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Both gene amplification and allelic loss occur at 14q13.3 in lung cancer

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Abstract

Purpose—Because loss of Nkx2-8 increases lung cancer in the mouse, we studied suppressive mechanisms in human lung cancer.

Experimental Design—*NKX2-8* is located within 14q13.3, adjacent to its close relative *TTF1/NKX2-1*. We first analyzed loss of heterozygosity (LOH) of 14q13.3 in 45 matched human lung cancer and control specimens. DNA from tumors with LOH was then analyzed with high-density SNP arrays. For correlation with this genetic analysis, we quantified expression of Nkx2-8 and TTF1 mRNA in tumors. Finally, suppressive function of Nkx2-8 was assessed via colony formation assays in 5 lung cancer cell lines.

Results—13/45 (29%) tumors had LOH. In 6 tumors, most adenocarcinomas, LOH was caused by gene amplification. The 0.8 Mb common region of amplification included MBIP, SFTA, TTF1, NKX2-8, and PAX9. In 4 squamous or adenosquamous cancers, LOH was caused by deletion. In 3 other tumors, LOH resulted from whole chromosome mechanisms (14^{-}, 14^{+}, or aneuploidy). The 1.2 Mb common region of deletion included MBIP, SFTA, TTF1, NKX2-8, PAX9, SLC25A21, and MIPOL1. Most tumors had low expression of Nkx2-8. Nevertheless, sequencing did not show *NKX2-8* mutations that could explain the low expression. TTF1 overexpression, in contrast, was common and usually independent of Nkx2-8 expression. Finally, stable transfection of Nkx2-8 selectively inhibited growth of H522 lung cancer cells.

Conclusions—14q13.3, which contains NKX2-8, is subject to both amplification and deletion in lung cancer. Most tumors have low expression of NKX2-8, and its expression can inhibit growth of some lung cancer cells.

Keywords

Nkx2-8; TTF1/Nkx2-1

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Introduction

NKX2-8 and *TTF1/NKX2-1* encode closely related homeodomain transcription factors in a family named for Drosophila *NK2*. The two genes are adjacent on 14q13.3 and both are expressed during lung development (1–3). TTF1 is oncogenic, overexpressed in many pulmonary adenocarcinomas (4–7). In contrast, studies of a mutant mouse suggest that Nkx2-8 suppresses tumor formation. The *Nkx2-8*-null mouse develops progressive bronchial pathology: hyperplasia at birth, dysplasia in adults, and invasive lung cancer in aged mice (3).

A suppressive function of Nkx2-8 is consistent with molecular studies that compared it with TTF1. The two factors showed identical DNA binding but different transcriptional effects on target genes (8). Moreover, the two proteins are expressed at different times and in distinct cell populations during lung development (3). TTF1 expression first appears in the early pharynx and then spreads downward with the formation of the trachea and lung buds. Its uniform expression in virtually every epithelial cell within these structures gradually fades away as development progresses (2,9). In mature lung, only weak expression of TTF1 is apparent, restricted to alveolar Type II cells. Nkx2-8 expression appears at a later stage of development in scattered cells of the distal lung buds, and then gradually spreads upward into the bronchi and trachea. Expression persists throughout life, primarily in a population of putative tracheobronchial stem cells (TBSC) (3). A second more peripheral cell population has weaker expression and shares the distribution of bronchioloalveolar stem cells (BASC) (10). The loss of Nkx2-8 causes expansion of the TBSC-like but not the BASC-like cell population. Nkx2-8 in the mouse therefore appears to suppress tumors through inhibition of precancerous cell populations, a mechanism that does not require suppression to persist in the eventual malignancy. Indeed, Nkx2-8 was first identified in hepatocellular carcinoma cell lines where its expression is compatible with a malignant phenotype (8,11).

The intriguing suppressive mechanism in the mouse studies led us to investigate whether *Nkx2-8* could also be a suppressor of human lung cancer. This possibility is consistent with several studies of lung cancer that showed frequent loss of heterozygosity (LOH) or cytogenetic deletion at 14q (12–14), including pericentromeric deletions that included 14q13.3 (15,16). Recent studies, however, detected a discrete region of gene amplification at 14q13.3 (5,17,18). Detection of LOH indicates that one allele predominates in a tumor, and is often considered synonymous with loss of an allele. However, gene amplification also causes one allele to predominate, so this gain of gene copies is also detected as LOH. In order to resolve these opposite mechanisms, we began by allelotyping 14q13.3. Tumors determined to have LOH were then analyzed by sequencing *NKX2-8*, and by hybridization of DNA to high-density SNP arrays. Further studies characterized tumor expression of Nkx2-8 and TTF1 mRNA. Finally, transfection experiments demonstrated that Nkx2-8 and TTF1 have opposite effects on transcription in lung cancer cells, and showed the potential suppressive function of Nkx2-8.

Materials and Methods

Clinical specimens and cell lines

45 paired tumor and control lung specimens—frozen specimens that were surplus from a previous study (19)—were collected from consenting patients under institutional review board approval. The individual case numbers were defined in the original accession process. No information was provided about the fraction of tumor cells within the specimens. Human lung cancer cell lines H322 (20), A549 (21), H1299 (22), H441 (23), and H522 (24) were obtained from the American Type Culture Collection and maintained in DMEM medium containing 5% fetal calf serum.

DNA and RNA analysis

DNA was purified from frozen tissue by SDS-proteinase K treatment, phenol extraction, and ethanol precipitation. Total RNA was isolated using the guanidium isothiocyanate method (TRIzol; Invitrogen, Carlsbad, CA) followed by treatment with deoxyribonuclease (DNase-free, Ambion, Austin, TX). For allelotyping, five PCR reactions of individual hypervariable CA-repeat markers were designed to give products of different sizes. Labeling with four fluorescent tags enabled simultaneous resolution in a DNA Sequencer (Supplementary Table S1A). Each marker was analyzed in a separate PCR reaction, and then aliquots from five reactions were combined for allelotyping. Allelic patterns were analyzed with GeneMapper software (Applied Biosystems, Foster City, CA).

Genomic array analysis used high-density Infinium 660W high-density SNP genotyping bead chips (Illumina, San Diego). Labeling and hybridization followed the manufactures protocols. Data were analyzed with BeadStudio software (Illumina).

cDNA was synthesized from 1 μg of RNA using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). PCR reactions contained cDNA synthesized from 0.05 μg of RNA and assays were done in triplicate. Studies of TTF1 expression, with a β-actin control, were carried out by the SYBR green method (Supplementary Table S1B). Studies of Nkx2-8 expression, with a separate β-actin control, utilized Taqman kits (Applied Biosystems). Amplification and detection were carried out with an ABI PRISM 7900HT Sequence Detection System. For DNA sequencing (Supplementary Table S1C), PCR reactions were carried out with the Expand Long Template PCR System (Roche, Indianapolis). Products were cleaned using QIAquick PCR purification columns (Qiagen, Hilden, GER) before sequencing.

DNA clones and transfection

Expression plasmids pCMV-Nkx2-8 and pCMV-TTF1 have been described previously (11). To construct a C-terminal deletion of Nkx2-8 (pCMV-Nkx2-8ΔC), the full length plasmid (239 codons) was amplified using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) with a reverse primer from codon 195 and a forward primer that started with the terminator (codon 240). The product was treated with polynucleotide kinase, blunt-end ligated, cloned, and verified by sequencing. To make pSV-NK2-Luc, an NK2-specific reporter plasmid, , an oligonucleotide containing 4 copies of the strong TTF1/NK2 binding site, GTCAAGTG, spaced at 20 nucleotide intervals, was cloned into the PstI and AgeI sites of pSV-Alb123-R-Luc (25). Transient transfection assays were carried out with the calcium phosphate method as described previously (25). Stable transfection of 3-cm diameter microwell cultures utilized 1 μg of total DNA, 500 ng of expression plasmid or empty vector plus 500 ng of pBabePuro (26) in Lipofectamine (Invitrogen). Cells were transfected at ~40% confluence and continuously selected with puromycin, starting 48 hr after transfection, cells. Dosages of puromycin, experimentally determined for each cell line, ranged from 0.375 to 0.75 μg/ml. Plates were fixed and stained with hematoxylin when colonies were discrete.

Results

Allelotyping

Prior studies of 14q showed a region of LOH that extended from 14q11.2 to D14S75 (14q13.3), an interval of 16.6 Mb (13,15). The region was flanked by D14S75, suggesting that the LOH interval contained *NKX2-8*. To provide higher resolution, we analyzed a 2.7 Mb region—from D14S1049 to D14S75—that approximated the smallest interval in the previous LOH studies, plus three additional markers within the interval. One was a new hypervariable repeat marker between *NKX2-8* and *PAX9* ("D14SNP"). In addition to the

repeat markers, SNPs in or near *NKX2-8* Exon 1 allowed direct discrimination of alleles via DNA sequencing. Figure 1 shows representative analysis of two cases.

We analyzed 45 lung cancer specimens obtained intraoperatively, along with matched noncancerous lung tissue from the same patients. Allelotyping demonstrated frequent LOH, with two general patterns (Fig. 2). Ten tumors showed continuous LOH throughout the entire region (Cases 9, 17, 18, 19, 20, 24, 38, 44, 75, and 82). Three tumors had more localized region of contiguous LOH that included *NKX2-8* (Cases 50, 51, and 73). Two additional adenocarcinomas (Cases 60 and 63) had LOH of single markers but were biallelic closer to *NKX2-8*.

Analysis with SNP arrays

To verify the allelotypes, and determine the causative chromosome abnormalities, we analyzed DNA from the 13 tumors with LOH by hybridization to SNP arrays (Fig. 3 and Supplementary Figs. S1–S3). The analysis confirmed LOH in all 13 cases, and showed that diverse mechanisms altered the allelic composition of individual tumors.

The 3 tumors with localized LOH (cases 50, 51, and 73) all had gene amplification, as did 3 additional tumors, which had amplified a larger chromosomal region (cases 9, 24, and 44). Five of these tumors were adenocarcinomas, and the other was a bronchioloalveolar carcinoma. Mapping of amplicons defined an 0.8 Mb common region of amplification extending from positions 36.6 Mb to 37.4 Mb on chromosome 14.

Deletion of a region that included 14q13.3 was observed in 4 tumors (cases 18, 20, 38, and 75). Among these, tumor 20 had a 1.3 Mb deletion (Figs. 3 and S2) that defined the minimal region common to all deletions, but this tumor apparently also had isodisomy 14. Nevertheless, the deletion occurred in only 1 chromosome copy. Three tumors with deletions were squamous cancers, and the fourth was adenosquamous. The 1.3 Mb common deletion interval includes Nkx2-8 and several other candidate tumor suppressor genes (see Fig. 3 map).

The remaining 3 LOH-containing tumors had changes that affected the entire chromosome (Fig. S3). Tumor 17, a squamous cancer, had only a single copy of chromosome 14 (14−), evidenced by reduced gene dosage and a monoallelic pattern of SNPs. This copy number loss mechanism was grouped with deletions. Tumor 19, a bronchioloalveolar carcinoma, was 14+, because it had uniform copy number increase and the SNP alleles were present in a ~2:1 ratio. This copy number gain mechanism was grouped with gene amplifications. Finally, tumor 82 had normal chromosome dosage but a ~2:1 allelic ratio, findings that indicate an aneuploid chromosome composition.

Absence of NKX2-8 mutations

Since deletion can indicate mutation of a suppressor gene, comprehensive sequencing of *NKX2-8* was carried out using the primers and amplimers listed in Supplementary Table 1C. Twenty-one paired tumor and control specimens, and five cell lines, were fully sequenced. These included all cases with deletion, LOH, or noninformative genotyping, and several others. Exon 1 was sequenced in nine additional cases. With only one exception, all sequences corresponded to the human reference sequence (GRCH37/hg19), or to known SNPs in the promoter and first exon. The exception was case 75, in which control tissue had the polymorphic promoter-region sequence …GGCCAGGCTAATCTTTACG(T/C)CCTCCTGAAACTTGTCAT… The T allele in this specimen probably represents a rare SNP, since the tumor retained the C allele that corresponds to the reference sequence. There was no evidence of inactivating mutation in any tumor or cell line. Mutation is not the only mechanism for inactivation of a tumor suppressor, and the following experiments evaluate

suppressive properties of Nkx2-8. Nevertheless, it is also important to consider other nearby genes co-deleted with Nkx2-8 as candidate tumor suppressors.

NKX2-8 and TTF1 gene expression

We next quantified expression of Nkx2-8 and TTF1 mRNA (Fig. 4). Nkx2-8 expression in control lung tissue was low or absent. These segments of noncancerous lung tissue were not characterized by anatomical location or pathology. RT-PCR amplification of Nkx2-8 was positive in only about 40% of these control specimens, and the strongest expressed Nkx2-8 at 0.003 X the level of β-actin (not illustrated). The interpretation of these "normal" levels is unclear, since Nkx2-8 is mainly expressed in a subset of bronchial cells (3). In the tumor analysis, 43/45 specimens yielded RNA—quantitative PCR detected some level of Nkx2-8 mRNA in 41 specimens and TTF1 mRNA in all 43. Nkx2-8 levels ranged from 0.001–0.19 X β-actin, while TTF1 levels ranged from 0.001–0.11 X β-actin. The weaker detections are hard to interpret, because they could represent minimal expression in all tumor cells, higher expression in a small fraction of tumor cells, or expression from lung tissue incorporated into the tumors. The expression data were fitted to normal Gaussian distributions. The 4 tumors with highest expression were outliers from this distribution and considered to overexpress Nkx2-8 (12%, range 0.04–0.19 X β-actin). For TTF1, 14 tumors were overexpressors (33%, 0.034–0.13 X β-actin). The two tumors with the highest expression of Nkx2-8 both had gene amplification, and also had the highest levels of TTF1 (cases 50 and 73). Case 19 (14+) also overexpressed both Nkx2.8 and TTF1. Such dual overexpression therefore appears to represent a distinctive phenotype associated with gain of 14q13.3. Nevertheless, 4 other tumors with gene amplification had low levels of Nkx2-8, although 3 of these overexpressed TTF1. Linear regression analysis compared expression of the following sets: all tumors, tumors with LOH, and tumors with two alleles. These were analyzed with and without the outlying cases 50 and 73 (Fig. S4). Similar analyses compared individual histological subtypes (not illustrated). These generally indicated a lack of correlation between Nkx2-8 and TTF1 expression. The two genes therefore appear to be independently controlled.

Functional studies of Nkx2-8

To find cell models for study of Nkx2-8 function, we began by allelotyping a panel of 11 lung cancer cell lines (not illustrated). Four of these lines (H441, H322, H522, and H1299) had a pattern of single alleles that was consistent with LOH at Nkx2-8 (Table S2), although matched normal tissues were not available for comparison. H441 has gene amplification, and the other 3 have normal gene dosage on SNP arrays (http://www.sanger.ac.uk/cgi-bin/genetics/CGP/cghviewer/CghViewer.cgi). For comparison, we also included biallelic A549 cells. Quantification of mRNA (Fig. 5A) showed that H441 overexpressed both Nkx2-8 and TTF1, and was thus similar to tumors 50 and 73. The other 4 cell lines had low to moderate expression of Nkx2-8 and did not express TTF1. It should also be noted that H522 and H322 have wild-type K-, H-, and N-ras genes, while the other lines have mutant K-ras (A549 and H441) or N-ras (H1299) (http://www.sanger.ac.uk/genetics/CGP/CellLines/).

In published studies, TTF1 and Nkx2-8 both had strong affinity to the same DNA motif, but with different effects on gene expression. TTF1 was a strong activator in numerous cell types while Nkx2-8 induced little or no transcriptional activation (8,11). The two proteins have very similar DNA binding domains, but the most conserved region of Nkx2-8 is a C-terminal domain that is divergent from TTF1 (Fig. 5B).

To compare the transcriptional function of Nkx2-8 and TTF1, we constructed pSV-NK2-Luc, a luciferase reporter plasmid sensitive to both activation and repression. It consists of 4

NK2-family binding sites upstream of a TATA-containing basal promoter, and has an SV40 enhancer that produces significant expression in the absence of NK2-family factors. In transfection assays (Fig. 5C), Nkx2-8 and TTF1 showed different behavior. TTF1 stimulated the reporter 3- to 6-fold in all 5 cell lines, a strong effect since the reporter plasmid has high basal activity. In contrast, Nkx2-8 had little effect on expression in H441, H322, and H1299 cells and repressed reporter expression in H522 and A549 cells. Deletion of the C-terminal domain eliminated repression and led to moderate transcriptional stimulation in all 5 cell lines. The two factors therefore functioned differently in the same cell lines, and this difference was influenced by the inhibitory C-terminal domain of Nkx2-8.

We then used stable transfection to determine the effects on cell growth (Fig. 5D, E). Nkx2-8, Nkx2-8ΔC, and TTF1 expression plasmids were cotransfected with a plasmid mediating resistance to puromycin. After selection, Nkx2-8 and Nkx2-8ΔC strongly reduced colony formation by H522 cells, and moderately reduced colony formation by H322, H1299, and A549 cells. In contrast, they moderately increased the number and size of colonies formed by H441 cells. TTF1 had weaker effects.

Discussion

This study of human lung cancer was initiated after we found that loss of *Nkx2-8* led to spontaneous lung carcinogenesis in the mouse (3). The mutant mice had bronchial hyperplasia at birth, and increased numbers of putative stem cells throughout life. The latter were revealed by a LacZ marker gene inserted into the deleted Nkx2-8 allele. At 12-months, the mutants had numerous dysplastic lesions in bronchi. Supplementary data included with this earlier paper showed tumor development at 18 months, with examples of both bronchial adenoma (a low-grade invasive tumor of mice) and squamous cancer. Subsequent unpublished studies showed that 80% of the mice developed spontaneous lung cancer by 22 months, and 40% had multiple tumors. Although 75% of the tumors were bronchial adenomas, 15% were high grade adenocarcinomas, and 10% were squamous cancers. Spontaneous adenocarcinomas and squamous cancers are both very unusual in mice. The LacZ marker gene was strongly expressed in dysplasia and silent in the majority of tumors. This pattern of expression suggests that the main effect of Nkx2-8 was to limit expansion of precancerous cells.

Consistent with suppression were prior studies that showed frequent LOH of 14q in lung cancer, findings that suggest deletions (12–14). Also consistent with suppression, we demonstrated that most human tumors have low expression of Nkx2-8 and that its forced expression inhibits growth of H522 lung cancer cells. Recent publications, however, showed gene amplification, a dominant oncogenic increase in copies of one allele (5,17,18). Since the amplified allele predominates, this change also produces "LOH". Our combination of allelotype and SNP array studies has now confirmed the high incidence of LOH at 14q13.3 and demonstrated diverse causes, some associated with oncogenic and others with suppressive mechanisms. Gene amplification is a dominant oncogenic mechanism and our data confirm recent studies of the 14q13.3 amplicon (7,17,18). Three tumors had true gene amplification localized to 14q13.3 (cases 50, 51, and 73). Other tumors (cases 9, 24, and 44) had larger regions of more moderate copy number increase. Both mechanisms seem to target *TTF1*, since almost all of these tumors overexpressed this gene. In contrast, only 2/6 overexpressed of *NKX2-8*, suggesting that it not the primary target of amplification. This is also consistent with the published characterization of an amplicon that included *TTF1* but not *NKX2-8* (18). Nevertheless, tumors 50 and 73, and cell line H441, strongly overexpressed both TTF1 and Nkx2-8. This distinctive phenotype may therefore represent a specific adenocarcinoma subtype.

In addition to cases with amplification, one tumor, a bronchioloalveolar carcinoma, had an extra copy of chromosome 14. Amplification and more modest copy increases were thus confined to adenocarcinoma and bronchioloalveolar carcinoma. Though considered a special tumor category, the latter are also adenocarcinomas that commonly overexpress TTF1, sometimes via gene amplification (17). In addition to gene amplification, there must be other mechanisms that activate TTF1 expression, since some of the highest levels occurred in tumors with two equal alleles. The prevalence of TTF1 expression has significantly impacted clinical management, with obvious utility as a lung cancer marker. Moreover, recent studies suggest that TTF1 expression imparts a specific biological behavior—positive tumors have a relatively favorable prognosis and better treatment responses than other lung adenocarcinomas (7,27).

Copy number loss consistent with inactivation of a tumor suppressor was present in 4 tumors. Three had deletions within 14q, and a fourth had lost one entire chromosome 14. These copy number reductions were more difficult to identify on SNP arrays than copy number increases, but the determinations were substantiated by the allelotypic demonstration of LOH (see the studies of tumor 18 in Figs. 1 and 3). All 4 tumors with loss had low expression of Nkx2-8. Nevertheless, the deletion intervals contained several additional candidate suppressor genes. Notably, all 4 tumors were squamous or adenosquamous cancers. The findings suggest two distinct pathways of tumor progression: amplification of 14q13.3 with overexpression of TTF1 (and sometimes Nkx2-8) in adenocarcinomas; or deletion of 14q13.2–13.3 and inactivation of a suppressor gene in squamous cancers. This correlation, however, differs from the spectrum of tumors in the Nkx2-8 mutant mouse, which developed adenocarcinomas and squamous carcinomas.

To further substantiate suppression by Nkx2-8, we studied transcriptional function and tested for growth inhibition. The main point of the transcriptional assays was to compare Nkx2-8 and TTF1, since the factors are so similar. The transient transfection assays showed clear differences and demonstrated that the distinctive C-terminal domain weakens transcriptional stimulation mediated by Nkx2-8. Nevertheless, Nkx2-8ΔC is incomplete, indeed missing the most conserved domain. The reporter gene transfections therefore reveal aspects of Nkx2-8, but do not fully define transcriptional function in vivo, which may be activating or repressing in different contexts (8,11).

We also studied effects on cell growth, and it is important to note that either transcriptional activators or repressors can inhibit cell growth, respectively by activation of an inhibitory, or repression of a stimulatory gene expression. Tumor phenotypes and their relationships to progenitor cells vary, so we studied a variety of cell lines, focusing on candidates for LOH at NKX2-8. Recently, Kendall et al. (5) described transfection of Nkx2-8 and TTF1 into immortalized bronchial epithelial cells. The individual factors had no effect, but the combination markedly increased colony size. Kendall also noted that expression of Nkx2-8 and TTF1 individually stimulated tumorigenicity of HCC15 cells, while shRNA-mediated inhibition of each factor inhibited the tumorigenicity of H2170 cells, a line with gene amplification that overexpresses both proteins. Their observations are consistent with the moderate stimulation we observed in H441 cells, and complementary to our analysis of H322, H522, and H1299 cells. Growth inhibition of H522 cells shows that Nkx2-8 can be suppressive in some lung cancer phenotypes. Interestingly, Nkx2-8ΔC also inhibits growth, which might indicate that the effect is mediated by competition with another transcription factor. In future studies, H522 cells will provide a resource for working out the mechanism of repression.

Since the case for tumor suppression by *NKX2-8* is tentative without a demonstration of inactivating mutations, the common deletion interval provides a list of other candidate

suppressor genes. Even so, our analysis does not rule out several possible mechanisms of Nkx2-8 inactivation, including regulatory mutations and DNA methylation. The gene is embedded within a 60-kb region with many highly conserved regions that could act as transcriptional enhancers, so sequence analysis of promoter and exons omits many possible mutations.

In contrast to TTF1, few lung cancers significantly express Nkx2.8. Nevertheless, strong expression of Nkx2-8 is compatible with some tumor phenotypes. Whether these tumors have distinctive properties remains to be determined, but our previous analysis of the mouse specifically links Nkx2-8 to bronchial stem/progenitor cells. Nkx2-8 apparently inhibits proliferation of these cells because the null mutation causes their numbers to increase. Loss or silencing of Nkx2-8 in progenitor cells might have a comparable effect on human carcinogenesis, even if expression were to reactivate at a later stage of tumor progression. Moreover, normal progenitor cells must have a mechanism for inactivating the growth-inhibitory effects of Nkx2-8 when they are stimulated to proliferate. Thus, reexpression of Nkx2-8 in a tumor does not rule out the possibility that reduced expression was important at an early stage of progression. There are precedents for this type of dual relationship to tumors. WT1, for example, suppresses formation of nephroblastomas but is also an activating regulator in many of these tumors (28).

Statement of Translational Relevance

Since an Nkx2-8-mutant mouse develops lung cancer, we studied whether the gene is also a suppressor of human lung cancer. The resultant analysis demonstrated that copy increase (gene amplification or chromosome gain) and decrease (deletion or chromosome loss) at 14q13.3 are independent mechanisms of lung cancer progression. Gain occurs in adenocarcinomas, while loss occurs predominantly in squamous carcinomas. Moreover, two related genes in this interval — *NKX2-8* and *TTF1*— show independent expression, controlled by gene copy number and other mechanisms. Adenocarcinomas frequently overexpress TTF1, and a few overexpress Nkx2-8. However, most tumors have low expression of Nkx2-8 and its forced expression can suppress lung cancer cells. The specific phenotypes apparently reflect different sequences of progression and may define distinct biological properties of individual tumors, with potential relevance to prognosis and treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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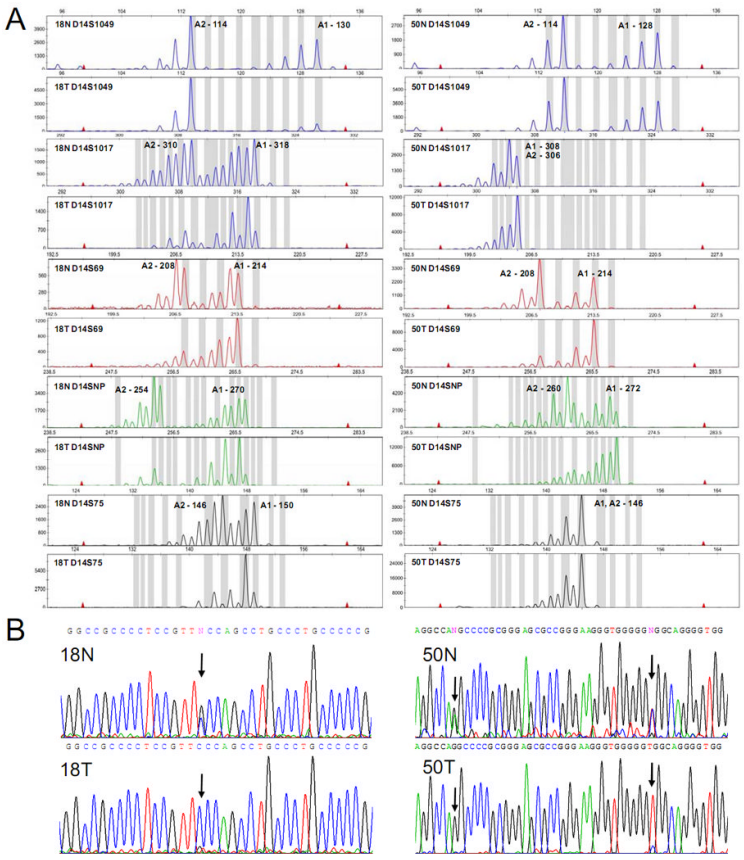

Fig. 1.

Analysis of 14q13.3 in two representative tumors. **A.** Tumor 18 has LOH of all 5 markers. Tumor 50 has two alleles at D14S1049, and LOH at D14S69 and D14SNP. D14S1017 and D14S75 are uninformative. **B.** Both tumors show loss of polymorphisms within *NKX2-8*. Tumor 18 has lost a G/C SNP (rsrs35669843), and Case 50 has lost G/A (rs3809433) and C/T (rs177324) SNPs.

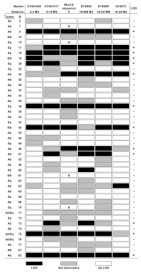

Fig. 2.

Allelic composition at 14q13.3 in 45 lung cancer specimens. Data were obtained by genotyping at 5 loci and direct sequencing of *NKX2-8*. Ad, adenocarcinoma; Sq, squamous carcinoma; BA, bronchioloalveolar carcinoma; Ad/Sq, adenosquamous carcinoma; NA, histopathological diagnosis not provided.

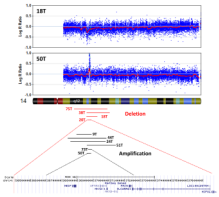

Fig. 3.

Analysis using SNP arrays. **Above**, plots of Log R Ratio of chromosome 14 from tumors 18 and 50, the same cases analyzed in Fig. 1. **Below**, mapping of the minimal regions of amplification and deletion. The map also displays the positions of 22 genes within the region common to the three deletions.

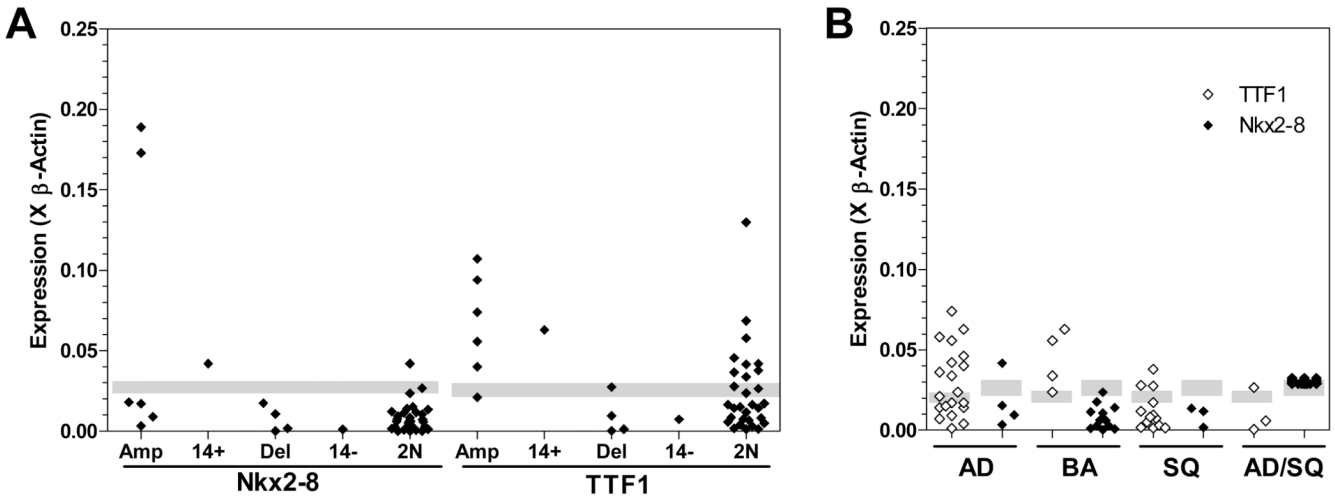

Fig. 4.

Nkx2-8 and TTF1 expression in lung cancer specimens. **A.** Correlation with allelic loss and gene amplification. Gene expression in 43 specimens was measured by real-time PCR of cDNA. Values were averaged from two triplicate determinations and fitted to a Gaussian distribution. Values above the gray bars mark overexpressors that were outliers from the distribution, and the widths of the gray bars mark borderline values. Amp, gene amplification; 14−, loss of 1 chromosome copy; Del, deletion; 14+, gain of 1 chromosome copy. **B.** Correlation of gene expression with tumor category. AD, adenocarcinoma; BA, bronchioloalveolar carcinoma; SQ, squamous cell carcinoma, AD/SQ, adenosquamous carcinoma.

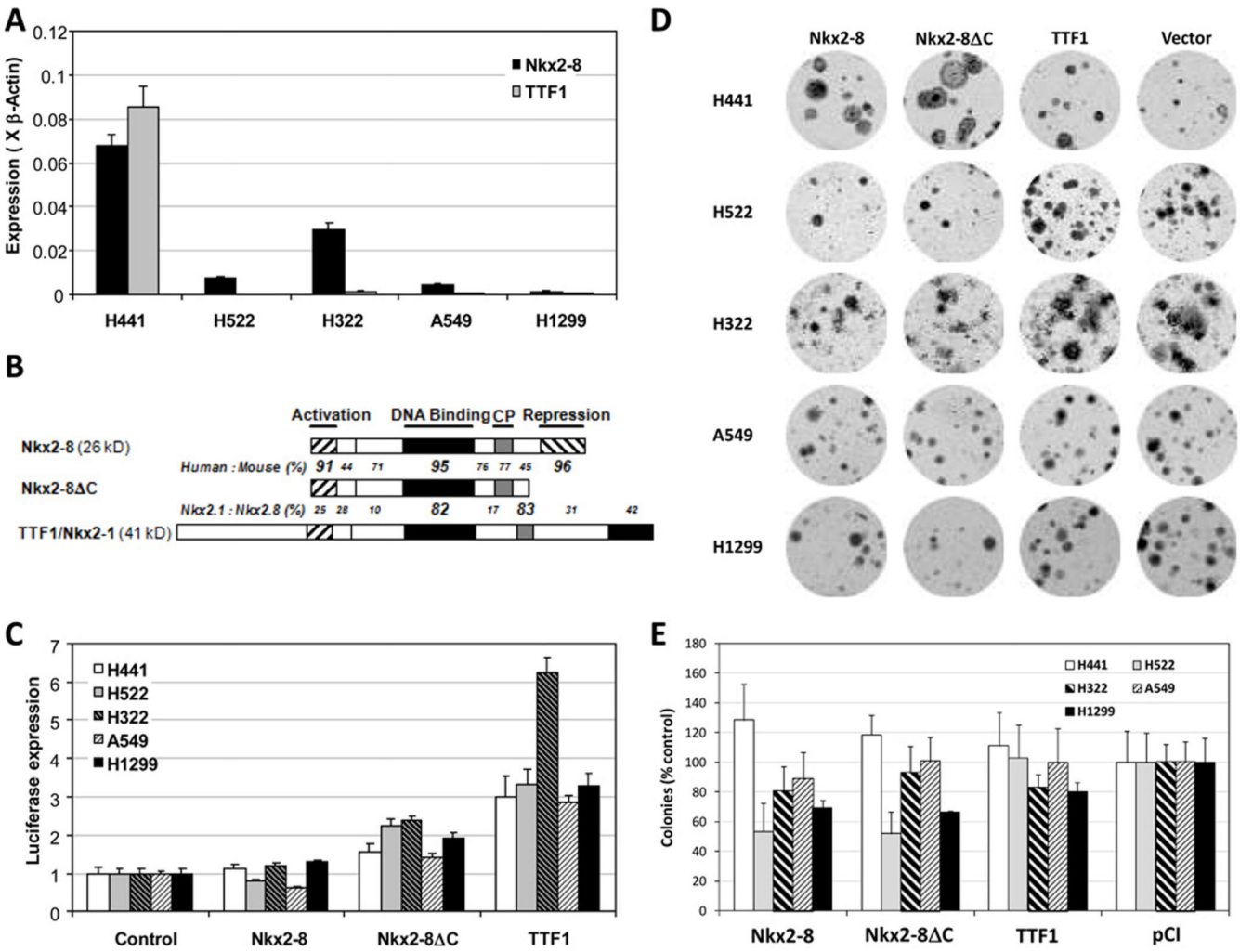

Fig. 5.

Effects of Nkx2-8 on transcription and cell growth. Nkx2-8 is a cell-specific repressor. **A.** Nkx2-8 and TTF1 expression in cell lines. The plot shows the averages of three real-time RT-PCR determinations, normalized to the level of β-actin mRNA. **B.** Domain structure of Nkx2-8. A C-terminal domain of Nkx2-8 is most highly conserved between the mouse and human proteins, while the homeodomain is most conserved between human Nkx2-8 and human TTF1/Nkx2-1. **C.** Transfection assays in 5 cell lines. A luciferase reporter gene contained four copies of an NK2 binding site upstream of a basal promoter, and the SV40 early enhancer downstream of the luciferase gene. This was cotransfected in a 5:1 ratio with plasmids expressing full length or C-terminal-deleted Nkx2-8, TTF1, or empty vector (Control). The plot shows the means and standard deviations of multiple determinations. **D.** Representative culture plates. Plasmids pCMV-Nkx2-8, pCMV-Nkx2-8ΔC, pCMV-TTF1, or pCI (empty vector control) were cotransfected with pBabePuro. Plates were then treated with Puromycin, and fixed and stained when colonies were well defined on the control plate. **E.** Quantification of colony formation. The values are averaged from two independent experiments, each with 3 replicate transfections for each plasmid.

Table 1

Classification of LOH

Type	Case	Abnormality
Adenocarcinoma	9	Amplification
Adenocarcinoma	24	Amplification
Bronchioloalveolar	44	Amplification
Adenocarcinoma	50	Amplification
Adenocarcinoma	51	Amplification
Adenocarcinoma	73	Amplification
Bronchioloalveolar	19	14+
Adenocarcinoma	82	Aneuploidy
Squamous	17	14−
Squamous	18	Deletion
Squamous	20	Deletion
Squamous	38	Deletion
Adenosquamous	75	Deletion