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GERMLINE GAIN-OF-FUNCTION MUTATIONS of *ALK* DISRUPT CENTRAL NERVOUS SYSTEM DEVELOPMENT

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Abstract

Neuroblastoma (NB) is a frequent embryonal tumour 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,* MIM 105590) 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 harbouring *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.

Introduction

Neuroblastoma (NB; MIM 256700) is the most frequent extra-cranial 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, a tyrosine kinase receptor gene of the insulin receptor family, is activated by fusion with various partners in anaplastic large cell lymphomas, inflammatory myofibroblastic tumours 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; Janoueix-Lerosey, et al., 2008; Mosse, et al., 2008; De Brouwer, et al., 2010]. 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 [Iwahara, et al., 1997; Vernersson, et al., 2006; Hurley, 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, behavioural 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 characterised 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

Case1, 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 (BW : 3100 g, BL : 46 cm, 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 three days of life. Levels of urinary catecholamine and its metabolites were raised. Rapid tumour progression led to chemotherapy by vincristine and cyclophosphamide with no improvement of the tumour mass or catecholamine excretion. Boli of corticosteroids were delivered and plasmapheresis performed with the hypothesis of a paraneoplasic 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 six weeks of age. Osteotendinous reflexes were present. A deceleration of the head circumference's growth was noticeable with OFC of 39 cm (5^{th} centile) at four

months. She died at age four and a half months from a severe apnea with no attempt at resuscitation. Necropsy was not performed.

The tumour 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 CSF. A computerised tomography (CT) scan showed no spinal cord compression. Meta-iodo-benzyl-guanidine (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 three 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 (Figure 1A).

Case 2, a female, was the first child born to unrelated healthy parents with no relevant family medical history. Intra-uterine growth retardation and sinusoidal cardiotocograph led to emergency Caesarean 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, gastro-oesophageal 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 three months. A thoraco-abdominal CT scan at age three weeks showed bilateral large heterogeneous and calcified adrenal masses. She

underwent four courses of chemotherapy leading to a reduction in the size of the tumours, 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 two 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 five 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 hypothesised. 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 five months. At nine 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 nine months following a decision to withdraw intensive care. Necropsy was not performed.

In retrospect, the brain MRIs performed at age six and 15 weeks showed a brainstem shape very similar to that observed in case 1 (Figure 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) 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), Figure 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, non epileptic encephalopathy with a fatal outcome. They were initially referred for possible central congenital hypoventilation syndrome (CCHS, Ondine's curse MIM209880) 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 Hirschsprung 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 tumour of the medulla (more often a pylocytic astrocytoma), 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 signalling 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 *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, tumour 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 tumours, 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 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 *HRAS* have been identified in 5/30 spermatocytic seminomas (a rare testicular germ cell tumour of late-age onset) [Goriely, et al., 2009]. We thus speculate that such mutations are to be found in the germline but probably lead to an extreme, possibly foetal lethal phenotype, distinct from Costello syndrome.

Here we report a novel syndrome with predisposition to NB due to constitutive *ALK* gain-of-function mutations. In doing so, we provide evidence that normal CNS development requires regulation of ALK activity, with a threshold being exceeded for some mutations only, and therefore we add *ALK* to the list of oncogenes with important roles in normal development.

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Table and Figures

Figure 1: Brain MRI of the two patients and three controls.

Note the abnormal shape of the brainstem with enlarged medulla oblongata eclipsing the ovoid form of the pons (arrows) on brain MRI (T1-weighted sagittal images) in both cases (top) as compared to controls (bottom, arrowheads).

A) patient 1, B) patient 2, C) antenatal MRI of a control foetus at 34 weeks gestation, D-E) controls.

Figure 2: *ALK* gene mutations.

2A. A constitutional heterozygous missense variation of the *ALK* gene having occurred *de novo* was identified in each case (c.3733T>G, p.F1245V in case 1 and c.3520T>G, p.F1174V in case 2, with numbering based on the cDNA sequence from NM_004304.3 (ALK_v001). There are no evidence that the *ALK* mutations are present in mosaic state. Indeed mutant allele is not under-represented compared to wild-type allele. Residue F1245 is located in the catalytic loop and residue F1174 in the C helix of the TKD [Bossi, et al., 2010; Lee, et al., 2010].

2B. Published *ALK* mutations in NB (adapted from (Janoueix-Lerosey, et al., 2010) with permission). Mutations are indicated by arrows, with the number of mutations identified at each position to date indicated underneath. The mutations are mainly located in the TK domain, with two hotspots at positions 1174 and 1275.

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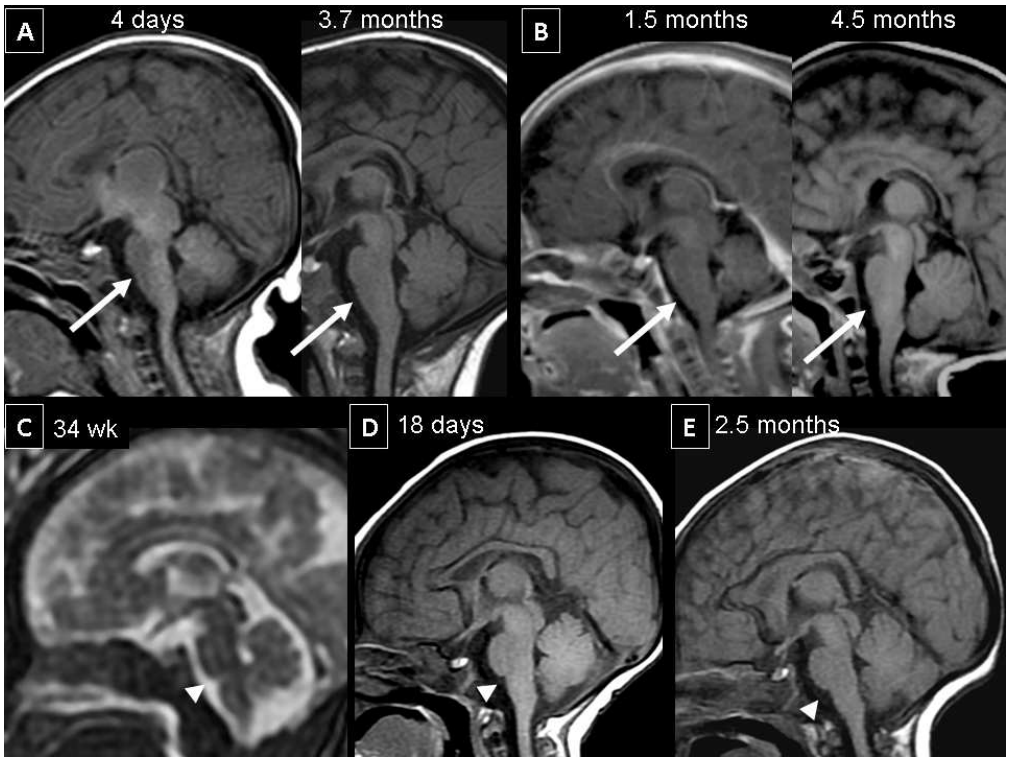

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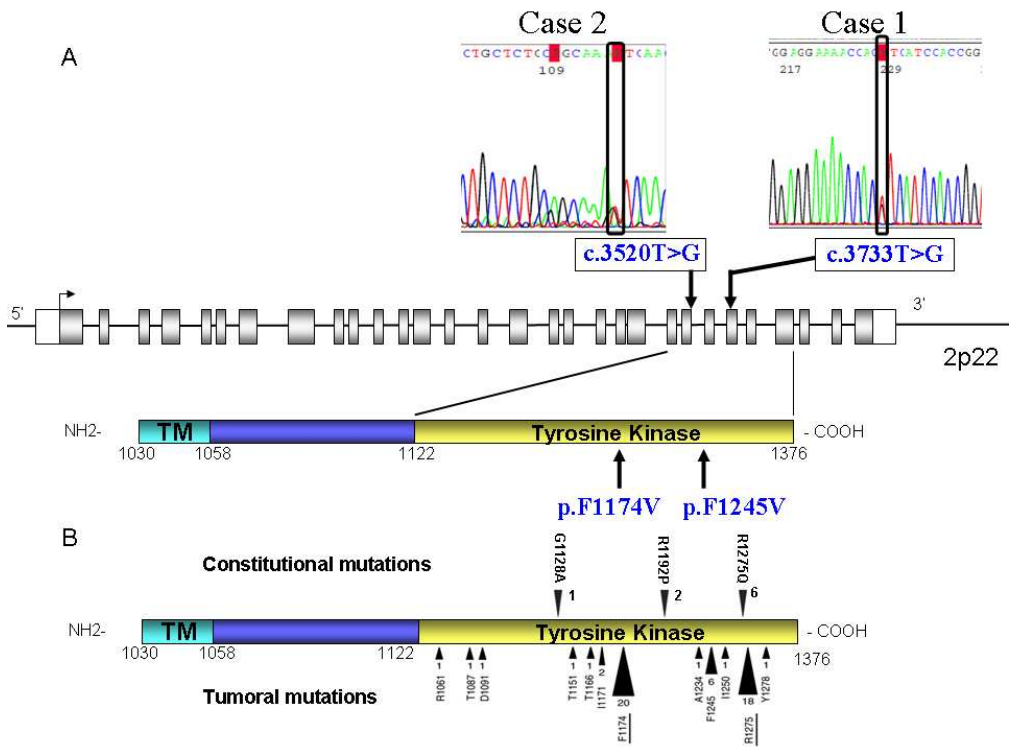

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Gene	MIM	Somatic mutation / tumour predisposition	Germline mutation / Syndromes	Reference
RET	164761	Thyroid	MEN2A / MEN2B*	[Mulligan, et al., 1993; Hofstra, et al., 1994]
FGFR3	134934	Bladder / Skin / Haematopoietic	Achondroplasia / TD	[Rousseau, et al., 1994]
FGFR2	176943	Uterus / Skin / Testicle	Crouzon / Apert / Pfeiffer	[Reardon, et al., 1994; Wilkie, et al., 1995]
HRAS	190020	Bladder / Thyroid / Skin	Costello*	[Aoki, et al., 2005]
KRAS	190070	Colon / Pancreas / Lung	Noonan / CFC	[Niihori, et al., 2006]
BRAF	164757	Colon / Thyroid / Skin	CFC	[Niihori, et al., 2006]
PTPN11	176876	Haematopoietic	Noonan	[Tartaglia, et al., 2001]
IDH2	147650	CNS / Haematopoietic	D2 Hydroxyglutaric Aciduria	[Kranendijk, et al.]
ALK	105590	PNS	Congenital encephalopathy	this report

Table 1: List of genes for which somatic and germline gain-of-function mutations have been reported in tumours and syndromes respectively.

Syndromes predisposing to tumours 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*.