Phenotypic abnormalities in childhood cancer patients; clues for molecular defects?
Merks, J.H.M.

Citation for published version (APA):
Frequent concurrence of neuroblastoma in Noonan and LEOPARD patients. Role for \textit{PTPN11} in sporadic neuroblastoma

Johannes H.M. Merks\textsuperscript{1}, Peter van Sluis\textsuperscript{2}, Rogier Versteeg\textsuperscript{2}, Raoul C.M. Hennekam\textsuperscript{3}, and Huib N. Caron\textsuperscript{1}.

\textsuperscript{1}Department of Pediatric Oncology, Emma Children’s Hospital, Academic Medical Center, Amsterdam, The Netherlands. \textsuperscript{2}Department of Human Genetics, Academic Medical Center, Amsterdam, The Netherlands. \textsuperscript{3}Department of Pediatrics and Institute for Human Genetics, Emma Children’s Hospital, Academic Medical Center, Amsterdam, The Netherlands.
Abstract

We present the concurrence of a neuroblastoma and LEOPARD syndrome. In the literature, five neuroblastomas have been reported to occur in Noonan syndrome, which is closely related to LEOPARD syndrome. Both syndromes can be caused by activating mutations in the PTPN11 gene, encoding the Src homology 2-containing protein tyrosine phosphatase (SHP2), a widely expressed phosphatase involved in intracellular signaling.

Based on the recent recognition of the leukemogenic role of PTPN11 mutations in juvenile myelomonocytic leukemia, and high incidence of neuroblastoma in both Noonan and LEOPARD syndrome, we analyzed the PTPN11 gene in a LEOPARD patient and in sporadic neuroblastoma samples. In the LEOPARD patient, we found a heterozygous Thr468Met mutation in exon 12 both in the germline and in the tumor. This mutation affects the active site of the PTP domain, and accounts for 33% of LEOPARD cases. PTPN11 sequencing in sporadic neuroblastoma tumor samples is still in progress. Up till now exons 8 and 12 are sequenced in 50 sporadic neuroblastoma samples, exon 3 in 109, and exon 13 in 135 neuroblastoma samples. All together these exons have been shown to account for 99% of somatic mutations in leukemia samples, 89% of germline mutations in Noonan, and 61% of germline mutations in LEOPARD patients, respectively. A Gly503Val mutation in exon 13 in the tumor of a stage IV neuroblastoma patient was found, affecting the active site of the PTP domain. Lymphocytes of the patient did not show the mutation.

This is the first evidence of an oncogenic role of PTPN11 in a solid tumor. The results show that the PTNP11 pathway can play a causative role in neuroblastoma oncogenesis. Further analysis of this pathway may therefore contribute to the understanding of this tumor.
Introduction

Neuroblastomas are the most common extracranial solid childhood tumors, accounting for 7% of all childhood cancers diagnosed under the age of 15 years. In neuroblastoma several genetic markers have been described, the most important markers being amplification of the N-Myc oncogene and loss of chromosome 1p, both indicating a bad prognosis. We recently encountered a patient with neuroblastoma, in whom we diagnosed LEOPARD syndrome. The term LEOPARD syndrome is an acronym introduced by Gorlin et al. for the combination of multiple Lentigines, ECG abnormalities, Ocular hypertelorism, Pulmonary stenosis, Abnormalities of the genitalia, Retardation of growth, and Deafness. LEOPARD syndrome is allelic to Noonan syndrome, and both syndromes have been shown to be caused by germline mutations in the PTPN11 gene, which encodes the protein tyrosine phosphatase SHP2. Several clinical manifestations of LEOPARD syndrome overlap those of Noonan syndrome, including facial anomalies, congenital heart defects, pectus carinatum et excavatum, and growth retardation. The variability of symptoms in LEOPARD syndrome is much more limited compared to Noonan syndrome, both inter- and intrafamilial. Both syndromes have skin pigmentary changes: in Noonan syndrome mainly café-au-lait spots are seen, in LEOPARD syndrome multiple lentigines are the major symptom, although café-au-lait spots also occur. Germline PTPN11 mutations have been found in 88% of LEOPARD cases, and 33 to 50% of Noonan cases.

Recently it was shown that somatic PTPN11 mutations occur in 32-34% of non-syndromic juvenile myelo-monocytic leukemia (JMML), and in a smaller percentage of cases with acute lymphoblastic leukemia (ALL), myelodysplastic syndrome (MDS), and acute myeloid leukemia (AML). The mutated PTPN11 gene was shown to encode for a tyrosine phosphatase acting as an oncoprotein in myeloid leukemias.

Literature search (Pubmed 1966-2004) showed that the combination of LEOPARD syndrome and neuroblastoma in a patient has not been reported before. Five cases with both neuroblastoma and Noonan syndrome have been published. We hypothesized that somatic PTPN11 mutations might play a role in neuroblastoma oncogenesis.

Patients and methods

From January 1, 2000 to March 1, 2003 a clinical morphological screening study was performed at the outpatient clinic for Late Effects of Childhood Cancer of our center. All consecutive patients who visited the clinic were invited to participate in the study. Written informed consent was obtained from all patients or their parents. Permission for the study was obtained from the Medical Ethical Committee of our hospital.

Neuroblastoma samples containing >80% of tumor cells of 135 non syndromic patients were randomly chosen out of a total of 250 tumor samples of consecutive patients, gathered between 1980 and 2004, and stored in the Emma Children’s Hospital-Academic Medical Center.
neuroblastoma tumor bank. High-molecular weight DNA was prepared from these 135 tumor samples as described by Mullenbach et al.\textsuperscript{23}. Exons 3, 8, 12, and 13 of the \textit{PTPN11} gene were first selected, as these exons have been shown to harbor 99\% (83/84 cases) of somatic mutations in leukemia cells\textsuperscript{15-17}, 89\% (81/91 cases) of germine mutations in Noonan syndrome\textsuperscript{11-14}, and 61\% (19/32 cases) of germine mutations in LEOPARD syndrome\textsuperscript{10}. Up till now exons 8 and 12 were sequenced in 50 sporadic neuroblastoma samples, exon 3 in 109, and exon 13 in 135 neuroblastoma samples. Sequencing of all other exons of the \textit{PTPN11} gene in a total of 135 sporadic neuroblastoma tumor samples is still in progress. All exons were amplified by polymerase chain reaction (PCR), using primers listed in table 1. Sequence reactions were performed with the ABI PRISM™Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). All exons were sequenced bi-directional with the same primers employed for initial PCR. PCR products were sequenced on an ABI 3730 (Applied Biosystems) automated sequencer. Sequences were base-called and assembled with the Staden-package\textsuperscript{24}, using sequence NM_002834.3 as a reference. All sequence assemblies and polymorphisms were manually reviewed to insure accuracy of variant identification.

<p>| Table 1. Primer pairs used to amplify the \textit{PTPN11} coding sequence of exons 3, 8, 12, and 13, and sizes of PCR products |</p>
<table>
<thead>
<tr>
<th>Exon</th>
<th>5' Primer</th>
<th>3' Primer</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>TCTTTATTTGTCCCTTGC C</td>
<td>TCACAAGCCCTTTGGAGTCAG</td>
<td>344</td>
</tr>
<tr>
<td>8</td>
<td>GACATCGAGGCAGTGTCCAGTTAC</td>
<td>CCTAAAGTCTTTTCAAGGACATG</td>
<td>350</td>
</tr>
<tr>
<td>12</td>
<td>GCCTCAAAGAGTAGACATTGTTC</td>
<td>GACTGTTTCGTCGACCTTTGC</td>
<td>250</td>
</tr>
<tr>
<td>13</td>
<td>CAACACTGTAGGCCATTGCAACA</td>
<td>CGTATGCAAGGCCCTAGCAAG</td>
<td>356</td>
</tr>
</tbody>
</table>

\textbf{Results}

Clinical morphology and constitutive \textit{PTPN11} analyses

In 2002 we investigated an 8-year old boy, who had presented in 1994, at the age of eight months, with an agressive stage IV neuroblastoma. The tumor showed N-Myc amplification and deletion of chromosome 1p. The proband was the only child of Caucasian, non-consanguineous and healthy parents, who did not show any phenotypic abnormalities. At clinical morphological examination he showed short stature (117.5 cm, age eight years: <P2), low set and posteriorly rotated ears, \textit{pectus excavatum et carinatum}, cryptorchism, multiple café-au-lait spots, and multiple lentigines (figure 1). The diagnosis LEOPARD syndrome was made. Sequencing of the \textit{PTPN11} gene in peripheral white blood cells showed a germline mutation in exon 12 at position 1403 compared to the reference sequence (NM_002834.3) resulting in a cytidine $\rightarrow$ thymidine transition (fig.2A). In the neuroblastoma tumor of our patient the same C1403T mutation was found. This mutation in exon 12 of the \textit{PTPN11} gene changes the threonine to a methionine codon at position 468 of the PTP domain of the protein. Parents were not investigated.
Figure 1. Clinical picture of the LEOPARD patient.
Note the proptosis, anteverted nares, macrostomia, low set and posteriorly rotated ears, pectus excavatum et carinatum, and multiple lentigines (with permission from the patient and his parents)

Sequencing PTPN11 in sporadic neuroblastoma
Clinical data were available for 99 of 135 patients; median age was 20 months (range 0–162 months), tumor stages of these 99 cases were stage I (n=13), stage II (n=15), stage III (n=12), stage IV (n=48), and stage IV-s (n=11). In all samples the N-Myc and chromosome 1p status was determined: 15 samples showed both N-Myc amplification and deletion of 1p, 2 samples showed only N-Myc amplification, and 9 harbored deletion of 1p only.
Up till this moment we sequenced exons 8 and 12 in 50 neuroblastoma samples, and exons 3 and 13 in 109 and 135 neuroblastoma samples, respectively. Sequencing was successful in all tumor samples. In patient N426, we found a mutation in exon 13 at position 1888 with a guanidine → thymidine transition in the neuroblastoma tumor sample (Fig.2C), affecting the active site of the PTP domain. The mutation was confirmed from an independent PCR. PTPN11 analysis of lymphocytes showed the wild type sequence (Fig.2D). The patient was a girl, who presented with a stage III neuroblastoma at the age of 18 months, and died of recurrent disease one year later.
For exons 3, 8, and 12 we found wild type sequences in all samples analyzed. We found the already described polymorphism of intron 7 close to exon 8 (∼21C→T) in five patients, four times heterozygous, and one homozygous. Furthermore we found a not earlier reported polymorphism of intron 12 (∼56C→G) in one patient, and a silent mutation in exon 3 (C635T) in another. No additional polymorphisms or mutations were detected so far.
Chapte rr  8

Figure 2. Sequence chromatogram of the \textit{PTPN11} mutation (exon 12: 1403 C\textrightarrow{}T) found in germline and in neuroblastoma of the LEOPARD patient (A), a reference control (B), the mutation (exon 13: 1888 G\textrightarrow{}T) found in the tumor of patient N426 (C), and not in her germline (D).

\textbf{Discussion}

The finding of a \textit{PTPN11} mutation in a patient with LEOPARD syndrome and neuroblastoma, the five earlier reported cases with Noonan syndrome and neuroblastoma \textsuperscript{18-22}, and the recent recognition of the leukemogenic role of \textit{PTPN11} mutations in JMML \textsuperscript{15}, prompted us to sequence the \textit{PTPN11} gene in sporadic neuroblastoma samples. So far we found a Gly503Val mutation in exon 13 in one tumor, while sequencing of all exons in a larger number of sporadic tumor samples is still in progress. This mutation affects the active site of the PTP domain. The mutation was not found in the germline of the patient.

The LEOPARD patient had a typical Thr468Met mutation in exon 12, which is present in one third of LEOPARD patients \textsuperscript{10}, and codes for the active site of the protein tyrosine phosphatase domain. The tumor of the patient showed the same germline mutation, and had an unfavorable biological profile.

In 1967, neuroblastoma was first reported in a 'male Turner patient' \textsuperscript{18}, the term used for Noonan syndrome patients before the recognition of Noonan syndrome as a separate entity. Later on, four other Noonan patients with neuroblastoma were published \textsuperscript{19-22}. Four cases had primary intra-thoracic tumors, one had an adrenal tumor. Two were indolent tumors, three others showed an aggressive behavior. As a causative gene for Noonan syndrome was only found in 2001 \textsuperscript{7}, \textit{PTPN11} status was only tested in the more recently reported case, showing a constitutional de
novo missense mutation (Ser502Thr) in exon 13. The PTPN11 status in the tumor of this patient was not reported. Biological properties of the tumors were not reported in any of the cases. Including the LEOPARD case, four out of six neuroblastomas were discovered because of clinical signs of the tumor. Although the total number of cases is small, this seems to contradict the fact that the tumors might be indolent, and only picked up because Noonan patients often get chest radiographs as part of their cardiologic work-up.

Acute lymphoblastic leukemia (ALL) has been reported to occur in Noonan syndrome patients several times \(^{25-28}\), and concurrence with JMML led to the elucidation of the leukemogenic role of somatic PTPN11 mutations \(^{15}\). Also combinations with other malignant tumors were reported: two cases with rhabdomyosarcoma \(^{29,30}\), two testicular carcinomas \(^{31,32}\), one malignant schwannoma \(^{33}\), one pheochromocytoma \(^{34}\), one Wilms tumor, and one non-Hodgkin lymphoma (the latter two unpublished own observations). However, the high concurrence rate with neuroblastoma is striking. Furthermore Noonan syndrome has been reported to concur with benign tumors, like vascular malformations and hemangiomas \(^{35}\), granula-cell tumors \(^{36}\), and giant cell lesions \(^{37}\). The latter was reported as Noonan-like/multiple giant cell lesion syndrome \(^{37}\). Tartaglia et al. later found the Asn308Ser mutation responsible for the phenotype in this family. The same Asn308Ser mutation was also found in another family with Noonan syndrome without bony involvement \(^{11,37}\).

The Gly503Val mutation in exon 13 in the tumor of our sporadic neuroblastoma patient is the first PTPN11 mutation found in a solid tumor. A mutation at the same locus has been reported in one case of JMML \(^{15}\). However this mutation at position 1888 (reference NM_002834.3) showed a guanidine → cytidine transition changing the glycine to an alanine, while the mutation in our neuroblastoma tumor sample had a guanidine → thymidine transition changing the glycine to a valine. Both mutations affect the active site of the PTP domain.

PTPN11 encodes the Src homology 2-containing protein tyrosine phosphatase (SHP2) \(^{38}\), and is a widely expressed phosphatase involved in intracellular signaling downstream to several growth factor, cytokine and hormone receptors. SHP2 plays an essential role during embryonic development. SHP2 participates in signaling pathways involved in gastrulation \(^{39,40}\), limb development \(^{41}\), semilunar valvulogenesis of the heart \(^{42}\), embryonic stem cell differentiation and hematopoiesis \(^{43,44}\). Transgenic mice with homozygous inactivated SHP2 are embryonic lethal \(^{45}\). Heterozygous activated mutants have decreased viability, surviving embryos showing the Noonan phenotype, and myeloproliferative disease \(^{46}\).

Intramolecular conformational switching controls the protein tyrosine phosphatase (PTP) activity of SHP2. Due to interactions between the N-SH2 and the PTP domains, SHP2 is usually inactive. Activation only arises after disruption of the N-SH2/PTP interaction, induced by conformational change of the SH2-domain as a result of binding to the phosphotyrosyl-containing motifs of its signaling partners \(^{47}\).

Mutations associated with Noonan syndrome and leukemia are not randomly distributed in SHP2.
The large majority of defects are located in or near the N-SH2 and PTP interacting surfaces \(^7,11,15,17\). Nonsense, splicing, or frame shift mutations were not identified in these large series of Noonan cases and leukemia samples, indicating that the causative amino acid changes induce a gain of function in SHP2, increasing the phosphatase activity, with concomitant downstream signaling through the RAS/MAP kinase pathway.

Since the first report by Tartaglia et al in 2003 \(^15\), the role of somatic \textit{PTPN11} mutations in leukemogenesis has been intensively investigated. The relative contribution of \textit{PTPN11} mutations differs among the different leukemia types; mutations of \textit{PTPN11} account for 32-34\% of JMML cases \(^15,16\), 10-28\% of MDS cases (the highest incidence being found in advanced MDS cases, i.e. children with an excess of blasts \(^15\)), 11 \% of common ALL cases \(^17\), 4-5.8\% of AML cases \(^15,17\) (with a relatively high contribution of acute monocytic leukemia cases; 4/12 \(^17\)), and 0\% of T-ALL cases \(^17\). Concurrence of somatic \textit{PTPN11} mutations with other leukemogenic gene mutations, and major gene rearrangements were tested; \textit{PTPN11}, \textit{NRAS}, \textit{KRAS}, and \textit{NF1} mutations were mutually exclusive \(^15,17\), suggesting that mutant SHP2 on its own is sufficient to deregulate myeloid growth through RAS/MAPK signaling. Also \textit{PTPN11} mutations and \textit{TEL-AML1}, \textit{E2A-PBX1}, \textit{BCR-ABL}, and \textit{AF4-MLL} rearrangements appeared almost mutually exclusive \(^17\).

In conclusion we found a Gly503Val mutation in exon 13 in a sporadic neuroblastoma tumor sample. This is the first evidence of the oncogenic role of a \textit{PTPN11} mutation in neuroblastoma. Sequencing of all exons in a larger number of sporadic neuroblastoma tumor samples will show the importance of \textit{PTPN11} mutations in neuroblastoma tumorigenesis. Further analysis of this pathway may therefore contribute to the understanding of this tumor.

\textbf{Acknowledgments}

The authors would like to thank our patient and his parents for their cooperation, and WM Nillesen and EA Sistermans (UMC,Nijmegen) for performing the initial \textit{PTPN11} mutational analysis in lymphocytes of our LEOPARD patient.
References