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Comprehensive genotyping and clinical characterisation reveal 27 novel NKX2-1 mutations and expand the phenotypic spectrum

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Contributors Genotyping experiments and analysis (Sanger sequencing and array CGH) as well as preparation of figures were done by AT. Extended phenotype data collection and analysis was carried out by SS-H. Further experimental data were generated by PS (mouse embryonic hybridisation experiments), IM and SJ (technical assistance on array CGH and Sanger sequencing), CD (EMSA experiments), HB (Mini Gene Reporter Assay & Cell Transfection experiments) and GK (NKX2-1 structural homology model). Furthermore, JK and MS assisted on analysis of extended phenotype data. GE, AS and H-HR contributed to interpretation of data and AG to study design, respectively. CB, KB, H-JC, PC, FZ, MG, JH, SI, CH, KK, BP, DR and IS did patients' phenotyping and sample collection. AT, RU and HK designed the study, interpreted data and wrote the manuscript, and are therefore the guarantors of the work.

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Abstract

Background—*NKX2-1* encodes a transcription factor with large impact on the development of brain, lung and thyroid. Germline mutations of *NKX2-1* can lead to dysfunction and malformations of these organs. Starting from the largest coherent collection of patients with a suspected phenotype to date, we systematically evaluated frequency, quality and spectrum of phenotypic consequences of *NKX2-1* mutations.

Methods—After identifying mutations by Sanger sequencing and array CGH, we comprehensively reanalysed the phenotype of affected patients and their relatives. We employed electrophoretic mobility shift assay (EMSA) to detect alterations of *NKX2-1* DNA binding. Gene expression was monitored by means of in situ hybridisation and compared with the expression level of *MBIP*, a candidate gene presumably involved in the disorders and closely located in close genomic proximity to *NKX2-1*.

Results—Within 101 index patients, we detected 17 point mutations and 10 deletions. Neurological symptoms were the most consistent finding (100%), followed by lung affection (78%) and thyroidal dysfunction (75%). Novel symptoms associated with *NKX2-1* mutations comprise abnormal height, bouts of fever and cardiac septum defects. In contrast to previous reports, our data suggest that missense mutations in the homeodomain of *NKX2-1* not necessarily modify its DNA binding capacity and that this specific type of mutations may be associated with mild pulmonary phenotypes such as asthma. Two deletions did not include *NKX2-1*, but *MBIP*, whose expression spatially and temporarily coincides with *NKX2-1* in early murine development.

Conclusions—The high incidence of *NKX2-1* mutations strongly recommends the routine screen for mutations in patients with corresponding symptoms. However, this analysis should not be confined to the exonic sequence alone, but should take advantage of affordable NGS technology to expand the target to adjacent regulatory sequences and the *NKX2-1* interactome in order to maximise the yield of this diagnostic effort.

INTRODUCTION

NKX2-1 (OMIM *600635), also known as *TITF-1*, *TTF-1* or *T/ebp*, is a member of the homeodomain-containing NK-2 gene family.¹ It encodes a transcription factor, which is expressed during early development of thyroid, lung and forebrain regions, particularly the basal ganglia and hypothalamus.²³ In thyrocytes, *NKX2-1* regulates the expression of thyroglobulin, thyrotropin receptor, thyroid peroxidase (TPO) and pendrin, and is therefore required for both development and maintenance of the thyroid gland. In the lung, *NKX2-1* is implicated in the branching of the lobar bronchi, and at later stages of embryonic development, it regulates the expression of surfactant proteins in type II pneumocytes. In the brain, it has a central role in proper specification and migration of interneuron subtypes in

the medial ganglionic eminence and telencephalon.⁴⁵ However, little is known about the direct and indirect transcriptional targets of *NKX2-1* in the central nervous system.

In mice, the disruption of the *Nkx2-1* gene resulted in a severe, lethal phenotype in homozygous animals, characterised by pulmonary hypoplasia, absence of functional thyroid tissue and complex malformations of brain structures, including the basal ganglia, hypothalamus and pituitary. Heterozygous littermates remained unaffected.⁴ Against this background and in the light of the multifaceted role of *NKX2-1* in normal lung, thyroid and brain development and function, a corresponding phenotype was also expected for germline mutations of *NKX2-1* in humans. Indeed, patients with large chromosomal deletions of chromosome 14 encompassing *NKX2-1* and several other genes showed malfunctioning of the thyroid, the lung and the central nervous system (CNS).⁶⁷ Yet, it was not before the identification of the first intragenic point mutations that these symptoms could be unequivocally assigned to a dysfunction of *NKX2-1*.^{8–10} Notably, in contrast to mice, where only inactivation of both alleles resulted in a disease phenotype, all patients in these studies were heterozygous for the mutation, therefore suggesting a dominant mode of inheritance in humans.

Considerable phenotypic variability in terms of organ involvement and expressivity was already evident in these first described patients and their affected relatives. All individuals suffered from a neurological disorder with profound muscular hypotonia and delay in motor development in their first months of life, followed by the onset of a movement disorder, mainly characterised by uncontrolled, jerky movements by means of a truncal chorea and peripheral athetosis. However, a functional defect of the thyroid was present in only half of the patients, variably manifesting as either severe congenital hypothyroidism (CH) or merely elevated thyroid-stimulating hormone (TSH) levels. Even fewer patients presented with additional pulmonary affection ranging from severe neonatal distress to the occurrence of frequent and severe pneumonias in the first two to three years of life.^{8–10}

Since then, 20 case reports and 7 series of patients with a total of 161 patients and 77 mutations have been reported (see online supplementary table 1),^{6–33} but altogether they have failed to reveal a correlation between type, size and position of mutations and a specific phenotype. The only exceptions were reports on oligodontia in individuals harbouring chromosomal deletions simultaneously encompassing *NKX2-1* and *PAX9*, reflecting the role of *PAX9* in odontogenesis.^{34–36} This overall lack of genotype–phenotype correlation was surprising given the fact that the two activating domains of *NKX2-1* are supposed to be differentially involved in thyroid and pituitary development, implying that at least different missense mutations might cause different phenotypes.³⁷

One reason for this unexpected finding could be a biased composition of patient cohorts in all studies published to date. This bias may be attributed to differences in the main emphasis of these studies, either on Benign hereditary chorea (BHC),¹⁵²²³⁸ CH¹³¹⁴ or chromosomal aberrations,¹⁶³⁹ respectively, or may have originated from particularities in disease naming,¹⁷ which could have led to exclusion of patients that do not display the full spectrum of possible symptoms. Therefore, a comprehensive description of mutation frequency and quality within a larger cohort of non-biased patients is still missing. We have taken

advantage of our 10-year experience in diagnosing *NKX2-1* deficiency⁸ to collect a cohort of 101 patients with BHC or the combination of thyroid, lung or neurological symptoms. Here we report the results of the most comprehensive screen for point mutations and DNA copy number variants (CNVs) affecting *NKX2-1* in patients with an indicative phenotype to date. Starting from the subset of index patients with confirmed *NKX2-1* mutations as well as their mutation-positive relatives, we have re-evaluated the spectrum of symptoms associated with this genotype. Moreover, we provide evidence for DNA copy number changes in next proximity to *NKX2-1* that do result in a phenotype comparable to that of intragenic mutations.

MATERIALS AND METHODS

Patients and DNA isolation

Over a period of 10 years, samples of 101 patients (57 male, 44 female, age 0–18 years) were referred for diagnosis characterised by clinical symptoms as follows: (i) triad of thyroid dysfunction, neurological deficit and pulmonary disease in 26 patients (25.5%); (ii) thyroid dysfunction and neurological deficit in 48 patients (47.1%); (iii) isolated neurological deficit in 16 patients (15.7%); (iv) neurological deficit and pulmonary malfunction in 8 patients (7.8%) and (v) thyroid dysfunction and pulmonary disease in 4 patients (3.9%). All patients have been classified by experienced clinicians from 35 medical centres in 18 countries. Genomic DNA of all subjects as well as of healthy controls was isolated from peripheral blood leucocytes using the Qiagen DNA blood mini kit (Qiagen, Hilden, Germany). Informed consent for the genetic diagnosis was obtained from the parents according to the local regulations (ethical approval no. EA2-089-06).

DNA sequencing analysis

The entire coding region of *NKX2-1* was amplified in two fragments from genomic DNA (forward primer: 5'-CGTCCCCAGACTCGCTCGCTC-3'/5'-CCTCCTCTTCCTTCCTCC-3'; reverse primer: 5'-CTCAGGAGGTGCGGCGAGAGCCGTC-3'/5'-GAGGGAAGCGGTGAGGCAGAG-3'). Exons 1–3 were sequenced using V3 kit and ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) applying appropriate primers. DNA of patients 16 and 24 were additionally cloned into a TOPO cloning vector (Invitrogen, Groningen, The Netherlands) as described previously to delineate allele distribution. If DNA was available, the inheritance of identified sequence variants was analysed in family members.

NKX2-1 structural homology model

For model design of the human *NKX2-1*, the already published NMR structure of the homologous rat thyroid transcription factor homeodomain (Protein-Database entry code 1FTT⁴⁰) served as a structural template. The interacting DNA fragment was arranged according to a nearly identical and superimposed X-ray structure of the *NKX2-5* homeodomain (gray backbone) bound with DNA (PDB entry code 3RKQ). The homeobox is constituted by three helices, which are connected by loops (figure 1C). For model preparation, Sybyl 8.1 was used (Tripos Inc., St. Louis, Missouri, 63144, USA) and the

image was produced with the PyMOL software (The PyMOL Molecular Graphics System, V.1.3 Schrödinger, LLC).

Electrophoretic mobility shift assay (EMSA)

Wild-type and mutant NKX2-1 proteins were in vitro translated from the pCMX expression vector using the TNT reticulocyte system (Promega, Southampton, UK). Synthetic oligonucleotide probes corresponding to the 3' NKX2-1 binding site on the thyroglobulin promoter were labelled with [³²P]dCTP by Kleenow polymerase and EMSA were performed as described previously.^{41,42} 5 µg of in vitro translated human wild-type NKX2-1 or mutant proteins (NKX2-1 p.Ser169X, NKX2-1 p. Gln182X, NKX2-1 p.Val205Phe, NKX2-1 p.Lys265LysfsX87, NKX2-1 p.Thr203Arg) were incubated with 1 µg BSA in the binding buffer (10 mmol/L TrisHCl pH 7.5, 1 mmol/L EDTA, 4% Ficoll 1 mmol/L dithiothreitol and 2 mmol/L PMSF). After incubation for 20 min at 30°C, 100 fmol of the end-radiolabeled oligonucleotide was added to the reaction and incubated for additional 45 min at 30°C. The oligonucleotide 5'-CACTGCCAGTCAAGTGTCTTGA-3' contains the rat NKX2-1 binding site, which is located at nucleotides -72 to -66 relative to the transcription start site of the thyroglobin gene (NCBI accession no. X06162).⁴³⁻⁴⁵ Electrophoresis was performed in a 5% native polyacrylamide gel in 0.5× TBE buffer pH 8.3 with 34 mA at 4°C for approximately 2.5 h. Protein complexes were visualised by autoradiography.

Mini gene reporter assay and cell transfection

The entire genomic region of NKX2-1 (Isoform 2 NM_003317.3) of a wild-type control DNA and two patients' (26 and 27) DNA was cloned into the eukaryotic expression vector pCS2+. CHO-K1 cells (10⁶/10 cm dish) were transfected with 5 µg plasmid DNA using metafectene (Biontex, Munich, Germany) according to the manufacturer's protocol. Three days after transfection, cells were harvested, total RNA was extracted using TRIzol (Sigma, Deisenhausen, Germany) and mRNA was isolated with the oligo-Tex mRNA kit (Qiagen, Hilden, Germany). Reverse transcription was performed using oligo-dT primer and M-MLV Reverse Transcriptase (Promega, Mannheim, Germany). Subsequent PCR was performed, and PCR products were directly sequenced.

Customised array CGH analysis

Sixty individuals negative for *NKX2-1* point mutations were selected for DNA copy number analysis by means of high-resolution array CGH employing a custom 44k oligonucleotide array (Agilent, Santa Clara, California, USA) representing genomic region chr14: 32055352-41215621 (HG18). As the 5' breakpoint was excluded in the identified deletion in case 5, results were verified by array CGH on a 244k oligonucleotide array from Agilent following the manufacturer's instructions (protocol no. G4410-90010). Labelling and hybridisation were performed according to the manufacturer's recommendations (protocol no. G4410-90010). Arrays were scanned with the G2565BA Agilent Microarray Scanner System (resolution 5 µm; PMT 100% for Cy3/Cy5, respectively) (Agilent Inc. Santa Clara, California, USA). Image processing was performed using Feature Extraction Software v9.1, and data were further analysed in CGH Analytics Software V.3.4.27 (Agilent) using the default settings. All genomic coordinates given in this paper refer to NCBI Build 36 (HG18). Confirmatory experiments were performed with breakpoint spanning PCR and direct

sequencing of amplicons. The sequence variant was analysed in family members if DNA specimens were available.

In situ hybridization

For in situ hybridisation, E11.5 NMRI wild-type embryos were used. Mice were mated, and noon of the day of the vaginal plug was considered E0.5. Embryos were dissected in ice-cold 1× PBS, fixed in 4% PFA, dehydrated and embedded in paraffin. 6 µm tissue sections were prepared on a microtome, mounted on silanized slides and dried at 37°C. For hybridisation, probes were labelled with digoxigenin (DIG). The *Nkx2-1* probe contained nucleotides c.494-992 (NM_009385.2), the *Mbip* probe c.574-145 (NM_145442.2), matching the probe used in Ref. ⁴⁶ Probes were amplified from cDNA by PCR, cloned into the pCRII-vector (Invitrogen, Carlsbad, USA), linearised and transcribed with T7 or Sp6 polymerase. The in situ hybridisation protocol was kindly provided by the Research Group Mundlos (Max Planck Institute for Molecular Genetics, Berlin, Germany) and has been adapted from Ref. ⁴⁷. Briefly, tissue sections were dewaxed, rehydrated, digested with Proteinase K and acetylated. Hybridisation with DIG-labelled probes was carried out at 68°C. Following posthybridisation washes, sections were incubated with an AP-conjugated anti-DIG-antibody (Roche Applied Science, Mannheim, Germany) and signal was detected with NBT/BCIP (Roche Applied Science, Mannheim, Germany).

Detailed phenotyping

For detailed phenotyping of mutation-positive individuals, a total of 28 patients (17 male, 11 female, age 1.5–44 years/18 index patients, 8 relatives) was reanalysed (ethical approval no. EA2-089-06). A questionnaire containing 187 items was used for obtaining clinical data and information on patient history. Video documentation of the patient's movement disorder was carried out according to standardised protocols, followed by independent classification by two experienced neurologists (using the Burke–Fahn–Marsden Dystonia Rating Scale).

For graphical representation of phenotype data, a heatmap was generated using 'R'⁴⁸ (<http://www.R-project.org/>) and 'ggplot2' (<http://ggplot2.org/>).

RESULTS

NKX2-1 intragenic mutations

Sequencing of 101 patients with a phenotype suggestive for a dysfunction of *NKX2-1* revealed 17 intragenic mutations in 17 patients, of which 15 are novel, whereas the remaining two have already been described in unrelated individuals.⁸ All mutations with respect to their location in the protein are depicted in figure 1A. As listed in more detail in table 1, we have identified six frameshift mutations due to insertion/deletion mutations (indels), one insertion and one deletion–insertion resulting in premature stop codons or elongated protein sequences, seven nonsense and one missense mutation. Furthermore, one compound-heterozygous missense mutation was identified, of which one allelic variant [c.703G>T] occurred de novo, whereas the other [c.698C>G] could also be identified in two siblings and the mother. We carried out EMSA to elucidate the effect of the two missense mutations on NKX2-1 DNA binding capacity in comparison to two nonsense-elongation and

one frameshift-elongation mutations. These experiments verified loss of DNA binding capacity for the three nonsense and frameshift mutations as well as for variant Val235Phe, whereas p. Thr233Arg appeared to have a DNA binding capacity equivalent to the wild-type protein (figure 1B). So far, all missense mutations within the homeobox domain were shown to interfere with DNA binding. We developed a homology structure model to delineate the binding capacity of p. Thr233Arg in more detail. The homeobox is constituted of three helices, which are connected by loops. The model demonstrates that the helix 3 is most important for DNA binding, whereby the exact adjustment between the helices 1 and 3 relative to each other is of general significance for NKX2-1 functions. In consequence, mutations at any amino acid in these helices either interfere with the correct fold of the box (intramolecular interactions) or they modulate intermolecular interactions. The wild-type positions of previously described mutations were introduced in the model as well as the side chains and potential orientations of two mutations described in this study—at positions 203 (p.-Thr203Arg) and 205 (p.Val205Phe) (figure 1C).

In addition, we performed mini gene reporter assays for the two intronic mutations that potentially affect splice site mutations (nos. 26 and 27). Both mutations result in splice variants that are each truncating the functional domains in exon 3 by a frame shift (figure 1D).

NKX2-1 CNV

DNA of 60 patients negative for *NKX2-1* point mutations was further investigated for the presence of copy number changes in the *NKX2-1* genomic region by high-resolution array CGH. By using a 44k customised oligonucleotide array, we identified 10 heterozygous deletions affecting the chromosomal region 14q13 in 10 patients. All 10 CNVs are differing in size and were verified by breakpoint spanning PCR and sequencing (table 1, figure 2B and online supplementary figure S2). The largest deletion is spanning 15.17 Mb (no. 6, chr14: 31766412-46945775) and comprises 45 RefSeq genes, including *NKX2-1*. The *NKX2-1* gene comprises two exon and one intron covering 3306 bp. Incomplete loss of *NKX2-1* has exclusively been observed in no. 4, where the deletion was limited to exon 1 and the distal *NKX2-1* promoter. Figure 2A displays the extent of all CNVs in their genomic context, according to the UCSC Genome Browser (HG18).

CNV affecting *MBIP*

Two CNVs, among them the smallest aberration encompassing 76.67 kb (no. 10, chr14: 35802212-35878882), did not include *NKX2-1*, but led to a heterozygous loss of the 5'-neighbouring gene *MBIP*, which encodes for the MAP3K12 binding inhibitory protein (figure 3A, table 1). Deletion no. 9 occurred de novo in a patient with chorea and mild hypothyroidism (3B.1) while deletion no. 10, which exclusively affects *MBIP*, was transmitted in a large kindred with chorea of dominant inheritance (figure 3B.2). We performed *Mbip* in situ hybridisation of early mice embryos and detected an identical pattern of expression in comparison to *NKX2-1*, including the basal ganglia, forebrain, thyroid and lung (figure 3C). We sequenced the coding region of *MBIP* in 74 patients negative for *NKX2-1* mutation, but did not detect any alteration (data not shown).

Phenotype spectrum in index patients

Altogether, we have identified 25 new cases of *NKX2-1* mutations and two additional patients with small deletions close to *NKX2-1* affecting the adjacent *MBIP* gene. Among them, 11 patients displayed the complete triad of movement, thyroid and lung symptoms, 13 patients the dual combination of movement defects and thyroid dysfunction, and 3 patients showed isolated movement disorder (figure 4A). Phenotype of cases with *NKX2-1* mutation did not differ from the two cases of *MBIP* mutations, as the latter were both affected by chorea associated with mild thyroid dysfunction in one of them. In all patients, neurological impairment was present, characterised by benign choreoathetosis preceded by muscular hypotonia in early infancy. Within the group of mutation-negative cases (figure 4B), 15 patients were affected by the triad of movement, thyroid and pulmonary symptoms, putatively caused either by variants in the *NKX2-1* enhancer and/or promoter region or by mutations in another yet unknown candidate gene.

Overall, the phenotypic spectrum of all 32 index mutation-positive patients that we identified in this report as well in our initial publication from 2002 (see online supplementary figure S3A) is dominated by the occurrence of a movement disorder in all patients. However, accompanying thyroid dysfunction was the most consistent combination of symptoms, followed by the triad of thyroid, lung and movement affection, whereas only three patients showed an isolated movement disorder. Moreover, comparison of the index cases who were initially examined for their presumably more severe phenotype with other affected family members who represent thereby an unbiased cohort of mutation-positive patients revealed an identical spectrum of symptom association (see online supplementary figure S3).

Extended phenotyping

In order to define a more detailed phenotype of *NKX2-1* deficiency in a larger cohort of patients, we performed a clinical study in 28 mutation-positive patients (7 deletions/21 point mutations) by recollecting all available clinical and history data as well as video documentation of individual movement statuses (figure 5A,B).

To date, a wide range of neurological descriptions has been used for the movement disorders in the available reports on *NKX2-1* deficiency, which is namely 'choreoathetosis' in the majority of cases. We aimed to gain an objective neurological classification through independent judgement. In 17 of 22 videos, movement disorder was classified as chorea, athetosis was stated in 9, ataxia in 7 and dystonia in 6. As either chorea or athetosis was present in all examined patients, our data are suggesting that choreoathetosis is in fact the most frequent form of movement disorder in *NKX2-1* deficiency.

In addition to the gross motor disorder, 23 patients were affected by dysarthria, while speech comprehension was normal in all. An attentiveness disorder was mentioned in 22 patients. Mental development was quantified by IQ testing in 12 patients, showing low normal levels in most cases (exact values were available in three individuals with 80, 116 and 119 IQ points, respectively (K-ABC test and CPM-test)). EEG recording was available in 16 patients, with 3 to have suspicion of focal lesions, while no seizures were reported in any of

them. MRI of the CNS was available in nine patients, with no major pathological findings (Rathkes pouches n=2 and hypoplastic pallidum n=1).

In 23 patients, thyroid defects were present as CH (n=11), hyperthyreotropinemia (n=12) and thyroid dysgenesis (n=14, among them athyreosis n=2, hemithyroid n=2, thyroid hypoplasia n=10).

Careful case history revealed a high incidence of lung affection, that is, in 22 of the 28 patients. In 13 patients, a respiratory distress syndrome with need for oxygen treatment during the neonatal period was reported. Fifteen patients were affected by recurrent pneumonias requiring antibiotic treatment (incidence ranging from 3 per annum to 12 per annum) in the first 6 years of life. In one patient, the pneumonic episodes persisted beyond that age, prior to diagnosis of a lung sequester. Spontaneous pneumothoraces (four relapses) occurred in another patient.

According to the murine phenotype in knockout animals with severe hypothalamic defects, we extended our exploration to various other symptoms, herein focusing on hypothalamic function. In 19 patients, bouts of fever episodes without evidence for infectious causes were recorded; 11 patients had increased appetite with reduced satiety, although BMI values were normal in all patients; in 14 patients, thirst was abnormally diminished, and in 10 patients a disturbed rhythm of sleep was reported.

Notably, patients' height standard deviation score (SDS) values revealed a trend for lower body length (median value -0.84 SDS, see figure 5A), and two patients even received growth hormone therapy (SDS values -3.89 and -2.08 , respectively).

In seven individuals, a congenital heart defect (ventricular or atrial septum defect, respectively) was present, of which none needed surgical intervention and three patients were seen with oligodontia. Furthermore, six pregnancies had breech presentation at birth, hypothetically giving evidence for intrauterine hypokinesia due to muscular hypotonia.

DISCUSSION

Despite the key role of *NKX2-1* in thyroid development,⁴⁹ several comprehensive screening approaches failed to detect *NKX2-1* mutations in patients with CH.⁵⁰⁻⁵² Only when the presumptive *NKX2-1* deficiency phenotype had been extended so that it also took into account the impact of *NKX2-1* on development and function of other organs such as lung and brain, the first mutations were identified.⁸⁹

Core phenotype associated with *NKX2-1* deficiency

Neurological impairment, mainly in the form of choreoathetosis, was present in all index patients identified in this study. This is in accordance with all reports in literature so far, apart from those neonatal cases with severe lung affection, who died before the onset of neurological symptoms.²⁰²⁸ The triad of symptoms (including defects of thyroid and lung) was significantly enriched in mutation-positive individuals, although non-neurological features were less frequent and their presence varied to a much higher extent. In line with literature, our analysis revealed a high rate of thyroid defects (75%) with mild TSH elevation

in the majority of patients. We detected a higher frequency of pulmonary disorder (78%) than previously reported, with neonatal respiratory distress (46%) and recurrent pneumonia with need for antibiotic treatment (53%) as the prevailing features. The reasons for the organ-specific differences in response to *NKX2-1* deficiency remain elusive, but some facts suggest that the lower tolerance towards *NKX2-1* deregulation in brain might be connected to the postnatal function of *NKX2-1* in this organ. *NKX2-1* is expressed in brain throughout life, especially in cholinergic and GABAergic neurons of the basal ganglia, both in mice and human.^{53–55} Moreover, structural brain defects have not been described in *NKX2-1* mutation-positive patients so far, and they are oligosymptomatic in the first years of their life. Importantly, *NKX2-1*-deficient patients in this cohort were predominantly not affected by epilepsy or severe mental retardation. This was unexpected given the phenotype of *Nkx2-1*-deficient mice with respect to cortical interneurons⁵ and recent bioinformatic analysis predicting *NKX2-1* as a candidate gene for epilepsy in humans.⁵⁶

New symptoms associated with *NKX2-1* deficiency: abnormal height, cardiac septum defects and asthma

Our comprehensive phenotypic characterisation revealed novel *NKX2-1*-associated hypothalamic symptoms, including frequent occurrence of fever without infection, dysrhythmic sleep and a trend towards lower body height SDS values. Altogether, this suggests an influence of *NKX2-1* on the hypothalamus–pituitary–growth axis in accordance with the severe disturbance of the hypothalamic function in the knockout mice.⁴ Furthermore, we confirmed the occurrence of attention deficit disorder as described by Gras and colleagues.¹⁵

Atrial and ventricular septum defects (VSD, ASD) were present in 7 out of 28 index patients with *NKX2-1* mutations. Congenital heart defects are a known comorbidity of thyroid malformations, with ASD as the most frequent manifestation.⁵⁷ Thus—at first sight—the cardiac findings observed in this study may reflect that proper heart development depends on normal thyroid hormone activity, for example, the postnatal surge of T3 levels, which normally triggers the switch from cardiac myosin heavy chain isoform MYH7 to MYH6.⁵⁸ Mutations in *MYH6* have already been identified in familial cases of atrial septum defects⁵⁹). However, cardiac defect frequency observed in *NKX2-1* mutation-positive patients of this study is much higher than the reported prevalence in patients with thyroid malfunction without genotype-based stratification (7/28 vs 13.8/1000^{57,60}). Moreover, one patient included in this study presented with congenital heart defect in the absence of any thyroidal defect. Both observations point at a direct role of *NKX2-1* in heart development and the aetiology of cardiac septum defects, which is independent of thyroid hormone activity. This idea is supported by the fact that mutations in *NKX2-5* have already been identified as a cause of ASD, and that both members of the NKX family share several direct and indirect downstream targets, including key regulators of heart development such as GATA4, GATA6 and HOP.^{61–63}

There is accumulating evidence for a role of *NKX2-1* in the development and progression of leukaemia, thyroid and bronchial carcinoma.^{64–69} Furthermore, an association of germline mutations and the development of neoplasiae, for example, multinodular goitre and papillary

thyroid carcinoma,⁶⁰ has been discussed. This may impact the use of congenital *NKX2-1* mutations as a prognostic marker in future. In principle, the identification of congenital *NKX2-1* mutations is a favourable result as it predicts that disease will hardly progress and it holds out the prospect that symptoms will improve at later stages of life.⁷⁰⁷¹ This positive aspect may be tarnished in future by uncertainties about an increased cancer risk that might be associated with congenital *NKX2-1* mutations. Unfortunately, the young average age of patients in this study did not allow any conclusive assessment of cancer risk associated with congenital *NKX2-1* mutations. However, one patient died of bronchial carcinoma at the age of 46 without having any history of smoking.

Quality of mutations and their correlation to phenotype

In line with previous studies, all intragenic point mutations identified in this study differ in position and quality. Thus, the exclusive clustering of missense mutations within the DNA binding homeodomain is unlikely to indicate a mutational hot spot, but rather reflects a lower tolerance towards single amino acid exchanges within the homeodomain compared with the surrounding transactivation domains. There, only nonsense or frame-shift mutations in the N-terminal part, and potentially also in the C-terminal part,³⁷ can compromise protein functionality.

We have performed accurate phenotyping in index patients and their affected relatives. This enabled us to monitor the symptoms caused by the very same *NKX2-1* mutation in different individuals. In general, intrafamilial phenotypic variability was smaller than the variability between unrelated patients carrying different mutations (figure 5B). Neurological symptoms turned out to be the most consistent feature within families, while the intrafamilial spectrum of thyroid and lung symptoms could range from no impairment at all to severe hypothyroidism with agenesis of the gland and severe neonatal respiratory distress, respectively.

Our family studies also highlighted the possible consequences of *NKX2-1* mutations not altering its ability to bind to DNA. So far all mutations located within the homeodomain have shown loss of function due to interference with DNA binding.^{8131521–252728} Here we report the first variant within the homeodomain (p.Thr203Arg) that does not affect DNA binding capacity. The mutation was present on the maternal allele of an index patient, who also carried a second missense mutation (p.Arg205Phe) mapping to the homeodomain encoded on his paternal allele. As can be inferred from the structural homology model, which depicts the intercalation of homeodomain helices and DNA binding groove (figure 1C), the p.Thr203Arg variant neither seems to play a role in the stabilisation of the helix conformation nor to interact directly with DNA. In line with this assumption, and in contrast to the p.-Arg205Phe mutation on the paternal allele, experimental validation by EMSA showed no deterioration of DNA binding capacity associated with the p.Thr203Arg variant.

Only the index patient who carried both mutations presented with the characteristic phenotype that is typically associated with *NKX2-1* deficiency, including neurological symptoms. His three relatives, who solely carried the mutation that had no effect on DNA binding (p.Thr203Arg), lacked most of these symptoms, but not all. Congenital heart defect was diagnosed in one of the three relatives, asthma or recurrent bronchial infection was

present in all of them. This observation can be interpreted in two ways: (i) only the second mutation (p.Arg205Phe) in the index patient is causative, while ASD and pulmonary problems in his relatives are not related to mutations in *NKX2-1*; or (ii) the mild pulmonary and cardiac symptoms are caused by impaired functionality of NKX2-1 that is not mediated by its DNA binding capacity. Although the observed haploinsufficiency of NKX2-1 argue in favour of the first hypothesis, there is also evidence in support of the second one. Minoo and colleagues recently discovered that the homeodomain of NKX2-1 physically interacts with FOXA1 in a DNA-independent manner in order to control the expression of surfactant protein C (SP-C) and Clara cell protein (Ccsp).⁷² Both SpC and Ccsp are essential for proper function of the lung, and their deregulation has already been implicated in the aetiology of asthma and recurrent pulmonary infections.^{73,74} In light of these facts, it is tempting to speculate that the characteristic *NKX2-1* phenotype and its variability are not only attributed to impaired DNA binding, but are also a reflection of altered protein–protein interaction characteristics of NKX2-1, or more precisely, one of its isoforms. Thus, preservation of DNA binding capacity of mutated NKX2-1 alone may not be an eligible argument to rule out disease association. This may especially apply to those rare cases that lack neurological symptoms, including the recently described missense mutation p.Phe198Leu, where, unfortunately, its functional implications have not been investigated.⁷⁵ Noteworthy, HOP, one of the shared downstream targets of NKX2-1 and NKX2-5 (see above), is a homeodomain protein that lacks any DNA binding potential.⁷⁶

CNVs outside of *NKX2-1* result in a phenotype similar to that of intragenic mutations

Given the impact of DNA CNVs on the aetiology of congenital disorders, we have complemented our sequence analysis by testing patients negative for *NKX2-1* point mutations by means of array CGH employing a high-resolution custom array. We have identified 10 novel heterozygous deletions. In line with previous reports,^{14,16} they all differed in size and position. This makes it unlikely that recurrence of *NKX2-1* deletions is the consequence of a specific genomic architecture that renders this region particularly prone to rearrangements. Our data also corroborate the reported lack of correlation between CNV size, which ranges from 76 kb to 15.17 Mb in our study, and severity of disease.¹⁴

Strikingly, two of the deletions did not encompass *NKX2-1* at all. Similar observations have been made recently and have fuelled speculations that removal of regulatory elements downstream of *NKX2-1* might produce a phenotype similar to intragenic loss.^{39,77} At least two prospective enhancer elements related to forebrain and thyroid function seem to exist with or in close proximity to the genomic interval encompassed by the two deletions identified in this study.^{78,79} For one of these conserved sequences, a forebrain expression has already been shown (http://enhancer.lbl.gov/cgi-bin/imagedb3.pl?form=presentation&show=1&experiment_id=1538&organism_id=1) and the other one has been highlighted as potential enhancer by a recent meta-analysis of GWAS data related to thyroid function and TSH levels.⁷⁹ Unfortunately, re-evaluation of public data on higher-order chromatin organisation and CTCF binding, a protein involved in DNA looping, did not reveal any particular chromosome conformation that conclusively indicates any connections of *NKX2-1* with a specific regulatory element in this region^{80–82} (see online supplementary figure S5). Nevertheless, there seems to exist selective pressure to keep *NKX2-1* in next

proximity to the interval deleted in the two patients as this region, together with *NKX2-1*, is part of one of the largest blocks of synteny in humans⁸³ and also represents the core region of the recurrent amplicon reported in thyroid and bronchial carcinomas.^{64–66} However, synteny and co-amplification could also signal the presence of genes, whose co-regulation with *NKX2-1* is essential for proper development and function of brain, thyroid and lung. Proceeding on this assumption, distortion of the fine-tuned stoichiometry of their products could mimic an *NKX2-1* deficiency like phenotype. Two genes recently discussed in the context of CNVs outside *NKX2-1* are *NPAS3* and *RALGAP1*.⁷⁷ Malfunction of these two genes can lead to respiratory distress and cognitive disorders, respectively. Yet, both genes are not mapping within the commonly deleted region of the two cases from our study. The only gene mapping to this interval is *MBIP* (MAP3K12 binding inhibitory protein 1), which encodes a component of the ATAC (Ada Two-A containing) complex. This histone acetyltransferase complex is supposed to translate extracellular cues into chromatin modifications and consequently altered gene expression.⁸⁴ Interrogation of protein interaction databases by means of String9.05⁸⁵ and literature mining did not reveal apparent commonalities between *MBIP* and *NKX2-1*, apart from the fact that both proteins are—directly or indirectly—involved in the regulation of protein acetylation. In order to elucidate more information on possible functional connections between the two proteins, we have evaluated expression patterns of *Mbip* in mouse embryos at embryonic day 11.5. In situ hybridisation revealed that the *Mbip* expression pattern spatially and temporally coincides with that of *Nkx2-1*, for example, in the thyroid anlage, lung and forebrain (figure 3C). This fits to the recent description of *Mbip* expression in progenitor cells of the murine lateral ganglionic eminence,⁴⁶ which is also known as the major expression domain of *Nkx2-1* in the forebrain at the same stage of embryonic development.^{4 53} This coincidence renders deregulation of *MBIP* a plausible cause of a *NKX2-1* deficiency like phenotype and has prompted us to sequence *MBIP* in those 74 patients of our cohort, who were negative for *NKX2-1* mutations. In contrast to what would have been expected under the assumption of a causative role of *MBIP* for this phenotype, no single mutation was identified in this gene.

In summary, our study expands the phenotypic spectrum recurrently associated with *NKX2-1* deficiency, with some of the symptoms maybe not exclusively linked to alterations of DNA binding capacity of this transcription factor. In our view, the high incidence of *NKX2-1* mutations justifies the regular molecular screening in corresponding patients in order to distinguish them from others with resembling phenotypes at early stages of disease, but considerable different outcomes. However, these diagnostic efforts should not be confined to the gene itself, but should also comprise the analysis of regulatory elements and interaction partners of *NKX2-1*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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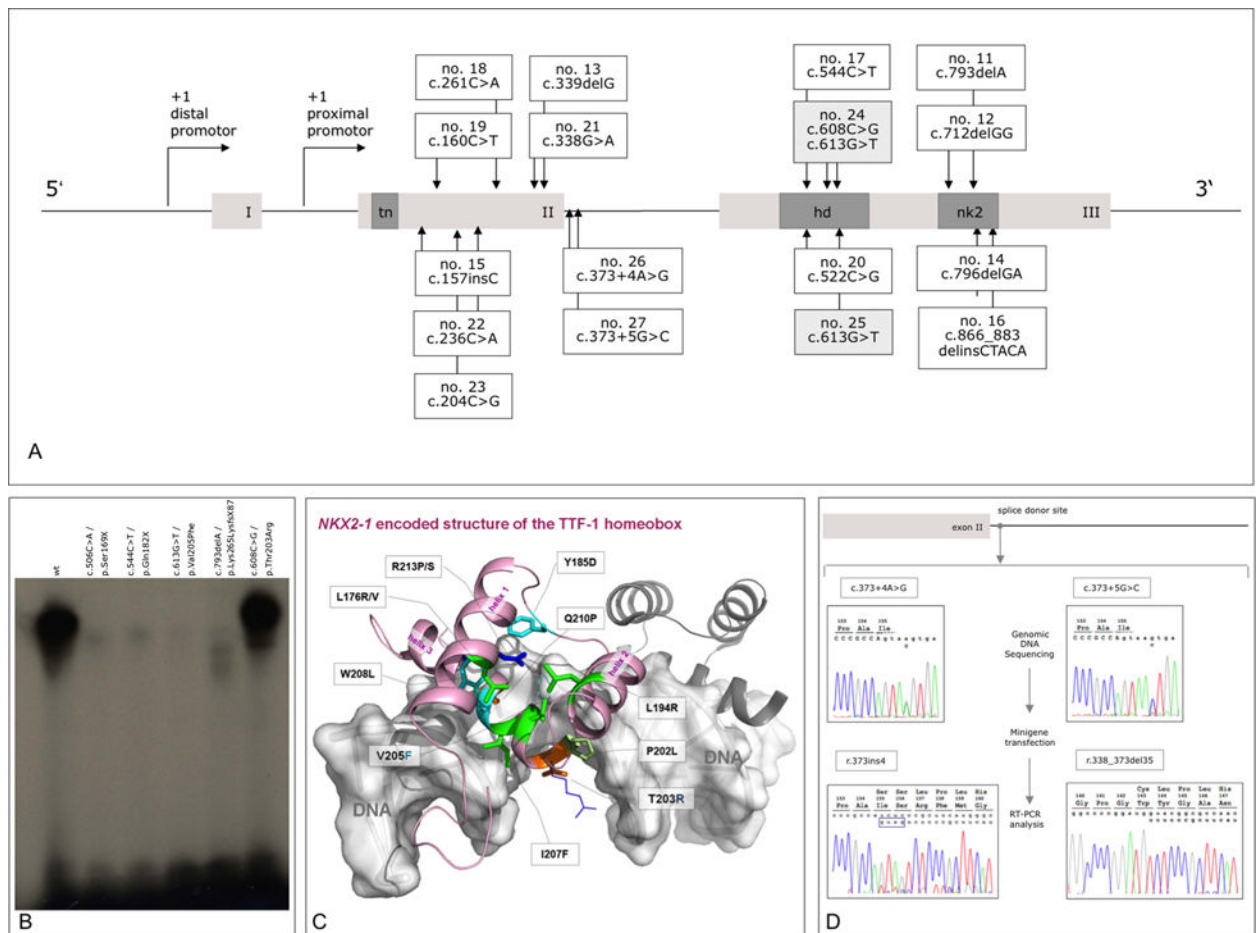
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**Figure 1.**

Position and functional consequences of *NKX2-1* mutations. (A) Distribution of 17 novel point mutations with respect to the functional domains of *NKX2-1* isoform 2 (REFSEQ NM_003317.3). Distal and proximal promoters⁸⁶⁸⁷ are indicated by arrows; functional domains are shown in dark grey (tn, tin domain; hd, homeodomain; nk2, NK2 domain). (B) DNA binding characteristics of mutant *NKX2-1*. Electromobility shift assay testing wild-type and mutant *NKX2-1* binding to a labelled probe corresponding to the *NKX2-1* binding site of the *TPO* promoter. wt (lane 1); c.506C>A/p.Ser169X (⁸ lane 2); c.544C>T/p.Gln182X (no. 17, lane 3); c.613G>T/p.Val205Phe (nos. 24/1 and 25, lane 4); c.793delA/p.Lys265LysfsX87 (no. 11, lane 5); c.608C>G/p.Thr203Arg (no. 24/2, lane 6); see text for details. (C) Structural homology model. Visualisation of the putative mode of binding between a human *NKX2-1* homeobox (light-magenta backbone) and a fragment of DNA (white, translucent surface). The wild-type positions of known naturally occurring mutations are shown as sticks (colour code: green, hydrophobic side chain properties; cyan, aromatic; orange, hydrophilic; blue, positively charged; light green, proline). The side chains and potential orientations of two mutations described in this study—at positions 203 (p. T203R) and 205 (p. V205F)—are additionally visualised as lines. (D) Splicing defects caused by intronic point mutations. Mutant nos. 26 and 27 were analysed by mini gene reporter assays. Sequencing chromatograms of genomic DNA (c.373+4A>G/c.373+5G>C variants) and

RNA after CHO cell transfection and reverse transcription (r.373ins4/r.338_373del35) are depicted in the upper and lower two boxes, respectively.

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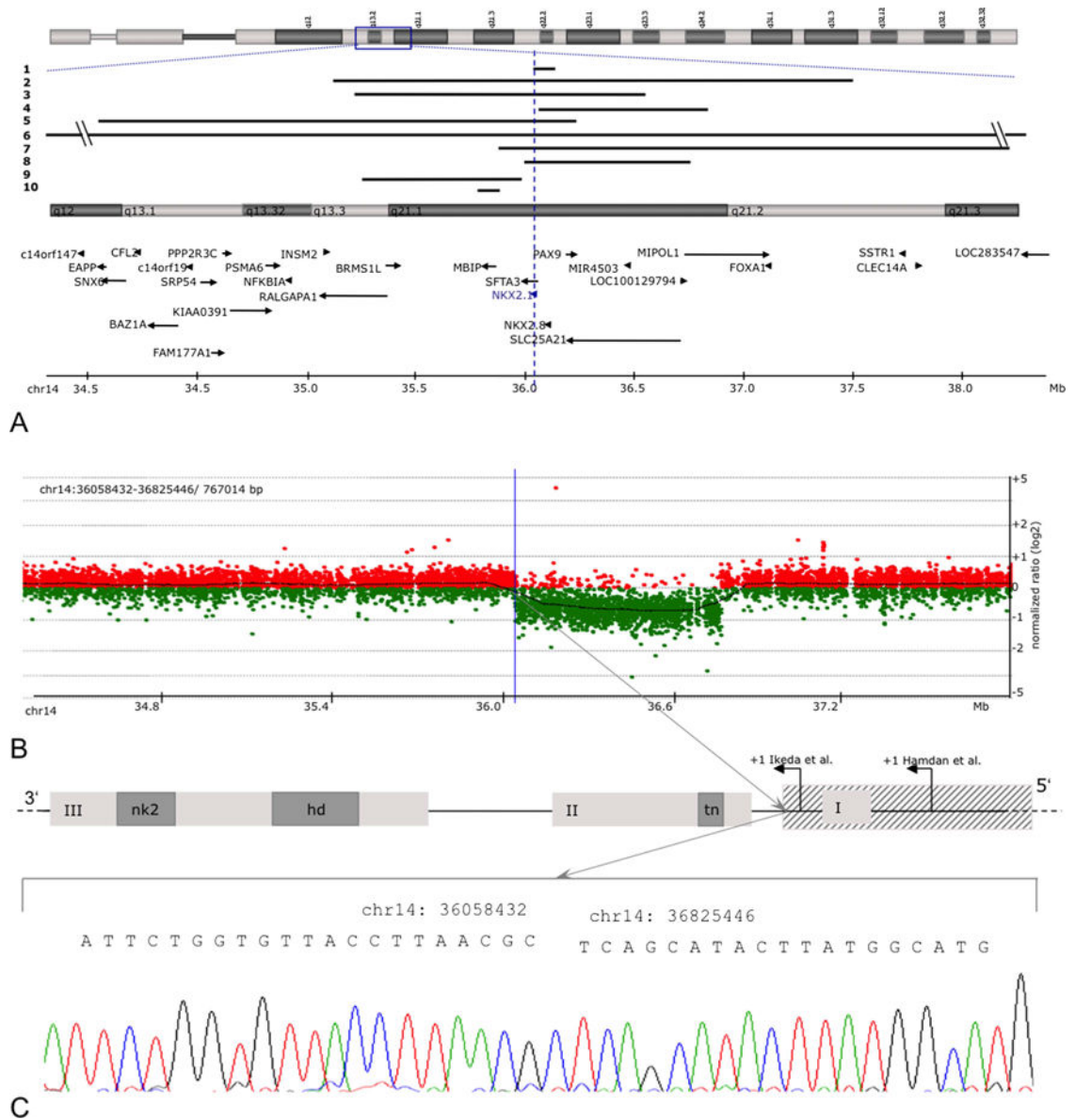
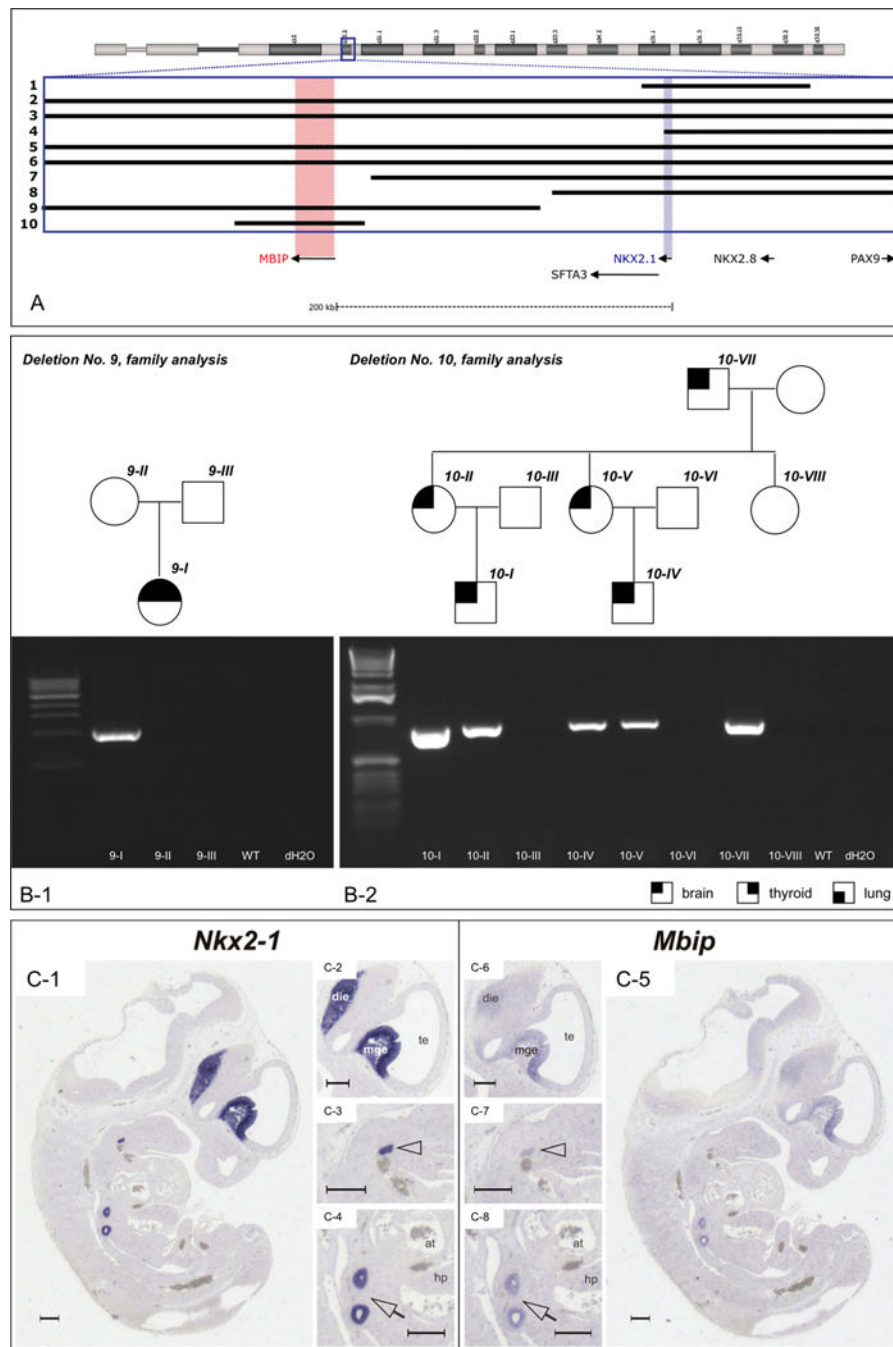


Figure 2. Disease-associated copy number variants (CNVs). (A) Localisation of all CNVs reported in this study. The vertical line highlights the position of *NKX2-1* on chromosome 14q13.3. (B) Array CGH profile of deletion no. 4 encompassing chr14: 35989750-36754641 (array CGH results of all other cases are summarised in online supplementary figure 2). (C) CNV verification by breakpoint spanning PCR and amplicon sequencing on the example of the proximal border of deletion no. 4 (hatched box).

**Figure 3.**

Copy number variants (CNVs) not including *NKX2-1*. (A) Close-up of chr14: 35,739,446-36,221,024 (Hg18). The positions of *NKX2-1* and *MBIP* are highlighted in blue and red, respectively. Note that CNVs nos. 9 and 10 do not affect *NKX2-1*. (B) Breakpoint spanning PCR verifying the deletion in index patients nos. 9 and 10 and their relatives; see legend at the bottom for phenotype symbols. All genomic coordinates are given for Hg18. (C) *Nkx2-1* and *Mbip* co-expression in mouse embryonic tissues. *Nkx2-1* and *Mbip* in situ hybridisations on adjoining sagittal sections of an E11.5 wild-type embryo. *Nkx2-1* and

Mbip have common expression domains in the diencephalon, the median ganglionic eminence, the lung anlage and the thyroid primordium. C1–C4, *Nkx2.1*; C5–C8, *Mbip* in situ hybridisation. Panels C2–C4 and C6–C8 represent enlargements of C1 and C5, respectively. Arrowheads, thyroid primordium; arrows, lung anlage; die, diencephalon; mge, median ganglionic eminence; te, telencephalic vesicle; at, atrium; hp, hepatic primordium. Scale bars represent 250 μm .

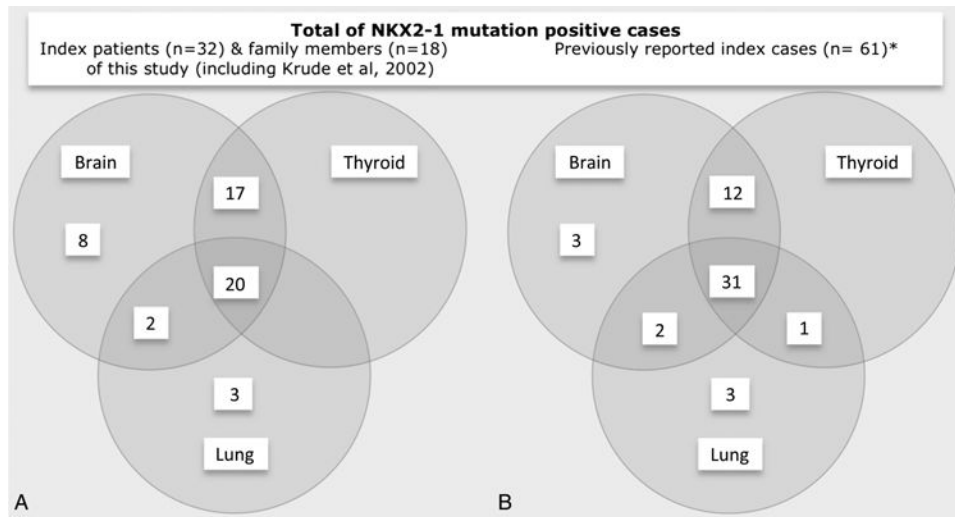


Figure 4.

Brain, thyroid and lung involvement in patients with and without NKX2-1 mutations. Venn diagrams demonstrating that the triad of symptoms is enriched in mutation-positive patients. (A) Symptoms in patients with *NKX2-1* deficiency (27/101). (B) Symptoms in patients without *NKX2-1* deficiency (74/101). Two further Venn diagrams, one focusing on the phenotype of 18 family members and another one including also the 5 index patients of our previous study,⁸ are given in online supplementary figure S3. Two further Venn diagrams demonstrating that symptom involvement is identical in index cases (n=32, including also the five index patients of our previous study⁸ to that of family members (n=18), except three patients with isolated asthma phenotype) are given in online supplementary figure S3.

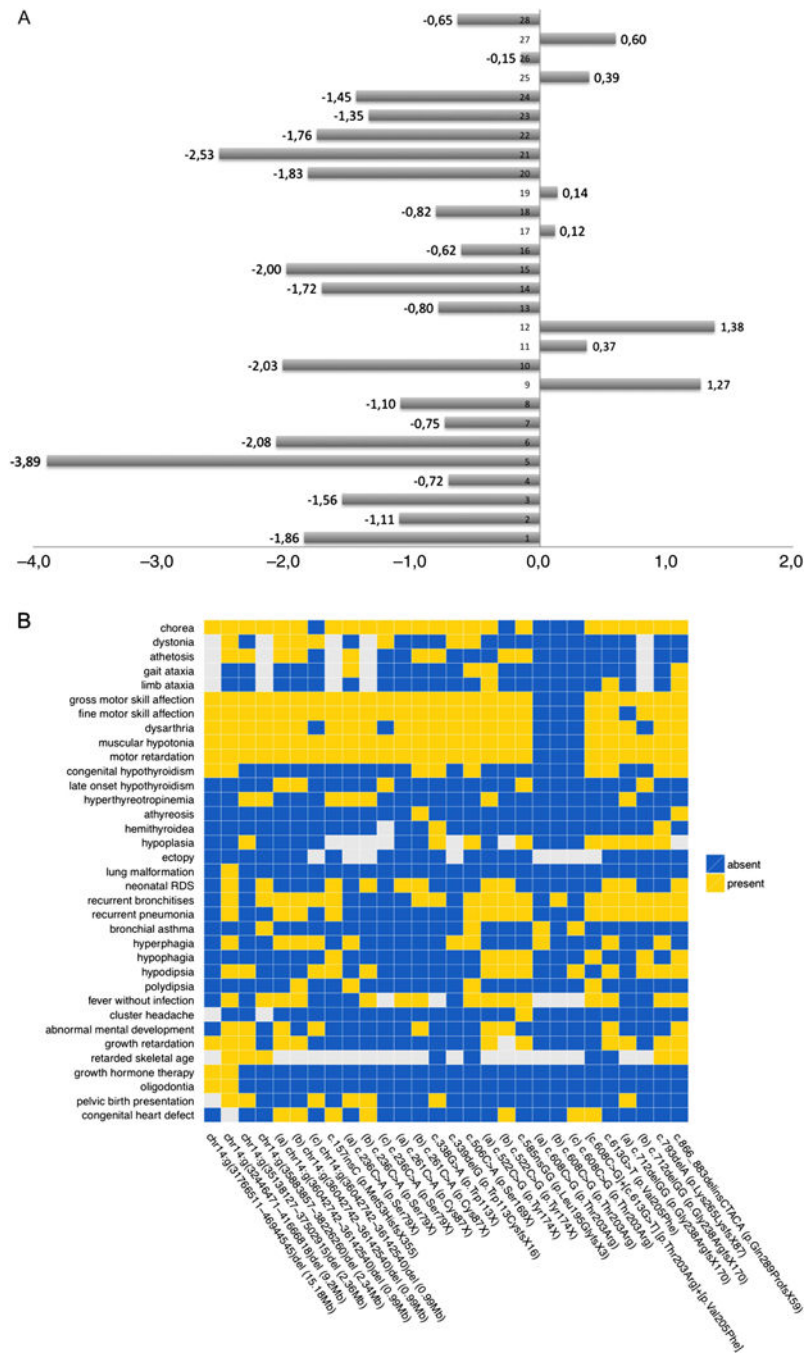


Figure 5. Additional symptoms in patients with *NKX2-1* mutations. (A) Body height standard deviation score (SDS) values of detailed phenotype cohort. Median value -0.84 SDS; patients 1–18 carrying *NKX2-1* mutations: 1, chr14:g (36042742_36142540)del; 2, chr14:g (36042742_36142540)del; 3, chr14:g (36042742_36142540)del; 4, chr14:g (35138127_37502915)del; 5, chr14:g (32446471_41666818)del; 6, chr14:g (31766511_46944545)del; 7, chr14:g (35883857_38226260)del; 8, c.522C>G_p.Tyr174X; 9, c.522C>G_p. Tyr174X; 10, c.585nsGG_p. Leu195GlyfsX3; 11, c.613G>T_p. Val205Phe;

12, [c.608C>G] +[c.613G>T]_[p.Thr203Arg]+[p. Val205Phe]; 13, c.608C>G_p. Val205Phe; 14, c.608C>G_p. Val205Phe; 15, c.608C>G_p. Val205Phe; 16, c.338G>A_p.Trp113X; 17, c.261C>A_p.Cys87X; 18, c.261C>A_p.Cys87X; 19, c.506C>A_p. Ser169X; 20, c.157insC_p. Met53HisfsX355; 21, c.236C>A_p. Ser79X; 22, c.236C>A_p.Ser79X; 23, c.236C>A_p.Ser79X; 24, c.866_883delinsCTACA_p. Gln289ProfsX59; 25, c.793delA_p. Lys265LysfsX87; 26, c.712delGG_p. Gly238ArgfsX170; 27, c.712delGG_p. Gly238ArgfsX170; 28, c.339delG_p. Trp113CysfsX16. (B) Heatmap summarising the results of detailed phenotyping in 18 index patients and 10 relatives with *NKX2-1* deficiency. The presence and absence of symptoms is indicated by yellow and blue boxes, respectively. Missing values are shown in grey. The first seven columns to the left depict the phenotypes of the patients affected by a copy number variant. Point mutations are sorted according to their genomic position. The letters (a), (b) and (c) heading the description of the mutation indicate family members sharing the same mutation.

Table 1

Overview of identified NKX2-1 deletions and intragenic mutations

No.	DNA sequence	Size	Protein localisation	Protein level	Inheritance		Phenotype				Sex
					Affected family members*	Thyroid dysfunction	Choreoathetosis/muscular hypotonia	Pulmonary malfunction			
1	chr14:g (36042742_36142540)del	99 798 bp	Entire protein	p.0	AD	M 2	Yes	Yes	Yes	M	
2	chr14:g (35138127_37502915)del	2 364 788 bp	Entire protein	p.0	AD	M 1	Yes	Yes	No	M	
3	chr14:g (35221080_36553158)del	1 332 078 bp	Entire protein	p.0	n/a	n/a	No	Yes	No	F	
4	chr14:g (36058432_36825446)del	767 014 bp	Entire protein	p.0	n/a	n/a	Yes	Yes	No	M	
5	chr14:g (34071433_36246414)del	2 174 981 bp	Entire protein	p.0	n/a	n/a	Yes	Yes	No	F	
6	chr14:g (31766511_46944545)del	15 178 034 bp	Entire protein	p.0	n/a	n/a	Yes	Yes	No	M	
7	chr14:g (35883857_38226260)del	2 342 403 bp	Entire protein	p.0	AD	F 2	Yes	Yes	Yes	M	
8	chr14:g (35989750_36754641)del	764 891 bp	Entire protein	p.0	De novo	Nil	Yes	Yes	Yes	F	
9	chr14:g (35257445_35257464 + 35257487_35984627)del	727 181 bp	Del excluding NKX2-1	p.?	De novo	Nil	Yes	Yes	No	F	
10	chr14:g (35801941_35878529)del	76 588 bp	Del excluding NKX2-1	p.?	AD	M 2/F 2	No	Yes	No	M	
11	c.793delA		NK2	p.Lys265LysfsX87	De novo	Nil	Yes	Yes	No	M	
12	c.712delGG		NK2	p.Gly238ArgfsX170	AD	F 2	Yes	Yes	Yes	M	
13	c.339delG		TN-HD	p.Tpp113CysfsX16	De novo	Nil	No	Yes	No	M	
14	c.796delGA		NK2	p.Asp266Argfs142X	De novo	Nil	Yes	Yes	No	F	
15	c.157insC		TN-HD	p.Met53HisfsX355	De novo	Nil	Yes	Yes	Yes	F	
16	c.866_883delinsCTACA		NK2-X	p.Gln289ProfsX59	De novo	Nil	Yes	Yes	Yes	M	
17	c.544C>T		HD	p.Gln182X	De novo	Nil	Yes	Yes	Yes	F	
18	c.261C>A		TN-HD	p.Cys87X	AD	F 2	Yes	Yes	Yes	M	
19	c.160C>T		TN-HD	p.Gln55X	Father n/a; mother WT	n/a	Yes	Yes	No	F	
20	c.522C>G		HD	p.Tyr174X	n/a	n/a	Yes	Yes	Yes	M	

No.	DNA sequence	Size	Protein localisation	Protein level	Inheritance			Phenotype			
					Affected family members*	Thyroid dysfunction	Choreoathetosis/muscular hypotonia	Pulmonary malfunction	Sex		
21	c.338G>A		TN-HD	p.Trp113X	n/a	n/a	Yes	Yes	No	M	
22	c.236C>A		TN-HD	p.Ser79X	AD	F 2	Yes	Yes	No	F	
23	c.204C>G		TN-HD	p.Tyr68X	AD	F 1	Yes	Yes	Yes	M	
24	[c.608C>G]+[c.613G>T]		HD	[p.Thr203Arg]+[p.Val205Phe]	AD/de	F 3	Yes	Yes	No	M	
25	c.613G>T		HD	p.Val205Phe	De novo	Nil	Yes	Yes	No	F	
26	c.373+4A>G/r.373ins4		TN-HD (splice donor site)	p.Ile155SerfsX126	De novo	Nil	Yes	Yes	Yes	M	
27	c.373+5G>C/r.338_373del35		TN-HD (splice donor site)	p.Trp143CysfsX138	n/a	n/a	Yes	Yes	No	M	
28	<i>chr14:g(32446471_41666818)del</i>	9.2 Mbp	<i>Entire protein</i>	<i>p.0</i>	<i>De novo</i>	<i>Nil</i>	Yes	Yes	Yes	<i>M</i>	
29	c.585msGG		<i>HD</i>	<i>p.Leu195GlyfsX3</i>	<i>De novo</i>	<i>Nil</i>	Yes	Yes	Yes	<i>M</i>	
30	c.506C>A		<i>HD</i>	<i>p.Ser169X</i>	<i>n/a</i>	<i>n/a</i>	Yes	Yes	<i>No</i>	<i>M</i>	
31	c.261C>A		<i>TN-HD</i>	<i>p.Cys87X</i>	<i>AD</i>	<i>F 1</i>	Yes	Yes	Yes	<i>M</i>	
32	c.613G>T		<i>HD</i>	<i>p.Val205Phe</i>	<i>De novo</i>	<i>Nil</i>	Yes	Yes	Yes	<i>M</i>	

Nomenclature for mutations according to recommendations of the Human Genome Variation Society (<http://www.hgvs.org/mutnomen/>); reference sequence REFSEQ Isoform 2 NM_003317.3; P, entire protein; HD, homeodomain; NK2, NK2-specific domain; AD, autosomal dominant; n/a, information not available; TN-HD, sequence between tin- and homeodomain; M, male; F, female;

* excluding index patient; mutations 29–32 as reported previously by authors.⁸

The italicised values refer to cases that have not been identified in the present genotyping study, but earlier in the Krude *et al* 2002 study⁸.