Germline Gain-of-Function Mutations of ALK Disrupt Central Nervous System Development

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ABSTRACT: Neuroblastoma (NB) is a frequent embryonal tumor of sympathetic ganglia and adrenals with extremely variable outcome. Recently, somatic amplification and gain-of-function mutations of the anaplastic lymphoma receptor tyrosine kinase (ALK) gene, either somatic or germline, were identified in a significant proportion of NB cases. Here we report a novel syndromic presentation associating congenital NB with severe encephalopathy and abnormal shape of the brainstem on brain MRI in two unrelated sporadic cases harboring de novo, germline, heterozygous ALK gene mutations. Both mutations are gain-of-function mutations that have been reported in NB and NB cell lines. These observations further illustrate the role of oncogenes in both tumour predisposition and normal development, and shed light on the pleiotropic and activity-dependent role of ALK in humans. More generally, missing germline mutations relative to the spectrum of somatic mutations reported for a given oncogene may be a reflection of severe effects during embryonic development, and may prompt mutation screening in patients with extreme phenotypes.


KEY WORDS: ALK; neuroblastoma; NB; neurodevelopment; syndrome with cancer

Introduction

Neuroblastoma (NB; MIM# 256700) is the most frequent extracranial solid tumour in children. Both familial cases with vertical transmission, and predisposition in chromosomal and monogenic syndromes, have long supported the involvement of genetic factors. Several NB predisposing genes were recently identified, such as PHOX2B, CREBBP, NSD1, HRAS, NF1, and ALK. The last three genes encode proteins involved in the RAS/MAPK pathway [Chiarle et al., 2008; Palmer et al., 2009] and ALK is a downstream target of PHOX2B [Bachetti et al., 2010].

ALK (MIM# 105590), a tyrosine kinase receptor gene of the insulin receptor family, is activated by fusion with various partners in anaplastic large cell lymphomas, inflammatory myofibroblastic tumors, and in some lung cancers [Chiarle et al., 2008]. Recently, somatic amplification and gain-of-function mutations of ALK were identified in about 2–4 and 7–10% of NB cases, respectively [Chen et al., 2008; De Brouwer et al., 2010; Janoueix-Lerosey et al., 2008; Mosse et al., 2008]. Germline gain-of-function mutations have also been reported in half of the familial cases of NB tested thus far [Janoueix-Lerosey et al., 2008; Mosse et al., 2008]. ALK is preferentially expressed in the central and peripheral nervous systems during development, but its role in the normal development of the nervous system remains speculative [Hurley et al., 2006; Iwahara et al., 1997; Vernersson et al., 2006]. Indeed, familial ALK gain-of-function mutations predispose to isolated NB, but are not associated with developmental anomalies, and ALK−/− mice have no obvious embryonic phenotype. However, behavioral impairment has been described in the ALK−/− mice, a phenotype attributed to neurochemical alterations in the hippocampi and basal cortex [Bilsland et al., 2008].

Here we report two unrelated cases with an association of congenital NB and severe encephalopathy characterized by a specific abnormal shape of the brainstem on brain magnetic resonance imaging (MRI). In both cases we identified a heterozygous, germline de novo missense mutation located in the tyrosine kinase domain (TKD) of ALK at positions previously identified as somatic mutational hot spots in NB and NB cell lines.

Patients and Methods

Case 1, a female, was the second child born to unrelated healthy parents, aged 29 and 31 years at the time of birth, with no relevant family medical history. She was born at term by Caesarean section...
with normal birth parameters following an uneventful pregnancy (birth weight [BW]: 3100 g, body length [BL]: 46 cm, occipito frontal circumference (OFC): 34 cm). She was hypotonic, hypomotile, and presented with major feeding difficulties, no sucking and swallowing reflexes, episodes of abdominal distension and apneas. Mechanical ventilation and tube feeding were required. An adrenal NB with pelvic extension was diagnosed at 3 days of life. Levels of urinary catecholamine and its metabolites were raised. Rapid tumor progression led to chemotherapy by vincristine and cyclophosphamide with no improvement of the tumor mass or catecholamine excretion. Boli of corticosteroids were delivered and plasmapheresis performed with the hypothesis of a paraneoplastic syndrome, but no neurological improvement was seen.

There was no congenital malformation or morphologic abnormality at clinical examination except for a high arched palate. Neurologic development was poor. She could fix and follow with normal eye movements and remained hypotonic with little spontaneous movements, sucking and swallowing were absent, she experienced severe episodes of desaturation and sweating, and she displayed hyperextension of the limbs. A tracheostomy tube was inserted at 6 weeks of age. Osteotendinous reflexes were present. A deceleration of the head circumference’s growth was noticeable with an OFC of 39 cm (fifth centile) at 4 months. She died at age 4.5 months from a severe apnea with no attempt at resuscitation. Necropsy was not performed.

The tumor was classified as stage 3 by histology [Brodeur et al., 1993]. Neither MYCN amplification nor 1p36 deletion were detected by FISH. No antineuronal antibodies were secreted in the cerebral spinal fluid (CSF). A computed tomography (CT) scan showed no spinal cord compression. Meta-iodo-benzylguanidine (MIBG) scintiscan showed no bone fixation. Electromyography and muscle histology were within the normal limits. Electroencephalography (EEG) showed slow activity without epilepsy. Auditory evoked potential was normal. Histological examination of a rectal biopsy showed normal enteric plexuses eliminating Hirschsprung disease as the cause of abdominal distension. Blood karyotype and a comparative genomic hybridization (CGH)-array with a 650-kb resolution showed normal chromosomes 46, XX. Brain magnetic resonance imaging (MRI) was performed at 3 days and again at 15 weeks of age. At the latter time point, an abnormal shape of the brainstem was noted with an enlarged medulla oblongata eclipsing the ovoid form of the pons. In retrospect, the same image was present from birth (Fig. 1A).

Case 2, a female, was the first child born to unrelated healthy parents with no relevant family medical history. Intrauterine growth retardation and sinusoidal cardiotocograph led to emergency Cesarean section at 31 weeks gestation (BW 1300 g, and a head circumference of 28.5 cm; both at approximately the 25th centile). Paternal and maternal ages at time of birth were 42 and 37 years, respectively. Hypotonia with little spontaneous movements, poor sucking, gastroesophageal reflux, and distended abdomen were noted at birth. She presented daily episodes of desaturation and tracheobronchomalacia necessitating respiratory support and a tracheostomy tube was inserted at age 3 months. A thoracoabdominal CT scan at age 3 weeks showed bilateral large heterogeneous and calcified adrenal masses. She underwent four courses of chemotherapy leading to a reduction in the size of the tumors, but a MIBG scintiscan showed uptake of dye in the right hemithorax that was later confirmed by CT scan. She had a patent foramen ovale with prolonged QT segments on electrocardiography. Bilateral hernias were surgically repaired at age 2 months. She was kept on nasogastric feeds for persistent difficulties in swallowing. Intermittent abdominal distension remained unexplained; a contrast enema showed no obstruction and endoscopic intestinal biopsies were normal. Temperature instability was also observed. At age 5 months, she developed...
abnormal movements of the right arm and leg. Repeated EEGs failed to show focal epileptiform activity and seizures arising from the brainstem were hypothesized. Although initially normal, cranial ultrasound showed an ischaemic cortical lesion on the right inferior parietal lobe. Growth parameters had all fallen below the 0.4th centile by age 5 months. At 9 months, she could fix, had a left convergent squint with normal fundi, and responded to sound. Sensory motor deficit was suspected. She died at age 9 months following a decision to withdraw intensive care. Necropsy was not performed.

In retrospect, the brain MRIs performed at age 6 and 15 weeks showed a brainstem shape very similar to that observed in case 1 (Fig. 1B). At histology, both adrenal biopsies showed infiltrating islands of undifferentiated neuroblasts. FISH analysis identified four copies of the MYCN gene, trisomy of chromosomes 1 and 9, and tetrasomy of chromosome 17.

Blood samples for both cases were obtained with informed consent and DNA was extracted according to standard protocols. Direct sequencing of the ALK and PHOX2B genes was performed on both strands as previously described using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Bedford, MA) and was analyzed on an ABI 3100 automated sequencer.

**Results**

No nucleotidic variation of the PHOX2B gene was found. A heterozygous variation of the ALK gene was identified in each case (c.3733T>G, p.F1245V in case 1 and c.3520T>G, p.F1174V in case 2; numbering is based on the cDNA sequence from NM_004304.3 (ALK_v001); Fig. 2). Each missense mutation altered a conserved amino acid within the intracellular TKD of the protein at a position already found mutated in several NB cell lines and tumours (reviewed in [Palmer et al., 2009] and [Janoueix-Lerosey et al., 2010]). Both mutations occurred de novo. A paternal contribution to the child genotype was confirmed for nine unlinked and polymorphic CA repeat microsatellite markers in case 1 and 2 (data available on request).

**Discussion**

In both cases described in this report, we identified a de novo heterozygous germline ALK gene mutation. Importantly, mutations
at position p.F1174 and p.F1245 have been reported already (with substitution for I, C, V, and L amino acids in both cases), but were invariably somatic [De Brouwer et al., 2010; Janoueix-Lerosey et al., 2010; Palmer et al., 2009]. However, the missense mutations p.G1128A, p.R1192P, and p.R1275Q, lying in the TKD of ALK, have been reported in familial cases segregating NB predisposition with incomplete penetrance and without presenting any neurological symptoms, and have not been reported as somatic mutations [Janoueix-Lerosey et al., 2008; Mosse et al., 2008]. Conversely, both children reported here presented with multifocal NB of neonatal onset and, severe, nonepileptic encephalopathy with a fatal outcome. They were initially referred for possible central congenital hypventilation syndrome (CCHS, Ondine’s curse; MIM #209880) due to episodes of apnoeas and desaturation, abdominal distension, and NB. However, these episodes were independent of the sleep–wake state and direct sequencing of the PHOX2B gene failed to identify a coding sequence mutation. Opsomyoclonic syndrome had also been considered but electroencephalographic recordings showed no epilepsy and eye movements were normal. Moreover, plasmapheresis and corticosteroids did not lead to neurological improvement. Compression by the abdominal mass and Hirshsprung disease were also considered as explanations for the episodes of abdominal distension. An alternative hypothesis is enteric nervous system dysfunction given that Alk is expressed in the developing gut in mice [Vernersson et al., 2006]. The brainstem anomaly in the two patients reported here does not seem progressive, although this could not be assessed fully, given that both patients died at an early age. Nonetheless, the medulla oblongata was enlarged from birth in both cases. The presence of this feature upon brain MRI may be a good indication of an ALK germline mutation in a newborn with severe encephalopathy and brainstem dysfunction of unknown cause with or without NB. Indeed, whether neonatal NB is a consistent feature of the syndrome remains to be defined. The differential diagnosis would be a tumor of the medulla (more often a pyloric acrocytoma), but enlargement would be asymmetric and presenting hypointensity on T1-weighted images.

There is a sharp contrast between the brain phenotype of the patients described in this report, and that of patients with Cardio-Facio-Cutaneous syndromes, in which germline gain-of-function mutations in several genes involved in the RAS signaling pathway have been described, and for whom absolute or relative macrocephaly is the rule (see [Tidyman and Rauen, 2009] for review). This is particularly true for Costello syndrome, which is ascribed to PTPN11, BRAF, and HRAS gain-of-function mutations, with amino acid substitution hotspots at codons p.G12 and p.G13 [Aoki et al., 2005]. Interestingly, a progressive enlargement of the cerebellum leading to posterior fossa crowding and cerebellar tonsilar herniation has been described in a majority of patients with Costello syndrome, while the shape of the brainstem remains normal [Gripp et al., 2010].

ALK is an extremely conserved tyrosine kinase receptor of the insulin receptor family with Midkine and Pleiotrophin as putative ligands in mammals. Ligand binding leads to ALK heterodimerisation, autophosphorylation, and activation of the RAS/MAPK, phosphoinositide-3 kinase (PI3K)/AKT, JAK/STAT3, or PLCγ pathways, promoting proliferation, differentiation or survival [Chiarle et al., 2008; Palmer et al., 2009; Wasik et al., 2009]. Fusion proteins arising from somatic rearrangements have been reported in anaplastic large cell lymphomas and other tumours (reviewed in [Palmer et al., 2009]). In NB and NB cell lines, both ALK amplification and gain-of-function missense mutations of conserved codons of the TKD have been reported [Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Passoni et al., 2009]. Some experimental data indicate variable oncogenic potential of ALK mutants with p.F1174L having an increased transforming capacity compared to p.R1275Q and p.K1062M [Chen et al., 2008; De Brouwer et al., 2010]. Altogether, these observations suggest different effects on ALK signalling for different mutations, with variable biological consequences. An interesting possibility is that there is an ALK activity threshold, above which CNS development would be impaired, but which is not reached by all ALK gain-of-function mutations reported thus far. Animal models are not yet available but knock-in mice bearing mutations at codon p.F1174 and p.R1245 are being generated in several groups. In the CNS of mice, ALK is expressed in several thalamic and hypothalamic nuclei, the pons, the medulla oblongata, and the ventral horn of the spinal cord [Vernersson et al., 2006]. It will be of high interest to explore the consequences of endogenous expression of mutant ALK on both neurological function and anatomic development of the pons, medulla, and motor neurons.

There is a growing list of genes for which somatic and germline gain-of-function mutations have been reported in tumours (of various types) and syndromes, respectively (Table 1). Interestingly, tumor predisposition burdens a minority of these syndromes. The repertoire of mutations and the relative proportion of each nucleotidic variation (and amino acid substitution) are different between somatic and germline cases. As a general rule, mutations exhibiting the highest activating effect in vitro are prevalent in the somatic repertoire and absent from its germline counterpart. The HRAS gene stands as a paradigm. Somatic gain-of-function mutations at codons p.G12, p.G13, and p.Q61 are found in various tumors, whereas germline mutations at codon p.Q61 have not been reported in patients with Costello syndrome. Moreover, when considering amino acid changes at codon 12, p.G12V is far more frequent somatically than p.G12S (and leading to a greater

<table>
<thead>
<tr>
<th>Gene</th>
<th>OMIM</th>
<th>Somatic mutation/tumour predisposition</th>
<th>Germline mutation/Syndromes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RET</td>
<td>164761</td>
<td>Thyroid</td>
<td>MEN2A/MEN2B*</td>
<td>[Hofstra et al., 1994; Mulligan et al., 1993]</td>
</tr>
<tr>
<td>FGFR3</td>
<td>134934</td>
<td>Bladder/Skin/Haematopoietic</td>
<td>AChondroplasia/TD</td>
<td>[Rousseau et al., 1994]</td>
</tr>
<tr>
<td>FGFR2</td>
<td>176943</td>
<td>Uterus/Skin/Testicle</td>
<td>Crouzon/Apert/Pfeiffer</td>
<td>[Reardon et al., 1994; Wilkie et al., 1995]</td>
</tr>
<tr>
<td>HRAS</td>
<td>190020</td>
<td>Bladder/Thyroid/Skin</td>
<td>Costello*</td>
<td>[Aoki et al., 2005]</td>
</tr>
<tr>
<td>KRAS</td>
<td>190070</td>
<td>Colon/Pancreas/Lung</td>
<td>Noonan/CFC</td>
<td>[Niibori et al., 2006]</td>
</tr>
<tr>
<td>BRAF</td>
<td>164757</td>
<td>Colon/Thyroid/Skin</td>
<td>CFC</td>
<td>[Tarqaglia et al., 2001]</td>
</tr>
<tr>
<td>PTPN11</td>
<td>176876</td>
<td>Haematopoietic</td>
<td>Noonan</td>
<td>[Niibori et al., 2006]</td>
</tr>
<tr>
<td>IDH2</td>
<td>147650</td>
<td>CNS/Haematopoietic</td>
<td>D2 Hydroxyglutaric Aciduria</td>
<td>[Kramendijck et al., 2010]</td>
</tr>
<tr>
<td>ALK</td>
<td>105590</td>
<td>PNS</td>
<td>Congenital encephalopathy</td>
<td>This report</td>
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</table>

Syndromes predisposing to tumors are indicated with an asterisk. Several cases of leukemia have been reported in CFC. A paternal age effect is observed for germline mutations of RET, FGFR2, FGFR3, HRAS, and PTPN11.
activation [Fasano et al., 1984]), while in Costello syndrome p.G12S is the most common substitution, with p.G12V having been reported only twice; both of these patients had a severe phenotype [van der Burgt et al., 2007]. Most interestingly, two “missing germline mutations” at codon 61 (Q61R and Q61K) of MYCN amplification. Clin Cancer Res 16:4353–4362.

References


