An integrative analysis of the tumorigenic role of TAZ in human non-small cell lung cancer


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Translational Relevance

TAZ has emerged as a key player in organ growth and tumorigenesis. We combined *in vitro, in vivo, and in situ* analyses to define the role of TAZ in human lung cancer. The oncogenic properties of TAZ were confirmed in cell culture experiments and in a mouse model. The analysis of a large cohort of non-small cell lung cancer (NSCLC) patients revealed inter-individual diversity in TAZ protein and mRNA levels, and that NSCLC patients with higher tumor TAZ expression levels have an unfavorable prognosis. Moreover, we demonstrated that TAZ regulates ErbB ligands, including amphiregulin, epiregulin, and neuregulin 1, and that the EGFR signaling pathway is activated in NSCLC tissues with high TAZ expression. The results not only confirm the clinical relevance of TAZ in NSCLC patients but also offer a conceptual basis for novel therapeutic strategies in subgroups of NSCLC patients.
Abstract

Purpose: TAZ, also known as WWTR1, has recently been suggested as an oncogene in non-small cell lung cancer (NSCLC). We investigated the clinical relevance of TAZ expression and its functional role in NSCLC tumorigenesis.

Experimental Design: We characterized TAZ at the DNA ($n = 192$), mRNA ($n = 196$), and protein levels ($n = 345$) in an NSCLC patient cohort. Gene expression analysis was complemented by a meta-analysis of public data sets ($n = 1,382$). The effects of TAZ on cell proliferation and cell cycle were analyzed in cell cultures and on tumor growth in mice. TAZ-dependent microarray-based expression profiles in NSCLC cells were combined with molecular profiles in human NSCLC tissues for in silico analysis.

Results: Higher TAZ mRNA and protein levels were associated with shorter patient survival. Transduction of TAZ enhanced cell proliferation and tumorigenesis in bronchial epithelial cells, while TAZ silencing suppressed cell proliferation and induced cell cycle arrest in NSCLC cells. Microarray and cell culture experiments showed that ErbB ligands (amphiregulin, epiregulin, and neuregulin 1) are downstream targets of TAZ. Our in silico analysis revealed a TAZ signature that substantiated the clinical impact of TAZ and confirmed its relationship to the epidermal growth factor receptor signaling pathway.

Conclusion: TAZ expression defines a clinically distinct subgroup of NSCLC patients. ErbB ligands are suggested to mediate the effects of TAZ on lung cancer progression. Our findings emphasize the tumorigenic role of TAZ and may serve as the basis for new treatment strategies.
Introduction

Lung cancer is the leading cause of cancer death worldwide. Despite the high histological heterogeneity, the overall prognosis for lung cancer is extremely poor, with non-small cell lung cancer (NSCLC) representing 85% of all diagnosed cases (1). Through the thorough characterization of human cancer tissues, distinct molecular aberrations have been identified in small subgroups of patients and are now being successfully exploited for therapeutic intervention, including epidermal growth factor receptor (EGFR) mutations, anaplastic lymphoma kinase, and c-ros oncogene 1 fusion proteins (2, 3). However, for the vast majority of patients, treatment options are scant and the overall prognosis remains poor, emphasizing the need to uncover additional molecular mechanisms important in lung cancer tumorigenesis.

The transcriptional coactivator with PDZ-binding motif (TAZ), also known as WW-domain containing transcription regulator-1 (WWTR1), was originally identified as a 14-3-3 interacting protein (4). The regulation of TAZ and its paralog, Yes-associated protein (YAP), occurs primarily via the Hippo pathway, which is conserved from flies to humans (5). TAZ acts mainly through the TEAD family of transcription factors to stimulate expression of genes that promote cell proliferation and control organ size (6). TAZ has also been suggested to be involved in other biological processes, including mesenchymal stem cell differentiation via modulation of Runx2- and PPARγ-dependent gene expression (7), self-renewal of human embryonic stem cells via regulation of the localization of Smad (8), and mechanotransduction (9). There is emerging evidence of crosstalk with other signaling pathways, such as those of the protease-activated receptors (PARs), G-protein-coupled receptors (GPCR), and Wnt/β-catenin (6, 10).
To investigate the physiological role of TAZ, we previously analyzed TAZ knockout mice (11). TAZ deficiency caused abnormal alveolarization during lung development, leading to enlarged airspaces with low elastance. Taz-heterozygous mice were more resistant to bleomycin-induced lung fibrosis. We identified connective tissue growth factor (CTGF) as a downstream target of TAZ. TAZ has also been shown to interact with thyroid transcription factor-1 (TTF-1) (12).

Despite its obvious role in lung morphogenesis and homeostasis under physiological conditions, recent studies showed that TAZ is overexpressed in various human cancers. TAZ contributes to the tumorigenesis of breast cancer cells by promoting cell migration, invasion, anchorage-independent growth, and epithelial-mesenchymal transition (EMT) (13, 14). In addition, TAZ confers cancer stem cell-related traits to breast cancer cells (15), further highlighting its importance in tumor initiation and progression. TAZ also plays a role in malignant glioma, colorectal cancer, and papillary thyroid cancer cells (16-18).

Few studies have analyzed the impact of TAZ in lung cancer, and these were restricted mainly to cell lines or included a limited number of NSCLC specimens (19-21). The aim of our study was to analyze the role of TAZ in NSCLC using an integrative strategy, combining results from in vitro and in vivo analyses with molecular and clinical information of human lung cancer tissues.

Materials and Methods

Global gene expression and gene copy number analyses in human NSCLC tissues

The study population consisted of NSCLC patients surgically treated between 1995
and 2005 with available fresh frozen tissue in the Uppsala Biobank in the Department of Pathology (Uppsala lung cancer cohort) (22). The study was approved by the Uppsala Ethical Review Board (2006/325). RNA and DNA were isolated from 196 frozen tissue samples and used for microarray analysis as described previously (GEO accession GSE37745) (22).

The meta-analysis was performed as described previously (22) and included nine publicly available Affymetrix expression data sets (the Uppsala cohort, GSE37745; GSE14814 (23); GSE3141 (24); GSE19188 (25); GSE4573 (26); GSE31210 (27); Shedden et al. 2008 (28); GSE29013 (29); GSE31547), together comprising 1,382 NSCLC patients and 22,277 probe sets overlapping the arrays.

Whole-genome SNP array experiments were performed according to standard protocols for Affymetrix Gene Chip Mapping 250K Nsp I arrays (Affymetrix, Santa Clara, CA, USA) on 192 of these cases, as described previously (30). Copy number estimates for each SNP were calculated as the ratio of the sample chip effect in the Uppsala cohort and the robust average across 90 HapMap samples as the reference (31). These non-log scale values were multiplied by two, and then CBS segmentation using default parameters was applied to the SNP-wise copy numbers (32). TAZ copy number values were matched with the gene expression probe sets using the mean chromosomal position of the probe sets. Subsequently, the Spearman’s rank correlation coefficient (rho) between TAZ gene expression and the copy number of the three probe sets was calculated.

Tissue microarray construction and immunohistochemistry

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A tissue microarray (TMA) encompassing 355 NSCLC cases was constructed as described previously (33). Of these cases, 189 were also analyzed for global gene expression and gene copy number changes. Immunohistochemistry was performed according to standard procedures (33). In brief, after antigen retrieval and blocking of endogenous peroxidase, slides were incubated with the anti-TAZ antibody (1:40 dilution, HPA007415, Sigma Aldrich, St. Louis, MO, USA) and the anti-amphiregulin antibody (1:10 dilution, AF262, R&D Systems, Minneapolis, MN, USA) for 30 min at room temperature (RT). After incubation with the secondary antibody (Dako REAL™ EnVision™, Dako, Glostrup, Denmark) for 30 min at RT, the staining was developed using diaminobenzidine solution for 10 min.

The immunostaining was evaluated under the supervision of a lung pathologist (PM). The staining intensity was scored manually using a 4-grade scale: negative (0), weak (1), moderate (2), and strong (3). The fraction of stained tumor cells was scored as follows: no positive cells (0), 1-9% (1), 10-49% (2), 50-100% (3). For all stainings, one score was used per duplicate, representing the same tumor sample, on the arrays. The ordinal scores for the staining intensity and the fraction of stained tumor cells were multiplied, obtaining values in the range of 0-9. For survival analysis, the TAZ staining score was dichotomized at the median value of 6 (low: 0-6, high: 9).

**Cell lines**

The human lung cancer cell lines A549 and NCI-H441 (H441) were purchased from the American Type Culture Collection (Rockville, MD, USA). The human bronchial epithelium cell line BEAS-2B was obtained from the European Collection of Cell
Cultures (Porton Down, UK). The 293FT cells were from Invitrogen (Carlsbad, CA, USA). All cell lines were cultured according to the manufacturer’s protocols.

**Small interfering RNA experiment**

Small interfering RNAs (siRNA) against human TAZ (Stealth RNAi, si TAZ #1: HSS119545; si TAZ #2: HSS119546) and the negative control (Stealth RNAi, si NTC: #12935-300) were purchased from Invitrogen. A549 and H441 cells were transfected with 20 nM siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions.

**Construction of the microRNA expression vectors**

The microRNA (miRNA) expression vectors were constructed using the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit with EmGFP (Invitrogen). Three pairs of sense and antisense oligonucleotides were designed for targeting TAZ (mi TAZ #1, #2, and #3; Supplementary Table S1) using BLOCK-iT™ RNAi Designer (http://rnadesigner.Invitrogen.com/rnaiexpress/). The designed oligonucleotides were annealed, followed by ligation into the pcDNA6.2-GW/EmGFP-miR vector (Invitrogen). As the control, pcDNA6.2-GW/EmGFP-miR negative control plasmid (mi NTC; Invitrogen) was used. The sequence containing the miRNA coding region was transferred to the lentivirus vector CSII-EF-RfA via the Gateway cloning system (Invitrogen) according to the manufacturer’s instructions.

**Cloning of human TAZ cDNA**

The open reading frame of TAZ, derived from the cDNA of A549 cells, was
amplified by PCR using high-fidelity DNA polymerase (PrimeSTAR Max; Takara Bio, Shiga, Japan). The purified PCR fragment was subcloned into the lentiviral expression vector CSII-EF-RfA. The final insert was confirmed by DNA sequencing using the ABI PRISM® 310 Genetic Analyzer. A green fluorescent protein (GFP) expression vector was used as the control.

Lentiviral infection

Recombinant lentiviruses were produced by transient transfection of 293FT cells with the lentiviral expression vectors, pCMV-VSV-G-RSV-Rev and pCAG-HIVgp, using Lipofectamine 2000 reagent (Invitrogen). After 72 h of transfection, the medium was collected, and A549 or BEAS-2B cells were incubated with lentivirus-containing medium.

Immunoblot analysis

The detailed procedures for immunoblot analysis were described previously (34). In brief, cells were lysed, followed by SDS gel electrophoresis and semi-dry transfer of the proteins to polyvinylidene difluoride membranes. They were probed with anti-TAZ (#2149; Cell Signaling, Beverly, MA, USA), anti-phospho-EGFR Y1068 (#3777; Cell Signaling), anti-total EGFR (#E12020; Transduction Laboratories, Lexington, KY, USA) or anti-α-tubulin (Sigma-Aldrich) antibodies and the appropriate horseradish peroxidasen conjugated secondary antibodies.

Cell proliferation analysis

The siRNA-transfected A549 and H441 cells and lentivirally transduced BEAS-2B
cells were seeded at a density of $7.5 \times 10^3$/well, $5 \times 10^4$/well, and $2 \times 10^4$/well, respectively, in quadruplicate wells of a 12-well plate. Cell numbers were counted on days 2, 4, and 6 after seeding.

**Colony formation assay**

The miRNA-transduced cells were suspended in culture medium containing 0.4% (wt/vol) agar (SeaPlaque GTG agarose; Lonza, Tokyo, Japan) and layered on top of culture medium containing 0.53% (wt/vol) agar at a density of $1.2 \times 10^4$/well in triplicate wells of a six-well plate. Colonies were allowed to form for 14 days and were then stained with MTT dye. Colonies $\geq 200 \mu m$ in diameter were counted using ImageJ software (http://imagej.nih.gov/ij/).

**Cell cycle analysis**

The siRNA-transfected A549 and H441 cells and lentivirally transduced BEAS-2B cells were seeded at a density of $4 \times 10^5$/well into triplicate wells of a six-well plate. After 48 h, cells were harvested, washed with phosphate-buffered saline (PBS), and fixed with 70% ethanol. After treatment with RNaseA (Sigma-Aldrich), the cells were stained with propidium iodide (Sigma-Aldrich), and flow cytometry analysis was performed.

**Apoptosis assay**

The siRNA-transfected A549 and H441 cells were seeded at a density of $4 \times 10^5$/well into a six-well plate. After overnight incubation, the cells were serum-starved for 24 h,
and annexin V-stained apoptotic cells were detected using the Annexin V-FITC Apoptosis Detection Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions.

**Tumorigenicity assay**

Male BALB/c nu/nu mice, 6-8 weeks old, were purchased from the Charles River Laboratories Japan, Inc. (Kanagawa, Japan). A total of $1 \times 10^7$ A549 cells transduced with miRNAs (6-8 mice per group) or BEAS-2B cells transduced with GFP or TAZ (10 mice per group) in 100-μL PBS were injected s.c. into mice. After 1 month or 6 months respectively, the mice were sacrificed and the tumors excised and snap frozen. The tumor volume was approximated as $a \times b \times c/2$ (where $a$, $b$, and $c$ represent the three different axes), and the tumor samples were examined for cell morphology using hematoxylin and eosin staining. The animal experiment was conducted in accordance with national guidelines and approved by the Ethics Committee for Animal Experiments at the University of Tokyo.

**Gene expression profiling in A549 and H441 cells**

Total RNA was extracted from A549 and H441 cells 48 h after NTC or TAZ siRNA transfection using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and subjected to the GeneChip® Human Gene 1.0 ST Array (Affymetrix). The expression levels of 19,734 genes were analyzed using the GeneSpring GX software (Agilent Technologies, Santa Clara, CA, USA). The data set was deposited in the Gene Expression Omnibus database (GSE51270). To identify the TAZ-regulated genes, we integrated two publicly available TAZ-dependent gene expression data sets derived from MCF10A (13) and SW480 cells
Gene ontology (GO) analysis and gene set enrichment analysis (GSEA) were performed as described previously (36, 37).

**Quantitative reverse transcription-PCR (RT-PCR)**

Total RNA was extracted from the cells, and first-strand cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR analysis was performed using the Mx-3000P qPCR System (Stratagene, La Jolla, CA, USA) and QuantiTect SYBR Green PCR (Qiagen) according to the manufacturer’s protocol. The expression levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR primers used are listed in Supplementary Table S2.

**ELISA**

The siRNA-transfected A549 cells were seeded at a density of $1.5 \times 10^5$/well into triplicate wells of a six-well plate. After 48 h, the culture medium was changed to serum-free medium. The cells were cultured for another 48 h, and the culture supernatant was collected. Lentivirally transduced BEAS-2B cells were seeded at a density of $1.5 \times 10^5$/well in epidermal growth factor (EGF)-deprived medium, and the culture medium was collected after 48 h. The concentration of amphiregulin was determined using the Human Amphiregulin DuoSet (R&D Systems) following the manufacturer’s protocol.

**Statistical analysis**

The correlations between TAZ expression levels and clinicopathological parameters
or between TAZ and amphiregulin protein expression levels were analyzed by the $\chi^2$-test. Kaplan-Meier plots were used to visualize the difference in survival between the two groups. The statistical significance of the differences was determined by the log-rank test. Furthermore, univariate and multivariate Cox models were fitted with inclusion of established prognostic parameters: age, patient performance status, and stage at diagnosis. Spearman’s rho was used to quantify the correlation between gene-gene and gene-protein expression levels. The differences between the experimental groups were examined for statistical significance using Student’s $t$-test, Wilcoxon’s rank-sum test, or analysis of variance (ANOVA) with Tukey’s post hoc test. The analyses were conducted using JMP, version 9.0.3 (SAS Institute Inc., Cary, NC, USA). $P < 0.05$ was considered significant. All data are expressed as means ± SEM.

RESULTS

TAZ gene expression and its clinical correlations in human NSCLC

Our study population was based on a clinically well-characterized lung cancer cohort comprising 196 patients (Uppsala cohort). The tumor tissues were annotated previously for KRAS and EGFR mutations, whole genome gene copy number changes, and gene expression profiles (22, 38). Three probe sets (202132_at, 202133_at, and 202134_s_at) with strong correlation (rho = 0.52-0.83) represent TAZ on the Affymetrix U133 Plus 2.0 array (GSE37745). Higher transcript levels of TAZ correlated with squamous cell carcinoma histology ($P = 0.002$) and younger age ($P = 0.011$, Supplementary Table S3). TAZ gene expression levels were lower in tumor samples harboring EGFR mutations than in EGFR/KRAS mutation-negative samples ($P = 0.046$, Supplementary Fig. S1A).
No correlations between TAZ mRNA expression levels and the other evaluated parameters (sex, smoking status, stage, and performance status) were found (Supplementary Table S3). The correlation between gene expression profiles and gene copy number levels, determined from the Affymetrix 250K SNP arrays, indicated that the mRNA levels can be explained in part by differences in the gene copy number of the corresponding TAZ gene locus \((P < 0.001, \text{Fig. 1A})\). Univariate survival analysis of the Uppsala cohort revealed that the 202134_s_at probe set was significantly associated with survival \((HR = 1.25; \text{confidence interval (CI)} = 1.04-1.50; P = 0.021)\). The expression of the other two TAZ probe sets did not show prognostic impact \((202132_at, P = 0.489; 202133_at, P = 0.339)\). The 202134_s_at association remained significant when TAZ expression levels were adjusted for important prognostic parameters, including stage, performance status, and age, in the multivariate analysis \((HR = 1.24; \text{CI} = 1.02-1.50; P = 0.035)\).

To substantiate the potential prognostic impact of the probe set 202134_s_at, a meta-analysis was performed. The analysis, which included nine independent, publicly available lung cancer data sets, confirmed the association of higher TAZ mRNA levels with shorter survival \((\text{Fig. 1B}, HR = 1.21; \text{CI} = 1.10-1.33; P < 0.001)\). This prognostic impact was pronounced in the adenocarcinoma subgroup \((\text{Fig. 1C}, HR = 1.33; \text{CI} = 1.16-1.52; P < 0.001)\) but could not be demonstrated when the squamous cell carcinoma subgroup was analyzed alone \((HR = 1.05; \text{CI} = 0.90-1.22; P = 0.568)\).

**TAZ protein levels in human NSCLC**

To transfer our findings from mRNA to *in situ* protein levels, immunohistochemistry
was performed on a TMA comprising tissue cores from 355 patients. Staining was evaluated for 345 cases and 18 additional non-malignant lung tissue samples.

In the non-malignant lung tissues, alveolar cells and bronchial epithelial cells showed positive nuclear staining. In addition, stromal cells, such as inflammatory cells, endothelial cells, and fibroblasts, demonstrated frequent nuclear positivity (Supplementary Fig. S2A). Lung cancer tissues revealed variable staining patterns, ranging from strongly positive (Fig. 2A) to completely negative (Fig. 2B). Immunohistochemical staining scores correlated well with gene expression levels in the same cancer sample, indicating the reliability of the antibody (Supplementary Fig. S2B).

For statistical analysis, the scores were dichotomized into low versus high TAZ protein levels in the NSCLC cases. Correlation with clinical parameters revealed that high levels were more common in squamous cell carcinoma (Supplementary Table S4). No correlations were observed between TAZ protein levels and other clinical parameters, including EGFR and KRAS mutation status (Supplementary Fig. S1B). In correspondence with the gene expression results, high TAZ protein levels were associated with shorter survival in the univariate analysis (Table 1, left: HR = 1.40, CI = 1.06-1.82, \( P = 0.019 \)), as illustrated by the Kaplan-Meier analysis (Fig. 2C). When we evaluated the adenocarcinoma and squamous cell carcinoma subgroups separately, a trend was observed in the univariate analysis (\( P = 0.160 \) and \( P = 0.087 \), respectively). The prognostic relevance in the whole cohort was retained in the multivariate analysis (Table 1, right: HR = 1.34, CI = 1.01–1.76, \( P = 0.040 \)).

In accordance with mRNA expression, gene copy numbers also correlated with the TAZ protein expression level (\( \rho = 0.17, P = 0.025 \)). Thus, TAZ expression could be
explained, at least in part, by gene copy number changes. These results indicated differential expression of TAZ in NSCLC and suggested that high TAZ expression at the genomic, mRNA, and protein levels might define a clinical subset of NSCLC patients.

**In vitro analysis of TAZ in lung cancer cell lines and bronchial epithelial cells**

To generate TAZ loss-of-function models, A549 and H441 human lung adenocarcinoma cells, which express endogenous TAZ, were used for the silencing experiments. Cells were transiently transfected with NTC or TAZ siRNA. A549 cells were also lentivirally transduced with artificial miRNAs against TAZ for colony formation assay and tumorigenicity assay. To establish TAZ gain-of-function models, immortalized human bronchial epithelial cells (BEAS-2B) were lentivirally transduced with control GFP or TAZ. Knockdown or overexpression of TAZ was confirmed by immunoblotting (Fig. 3A and Supplementary Fig. S3A). The cells did not show morphological changes after siRNA transfection or lentiviral transduction.

We investigated the effect of TAZ on cell proliferation. TAZ knockdown in A549 and H441 cells inhibited cell proliferation (Fig. 3B), while TAZ overexpression in BEAS-2B cells enhanced cell proliferation slightly (Fig. 3C). Soft agar colony formation assays showed that A549 cells with miRNA-mediated TAZ knockdown exhibited a reduction in anchorage-independent growth on soft agar (Supplementary Fig. S3B).

To explore the mechanisms by which TAZ promotes lung cancer cell proliferation, we carried out cell cycle analysis. In line with the findings of the cell proliferation assay,
the G0/G1 fraction was increased by TAZ knockdown in A549 cells (Fig. 3D), while G1/S transition was promoted by TAZ overexpression in BEAS-2B cells (Fig. 3E).

To investigate the involvement of TAZ in apoptosis, A549 and H441 TAZ-knockdown cells were serum-starved, and apoptotic cells were detected by annexin V staining. We found a significant increase in apoptosis induced by serum deprivation in A549 and H441 cells after TAZ knockdown (Supplementary Fig. S3C). These results supported that the TAZ oncogene promotes lung cancer proliferation and survival.

**Tumorigenic impact of TAZ in a mouse model**

To assess the potential tumorigenicity of TAZ *in vivo*, a xenograft mouse model was generated by injecting nude mice with miRNA-transduced A549 cells or TAZ-transduced BEAS-2B cells. All the mice injected with miRNA-transduced A549 cells developed tumors, and TAZ knockdown reduced tumor growth (Fig. 3F). On the other hand, during the observation period, 5 of 10 mice injected with TAZ-transduced BEAS-2B cells developed tumors, while only one mouse demonstrated minimal tumor growth after 6 months in the control group (Supplementary Fig. S3D and E). Tumor samples showed similar cell morphology (Supplementary Fig. S3F). These results indicated the potential role of TAZ in tumorigenesis of bronchial epithelial cells.

**Gene expression profiling in TAZ knockdown lung cancer cells**

To identify the downstream targets of TAZ in lung cancer cells, we performed microarray-based expression profiling analysis in A549 cells after TAZ knockdown.
Following TAZ siRNA transfection, TAZ expression was less than 20%. TAZ suppression led to increased expression (>1.5-fold) of 227 transcripts and decreased expression (<0.67-fold) of 131 transcripts. Key molecules involved in lung epithelial cell differentiation, survival, and proliferation are shown in Fig. 4A and Supplementary Fig. S4A. Similar TAZ knockdown experiments were performed in H441 cells, which demonstrated increased expression (>1.5-fold) of 69 transcripts and decreased expression (<0.67-fold) of 138 transcripts. The differentially expressed genes after TAZ knockdown in A549 and H441 cells showed remarkable overlap (30 transcripts).

To validate our results, we integrated two additional gene lists from cell lines with altered TAZ expression, yielding a total of four gene lists: the genes upregulated (>2.0-fold) in MCF10A cells (breast epithelial cell) by retroviral transduction of TAZ (13) and the genes downregulated (<0.67-fold) in A549, H441, and SW480 (colon cancer) cells by siRNA-mediated TAZ knockdown (35). There were 259 overlapping genes in at least two cell lines, defined as TAZ-regulated genes (Supplementary Table S5). To characterize these 259 genes, we performed GO analysis and found that a large portion of the TAZ-regulated genes is associated with cell cycle regulation (Fig. 4B). The in vitro findings were recapitulated in human NSCLC tissues of the Uppsala cohort using GSEA. TAZ-regulated genes were clearly enriched in NSCLC with high TAZ expression (Fig. 4C). These genes were also enriched in NSCLC tissues of patients with poor prognosis (Fig. 4D), suggesting that TAZ target genes might be responsible for the aggressive phenotype of NSCLC.

The downregulated genes in the A549 cells after TAZ knockdown included the cell cycle regulators, cyclin D1 and D3 (CCND1 and CCND3), as well as ErbB ligands, amphiregulin (AREG), epieregulin (EREG), and neuregulin 1 (NRG1), which were also
listed as TAZ-regulated genes in other cell types (Supplementary Table S5). Since the EGFR family and its ligands play an important role in lung cancer, these findings prompted us to validate the microarray data and to explore the TAZ-mediated regulation of the three ErbB ligands in detail.

**TAZ regulates expression of ErbB ligands in lung epithelial cells**

The gene expression differences determined by microarray analysis of the ErbB ligands in A549 cells were confirmed by quantitative RT-PCR (Fig. 5A). We also found that they were upregulated in TAZ-overexpressing BEAS-2B cells. ELISA assays showed that TAZ knockdown led to decreased amphiregulin protein levels in the culture medium of A549 cells (Fig. 5B). In line with this, TAZ overexpression increased amphiregulin protein levels in the supernatant of cultured BEAS-2B cells. We found that the promoter regions of the three ErbB ligands contain putative recognition motifs for TEAD, a major transcription factor that binds TAZ (Supplementary Fig. S4B and C).

**Correlation between TAZ and ErbB ligand expression in NSCLC**

We next investigated whether there is a correlation between TAZ and ErbB ligand expression using publicly available microarray data sets. There were positive correlations in the NSCLC cell lines according to two data sets, GSE4127 (39) and GSE4824 (40), except for neuregulin 1 in GSE4127, in which only a trend towards a positive correlation was observed (Fig. 5C). The correlation between TAZ and amphiregulin expression was also analyzed in human cancer tissues using
immunohistochemistry in 344 evaluable cases of the Uppsala cohort (Supplementary Fig. S5). Amphiregulin staining correlated well with gene expression levels within the same cancer samples (rho = 0.22, P = 0.003), confirming the validity of the antibody. Furthermore, a positive correlation between TAZ and amphiregulin protein levels was found (P < 0.001, Fig. 5D).

To evaluate whether the EGFR pathway was activated in NSCLC with high TAZ expression, we again used GSEA. An EGFR signaling-related gene set from a previous study (41) was enriched in NSCLC with high TAZ expression in the Uppsala cohort (P = 0.004, Fig. 5E). Three other EGFR signaling-related gene sets also showed positive correlations with high TAZ expression, but they did not reach statistical significance (Supplementary Fig. S6A) (42, 43). Immunoblot analysis showed that TAZ overexpression increased EGFR phosphorylation in BEAS-2B cells, although we failed to observe decreased EGFR phosphorylation after siRNA-mediated TAZ knockdown (Supplementary Fig. S6B). Altogether, these findings suggested that TAZ activates the EGFR signaling pathway through the induction of EGFR ligands.

**Correlation between TAZ expression and sensitivity to EGFR inhibitors**

To investigate whether EGFR inhibition is a reasonable treatment strategy for NSCLC with high TAZ expression, drug sensitivity data and gene expression profiles in NSCLC cells were obtained from the Cancer Cell Line Encyclopedia (CCLE) database (44). We found that TAZ expression tended to be higher in NSCLC cell lines with high sensitivity to two EGFR tyrosine kinase inhibitors (EGFR-TKIs), while there were no apparent associations between TAZ expression and sensitivity to other anti-cancer drugs (Supplementary Fig. S7A). In addition, 139 genes that define gefitinib sensitivity (45)
were enriched in NSCLC with high TAZ expression (Supplementary Fig. S7B). Thus, the \textit{in silico} analysis indicated a rational strategy for EGFR inhibition in TAZ-positive NSCLC.

**DISCUSSION**

Using an integrated approach, we characterized TAZ expression at the genomic, mRNA, and protein levels in a large, well-annotated NSCLC patient cohort to combine comprehensive molecular data with clinical parameters and to identify TAZ as an independent prognostic marker. Consistent with the \textit{in situ} findings in human lung cancer tissue, TAZ promoted lung cancer/epithelial cell proliferation \textit{in vitro} and tumor development in an \textit{in vivo} mouse model. Finally, based on multiple experimental lines, tumorigenic effects might be regulated through EGFR signaling.

The association of higher TAZ expression with shorter survival in lung cancer patients was described previously in one study (20). However, there were certain differences of interest. First, the staining pattern of the antibody used in our study clearly indicated TAZ expression in normal alveolar and bronchial epithelial cells as well as in stromal cells. TAZ protein expression in non-neoplastic lung tissue was not described in the previous report. Secondly, as opposed to the previous study, TAZ expression levels were in general higher in squamous cell carcinoma in our study, which was also supported by analyses of all other microarray data sets (data not shown). This discrepancy might be caused by differences in the clinicopathological features of the study cohorts and/or the methods used for immunohistochemistry, including the use of different antibodies.
We found a clinical subset of NSCLC patients with high TAZ expression at the genomic, mRNA, and protein levels in the Uppsala cohort. The previous studies showed that high level of TAZ gene amplification analyzed by GISTIC algorithm was observed in 35 of 178 squamous cell carcinomas (19.7%) (46) and one of 182 adenocarcinomas (0.5%) (47). This is consistent with the finding that TAZ expression levels were higher in squamous cell carcinoma in our study. They also showed that TAZ mutation was found in only one case (0.6%) of squamous cell carcinomas, and two cases (1.1%) of adenocarcinomas, suggesting TAZ activation may not be generally caused by genetic mutations.

We showed that miRNA-mediated TAZ knockdown in A549 cells exhibited tumor growth reduction in a xenograft model, which was consistent with the previous study (19). Furthermore, we found that TAZ-transduced BEAS-2B cells showed a higher frequency of tumor formation in nude mice. Because lentiviral transduction of BEAS-2B cells may itself induce tumorigenesis (48), the results in this model should be interpreted with caution and will require further investigation.

To further explore which signaling pathways TAZ regulates, we performed a comparative gene expression analysis and identified amphiregulin, epiregulin, and neuregulin 1 as candidate genes. We demonstrated that TAZ induces amphiregulin secretion from lung epithelial cells. Our results were consistent with a previous report that TAZ increased amphiregulin production in mammary epithelial cells (49). Epiregulin and neuregulin 1 were also among the potential targets found in the microarray screening of A549 cells, as well as MCF10A and SW480 cells, with TAZ knockdown (13, 35). Importantly, their promoter regions were likely to harbor TEAD-binding sites, suggesting direct regulatory interactions.
EGFR-TKIs are now widely accepted as effective therapeutic strategies for EGFR mutation-positive NSCLC patients. However, some EGFR mutation-negative patients also benefit from EGFR-TKI treatment (50). Indeed, a previous study linked increased expression of the EGFR ligand amphiregulin to improved clinical outcomes in EGFR mutation-negative patients treated with EGFR-TKI (51). TAZ expression may help to identify those patients who would benefit from treatment with EGFR-TKIs. We report here supportive findings using data from the CCLE database. However, it should be stressed that other strategies have been suggested to inhibit oncogenic TAZ activation, including direct targeting of TAZ, Rho and Rock inhibitors, and upstream WNT inhibition (6).

Indeed, our integrative microarray analysis indicated several other mechanisms of TAZ-induced tumorigenesis. TAZ may facilitate cancer progression through activation of tumor-promoting genes, such as CTGF, Cyr61, and cyclins (CCND1 and CCND3). Further studies are clearly warranted to identify the TAZ-dependent regulatory network in cancer.

In summary, our study confirms the tumorigenic impact and clinical significance of TAZ expression in NSCLC and sheds light on the regulatory mechanisms and the potential for TAZ as a therapeutic target in the era of personalized medicine.

Acknowledgments

We are grateful to Asayo Imaoka and Makiko Sakamoto for their technical assistance. We also thank Akihisa Mitani for the useful discussions.
REFERENCES


Table 1. Univariate and multivariate Cox regression model of 345 NSCLC cases including TAZ expression (high expression vs. low expression) and the most important prognostic parameters (dichotomized stage II–IV vs. I, age >70 vs. ≤70 years, performance status I–III vs. 0).

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Figure legends

Figure 1. Correlation between TAZ gene copy number and gene expression level, and a meta-analysis of the prognostic impact of TAZ gene expression.

A, scatter plots for TAZ gene copy numbers and gene expression levels. Spearman’s correlation coefficients (rho) and \( P \) values were calculated for each probe set. B and C, forest plots visualizing the results from the meta-analysis of the TAZ probe set 202134_s_at, including 1,382 NSCLC (B) or 981 adenocarcinoma (C) patients from nine studies. The overall effect was quantified by the \( P \) value of the random effects model.

Figure 2. TAZ protein expression in NSCLC.

A and B, the tissue microarray, consisting of 345 evaluable NSCLC cases, was stained with a polyclonal antibody against TAZ, and representative immunostaining images are presented for high (A) and low TAZ expression (B). C, the staining scores (0–9 as a product of the staining intensity and the proportion of stained tumor cells) were dichotomized (0–6 vs. 9) and used for stratification in the Kaplan-Meier analysis. \( P \) value was obtained using a log-rank test.

Figure 3. Effects of TAZ on lung cell proliferation, cell cycle progression, and tumor formation.
A, immunoblot analysis showed the effects of TAZ knockdown and overexpression. A549 and H441 cells were transfected with si NTC (control siRNA) or si TAZ (siRNA for TAZ). BEAS-2B cells were transduced with GFP or TAZ. α-tubulin was used as the loading control. B and C, numbers of siRNA-transfected A549 and H441 cells (B) and lentivirally transduced BEAS-2B cells (C) were counted on days 2, 4, and 6 after seeding. D and E, A549 cells transfected with si NTC or si TAZ (D) and BEAS-2B cells transduced with GFP or TAZ (E) were stained with propidium iodide, and flow cytometry analysis was performed. Data are representative of three independent experiments. Error bars: SEM. F, a total of $1 \times 10^7$ A549 cells transduced with miRNAs were injected subcutaneously into nude mice. Tumor volume and tumor weight were measured 1 month later. *$P < 0.05$, Student’s *t*-test, si NTC versus si TAZ #1 or #2 (B, D), GFP versus TAZ (C, E), mi NTC versus mi TAZ #1, #2 or #3 (F).

**Figure 4.** Analyses of TAZ-regulated genes.

A, selected TAZ-regulated genes in A549 cells. B, GO analysis of TAZ-regulated genes obtained from merging of the data from the A549, H441, MCF10A, and SW480 cells. C and D, GSEA was performed to examine the enrichment of TAZ-regulated genes in NSCLC samples of the Uppsala cohort. Patients were divided into two groups according to the expression level of TAZ or overall survival. The enrichment of TAZ-regulated genes is shown schematically with the genes that correlated best with high TAZ expression (C) or poor prognosis (D) on the left and the genes that correlated best with low TAZ expression (C) or good prognosis (D) on the right.
Figure 5. TAZ regulates the expression of ErbB ligands.

A, the relative expression levels of the indicated transcripts in A549 cells transfected with si NTC or si TAZ and in BEAS-2B cells transduced with GFP or TAZ. B, the culture media were collected from A549 cells transfected with si NTC or si TAZ and BEAS-2B cells transduced with GFP or TAZ. The concentration of amphiregulin was measured by ELISA. The results represent the concentration per $1 \times 10^6$ cells. Error bars: SEM. *$P < 0.05$, Student’s $t$-test. C, the correlations between TAZ and ErbB ligand expression from two microarray data sets from NSCLC cell lines, GSE4127 and GSE4824. Probe sets: TAZ, 202132_at; amphiregulin, 205239_at; epiregulin, 205767_at; neuregulin 1, 208241_at. Spearman correlation coefficients (rho) and the $P$ values were calculated. D, the correlation between TAZ protein expression and amphiregulin in the TMA. E, GSEA was performed to examine the enrichment of EGFR signaling-related genes in NSCLC samples of the Uppsala cohort. Patients were divided into two groups according to the TAZ expression level. The EGFR signaling signature was based on the genes whose expression peaked 8 h after stimulation of MCF10A cells with EGF. The enrichment of EGFR signaling-related genes is shown schematically with the genes that correlated best with high TAZ expression on the left and those that correlated best with low TAZ expression on the right.
**Figure 1**

A

![Graphs showing TAZ gene expression vs. TAZ gene copy number for different experiments.](image)

- **202132_at**
  - $P < 0.001$
  - Rho = 0.37

- **202133_at**
  - $P < 0.001$
  - Rho = 0.35

- **202134_s_at**
  - $P < 0.001$
  - Rho = 0.44

B

**All NSCLC**

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C

**Adenocarcinoma**

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Fixed effect model
Random effects model

- HR: Hazard Ratio
- 95% CI: 95% Confidence Interval
- $P < 0.001$
Figure 3

A

B

C

D

E

F
Figure 4

A

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B

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C

Gene set: TAZ-regulated genes
TAZ-high vs. TAZ-low, P = 0.002

D

Gene set: TAZ-regulated genes
poor prognosis vs. good prognosis, P = 0.014
Figure 5

A

AREG

A549

si NTC  si TAZ #1  si TAZ #2

Relative expression

BEAS-2B

GFP  TAZ

Relative expression

B

AREG (pg/mL)

A549

si NTC  si TAZ #1  si TAZ #2

GFP  TAZ

AREG (pg/mL)

BEAS-2B

C

GSE4127

(n = 19)

AREG gene expression

TAZ gene expression

P = 0.003

rho = 0.64

EREG gene expression

TAZ gene expression

P = 0.001

rho = 0.69

NRG1 gene expression

TAZ gene expression

P = 0.647

rho = 0.11

GSE4824

(n = 52)

AREG gene expression

TAZ gene expression

P = 0.002

rho = 0.43

EREG gene expression

TAZ gene expression

P = 0.005

rho = 0.39

NRG1 gene expression

TAZ gene expression

P = 0.017

rho = 0.33

D

Gene set: EGFR signaling-related genes

TAZ-high vs. TAZ-low, P = 0.004

E

Gene set: EGFR signaling-related genes

TAZ-high vs. TAZ-low, P = 0.004
An integrative analysis of the tumorigenic role of TAZ in human non-small cell lung cancer

Satoshi Noguchi, Akira Saito, Masafumi Horie, et al.

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