Transcription start site–level expression of thyroid transcription factor 1 isoforms in lung adenocarcinoma and its clinicopathological significance

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Abstract

There are multiple transcription start sites (TSSs) in agreement with multiple transcript variants encoding different isoforms of NKX2-1/TTF-1 (thyroid transcription factor 1); however, the clinicopathological significance of each transcript isoform of NKX2-1/TTF-1 in lung adenocarcinoma (LAD) is unknown. Herein, TSS-level expression of NKX2-1/TTF-1 isoforms was evaluated in 71 LADs using bioinformatic analysis of cap analysis of gene expression (CAGE)-sequencing data, which provides genome-wide expression levels of the 5′-untranslated regions and the TSSs of different isoforms. Results of CAGE were further validated in 664 LADs using in situ hybridisation. Fourteen of 17 TSSs in NKX2-1/TTF-1 (80% of known TSSs in FANTOM5, an atlas of mammalian promoters) were identified in LADs, including TSSs 1–13 and 15; four isoforms of NKX2-1/TTF-1 transcripts (NKX2-1_001, NKX2-1_002, NKX2-1_004, and NKX2-1_005) were expressed in LADs, although NKX2-1_005 did not contain a homeodomain. Among those, six TSSs regulated NKX2-1_004 and NKX2-1_005, both of which contain exon 1. LADs with low expression of isoforms from TSS region 11 regulating exon 1 were significantly associated with poor prognosis in the CAGE data set. In the validation set, 62 tumours (9.3%) showed no expression of NKX2-1/TTF-1 exon 1; such tumours were significantly associated with older age, EGFR wild-type tumours, and poor prognosis. In contrast, 94 tumours, including 22 of 30 pulmonary invasive mucinous adenocarcinomas (IMAs) exhibited exon 1 expression without immunohistochemical TTF-1 protein expression. Furthermore, IMAs commonly exhibited higher exon 1 expression relative to that of exon 4/5, which contained a homeodomain in comparison with EGFR-mutated LADs. These transcriptional and clinicopathological results reveal that LAD use at least 80% of NKX2-1 TSSs and expression of the NKX2-1/TTF-1 transcript isoform without exon 1 (NKX2-1_004 and NKX2-1_005) defines a distinct subset of LAD characterised by aggressive behaviour in elder patients. Moreover, usage of alternative TSSs regions regulating NKX2-1_005 may occur in subsets of LADs.

Keywords: lung adenocarcinoma; TTF-1; NKX2-1; promoter; 5′-UTR; TSS; isoforms

Introduction

Thyroid transcription factor 1 (TTF-1), also known as NKX2-1, is a member of the highly conserved homeodomain-containing transcription factor family, which activates the expression of selected genes in the lung and thyroid, as well as a restricted part of the brain, and is essential for the development and differentiation of these organs [1–3]. The cellular mechanisms regulating lung homeostasis are not completely

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understood; however, different epithelial regions and compartments in the lung are known to be maintained by distinct resident stem cell populations [4]. Notably, a series of peripheral lung cells defined as the terminal respiratory unit (TRU), in which gas exchange occurs, is under the control of NKX2-1/TTF-1 [5]. Furthermore, approximately 70% of lung adenocarcinomas (LADs) express TTF-1 independent of disease stage and retain features of the TRU to a certain extent [6], strongly suggesting that NKX2-1/TTF-1 is a potential lineage survival oncogene in lung cancer [7]. Currently, TTF-1 is widely used as the most specific marker for LAD diagnosis in routine pathological examination [8], which leads to the detection of actionable alterations, such as EGFR, KRAS, and BRAF mutations; gene fusions involving anaplastic lymphoma kinase (ALK); rearranged during transfection (RET); and proto-oncogene tyrosine-protein kinase ROS1 (ROS1) or tyrosine-protein kinase Met (MET) exon 14 skipping [9,10]. Despite the discovery of these oncogene mutations, at least 12% of patients with LAD do not possess any of these genetic alterations [10,11], suggesting that other molecular changes likely contribute to lung cancer development.

Epigenomic features do not affect the DNA sequence, but may affect the transcripational output of genes in a cell-type specific manner by altering the activity of regulatory elements, including promoters, which are located proximal to the transcription start site (TSS) of genes [12]. Alternative splicing is the process by which a single gene may produce many different transcripts that may show a wide range of activities, and is responsible for much of the diversity of the human proteome [12]. TSS determination of NKX2-1/TTF-1 transcription shows multiple TSSs, in agreement with multiple transcript variants encoding different isoforms [5]. In humans, two complementary DNAs (cDNAs) were initially identified that translate into the 42-kDa ‘major isoform’ and the 44-kDa ‘minor protein isoform’. These isoforms were differentially expressed during mouse fetal lung development, with the onset of accumulation of the longer transcript occurring at a later stage than that of the shorter transcript [13,14]. These two transcripts have differences in their capacity to activate the surfactant protein-C promoter, which is a pulmonary differentiation-specific gene, indicating functional differences [13]. Furthermore, NKX2-1/TTF-1 shows different functions depending on cell conditions, being considered a double-sword gene with lineage-dependent tumour cell survival and tumour suppression activities depending on the context [7]. This suggests that each isoform has differential functions in lung carcinogenesis. However, the clinicopathological significance of each promoter and the concordant isoform in LAD remains largely unknown.

In recent studies, an atlas of human cellular states based on regulatory element activities across the genome, such as promoters [15] and enhancers [16], has been built by monitoring transcription initiation activities with cap analysis of gene expression (CAGE) [17]. The method determines 5'end sequences of messenger RNA (mRNA) using next-generation sequencing, where cDNAs are synthesised from extracted RNA, and cDNAs corresponding to 5'-ends of RNA are selected using the cap-trapper method [18] and sequenced. Obtained reads are aligned with genome sequences and their 5'-ends indicate frequencies of TSSs at single-base resolution [19]. Herein, the ability of this technology to elucidate the role of each TSS and transcript isoform of NKX2-1/TTF-1 in LAD was examined, with special emphasis on its prognostic impact. The clinicopathological significance of NKX2-1/TTF-1 exon 1 expression in a large cohort of Japanese patients was further evaluated using RNAscope, a novel in situ hybridisation assay. We used the NKX2-1 probe that was designed to target exon 1 in NKX2-1.004 (ENST00000518149.5_4). This study expands the understanding of the role of NKX2-1/TTF-1 in LAD.

Materials and methods

Study population

The archives of the Department of Human Pathology, Juntendo University School of Medicine, were screened for all patients who had undergone a complete resection of primary LAD from February 2010 to July 2016. Clinicopathological data were obtained, including age, gender, smoking status, tumour size, lymphovascular invasion, lymph node and distant metastases, resection type, adjuvant therapy, and mutation status of EGFR and KRAS. The archives contained data for 1,124 patients with LAD. Of the 1,124 LAD samples of the cohort, 71 cases were assigned to the discovery set used to perform CAGE assay [20], while full-length RNA sequencing (RNA-seq) was also performed in seven cases [9]. Among the remaining 1,053 cases, adenocarcinoma in situ, minimally invasive adenocarcinoma, and lepidic adenocarcinoma were excluded to clarify the prognostic impact of NKX2-1 exon 1 expression. Invasive LADs with intermediate- to high-grade clinical aggressiveness including acinar, papillary, solid, micropapillary, or other invasive adenocarcinomas of a special type [21] were assigned to the validation set. Follow-up was conducted for all patients.
via regular physical and blood examination, with mandatory X-ray, computed tomography, or magnetic resonance imaging. Informed consent was obtained from all involved patients. The study design was ethically approved by the institutional review board of Juntendo University (Approval No. 20200996).

Bioinformatics analysis of the CAGE data set
CAGE data were obtained from a previous study [20]. In brief, the CAGE reads were aligned to the reference genome (hg19) with a high mapping quality of ≥20. The aligned CAGE reads were counted in each region of the FANTOM5 robust peaks [15], a reference set of TSS regions, as raw signals for promoter activities. Expression levels of individual TSSs were quantified as counts per million (CPM). Inactive TSS regions, with CPM ≤ 1 in more than 77% of samples, were filtered out [22]. Associations among the TSS regions were assessed by Spearman’s rank correlation. The distances between the samples in the NKK2-1 TSS regions were calculated as Euclidean distances for CPM, and the average linkage clustering was performed using R (version 3.6.3, https://www.r-project.org/). Based on expression levels, survival analyses of individual TSS regions were performed using the survival package in R (https://cran.r-project.org/web/packages/survival/).

Histological and immunohistochemical analyses
All tissues were fixed in 10% formalin-fixed paraaffin-embedded (FFPE) after routine processing. Haematoxylin and eosin (H&E)-stained slides and Elastica van Gieson-stained slides were available for all patient samples. All tumours measuring 3 cm or less in diameter were submitted in their entirety, and larger tumours were sampled extensively. Pathological diagnoses were based on the 2015 World Health Organization classification [23]. For immunohistochemical analyses of TTF-1 (clone 8G7G3/1; DAKO, Glostrup, Denmark), tumours were assembled into tissue microarrays (TMAs), using 1.5–2.0 mm cores sampled from one or two different representative areas of each FFPE tissue block (Pathology Institute Corp., Toyama, Japan), as previously described [24]. TTF-1 was considered positive if 1% or greater of tumour cells were stained.

RNAscope assay and image analysis
In situ detection of NKK2-1 transcript was performed with a RNAscope Assay using the RNAscope Duplex Reagent Kit (#322430; Advanced Cell Diagnostics Inc., Newark, CA, USA), according to the manufacturer’s instructions. The NKK2-1 probe was designed to target exon 1 in NKK2-1_004 (ENST00000518149.5_4) (Advanced Cell Diagnostics Inc.) (see supplementary material, Table S1). For the RNAscope assay, TMA slides from FFPE tissue blocks were used. RNAscope and immunohistochemistry for TTF-1 were performed on serial sections. The 664 cases of the validation cohort and 33 cases of the discovery cohort were submitted to RNAscope assay. To ensure result interpretability, a positive (#313901, RNAscope Positive Control Probe-Hs-PPIB; Advanced Cell Diagnostics Inc.) and a negative control probe (#310043, RNAscope Negative Control Probe-Hs-DapB; Advanced Cell Diagnostics Inc.) were used. After staining, TMA slides were scanned using the Nuance Multispectral Imaging System (version 3.0.2; Perkin Elmer Inc., Waltham, MA, USA), inForm Advanced Image Analysis Software (version 2.4.0; Perkin Elmer Inc.) was used for quantitative image analysis. Four random areas (0.09048 mm<sup>2</sup> each) in each sample were analysed at ×400 total magnification. The data are expressed as optical density (average signal levels per area). An optical density of ≥66.56 was considered positive for expression based on the optical density distribution data in the 664 samples examined in this study (see supplementary material, Figure S1).

Quantitative polymerase chain reaction
Quantitative polymerase chain reaction (qPCR) was performed on 20 cases of invasive mucinous adenocarcinoma (IMA) of the lung whose genetic alterations were previously described [25], as well as three EGFR-mutated LAD cases that were immunohistochemically positive for TTF-1, consisting of two cases of papillary adenocarcinoma and one case of acinar adenocarcinoma. RNA was extracted from FFPE tissue using the RNeasy FFPE Kit (Qiagen, Hilden, Germany). qPCR was performed using inventoried Taqman assays (Applied Biosystems, Carlsbad, CA, USA) corresponding to exon 1 in the NKK2-1_004 isoform (forward: 5'-GCCATTTACGCCACCACTTTAA-3'; reverse: 5'-GCCATTTACGCCACCACTTTAA-3'; probe: AAGATATT TGGTTATTCCCG); TTF-1 exon 4/5 (Assay ID: Hs00968940_m1; Thermo Fisher Scientific, Waltham, CA, USA), myosin-binding protein H (MYBPH) (Assay ID: Hs00192226_m1; Thermo Fisher Scientific), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Assay ID: Hs02786624_g1; Thermo Fisher Scientific). All PCRs were performed with a TaqMan Fast Advanced Master Mix (Applied Biosystems) on an Applied Biosystems Step One Plus Real-Time PCR System in accordance with the standard protocols. The amount of each target gene relative to the GAPDH housekeeping gene was determined using the comparative threshold cycle
Method. Data are the mean of values from three separate experiments.

Cell lines and cell culture

EGFR-mutated cell lines (H3255, 11–18, H4006, HCC827, PC9, and H1650) and KRAS-mutated cell lines (HCC44, H23, H2030, and A549) were purchased from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin–streptomycin in an atmosphere of 5% CO₂ at 37 °C. All cell lines were routinely tested for Mycoplasma and were found to be negative.

Western blot assays

Protein samples were separated by SDS-PAGE and subsequently blotted onto a polyvinylidene fluoride membrane. An iBind Western Device (Life Technologies Corporation, Carlsbad, CA, USA) was used for the antigen–antibody reaction. The membrane was incubated with antibodies against TTF-1 (sc-53136; Santa Cruz, Dallas, TX, USA) and GAPDH (sc-3223; Santa Cruz). Bound antibodies were detected with horseradish peroxidase-conjugated secondary mouse antibody (GE Healthcare Biosciences, Little Chalfont, UK), and images were taken using the Amersham Imager 680 (GE Healthcare Biosciences).

Statistical analysis

Categorical variables were analysed using a Fisher’s exact or chi-square test. To determine prognosis, Kaplan–Meier survival analysis was performed. The date of surgical resection was set as the starting point and the
date of death, date of recurrence, or last date of follow-up was used as the end point. Statistical analyses were performed using GraphPad Prism® software version 7.0a (GraphPad, San Diego, CA, USA). *P* value of <0.05 was considered significantly different.

**Results**

Clinicopathological characteristics in the study cohort

Clinicopathological characteristics of 71 and 664 patients with LADs examined in the discovery and validation data sets, respectively, are shown in Table 1. The median age of the 71 cases in the discovery data set was 66.7 years, 29 (40.8%) of which were female and 37 (52.9%) were never or light smokers (the smoking index was $\leq 400$); the median age of the 664 cases in the validation data set was 67.8 years, 314 (47.2%) of which were female and 367 (55.4%) were never or light smokers. Overall, comparison of the discovery and validation data sets revealed no significance differences in clinicopathological features, such as age, gender, smoking status, and the pathological stage at presentation. As expected, histological subtypes were significantly different among the two groups as the validation data set consisted of invasive LADs, including...
CAGE assay demonstrated TSS-level expression of \textit{NKX2-1/TTF-1} in LAD

Quantitative TSS-level expression profiles were obtained from 71 LADs in the discovery data set in which the CAGE assay was performed as previously described [20]. Initially, genome databases were explored, such as Ensemble, UCSC Genome Browser, and NCBI Human Genome Resources, to obtain the location and gene structure of human \textit{NKX2-1/TTF-1}. Human \textit{NKX2-1/TTF-1} is located on chromosome 14 and produces five transcripts (ENST00000498187.6_4, \textit{NKX2-1}_001; ENST00000354822.7_5, \textit{NKX2-1}_002; ENST00000522719.2_4, \textit{NKX2-1}_003; ENST00000518149.5_4, \textit{NKX2-1}_004; and ENST00000546983.1_3, \textit{NKX2-1}_005) containing different 5’-untranslated region (5’-UTR) first exons. Expression profile according to Genotype-Tissue Expression (GTEx) database (release V6p: dpGap Accession phs000424.v6.p1) showed that all isoforms except \textit{NKX2-1}_003 were highly expressed in the normal lung and thyroid gland, suggesting site-specific expression. The transcripts \textit{NKX2-1}_001, \textit{NKX2-1}_002, and \textit{NKX2-1}_004 contained homeodomains, while \textit{NKX2-1}_005 did not. With respect to expression level, \textit{NKX2-1}_001 had the highest expression, followed by \textit{NKX2-1}_002, whose expression level was nearly equal to that of \textit{NKX2-1}_004 (Figure 1A). Further computational analysis using FANTOM5 data revealed 17 TSSs in the human genome. Among them, 14 TSS regions (82%), including the TSSs 1–12, 13, and 15, were detected in LADs examined in this study (Figure 1B and supplementary material, Table S2). Thus, CAGE assay combined with full-length RNA-seq demonstrated that

Figure 2. TSS expression level of TTF-1 (\textit{NKX2-1/TTF-1}) in LAD. (A) Seventy-one LADs are clustered based on enrichment of the expression of 14 \textit{NKX2-1/TTF-1} TSSs. (B) Box plots of the TSS regions of \textit{NKX2-1/TTF-1} in each histological grade. Expression was quantified as CPM. Each box indicates the mean and upper and lower quartiles, whereas the bar indicates the range. Both low- and high-grade adenocarcinoma as well as special type (IMA) expressed 14 TSSs. (C) Comparison of OS in tumours with each TSS expression level. Note that the expression level of TSS region 11 is the most associated with prognosis in patients with LAD (log-rank \(p = 0.00810\)).

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all transcript isoforms except \textit{NKX2-1}_003 were expressed in LADs. \textit{NKX2-1}_001 was regulated by TSSs 7 and 13, whereas \textit{NKX2-1}_002 was regulated by TSSs 1, 3, 5, 8, 12, and 15. Furthermore, six TSSs (TSSs 2, 4, 6, 9, 10, and 11) regulated \textit{NKX2-1}_004 and \textit{NKX2-1}_005, containing exon 1 (Figure 1C). Expression levels of TSSs that regulated the same 5'-UTR first exons were correlated with each other (Figure 1D).
TSS-level expression of \( \text{NKX2-1}/\text{TTF-1} \) was associated with LAD prognosis

In the discovery data set, 71 LADs were clustered based on enrichment of the expression of isoforms from 14 TSSs of \( \text{NKX2-1}/\text{TTF-1} \). A subset of isoforms from TSSs was highly expressed or decreased in each case (Figure 2A). Furthermore, conventional LAD as well as IMA, LAD of special type, used all 14 of the TSSs (Figure 2B). With respect to the malignant potential of TSSs, LADs with low expression of isoforms from TSS region 11, which regulated exon 1 in \( \text{NKX2-1}/\text{TTF-1} \), were the most significantly associated with poor prognosis (\( p = 0.00810 \)). Furthermore, low expression of isoforms from other TSSs that regulate exon 1, including TSSs 2 (\( p = 0.02087 \)), 4 (\( p = 0.02249 \)), and 10 (\( p = 0.02274 \)) was significantly associated with poor prognosis. In contrast, expression of isoforms from other TSSs, including those regulating \( \text{NKX2-1}_001 \) and \( \text{NKX2-1}_002 \) was not significantly correlated with prognosis, with the exception of TSS region 7 (\( p = 0.02024 \)) (Figure 2C).

Detection of \( \text{NKX2-1}/\text{TTF-1} \) exon 1 in FFPE LAD tumour tissues

The clinicopathological impact of expression of \( \text{NKX2-1}/\text{TTF-1} \) exon 1 was further examined. Expression of \( \text{NKX2-1}/\text{TTF-1} \) exon 1 was evaluated using RNAscope and a designed probe targeting \( \text{NKX2-1}/\text{TTF-1} \) exon 1 in FFPE tissues. First, the association of expression of \( \text{NKX2-1}/\text{TTF-1} \) exon 1 was examined, which was detected by CAGE and RNAscope in the discovery data set, and whose tumours exhibited high- to intermediate-grade histology (acinar, papillary, and solid adenocarcinoma), indicating that there were correlations among them (\( p = 0.0020 \)) (see supplementary material, Figure S2). Next, RNAscope was performed in the validation data set

| Table 2. Clinicopathological characteristics of LAD with \( \text{NKX2-1}/\text{TTF-1} \) exon 1 expression. |
|-----------------------------------------------|
| **Tumour without exon 1 expression** (\( n = 62 \)) | **Tumour with exon 1 expression** (\( n = 602 \)) | **Correlation** (\( p \)) |
| **Median age (range)** | 71.66 (41–85) | 67.38 (28–89) | 0.0006 |
| **Gender** | | | 0.0610 |
| Female | 22 | 292 | |
| Male | 40 | 310 | |
| **Smoking index** | | | 0.2827 |
| \( \leq 100 \) | 22 | 275 | |
| 101–400 | 7 | 63 | |
| \( \geq 401 \) | 33 | 262 | |
| Unknown | 0 | 2 | |
| **Size (mm)** | | | 0.4728 |
| \( \leq 20 \) | 17 | 219 | |
| 21–30 | 20 | 180 | |
| 31–50 | 16 | 142 | |
| \( \geq 51 \) | 9 | 61 | |
| **Nodal status** | | | 0.2544 |
| NO | 40 | 430 | |
| N1/N2/N3 | 22 | 172 | |
| **TNM stage** | | | 0.2967 |
| 0–I | 34 | 371 | |
| II–IV | 28 | 231 | |
| **Histological subtype** | | | 0.0618 |
| Acinar adenocarcinoma | 30 | 275 | |
| Papillary adenocarcinoma | 12 | 194 | |
| Solid adenocarcinoma | 13 | 98 | |
| Microcystic adenocarcinoma | 0 | 7 | |
| IMA | 7 | 23 | |
| Enteric adenocarcinoma | 0 | 3 | |
| Fetal adenocarcinoma | 0 | 2 | |
| **Histological grade** | | | 0.0259 |
| Intermediate/high grade | 55 | 574 | |
| Special | 7 | 28 | |
| **Lymphovascular invasion** | | | 0.2425 |
| Absent | 40 | 342 | |
| Present | 22 | 260 | |
| **Pleural invasion** | | | 0.1648 |
| Absent | 34 | 384 | |
| Present | 28 | 218 | |
| **Micropapillary pattern** | | | 0.3173 |
| Absent | 56 | 516 | |
| Present | 6 | 86 | |
| **Cribriform pattern** | | | 0.0871 |
| Absent | 52 | 546 | |
| Present | 10 | 56 | |
| **Clear cell features** | | | 0.1570 |
| Absent | 48 | 508 | |
| Present | 14 | 94 | |
| **Signet ring cell features** | | | 0.8539 |
| Absent | 61 | 594 | |
| Present | 1 | 8 | |

(Continues)
consisting of 664 LADs. The median optical density for NKX2-1/TTF-1 exon 1 in the validation data set was 314.8, and 602 (90.7%) tumours expressed NKX2-1/TTF-1 exon 1 (Figure 3).

LAD clinicopathological characteristics of NKX2-1/TTF-1 exon 1 expression

To clarify LAD characteristics with no NKX2-1/TTF-1 exon 1 expression, the clinicopathological factors in 664 LADs were evaluated. The clinicopathological characteristics of 62 LADs with no expression of NKX2-1/TTF-1 exon 1 are summarised in Table 2. LADs with no expression of NKX2-1/TTF-1 exon 1 were significantly associated with older age ($p = 0.0006$), no TTF-1 immunoreactivity ($p = 0.0009$), and EGFR wild-type tumours ($p = 0.0009$). However, there was no significant correlation between expression of NKX2-1/TTF-1 exon 1 and LAD histological features, such as micropapillary or cribriform...
patterns, that are unfavourable prognostic factors in LAD [24]. Furthermore, 49 (79%) and 51 (82%) LADs with no NKX2-1/TTF-1 exon 1 expression exhibited an EGFR and KRAS wild-type genotype, respectively. In contrast, 94 (14%) LADs exhibited NKX2-1/TTF-1 exon 1 expression without TTF-1 protein expression. Of those, solid adenocarcinoma was the most frequent histological subtype (27%), followed by acinar, papillary adenocarcinoma, and IMA (23%). Interestingly, IMA frequently (73%) exhibited the same expression pattern, and other LADs of a special type, including one fetal adenocarcinoma and two enteric adenocarcinomas, also exhibited a similar expression pattern (Figure 4A). Whether IMA exhibited NKX2-1/TTF-1 exon 1 expression was further validated using qPCR, revealing that 19 of 20 (95%) cases exhibited higher exon 1 expression relative to that of exon 4/5, compared to EGFR-mutated adenocarcinoma that was immunohistochemically positive for TTF-1 (Figure 4B).

Figure 5. Kaplan–Meier curves of OS of 664 patients with LAD after surgical resection. Comparison of OS according to (A) stage, (B) histological grade, (C) tumour TTF-1 protein expression, and (D) tumour NKX2-1/TTF-1 exon 1 expression. (E and F) Comparison of OS stratified by NKX2-1/TTF-1 exon 1 expression in patients with immunohistochemically TTF-1-positive (E) or -negative (F) tumours.
A549 cells expressed *NKX2-1/ TTF-1* exon 1

To validate the disproportionate expression levels between *NKX2-1/ TTF-1* exon 1 and exon 4/5 observed in FFPE tumour tissues, expression of both *NKX2-1/ TTF-1* exon 1 and exon 4/5 was evaluated in a panel of 10 lung cancer cell lines, consisting of six *EGFR*- and four *KRAS*-mutated cell lines. H3255 expressed the highest level of *NKX2-1/ TTF-1* exon 4/5 mRNA, which is consistent with a previous study [26]. Overall, the expression levels of *NKX2-1/ TTF-1* exon 1 were positively correlated with those of exon 4/5. However, A549, a *KRAS*-mutated cell line, expressed *NKX2-1/ TTF-1* exon 1, despite lacking the expression of *NKX2-1/ TTF-1* exon 4/5, MYBPH, and TTF-1 protein expression (Figure 4C).

Clinical outcomes

The median follow-up period after surgery for all patients in the validation data set was 53.2 months. Overall survival (OS) rate was significantly associated with pathological stage (log-rank test, *p* < 0.0001; Breslow–Wilcoxon test, *p* < 0.0001) and pathological grade (log-rank test, *p* = 0.0008; Breslow–Wilcoxon test, *p* < 0.0001) (Figure 5A,B). Patients with TTF-1-positive tumours had significantly favourable OS (log-rank test, *p* < 0.0001; Breslow–Wilcoxon test, *p* < 0.0001) (Figure 5C), which is consistent with previous reports [27]. In addition, patients whose tumour exhibited no expression of *NKX2-1/ TTF-1* exon 1 had significantly shorter median OS (log-rank test, *p* = 0.0306; Breslow–Wilcoxon test, *p* = 0.0032); however, these differences were barely significant compared to TTF-1 protein expression (Figure 5D). Furthermore, among tumours with TTF-1 protein expression, median OS was shorter in those patients whose tumours exhibited no expression of *NKX2-1/ TTF-1* exon 1 than in those patients with expression of *NKX2-1/ TTF-1* exon 1, although these differences were not significantly different; similar results were obtained among TTF-1-negative LADs, suggesting that a tumour suppressive role of *NKX2-1/ TTF-1* transcript isoforms lacking exon 1 may be independent of TTF-1 protein expression (Figure 5E,F). In subgroup analyses of *EGFR*- and *KRAS*-mutated cases, there was no significant difference in survival between tumours with and without expression of *NKX2-1/ TTF-1* exon 1, although the latter had shorter OS (41 months in *EGFR*-mutated cases and 36 months in *KRAS*-mutated cases) (see supplementary material, Figure S3).

Discussion

To the best of our knowledge, this is the first study to assess the association between *NKX2-1/ TTF-1* isoforms and various clinicopathological parameters in LAD. These results of TSS-level expression of *NKX2-1/ TTF-1* revealed that LAD cells use at least 80% of *NKX2-1* TSSs, suggesting that each TSS and transcript isoform could play a distinct role in lung tumourigenesis, which contributes to the degree of heterogeneity of tumours. Furthermore, it was demonstrated that LAD with low expression of isoforms from TSS region 11, as well as from TSSs 2, 4, and 10, which regulate *NKX2-1_004* and *NKX2-1_005* containing exon 1, exhibited poor prognosis. Among *NKX2-1/ TTF-1* isoforms, *NKX2-1_001* and *NKX2-1_002* have been the focus of intense research activities. ‘The proximal major promoter’ that regulates *NKX2-1_001* contains a TATA-like element and binding site for Forkhead box A1 (FOXA1) (HNF3α), FOXA2 (HNF3β), and GATA-binding protein 6 (GATA6), all of which are known to be crucially involved in lung development, whereas ‘the minor distal promoter’ that regulates *NKX2-1_002* is modulated by the transcription factors SPI1 and SP3 [28]. However, the role of *NKX2-1_004* and *NKX2-1_005* containing exon 1 in lung development, homeostasis, and tumourigenesis remains largely unclear. Interestingly, *NKX2-1/ TTF-1* exon 1 is conserved across multiple species, including non-primate species, except fish, in contrast to other exons in *NKX2-1/ TTF-1* that are highly to completely conserved, regardless of species, on the University of California Santa Cruz Genome Browser [29], suggesting that *NKX2-1_004* and *NKX2-1_005*, especially *NKX2-1_004* containing a homeodomain, may play a pivotal role in the development and regulation of homeostasis of peripheral lung epithelial cells.

In the present study, LADs with no expression of *NKX2-1/ TTF-1* exon 1 were associated with poor survival outcomes. As *NKX2-1_005* lacks a homeodomain that binds DNA in a sequence-specific manner and transcriptionally activates target genes, reduced expression of *NKX2-1_004* rather than *NKX2-1_005* may induce aggressiveness in LAD, suggesting a tumour suppressive role of *NKX2-1_004*. Likewise, previous reports [27] along with these data show that reduced expression of TTF-1 is significantly associated with unfavourable prognosis in patients with LAD, indicating a tumour suppressive function of *NKX2-1/ TTF-1* in lung tumourigenesis. However, loss-of-function and gain-of-function studies in human lung carcinoma and transformed cells support a role of *NKX2-1* as an oncogene [7,30–33]. Furthermore, haploinsufficiency or conditional knockout of Nkx2-1/ Ttf-1 in a transgenic mouse model leads to enhanced...
development of *Kras*-mutated lung tumours, in contrast to suppression of *Egfr*-mutated lung tumours [26]. Notably, MYBPH, a direct transcriptional target of NKX2-1/TTF-1, reduces cell motility and metastasis in *Kras*-mutated cell lines [34]. In addition, NKX2-1/TTF-1-regulated microRNA-532-5p has a tumour suppressive role by targeting *Kras* in LADs [35]. These data suggested that NKX2-1/TTF-1 had both an oncogenic and suppressive role in lung tumourigenesis, which could be dependent on mitogenic driver mutations. Although the specific function of NKX2-1_004 in both LAD and normal lung tissues remains unclear at present, recent comprehensive epigenome and transcriptome analyses using Tracing Enhancer Networks using Epigenetic Traits (TENET) reveals that NKX2-1 is the top transcriptional regulator inactivated in LAD, and is linked to over a hundred inactivated enhancers [36]. Further studies by other approaches will be needed to elucidate transcriptional regulation of NKX2-1 in lung cancer development and the role of each splice variant in different genetic backgrounds. Nevertheless, it was demonstrated that no expression of NKX2-1/TTF-1 exon 1 was frequently detected in *EGFR* and *KRAS* wild-type tumours, suggesting that a tumour suppressive role of NKX2-1_004 might be independent of such oncogenic alterations.

IMA is a unique histological variant of LAD, which commonly lacks TTF-1 expression and expresses hepatocyte nuclear factors (HNFs), including HNF4α [25]. An inactivating mutation or epigenetic silencing of NKX2-1/TTF-1 downregulates its protein expression. Recently, while NKX2-1/TTF-1 inactivation mutations are rare, they are found in TTF-1-negative LADs, especially in IMA (33–43%) [37,38]. Furthermore, NKX2-1/TTF-1 is hypermethylated in the remaining TTF-1-negative cases; however, neither inactivation mutations nor hypermethylation is detected in some TTF-1-negative LADs [37], suggesting that other mechanisms of epigenetic silencing, such as microRNA and histone modification, may be involved in the downregulation of NKX2-1/TTF-1. In the present study, A549 cells exhibited expression of NKX2-1/TTF-1 exon 1, despite lack of expression of NKX2-1/TTF-1 exon 4/5 and subsequent TTF-1 protein expression in vitro, which is consistent with a previous study showing that neither NKX2-1_001 nor NKX2-1_002 transcripts are detected in A549 cells [14]. Moreover, it was identified that most IMA exhibited the same expression pattern. Thus, it is possible that a subset of LADs, including IMA, use an alternative TSS and subsequently express NKX2-1_005 lacking a homeodomain. It still unclear whether NKX2-1_005 translates into protein. However, accumulated evidence shows that long non-coding RNAs play a pivotal role in gene regulation [39]. RNAs insufficiently spliced from NKX2-1_005 are retained in the nucleus and might be linked with their specific subcellular localisations and functions in IMA, and may distinguish the biological behaviour of IMA from that of other conventional LADs. Alternatively, the protein translated from NKX2-1_005 might act in a dominant-negative manner, affecting interactions of other isoforms with cofactors, and thus affecting TTF-1 binding to its cognate sites.

However, this study has some limitations including undetermined transcriptome profiles of NKX2-1 in lymph nodes or in distant metastases, as NKX2-1/TTF-1 shows different functions depending on cell conditions [7]. Additional studies are required to clarify the clinicopathological impact of NKX2-1/TTF-1 exon 1 expression in LAD using samples from metastatic sites or recurrent disease.

In summary, these transcriptome and clinicopathological analyses reveal that LADs harbour at least 14 TSSs of NKX2-1/TTF-1, and decreased expression of NKX2-1/TTF-1 transcript isoforms with exon 1, such as NKX2-1_004, lead to poor prognosis in patients with LAD, most of which had a *EGFR/KRAS* wild-type genotype. *In situ* hybridisation for Epstein–Barr virus-encoded RNA is practically used in the pathology laboratory. Likewise, detection of specific exons or exon junctions by *in situ* hybridisation may be useful to further classify LADs. While these results are valuable as an indicator of a patient’s prognosis, further investigation targeting cancer-specific splice variants, such as NKX2-1_005 in IMA, may be novel potential targets for LAD.

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Author contributions statement

KSa, TH, SKi, MK, TY and TS provided pathological information. KT and KSu provided patient’s clinical information. KSa and SS carried out RNAscope assay and image analysis. MH, KT and SKo carried out bioinformatics analysis of the CAGE data set. KSa, TH, YS, KK, MI and TS conceived experiments and analysed data. All authors were involved in writing the paper and had final approval of the submitted and published versions. TH takes full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript.

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**SUPPLEMENTARY MATERIAL ONLINE**

**Figure S1.** Correlation of optical density and survival

**Figure S2.** Correlation analysis between CAGE and RNAscope

**Figure S3.** Kaplan–Meier curves of OS of 664 patients with LAD after surgical resection

**Table S1.** RNAscope probe design

**Table S2.** Expression level of NKX2-1/TTF-1 promoters in 71 LADs