occurs, chain elongation stops because the ddNTP lacks a 3' hydroxyl. In time, each reaction mixture ending at every occurrence of the ddNTP (yellow).
(b) Three of the labeled chains that would be generated from the specific DNA sequence shown in the presence of ddGTP.
(c) An actual audioradiogram of a polyacrylamide gel in which more than 300 bases can be road. Each reaction was carried out in duplicate using a commercial preparation of the DNA polymerase.

Procedure:

The Amplicycle sequencing kit (Perkin Elmer) was used to verify and identify suspicious SSCP changes. Each reaction tube contained 2 µl of the base-specific terminator mix, 2 µl of template 0.2 µl of the forward or reverse primer for this region, 0.8 µl of 10X cyclin and 0.1 µl of ^{32}P-dATP. PCR conditions were 94 °C for 2 min, then 94 °C for 30 sec, 55 °C for 1 min, and 72 °C for 30 sec for 30 cycles.

Glass plates, comb and spacers were cleaned and put together just like for an SSCP gel. 8% polyacylamid gel was carefully poured into the form using a mix of 50 ml gel mix 8 (Life Technologies) and 300 µl of APS 10%. After polymerization and before electrophoresis, the gel was warmed up by applying 55 W for 30 min. 3 µl of dye was added to each PCR reaction product, and the gel lanes were loaded with 10 µl of product. The gel was run at 55 W for approximately 2.5 hours, dried for another 2 hours and placed in a film box for autoradiography over night.

Allelic Expression Study

Plan and description of method:

When applying the SSCP analysis technique described above to tumor cDNA instead of DNA, judgements can be made if one and which or if both alleles were expressed in the original cells. This is important, since an alteration on the DNA level is only relevant, if it is expressed into RNA, and eventually formed into protein.

Procedure:

For allelic expression studies, SSCP analysis was performed on tumor cDNA using the primer pairs P4-4CF (5' GGA CTT GAA GAG TGC TCC AA) and P4-4CR (5' ATT CCA AGG GAC ACC ACG AA) for exon 4; and p4.exon 19F (5'ATC ACA TCT GAG ACC CCA AGC) and p4.exon22-R (5' GAA AGT CTG AAG TCT GAC TAT GGC) for exon 21 and intron 21. All PCR reactions on each tumor sample were carried out in triplicate.

Semiquantitative RT-PCR Analysis

Plan:

To further assess the involvement of p4.1 on the RNA level, competitive semiquantitative RT-PCR Analysis was performed on the cDNA of the 78 tumor samples. With this method, if and how much p4.1 RNA is expressed in the tumor samples can be assessed, and answers the question whether p4.1 RNA is generally downregulated in neuroblastoma tumors and/or if aberrations detected on the DNA-level lead to reading and copying errors, resulting in a decrease, or absence of p4.1 RNA in these tumors.

Description of method:

RNA is reverse transcribed by Reverse Transcriptase as described in the section "Exclusion of Exon 3 and alternative splicing pattern". This time, advantage is taken of the fact that amplification efficiency of the desired product differs among the samples, so that the levels of RNA expression are made comparable by including a titered amount of a distinguishable template in every sample. Housekeeping genes, such as ß-actin, are equally expressed and present in every single cell of the body, and therefore ideal as templates, which act as

competitors to the sample DNA. This is why this method is called competitive PCR. The ideal concentration for the competing primer pairs must be evaluated to achieve an equal amplification of product in a control sample. Again, radioactive dNTPs are used, and the uptake of the radioactive isotope correlates directly to the amount of PCR product. The different intensities of the bands on the developed film therefore can be used to compare and quantify the amount of RNA among the different samples (Emery Alan E.H. and Mueller Robert F., 1993a).

Procedure:

In this study, cDNA from all 78 neuroblastoma samples was subjected to PCR analysis using the p4.1 primers (5' CCA GTT ACC GAG CAG CTA AG and 5'CTT GCT GTA CGC TCA AAG TG) amplifying a 206 bp product and the ß-actin primers (5' TCA TGA AGT GTG ACG TGG ACA TCC and 5' CCT AGA AGC ATT TGC GGT GGA CGA TG), amplifying a 287 bp fragment for internal control. To assess the optimal ß-actin primer concentration in the reaction mix, cDNA of 3 different tumors was amplified using various ß-actin primer concentrations ranging from 1/1 µM to 1/32 µM. PCR conditions were: denaturation at 94 °C for 10 min, then 35 cycles at 94 °C for 60 sec, 55 °C for 60 sec (annealing), and 72 °C for 60 sec, with final extension at 72 °C for 10 min. The optimal dilution was 1/8 µM. Consequently, each reaction for the competitive PCR analysis included 1 µM p4.1 primers, 0.125 µM actin primers, 1 µl of cDNA 1X reaction buffer, 5% DMSO, 200 µM dNTPs, 0.1 µCi/µl [α-^{32}P] dCTP (Dupont), and 50 U/ml Taq polymerase (Perkin Elmer). All RT-PCR reactions were performed in triplicate. 5 µl of loading dye were added to each sample after the amplification process and the PCR products were resolved on a 6% polyacrylamide gel, which was prepared just like a sequencing gel with the only difference, that gel mix 6 solution (Life technologies) was being used. As for the sequencing gel, the gel was pre run at 55 Watts for

30 min, the samples were then loaded and electrophoresis took place at 60 Watts for 3 hours. The gel was transferred from the glass plates to paper and dried in an automated gel dryer for 2 hours. Results were analyzed using a Phophoimager (Molecular Dynamics).

Isolation of and expression of EPB4.1 transcript from the Lan-5 cells

Plan:

In order to learn more about the localization of p4.1 protein in neuroblastoma tumors, it was necessary to isolate an EPB4.1 transcript and to insert it into mammalian cells.

To achieve this goal, EPB4.1 coding sequence was isolated from the Lan-5 cell line by producing cDNA from total RNA. That fragment was subsequently amplified and cloned into a bacterial vector. A vector is the term used for the carrier of the foreign DNA fragment used in the cloning process. Essentially there are three main types of vector: plasmids, bacteriophages and cosmids. Plasmids occur naturally in bacteria, to which they confer resistance to various antibiotics, heavy metals, etc. They are stably inherited in an extrachromosomal state and consist of a circular duplex of DNA. The advantage as vectors is that they possess a limited number of unique restriction sites and carry resistance to particular antibiotics, a characteristic, which is used to identify recombinant clones. Restriction endonucleases are certain microbial enzymes that cleave DNA at sequence specific sites. They were discovered by Hamilton Smith and his colleagues in 1970 for which he was later awarded the Nobel Prize. Because of the complementary pairing of bases in the DNA molecule, the restriction endonucleases always create double-stranded breaks. Depending on the particular base sequence which is cleaved either a staggered or blunt end result (Emery Alan E.H. and Mueller Robert F., 1993b). When DNA has been cleaved by a restriction

enzyme, which produces staggered termini with complementary nucleotide sequences, these termini are referred to as being 'sticky' since they will unite with complementary sequences produced by the same enzyme on DNA of a suitable vector. The cohesive termini are held together by hydrogen bonding but are then sealed and stabilized with an enzyme called DNA ligase. The union of the foreign DNA fragment with that of the vector produces the desired recombinant molecule.

After the foreign DNA has been incorporated into the plasmid the plasmid is introduced into the host bacterial cell by making the cell membrane permeable to the plasmid by a variety of methods, such as exposing the bacteria to calcium salts. This is referred to as transformation.

The next step is to grow the host-vector in culture medium to produce clones, i.e. multiple identical copies of the various individual recombinant DNA molecules generated.

If the restriction enzyme used to insert the DNA fragment cuts within the drug resistance gene of the vector then this can be used as a screening procedure. This would allow one to differentiate the recombinant clones from those without inserts in which the vector has merely been religated to itself, since any host cells transformed by the latter will still be resistance to both antibiotics (Emery Alan E.H. and Mueller Robert F., 1993c).

Once the gene for the p4.1 protein has been cloned it was to be inserted into the pcDNA3.1/His C plasmid to create a construct with an X-press epitope promotor sequence fused to the 5' end. Such a construct is called an expression vector because it is designed to express large amounts of protein. For In Vitro Transcription and Translation (TNT) analysis, the Lan-5 p4.1 protein coding region was subcloned into pSPT18 vector lacking the first methionine. Different promoters sequences are used depending upon whether the protein is to be made in bacterial, yeast or mammalian cells. For inducible expression and localization

studies of p4.1 in human 293 epithelial cells, it was therefore also necessary to insert the DNA fragment into the pind.sp1.V5 expression vector, which is thereafter stably transfected into the genome of the cultured 293 mammalian epithelial cells, which then produce the protein (Figure 6).

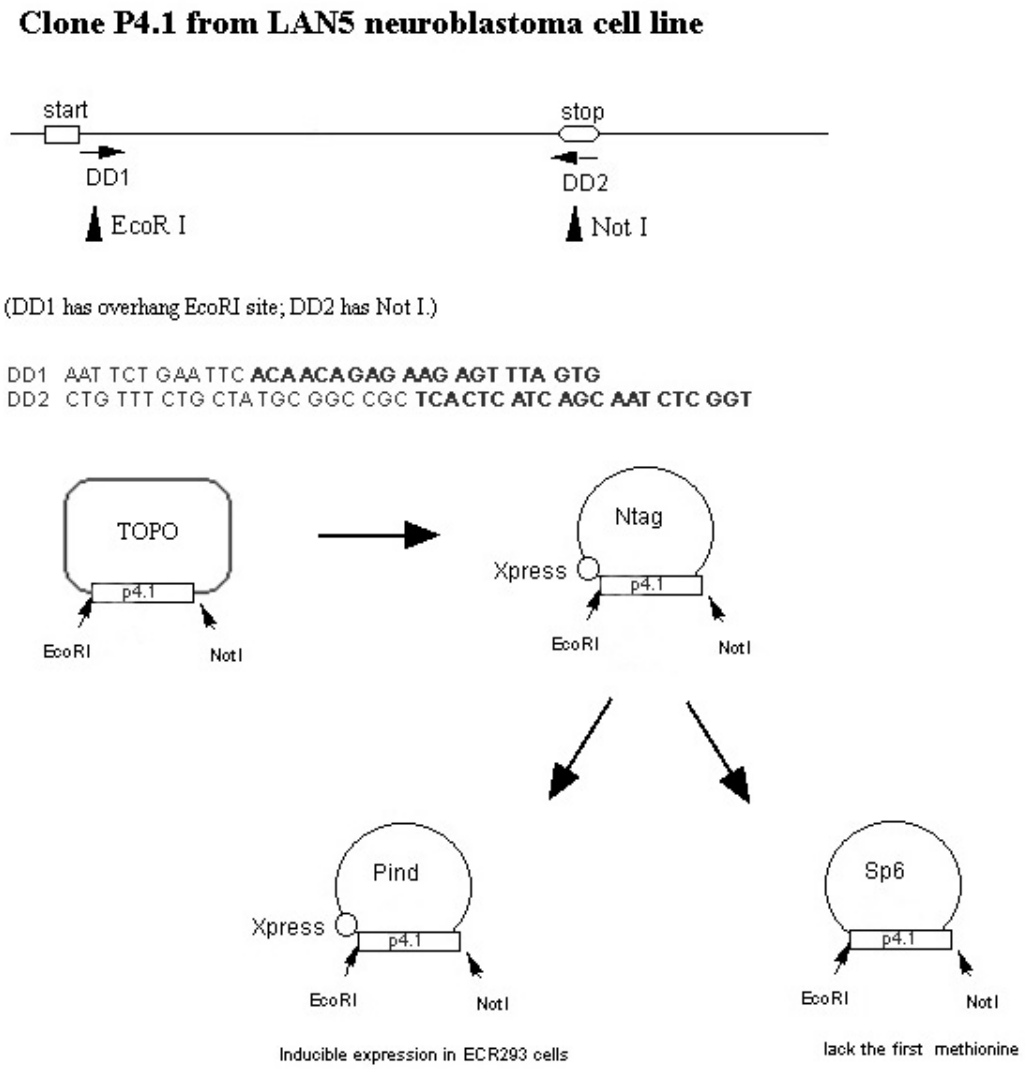


Figure 6: Cloning of the Protein 4.1 Construct

1. amplify p4.1 on Lan-5 (-) cDNAs
2. clone p4.1 coding sequence into TOPO vector
3. subclone insert from TOPO vector into Ntag vector
4. subclone insert from Ntag vector into Pind vector (pind.sp1) and sp6 vector

Procedure:

Isolation of Lan-5 p4.1 protein coding sequence

Extraction of total RNA

Total RNA of the Lan-5 cell line was extracted from a 5 ml Lan-5 neuroblastoma cell line culture flask by discarding 3.5 ml of medium, before scraping the cells and transferring them in the remaining content in a 1.7 ml tube. The cells were pelleted by spinning them at 2000 rpm for 5 min, resuspended in 1 ml RNA STAT, and pipeted up and down for approximately 20 times until viscosity drops occurred. 200 µl of Chloroform were added. The tube was vigorously shaken for 15 sec, before leaving it for 2 – 3 min at room temperature. Centrifugation at 14000 rmp for 15 min and transfer of the upper phase to a Rnase free tube were the next steps. 500 µl of isopropanol were added to the Rnase free tube. It was then vortexed and spun at 14000 rpm for 10 min. The resulting pellet was washed with 500 µl 75% ethanol. After a short spin, the pellet was air dried for 10 min and then resuspended in 20 µl of DEPC water. Extraction of the total RNA was confirmed by running 1 µl of reaction on a mini-agarose RNA gel, which was produced by mixing 0.25 g of Agarose with 22 ml of DEPC water, microwaving theis mixture for 50 sec, followed by a cooling period of 10 min at room temperature, before 2.5 ml of 10X mops and 0.9 ml of formaldehyde were added and the gel was poured. 1 µl of RNA and 1 µl of RNA markers were loaded onto the gel, after the have been mixed with 3 µl of a mixture which consisted of 2 µl of 10X mops, 3.5 µl of formaldehyde, 10 µl of formamide, 0.2 µl of Ethium bromide and 2 µl of RNA loading dye. Before loading, the tubes were topped with oil and heated to 65 °C for 15 min and put on ice for 5 min.

c-DNA (-) strand synthesis

Complementary c-DNA (-) strand was produced using the Cicol BRL kit. 2 µl of total RNA was pipetted into a sterile 0.5 µl tube. 1 µl of 10X PCR buffer containing 15 mM of $MgCl_2$ (Perkin Elmer), 0.8 µl of 25 mM $MgCl_2$, 1 µl of Dnase I, and 5.2 µl of Depc water were added. Tube content was mixed and left at room temperature for 15 min. Thereafter 1 µl of 25 mM EDTA was added and the tube was heated to 65 °C for 15 min and afterwards put on ice for 1 min to inactivate Dnase I.

To anneal the oligo dT, 1 µl of Oligo dT and 2 µl of Depc water were added, and the tube was heated to 70 °C for 10 min and placed on ice right away for 2 min to denature RNA secondary structures. After a short centrifugation, 2 µl of 10X PCR buffer, 2 µl of 25 MM mgCl2, 2 µl of 0.1 M DDT and 1 µl of dNTPs were added. The content was mixed and the tube heated to 42 °C for 5 min to allow the oligo dT to anneal to the RNA polyA tail.

c-DNA synthesis was then initiated by adding 1 µl of Superscript IIRT enzyme and heating the tube to 42 °C for 50 min. For enzyme deactivation, the tube was heated to 70 °C for 15 min and then chilled on ice for 2 min afterwards. After a short spin, total RNA was degraded by adding 1 µl of RNase H and keeping the tube at 37 °C for 20 min. c-DNA product was confirmed on an agarose gel by performing regular PCR on ß-actin using PCR conditions mentioned above.

Preparation of the Insert

Primers 5’ AAT TCT GAA TTC ACA ACA GAG AAG AGT TTA GTG and 5’ CTG TTT CTG CTA TGC GGC CGC TCA CTC ATC AGC AAT CTC GGT were used to PCR amplify the protein coding sequence from the first methionine in exon 2 to the stop codon in exon 21 of p4.1 in the Lan-5 cell line. PCR conditions were: denaturation at 94 °C for 10 min, then

35 cycles at 94 °C for 60 sec, 55 °C for 60 sec (annealing), and 72 °C for 60 sec, with final extension at 72 °C for 10 min. Products were analyzed in a UV-light box after they were electrophoresed on a 1% agarose gel as described earlier.

Cloning of the Insert into a TOPO-Vector

The product was inserted into the TOPO TA Cloning Vector (Invitrogen) according to the manufacturers protocol. For that reason, 2 µl of PCR sample were mixed with 2 µl of sterile water and 1 µl of PCR-TOPO Vector. The mixture was flipped gently and incubated for 5 min at room temperature. After a brief centrifugation, tube was placed on ice.

Transformation took place by adding 2 µl of 0.5 M ß-mercaptoethanol to a vial of competent cells. After stirring with a pipette tip, 2 µl of the TOPO-Cloning reaction were added into the vial of One Shot cells and mixed gently. After an incubation time on ice for 30 min, cells were heat shocked for 30 seconds at 42 °C without shaking. The tube was transferred to ice immediately for 2 min. Then, 250 µl of room temperature SOC medium were added and the tube was incubated at 37 °C for 1 hour. 150 µl of the tube content was spread on prewarmed LB Agar Plates containing 100 µg/ml Ampicillin (Digene) and the plate was incubated at 37 °C overnight.

The next day, 4 colonies were picked and cultured separately overnight in Falkon Tubes containing in 2 ml LB medium also containing 100 µg of Amp/ml.

After another 24 hours, the plasmid was isolated following the Midi Prep protocol (Quiagen): Falcon tubes were spun at 3000 rpm for 10 min and the supernatant was discarded. The pellets was resuspended in 4 ml of Buffer P1 (Rnase A) and 4 ml Buffer P2 was added. Tubes were inverted 6 times and left at room temperature for 5 min. Next, 4 ml of Buffer P3 was added to each tube and they were again inverted 6 times, before cooling them to 4 °C for 15 min and

centrifuging them at 4000 rpm for 30 min. 4 columns were set up and filled with 4 ml of QBT Buffer. Content of Falkon tubes was poured into columns using a cell strainer. Columns were then filled twice with 10 ml QF Buffer. Plasmid could then be eluted into new Falkon tubes by adding 5 ml of Buffer QF to the columns. 3.5 ml of isopropanol was added and the tubes were vortexed before they were centrifuged at 4000 rpm for 30 min. The pellets were washed with 1 ml of 70% ethanol and transferred to 1.5 ml tubes. They were then centrifuged briefly at top speed and the pellet was resuspended in 50 µl of DEPC water.

Cloning of the p4.1 Insert into a pcDNA3.1/His C Vector

To create a construct with an X-press epitope promotor sequence fused to the 5' end and for selection purposes, the TOPO plasmid and the bacterial vector pcDNA3.1/His C (Invitrogen) were cut by restriction digestion at ECOR I and Not I sights in two separate reactions by mixing 1 µl of 10X Buffer (Qiagen), 7 µl of plasmid, 1 µl of ECOR I and 1 µl of Not I restriction enzyme. The mixture was incubated at 37 °C for 1 hour. The insert of the TOPO vector and the pcDNA3.1/His C vector were gel purified as described above and then ligated using mixing 1 µl of 10X Ligase Buffer, 1 µl of Ligase, 4 µl of plasmid and 4 µl of insert. Mixture was incubated at 37 °C for 40 min. The new construct was cultured and isolated as described above.

Sequencing of the p4.1 Construct

Next, the construct was entirely sequenced using the following primers: p4seq-1F (5' CAG TCT TGA TGA AGA GAT CA); p4seq-2F (CTC CAT GGC GTG GAT TAT G); P4seq-3R (5' ACC TTC CAT GCT TCT GTG); P4seq-4F (5' CCA CAC TTC GAG CGT ACA); P4seq-5F (5' CAC TCA CTC ACC CTTCCG); P4seq-6R (5' TGT CAG CAA GAC TCC

TGG); P4seq-7F (5' CAG ACA TAG CAA TTT AAT G); P4seq-8F (5' GAG CCA GAG CCC ACA GAA GCA T); P4-4CF (5' GGA CTT GAA GAG TGC TCC AA); P4-4CR (5' ATT CCA AGG GAC ACC ACG AA); BamHF (5' CCA GTT ACC GAG CAG CTA AG); and BamHR (5' CTT GCT GTA CAC TCA AAG TG), using the sequencing protocol described above.

In Vitro Transcription and Translation (TNT) of Lan--p4.1 protein

For this study, the Lan-5 p4.1 protein coding region was subcloned into pSPT18 (Boehringer Mannheim) at Hind III and Not I sites in the same way described above to have the expression control under a sp6 promoter. The TNT SP6-coupled Reticulocyte Lysate System (Promega) was used to synthesize in vitro transcribed and translated Protein 4.1 according to the manufacrures's protocol. [^{35}S]-methionine (Amersham Life Science) was used to label the synthesized protein. In detail, 25 µl of TNT Rabbit Reticulocyte Lysate was mixed with 2 µl of TNT Reaction Buffer, 1 µl of TNT RNA Polymerase, 1 µl of Amino Acid Mixture Minus Methionine (Plasmid-DNA), 4 µl of [^{35}S]-methionine and 17 µl of sterile water. The mixture was incubated at 30 °C for 70 min. The protein products were analyzed by autoradiographie in a Nu-PAGE gel (Nowvex).

Subcellular localization of Lan-5 p4.1

Lan-5 p4.1 sequence was subcloned into Pind.sp1.V5 expression vector (Invitrogen) for inducible expression. The inducible construct was transfected into 293 mammalian cells using lipofectamine plus (Gibco BRL) by adding 2 µl of DNA into a tube containing 500 µl of OPTIMEM. 10 µl of lipofectin was added into another tube containing 50 µl OPTIMEM. The tube was vortexed and incubated for 15 min at room temperature. Cells were washed two

times with OPTIMEM. They were seeded 2 days before transfection and were 50 – 80% confluent. Next, the DNA-lipofectin mixture was poured over the cells and the plate was shaken gently at least 3 times during the first hour of transfection. After 24 hours, the Lipofectin/DNA containing medium was exchanged for 2 ml of normal growth medium. 3 days post-transfection, antibiotics were added to the medium for cell selection and cells were cultured in 15 ml flasks.

Transfected cells wee seeded on the slide chambers (Nunc, Naperville, IL) in RPMI 1640/10% calf serum (Life Technologies) for 24 h in the presence of 1.25 μg/ml muristerobe A (Invitrogen). Then the cells were washed with PBA (PBS with 0.1% NaN_3 and 0.1% BSA, pH 7.4) before staining with antibody. Fix & Perm Permeabilization medium (Caltag, Burlingame,CA) was used for fixaton and permeabilization of the cells. Primary antibody, Ani-Xpress antibody (Invitrogen, Carlsbad, CA), and secondary antibody, FITC-conjugated goat anti-mouse IgG-1 (Caltag, Burlingame, CA) were used to detect the X-press fused p4.1 under a Zeiss Axiophot epifluorescence microscope. Two-color images were captured on a Photometrics CCD camera (Photometrics) using IP Lab image software (Scan Analytics Corporation). Cells were stained with DAPI (Oncor) for visualizing the nucleus of cells.

RESULTS

Alternative splicing pattern analysis

As described above uses EPB4.1 a complex alternative splicing machinery to produce several isoforms depending on the inclusion or exclusion of various exons. The incorporation of exon 3 (the stop codon-rich) renders the 80 kDa erythroid product by utilizing a start codon in exon 4, whereas the exclusion of exon 3 leads to usage of a start codon in exon 2, resulting in the synthesis of the nonerythroid 135 kDa isoform (Figure 7).

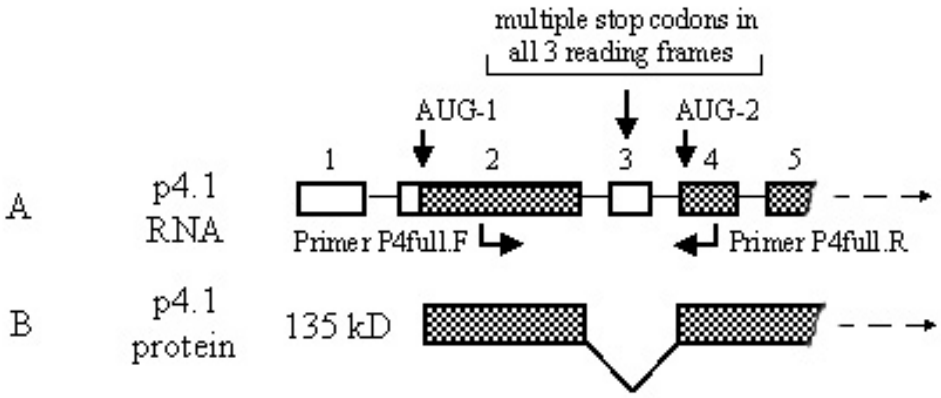


Figure 7: Alternative Splicing pattern of nonerythroid p4.1
Exclusion of exon 3 (A) leads to the 135 kDa nonerythroid isoform (B)

To investigate if p4.1 is expressed at all, and also for protein functional study purposes, exploring and understanding the splicing pattern of exon 3 in all tumors, including the Lan-5 construct was important. The issue was addressed by performing RT-PCR with primers flanking this exon. The results demonstrated that all EPB4.1 neuroblastoma transcripts lacked exon 3, and hence produced the expected 135 kDa isoform (Figure 8). DNA sequence analysis

of the Lan-5 p4.1 clone also confirmed the exclusion of exon 3. All the other exons of EPB4.1 were present in this transcript.

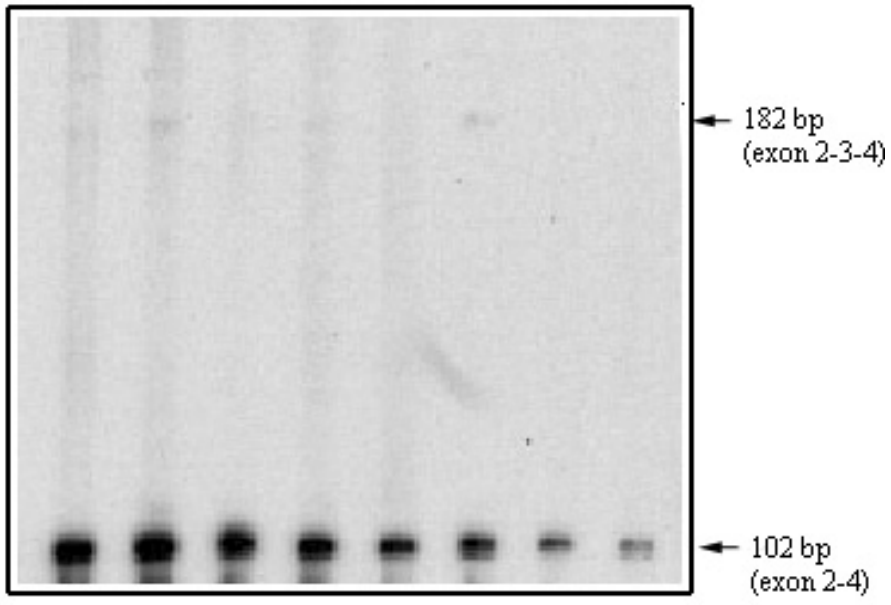


Figure 8:
Exon 3 exclusion from the EPB4.1 transcript in neuroblastome tumors. A representative analysis this is shown in 8 tumors. An exon 2 forward primer and an exon 4 reverse primer were used to perform PCR on the tumor cDNA. The expected size for the PCR product including exon 3 is 182 bp. A 102 bp product is expected from the transcript lacking exon 3. No tumor analyzed shows the 182 bp product.

Mutation analysis

Genomic DNA from 78 neuroblastoma tumors and 5 neuroblastoma cell lines was subjected to SSCP analysis using 22 sets of primers (Table 1) that target the 5' untranslated region and the protein coding exons of EPB4.1. The Lan-5 transcript of EPB4.1 was also subjected to DNA sequencing for sequence comparison with the wild-type. SSCP analysis revealed the following sequence changes mutations:

Missense mutations – mutations that alter the resulting amino acid sequence and consequently the final protein product – were detected in exon 4 (813 G/A, V/I) in three tumors (Fig 9a upper panel),, and one cell line, and in exon 8 (1212 G/A, V/M) in one tumor.

Mutations leading to the replacement of single nucleotides in exons without affecting the resulting amino acid sequence are called Silent mutations and were found in exon 21 (2801 C/T) in six tumors, and exon 16(2144 A/G, G/G) in one tumor. Intronic aberrations – changes in the intron sequence with unknown consequences - (introns 9, 10, 17, 18, and 21 respectively) could be found in 9 tumors; 7 carrying point muatations, 2 with deletions (data not shown).

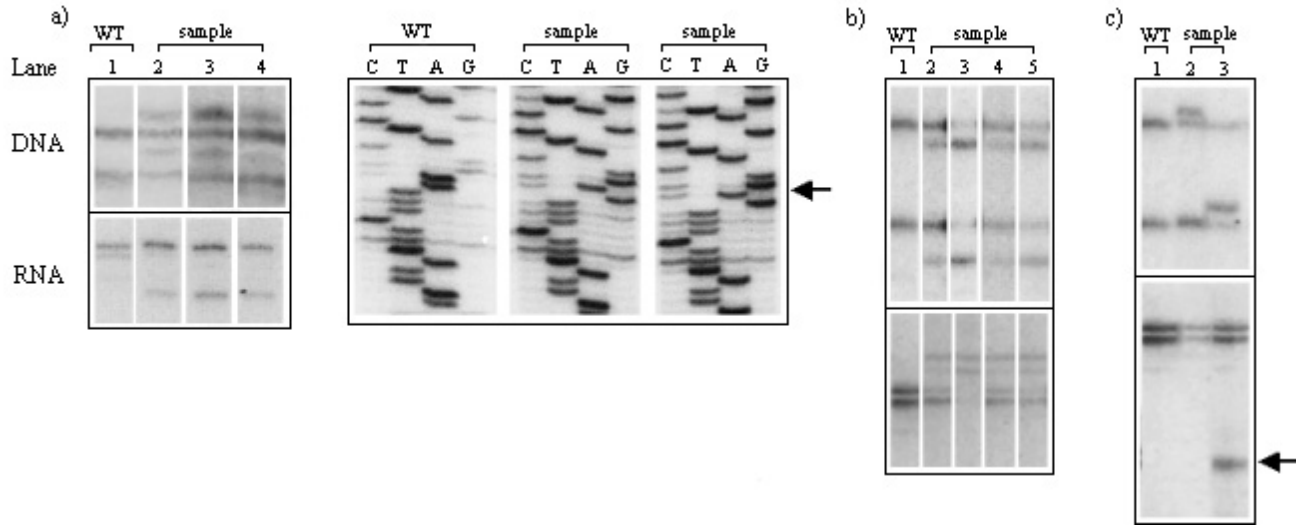


Figure 9: Extract of Mutation Analysis
(a) Absence of wild-type EPB4.1 expression. SSCP analysis of genomic DNA and cDNA revealed the absence of wild-type transcrips. Upper panel, SSCP of genomic DNA; lower panel, SSCP of RT-PCR; lane 1, tumor with wild-type EPB4.1.
(b) Biallelic expression of EPB4.1. SSCP analysis of genomic DNA (upper panel) and cDNA (lower panel) from tumors with wild-type EPB4.1 (lane 1), with both wild-type and silent mutation alleles (lane 2, 4, and 5), and with only silent mutation (lane 3).
(c) Detection of an aberrant transcript. SSCP analysis of genomic DNA (upper panel) and cDNA (lower panel) from tumors with wild-type EPB4.1 (lane 1), with 13 bp deletion in intron 21 (lane 2), and with point mutation 10 bp downstream of exon 20 (lane 3). An aberrant transcript is seen in lane 3.

Sequence analysis of the Lan-5 EPB4.1 transcript confirmed the aberrations detected by SSCP analysis, but revealed a additional ones, also. In total: Silent mutations in exon 2 (246 C/T,

H/H), and exon 4 (650 G/A, Q/S); 6 missense mutations in exon 2 (250 G/C, V/L; 320 G/A, S/N), exon 4 (676 C/T, S/F), exon 5 (948 G/A, A/T), exon 16 (2167, A/G, N/S), and exon 17 (2208 A/G, K/E). All detected sequence variations are summarized in a compact form in Table 2. A detailed list can be found in the Appendix-Section.

Table 2: Results Compact

Turmor ID	Expression	LOH	Wild Type Allele	Missense Sequence Changes	Silence Sequence Changes	Intronic Sequence Changes
2750/ 1627	77/23	--	Ø	Ø	Ø	Intron 21 (+13 of Exon 21, C/T)
2757/ 1634	70/30	LOH 1p	Ø	Ø	Ø	Intron 18 (-19 of Exon 19, G/C)
2759/ 1636	66/33	--	Presence of WT allele	Exon 8 (1212 G/A, V/M)	Exon 21 (2801 C/T, T/T)	Ø
2764/ 1641	71/29	--	Absence of WT allele	Ø	Exon 21 (2801 C/T, T/T)	Ø
2765/ 1642	78/22	Homo-zygous	Ø	Ø	Ø	Ø
2767/ 1644	80/20	--	Presence of an aberrant transcript	Ø	Ø	Intron 21 (+10 of Exon 21, G/A)
2768/ 1645	66/34	--	Ø	Ø	Exon 16 (2144 A/G, G/G)	Ø
2790/ 1655	73/27	--	Presence of WT allele Absence of WT expression	Exon 4 (813 G/A, V/I)	Ø	Ø
2794/ 1659	95/5	Hetero-zygous	Presence of WT allele	Ø	Exon 21 (2801 C/T, T/T)	Ø
2795/ --	--/--	--	Presence of WT allele	Ø	Exon 21 (2801 C/T, T/T)	Ø
2798/ 1664	93/7	--	Ø	Ø	Ø	Intron 18 (-19 of Exon 19, G/C) Intron 21 (13nt del) (+10 to +22 of Exon 21)
2800/ 1666	73/27	--	Presence of WT allele	Ø	Exon 21 (2801 C/T, T/T)	Ø
2801/ 1667	88/12	--	Presence of WT allele	Ø	Exon 21 (2801 C/T, T/T)	Ø

Turmor ID	Expression	LOH	Wild Type Allele	Missense Sequence Changes	Silence Sequence Changes	Intronic Sequence Changes
2857/ 1723	67/33	Hetero-zygous	Ø	Ø	Ø	Intron 10 (4 nt del) (-16 to –13 of Exon 11)
2859/ 1725	65/55	--	Presence of WT allele Absence of WT expression	Exon 4 (813 G/A, V/I)	Ø	Intron 9 (+36, G/A of Exon 9)
2873/ 1739	60/40	--	Presence of WT allele Absence of WT expression	Exon 4 (813 G/A, V/I)	Ø	Ø
2886/ 1752	67/32	--	Ø	Ø	Ø	Intron 10 (-19 of Exon 11, T/G)
LAN5		--	Ø	Exon 2 (250 G/C, V/L; 320 G/A, S/N) Exon 4 (676 C/T, S/F) Exon 5 (948 G/A, A/T) Exon 16 (2167, A/G, N/S) Exon 17 (2208 A/G, K/E)	Exon2 (246 C/T, H/H) exon 4 (650 G/A, Q/Q)	Ø
CHP-134B		--	Presence of WT allele	Exon 4 (813 G/A, V/I)	Ø	Ø
AS		--	Ø	Ø	Ø	Intron 18 (-19 of Exon 19, G/C)

Expression analysis

To analyze the significance of the SSCP- and sequencing-findings, allelic expression of EPB4.1 on those seven tumors that had sequence changes in exon 4 or exon 21 by RT-PCR-SSCP analysis were evaluated.

All the 3 tumors with a G/A change in exon 4 displayed absence of wild-type expression (Fig. 9 a lower panel), although wild-type alleles were detectable in the genome (Fig. 9a upper panel). The SSCP bands were excised and subjected to direct DNA sequencing for final confirmation.

Analysis of the allelic expression of the other four tumors harboring a silent mutation in exon 21, demonstrated biallelic expression except one, that had lost the wild-type allele in the genome (Fig. 9b).

The effect of intronic aberrations found in 2 tumors - one with a 13-bp deletion and another with a point mutation 10 nucleotides downstream of exon 22 in intron 21 - on EPB4.1 expression was evaluated by performing SSCP analysis on the RT-PCR products. In the tumor with a 13-bp deletion, no splicing errors in exons 20 to 21 were detected. However, in the case of the tumor with the point mutation, an aberrant transcript was observed (Fig. 9c). The subsequent DNA sequence analysis revealed, that an absence of 81 nucleotides from exon 20 was associated with this aberrant transcript (data not shown).

Since the high frequency of mutations detected in the Lan5-p4.1 clone by direct DNA sequencing suggests that the SSCP analysis might underestimate the actual number of mutations in the tumors, and since the selective suppression of p4.1 expression was observed in the tumors with missense mutations in exon 4, it became essential to gain knowledge of the expression level of

p4.1 in the other tumors as well. Therefore the project was extended and the level of protein 4.1 transcripts in all 72 tumors was assessed in comparison to a housekeeping gene by semiquantitative duplex RT-PCR, using one set of primers amplifying a 276 bp fragment from EPB4.1 transcript and the other set amplifying a 285 bp fragment from beta-actin.

Nine out of 72 tumors displayed either absence or significantly reduced EPB4.1 expression (Fig. 10 i-ii). A set of 11 tumors displayed low level of EPB4.1 expression (<0.25), as compared with a major normal distribution interpolated by using CA-Cricket Graph III (Fig. 10 iii).

However, tumors with reduced p4.1 expression were subsequently compared to the ones found to carry p4.1 mutations, but no homology or correlation could be detected. In fact, not a single sample with aberrations showed reduced p4.1 expression!

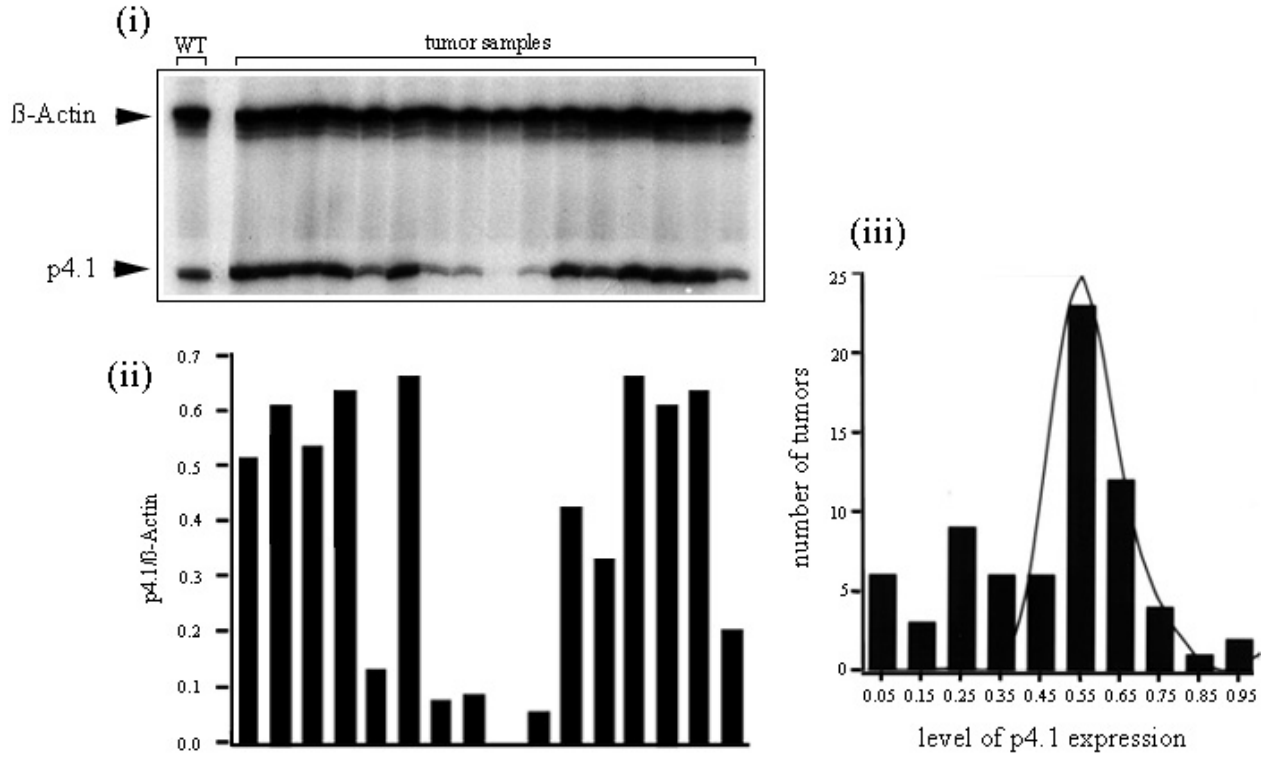


Figure 10: Expression Analysis
A representative analysis of level of EPB4.1 expression is shown in 16 tumors (i). Amplifications from EPB4.1 and beta-action are 206 bp and 285-bp, respectively. Levels of EPB4.1 expression were analyzed by a Phosphoimager (Molecular Dynamics) and the relative levels of EPB4.1 after normalized to beta-actin is shown (ii). Distribution of the levels of EPB4.1 expression in neuroblastomas is shown and a major normal distribution curve is interpolated (iii).

After unblinding the patients' data, the expression level of each neuroblastoma tumor was also compared to its clinical staging. There were 25 stage I classified tumors with a mean p4.1/ß-actin ratio of 0.397, 5 tumors classified stage II and a mean ratio of 0.391, 3 tumors classified stage IIb with a mean ratio of 0.160, 11 tumors in stage III with a mean ratio of 0.322, 16 tumors in stage IV with a mean ratio of 0.491 and 10 tumors classified stage IVs with a mean p4.1/ß-actin ratio

of 0.351. Displaying the ratio of every tumor stage in a graph, no characteristic pattern, or correlation could be observed (Figure 11).

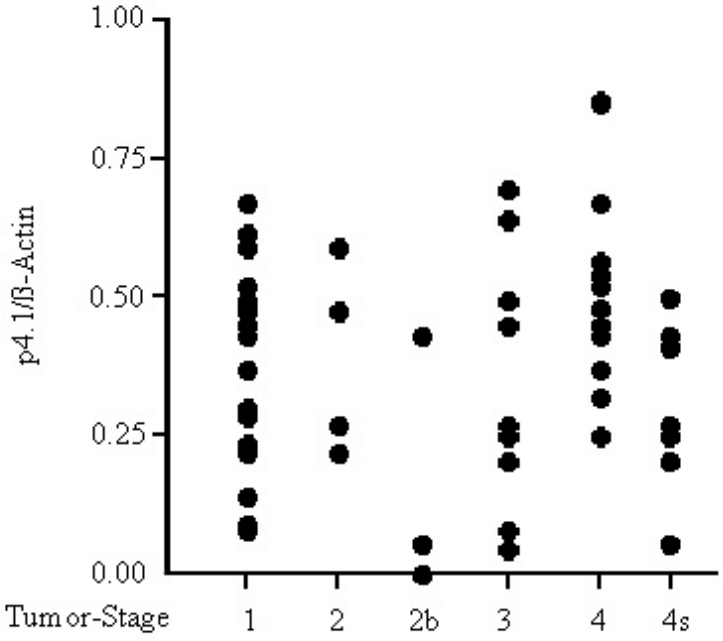


Figure 11:
Clinical Correlation
No pattern or correlation could be observed by comparing the EPB4.1 expression level of each primary neuroblastoma tumor with the clinical stage of the patient.

Membrane localization of Lan-5 EPB4.1 gene product

Previous studies of EPB4.1's 135 kDa isoform indicated that the protein is localized to the nucleus and interacts with the nuclear mitotic apparatus (nuMA) through the last 62 C-terminal amino acids (exon 20 and 21), although in the same report, by using a anti-headpiece antibody (specific for exon 2 and 4), anti-24 kD (specific for exon 19), and anti-10 kDa antibodies (specific for exon 17), the 135 dDa isoform can also be detected in the cytoplasm as well as nuclear matrix fractions of MDCK cells. For localization studies in neuroblasomas, the

expression of traceable tumor-p.4.1, particularly the 135 kDa isoform, would be ideal. A construct containing full-length p4.1 was not known in the literature and was therefore created and submitted to Gene Bank (Genebank Accession number: AF 156225). Sequencing revealed that the transcript included the protein coding sequence from exon 2 and 4-21, and carried the numerous alterations summarized in table 2.

In vitro transcription and translation studies showed that this transcript was able to express the expected 135 kDa as well as 80 kDa polypeptides, presumably through the usage of the initiation codons in exon 2 and 4, respectively (Figure 12).

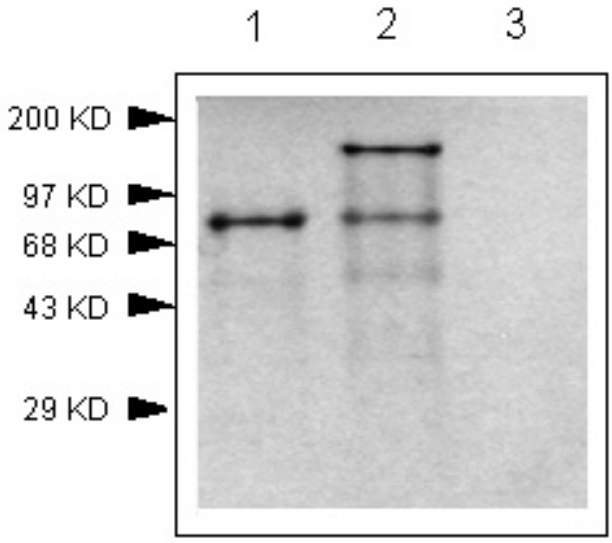


Figure 12: TNT Analysis
Full length *in vitro* transcription and translation of the Lan-5 EPB4.1 construct. The resulting protein products represent the expected 135 kDa and 80 kDa isoforms.

The presence of X-press tag in the Lan-5 p4.1 construct allowed to specifically detecting the 135 kDa isoform using an X-press antibody. X-press-Lan-5 p4.1 was subcloned into an inducible

expression vector and transfected into 293 epithelial cells. Immunfluorescence studies showed that the 135 kDa protein was localized in the membrane compartment of transfected cells. No nuclear localization staining of this 135 kDa isoform was detected under any fixation conditions (Figure 13).

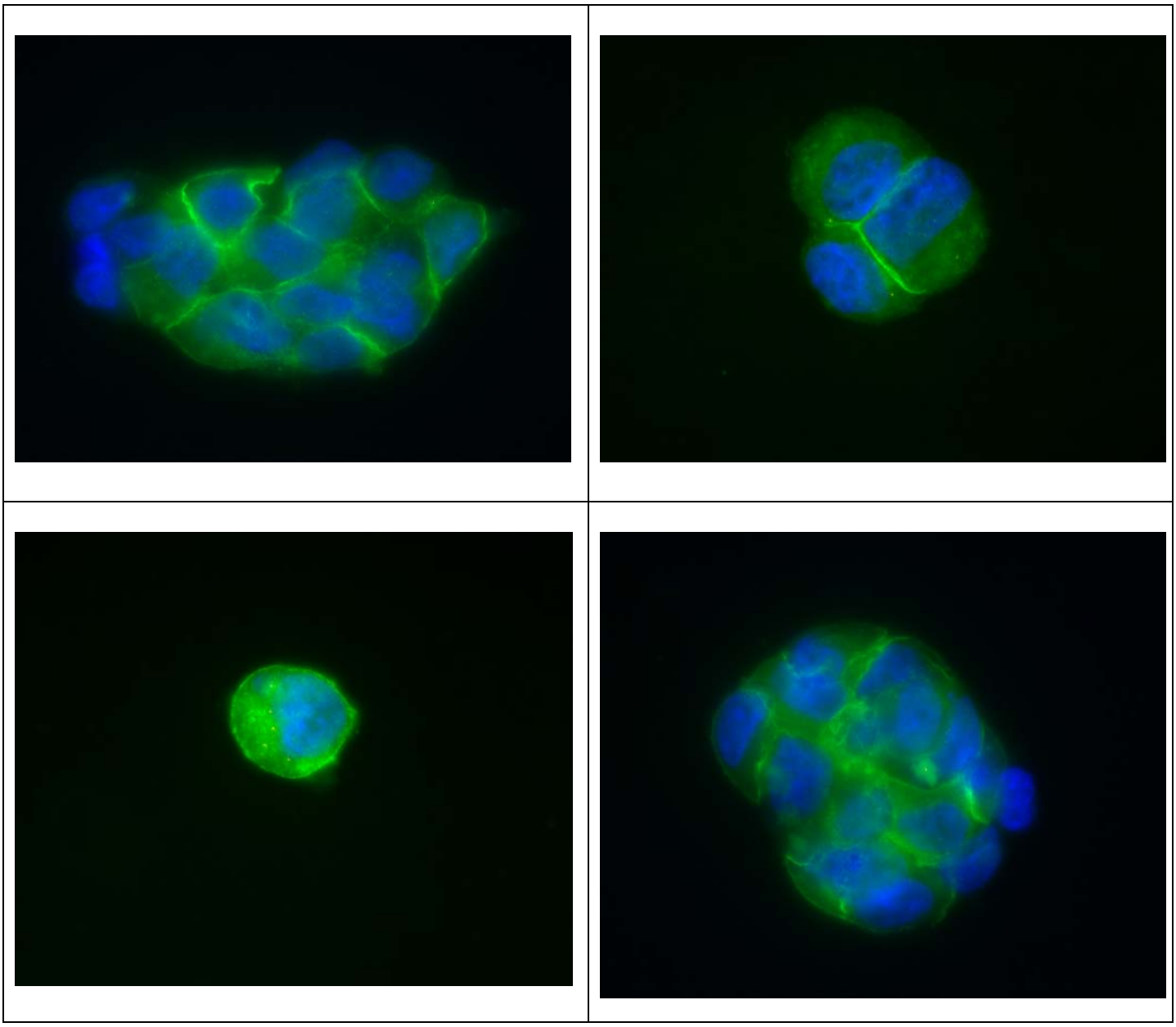


Figure 13: Protein 4.1 Localization
Subcellular localization of Lan-5 135 kDa isoform. 293 cells transfected with X-press tagged Lan-5 EPB4.1 cDNA displays a cytoplasm/membrane localization (green). The nuclei of the cells are shown in blue.

DISCUSSION

The telomeric end of chromosome 1 is a region of intense scrutiny because of its propensity to undergo deletions in a variety of tumors. While mutations of the NF2 tumor suppressor gene account for a majority of meningioma cases, up to 40% of them are of unknown origin (Ruttledge et al., 1994). Loss of heterozygosity (LOH) studies have implicated that the chromosomal region 1p is containing a second meningioma tumor suppressor gene (Bostrom et al., 1997; Carlson et al., 1997; Simon et al., 1995). Similar studies have correlated 1p LOH with a recurring and malignant grade of meningioma. Together, these data suggest a tumor suppressor gene of even greater consequence than the NF2 gene is associated with the development of aggressive meningiomas originating upon genetic inactivation of the chromosome 1p region (Ishino et al., 1998; Simon et al., 1995). Similarly, deletions of 1p36 are the most common genetic abnormalities in neuroblastomas, again suggesting the presence of a tumor suppressor gene. This hypothesis is strengthened by the observation that the micro cell transfer of chromosome 1p into neuroblastoma cells abolishes their growth (Bader et al., 1991).

The recent cloning of p73 generated much initial excitement as a putative neuroblastoma tumor suppressor at 1p36, by virtue of its striking homology to the p53 tumor suppressor (Kaghad et al., 1997). However, unlike p53, a gene mutated in 50% of human malignancies, no mutations of the p73 gene have yet been identified in neuroblastomas, or in any other human cancers, although – over the years - there have been numerous attempts by various research groups (Barrois et al., 2001; Nomoto et al., 1998; Norris et al., 2001). Knockout mice null for p73 corroborate this observation in that they are viable and do not develop tumors (Kaelin, Jr., 1998). Another

potential tumor suppressor gene AML2, and CDKN2, which is often mutated in melanomas and other cancers, are both located on 1p36 could also be ruled out to be involved in neuroblastomas (Beltinger et al., 1995; Spieker et al., 2001). In another attempt, Human Krüppel-Related 3 (HKR3) was assessed as a tumor suppressor gene in neuroblastomas. Although the loss of 1p36 was present in many tumor samples, HKR3 was always expressed, and no mutations, or gross genomic DNA rearrangements could be detected (Maris et al., 1997). More recently, the CDC2L1 (p58) gene, also mapped to 1p36, could be ruled out as the responsible tumor suppressor using SSCP analysis and sequencing on 15 1p LOH neuroblastoma tumors (Martinsson et al., 1997).

Understanding Protein 4.1's complicated splicing machinery is essential in respect to the various possible isoforms, their discrete functions and involvement in various diseases.
For example, the genetic basis of erythroid p4.1R deficiency in a patient with 4.1 (-) hereditary elliptocytosis (HE) has been elucidated. In this patient, a homozygous deletion of the downstream ATG in exon 4 causes the total absence of the 80 kDa isoform of protein 4.1R, which is usually predominantely expressed in red blood cells, in both erythroid and non-erythroid cells. However, in the same 4.1 (-) HE patient, the expression of nonerythroid p4.1 remains unaltered (Conboy et al., 1993; Davies and Lux, 1989).

Nonerythroid p4.1 is exemplified by the 135 kDa isoform translated from the upstream ATG located in exon 2 (Conboy, 1993). Its main function is yet to be determined, but it is involved in the spindle apparatus, possibly playing a critical role in the cell turnover rate. Again, it shares

strong homology to the NF2 tumor suppressor gene, which suggests an important role for nonerythroid protein 4.1, in that a mutation causing total 4.1 deficiency may be critically harmful. Splicing pattern analysis revealed that EPB4.1 transcripts are generally expressed in neuroblastoma tumors investigated. Moreover, all transcripts excluded exon 3, suggesting – as expected – that the predominant EPB4.1 gene product in neuroblastoma is the nonerythroid 135 kDa isoform.

All this evidence is supporting the theory, that protein 4.1 could play a significant and fundamental role in neuroblastoma development, and set the basis for this doctorial thesis work.

78 DNA and 78 RNA primary neuroblastoma tumor samples plus 5 neuroblastoma cell lines were analyzed for sequence alterations in the p4.1 gene (EPB4.1). Since neuroblastoma is a rare disease, constitutive DNA of the patients was unfortunately not available for LOH analysis on the 1p36 locus for the majority of tumors. Therefore and in compensation, SSCP analysis had to be directly employed on a large number of tumor samples.

Out of the 78 Tumors examined, ten contained missense mutations (Table 2). The exonic distribution of these mutations included one in exon 3 (I/S), four in exon 4 (all showed the same mutation: V/I), one in exon 8 (V/M) and five different mutations in the cell line Lan-5.

The four identical alterations observed in exon 4 (V/I) initially seemed to be a promising finding. But when 52 samples of normal blood genomic DNA from random individuals were screened for genomic changes in exon 4 of EPB4.1, seven carried the same alteration, which therefore must be interpreted as polymorphism. This finding was also submitted to Gene Bank (Genebank Accession number: AF 156226). Exon 8 of EPB4.1 encodes part of the FERM domain of protein 4.1R. Alterations in this region could be crucial, since members of this family include the ERMs

(ezrin, radixin, moesin), merlin/schwannomin, novel protein 4.1 homologues, and protein-tyrosine phosphatases (Arpin et al., 1994; Chishti et al., 1998; Parra et al., 1998; Walensky et al., 1999) There is considerable evidence suggesting that this domain is involved in the regulation of cell growth. Inactivating mutations in the gene encoding merlin/schwannomin occur in neurofibromatosis type 2 (NF2) disease, whose hallmark is the development of schwannomas and meningiomas (Rouleau et al., 1993). Additionally, overexpression of the NF2 gene suppresses the growth of NIH 3T3 fibroblasts (Lutchman and Rouleau, 1995). Genetic studies in Drosophila implicate a regulatory role in cell proliferation for the merlin homologue Expanded and the protein 4.1R homologue Coracle (Boedigheimer et al., 1997; LaJeunesse et al., 1998). A novel mammalian homologue of protein 4.1R, termed DAL-1, has been recently shown to have growth suppressing properties in lung cancer (Tran et al., 1999). Shimizu et al. (Shimizu et al., 1998) have also noted a decreased expression of protein 4.1 R in basal cell carcinoma and nearly complete loss of protein 4.1R in squamous cell carcinoma. Similarly, the FERM domain of radixin is known to interact with the Dbl oncogene product, a guanine nucleotide exchange factor for the Rho GTPase (Takahashi et al., 1998). Interestingly the Rho family of GTPases are key regulators of the actin cytoskeleton (Hall, 1998). Taken together, these observations suggest that the members of the protein 4.1 superfamily may modulate cell proliferation pathways via regulating the cytoskeletal-membrane interactions.

Biochemical similarities between protein 4.1R and brain tumor suppressor merlin/schwannomin allude to a conserved mechanism of action in vivo. While protein 4.1R's association with the actin cytoskeleton and the plasma membrane in the red cell is well established, merlin's interactions in vivo are less well understood. There is evidence that merlin is also localized beneath the plasma membrane (Deguen et al., 1998). However, observations suggest that merlin

may similarly function as a molecular linker between the membrane and cytoskeleton. The effect of the V/M mutation of exon 8 in the FERM domain observed in this study on the function of that domain is not known and remains to be investigated.

SSCP analysis also revealed that 6 missense mutation-carrying tumors also contained the wild type allele (Table 2). Interestingly, the expression of wild-type p4.1 was selectively suppressed (Fig. 9). This inhibition is unlikely to be related to imprinting since the expression of both alleles was observed in tumors carrying silent mutations (Fig. 9b). Nevertheless, the number of tumors carrying this aberration was too small to hypothesis that these changes play a fundamental role in neuroblastoma tumor development.

We also observed that the EPB4.1 displays splicing errors in the intronic sequence (#2798, +10 of exon 21, G/A)(Fig. 9c). However, these variations could eventually be found in the blood of random healthy individuals, and consequently these changes are not tumor-specific. Also, since all 3 tumors that only express mutant alleles share a constitutive amino acid change, the absence of the wild-type expression is unlikely to be significant.

We have further examined the expression of the 135 kDa isoform of protein 4.1 from the neuroblastoma cell line Lan-5 using PCR, cloning, and transfection techniques. Sequence analysis of the resulting EPB4.1 construct revealed a composition of exons 2, plus exon 4 to exon 21. Multiple missense mutations were detected in exon 2, exon 5, exon 16 and exon 17. Using an N-terminal tag specific antibody, the 135 kDa isoform of protein 4.1 was localized to the plasma membrane of the transfected epithelial cells. (Fig. 10) It is noteworthy here that although both 135 kDa and 80 kDa isoforms will be produced in the transfected cells, only the 135 kDa isoform will be detected by the N-terminal tag antibody. The subcellular localization of

nonerythroid p4.1 is complicated by its alternative exon usage. In the 80 kDa isoform with exon composition 4 to 21, exon 16 was demonstrated to be important for protein nuclear targeting presumably through a cluster of basic amino acids, KKKR, generated by joining exon 13 and 16 (Luque et al., 1998). It is also known that the nonerythroid 135 kDa isoform of p4.1R localized to the nucleus and interacted with the nuclear mitotic apparatus (NuMA) through the last 62 c-terminal amino acids (exon 20 and 21) (Luque et al., 1998). But the 135 kDa isoform can be detected in the cytoplasmic as well as the nuclear matrix fractions of MDCK cells by anti-headpiece domain (specific for exons 2 and 4), anti-24 kD (specific for exon 19), and anti-10Kda antibodies (specific for exon 19) (Luque et al., 1998). All in all, most of the available evidence supports the notion that the wild-type 135 kDa isoform with the exon composition reported in Lan-5 p4.1 should display a nuclear localization. Whether the exclusive membrane association of the Lan-5 p4.1 construct is a consequence of neuroblastoma specific mutations remains to be established by further localization of additional mutants and wild-type p4.1 constructs with comparable exon composition, especially since the cytoplasmic localization observed could be related to malfunctioning Protein 4.1 secondary to changes caused by extensive culturing of the Lan-5 cell line.

Semi-quantitative RT-PCR analysis indicated inhibition of p4.1 expression in nine tumors, though SSCP analysis failed to detect any mutations in these tumors (except #2798). Furthermore, after unblinding the patients' clinical data, no correlation could be found between the tumor stage and the level of p4.1 expression, or the tumor stage and the presence of detected gene alterations. Last, LOH data was only available on very few tumor samples, but among the five that did show LOH in the 1p36 region, only one actually displayed EPB4.1 alterations.

Taken together, it is now clear that Protein 4.1 does not play a critical role in human neuroblastomas. However, there is no doubt, that 1p36 is the crucial chromosomal region for neuroblastoma alterations. To date, despite various attempts, still, neither the responsible gene, or set of genes, nor a new promising candidate neuroblastoma tumor suppressor has been reported or proposed in the literature (Hiyama et al., 2001; Spieker et al., 2001).

SUMMARY

In summary, EPB4.1's exon 3 is spliced out – as expected – and nonerythroid 135 kDa p4.1 is the predominant isoform in neuroblastoma tumors. SSCP DNA and cDNA analysis on the entire coding region of EPB4.1 in 78 neuroblastoma samples and 5 neuroblastoma cell lines failed to reveal evidence of homozygous deletion or mutation of all or part of the gene: Lan-5 sequence variations have probably arisen during extended culturing, and suggest that either the gene doesn't perform an essential function or that the primary amino acid sequence can be substantially modified without loss of function. The 3 tumors that express only mutant alleles, share a constitutive amino acid change, which is also present in random normal blood DNA, the absence of the wild-type expression is unlikely to be significant. Sequence changes found in intron 21 must be interpreted as polymorphism. Splicing errors found in the intron 21 sequence are of unknown consequence, but they were too rare to play a major role in neuroblastoma tumor development. Neither the expression level of p4.1 versus ß-actin, nor the comparison of the expression level with the clinical tumor stage revealed abnormalities to further suggest EPB4.1 as the responsible tumor suppressor in neuroblastomas.

The entire EPB4.1 gene has been cloned and published in Gene Bank. The construct is being used in various research projects. Whether any alteration in subcellular localization of EPB4.1 is associated with neuroblastoma development is questionable and requires further investigation.

REFERENCE LIST

1. Alloisio,N., Dalla,V.N., Rana,A., Andrabi,K., Texier,P., Gilsanz,F., Cartron,J.P., Delaunay,J., and Chishti,A.H. (1993). Evidence that red blood cell protein p55 may participate in the skeleton-membrane linkage that involves protein 4.1 and glycophorin C. Blood *82*, 1323-1327.

2. Amler,L.C., Corvi,R., Praml,C., Savelyeva,L., Le Paslier,D., and Schwab,M. (1995). A reciprocal translocation (1;15) (36.2;q24) in a neuroblastoma cell line is accompanied by DNA duplication and may signal the site of a putative tumor suppressor-gene. Oncogene *10*, 1095-1101.

3. Anderson,R.A., Correas,I., Mazzucco,C., Castle,J.D., and Marchesi,V.T. (1988). Tissue-specific analogues of erythrocyte protein 4.1 retain functional domains. J. Cell Biochem. *37*, 269-284.

4. Anderson,R.A. and Marchesi,V.T. (1985). Regulation of the association of membrane skeletal protein 4.1 with glycophorin by a polyphosphoinositide. Nature *318*, 295-298.

5. Arpin,M., Algrain,M., and Louvard,D. (1994). Membrane-actin microfilament connections: an increasing diversity of players related to band 4.1. Curr. Opin. Cell Biol. *6*, 136-141.

6. Bader,S.A., Fasching,C., Brodeur,G.M., and Stanbridge,E.J. (1991). Dissociation of suppression of tumorigenicity and differentiation in vitro effected by transfer of single human chromosomes into human neuroblastoma cells. Cell Growth Differ. *2*, 245-255.

7. Baklouti,F., Huang,S.C., Tang,T.K., Delaunay,J., Marchesi,V.T., and Benz,E.J., Jr. (1996). Asynchronous regulation of splicing events within protein 4.1 pre-mRNA during erythroid differentiation. Blood *87*, 3934-3941.

8. Baklouti,F., Huang,S.C., Vulliamy,T.J., Delaunay,J., and Benz,E.J., Jr. (1997). Organization of the human protein 4.1 genomic locus: new insights into the tissue-specific alternative splicing of the pre-mRNA. Genomics *39*, 289-302.

9. Barrois,M., Eychenne,M.K., Terrier-Lacombe,M.J., Duarte,N., Dubourg,C., Douc-Rasy,S., Chompret,A., Khagad,M., Hartmann,O., Caput,D., and Benard,J. (2001). Genomic and allelic expression status of the p73 gene in human neuroblastoma. Med. Pediatr. Oncol. *36*, 45-47.

10. Belliveau,M.J., Lutchman,M., Claudio,J.O., Marineau,C., and Rouleau,G.A. (1995). Schwannomin: new insights into this member of the band 4.1 superfamily. Biochem. Cell Biol. *73*, 733-737.

11. Beltinger,C.P., White,P.S., Sulman,E.P., Maris,J.M., and Brodeur,G.M. (1995). No CDKN2 mutations in neuroblastomas. Cancer Res. *55*, 2053-2055.

12. Biegel,J.A., White,P.S., Marshall,H.N., Fujimori,M., Zackai,E.H., Scher,C.D., Brodeur,G.M., and Emanuel,B.S. (1993). Constitutional 1p36 deletion in a child with neuroblastoma. Am. J. Hum. Genet. *52*, 176-182.

13. Boedigheimer,M.J., Nguyen,K.P., and Bryant,P.J. (1997). Expanded functions in the apical cell domain to regulate the growth rate of imaginal discs. Dev. Genet. *20*, 103-110.

14. Bostrom,J., Muhlbauer,A., and Reifenberger,G. (1997). Deletion mapping of the short arm of chromosome 1 identifies a common region of deletion distal to D1S496 in human meningiomas. Acta Neuropathol. (Berl) *94*, 479-485.

15. Brodeur,G.M. (1994). Molecular pathology of human neuroblastomas. Semin. Diagn. Pathol. *11*, 118-125.

16. Brodeur,G.M. (2003). Neuroblastoma: biological insights into a clinical enigma. Nat. Rev Cancer *3*, 203-216.

17. Bunin,G.R., Ward,E., Kramer,S., Rhee,C.A., and Meadows,A.T. (1990). Neuroblastoma and parental occupation. Am. J. Epidemiol. *131*, 776-780.

18. Carlson,K.M., Bruder,C., Nordenskjold,M., and Dumanski,J.P. (1997). 1p and 3p deletions in meningiomas without detectable aberrations of chromosome 22 identified by comparative genomic hybridization. Genes Chromosomes. Cancer *20*, 419-424.

19. Caron,H., van Sluis,P., van Hoeve,M., de Kraker,J., Bras,J., Slater,R., Mannens,M., Voute,P.A., Westerveld,A., and Versteeg,R. (1993). Allelic loss of chromosome 1p36 in neuroblastoma is of preferential maternal origin and correlates with N-myc amplification. Nat. Genet. *4*, 187-190.

20. Cassoly,R. (1983). Quantitative analysis of the association of human hemoglobin with the cytoplasmic fragment of band 3 protein. J. Biol. Chem. *258*, 3859-3864.

21. Chasis,J.A., Coulombel,L., Conboy,J., McGee,S., Andrews,K., Kan,Y.W., and Mohandas,N. (1993). Differentiation-associated switches in protein 4.1 expression. Synthesis of multiple structural isoforms during normal human erythropoiesis. J. Clin. Invest *91*, 329-338.

22. Chishti,A.H., Kim,A.C., Marfatia,S.M., Lutchman,M., Hanspal,M., Jindal,H., Liu,S.C., Low,P.S., Rouleau,G.A., Mohandas,N., Chasis,J.A., Conboy,J.G., Gascard,P., Takakuwa,Y., Huang,S.C., Benz,E.J., Jr., Bretscher,A., Fehon,R.G., Gusella,J.F., Ramesh,V., Solomon,F., Marchesi,V.T., Tsukita,S., Tsukita,S., and Hoover,K.B. (1998). The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. Trends Biochem. Sci. *23*, 281-282.

23. Cohen,C.M., Foley,S.F., and Korsgren,C. (1982). A protein immunologically related to erythrocyte band 4.1 is found on stress fibres on non-erythroid cells. Nature *299*, 648-650.

24. Conboy,J., Mohandas,N., Tchernia,G., and Kan,Y.W. (1986). Molecular basis of hereditary elliptocytosis due to protein 4.1 deficiency. N. Engl. J. Med. *315*, 680-685.

25. Conboy,J.G. (1993). Structure, function, and molecular genetics of erythroid membrane skeletal protein 4.1 in normal and abnormal red blood cells. Semin. Hematol. *30*, 58-73.

26. Conboy,J.G., Chan,J.Y., Chasis,J.A., Kan,Y.W., and Mohandas,N. (1991). Tissue- and development-specific alternative RNA splicing regulates expression of multiple isoforms of erythroid membrane protein 4.1. J. Biol. Chem. *266*, 8273-8280.

27. Conboy,J.G., Chasis,J.A., Winardi,R., Tchernia,G., Kan,Y.W., and Mohandas,N. (1993). An isoform-specific mutation in the protein 4.1 gene results in hereditary elliptocytosis and complete deficiency of protein 4.1 in erythrocytes but not in nonerythroid cells. J. Clin. Invest *91*, 77-82.

28. Correas,I. and Avila,J. (1988). Erythrocyte protein 4.1 associates with tubulin. Biochem. J. *255*, 217-221.

29. Correas,I., Leto,T.L., Speicher,D.W., and Marchesi,V.T. (1986). Identification of the functional site of erythrocyte protein 4.1 involved in spectrin-actin associations. J. Biol. Chem. *261*, 3310-3315.

30. Cotran,R.S., Kumar,V., and Collins,T. (1999a). Robbins Pathologic Basis of Disease. (W.B. Saunders Company), p. 487.

31. Cotran,R.S., Kumar,V., and Collins,T. (1999b). Robbins Pathologic Basis of Disease. W.B. Saunders Company), p. 607.

32. Cotran,R.S., Kumar,V., and Collins,T. (1999c). Robbins Pathologic Basis of Disease. (W.B. Saunders Company), p. 286.

33. D'Angio,G.J., Evans,A.E., and Koop,C.E. (1971). Special pattern of widespread neuroblastoma with a favourable prognosis. Lancet *1*, 1046-1049.

34. Davies,K.A. and Lux,S.E. (1989). Hereditary disorders of the red cell membrane skeleton. Trends Genet. *5*, 222-227.

35. De Carcer,G., Lallena,M.J., and Correas,I. (1995). Protein 4.1 is a component of the nuclear matrix of mammalian cells. Biochem. J. *312 (Pt 3)*, 871-877.

36. Deguen,B., Merel,P., Goutebroze,L., Giovannini,M., Reggio,H., Arpin,M., and Thomas,G. (1998). Impaired interaction of naturally occurring mutant NF2 protein with actin-based cytoskeleton and membrane. Hum. Mol. Genet. *7*, 217-226.

37. Donehower,L.A., Harvey,M., Slagle,B.L., McArthur,M.J., Montgomery,C.A., Jr., Butel,J.S., and Bradley,A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215-221.

38. Emery Alan E.H. and Mueller Robert F. (1993b). Elements of Medical Genetics. Churchill Livingstone), pp. 27-28.

39. Emery Alan E.H. and Mueller Robert F. (1993a). Elements of Medical Genetics. Churchill Livingstone), pp. 75-76.

40. Emery Alan E.H. and Mueller Robert F. (1993c). Elements of Medical Genetics. Churchill Livingstone), pp. 28-30.

41. Gascard,P., Lee,G., Coulombel,L., Auffray,I., Lum,M., Parra,M., Conboy,J.G., Mohandas,N., and Chasis,J.A. (1998). Characterization of multiple isoforms of protein 4.1R expressed during erythroid terminal differentiation. Blood *92*, 4404-4414.

42. Gascard,P., Nunomura,W., Lee,G., Walensky,L.D., Krauss,S.W., Takakuwa,Y., Chasis,J.A., Mohandas,N., and Conboy,J.G. (1999). Deciphering the nuclear import pathway for the cytoskeletal red cell protein 4.1R. Mol. Biol. Cell *10*, 1783-1798.

43. Granger,B.L. and Lazarides,E. (1984). Membrane skeletal protein 4.1 of avian erythrocytes is composed of multiple variants that exhibit tissue-specific expression. Cell *37*, 595-607.

44. Granger,B.L. and Lazarides,E. (1985). Appearance of new variants of membrane skeletal protein 4.1 during terminal differentiation of avian erythroid and lenticular cells. Nature *313*, 238-241.

45. Grovas,A., Fremgen,A., Rauck,A., Ruymann,F.B., Hutchinson,C.L., Winchester,D.P., and Menck,H.R. (1997). The National Cancer Data Base report on patterns of childhood cancers in the United States. Cancer *80*, 2321-2332.

46. Gurney,J.G., Ross,J.A., Wall,D.A., Bleyer,W.A., Severson,R.K., and Robison,L.L. (1997). Infant cancer in the U.S.: histology-specific incidence and trends, 1973 to 1992. J. Pediatr. Hematol. Oncol. *19*, 428-432.

47. Haest,C.W. (1982). Interactions between membrane skeleton proteins and the intrinsic domain of the erythrocyte membrane. Biochim. Biophys. Acta *694*, 331-352.

48. Hall,A. (1998). Rho GTPases and the actin cytoskeleton. Science *279*, 509-514.

49. He,D., Zeng,C., and Brinkley,B.R. (1995). Nuclear matrix proteins as structural and functional components of the mitotic apparatus. Int. Rev Cytol. *162B*, 1-74.

50. Hemming,N.J., Anstee,D.J., Staricoff,M.A., Tanner,M.J., and Mohandas,N. (1995). Identification of the membrane attachment sites for protein 4.1 in the human erythrocyte. J. Biol. Chem. *270*, 5360-5366.

51. Hiyama,E., Hiyama,K., Ohtsu,K., Yamaoka,H., Fukuba,I., Matsuura,Y., and Yokoyama,T. (2001). Biological characteristics of neuroblastoma with partial deletion in the short arm of chromosome 1. Med. Pediatr. Oncol. *36*, 67-74.

52. Horne,W.C., Huang,S.C., Becker,P.S., Tang,T.K., and Benz,E.J., Jr. (1993). Tissue-specific alternative splicing of protein 4.1 inserts an exon necessary for formation of the ternary complex with erythrocyte spectrin and F-actin. Blood *82*, 2558-2563.

53. Horne,W.C., Leto,T.L., and Marchesi,V.T. (1985). Differential phosphorylation of multiple sites in protein 4.1 and protein 4.9 by phorbol ester-activated and cyclic AMP-dependent protein kinases. J. Biol. Chem. *260*, 9073-9076.

54. Horne,W.C., Prinz,W.C., and Tang,E.K. (1990). Identification of two cAMP-dependent phosphorylation sites on erythrocyte protein 4.1. Biochim. Biophys. Acta *1055*, 87-92.

55. Huang,J.P., Tang,C.J., Kou,G.H., Marchesi,V.T., Benz,E.J., Jr., and Tang,T.K. (1993). Genomic structure of the locus encoding protein 4.1. Structural basis for complex combinational patterns of tissue-specific alternative RNA splicing. J. Biol. Chem. *268*, 3758-3766.

56. Ishino,S., Hashimoto,N., Fushiki,S., Date,K., Mori,T., Fujimoto,M., Nakagawa,Y., Ueda,S., Abe,T., and Inazawa,J. (1998). Loss of material from chromosome arm 1p during malignant progression of meningioma revealed by fluorescent in situ hybridization. Cancer *83*, 360-366.

57. Jacks,T., Remington,L., Williams,B.O., Schmitt,E.M., Halachmi,S., Bronson,R.T., and Weinberg,R.A. (1994). Tumor spectrum analysis in p53-mutant mice. Curr. Biol. *4*, 1-7.

58. Jons,T. and Drenckhahn,D. (1992). Identification of the binding interface involved in linkage of cytoskeletal protein 4.1 to the erythrocyte anion exchanger. EMBO J. *11*, 2863-2867.

59. Kaelin,W.G., Jr. (1998). Another p53 Doppelganger? Science *281*, 57-58.

60. Kaghad,M., Bonnet,H., Yang,A., Creancier,L., Biscan,J.C., Valent,A., Minty,A., Chalon,P., Lelias,J.M., Dumont,X., Ferrara,P., McKeon,F., and Caput,D. (1997). Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell *90*, 809-819.

61. Knudson,A.G., Jr. and Strong,L.C. (1972). Mutation and cancer: neuroblastoma and pheochromocytoma. Am. J. Hum. Genet. *24*, 514-532.

62. Kontrogianni-Konstantopoulos,A., Frye,C.S., Benz,E.J., Jr., and Huang,S.C. (2001). The prototypical 4.1R-10-kDa domain and the 4.1g-10-kDa paralog mediate fodrin-actin complex formation. J. Biol. Chem. *276*, 20679-20687.

63. Krauss,S.W., Chasis,J.A., Rogers,C., Mohandas,N., Krockmalnic,G., and Penman,S. (1997a). Structural protein 4.1 is located in mammalian centrosomes. Proc. Natl. Acad. Sci. U. S. A *94*, 7297-7302.

64. Krauss,S.W., Heald,R., Lee,G., Nunomura,W., Gimm,J.A., Mohandas,N., and Chasis,J.A. (2002). Two distinct domains of protein 4.1 critical for assembly of functional nuclei in vitro. J. Biol. Chem. *277*, 44339-44346.

65. Krauss,S.W., Larabell,C.A., Lockett,S., Gascard,P., Penman,S., Mohandas,N., and Chasis,J.A. (1997b). Structural protein 4.1 in the nucleus of human cells: dynamic rearrangements during cell division. J. Cell Biol. *137*, 275-289.

66. LaJeunesse,D.R., McCartney,B.M., and Fehon,R.G. (1998). Structural analysis of Drosophila merlin reveals functional domains important for growth control and subcellular localization. J. Cell Biol. *141*, 1589-1599.

67. Lallena,M.J. and Correas,I. (1997). Transcription-dependent redistribution of nuclear protein 4.1 to SC35-enriched nuclear domains. J. Cell Sci. *110 (Pt 2)*, 239-247.

68. Leto,T.L. and Marchesi,V.T. (1984). A structural model of human erythrocyte protein 4.1. J. Biol. Chem. *259*, 4603-4608.

69. Leto,T.L., Pratt,B.M., and Madri,J.A. (1986). Mechanisms of cytoskeletal regulation: modulation of aortic endothelial cell protein band 4.1 by the extracellular matrix. J. Cell Physiol *127*, 423-431.

70. Lorenzo,F., Dalla,V.N., Morle,L., Baklouti,F., Alloisio,N., Ducluzeau,M.T., Roda,L., Lefrancois,P., and Delaunay,J. (1994). Protein 4.1 deficiency associated with an altered

binding to the spectrin-actin complex of the red cell membrane skeleton. J. Clin. Invest *94*, 1651-1656.

71. Lue,R.A., Marfatia,S.M., Branton,D., and Chishti,A.H. (1994). Cloning and characterization of hdlg: the human homologue of the Drosophila discs large tumor suppressor binds to protein 4.1. Proc. Natl. Acad. Sci. U. S. A *91*, 9818-9822.

72. Luque,C.M., Lallena,M.J., Alonso,M.A., and Correas,I. (1998). An alternative domain determines nuclear localization in multifunctional protein 4.1. J. Biol. Chem. *273*, 11643-11649.

73. Lutchman,M. and Rouleau,G.A. (1995). The neurofibromatosis type 2 gene product, schwannomin, suppresses growth of NIH 3T3 cells. Cancer Res. *55*, 2270-2274.

74. Marfatia,S.M., Lue,R.A., Branton,D., and Chishti,A.H. (1994). In vitro binding studies suggest a membrane-associated complex between erythroid p55, protein 4.1, and glycophorin C. J. Biol. Chem. *269*, 8631-8634.

75. Marfatia,S.M., Morais Cabral,J.H., Lin,L., Hough,C., Bryant,P.J., Stolz,L., and Chishti,A.H. (1996). Modular organization of the PDZ domains in the human discs-large protein suggests a mechanism for coupling PDZ domain-binding proteins to ATP and the membrane cytoskeleton. J. Cell Biol. *135*, 753-766.

76. Maris,J.M. (1999). Molecular Biology of Neuroblastoma. Journal of Clinical Oncology.

77. Maris,J.M., Jensen,J., Sulman,E.P., Beltinger,C.P., Allen,C., Biegel,J.A., Brodeur,G.M., and White,P.S. (1997). Human Kruppel-related 3 (HKR3): a candidate for the 1p36 neuroblastoma tumour suppressor gene? Eur. J. Cancer *33*, 1991-1996.

78. Maris,J.M., White,P.S., Beltinger,C.P., Sulman,E.P., Castleberry,R.P., Shuster,J.J., Look,A.T., and Brodeur,G.M. (1995). Significance of chromosome 1p loss of heterozygosity in neuroblastoma. Cancer Res. *55*, 4664-4669.

79. Martinsson,T., Sjoberg,R.M., Hallstensson,K., Nordling,M., Hedborg,F., and Kogner,P. (1997). Delimitation of a critical tumour suppressor region at distal 1p in neuroblastoma tumours. Eur. J. Cancer *33*, 1997-2001.

80. Mascio,L.N., Verbeek,P.W., Sudar,D., Kuo,W.L., and Gray,J.W. (1995). Semiautomated DNA probe mapping using digital imaging microscopy: I. System development. Cytometry *19*, 51-59.

81. Mattagajasingh,S.N., Huang,S.C., Hartenstein,J.S., Snyder,M., Marchesi,V.T., and Benz,E.J. (1999). A nonerythroid isoform of protein 4.1R interacts with the nuclear mitotic apparatus (NuMA) protein. J. Cell Biol. *145*, 29-43.

82. Mori,K., Iwao,K., Miyoshi,Y., Nakagawara,A., Kofu,K., Akiyama,T., Arita,N., Hayakawa,T., and Nakamura,Y. (1998). Identification of brain-specific splicing variants of the hDLG1 gene and altered splicing in neuroblastoma cell lines. J. Hum. Genet. *43*, 123-127.

83. Morris,M.B. and Lux,S.E. (1995). Characterization of the binary interaction between human erythrocyte protein 4.1 and actin. Eur. J. Biochem. *231*, 644-650.

84. Muresu,R., Casciano,I., Volpi,E.V., Siniscalco,M., and Romani,M. (1995). Cytogenetic and molecular studies on the neuroblastoma cell line NGP: identification of a reciprocal t(1;15) involving the "consensus region" 1p36.1. Genes Chromosomes. Cancer *13*, 66-71.

85. Murthy,S.N., Liu,T., Kaul,R.K., Kohler,H., and Steck,T.L. (1981). The aldolase-binding site of the human erythrocyte membrane is at the NH2 terminus of band 3. J. Biol. Chem. *256*, 11203-11208.

86. Nomoto,S., Haruki,N., Kondo,M., Konishi,H., Takahashi,T., Takahashi,T., and Takahashi,T. (1998). Search for mutations and examination of allelic expression imbalance of the p73 gene at 1p36.33 in human lung cancers. Cancer Res. *58*, 1380-1383.

87. Norris,M.D., Gilbert,J., Smith,S.A., Marshall,G.M., Salwen,H., Cohn,S.L., and Haber,M. (2001). Expression of the putative tumour suppressor gene, p73, in neuroblastoma and other childhood tumours. Med. Pediatr. Oncol. *36*, 48-51.

88. Parra,M., Gascard,P., Walensky,L.D., Gimm,J.A., Blackshaw,S., Chan,N., Takakuwa,Y., Berger,T., Lee,G., Chasis,J.A., Snyder,S.H., Mohandas,N., and Conboy,J.G. (2000). Molecular and functional characterization of protein 4.1B, a novel member of the protein 4.1 family with high level, focal expression in brain. J. Biol. Chem. *275*, 3247-3255.

89. Parra,M., Gascard,P., Walensky,L.D., Snyder,S.H., Mohandas,N., and Conboy,J.G. (1998). Cloning and characterization of 4.1G (EPB41L2), a new member of the skeletal protein 4.1 (EPB41) gene family. Genomics *49*, 298-306.

90. Parra,M.K., Gee,S.L., Koury,M.J., Mohandas,N., and Conboy,J.G. (2003). Alternative 5' exons and differential splicing regulate expression of protein 4.1R isoforms with distinct N-termini. Blood *101*, 4164-4171.

91. Pasternack,G.R., Anderson,R.A., Leto,T.L., and Marchesi,V.T. (1985). Interactions between protein 4.1 and band 3. An alternative binding site for an element of the membrane skeleton. J. Biol. Chem. *260*, 3676-3683.

92. Pasternack,G.R. and Racusen,R.H. (1989). Erythrocyte protein 4.1 binds and regulates myosin. Proc. Natl. Acad. Sci. U. S. A *86*, 9712-9716.

93. Peters,L.L., Weier,H.U., Walensky,L.D., Snyder,S.H., Parra,M., Mohandas,N., and Conboy,J.G. (1998). Four paralogous protein 4.1 genes map to distinct chromosomes in mouse and human. Genomics *54*, 348-350.

94. Reid,M.E., Takakuwa,Y., Tchernia,G., Jensen,R.H., Chasis,J.A., and Mohandas,N. (1989). Functional role for glycophorin C and its interaction with the human red cell membrane skeletal component, protein 4.1. Prog. Clin. Biol. Res. *319*, 553-571.

95. Rothstein,A., Knauf,P.A., Grinstein,S., and Shami,Y. (1979). A model for the action of the anion exchange protein of the red blood cell. Prog. Clin. Biol. Res. *30*, 483-496.

96. Rouleau,G.A., Merel,P., Lutchman,M., Sanson,M., Zucman,J., Marineau,C., Hoang-Xuan,K., Demczuk,S., Desmaze,C., and Plougastel,B. (1993). Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. Nature *363*, 515-521.

97. Ruttledge,M.H., Sarrazin,J., Rangaratnam,S., Phelan,C.M., Twist,E., Merel,P., Delattre,O., Thomas,G., Nordenskjold,M., and Collins,V.P. (1994). Evidence for the complete inactivation of the NF2 gene in the majority of sporadic meningiomas. Nat. Genet. *6*, 180-184.

98. Saunders,R.D., Avides,M.C., Howard,T., Gonzalez,C., and Glover,D.M. (1997). The Drosophila gene abnormal spindle encodes a novel microtubule-associated protein that associates with the polar regions of the mitotic spindle. J. Cell Biol. *137*, 881-890.

99. Schischmanoff,P.O., Winardi,R., Discher,D.E., Parra,M.K., Bicknese,S.E., Witkowska,H.E., Conboy,J.G., and Mohandas,N. (1995). Defining of the minimal domain of protein 4.1 involved in spectrin-actin binding. J. Biol. Chem. *270*, 21243-21250.

100. Sheffield,V.C., Beck,J.S., Kwitek,A.E., Sandstrom,D.W., and Stone,E.M. (1993). The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. Genomics *16*, 325-332.

101. Shi,Z.T., Afzal,V., Coller,B., Patel,D., Chasis,J.A., Parra,M., Lee,G., Paszty,C., Stevens,M., Walensky,L., Peters,L.L., Mohandas,N., Rubin,E., and Conboy,J.G. (1999). Protein 4.1R-deficient mice are viable but have erythroid membrane skeleton abnormalities. J. Clin. Invest *103*, 331-340.

102. Shimizu,T., Takakuwa,Y., and Ohkawara,A. (1998). Distribution of protein 4.1 in basal and squamous cell carcinoma. Arch. Dermatol. Res. *290*, 226-228.

103. Simon,M., von Deimling,A., Larson,J.J., Wellenreuther,R., Kaskel,P., Waha,A., Warnick,R.E., Tew,J.M., Jr., and Menon,A.G. (1995). Allelic losses on chromosomes 14, 10, and 1 in atypical and malignant meningiomas: a genetic model of meningioma progression. Cancer Res. *55*, 4696-4701.

104. Spieker,N., Beitsma,M., van Sluis,P., Chan,A., Caron,H., and Versteeg,R. (2001). Three chromosomal rearrangements in neuroblastoma cluster within a 300-kb region on 1p36.1. Genes Chromosomes. Cancer *31*, 172-181.

105. Takahashi,K., Sasaki,T., Mammoto,A., Hotta,I., Takaishi,K., Imamura,H., Nakano,K., Kodama,A., and Takai,Y. (1998). Interaction of radixin with Rho small G protein GDP/GTP exchange protein Dbl. Oncogene *16*, 3279-3284.

106. Tang,C.J. and Tang,T.K. (1991). Rapid localization of membrane skeletal protein 4.1 (EL1) to human chromosome 1p33----p34.2 by nonradioactive in situ hybridization. Cytogenet. Cell Genet. *57*, 119.

107. Tang,C.J. and Tang,T.K. (1998). The 30-kD domain of protein 4.1 mediates its binding to the carboxyl terminus of pICln, a protein involved in cellular volume regulation. Blood *92*, 1442-1447.

108. Tang,T.K., Qin,Z., Leto,T., Marchesi,V.T., and Benz,E.J., Jr. (1990). Heterogeneity of mRNA and protein products arising from the protein 4.1 gene in erythroid and nonerythroid tissues. J. Cell Biol. *110*, 617-624.

109. Tran,Y.K., Bogler,O., Gorse,K.M., Wieland,I., Green,M.R., and Newsham,I.F. (1999). A novel member of the NF2/ERM/4.1 superfamily with growth suppressing properties in lung cancer. Cancer Res. *59*, 35-43.

110. Walensky,L.D., Blackshaw,S., Liao,D., Watkins,C.C., Weier,H.U., Parra,M., Huganir,R.L., Conboy,J.G., Mohandas,N., and Snyder,S.H. (1999). A novel neuron-enriched homolog of the erythrocyte membrane cytoskeletal protein 4.1. J. Neurosci. *19*, 6457-6467.

111. Walensky,L.D., Shi,Z.T., Blackshaw,S., DeVries,A.C., Demas,G.E., Gascard,P., Nelson,R.J., Conboy,J.G., Rubin,E.M., Snyder,S.H., and Mohandas,N. (1998). Neurobehavioral deficits in mice lacking the erythrocyte membrane cytoskeletal protein 4.1. Curr. Biol. *8*, 1269-1272.

112. White,P.S., Maris,J.M., Sulman,E.P., Jensen,S.J., Kyemba,S.M., Beltinger,C.P., Allen,C., Kramer,D.L., Biegel,J.A., and Brodeur,G.M. (1997). Molecular analysis of the region of distal 1p commonly deleted in neuroblastoma. Eur. J. Cancer *33*, 1957-1961.

113. Williams,B.O., Morgenbesser,S.D., DePinho,R.A., and Jacks,T. (1994). Tumorigenic and developmental effects of combined germ-line mutations in Rb and p53. Cold Spring Harb. Symp. Quant. Biol. *59*, 449-457.

114. Woods,D.F. and Bryant,P.J. (1989). Molecular cloning of the lethal(1)discs large-1 oncogene of Drosophila. Dev. Biol. *134*, 222-235.

115. Yu,J. and Steck,T.L. (1975). Isolation and characterization of band 3, the predominant polypeptide of the human erythrocyte membrane. J. Biol. Chem. *250*, 9170-9175.

APPENDIX

sample #	DNA/ RNA #	Ratio actin/p4.1	stage INSS/ Evans	known LOH	WT allele	missense mutation	silent mutation	intron aberration
1	2750/ 1627	77/23	IV	--	Ø	Ø	Ø	Intron 21 pt (+13 of exon 21, C/T)
2	2751/ 1628	70/29	II	--	Ø	Ø	Ø	Ø
3	2752/ 1629	71/29	III	--	Ø	Ø	Ø	Ø
4	2753/ 1630	65/35	IVs	--	Ø	Ø	Ø	Ø
5	2754/ 1631	61/39	3	--	Ø	Ø	Ø	Ø
6	2755/ --	--/--	3	--	Ø	Ø	Ø	Ø
7	2756/ 1633	67/33	3	--	Ø	Ø	Ø	Ø
8	2757/ 1634	70/30	2b	LOH 1p	Ø	Ø	Ø	Intron 18 pt (-19 of exon 19, G/C)
9	2758/ 1635	64/36	4	--	Ø	Ø	Ø	Ø
10	2759/ 1636	66/33	4s ?	--	Presence of WT allele	Exon 8 (1212 G/A, V/M)	Exon 21 (2801 C/T, T/T)	Ø
11	2763/ --	--/--	3	--	Ø	Ø	Ø	Ø

sample #	DNA/ RNA #	Ratio actin/p4.1	stage INSS/ Evans	known LOH	WT allele	missense mutation	silent mutation	intron aberration
12	2764/ 1641	71/29	4s	--	Absence of WT allele	Ø	Exon 21 (2801 C/T, T/T)	Ø
13	2765/ 1642	78/22	1	Homo-zygous	Ø	Ø	Ø	Ø
14	2766/ 1643	76/24	4	--	Ø	Ø	Ø	Ø
15	2767/ 1644	80/20	3	--	Presence of an aberrant transcript	Ø	Ø	Intron 21 pt (+10 of exon 21, G/A)
16	2768/ 1645	66/34	1	--	Ø	Ø	Exon 16 (2144 A/G, G/G)	Ø
17	2769/ 1646	70/30	1	--	Ø	Ø	Ø	Ø
18	2770/ 1647	80/20	4	--	Ø	Ø	Ø	Ø
19	2771/ 1648	80/20	3	Hetero-zygous	Ø	Ø	Ø	Ø
20	2772/ 1649	65/35	4	Hetero-zygous	Ø	Ø	Ø	Ø
21	2790/ 1655	73/27	4	--	Presence of WT allele Absence of WT expression	Exon 4 (813 G/A, V/I)	Ø	Ø
22	2791/ 1656	69/31	4	--	Ø	Ø	Ø	Ø
23	2792/ 1657	79/21	4s	--	Ø	Ø	Ø	Ø

sample #	DNA/ RNA #	Ratio actin/p4.1	stage INSS/ Evans	known LOH	WT allele	missense mutation	silent mutation	intron aberration
24	2793/ 1658	70/30	4s	--	Ø	Ø	Ø	Ø
25	2794/ 1659	95/5	2b	Hetero-zygous	Presence of WT allele	Ø	Exon 21 (2801 C/T, T/T)	Ø
26	2795/ --	--/--	3	--	Presence of WT allele	Ø	Exon 21 (2801 C/T, T/T)	Ø
27	2796/ 1661	100/0	2b	Hetero-zygous	Ø	Ø	Ø	Ø
28	2797/ --	--/--	3	Hetero-zygous	Ø	Ø	Ø	Ø
29	2798/ 1664	93/7	1	--	Ø	Ø	Ø	Intron 18 pt (-19 of exon 19, G/C) intron21 (13nt del) (+10 to +22 of exon 21)
30	2799/ 1665	92/8	1	--	Ø	Ø	Ø	Ø
31	2800/ 1666	73/27	1	--	Presence of WT allele	Ø	Exon 21 (2801 C/T, T/T)	Ø
32	2801/ 1667	88/12	1	--	Presence of WT allele	Ø	Exon 21 (2801 C/T, T/T)	Ø
33	2802/ --	--/--	3	--	Ø	Ø	Ø	Ø
34	2803/ 1669	70/30	1	--	Ø	Ø	Ø	Ø
35	2804/ 1670	83/17	3	--	Ø	Ø	Ø	Ø
36	2805/ 1671	70/30	1	--	Ø	Ø	Ø	Ø

sample #	DNA/ RNA #	Ratio actin/p4.1	stage INSS/ Evans	known LOH	WT allele	missense mutation	silent mutation	intron aberration
37	2806/ 1672	70/30	4	--	Ø	Ø	Ø	Ø
38	2807/ 1673	70/30	4	--	Ø	Ø	Ø	Ø
39	2839/ 1704	63/37	1	--	Ø	Ø	Ø	Ø
40	2840/ 1705	69/31	3	--	Ø	Ø	Ø	Ø
41	2841/ 1706	66/34	1	--	Ø	Ø	Ø	Ø
42	2842/ 1707	93/7	3	--	Ø	Ø	Ø	Ø
43	2843/ 1708	82/18	1	--	Ø	Ø	Ø	Ø
44	2844/ 1709	68/32	2	Hetero-zygous	Ø	Ø	Ø	Ø
45	2845/ 1710	79/21	2	--	Ø	Ø	Ø	Ø
46	2846/ 1711	63/37	1	--	Ø	Ø	Ø	Ø
47	2847/ 1712	63/37	2	--	Ø	Ø	Ø	Ø
48	2848/ 1713	69/31	1	Hetero-zygous	Ø	Ø	Ø	Ø
49	2850/ 1716	65/35	4	LOH 1p	Ø	Ø	Ø	Ø
50	2851/ 1717	--/--	3	LOH 1p	Ø	Ø	Ø	Ø

sample #	DNA/ RNA #	Ratio actin/p4.1	stage INSS/ Evans	known LOH	WT allele	missense mutation	silent mutation	intron aberration
51	2852/ 1718	70/30	4s	--	Ø	Ø	Ø	Ø
52	2853/ 1719	77/23	1	--	Ø	Ø	Ø	Ø
53	2854/ 1720	70/30	4s	--	Ø	Ø	Ø	Ø
54	2855/ 1721	67/33	1	--	Ø	Ø	Ø	Ø
55	2856/ 1722	82/18	2	--	Ø	Ø	Ø	Ø
56	2857/ 1723	67/33	1	Hetero-zygous	Ø	Ø	Ø	Intron 10 (4 nt del) (-16 to –13 of exon 11)
57	2858/ 1724	95/5	4s	Hetero-zygous	Ø	Ø	Ø	Ø
58	2859/ 1725	65/55	4	--	Presence of WT allele Absence of WT expression	Exon 4 (813 G/A, V/I)	Ø	Intron 9 pt (+36, G/A of exon 9)
59	2865/ 1731	81/19	1	--	Ø	Ø	Ø	Ø
60	2866/ 1732	65/35	4	Hetero-zygous	Ø	Ø	Ø	Ø
61	2867/ 1733	60/40	4	--	Ø	Ø	Ø	Ø
62	2868/ 1734	92/8	1	Hetero-zygous	Ø	Ø	Ø	Ø

sample #	DNA/ RNA #	Ratio actin/p4.1	stage INSS/ Evans	known LOH	WT allele	missense mutation	silent mutation	intron aberration
63	2869/ 1735	68/32	1	--	Ø	Ø	Ø	Ø
64	2870/ 1736	62/38	1	Hetero-zygous	Ø	Ø	Ø	Ø
65	2871/ 1737	59/41	3	--	Ø	Ø	Ø	Ø
66	2872/ 1738	54/46	4	LOH 1p	Ø	Ø	Ø	Ø
67	2873/ 1739	60/40	1	--	Presence of WT allele Absence of WT expression	Exon 4 (813 G/A, V/I)	Ø	Ø
68	2874/ 1740	70/30	1	--	Ø	Ø	Ø	Ø
69	2876/ 1742	83/17	4s	--	Ø	Ø	Ø	Ø
70	2878/ 1744	80/20	4s	--	Ø	Ø	Ø	Ø
71	2879/ 1745	79/21	3	--	Ø	Ø	Ø	Ø
72	2880/ 1746	67/33	--	--	Ø	Ø	Ø	Ø
73	2881/ 1747	96/4	3	Hetero-zygous	Ø	Ø	Ø	Ø
74	2882/ 1748	70/30	1	Homo-zygous	Ø	Ø	Ø	Ø
75	2883/ 1749	63/37	1	Hetero-zygous	Ø	Ø	Ø	Ø

sample #	DNA/ RNA #	Ratio actin/p4.1	stage INSS/ Evans	known LOH	WT allele	missense mutation	silent mutation	intron aberration
76	2884/ 1750	68/32	--	--	Ø	Ø	Ø	Ø
77	2885/ 1751	66/34	4	LOH 1p	Ø	Ø	Ø	Ø
78	2886/ 1752	67/32	4	--	Ø	Ø	Ø	Intron 10 (-19 of exon 11, T/G)
I	LAN5 P4.1		--	--	Ø	Exon 2 (250 G/C, V/L; 320 G/A, S/N) Exon 4 (676 C/T, S/F) Exon 5 (948 G/A, A/T) Exon 16 (2167, A/G, N/S) Exon 17 (2208 A/G, K/E)	Exon2 (246 C/T, H/H) Exon 4 (650 G/A, Q/Q)	Ø
II	SKNDz		--	--	Ø	Ø	Ø	Ø
III	NPG		--	--	Presence of WT allele	Ø	Ø	Ø
IV	CHP-134B		--	--	Presence of WT allele	Exon 4 (813 G/A, V/I)	Ø	Ø
V	AS		--	--	Ø	Ø	Ø	Intron 18 (-19 of exon 19, G/C)

ACKNOWLEDGEMENTS

Mein ganz besonderer Dank gebührt Prof. Dr. med. Christopher Poremba, für dessen Einsatz, Förderung und außergewöhnliche Betreuung sowie für die Herstellung des Kontakts zum NIH, USA. Ohne ihn wäre diese Dissertation nicht möglich gewesen. Ich danke auch dem Team um Dr. rer nat. Karl-Ludwig-Schäfer mit Dagmar Haves, Frauke Schmidt, Petra Meier und Lyndia Grote für technische Unterweisungen und Unterstützung, insbesondere bei meinen ersten Labor- und Pipetiererfahrungen sowie Dr. Zheng Ping Zhuang, M.D. und Dr. med. Alexander Vortmeyer, M.D., PhD vom National Cancer Institute, NIH, USA für deren freundliche Aufnahme und Förderung während des USA-Aufenthaltes. Von ganzem Herzen sei in diesem Zusammenhang auch Dr. Steve Huang, Ph.D. für seine 24h-pro-Tag und 7-Tage-die-Woche Betreuung und Anleitung im Labor ganz besonders gedankt. Auch sollen an dieser Stelle Dr. Svetlana Pack, Ph.D., NCI, NIH und Prof. Athar Chishti, Ph.D., Chief, Section of Hematology and Oncology Research, Tufts Univerisity, Boston, deren Forschung die Grundlage für diese Dissertation bildete, für die lehrreichen und konstruktiven Diskussionen und Besprechungen nicht unerwähnt bleiben. Auch gedankt sei Prof. Dr. med. Holger Christiansen der Klinik für allgemeine Kinderheilkunde, Uniklinik Marburg für Überlassung von Gewebe und Patientendaten aus der Deutschen Neuroblastoma Bank. Herrn Priv.-Doz. Dr. med. Dominik Schneider vom Institut für Kinder-Okologie, -Hämatologie, und –Immunologie der HHU Düsseldorf danke ich für die Übernahme des Korreferates.

Abschließend bin ich meiner Familie für ihre ständige emotionale und auch materielle Unterstützung zum Erreichen meiner Träume zu tiefster Dankbarkeit verpflichtet.

CURRICULUM VITAE

Name: Urs Daniel Lichtenauer
Geburtsdatum: 11.05.1974
Geburtsort: Münster/Westf.
Addresse: Esmarchstr. 14; 40223 Düsseldorf
Telefon: 0211-3020747
Email: lichteu@gmx.net

Ausbildung

1981 - 1985	Mariengrundschule Appelhülsen
1985 - 1991	Johann-Conrad-Schlaun-Gymnasium Münster
1991 - 1992	Wooster Senior High, Wooster, Ohio, USA
1992 - 1994	Johann-Conrad-Schlaun-Gymnasium Münster
1994 - 1995	Zivildienst: Raphaelsklinik, Münster
1995 - 1998	Medizinstudium Universität Münster
1999 - 2000	Medizinstudium Universität Wien, Österreich
2000 - 2001	Medizinstudium Universität Münster
2001 - 2002	Praktisches Jahr 1. Tertial: Innere Medizin, Georgetown University, Washington DC, USA (mit Reisestipendium des DFA) 2. Tertial: Chirurgie, King's College of London, Gillingham, Kent, England 3. Tertial: Anästhesie, Universitätskliniken Münster
2002 - 2003	Arzt im Praktikum, Deutsches Diabetes Forschungsinstitut, Düsseldorf
2004 - heute	Assistenzarzt, Deutsches Diabetes Forschungsinstitut, Düsseldorf

Abschlüsse

Herbst 1992	American High School Diploma
Herbst 1994	Abitur
Herbst 1997	Physikum
Herbst 1998	1. Staatsexamen
Frühjahr 2001	2. Staatsexamen
Sommer 2003	3. Staatsexamen

Dissertationsarbeit

Westfälische Wilhelms Universität Münster und
National Cancer Institute, National Institutes of Health (NIH), Bethesda, Maryland, USA und
Heinrich Heine Universität Düsseldorf
Thema: 'Assessment of Protein 4.1 in Neuroblastoma Tumors'

Veröffentlichungen

2004	UD Lichtenauer, J Seissler, WA Scherbaum. Die Diabetischen Folgeerkrankungen. Mikro- und makroangiopathische Organschäden, *Internist (Berl)* 2003 Jul;44(7):840-6, 848-52. Review.
2001	Huang SC*, UD Lichtenauer*, SD Pack, C Wang, AC Kim, M Lutchman, CA Koch, S Huang, EJ Benz, H Christiansen, B Dockhorn-Dworniczak, C Poremba, AO Vortmeyer, AH Chishti, Z Zhuang. Reassignment of the EPB4.1 gene to 1p36 and assessment of its involvement in neuroblastomas, *European Journal of Clinical Investigation* 2001 Oct;31(10):907-14.
2000	Huang SC*, CA Koch*, AO Vortmeyer, SD Pack, UD Lichtenauer, P Mannan, IA Lubensky, GP Chrousos, RF Gagel, K Pacak, Z Zhuang. Duplication of the mutant RET allele in trisomy 10 or loss of the wild-type allele in multiple endocrine neoplasia type 2-asssociated pheochromocytomas, *Cancer Research* 2000 Nov 15;60(22):6223-6.

* = Erstautoren

Forschung

1997 - 1998	Gerhard-Domagk-Institut für Pathologie, Universitätskliniken Münster
1998 - 1999	National Cancer Institute, NIH, USA
1999 - 2000	Institut für klinische Pharmakologie, AKH Wien, Österreich
2000 - 2001	Gerhard-Domagk-Institut für Pathologie, Universitätskliniken Münster
2001	Department of Surgery, Gillingham, Kent, England
2002 - heute	Deutsches Diabetes Forschungsinstitut, Düsseldorf
2003	University of Michigan, Ann Arbor, USA

Famulaturen

1998	Molekularpathologie, Universitätskliniken Münster
	Orthopädie u. Sportmedizin, Praxis Dr. Koch, Münster
	Unfallchirurgie, Rotes Kreuz Krankenhaus, Bremen

	Kardiologie, St. Vinzenz Hospital, Coesfeld
1999	Allergy and Infectious Diseases, NIH, Bethesda, Maryland, USA
	Endocrinology, Washington Hospital Center, Washington DC, USA
	Neurochirurgie, Universitätsklinikum Wien (AKH), Österreich
2000	Infektionologie und Chemotherapie, Universitätsklinikum Wien, Österreich
	Tropenmedizin, Bernhard-Nocht-Institut für Tropenmedizin, Hamburg

Tutorentätigkeit

seit 1997	Ausbilder beim DRK
seit 1998	Tutor bei Ausbilderlehrgängen der DLRG
1997 - 1998	Tutor für mikroskopische und makroskopische Anatomiekurse, Institut für Anatomie, Münster
	Tutor für mikroskopische Anatomie, Institut für Anatomie, Münster
2001	Tutor im Kurs “Anatomie in der klinischen Praxis”, Institut für Anatomie, Münster

Zusatzqualifikationen

Sprachkurse

1989	Schüleraustausch mit York, England
1996 - 1998	weiterführende Englischkurse
	- Medical English I
	- Medical English II
	- Conversation in English
	- Communication Skills for Medicine
	- General Pathology
	- Advanced General Pathology

Fachkurse

1996 - 1998	- Gastroenterologie am Krankenbett
	- Einführung in die Sonographie
	- Einführung in die Echokardiographie
2000	- Actronics und Megacode Reanimationstraining
2001	- Einführung ins Internet Publishing
	- Radiology Tutorials, England
2002	- Strahlenschutzeinführungskurs
	- Abdomen- und Schilddrüsensonographiegrundkurse
2003	- Strahlenschutzgrund- und Spezialkurs
	- Notarztkurs

Ausbildungen

1995	Sanitäterausbildung
1998	Ausbildung zum Rettungstaucher

Computerkenntnisse

Schreibmaschinenkurs, Winperfect, Winword, Excel, Adobe Photoshop, IRC, DNA-Strider, Genbank Datenbasen, Internet, Web Design

Hobbies

Lesen, Fechten, Laufen, Schwimmen, Tauchen, Segeln, Basketball, Tanzen