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SHORT REPORT

Dissection of the *MYCN* locus in Feingold syndrome and isolated oesophageal atresia

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Feingold syndrome (FS) is a syndromic microcephaly entity for which *MYCN* is the major disease-causing gene. We studied the expression pattern of *MYCN* at different stages of human embryonic development and collected a series of 17 FS and 12 isolated oesophageal atresia (IOA) cases. An *MYCN* gene deletion/mutation was identified in 47% of FS cases exclusively. We hypothesized that mutations or deletions of highly conserved non-coding elements (HCNEs) at the *MYCN* locus could lead to its misregulation and thereby to FS and/or IOA. We subsequently sequenced five HCNEs at the *MYCN* locus and designed a high-density tiling path comparative genomic hybridization array of 3.3 Mb at the *MYCN* locus. We found no mutations or deletions in this region, supporting the hypothesis of genetic heterogeneity in FS.
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INTRODUCTION

Feingold syndrome (FS, MIM164280) combines characteristic digital anomalies (ie, brachymesophalangy of the second and fifth fingers and brachysyndactyly of the toes), microcephaly, oesophageal/duodenal atresia, and variable learning disabilities.[1] FS has been mapped to 2p23–24[2] and is the consequence of *MYCN* gene (MIM 164840) loss-of-function either by germline deletions or by coding-sequence mutations.[3,4] Conversely, *MYCN* amplification is a prognostic factor for a bad outcome and is found in about 10% of neuroblastomas.[5]

In this study, we studied the expression pattern of *MYCN* at different stages of human embryonic development, and screened a cohort of 17 patients suspected of FS and 12 patients with isolated oesophageal atresia (IOA). We identified a heterozygous mutation/deletion in seven FS cases (47%) and no mutation or deletion in IOA. Some highly conserved non-coding elements (HCNEs), able to direct *N-myc* expression, have been identified in transgenic mice[6–8] We hypothesized that deregulation of tissue- or stage-specific *MYCN* expression following mutation or disruption of regulatory HCNEs at the *MYCN* locus could lead to FS and/or IOA. We subsequently sequenced five HCNEs at the *MYCN* locus and searched for small deletions in the 3.3-Mb vicinity of *MYCN*.

PATIENTS AND METHODS

A total of 29 patients were included in the study: 17 patients with possible FS (Table 1) and 12 patients with IOA. Diagnostic criteria for FS were the presence of three or more of the core features: (i) microcephaly, (ii) brachymesophalangy of the second and fifth finger, (iii) 2/3 or 4/5 toe syndactylies, and (iv) oesophageal or duodenal atresia. Whereas postnatal microcephaly was constant after 3 years of age, head circumference was normal at birth in three cases. All patients showed mild-to-moderate mental retardation and eight developed postnatal growth retardation. Brachymesophalangy of the second and fifth finger was noted in 15 cases, syndactylies in 12 cases, and oesophageal atresia in 14 of the 17 cases (Figure 1, Table 1). Additional features are listed in Table 1. All IOA cases were sporadic (10 type III and 2 type I), with no additional malformations.

Blood samples were obtained with informed consent and DNA was extracted according to standard protocols. DNA sequencing of the three coding exons and intronic flanking regions was performed by the fluorometric method on both strands (ABI BigDye Terminator Sequencing Kit V.2.1, Applied Biosystems). Comparative genomics analysis of the *MYCN* locus indicated five HCNEs with >75% identity over 350 bp across humans, rhesus, dog, and mouse (Figure 1). These HCNEs were studied by direct sequencing in all patients with no coding-sequence mutation (primers available on request).

A 3.3-Mb region extending 1.94 Mb centromeric (5′) and 1.36 Mb telomeric (3′) to *MYCN* (chr2: 12 800 000–16 590 000; NCBI Build 36.1) was studied by fine-tiling array-based comparative genomic hybridization (CGH; NimbleGen Systems, http://www.nimblegen.com/products/cgh/human.html#cnv) on 6 FS and 10 IOA patients with no *MYCN* coding-sequence mutation, as well as 550 normal-banded chromosomes on blood karyotype. The average spacing of probes in Nimblegen fine-tiling array is 52 bp. A deletion was considered when at least 10 probes were abnormal, giving a deletion detection resolution of about 500 bp at the *MYCN* locus. Genome-wide array-CGH with a resolution of 50 kb was performed in the five FS patients with no *MYCN* mutation and normal Nimblegen fine-tiling array, using the Agilent Human Genome CGH Microarray Kit 244 K (Agilent Technologies, Santa Clara, CA, USA).

To study *MYCN* expression during human development, embryos were collected from terminated pregnancies in agreement with French bioethics laws

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Table 1 Clinical features in the series of 17 FS patients with and without *MYCN* mutation

Mutated patients	*AO2*	*AO28*	*AO37*	*AO56*	*AO60*	*AO65*	*AO67*	*AO68*	*Total*
Sex	F	F	M	M	F	M	F	M	4M/4F
Familial history	−	+	−	−	+	−	+	−	3/8
Head circumference at birth	−2	−3	−4	−3	−2	−4	−3	−2	8/8
Postnatal microcephaly (SD)	−3	−3	−4	−3	−2	−4	−3	−2	8/8
Weight and size at birth	50th c.	25th c.	25–50th c.	2550th c.	25–50th c.	25–50th c.	25–50th c.	25–50th c.	
Postnatal growth retardation (SD)	−2	−2.5	−2	−2	−1	−2	0	0	5/8
Mental retardation	Mild	Moderate	Mild	Mild	Mild	Moderate	Mild	Mild	8/8
Micrognatia	−	−	−	−	−	+	+	−	2/8
Brachymesophalangy II et V	+	+	+	+	+	+	+	+	8/8
Toe syndactyly 2/3	−	+	−	+	+	+	+	−	5/8
Toe syndactyly 4/5	+	+	+	−	−	+	+	−	5/8
Oesophageal atresia	+	+	+	+	+	+	+	−	7/8
Duodenal atresia	−	−	−	−	−	−	−	−	0/8
Renal hypoplasia	+	−	+	−	−	−	−	−	2/8
Congenital cardiac defect	ASD	VSD	−	−	−	−	−	−	2/8
Deafness	−	−	−	−	−	−	−	−	0/8
Asplenia	−	+	−	−	−	−	−	−	1/8
Result of *MYCN* gene screening	c.1180G>A	c.1293delC	c.1110insG	c.928-930insGT	c.474-514del	c.1177C>T	c.134dupC	del 2p24.3	8/8

Non-mutated patients	*AO3*	*AO4*	*AO22*	*AO35*	*AO36*	*AO39*	*AO41*	*AO42*	*AO43*	*Total*
Sex	M	F	F	M	M	F	M	F	F	4M/5F
Familial history	−	−	−	−	−	+[a]	+[b]	−	−	2/9
Head circumference at birth	–2.5	0	−2	0	−2.5	0	–3	−4	−1	5/9
Postnatal microcephaly (SD)	–2.5	−2	−2.5	−2	−2.5	–3	–3	−4	−2	9/9
Weight and size at birth	25–50th c.	50th c.	50th c.	50th c.	50th c.	50th c.	25–50th c.	50th c.	50th c.	
Postnatal growth retardation (SD)	−1	−1	−	−2	−1.5	0	−2.5	−3	0	3/9
Mental retardation	Mild	Moderate	Mild	Mild	Mild	Mild	Moderate	Mild	Mild	9/9
Micrognatia	−	+	−	+	−	−	−	−	−	2/9
Brachymesophalangy II et V	+	−	+	+	+	+	+	+	−	7/9
Toe syndactyly 2/3	−	+	+	−	−	+	−	+	−	4/9
Toe syndactyly 4/5	−	−	−	+	−	+	−	−	−	2/9
Oesophageal atresia	+	+	−	+	+	+	−	+	+	7/9
Duodenal atresia	−	−	−	+	−	−	−	−	−	1/9
Renal hypoplasia	−	−	−	−	−	−	−	−	−	0/9
Congenital cardiac defect	−	−	−	−	VSD, MA, AC	−	−	−	VSD	1/9
Deafness	−	−	−	+	−	−	−	−	−	0/9
Asplenia	−	−	−	−	−	−	−	−	−	0/9
Result of *MYCN* gene screening	−	−	−	−	−	−	−	−	−	0/9
Result of Nimblegen fine-tiling array	Normal	Normal	Normal	Normal	Normal	Normal	NP	NP	NP	0/6
Result of 244K genome wide array	Normal	Normal	Normal	Normal	Normal	NP	NP	NP	NP	0/5

Abbreviations: AC, aortic coarctation; ASD, atrial septal defect; del, deletion; F, female; M, male; MA, mitral atresia; VSD, ventricular septal defect.
[a]The father and a sister of AO39 are microcephalic and have digital anomalies (brachymesophalangy of the second and fifth fingers and brachysyndactyly of the toes). The sister has also learning disabilities.
[b]The mother of AO41 is microcephalic and has anomalies in the hand (brachymesophalangy of the second and fifth fingers).

(94-654 and 04-800) and the Necker Hospital ethics committee. Probe synthesis and hybridization were carried out as described previously.[9]

RESULTS

Direct sequencing and searching for deletion in *MYCN* locus

We identified a heterozygous coding-sequence mutation in seven patients (five novel, Table 1). All mutations resulted in a premature stop codon that removed the basic helix-loop-helix (b-HLH) and the leucine-zipper (LeuZ) domains or modified a conserved amino acid essential for DNA binding (Figure 2). One patient had a deletion of 425 kb encompassing the *MYCN* gene alone. Six mutations occurred *de novo* and one was inherited from the affected father (AO28, Table 1), who showed brachymesophalangy of the second and fifth fingers, syndactyly between the fourth and fifth toes, microcephaly, and mild mental retardation. Additional features observed in patients harbouring a *MYCN* coding-sequence mutation or deletion were congenital heart malformations (two cases), kidney hypoplasia (two cases), asplenia (one case), and diaphragmatic hernia (one case). This last malformation had never been reported previously and CGH analysis showed no additional rearrangements in this patient. The *MYCN* locus was further investigated in patients with no coding-sequence mutations; we sequenced five HCNEs identified in the *MYCN* locus (Figure 2) and identified no nucleotide variations in either FS or IOA patients. Fine-tiling array-based CGH identified no micro-rearrangements in the 3.3-Mb region encompassing *MYCN*. Genome-wide array-CGH 244 K was normal in the five patients with no *MYCN* coding-sequence mutation and normal Nimblegen fine-tiling array (Table 1).

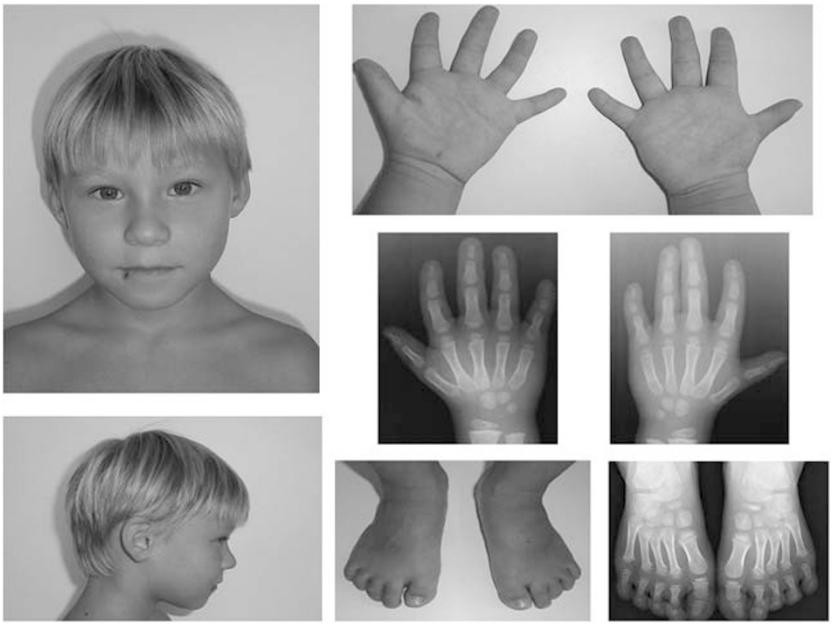

Figure 1 Patient AO68 with FS and *MYCN* deletion. Note the round face, brachymesophalangy of the second and fifth fingers, and short feet with brachydactyly.

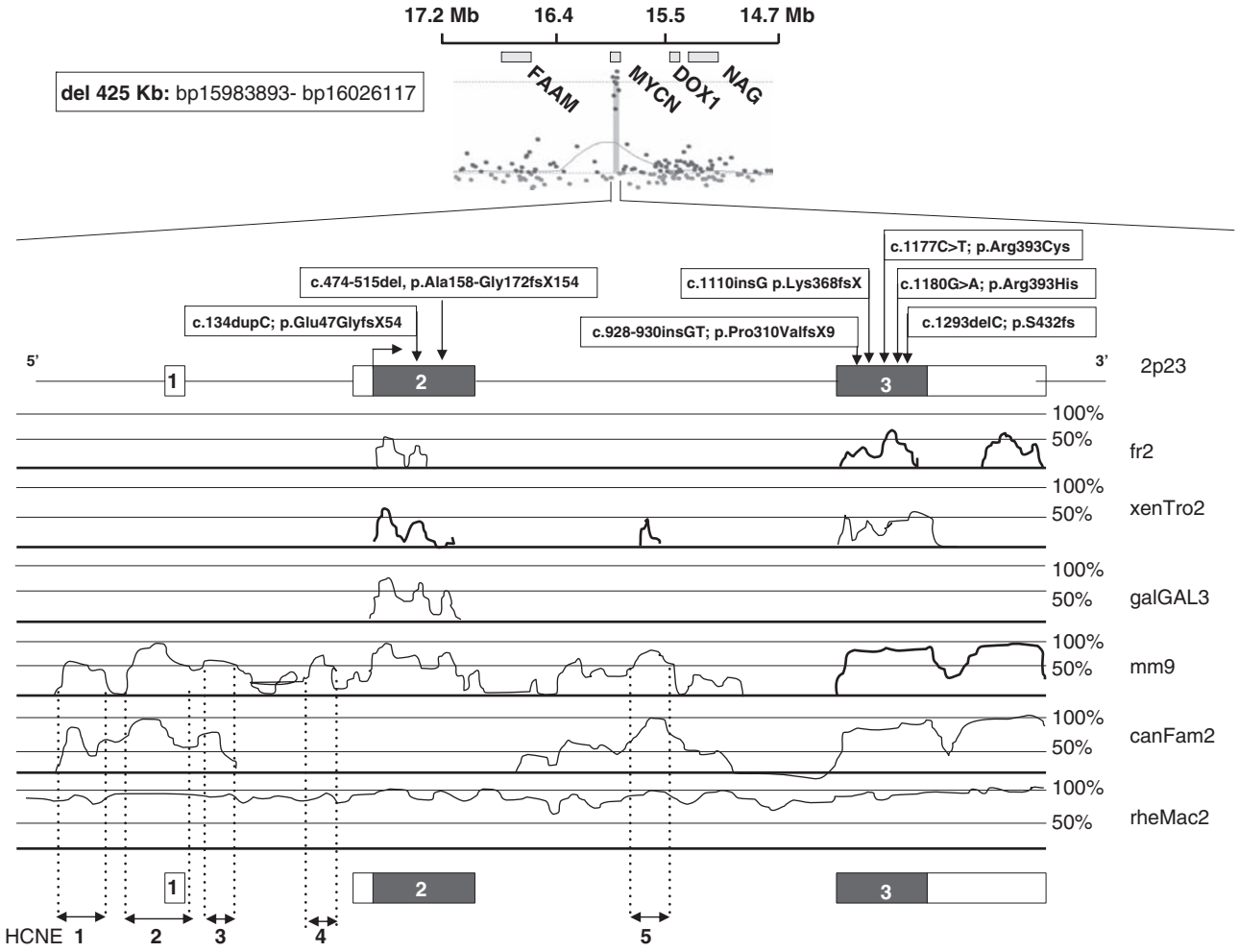

Figure 2 Schematic representation of the *MYCN* locus (6647 bp). The deletion and mutations identified in five FS patients and HCNEs with >75% identity over 350 bp across humans, rhesus, dog, and mouse (ECR browser software) are shown.

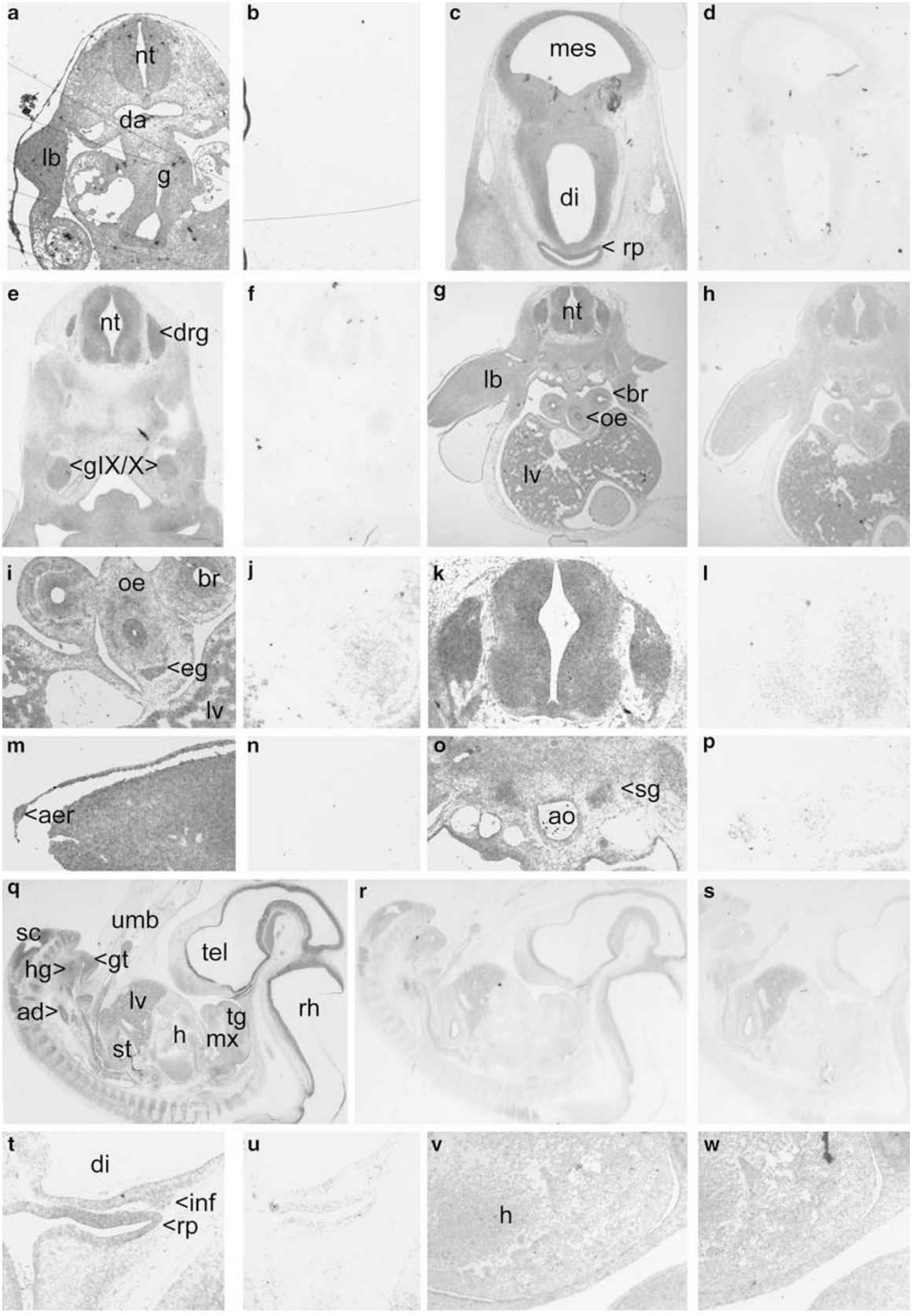

Figure 3 For caption see next page.

Figure 3 *MYCN* expression in early human development. Antisense and sense (negative control, **b**, **d**, **f**) riboprobes are presented side by side in panels **a** to **p**. (**a**) At CS 13 (37 days' gestation) *MYCN* is ubiquitously expressed. (**c**) CS 15 (36 days' gestation), transverse section in head, showing *MYCN* expression is still widespread but is particularly strong in the brain and craniofacial mesenchyme, as well as in the precursors to the pituitary gland. (**e**) CS 15, transverse section through the cervical neural tube. (**g**) CS 15, section at the level of the forelimb. As compared with the control (**h**), no expression is observed in the liver, but is specific to both epithelia and mesenchyme of bronchi, oesophagus (**i**, **j**), and forelimb bud (**m, n**), as well as at levels of the central and peripheral nervous system (CNS/PNS; **i–l**, **o**, **p**). (**q**) Hematoxylin–eosin stain of sagittal section at CS 17 and 18 (42–45 days' gestation). *MYCN* expression in an adjacent section (**r**) shows continued but diminished expression in the CNS/PNS and craniofacial mesenchyme, and presence of transcripts in the adrenal gland, hindgut, and genital tubercule. (**t**) *MYCN* continues to be expressed in the developing pituitary, unlike in the ventricular myocardium (**v** *vs* **w**). Abbreviations: ad, adrenal gland; aer, apical ectodermal ridge; ao, aorta; br, bronchi; da, dorsal aorta; di, diencephalon; drg, dorsal root ganglia; eg, enteric ganglia; g, gut; g/IX/X, cranial ganglia IX/X; gt, genital tubercule; h, heart; hg, hindgut; inf, infundibulum; lb, limb bud; lv, liver; mes, mesencephalon; mx, maxilla; nt, neural tube; oe, oesophagus; rp, rathke's pouch; rh, rhombencephalon; sc, spinal cord; sg, sympathetic ganglion; st, stomach; tel, telencephalon; tg, tongue; umb, umbilical cord. Scale bars: 0.5 mm, except for **q–s**, 1 mm.

MYCN expression in early human development

Additional features observed in patients with an *MYCN* mutation motivated the study of *MYCN* expression at different stages of human embryonic development (Figure 3). At Carnegie stage (CS) 13, *MYCN* appears ubiquitously expressed, with higher expression in the limb-bud mesenchyme (Figures 2a and b). At CS 15, *MYCN* is differentially and highly expressed in the CNS/PNS, the oesophageal and bronchic epithelia, Rathke's pouch, sympathetic ganglia, and both ectodermal and mesenchymal components of the forelimb. At CS 17 and 18, *MYCN* is highly expressed throughout the CNS/PNS and in both Rathke's pouch and the corresponding precursor of the neurohypophysis, the infundibulum (Figures 3t and u), the smooth muscle of the umbilical arteries, the adrenal gland, and the hindgut as well as other sites (Figures 3q–u). However, despite low levels of cardiac expression seen *in situ* at CS 13, we no longer observed any cardiac expression at CS 18 (Figures 3v and w).

DISCUSSION

We identified an *MYCN* mutation in 50% of our cases (8/17). No major phenotypic differences could be found among the core features of FS retrospectively, between patients with and without a *MYCN* mutation (Table 1). Only syndactyly of toes 4 and 5 was more frequent in the group with *MYCN* mutations. The high frequency of oesophageal atresia in our series is due to a recruitment bias through paediatric surgeons. Importantly, no additional malformations were present in the group of patients without mutations. Although head circumference can be normal at birth, postnatal microcephaly is constant in our series. Most patients were sporadic cases, contrasting with a previous report.[4] This discrepancy could be ascribed to both a recruitment bias for familial cases before the gene was identified, and the fact that, clinically, the entity is better recognized since then. Several additional congenital malformations have been reported in FS; ie, vertebral malformations, congenital cardiac defects, and renal hypoplasia.[4] Renal hypoplasia needs to be detected early on in order to prevent renal failure.[2] One of our patients presented asplenia. This has not hitherto been reported in FS but is present in the *N-myc* hypomorphic mouse model.[10] A diaphragmatic hernia was detected in the same patient at birth. Facial features reported in FS are tenuous and combine short palpebral fissures, broad nasal bridge, and micrognathia.

We studied the pattern of expression of *MYCN* at different stages of normal human embryonic development. *MYCN* is widely expressed in forelimb mesenchyme at the stages we studied, consistent with the constant distal bone malformations observed in FS. Expression in Rathke's pouch raises the question of involvement of the pituitary gland in the growth deficit. We observed *MYCN* expression in both bronchial tubes and the oesophagus at CS 15, but not in the diaphragm at CS 17 and 18. *N-myc* knockout mice had been generated concomitantly by three independent groups.[11–13] Embryonic lethality was consistently observed between embryonic days E10.5 and E12.5 of gestation, with developmental delay and small size of mesonephros, lung, heart, and gut. Interestingly, mutant mice with 25% of wild-type levels of N-myc protein die at birth and are unable to breathe because of a severe deficiency in lung-branching morphogenesis.[10]

The molecular mechanisms underlying the regulation of *MYCN* expression have not been totally elucidated. It has been shown, by replacing endogenous *N-myc* coding sequences by the *c-myc* ones, that *c-myc* can complement *N-myc* functions.[14] Therefore, the specificity of both genes resides in their controlled expression patterns. No mutation/deletion of *MYCN* regulatory elements could be identified in humans. Altogether, these results are suggestive of genetic heterogeneity in FS.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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