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REVIEW ARTICLE

NK2 homeobox gene cluster: Functions and roles in human diseases

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Abstract NK2 genes (*NKX2* gene cluster in humans) encode for homeodomain-containing transcription factors that are conserved along the phylogeny. According to the most detailed classifications, vertebrate *NKX2* genes are classified into two distinct families, *NK2.1* and *NK2.2*. The former is constituted by *NKX2-1* and *NKX2-4* genes, which are homologous to the *Drosophila scro* gene; the latter includes *NKX2-2* and *NKX2-8* genes, which are homologous to the *Drosophila vnd* gene. Conservation of these genes is not only related to molecular structure and expression, but also to biological functions. In *Drosophila* and vertebrates, NK2 genes share roles in the development of ventral regions of the central nervous system. In vertebrates, *NKX2* genes have a relevant role in the development of several other organs such as the thyroid, lung, and pancreas. Loss-of-function mutations in *NKX2-1* and *NKX2-2* are the monogenic cause of the brain-lung-thyroid syndrome and neonatal diabetes, respectively. Alterations in *NKX2-4* and *NKX2-8* genes may play a role in multifactorial diseases, autism spectrum disorder, and neural tube defects, respectively. *NKX2-1*, *NKX2-2*, and *NKX2-8* are expressed in various cancer types as either oncogenes or tumor suppressor genes. Several data indicate that evaluation of their expression in tumors has diagnostic and/or prognostic value.

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Introduction

Homeobox-containing genes are defined by the presence of a conserved 180 bp sequence called homeobox, whose name derives from its presence within several homeotic genes in *Drosophila* melanogaster.[1] Homeobox-containing genes encode transcription factors that have a pivotal role during animal development.[2,3] Homeobox sequences encode for the homeodomain (HD) which is the DNA-binding domain of these transcription factors. The HD is extremely conserved along the phylogeny and is constituted by three α-helices folding together to generate a relatively rigid structure, in which major sequence-specific DNA contact is established by the third (recognition) helix located on the DNA major groove.[4–7]

In humans, more than 200 homeobox-containing genes have been identified[8] and, for most of them, a specific role during development has been delineated.[9] Mutations in several homeobox-containing genes are either causative of monogenic diseases or risk factors for polygenic ones.[10,11] Based on sequence conservation, homeobox-containing genes are grouped into different classes, subclasses, and families.[8] Referring to the work by Holland and colleagues,[8] NK2 genes are part of the Antennapedia (antp) class and include two distinct families, *NK2.1* and *NK2.2*. The former is constituted by *NKX2-1* and *NKX2-4* genes, which are homologous to the *Drosophila scro* gene; the latter includes *NKX2-2* and *NKX2-8* genes, which are homologous to the *Drosophila vnd* gene. *Drosophila*-related genes are much bigger than human genes, mostly for differential intron length. The homeobox coding sequence is contained within a single exon in all genes, except for *scro*. Together with the NK4 family members, NK2.1/2 proteins interact with DNA by contacting sequences having a 5'-CAAT-3' core.[6,12] In addition to the HD, NK2.1/2 proteins share two other domains, the tinman (TN) domain, located near the amino-terminal, and the NK2-specific domain (SD), located at the C-terminal.[13–15] Similarities between NK2 proteins are shown in Figure 1. Both the HD and the SD are extremely conserved in *scro* and *vnd* homologous proteins. The TN domain is highly conserved among *scro*-homologous proteins but less among *vnd*-homologous ones.

Drosophila scro and *vnd* genes are located in different chromosomes. Instead, chromosomal co-localization and genomic proximity are present for human *NKX2-1* and *NKX2-8* on chromosome 14 and *NKX2-2* and *NKX2-4* on chromosome 20. This peculiar localization into clusters suggests that the members of each cluster share the same regulation pattern. Indeed, *NKX2-1* and *NKX2-8* are co-expressed in the thyroid gland while *NKX2-2* and *NKX2-4* are co-expressed in the hypothalamus and the pituitary gland. By using the TADKB tool (http://dna.cs.miami.edu/TADKB/browse.php), it is evident that each couple is part of the same topological associating domain (TAD) in different cell lines.

Alterations in the NKX2 family are related to distinct human diseases. Here, after delineating the major biological functions of these genes, their roles in human genetic disorders are reviewed.

Expression and biological functions of *NK2.1* and *NK2.2* genes

NK2 gene expression and its functions in different species indicate remarkable conservation along the phylogeny. In particular, the identity of the ventral regions of the central nervous system (CNS), from *Drosophila* to humans, appears to be regulated by these genes. As stated in the introduction, compared to *Drosophila*, NK2 genes are duplicated in vertebrates.[16,17] Gene duplication is a major driver of molecular evolution, being responsible for the origin of new genes.[18] Since functional divergence among duplicated genes is required for their maintenance within the genome, "neo-functionalization" occurs when one of the duplicated copies acquires new functions.[19] Alternatively, "sub-functionalization" occurs when ancestral functions of the progenitor gene are partitioned between duplicated copies.[20] Both mechanisms have been likely involved in NK gene evolution. Indeed, a model including neo- and sub-functionalization together has been proposed.[21]

To understand the aforementioned concept, the peculiar expressions and functions of *Drosophila scro* and *vnd* will be initially summarized, and then the description of human orthologues will be discussed.

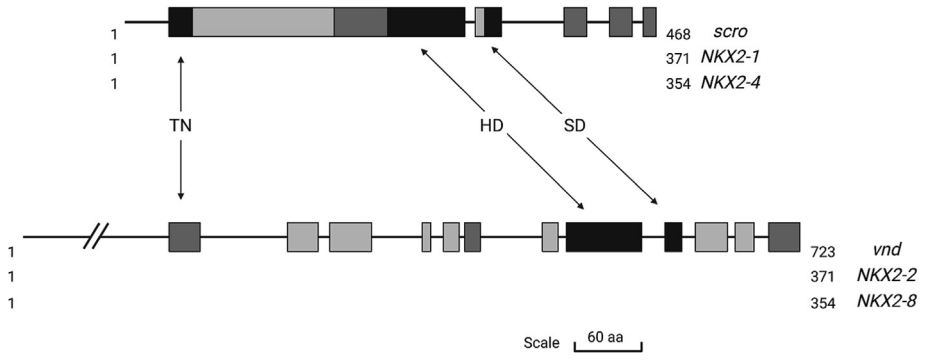

Figure 1 Structural similarity among NK2.1 and NK2.2 proteins. Schematic representation of the similarity among scro/NKX2-1/NKX2-4 and vnd/NKX2-2/NKX2-8 proteins. Colors represent the percentage of sequence homology: light gray boxes represent sequences with homology <20%; dark gray boxes represent sequences with homology between 20% and 80%; black boxes represent sequences with homology >80%. Black lines represent fragments of *Drosophila* proteins not present in human NK proteins. Numbers represent protein length. HD: homeodomain; SD: NK2-specific domain; TN: tinman domain. Created with Biorender.com.

Drosophila melanogaster

Scro

During *Drosophila* embryogenesis, *scro* is expressed in the pharyngeal primordia and later maintained in the pharynx. This gene is also expressed in bilateral clusters of procephalic neuroblasts and segmental clusters of neuronal precursors in the ventral nerve cord. Then, *scro* expression is detected in the larval and adult optic lobes.[22] Homozygous loss-of-function mutations of *scro* are lethal during both embryonic and early larval development. Deformed neuroepithelial cells in the developing optic lobe and severely malformed adult optic lobes are present in mutants,[23] indicating that *scro* plays a role in the development and/or function of this tissue. Indeed, one of the *scro* targets is the pigment-dispersing factor (PDF) encoding gene, which is expressed in ventral lateral neurons (LNvs), acting as a central pacemaker for circadian locomotor activity rhythms. *Scro* acts as a repressor, binding to a cis-acting element of the *pdf* gene.[24]

Vnd

During *Drosophila* development, *vnd* is expressed in the ventral column of neuroectoderm and neuroblasts and is maintained in a subset of neurons derived from these neuroblasts.[25–27] Loss of *vnd* transforms the ventral column into the intermediate column identity, and ectopic *vnd* transforms their identity the other way around. Therefore, this gene is critical in specifying the dorsoventral axis during *Drosophila* CNS development.[27] The major known molecular function of *vnd* is transcriptional repression. By performing misexpression experiments in *Drosophila* larva, it has been shown that vnd represses *ind* and *msh* gene expression and that the addition of *vnd* binding sites to a heterologous enhancer is sufficient to mediate repression. Moreover, the authors show that transcriptional repression occurs by interaction with Groucho protein in the TN domain.[28]

Homo sapiens

Human orthologues retain some features of *Drosophila* genes but acquire new and tissue-specific ones. Indeed, studies investigating the expression and functions of NK2 genes have been performed using different animal models. In this section, we will refer to the vertebrate homeobox-containing genes with the Homo sapiens-related name, notwithstanding several of the reviewed data that have been obtained in mouse, zebrafish, or other species.

NKX2-1

NKX2-1 (also named *TTF-1*, *TITF-1*, *T-EBP*) is expressed during the development of the thyroid, lungs, and several regions of the ventral forebrain.[29] During thyroid development, *NKX2-1* expression initiates at the very beginning of gland morphogenesis in the ventral endoderm of the primitive pharynx (thyroid anlage) and continues up to adulthood.[30] In addition to the follicular thyroid cells, *NKX2-1* is expressed in the C-cells as well as in parathyroid glands.[31] *NKX2-1* contributes to the expression of several genes related to the differentiation of thyroid follicular cells such as thyroglobulin (*TG*), thyroid peroxidase (*TPO*), and thyrotropin receptor (*TSHR*).[11,32] Indeed, experiments using conditional *NKX2-1* knockout mice suggest that this gene is required for the maintenance of the normal architecture and functions of differentiated thyroids.[33]

As for airway development, NKX2-1 represents the earliest marker of lung fate. It is later confined to the alveolar and bronchial epithelium.[34] Indeed, it is first expressed in the prospective lung domain of the developing foregut endoderm at E9 during mouse embryogenesis. Ikonomou and colleagues demonstrated that Nkx2-1-positive lung epithelial primordial progenitors express Wnt and Tgf-β superfamily pathways shaping their cell-fate determination from pre-specified embryonic foregut.[35] This transcription factor is also essential for the induction of lung branching morphogenesis.[36–38] NKX2-1 appears essential also in the septation of the tracheoesophageal primordium.[38] Kuwahara and colleagues demonstrated by single-cell RNA-sequencing that tracheal commitment is NKX2-1 independent, since Nkx2-1$^{-/-}$ mutant embryos did not experience a transcriptome-wide tracheal-to-esophageal conversion.[39] Some NKX2-1 independent mechanisms contribute to tracheal cartilage specification, as some disorganized cartilage still forms in Nkx2-1$^{-/-}$ mutants. Moreover, they highlighted that NKX2-1 is able to bind the regulatory sequences of *sonic hedgehog* (*Shh*) and *Wnt Family Member 7B* (*Wnt7b*) regulating their expression to control mesenchymal specification to cartilage and smooth muscle, coupling epithelial identity with mesenchymal specification.[39]

In adulthood, elevated *NKX2-1* expression is detected in lung alveolar type II epithelial cells as well as in epithelial cells-based lining bronchi and bronchioles.[40] This transcription factor is essential for the expression of distinct surfactant proteins (A, B, C, D), Clara cell secretory proteins, and others.[41] By using conditional knockout in mouse, Snyder and co-workers have demonstrated that *NKX2-1* deletion induces loss of pulmonary commitment and conversion to gastric lineage; such an effect is mediated by recruitment of transcription factors Foxa1 and Foxa2 to lung-specific promoters, preventing activation of the gastric-specific transcription program.[42]

To date, several different NKX2-1 interactors have been so far identified in lung-related cells including Pax8, GATA6, STAT3, retinoic acid receptor (RAR) and associated co-factors, nuclear factor-I (NFI—B), AP1 family members, and BR22.[43–49] Moreover, in the murine respiratory epithelial cell within the lungs, NKX2-1 interacts with the surfactant protein C promoter together with the TAZ protein.[50] By using immortalized lung epithelial cells, the interaction between NKX2-1, PARP1, and PARP2 proteins has been shown as well as the enhancement in the activity of the promoter of surfactant protein B gene.[51]

During development, *NKX2-1* is expressed in restricted regions of the brain within the diencephalon, in some regions of the hypothalamus and the neurohypophysis, and in the telencephalon.[34] In preoptic and hypothalamic areas, *NKX2-1* is expressed in the ventral region of the ventricular neuroepithelium. Though this gene is not expressed in Rathke's pouch (derived from the oral epithelium and primordium of the anterior and the intermediate parts of the pituitary), *NKX2-1* null mice lack completely the pituitary

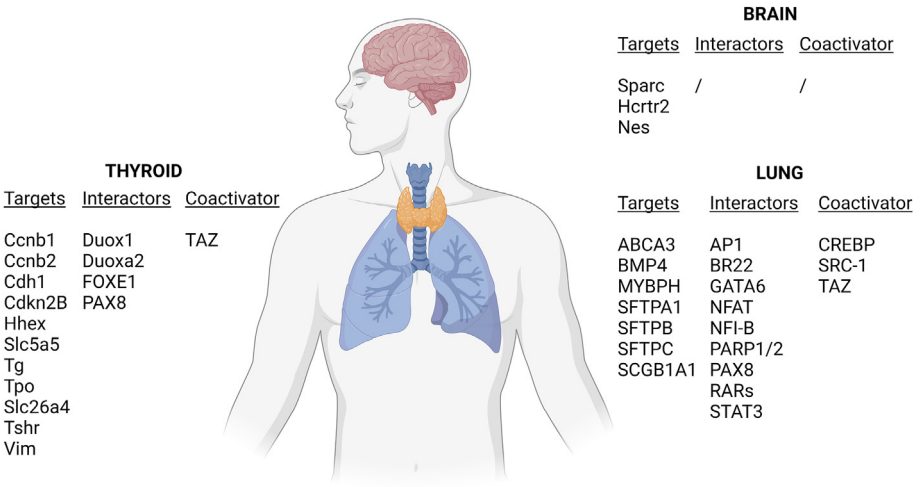

Figure 2 NKX2-1 transcriptional web. Representation of NKX2-1 targets, interactors, and co-activators in the brain, lungs, and thyroid. Created with Biorender.com.

gland. Indeed, this indicates that *NKX2-1* expression in the floor of the diencephalon and in the infundibulum is required for a fully development of the anterior and intermediary pituitary from Rathke's pouch.[37] It has been shown that *NKX2-1* is expressed in hypothalamic proopiomelanocortin (POMC) neurons from early development to adulthood. Being essential for *POMC* expression, it is considered a member of the multi-component system contributing to body weight regulation.[52] Moreover, by controlling angiotensinogen expression in the subfornical organ, NKX2-1 contributes to the regulation of body fluids homeostasis.[53] In the cortex, NKX2-1 has been shown to contribute to the interneuron subtype specification.[54] NKX2-1 interactors, targets, and coactivators in the brain, lungs, and thyroid are summarized in Figure 2.

NKX2-4

NKX2-4 results from the duplication of *NKX2-1*.[16,55] *NKX2-4* is indeed expressed in a restricted area of the hypothalamus. Differently from *NKX2-1*, *NKX2-4* is not expressed in the telencephalon. Like *NKX2-1*, instead, *NKX2-4* is expressed in the most ventral region of the hypothalamic area.[55] Thus, in this region, NKX2-1 and NKX2-4 appear to have redundant functions. In fact, experiments in zebrafish highlighted that the combined morpholino gene silencing, but not a single one, is required to obtain a significant modification of hypothalamus development.[56] By genetic experiments in zebrafish, it has been shown that, in several hypothalamic neurons, NKX2-4 (as well as NKX2-1) controls *bsx*, a gene encoding a homeodomain-containing protein, which plays a critical role in the differentiation of multiple neuromodulatory cell types in the forebrain.[57] NKX2-4 has been detected in the thyroid gland only in the rainbow trout. Experiments in HeLa cells highlighted that NKX2-4 is able to transactivate the thyroglobulin promoter, even if to a lesser extent compared to NKX2-1.[58]

NKX2-2

During development, *NKX2-2* is expressed in the CNS, pancreas, and intestine, in which regulates the cell fate of several cell lineages.[59–61] In the developing pancreas, endocrine progenitors show the *NKX2-2* highest expression.[62] In adulthood, NKX2-2 pancreatic expression is detected in the alpha, the beta, and the pancreatic polypeptide (PP) cells but not in delta ones.[61] *NKX2-2* null mice die shortly after birth developing severe hyperglycemia, thus indicating that this transcription factor is essential for the final differentiation of pancreatic beta cells.[61,63] The insulin encoding gene has been identified as a direct target of NKX2-2.[64] Using rat cells, the interaction between NKX2-2 and calmodulin-binding transcription activator 1 (Camta1) has been demonstrated. Indeed, this interaction controls the promoter activity of the miR-212/miR-132 cluster, whose overexpression increases insulin secretion.[65] During both intestinal development and adulthood, *NKX2-2* is expressed in epithelial enteroendocrine cells located in the villus and crypt. These cell types are thus able to give rise to all intestinal epithelial cell types, thus having stem cell properties during normal intestinal cell renewal.[66]

In the early development of the neural tube, *NKX2-2* is expressed in most ventral progenitors. In particular, this gene is expressed in the ventral regions of the diencephalon, the hypothalamus, and the thalamus, partially overlapping with *NKX2-1*-expressing regions. Indeed, both contribute to the brain morphological differentiation.[13] In the ventral neural tube, the identity specification of neurons is dependent on the phased activity of the signaling protein Shh. By analyzing neurogenesis in the ventral spinal cord and hindbrain, it has been shown that *NKX2-2* plays a pivotal role in ventral neuronal patterning; in these regions, it triggers neuronal identity by interpreting Shh phased signals.[59] By using cells derived from primary cultures of rat oligodendrocyte precursors, Wei and co-workers have found that *NKX2-2* represses the expression of myelin basic protein by interacting with two in-cis regulatory elements.[67] They also showed that the transcription factor Sp1 reversed the repressive effect of *NKX2.2* by competing with NKX2-2 for its binding sites.

NKX2-8

NKX2-8 (named *Nkx2.9* in mouse) has been identified through the binding of its protein product to the regulatory

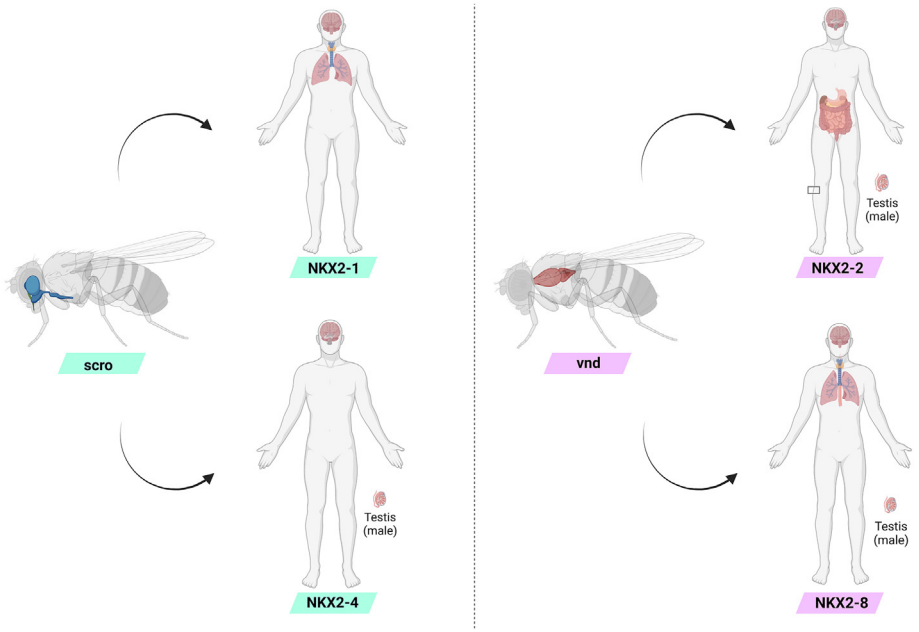

Figure 3 Tissue expression of *Drosophila* scro and vdn together with their human orthologues. Left panel: *scro* is expressed in *Drosophila*'s central nervous system and pharynx, while its human orthologues are expressed in the brain, lung, and thyroid (NKX2-1) and brain and testis (NKX2-4), respectively. Right panel: *vnd* is expressed in *Drosophila*'s ventral nerve cord, while its human orthologues are expressed in the brain, skin, gastrointestinal system, and testis (NKX2-2), and brain, lungs, thyroid, esophagus, and testis (NKX2-8), respectively. Created with Biorender.com.

elements of the α-fetoprotein gene.[68] However, it has been shown that *NKX2-8* expression is not detectable in the liver at any developmental stage as well as in adulthood, with the absence of morphological anomalies in this organ.[69] In contrast, *NKX2-8* is expressed in developing thyroid, pharynx, and lung. In the latter, *NKX2-8* is detected in tracheobronchial stem cells, in which it downregulates the proliferation activity. In fact, when this gene is inactivated, abnormal expansion of this cell type is observed, inducing bronchial dysplasia.[69] It has been proposed that in developing lungs *NKX2-8* downregulates *NKX2-1* gene and antagonizes the transcriptional activity of the latter on its target genes.[69] As *NKX2-1* and *NKX2-2*, *NKX2-8* is expressed in the ventral region of the developing neural tube. In particular, its expression is restricted to V3 interneurons of the spinal cord and visceral motoneurons in the hindbrain. However, because of functional redundancy with *NKX2-2*, inactivation of *NKX2-8* has no effects on spinal cord physiology. Instead, its inactivation in the hindbrain affects the development of branchial motoneurons, always determining morphological alteration of the accessory nerve (11th) and, only in some embryos, the abnormalities of the glossopharyngeal (9th) and vagal (10th) nerves.[70] Thus, though overlapping expression of the two genes, NKX2-2 is not able to fully substitute NKX2-8 for motoneuron differentiation of the ventral hindbrain. Chromatin immunoprecipitation (ChIP) experiments suggest that NKX2-8 binds to and regulates genes having a role in apoptosis and fatty acid oxidation, including *CPT1A* and *CPT2* genes; moreover, demonstrated that NKX2-8 can interact with Sin3A/HDAC1/SAP18 complex binding with their promoters.[71] By performing cell line transfection experiments, a synergic effect between NKX2-8 and serum response factor on a minimal cardiac alpha-actin promoter has been demonstrated.[72]

A summary of the tissue expression of *Drosophila scro* and *vnd* together with human NK2.1/2 family members is provided in Figure 3. Moreover, the differential localization of human NK2.1/2 genes expression during development and adulthood is summarized in Table 1.

Table 1 Differential localization of human NK2 genes expression during development and adulthood.

NKX2 gene	Major Tissues Expression during development	Major Tissues Expression during adulthood
NKX2-1	lung, tracheoesophageal primordium, thyroid, diencephalon, hypothalamus, neurohypophysis, telencephalon, hypothalamus	lung, thyroid, hypothalamus, pituitary gland
NKX2-2	pancreas, intestine, diencephalon, hypothalamus, thalamus	pancreas, intestine, hypothalamus, pituitary gland
NKX2-4	telencephalon, hypothalamus	hypothalamus, pituitary gland
NKX2-8	thyroid, pharynx, lung, spinal cord, hindbrain	lung, thyroid, esophagus, hypothalamus, pituitary gland

NKX2 genes in human diseases

NKX2-1

NKX2-1 heterozygous alterations have been identified and associated with a syndromic form initially named brain-lung-thyroid syndrome due to the broad phenotypic spectrum including a variable combination of lungs, thyroid, and neurological defects. Recently, since the varied manifestations of this syndrome, the disorders have been referred to as NKX2-1-related disorders.[73] The main characteristics of this disease are represented by benign hereditary chorea, respiratory distress syndrome, and congenital hypothyroidism (Fig. 4). Indeed, the hallmark of brain-lung-thyroid syndrome (BLT) is childhood-onset chorea, commonly beginning in early infancy (most commonly around the first year of life) or, less frequently, in late childhood or adolescence. Progression occurs up to the second decade and then remains static. Lung disease is the second most common manifestation varying from respiratory distress syndrome in newborns to interstitial lung disease in young children. In the elders, pulmonary fibrosis may occur. Hypothyroidism is usually mild, often subclinical, due to various forms of thyroid abnormalities including hypoplasia, haemiagenesis, or athyreosis.[32,74]

NKX2-1 alterations exhibit autosomal dominant inheritance with variable expressivity and penetrance but frequently occur *de novo*. So far, about 100 alterations involving the *NKX2-1* gene have been reported, the majority of which are point mutations.[75] Notwithstanding, whole gene deletions and deletions proximal to NKX2-1 have been found in subjects with BLT syndrome, suggesting the presence of an upstream regulatory region.[76]

The reported mechanism of disease is haploinsufficiency.[77] Indeed, truncating mutations removing, for example, the nuclear localization signal produces a protein that is unable to translocate into the nucleus, while missense variant impaired NXK2-1 binding to its targets, thus corroborating dosage imbalance.[78] To date, no genotype—phenotype correlations have been observed, as the manifestations of NKX2-1-related disorders vary among individuals with the same pathogenic variant even within the same family.[78] The severity of the phenotype is not consistent with NKX2-1 mutation type (missense or nonsense), deletion size, or location within functional domains (homeobox and transcription regulation). Larger contiguous gene deletions have been associated with a more severe spectrum of the brain—lung—thyroid triad (often with additional clinical characteristics), while point nonsense mutations, affecting the terminal regions of the protein, are reported in those with a milder clinical phenotype.[73,74,79] However, this putative genotype—phenotype association is not seen in all cases, as reported by a colleague reporting whole gene deletions triggering a milder disease.[80] All these data underpin a high degree of variability, suggesting that there may be other contributory environmental and/or epigenetic factors.

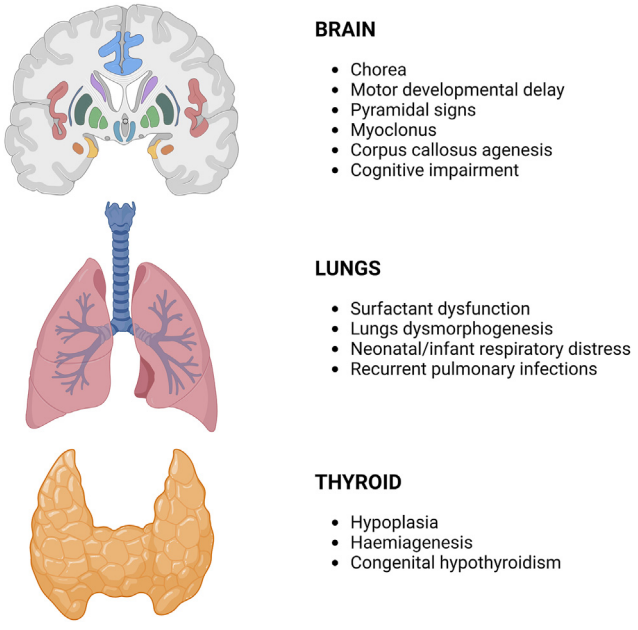

Figure 4 Main characteristics of the brain-lung-thyroid syndrome (BLTS). Illustration summarizes the main features of BLTS, sorted by organs involved. Created with Biorender.com.

NKX2-1 plays also a role in neoplastic disease, especially in thyroid and lung cancer. In thyroid follicular cells-derived neoplasia, *NKX2-1* is highly expressed in differentiated carcinomas (both papillary and follicular subtypes) but not in the undifferentiated (i.e., anaplastic) ones.[81,82] Anaplastic carcinoma often arises in the context of differentiated thyroid carcinoma and it has been recently shown that *NKX2-1* expression is lost during the transition from differentiated to anaplastic carcinoma.[83,84] Moreover, *NKX2-1* silencing in the non-tumorigenic FRTL5 rat cell line reduces proliferation.[85] Thus, it seems that during thyroid tumorigenesis NKX2-1 could have an "oncogenic" or "tumor suppressor" effect depending on cell context, differentiated or not, respectively. In addition to tumors originating from follicular cells, *NKX2-1* is expressed in medullary thyroid carcinomas (originating from parafollicular cells).[86] Data suggesting the NKX2-1 bivalent functions (pro or anti-oncogenic) has been gained also for lung cancer.

About 70% of adenocarcinomas express *NKX2-1* and retain features of the terminal respiratory unit (TRU) to a certain extent.[87—89] Despite its role as a lineage-survival oncogene in lung adenocarcinomas, *NKX2-1* expression is also known to be associated with favorable prognosis in affected patients.[90] It has been demonstrated that NKX2-1 regulated genes involved in lung cytoskeletal and cell—cell organization, negatively affecting cell motility, invasion, and metastasis.[91] On the other hand, a study by Winslow et al. highlighted that *Nkx2-1* downregulation represses Hmga2 expression and acquisition of metastatic ability in a mouse model of lung adenocarcinoma with conditionally activated Kras and loss-of-function p53 mutant alleles.[92]

Furthermore, *NKX2-1* represses TGF-b-induced epithelial-to-mesenchymal transition (EMT) by impairing TGF-b-related induction of Snail and Slug, as well as by reducing TGF-b production.[93] All these data points to the notion that NKX2-1 plays a double-edged role in cancer.[91,94] NKX2-1 is also expressed in small cell lung cancer (SCLC) where it represents a powerful diagnostic tool.[95—97] According to the knowledge that NKX2-1 represses the gastric-specific gene expression (see above), NKX2-1 negative murine lung tumors express gastrointestinal transcriptome.[42]

Finally, rearrangements of *NKX2-1* with T cell receptor or immunoglobulin heavy chain loci were identified in T-cell acute lymphoblastic leukemia[98] and ectopic expression of NKX2-1 has been observed in diffuse large B-cell lymphoma.[99,100]

NKX2-4

To the best of our knowledge, the only disease in which *NKX2-4* appears to be involved is autism spectrum disorder (ASD). Meta-analysis-based studies have shown that variants of *NKX2-4* could represent a significant genetic risk factor for ASD.[101] Regarding to neoplastic diseases, hypermethylation in the *NKX2-4*-containing area has been found in adenoid cystic carcinoma of the salivary gland, although the exact meaning of these data is not fully clear.[102] Aberrant activation of *NKX2-4* has been found in erythroblastic AML determining the deregulation of genes involved in differentiation events and, therefore, contributing to the generation of specific AML subtypes.[103] The partial redundancy between NKX2-1 and NKX2-4 may partially explain the apparently slight effects of *NKX2-4* gene variants in human diseases. Accordingly, in the gnomAD database (gnomad.broadinstitute.org) this gene seems not to be intolerant to loss-of-function mutations.

NKX2-2

In agreement with the role of *NKX2-2* in pancreas development, mutations of this gene are causative of neonatal diabetes. By studying subjects burdened by this disease and born by consanguineous parents, Flanagan and co-workers have found homozygous loss-of-function mutations of *NKX2-2*.[104] These patients also show several features of developmental delay (affecting intellectual, sensory, and motor functions) consistent with the role of *NKX2-2* in the development of several brain regions. In addition to diabetes, *NKX2-2* mutations are associated with obesity. Indeed, an infant with a homozygous *NKX2-2* loss-of-function mutation suffering severe obesity has been described.[105] Interestingly, in this subject, obesity was ascribed to elevated ghrelin levels; in fact, he showed a paradoxical increase of this orexigenic hormone after an oral glucose tolerance test. The absence of *NKX2-2* function could increase ghrelin levels by the fate change of pancreatic islet cell progenitors from insulin-to ghrelin-producing cells.

Ewing sarcoma is a malignant tumor with metastatic potential, affecting the bone and soft tissues of children and young adults. Most cases of Ewing sarcoma show the presence of the recurrent chromosomal translocation t(11; 22) (q24; q12), which encodes the aberrant fusion protein EWS/FLI that acts as a transcription factor able to dysregulate the expression of relevant genes involved in the development of this tumor type.[106,107] By using a sophisticated experimental approach, it has been demonstrated that NKX2-2 is a fundamental factor in the pathogenesis of Ewing sarcoma.[108] In addition to the relevance in the pathogenesis of Ewing sarcoma, the expression of this transcription factor has a relevant role in routine pathological discrimination of round cell sarcomas. In fact, Yoshida and co-workers have found that evaluation of NKX2-2 expression by immunohistochemistry is a marker tool for Ewing sarcoma with a sensitivity of 93% and a specificity of 89%, in the differential diagnosis of small round cell tumors.[109] When combined with the expression of CD99, the specificity is increased to 98%.[110]

Nagel and co-workers found overexpression of *NKX2-2* in Hodgkin lymphoma; they demonstrated that the IL17 receptor gene *IL17*RB activated *NKX2-2* transcription. Downstream analysis indicates that NKX2-2 disturbs B-cell differentiation processes inhibiting transcription of homeobox gene *MSX1* while activating expression of basic helix-loop-helix factor *NEUROD*1.[100]

NKX2-8

The only human non-cancerous diseases with which *NKX2-8* has been so far associated are neural tube defects (NTDs), a group of common congenital defects characterized by failure of neural tube closure.[111] NTDs are multifactorial in their etiology with genetic factors playing a significant role (heritability up to 70%).[112] By analyzing the Weimaraner breed dogs affected by spinal dysraphism, it was shown that these animals harbor a homozygous loss-of-function mutation in *NKX2-8*.[113] In the same study, Safra and co-workers evaluated 149 patients with lumbosacral myelomeningocele for *NKX2-8* mutations and found six subjects heterozygous for the p.S62T and one for the p.A94T missense mutations. By comparing this cohort with a control population from the Exome Variant Server (EVS), the authors found a significant difference although substitutions in these genes are quite frequent in the gnomAD database, suggesting a potential low predisposing effect (if any) on NTDs.

The functional redundancy with other *NKX* genes may explain the lack of data relating to *NKX2-8* and non-cancerous human diseases. Instead, this gene is frequently involved in various cancer types. Coactivation of *NKX2-1* and *NKX2-8* is associated with poor prognosis in non-small-cell lung cancer (NSCLC), and resistance to cisplatin *in vitro*.[114] Indeed, *NKX2-8* seems to foster clinical aggressiveness in this disease. However, such an effect could be context-dependent. In fact, data have been produced indicating NKX2-8 as a potential tumor suppressor in squamous cell tumors which are generally *NKX2-1* negative.[115] Indeed, data from many other neoplasms suggests an oncosuppressor activity for *NKX2-8* gene. In fact, downregulation of this gene determines i) the decrease in survival and angiogenesis promotion in oesophageal cancer,[116] ii) tumor progression and prognosis in hepatic cell carcinoma,[117] iii) the higher recurrence rate and platinum resistance in epithelial ovarian cancer, and iv) the increase in lymph node metastasis and worse prognosis in urothelial carcinoma.[118] Conversely, it has been demonstrated that *NKX2-8* upregulation inhibits epithelial-to-mesenchymal transition and reduces motility as well as invasiveness in cell lines from urothelial carcinoma.[119]

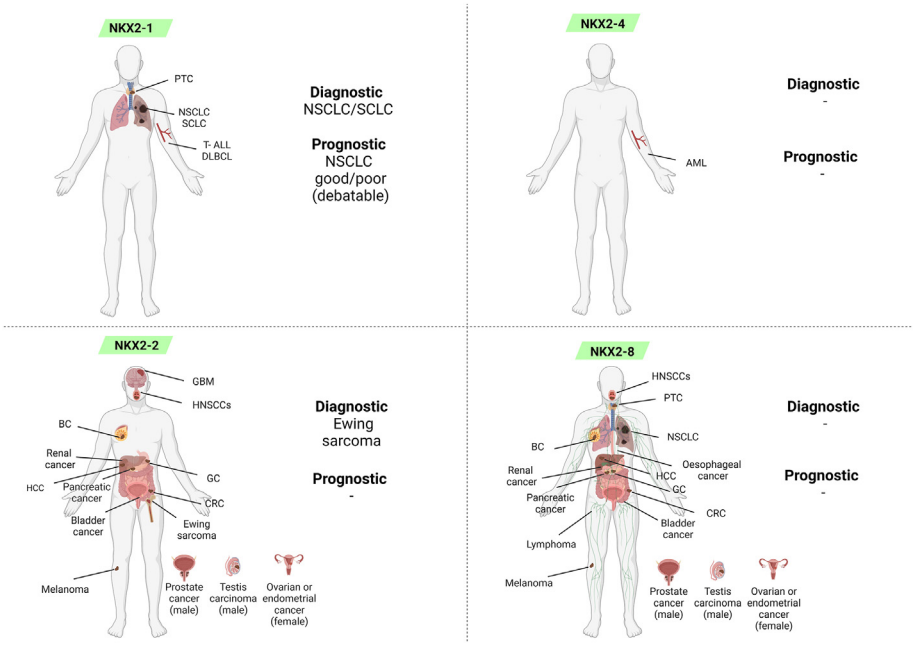

Figure 5 NK2.1/2 expression in cancer. Illustration summarizes NK2.1 and NK2.2 involvement in cancer, together with data regarding diagnostic and/or prognostic potential as cancer markers. AML: acute myeloid leukemia; BC: breast cancer; CRC: colorectal cancer; DLBCL: diffuse large B cell lymphoma; GBM: glioblastoma; GC: gastric cancer; HCC: hepatocellular carcinoma; HNSCCs: head and neck squamous cell carcinomas; NSCLC: non-small cell lung cancer; PTC: papillary thyroid cancer; SCLC: small cell lung cancer; T-ALL: T-cell acute lymphoblastic leukemia. Created with Biorender.com.

Conclusions

The discovery of homeobox-containing genes, which includes the NKX2 family, represents a dramatic leap forward in the understanding of the molecular genetics of embryogenesis. Indeed, NKX2 genes exert essential functions in normal developmental patterning since they regulate tissue maturation in a spatially and temporally defined order. As they regulate a series of vital cellular functions, any alteration (i.e., mutation) dysregulates identifiable tissue-specific pathways.

No matter the similarity of gene sequences, NKX2 genes have been associated with diverse human conditions. *NKX2-1* haploinsufficiency has a cardinal role in the brain-lung-thyroid syndrome; *NKX2-8* is associated with NTDs; *NKX2-2* plays a role in the onset of diabetes and obesity; *NKX2-4* alteration is related to ASD. NK2.1/2 expression in cancer tissues is summarized in Figure 5.

All these data contribute to fostering the idea that NK genes evolved by both neo- and sub-functionalization. However, notwithstanding the heterogeneity, NKX2 genes represent a valuable biomarker in cancer (such as lung cancer).

Author contributions

Conceptualization: G.D.; Writing — original draft: C.M.; Writing — review and editing: C.M., F.B. and G.D.

Conflict of interests

The authors declare that there is no competing interests.

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