Comparative analysis of TTF-1 binding DNA regions in small-cell lung cancer and non-small-cell lung cancer

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Thyroid transcription factor-1 (TTF-1, encoded by the NKX2-1 gene) is highly expressed in small-cell lung carcinoma (SCLC) and lung adenocarcinoma (LADC), but how its functional roles differ between SCLC and LADC remains to be elucidated. Here, we compared the genome-wide distributions of TTF-1 binding regions and the transcriptional programs regulated by TTF-1 between NCI-H209 (H209), a human SCLC cell line, and NCI-H441 (H441), a human LADC cell line, using chromatin immunoprecipitation-sequencing (ChIP-seq) and RNA-sequencing (RNA-seq). TTF-1 binding regions in H209 and H441 cells differed by 75.0% and E-box motifs were highly enriched exclusively in the TTF-1 binding regions of H209 cells. Transcriptome profiling revealed that TTF-1 is involved in neuroendocrine differentiation in H209 cells. We report that TTF-1 and achaete-scute homolog 1 (ASCL1, also known as ASH1, an E-box binding basic helix–loop–helix transcription factor, and a lineage-survival oncogene of SCLC) are coexpressed and bound to adjacent sites on target genes expressed in SCLC, and cooperatively regulate transcription. Furthermore, TTF-1 regulated expression of the Bel-2 gene family and showed antiapoptotic function in SCLC. Our findings suggest that TTF-1 promotes SCLC growth and contributes to neuroendocrine and antiapoptotic gene expression by partly coordinating with ASCL1.

1. Introduction

Thyroid transcription factor-1 (TTF-1, encoded by the NKX2-1 gene) is a homeodomain-containing master transcription factor (TF) of lung morphogenesis and differentiation of pulmonary epithelial cells (Kimura et al., 1996; Minoo et al., 1999). TTF-1 is expressed in 75–80% of lung adenocarcinoma (LADC), a non-small-cell lung cancer (NSCLC) subtype, and is a marker of the terminal respiratory unit (TRU) subtype (Yatabe et al., 2002). TTF-1-positive LADC patients show better prognosis than TTF-1-negative LADC

Abbreviations
ASCL1, achaete-scute homolog 1; CCLE, cancer cell line encyclopedia; ChIP-seq, chromatin immunoprecipitation-sequencing; EMT, epithelial–mesenchymal transition; GO, gene ontology; IB, immunoblotting; IHC, immunohistochemistry; IP, immunoprecipitation; LADC, lung adenocarcinoma; NSCLC, non-small-cell lung cancer; PLA, proximity ligation assay; qRT-PCR, quantitative real-time reverse transcription–PCR; RNA-seq, RNA-sequencing; SCLC, small-cell lung cancer; TF, transcription factor; TGF-β, transforming growth factor-β; TRU, terminal respiratory unit; TTF-1, thyroid transcription factor-1.
patients (Kim et al., 2018; Zhang et al., 2015). TTF-1 reduces invasion and metastasis in LADC (Hosono et al., 2012; Winslow et al., 2011); TTF-1 inhibits TGF-β-induced epithelial–mesenchymal transition (EMT) in LADC cells (Isogaya et al., 2014; Saito et al., 2009). In contrast, the NKK2-1 gene is amplified in 10–15% of LADCs and acts as a lineage-survival oncogene (Kwei et al., 2008; Tanaka et al., 2007). TTF-1 has a prosurvival function in cancer cells via ROR1 induction and LMO3 oncogene regulation (Watanabe et al., 2013; Yamaguchi et al., 2012). Thus, TTF-1 plays a double-edged role in LADC (Yamaguchi et al., 2013).

Although small-cell lung cancer (SCLC) has primitive neuroendocrine features distinct from LADC, 80–90% of SCLC tumors express pathologically high levels of TTF-1 (Misch et al., 2015). TTF-1 is expressed not only in SCLC but also in small-cell carcinoma of other organs, such as prostate (Wang and Epstein, 2008). Similar to achaete-scute homolog 1 (ASCL1, also known as ASH1), the NKX-homeodomain family TFs play a critical role in reprogramming normal human epithelial tissues to a neuroendocrine cancer lineage (Park et al., 2018), suggesting a critical function of TTF-1 in SCLC other than promoting epithelial cell differentiation. Conversely, the majority of SCLCs are of the peripheral type, and the peripheral-type SCLC expresses TTF-1 more frequently than does the central-type (Miyauchi et al., 2015), indicating that most SCLCs are derived from TRU cells expressing TTF-1. A recent report revealed that the expression of TTF-1 is positively regulated by ASCL1 in SCLC cell lines to induce nuclear factor I B-type (NFIB) (Horie et al., 2018).

The aforementioned studies strongly suggest a central role of TTF-1 in SCLC pathology. However, the difference in the roles of TTF-1 between SCLC and LADC remains to be elucidated. Employing chromatin immunoprecipitation-sequencing (ChIP-seq) and RNA-sequencing (RNA-seq), here we compared the genome-wide TTF-1 binding profiles and the TTF-1-mediated transcriptional programs in SCLC and LADC cell lines.

2. Materials and methods

2.1. Cell culture

Human SCLC NCI-H209 (H209) and NCI-H345 (H345), and NSCLC A549 and NCI-H441 (H441) were obtained from American Type Culture Collection (Manassas, VA, USA). Human SCLC Lu-135 and STC-1 cells were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). H209, H441, Lu-135, and STC-1 cells were cultured in RPMI 1640 (#11875; Thermo Fisher Scientific, Waltham, MA, USA). H345 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/Nutrient Mixture F-12 (1 : 1) medium (#11330; Thermo Fisher Scientific) with 5 μg·mL⁻¹ insulin, 5 μg·mL⁻¹ transferrin, 30 nM sodium selenite (#11884; Sigma-Aldrich, St. Louis, MO, USA), 10 nM β-estradiol (#E2257; Sigma-Aldrich), and 10 nM hydrocortisone (#H0135; Sigma-Aldrich). A549 cells were cultured in DMEM (#11965; Thermo Fisher Scientific). All culture media included 10% FBS (#SH30910.03; Thermo Fisher Scientific), 100 U·mL⁻¹ penicillin G, and 100 μg·mL⁻¹ streptomycin. All cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C.

2.2. Clinical samples

This study was certified by Ethics Committee in the University of Tokyo and in the Cancer Institute and was carried out in accordance with the Helsinki Declaration. Primary SCLC samples were obtained from patients undergoing pulmonary resection at the Department of Surgery, Cancer Institute Hospital, with written informed consent. These samples were immediately frozen with liquid nitrogen and stored at −80 °C. The frozen materials were microscopically examined by two independent pathologists and were dissected to enrich cancer cells when necessary. Total RNA was extracted using TRIzol (Thermo Fisher Scientific), confirming high quality of RNA with RNA integrity number ≥ 7.0, and expression array analysis using Affymetrix GeneChip Human Genome U133 Plus 2.0 oligonucleotide arrays (Fremont, CA, USA) was conducted previously (Sato et al., 2013). In overall survival analysis, the Kaplan–Meier curve was drawn and P-value was calculated by log-rank test using R (version 2.15.3) with ‘survival’ package (version 2.41.3) (http://www.R-project.org/). The end of follow-up period was 142 months from the primary surgery, and the mean follow-up time of the cases was 92 months. Death as a result of SCLC was the primary end point, and deaths by other causes were censored.

2.3. Antibodies

The following antibodies were used: anti-TTF-1 [for immunoblotting (IB) and ChIP, 1 : 1000 and 5 μg, respectively, #MS-699-P; Lab Vision Corporation, Fremont, CA, USA; for immunohistochemistry (IHC)]
2.4. Immunohistochemistry of tissue microarray

A tissue microarray of SCLC (LC818a) was obtained from US Biomax (Rockville, MD, USA). The array was deparaffinized and rehydrated followed by antigen retrieval using 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by 3.0% hydrogen peroxide. The array was then blocked with Blocking One reagent (Nacalai Tesque, Kyoto, Japan) and incubated with anti-TTF-1, anti-MASH1/ASCL1, and ChIP (5 µg), #556604; BD, Franklin Lakes, NJ, USA; anti-Bim (1 : 1000, #2933; Cell Signaling Technology, Danvers, MA, USA), and anti-Bcl-2 (1 : 100 for IHC, 1 : 1000 for IB, and 1 : 400 for immunofluorescence, #15071; Cell Signaling Technology).

2.5. Immunofluorescence

Paraffin-embedded H209 cells were treated as described above. The cells were stained with anti-TTF-1 and anti-Bcl-2 antibodies. Stained cells were visualized using anti-mouse IgG H&L (Alexa Fluor 594; Thermo Fisher Scientific), anti-rabbit IgG H&L (Alexa Fluor 488; Thermo Fisher Scientific), and DAPI. Images were captured with the all-in-one fluorescence microscope, BZ-X710 (Keyence, Osaka, Japan).

We evaluated three spots per tumor sample with a 20× objective. For TTF-1 and ASCL1 IHC, the fraction of stained tumor cells was scored as follows: 0, 0%; 1, 1–20%; 2, 21–50%; 3, 51–80%; and 4, > 81%. For Bel-2 IHC, the intensity of staining was scored as follows: 0, negative; 1, weak; 2, moderate; 3, strong; and 4, very strong. The IHC scores of each array spot were evaluated by a pulmonologist (S.H.).

2.6. In situ proximity ligation assay

We used Duolink kit (Olink, Uppsala, Sweden) for in situ PLA assay as previously described (Isogaya et al., 2014). The anti-TTF1 and anti-MASH1/ASCL1 were used as primary antibodies. Combination of the primary antibodies was determined so that no antibody cross-reacted with the PLA probe-conjugated secondary antibody to other primary antibodies. Vectashield mounting medium with DAPI (Vector Laboratories) was used as a nuclear counterstain. The experiment was performed twice with essentially the similar results.

2.7. Immunoblotting and immunoprecipitation

For IB, cells were rinsed with ice-cold PBS and lysed with RIPA buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and 0.5% sodium deoxycholate] that included cOmplete EDTA-free protease inhibitor (Roche Diagnostics, Basel, Switzerland). After centrifugation at 15 000 r.p.m. (20 400 g) and 4 °C for 10 min, protein concentrations were estimated using the BCA Protein Assay Kit (Thermo Fisher Scientific). The same amount of proteins was subjected to SDS/polyacrylamide gel electrophoresis and transferred to Fluoro Trans W membranes (Pall, Port Washington, NY, USA). For immunoprecipitation (IP), cultured cells were lysed with lysis buffer [1% Nonidet P-40, 150 mM NaCl, 20 mM Tris/HCl (pH 7.5), and cOmplete EDTA-free protease inhibitor]. Co-IP was performed as previously described (Koinuma et al., 2009a). IB was carried out as described previously (Katsura et al., 2017; Koinuma et al., 2011) and imaged with a LAS-4000 lumino Image analyzer (FUJIFILM, Tokyo, Japan). The experiments were repeated, and the representative data are shown in the figures.

2.8. RNA interference

Reverse transfection of Stealth Select siRNA (Thermo Fisher Scientific) was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific). We used two sets of siRNA: TTF-1 (siTTF-1) (#1: HSS144277 and #2: HSS144278) and ASCL1 (siASCL1) (#1: HSS100745 and #2: HSS181121). Medium GC Complex #2: 12935-112 (Thermo Fisher Scientific) was used as negative control siRNA (siNC).

2.9. RNA extraction and quantitative real-time reverse transcription–PCR

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNAs were
synthesized using PrimeScript II reverse transcriptase and oligo dT primers (Takara Bio, Shiga, Japan) according to the manufacturer’s instructions. Quantitative real-time reverse transcription–PCR (qRT-PCR) was performed with the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) and the FastStart Universal SYBR Green Master Mix (ROX) (Roche Diagnostics). All samples were run in duplicate, and results were averaged and normalized to the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Primer sequences are shown in Table S1.

2.10. Chromatin immunoprecipitation, ChIP-seq, and data analysis

ChIP-qPCR and ChIP-seq of H441 and H209 cells were performed using anti-TTF-1 antibody or anti-ASCL1 antibody as described previously (Koinuma et al., 2009b). Data were obtained as two biological replicates. The TTF-1 ChIP-seq of H441 cells has been published [available at Gene Expression Omnibus (GEO; GSE51510)] (Isogaya et al., 2014), and we additionally obtained new data as a biological replicate in this study. For ChIP-seq data analysis, reads were trimmed down to 50 bp to compare with the published datasets and were aligned against the human reference genome (NCBI Build 36, hg19) with BOWTIE (Langmead et al., 2009). Peaks of TTF-1 and ASCL1 ChIP-seq were called using MACS2 (Zhang et al., 2008) by two-sample analysis using default parameters, where input genomic DNA was used as a negative control. Mapped sequence data were visualized using Integrative Genomics Browser. Gene annotations and gene ontology (GO) analysis for TTF-1 and ASCL1 ChIP-seq data were performed using GREAT version 3.0.0 with default parameters and whole genome as background (McLean et al., 2010). De novo motif discovery and motif centrality analysis for TTF-1 and ASCL1 ChIP-seq data were performed using GREAT version 3.0.0 with default parameters and whole genome as background (Langmead et al., 2009). De novo motif discovery and motif centrality analysis for TTF-1 and ASCL1 ChIP-seq were conducted with MEME-CHEP ver 5.0.5 (Machanick and Bailey, 2011), which internally used DREME version 5.0.5 and CENTRIMO version 5.0.5 (Bailey and Machanick, 2012). The 500-bp genomic sequences flanking the peak summits of the binding regions were used for calculation. Default parameters were used except for the number of motifs (8) and the minimal length of the motif (5) for MEME. Primer sequences for ChIP-qPCR are shown in Table S2. The full list of the motifs reported by DREME is available as Data S1–S3.

2.11. RNA-seq and data analysis

RNA-seq was performed as described previously (Isogaya et al., 2014; Kawasaki et al., 2018). For RNA-seq data analysis, reads were aligned against the human genome (NCBI Build 36, hg19) using tophat2 (https://ccb.jhu.edu/software/tophat/). Differential expression was evaluated using the Cuffdiff function of CUFFLINKS (http://cufflinks.cbcb.umd.edu/). Gene set enrichment analysis (GSEA) (Subramanian et al., 2005) was used for gene functional classification.

2.12. Plasmid construction and cDNA transfection

Plasmids encoding human TTF-1 and ASCL1 were constructed by PCR amplification. Fragments were subcloned into pcDNA3-6xMyc vector (TTF-1) or pcDNA3-FLAG vector (ASCL1). All cDNAs were verified by sequencing. Transient transfection into cells was performed using Lipofectamine 3000 reagent (Thermo Fisher Scientific), as recommended by the manufacturer’s protocol. Plasmids for adenoviral expression vectors of LacZ and TTF-1 were prepared as previously described (Saito et al., 2009).

2.13. Adenovirus production and infection

Adenoviruses for transduction of LacZ (Ad-LacZ) or TTF-1 (Ad-TTF-1) were generated using ViraPower Adenoviral Expression System (Thermo Fisher Scientific). Adenovirus titer was determined using the Adeno-X Rapid Titer Kit (Takara-Clontech, Shiga, Japan). Multiplicity of infection 200 was used for infection using Ad-TTF-1. The same corresponding multiplicity of infection for Ad-LacZ was used as control.

2.14. Cell proliferation assay

H209 cells were seeded at a density of $1 \times 10^5$ per well into a 24-well plate with siRNA transfection. After 72 h, cell proliferation was evaluated with Cell Count Reagent SF (Nacalai Tesque). Absorbance at 450 nm was measured with a Model 680 Microplate Reader (Bio-Rad, Melville, NY, USA), and the absorbance at 595 nm was deducted from it.

2.15. Apoptosis assay

H209 cells were seeded at a density of $5 \times 10^5$ per well into a 6-well plate with siRNA transfection. After 72 h, cells were stained with Annexin V and propidium iodide (eBioscience, Vienna, Austria) for 15 min at room temperature prior to detection in Gallios (Beckman Coulter, Brea, CA, USA). Results were analyzed using the FLOWJO software (BD).
Fig. 1. High expression of NKX2-1 in a subset of SCLC. (A) Expression of NKX2-1 mRNA (encoding TTF-1) in various cancers from the CCLE database. Normalized expression of the microarray data was calculated by robust multichip analysis (RMA). (B) Lung cancer cell datasets from CCLE were divided into SCLC ($n=52$), LADC ($n=73$), and squamous cell carcinoma ($n=28$). $*P<0.05$, one-way ANOVA with Dunnett’s test. (C) Expression of NKX2-1 in 23 clinical SCLC tumors and 42 normal tissues (GSE43346). Red dotted bar indicates the average expression of NKX2-1 in the normal tissues. $***P<0.001$, unpaired $t$-test. (D) Comparison of NKX2-1 expression in the clinical SCLC samples (GSE43346) between the classic ($n=12$) and variant ($n=11$) subtypes. Bars indicate the mean and S.E. $***P<0.001$, unpaired $t$-test. (E) Relationship between NKX2-1 expression and overall survival in SCLC patients (GSE43346) (Sato et al., 2013) analyzed by the Kaplan–Meier plot. Patients were divided into NKX2-1low (GeneChip score < 250, $n=13$) and NKX2-1high (score > 250, $n=10$). $P$-value was calculated by log-rank test. (F) qRT-PCR analysis of NKX2-1 mRNA in lung cancer cells used in this study. Data represent means of the two biological replicates. Error bars, SE. (G) IB for TTF-1 in the lung cancer cell lines.
2.16. Statistical analysis

Student’s t-test was used for two-sample analyses. Comparisons of the multiple experimental groups were made using one-way ANOVA with Dunnett’s test. The Mann–Whitney U-test was used for IHC score data. Comparisons of the frequency were made using the chi-square test. Statistical analyses were conducted with PRISM 7.00 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. TTF-1 is highly expressed in a subset of SCLC in association with poor prognosis

Investigation of microarray data from the Cancer Cell Line Encyclopedia (CCLE) (Barretina et al., 2012) showed that the median values of NKX2-1 expression in lung cancers were higher among cancers originating from different tissues (Fig. 1A), and SCLC showed significantly higher expression than other types of lung cancers (Fig. 1B). The expression of NKX2-1 in clinical SCLC tumors was also higher than that in normal tissues except in thyroid and lung, which physiologically express TTF-1 (Fig. 1C) (Sato et al., 2013). Our previous microarray dataset of SCLC tumors revealed that the classic-type SCLC was characterized with lower AJUBA (also known as JUB) and higher GRP expression when compared with the variant SCLC group. Classic-type SCLC was associated with poor prognosis, and NKX2-1 expression was significantly higher in this type (Fig. 1D) (Sato et al., 2013). Although a recently published paper reported the association of high NKX2-1 expression and poor SCLC patient prognosis (Yan et al., 2019), NKX2-1 expression was not significantly associated with overall survival in this cohort (Fig. 1E) (log-rank test, \( P = 0.09 \)).

We then investigated the expression of NKX2-1 in LADC and SCLC cell lines. H209 and H345 cells (classified as classic-type SCLC cell lines according to the neuroendocrine feature) (Horie et al., 2016) highly expressed TTF-1 mRNA and protein (Fig. 1F,G).

3.2. TTF-1 binding regions in LADC and SCLC cells show little overlap in distribution

Genome-wide distribution of TTF-1 binding regions in SCLC and LADC cells was compared by ChIP-seq in the H209 and H441 cell lines. Binding regions of 48,421 and 26,752 were identified from each of the two biological replicates in H209 cells \( (q < 10^{-5}) \). In H441 cells, 58,099 and 74,258 binding regions were identified in each of the two biological replicates \( (q < 10^{-5}) \). We calculated the intersection of the biological replicates for each cell line and used 21,871 (H209) and 51,454 (H441) TTF-1 binding regions in the following analyses, respectively. The known NKX2-1 motif was enriched and had a centrality for both cell lines (Fig. 2A), supporting the validity of the data. Significant peaks were found at known TTF-1 binding sites in H441 LADC cells, for example, the SFTPB gene (encoding surfactant protein B) locus but not at the HBB gene locus used as a negative control (Fig. S1A). In contrast, TTF-1 binding at SFTPB locus in H209 cells was not significant at the present settings (Fig. S1A) and ChIP-qPCR suggested very weak binding compared to H441 cells (Fig. S1B).

Surprisingly, comparisons between H441 and H209 cells revealed little overlap of the TTF-1 binding regions at the genome-wide level (Fig. 2B). KRT4 and SDPR gene loci, which were TTF-1-bound genes in LADC cells (Isogaya et al., 2014), had peaks only in H441 cells, whereas NFIB and NCAM1 gene loci, which are known as oncogenes for SCLC (Calbo et al., 2011; Semenova et al., 2016), had peaks mainly in H209 cells (Fig. 2C). We confirmed the similar tendency by ChIP-qPCR (Fig. 2D). Furthermore,
Differential TTF-1 binding regions in lung cancer

A. TTF-1 binding motif

B. TTF-1 ChIP-seq peaks

C. Position of best site in sequence

D. TTF-1 ChIP-qPCR

E. Adenocarcinoma

F. H441 cells

H209 cells

| Rank | Calculated motif (E-value (DREME)) | Similar motifs | Distribution |
|------|-----------------------------------|----------------|--------------|
| 1    | AGGCA   | AP-1           | Forkhead     |
| 2    | GGGG    | NKX-homeodomain| E-box        |
| 3    | TCC     | STAT           | SP1          |
| 4    | AAAA    | Forkhead       |              |

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comparisons using published TTF-1 ChIP-seq data of other NSCLC cell lines (SRP045118) (Clarke et al., 2015) revealed that while TTF-1 binding regions in NCI-H1819 and HCC1195 cells showed more overlap with H441 cells, TTF-1 binding regions in HCC1833 cells, which were derived from LADC with neuroendocrine features (Kosari et al., 2014), showed comparable ratios of overlap in H209 and H441 cells (Fig. 2E). These findings suggest that TTF-1 plays highly different roles in LADC and SCLC.

De novo motif analysis of the TTF-1 binding regions in H441 and H209 cells was performed to understand the differences in their binding sequence preferences. Predictably, NKX-homeodomain motif emerged as one of the top preferred motifs in both cell lines, while the Forkhead family TFs (the known cooperative TFs of TTF-1) binding motifs were also commonly identified (Fig. 2F). Among differentially preferred motifs, AP-1 was strongly enriched in H441 cells, consistent with a previous report (Maeda et al., 2012), whereas the E-box motif was enriched specifically in H209 cells. Both ASCL1 and NEUROD1, members of the E-box binding TFs, were the lineage-specific TFs and differentially regulate key oncogenes in SCLC (Borromeo et al., 2016), suggesting that TTF-1 plays a role in the development of SCLC through these TFs.

Gene ontology analysis of nearby genes calculated from the ChIP-seq data indicated that in H209 cells, the TTF-1-bound genes were related to the biological process terms related to neuron differentiation and aorta morphogenesis (Fig. S1C), suggesting the involvement of TTF-1 in cellular morphology and differentiation in SCLC. In addition, the molecular function terms included BH3/BH domain binding (Fig. S1C), indicating that TTF-1 may associate with Bel-2 family genes, which regulate apoptosis. In contrast, TTF-1-bound genes in H441 cells were related to the MAP kinase pathway and cell-to-cell junction and no terms in the top 20 list were shared by the two cell lines (Fig. S1C).

### 3.3. TTF-1 and ASCL1 bind to common genomic regions in SCLC cells

Gene expression profiling of SCLC cell lines revealed that the expression of NKX2-1 showed positive correlation with ASCL1 but not with NEUROD1, whereas the expression of MYC, a target of NEUROD1, and NKX2-1 exhibited negative correlation (Fig. S2A). Likewise, NKX2-1 and ASCL1 were coordinately expressed in SCLC tissue samples (GSE43346) (Fig. S2B). Investigations of the ASCL1, NEUROD1, and MYC expressions in several SCLC cell lines revealed a similar pattern (Fig. S2C). These findings suggest that expressions of TTF-1 and ASCL1 are strongly related.

Although the coexpression of TTF-1 and ASCL1 and their binding motif-based analyses have been reported in SCLC (Gazdar et al., 2017; Park et al., 2018), direct relationship between these TFs has not been fully investigated at a genome-wide level. To this end, we obtained ASCL1 ChIP-seq data from other SCLC cell lines (GSE69398). According to ASCL1 ChIP-seq data in the ASCL1-expressing cell lines (NCI-H128, NCI-H2107, and NCI-H889) and NEUROD1 ChIP-seq data in the NEUROD1-expressing cell lines (NCI-H82 and NCI-H524), TTF-1 binding regions in H209 cells had more overlap with the ASCL1 binding regions than with the NEUROD1 binding regions (Fig. 3A). We next carried out ASCL1 ChIP-seq in H209 cells and identified 13,920 and

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**Fig. 3.** Interaction between TTF-1 and ASCL1 proteins in SCLC. (A) Charts showing the overlaps with TTF-1 binding regions in ASCL1 or NEUROD1 binding regions. The binding regions of ASCL1/NEUROD1 were identified by ChIP-seq data of SCLC cell lines (GSE69398). (B) A chart showing the overlaps with TTF-1 binding regions in ASCL1 binding regions in H209 cells. (C) A heat map representation of TTF-1 and ASCL1 ChIP-seq peaks. The 500-bp sequences flanking the summit position of each TTF-1 or ASCL1 binding region were used for the analysis. The known NKX-homeodomain binding motif (Nkx2-5, MA0503.1) and ASCL1 binding motif (ASCL1, MA1100.1) were used for calculation. The x-axis indicates the relative position (bp) of the best site from the peak summit of each binding region. (E) Co-IP assay of HEK293T cells transfected with expression plasmids for 6xMyct-TTF-1 and FLAG-ASCL1. (F) In situ PLA using TTF-1 and ASCL1 antibodies in H209 cells to show their proximity in the nucleus. H209 cells treated only with the TTF-1 antibody were used as a control. Proximity between TTF-1 and ASCL1 was detected as signals (red) in the nuclei (DAPI, blue). Scale bars, 50 µm. (G) Anti-TTF-1 and ASCL1 IHC on a tissue microarray of SCLC. The fraction of stained cancer cells was scored as shown in Fig. S4A. Representative images of TTF-1 and ASCL1 IHC staining scores in the SCLC tissue microarray. (H) Correlations of TTF-1 and ASCL1 IHC staining scores in the SCLC tissue microarray. r, Spearman’s correlation coefficient. (I) Scatter plots of TTF-1 (left) and ASCL1 (right) staining scores in a SCLC tissue microarray divided into two groups according to the SCLC stage. Data are represented as mean ± SE (*P < 0.05, **P < 0.01, Mann-Whitney U-test).
Differential TTF-1 binding regions in lung cancer

A. ASCL1 ChIP-seq

- H128: Total peaks = 5662 (q < 10^-5)
- H2107: Total peaks = 6535 (q < 10^-5)
- H889: Total peaks = 28,645 (q < 10^-5)

B. ASCL1 ChIP-seq

- H209: Total peaks = 8949 (q < 10^-5)

C. TTF-1 binding regions

D. H209 ASCL1 TTF-1

E. 6xMyc-TTF-1 – + + – + – + + 10% input
   FLAG-ASCL1 – – + – – +

F. H209 Control TTF-1 + ASCL1

G. IHC (TTF-1) IHC (ASCL1)

H. Average scores (ASCL1) vs. Average scores (TTF-1)

I. Average scores (TTF-1) Average scores (ASCL1)

r = 0.7741
P < 0.001

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11 141 ASCL1 binding regions from each of the two biological replicates \((q < 10^{-5})\). The 8949 common binding regions were then used in the following analyses. The known ASCL1 binding motif was significantly enriched and had a clear centrality (Fig. S3A), supporting the validity of the data. ASCL1 binding regions largely overlapped with the TTF-1 binding regions in H209 cells but not with those in H441 cells (Fig. 3B,C). Moreover, motif centrality analysis in H209 cells showed unimodal distribution of the NKX-homeodomain motif and bimodal distribution of the ASCL1 motif in the overlapping regions between TTF-1 and ASCL1 ChIP-seq peaks (Fig. 3D), suggesting that TTF-1 and ASCL1 are closely located in the genomic DNA.

### 3.4. TTF-1 physically and functionally interacts with ASCL1

We then found the physical interaction between TTF-1 and ASCL1 using HEK293T cells ectopically expressing both TFs (Fig. 3E). Formation of the endogenous TTF-1-ASCL1 complex in H209 cells was also observed by in situ PLA to find their nuclear distribution (Fig. 3F). The predicted GO terms of the ASCL1-bound genes were in part common to those of the TTF-1-bound genes, such as 'BH domain binding' and 'aorta morphogenesis' (Fig. S3B). Furthermore, de novo motif analysis in the overlapping regions between TTF-1 and ASCL1 ChIP-seq peaks identified both E-box and NKX-homeodomain motifs (Fig. S3C). These findings suggest that in SCLC cells, TTF-1 interacts physically and functionally with ASCL1.

We also examined the relationship between TTF-1 and ASCL1 expression in clinical SCLC tumors using a tissue array. IHC revealed that the expression of TTF-1 or ASCL1 was then silenced to target gene expression in neuronal system, for example, \(\text{DLL3}\), \(\text{SYTL3}\), \(\text{TACR1}\), and \(\text{TESC}\), suggesting that both TTF-1 and ASCL1 were involved in the maintenance of neuroendocrine features in SCLC cells. One of the genes upregulated by both siRNAs was \(\text{BCL2L11}\) (encoding BIM) of the proapoptotic \(\text{BCL2}\) family gene.

Additionally, both TTF-1 and ASCL1 scores were significantly higher in advanced-stage tumors than in earlier stage ones (Fig. 3I).

### 3.5. TTF-1 and ASCL1 cooperatively regulate target gene expression

Expression of TTF-1 or ASCL1 was then silenced to evaluate their effects on target gene expression. We noticed that TTF-1 knockdown led to ASCL1 upregulation (Fig. 4A,B), and ASCL1 knockdown led to upregulation of TTF-1 protein in H209 cells (Fig. 4C,D). Although TTF-1 and ASCL1 were coexpressed in SCLC (Fig. 3G,H), these findings suggested a tight regulation of the amounts of both TFs.

Significant TTF-1 binding and ASCL1 binding were observed in the promoter region of \(\text{CALCA}\) (encoding \(\text{CGRP}\)) and \(\text{DLL3}\) genes (Fig. 4E,F), which are the genes characteristic of the neuroendocrine phenotype. We then conducted the knockdown experiments with siRNAs for TTF-1 and ASCL1 using H209 cells (Fig. S5A,B) and assessed mRNA expression of these genes. The limited effect of each siRNA on the expression of \(\text{CALCA}\) and \(\text{DLL3}\) possibly reflected the mutual regulation between TTF-1 and ASCL1, and enhanced inhibition of \(\text{CALCA}\) and \(\text{DLL3}\) expression was observed when both TTF-1 and ASCL1 were depleted (Fig. 4G).

RNA-seq was then performed following the knockdown of TTF-1 or ASCL1 to find their targets in H209 cells (Fig. 4H). Some genes downregulated by both TTF1 and ASCL1 siRNAs were expressed in neuronal system, for example, \(\text{LRRN4}\), \(\text{SYTL3}\), \(\text{TACR1}\), and \(\text{TESC}\), suggesting that both TTF-1 and ASCL1 were involved in the maintenance of neuroendocrine features in SCLC cells. One of the genes upregulated by both siRNAs was \(\text{BCL2L11}\) (encoding BIM) of the proapoptotic \(\text{BCL2}\) family gene.

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**Fig. 4.** Cooperative regulation of the expression of target genes by TTF-1 and ASCL1 in SCLC. (A) Expression of \(\text{NKX2-1}\) (encoding TTF-1; left) or \(\text{ASCL1}\) (right) mRNA in H209 cells treated with negative control (siNC) or TTF-1 siRNAs (siTTF-1) by qRT-PCR. (B) IB for TTF-1 and ASCL1 in H209 cells treated with siNC or siTTF-1. (C) Expression of \(\text{NKX2-1}\) and \(\text{ASCL1}\) mRNAs in H209 cells treated with siNC and ASCL1 siRNAs (siASCL1), (D) IB for TTF-1 and ASCL1 in H209 cells treated with siNC or siASCL1. (E) Visualization of the TTF-1 and ASCL1 ChIP-seq data at the \(\text{CALCA}\) and \(\text{DLL3}\) gene loci. The upper two lanes show TTF-1 binding signals in H441 cells. The middle or lower two lanes show TTF-1 or ASCL1 binding signals in H209 cells, respectively. Rep 1 and Rep 2 indicate biological replicates 1 and 2. The kb sizes denote the ranges shown in the panels. Arrows show the position of ChIP-qPCR analysis evaluated in (F). (F) ChIP-qPCR analysis of TTF-1 and ASCL1 at the genomic regions shown in (E). %input values at the target regions were normalized to those in \(\text{HBB}\) locus. (G) Fold change in the expression of \(\text{CALCA}\) and \(\text{DLL3}\) mRNA in H209 cells treated with siTTF-1, siASCL1, or both, relative to those treated with siNC. Expression of mRNAs was quantified by qRT-PCR. (H) Genes down- or upregulated by both TTF-1 and ASCL1 in H209 cells. The genes with overlapping peaks between TTF-1 and ASCL1 ChIP-seq data were selected from the commonly regulated genes of TTF-1 and ASCL1 determined by RNA-seq of TTF-1- or ASCL1-depleted cells. These genes are listed in the boxes. qRT-PCR data represented as mean ± SE of the three independent experiments. **\(P < 0.01\), ***\(P < 0.001\), one-way ANOVA with Dunnett’s test.
**A**

Relative expression of NKK2-1 and ASCL1 with siNC and siTTF-1 for H441 and H209 TTF-1.

**B**

Western blot analysis of TTF-1 and ASCL1 for IB; TTF-1 and IB; Tubulin.

**C**

Relative expression of NKK2-1 and ASCL1 with siNC and siASCL1 for H441 and H209 TTF-1.

**D**

Western blot analysis of TTF-1 and ASCL1 for IB; TTF-1 and IB; Tubulin.

**E**

Enrichment of ChIP-qPCR for TTF-1 and ASCL1 in H441 and H209 TTF-1 and H209 ASCL1.

**F**

Enrichment of TTF-1 ChIP-qPCR and ASCL1 ChIP-qPCR in H441 and H209.

**G**

Relative mRNA expression of CALCA and DLL3 with siRNA for H441 and H209.

**H**

Common target genes of TTF-1 and ASCL1:

- **Transcriptional activation**
  - DEGS2
  - KCNH6
  - KIFC3
  - LRRN4
  - POU6F2
  - RNF183
  - SYTL3
  - TACR1
  - TESC
  - TMEM158
  - TSPEAR

- **Transcriptional repression**
  - ADCYAP1
  - BCL2L11
  - CNN2
  - DLK1
  - FN1
  - GABRB3
  - RBFOX2
  - SDC2
  - SHISA2
  - FOXN3
We confirmed that BIM protein was upregulated by TTF-1 and/or ASCL1 knockdown in H209 cells (Fig. S6A,B).

3.6. TTF-1 positively regulates the expression of Bcl-2

We further focused on BCL2 expression as a TTF-1-bound target gene. Gene expression profiling of SCLC cell lines in CCLE dataset revealed that BCL2 exhibited positive correlation with NKX2-1 (Fig. 5A). Moreover, TTF-1 knockdown downregulated the mRNA expression of BCL2 in H209 cells (Fig. 5B). We also confirmed that knockdown of TTF-1 decreased the Bcl-2 expression by the immunofluorescent assay in H209 cells (Fig. 5C). IHC of a SCLC tissue array showed the expression of TTF-1 positively correlated with Bcl-2 (Fig. 5D,E and Fig. S4B). Although ASCL1 also reportedly upregulates BCL2 in some of the SCLC, the effect of ASCL1 siRNA on BCL2 was not remarkable in H209 cells (data not shown). These results suggest a complementary role of TTF-1 in the regulation of BCL2.

3.7. TTF-1 promotes survival of SCLC cells and regulates the genes associated with poor prognosis

We postulated that TTF-1 promotes survival of SCLC cells through inhibition of apoptosis, similar to ASCL1 (Murai et al., 2015). Using WST-8 assay, we revealed that TTF-1 silencing in H209 cells resulted in decreased cell viability (Fig. 5F). On the contrary, ectopic TTF-1 by adenovirus vector increased cell viability in TFF-1-low Lu-135 cells (Fig. 5G and Fig. S7). We further investigated whether TTF-1 knockdown could enhance apoptosis of H209 cells. As a nature of SCLC cell lines, baseline population of apoptotic cells in the culture was high (Horie et al., 2018). However, the fractions of Annexin-positive apoptotic cells marginally but significantly increased after TTF-1 knockdown (Fig. 5H).

Finally, we examined enrichment of 166 genes, which were downregulated by TTF-1 knockdown in H209 cells, using expression arrays of clinical SCLC samples (GSE43346) to explore its relationship to patient prognosis. When this SCLC cohort was divided into good and poor prognosis groups, TTF-1-regulated genes were significantly enriched in the poor prognosis group (Fig. 5I), which also suggested the tumor-promoting role of TTF-1 in clinical SCLC tumors.

4. Discussion

In this study, we clarified the distinct properties of TTF-1 binding regions between SCLC and LADC. Our findings suggested that TTF-1 promotes SCLC growth and contributes to neural differentiation by partly coordinating with ASCL1. One of the representative motifs commonly enriched in the TTF-1 binding regions in both H441 and H209 cells was that of the Forkhead family genes. Forkhead family TFs are pioneer factors that target enhancers for tissue-specific gene activation during development and cellular reprogramming (Iwafuchi-Doi et al., 2016). In a murine LADC model, TTF-1 physically binds and interacts with FOXA, providing a direct connection between transcriptional lung differentiation programs and tumor initiation (Snyder et al., 2013). Therefore, the differential TTF-1 binding regions between SCLC and LADC appear to be determined epigenetically by Forkhead TFs.

Previous comprehensive genomic studies indicate that SCLC harbors several somatic mutations, such as deletion of the TP53 and RB1 genes (Peifer et al., 2012). Moreover, several TF genes are amplified,
including MYC, NFIB, and SOX2 (George et al., 2015; Kim et al., 2006; Rudin et al., 2012). Among them, ASCL1 is believed to be a key regulator of neuroendocrine differentiation (Borges et al., 1997; Osada et al., 2008) and a lineage-survival oncogene of SCLC (Borromeo et al., 2016). ASCL1 is also known
as a repressive target of Smad TFs downstream of transforming growth factor-β (TGF-β) and is required for tumor formation by suppressing apoptosis in SCLC cells (Murai et al., 2015). ASCL1, insulinoma-associated 1 (INSM1) zinc finger transcription factor, and BRN2 collaborate to form regulatory circuitry involved in neuroendocrine differentiation of SCLC (Borromeo et al., 2016; Fujino et al., 2015; Sakaeda et al., 2013). Furthermore, concomitant enrichment of proneural TFs (including ASCL1) and NKX-homeodomain TFs (including TTF-1) is critical for transformation to small-cell neuroendocrine carcinoma (Park et al., 2018). Consistently, our genome-wide analysis of SCLC cells revealed that TTF-1 binding regions and TTF-1-regulated genes are associated with cellular differentiation and neural development. Of note, LADC that expresses ASCL1 shows neuroendocrine phenotype (Miyashita et al., 2018). Other reports indicate that LADC transforms into SCLC during the course of clinical treatments (Ferrer et al., 2019; Marcoux et al., 2019). In LADC cells, TTF-1 interacts with the Smad family TFs downstream of TGF-β signaling to inhibit EMT, providing a mechanism of tumor suppressor function of TTF-1 (Isogaya et al., 2014). TGF-β signaling is frequently silenced in SCLC (Murai et al., 2015). The study of dynamics of changes in genomic distribution of TTF-1 and its co-TFs, during the transformation of LADC into SCLC, could help clarify its complex roles during tumor progression and differentiation.

Bcl-2, one of the transcriptional targets of ASCL1, is known as an antiapoptotic regulator and acts as an oncogene in neuroendocrine lung cancers (Augustyn et al., 2014). Our results revealed that TTF-1 positively regulated the expression of Bcl-2 in SCLC cells and was coexpressed in clinical tissues. Bcl-2 expression can be presumably enhanced by TTF-1 in clinical tumors, which in turn may participate in SCLC progression. Consistently, Cardnell and colleagues have reported the association between TTF-1 expression and sensitivity to a Bcl-2 inhibitor (Cardnell et al., 2017). Considering the cooperative regulation of proapoptotic BIM expression, relationship between TTF-1 and ASCL1 might be of special clinical significance as a predictive marker of SCLC treatment.

5. Conclusions

Our results revealed distinct properties of TTF-1 distribution on the genome in SCLC. Our genome-wide analysis unraveled different roles of TTF-1 between LADC and SCLC and revealed its transcriptional regulatory programs related to antiapoptotic and neuroendocrine gene expression in SCLC.

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Conflict of interest

KM and SE were partly supported by Eisai, Co., Ltd. The remaining authors declare no conflict of interest.

Data availability

The raw ChIP-seq and RNA-seq data have been deposited to GEO (GSE129341).

Author contributions

SH performed most of the in vitro experiments together with YT and A Katsura. SH performed the immunohistochemistry. FM and SE determined the experimental conditions of the SCLC cell lines. SH, YT, A Kaneda, MM, ST, HA, and DK acquired and analyzed the high-throughput sequencing data. SH, DK, and KM conceived and designed the project. A Kaneda, YI, and HA obtained and analyzed the patient samples. SH, DK, and KM wrote the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Comparison of TTF-1 ChIP-seq data in H441 and H209 cells.

Fig. S2. Positive correlation between NKX2-1 and ASCL1 expression in small cell lung cancer cell lines and tissue samples.

Fig. S3. Characteristics of ASCL1 ChIP-seq data in H209 cells.

Fig. S4. Immunohistochemistry (IHC) scores of TTF-1 and Bcl-2.

Fig. S5. Validation of ASCL1 siRNA and double knockdown with TTF-1 and ASCL1 siRNAs.

Fig. S6. Regulation of BIM expression by TTF-1 and ASCL1 in H209 cells.

Fig. S7. Validation of TTF-1 adenoviral expression vector.

Table S1. Primer sequences for qRT-PCR analyses of human mRNA.

Table S2. Primer sequences for ChIP-qPCR analyses.

Data S1. Output data of the motif analysis of TTF-1 binding regions in H209 cells using DREME, supporting data for Figure 2F.

Data S2. Output data of the motif analysis of TTF-1 binding regions in H441 cells using DREME, supporting data for Figure 2F.

Data S3. Output data of the motif analysis of TTF-1/ASCL1 co-binding regions in H209 cells using DREME, supporting data for Figure S3C.