Integrative genomics analysis reveals the multilevel dysregulation and oncogenic characteristics of TEAD4 in gastric cancer

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Tumorigenesis is a consequence of failures of multistep defense mechanisms against deleterious perturbations that occur at the genomic, epigenomic, transcriptional and proteomic levels. To uncover previously unrecognized genes that undergo multilevel perturbations in gastric cancer (GC), we integrated epigenomic and transcriptomic approaches using two recently developed tools: MENT and GENT. This integrative analysis revealed that nine Hippo pathway-related genes, including components [FAT, JUB, LAT52, TEA domain family member 4 (TEAD4) and Yes-associated protein 1 (YAP1)] and targets (CRIM1, CYR61, CTGF and ITGB2), are concurrently hypomethylated at promoter CpG sites and overexpressed in GC tissues. In particular, TEAD4, a link between Hippo pathway components and targets, was significantly hypomethylated at CpG site cg21637033 (P = 3.8 × 10⁻²⁰) and overexpressed (P = 5.2 × 10⁻⁹) in 108 Korean GC tissues compared with the normal counterparts. A reduced level of methylation at the TEAD4 promoter was significantly associated with poor outcomes, including large tumor size, high-grade tumors and low survival rates. Compared with normal tissues, the TEAD4 protein was more frequently found in the nuclei of tumor cells along with YAP1 in 53 GC patients, demonstrating the posttranslational activation of this protein. Moreover, the knockdown of TEAD4 resulted in the reduced growth of GC cells both in vitro and in vivo. Finally, chromatin immunoprecipitation-sequencing and microarray analysis revealed the oncogenic properties of TEAD4 and its novel targets (ADM, ANG, ARID5B, CALD1, EDN2, FSCN1 and OR52), which are involved in cell proliferation and migration. In conclusion, the multilevel perturbations of TEAD4 at epigenetic, transcriptional and posttranslational levels may contribute to GC development.

Introduction

Tumorigenesis results from the collapse of diverse homeostatic mechanisms that maintain controlled cell division and growth. These homeostatic mechanisms are achieved through multiple molecular processes, which occur at the genomic, epigenomic, transcriptomic and proteomic levels (1). Hence, deleterious hits on these multilevel processes lead to the breakdown of cellular homeostasis. Because of the multilevel perturbations, cancer development is highly heterogeneous, making the identification of causal genes and underlying mechanisms difficult.

Due to the complexity of cancer, integrative approaches are required to discover multilevel perturbations in cancer genes (2). Recent advances in biotechnology have made it possible to generate many public datasets, thereby facilitating the use of this information. As part of this trend, we recently developed two databases: GENT (Gene Expression across Normal and Tumor tissue) and MENT (Methylation and Expression database of Normal and Tumor tissues) (3,4), which provide comprehensive transcriptomic and epigenomic profiles, respectively, across various cancers and normal tissues. GENT was built by collecting >34 000 samples from the GEO (Gene Expression Omnibus), Array Express and the Expression Project for Oncology (3). MENT was developed by collecting >6000 samples from GEO and TCGA (The Cancer Genome Atlas) (4).

The Hippo pathway is a conserved tumor suppressor pathway and plays roles in biological processes, including organ size control, tissue regeneration, stem cell self-renewal and cancer development (5). Although it has not been well demonstrated, cell polarity, adhesion and junctional proteins, including NF2/merlin, KIBRA, FRMD1/6, FAT and JUB, have been known as upstream regulators for the Hippo pathway (6). These upstream signals converge on a core kinase cassette composed of MST1/2, WW45, LATS1/2 and MOB1. MST1/2-WW45 phosphorylates and activates LATS1/2-MOB1, which in turn phosphorylates and inhibits the transcriptional coactivators Yes-associated protein 1 (YAP1) and TAZ (5,6). YAP1 or TAZ interacts with the TEA domain (TEAD)/TEF family of transcription factors (TEAD1–4) to transcriptionally regulate the target genes (5,7). However, it is largely unknown whether the Hippo pathway is dysregulated by multilevel perturbations in cancer.

Here, by screening differential DNA methylation (DNAm) and gene expression between normal and gastric cancer (GC) tissues using MENT and GENT, we identified the concurrent DNA hypomethylation and overexpression of Hippo pathway-related genes, including TEAD4. We further evaluated the functional relevance of TEAD4 to GC development through clinical validation, cell biological analysis and genome-wide approaches.

Materials and methods

Patients

A total of 108 paired GC/normal tissues were obtained from Chgunnam National University Hospital in Korea (Supplementary Table S1, available at Carcinogenesis Online). Samples were obtained with the informed consent, and the study was approved by the Internal Review Board at Chgunnam National University Hospital.

Pyrosequencing

Genomic DNA was prepared using the Puregene™ DNA purification kit (Qiagen) and modified using the EZ DNA Methylation kit (ZYM0 Research). A TEAD4 promoter was amplified using a 2× Taq Plus Premix (Noble bio). The pyrosequencing primers were designed by PSQ Assay Design (Biotage AB): forward: 5-GGTTTATAGTGTTAAGGGTTAG-3; biotinylated reverse: 5-CCCCCCCCCAACTCCCTCTTC-3; sequencing primer: 5-GAGTTATGGTTAAGGGTTAG-3. Pyrosequencing was performed with the PSQ HS 96A System (Biotage AB).

Cell culture

AGS, MKN28, MKN45, SNU16 and SNU216 GC cell lines and 293T were purchased from Korean Cell Line Bank. GC cell lines and the 293T were maintained in RPMI 1640 and Dulbecco’s modified Eagle’s medium (WELGENE).

Abbreviations: ADM, adrenomedullin; ANG, angiogenin; ARID5B, AT-rich interactive domain 5B; CALD1, caldesmon 1; ChIP-seq, chromatin immunoprecipitation-sequencing; DNAm, DNA methylation; EDN2, endothelin 2; FSCN1, Fascin homolog 1; FTX8, fucosyltransferase 8; GC, gastric cancer; GENT, Gene Expression across Normal and Tumor tissue; MENT, Methylation and Expression Database of Normal and Tumor tissues; OSR2, odd-skipped related 2; siRNA, small interfering RNA; TEAD4, TEA domain family member 4; YAP1, Yes-associated protein 1.
supplemented with 10% fetal bovine serum (HyClone), respectively, at 37°C in a humidified atmosphere with 5% CO₂.

Tissue microarray and immunohistochemistry
The tissue microarray and immunohistochemistry were performed using 59 paired GC samples as described previously (SuperBioChips) (8). After excluding 6 tissue pairs that failed to stain, 53 pairs were analyzed. Antibodies against TEAD4 (H00007004-M01) and YAP1 (#9192) were purchased from Abnova and Cell Signaling Technology, respectively.

Quantitative reverse transcription–PCR
Total RNA was isolated using an RNasey® Mini kit (Qiagen) and cDNA was synthesized using the iScript™ cDNA Synthesis kit (Bio-Rad Laboratories). Expression levels were quantified using a C1000™ Thermal Cycler and the iQ™ SYBR® Green supermix (Bio-Rad Laboratories). The measurements were normalized by GAPDH level.

Western blot analysis
The harvested cells were lysed with radioimmunoprecipitation assay buffer. Twenty micrograms of cell extracts were loaded onto a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel, and the gel was then subjected to western blot analysis using a Bio-Rad western blot system.

Generation of stable cell lines
For gene knockdown, the 293T cell line was cotransfected with the MISSION® Lentiviral Packaging Mix and either a non-silencing control vector, a TEAD4 shRNA vector or a YAP1 shRNA vector (Sigma–Aldrich). For overexpression, a TEAD4 overexpression vector cloned into pCDH-CMV-MCS-EF1-Puro (System Biosciences) was used. After 6 h, the medium was changed to Dulbecco’s modified Eagle’s medium supplemented with 2% fetal bovine serum. After 24 h, the GC cell lines for the knockdown experiments were seeded onto 6-well culture plates (SPL). The next day, the supernatants containing the lentivirus were collected from the 293T cells, filtered and applied to the target cells for lentiviral transduction. After 10 h, the medium was changed to complete RPMI medium. After 2 weeks of puromycin selection (Invitrogen), the knockdown was confirmed with quantitative reverse transcription–PCR and western blotting.

RNA interference with small interfering RNA
Small interfering RNA (siRNAs) for targeting TEAD4, YAP1 or Fascin homolog 1 (FSCN1) were purchased from Bioneer. 2 x 10⁵ cells on a 6-well-plate (SPL) were transfected with 100 pmol of siRNA using Lipofectamine® RNAiMAX (Invitrogen). After 48 h, the knockdown was assessed with quantitative reverse transcription–PCR.

Cell proliferation and xenograft assay
2 x 10⁴ cells were plated onto a 96-well plate (SPL) and the proliferation over a given time course was measured with the EZ-Cytox Cell Viability Assay Kit (ITSBIO) using a microplate reader (Molecular Devices) at a 450 nm. For xenograft assay, parental or TEAD4-knockdown MKN45 cells were subcutaneously injected into nude mice (3 x 10⁴ cells per mouse). Tumor volume was measured with calipers during 3 weeks and calculated by the formula, (width³ x length)/2.

Microarray analysis
The quality of the RNAs extracted from the control or the TEAD4-knockdown cell lines of SNU216 was tested with the Experion™ RNA StdSens Analysis Kit (Bio-Rad Laboratories), and cRNA was synthesized using the Illumina® TotalPrep™ RNA Amplification Kit. The hybridization and image acquisition were performed with an Illumina BeadArray System, and the data were quantile normalized.

Chromatin immunoprecipitation and sequencing
MKN28 and SNU216 cells were 1% formaldehyde fixed, lysed and sonicated using a Model 100 Sonic Dismembrator (Fisher scientific). The chromatin fragments were immunoprecipitated with a TEAD4 antibody. The remaining steps were performed with an EZ-ChIP™-Chromatin Immunoprecipitation Kit (Millipore). For chromatin immunoprecipitation–sequencing (ChIP-seq), 200–300bp sized genomic libraries were generated with the input and the TEAD4–immunoprecipitated chromatin fragments using the Illumina TruSeq ChIP Sample Prep Kit and sequenced them using the Illumina Genome Analyzer IxL, producing 76pb single reads.

ChIP-seq data analysis
Sequencing reads were aligned on human reference genome 19 with Bowtie2. Chip-peak calling was performed using a Model-based Analysis of Chip-Seq (MACS), and the Chip-peak annotation was performed with windowBed of BEDTools. The Chip-peaks with a −2.0 to +0.5kb region relative to transcription start sites were considered as TEAD4 binding sites in the promoter of corresponding genes. Consensus motifs were analyzed within a ±50bp region on either side of the peak summits using STAMP.

Statistical analysis
To compare means, experiments were performed in three replicates and analyzed with the t-test using SPSS 11.5. The data were presented as the means ± SD, and P < 0.05 was considered to be statistically significant.

Data access
The data of ChIP-seq and microarray is available from the NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) via accession numbers GSE44416 and GSE44383, respectively.

Results
Identification of concurrent DNA hypomethylation and overexpression of multiple Hippo pathway-related genes in GC
To uncover previously unrecognized GC genes that undergo multi-level perturbations during tumorigenesis, we integrated the GENT and MENT, which encompass 432 samples (357 GC/75 normal) and 205 samples (114 GC/91 normal), respectively (3,4). Based on the threshold criteria for differential DNAm (>10% difference) and for differential gene expression (>0.5-fold difference in Log₂ scale) between GC and normal tissues, the integration of GENT and MENT revealed 1968 hypermethylated/downregulated probe combinations (Hyper/Down) for 818 genes and 321 hypomethylated/upregulated probe combinations (Hypo/Up) for 208 genes in GC (Figure 1A and B and Supplementary Table S2, available at Carcinogenesis Online). To determine the dysregulated cancer pathway(s) associated with these gene sets, we overlapped these genes with previously reported cancer pathway signatures (9,10). This analysis revealed that Hypo/Up targets significantly overlap with the signatures of YAP1 and RAS, but not with the signatures of MYC, E2F3, SRC and β-catenin (Supplementary Table S3, available at Carcinogenesis Online). Because RAS, a well-known oncogene, is expected to be upregulated in several cancers including GC, we concentrated on the YAP1 signature. A total of 19 among the 321 Hypo/Up targets significantly overlapped with the YAP1 signature (P < 0.012). Moreover, the Hypo/Up targets contained known Hippo pathway components, such as FAT, JUB, LAT2, TEAD4 and YAP1 (Figure 1B). Thus, nine Hippo pathway-related genes, including components (FAT, JUB, LAT2, TEAD4 and YAP1) and targets (CTGF, CYR61, CRIM1 and ITGB2), were concurrently hypomethylated and overexpressed in GC.

To confirm the screening results, the DNAm patterns of the nine Hippo pathway-related genes were individually examined using MENT. Although various cancer types displayed no differences in the DNAm of the Hippo pathway-related genes between normal and tumor tissues, GC showed clear DNA hypomethylation (Figure 1C and Supplementary Figure S1, available at Carcinogenesis Online). Notably, because the patterns of DNAm found among the Hippo pathway-related genes appeared to be similar (Supplementary Figure S1, available at Carcinogenesis Online), we measured the pair-wise correlation of the DNAm levels between the Hippo pathway-related genes. In normal tissues, the DNAm level at the CpG site cg26369058 of the TEAD4 promoter was highly correlated with the DNAm levels at the promoter CpGs from other Hippo pathway components, including YAP1 (r = 0.92, cg26369058), LAT2 (r = 0.87, cg05714479), FAT (r = 0.91, cg19428336) and JUB (r = 0.94, cg17559711), and the Hippo pathway targets, including CTGF (r = 0.92, cg24311382), CYR61 (r = 0.94, cg04453065) and CRIM1 (r = 0.90, cg10881306) but not ITGB2 (r = −0.46, cg24167037) (Figure 1D). However, in GC, the correlations were decreased as follows: YAP1 (r = 0.81), LAT2 (r = 0.20), FAT (r = 0.58), JUB (r = 0.82), CTGF (r = 0.54), CYR61 (r = 0.48), CRIM1 (r = 0.37) and ITGB2 (r = −0.17) (Figure 1D). These results raised the possibility that multiple Hippo pathway-related genes may be coregulated in normal tissues at the epigenetic level, but this coregulation is slightly disrupted in GC.

To examine the DNA hypomethylation of the nine Hippo pathway-related genes in broader regions, we used the TCGA methylation database, which was generated using 450K methylation array. Compared with
normal tissues \((n = 2)\), GC tissues \((n = 261)\) showed the hypomethylation patterns in promoter and 5′UTR regions of the nine Hippo pathway-related genes \((\text{Supplementary Figure S2}, \text{available at Carcinogenesis Online})\). This means that the nine Hippo pathway-related genes were hypomethylated at multiple CpG sites around regulatory regions.

**Clinical importance of DNA hypomethylation at the TEAD4 promoter in GC**

To confirm the screening results using our Korean GC samples, we conducted pyrosequencing. In this part, we focused on TEAD4, a molecular bridge linking the Hippo pathway components to the Hippo pathway targets. Consistent with the MENT analysis, 108 GC tissues exhibited significant DNA hypomethylation at a CpG cg21637033 in the TEAD4 promoter compared with their normal counterparts \((\text{Figure 2A}; P = 3.82 \times 10^{-20})\). Notably, the methylation level at the TEAD4 promoter was negatively correlated with the tumor size \((\text{Figure 2B}; r = -0.23, P = 0.017)\). Because the Hippo pathway has been known to coordinate tissue size by recognizing cell density and position \((6)\), the negative correlation between the TEAD4 DNAm and the tumor size is likely to reflect the ability of the Hippo pathway to restrict tissues outgrowth.

Also, we observed that the difference in the DNAm levels between the normal and GC tissues was higher in the stage IV group compared with the stage I group \((\text{Figure 2C}; P = 0.043)\). In other words, a larger reduction in the amount of DNAm at the TEAD4 promoter may be associated with an increased tumor progression. Furthermore, a low methylation level at the TEAD4 promoter resulted in a poor survival of the GC patients compared with a high methylation level \((\text{Figure 2D}; P = 0.048)\). Accordingly, these results showed that the reduced DNAm level at the TEAD4 promoter is correlated with poor disease outcomes, such as high-grade tumor and poor survival rate.

**The relationship between the DNAm and the expression level of TEAD4 in GC tissues**

Given the above clinical implications of the TEAD4 DNAm level, the alteration of the DNAm level could affect the gene expression of TEAD4. Therefore, we measured the expression level of TEAD4 using 108 paired GC/normal tissues. Indeed, the GC tissues had significantly higher expression levels of TEAD4 compared with the normal tissues \((\text{Figure 2E}; P = 5.15 \times 10^{-10})\). Moreover, the expression level of TEAD4 was inversely correlated with the DNAm level at the TEAD4 promoter \((\text{Figure 2F}; r = -0.25, P = 0.00027)\), indicating that the DNA hypomethylation may be involved in the TEAD4 overexpression. Consistent with this, the inverse correlation was also observed in 10 GC cell lines \((\text{Figure 2G}; r = -0.78, P = 0.0081)\). Because the differential DNAm between normal and tumor tissues was higher in high-grade GC \((\text{Figure 2C})\), we expected the increased expression level of TEAD4 in high-grade GC. Indeed, the higher expression level of TEAD4 was observed in high-grade GC \((\text{Figure 2H})\).

**Enhanced nuclear localization of TEAD4 accompanied by YAP1 in GC**

Previously, YAP1 was markedly accumulated in the nuclei of GC cells \((11)\). Because YAP1 cooperates with TEAD4 as a TEAD-binding coactivator, the nuclear localization of YAP1 in GC was expected to be accompanied by nuclear localization of TEAD4. To examine the protein expression levels and patterns of TEAD4 and YAP1, we...
Fig. 2. The clinical significance of DNA methylation and gene expression of TEAD4. (A) The TEAD4 DNA methylation level comparing 108 GC tissues to the normal counterparts. (B) The correlations between the TEAD4 DNA methylation level and tumor size (mm). (C) Differences in the TEAD4 DNA methylation level between normal and GC tissues are plotted according to tumor stages. A P value is shown for the comparison of stage I with stage IV. (D) A Kaplan–Meier survival curve analyzed according to the DNA methylation level of TEAD4. High or low methylation groups were divided based on median DNA methylation level. Log rank P value is shown. (E) The TEAD4 expression level comparing 108 GC tissues to the normal counterparts. (F) The correlations between the TEAD4 DNA methylation level and TEAD4 expression level in GC and normal tissues. (G) The correlations between the TEAD4 DNA methylation level and the TEAD4 expression level in 10 GC cell lines. (H) The expression levels of TEAD4 according to tumor stages.

Fig. 3. Frequent nuclear localization of TEAD4 accompanied by YAP1 in GC. (A) Representative immunohistochemistry images for TEAD4 or YAP1 are presented for the GC and the matched normal tissues; 20-fold magnified figures are shown. The arrows indicate notable expression correlations between TEAD4 and YAP1. (B) The percentage of nuclear or cytoplasmic expression of TEAD4 in 53 paired GC/normal tissues. (C) The statistics for the expression correlations between TEAD4 and YAP1. ‘Positive correlation’ indicates parallel patterns for expression levels or localization patterns between TEAD4 and YAP1. ‘Negative correlation’ indicates opposite expression patterns between TEAD4 and YAP1. ‘Independent’ means that the patterns are not correlated between TEAD4 and YAP1.
performed immunohistochemistry using 53 paired GC/normal tissues. In normal tissues, both TEAD4 and YAP1 were predominantly found in the cytoplasmic fractions of gastric glands (Figure 3A). In intestinal metaplasia, goblet cells highly expressed TEAD4, but not YAP1, indicating that TEAD4 may play roles in a YAP1-independent manner in goblet cells (Supplementary Figure S3, available at Carcinogenesis Online). Given that intestinal metaplasia can be considered to be a preneoplastic lesion for intestinal-type GC (12,13), TEAD4 could be involved in an early step of intestinal-type GC development.

In GC tissues, TEAD4 was observed more frequently in the nuclear fractions of the tumor cells than the matched normal tissues (Figure 3A and B, P = 0.032), indicating the posttranslational activation of TEAD4. Consistent with a previous report that YAP1 is accumulated in the nuclei of GC cells (11), YAP1 was also frequently found in the nuclei of our GC tissues (Figure 3A and Supplementary Figure S4, available at Carcinogenesis Online). The nuclear localization of TEAD4 and YAP1 was observed irrespective of the GC differentiation states (Supplementary Figure S4, available at Carcinogenesis Online). In addition, the tissue region with higher expression of TEAD4 showed higher amounts of YAP1 (Figure 3A), displaying a positive expression correlation between TEAD4 and YAP1 (Figure 3C). Therefore, TEAD4 localizes into the nucleus more frequently and accompanies with YAP1 in GC.

Requirement of TEAD4 expression for the growth and maintenance of GC cells

To date, studies on the functional roles of TEAD4 have mainly concentrated on trophoderm specification and muscle development (14–16). In cancer field, a weak transforming activity of TEAD4 was reported in MCF-10A mammary epithelial cells (17). Because we discovered several perturbations of TEAD4 in GC and the functional role of TEAD4 has not yet been examined in GC, we decided to test the functional impact of TEAD4 using GC cells.

To evaluate the oncogenic activity of TEAD4, we generated TEAD4 stable knockdown cell lines using AGS, MKN28, MKN45, SNU16 and SNU216 cell lines (Figure 4A). YAP1-knockdown cell lines were also generated for the comparison with the function of TEAD4 (Figure 4A). A TEAD4 or YAP1 knockdown significantly decreased the proliferation of all GC cells tested compared with the controls (Figure 4B). Two different TEAD4 shRNAs consistently reduced proliferation, excluding an off-target effect. In addition, when TEAD4 was stably overexpressed to assess the gain-of-function effect of it, the cell proliferation of SNU216 was highly increased (Figure 4C). Besides, when the TEAD4-depleted MKN45 cell line was injected into nude mice, we observed that the tumorigenic activity of the MKN45 was significantly decreased in vivo (Figure 4D). Collectively, these data indicate that the TEAD4 is required for the growth of GC cells both in vitro and in vivo.

ChIP-seq and microarray analysis reveal the oncogenic characteristics of TEAD4 in GC

To assess the detailed functional roles of TEAD4 as a transcription factor in GC, ChIP-seq was performed with the MKN28 and SNU216 cell lines, both of which had dramatic phenotypic effects on proliferation with the TEAD4 knockdown. When we analyzed the ±50bp sequences relative to summits of the TEAD4 ChIP-peaks, a conserved TEAD-binding motif (5′-CATTCC-3′) was the most significantly

Fig. 4. Requirement of TEAD4 for the growth of GC cells. (A) The confirmation of TEAD4 or YAP1 knockdown by western blotting. (B) The relative proliferation of TEAD4- or YAP1-knockdown cell lines was measured during 4 days by comparing with the non-silencing shRNA control (NSC). The mean values of three times measurement were plotted for proliferation. (C) The relative proliferation of SNU216 GC cells, which were generated with TEAD4 expression vector (TEAD4 over) or control empty vector (Con), was measured. (D) The in vivo tumor growth of TEAD4-depleted MKN45 into nude mice. The tumor volume (mm3) generated from NSC (n = 7) or TEAD4-knockdown (n = 7) cells was measured during 20 days. *P < 0.05.
TEAD4 dysregulation in gastric cancer

Defining the promoter region as −2.0 to +0.5 kb relative to transcription start sites, we examined the TEAD4 target promoters. This analysis revealed that TEAD4 binds to the promoter regions corresponding to 3246 genes in MKN28 and 1162 genes in SNU216 (Figure 5C). A total of 876 genes were identified as common TEAD4-binding genes in both cells. A gene ontology analysis of the 876 common TEAD4-binding genes indicated that TEAD4 binds to genes involved in several cancer-related categories, including ‘Small cell lung cancer’, ‘Apoptosis’, ‘Pathways in cancer’, ‘Positive regulation of cell motion’ and ‘WNT receptor signaling pathway through beta-catenin’ (Supplementary Figure S5A, available at Carcinogenesis Online). In addition, YAP1 was predicted as the most significantly enriched upstream regulator of the 876 common TEAD4-binding genes (Supplementary Figure S5B, available at Carcinogenesis Online), again indicating the cooperation of TEAD4 with YAP1.

Interestingly, TEAD4 ChIP-seq revealed the potential feedback regulation in the Hippo pathway. TEAD4 ChIP-peaks were found in the promoters of TEAD1 and TEAD4, indicating that positive feedback or autoregulation may occur (Supplementary Figure S6A, available at Carcinogenesis Online). Also, TEAD4 ChIP-peaks were found in the promoters of PTPN14, AMOTL2 and LATS2, which were ranked in the top 10 strongest TEAD4-binding genes (Supplementary Figure S6B and C, available at Carcinogenesis Online) and have been reported as negative regulators for YAP1 (18–22), indicating the possibility for negative feedback regulation.

To discover novel targets regulated by TEAD4, we performed microarray analysis using the SNU216-knockdown cells. A total of 1017 genes were up- or downregulated >1.5-fold in the TEAD4-knockdown cells. The integration of these genes with the TEAD4-binding genes revealed 87 putative TEAD4 target genes (Figure 5D and Supplementary Table S4, available at Carcinogenesis Online). The functional categorization of these 87 genes revealed that the most significantly enriched category is ‘Female pregnancy’. This may reflect the role of TEAD4 in the specification of trophectoderm and inner cell mass during preimplantation mammalian development (14). The categories, ‘Anion transport’ and ‘Response to nutrient levels’, may support the role of TEAD4 to provide nutrients to embryos in trophoblast. The ‘Muscle contraction’ is also reasonable because TEAD4 is involved in muscle development (16,23). Importantly, the categories, ‘Positive regulation of cell proliferation’ and ‘Cell motion’, were also significantly enriched, directly connecting the functional relevance of TEAD4 to GC (Figure 5D).

A total of seven genes, angiogenin (ANG), adrenomedullin (ADM), endothelin 2 (EDN2), odd-skipped related 2 (OSR2), AT-rich interactive domain 5B (ARID5B), caldesmon 1 (CALD1) and fucosyltransferase 8 (FUT8), were included in the cancer-related categories, “Positive regulation of cell proliferation” and “Cell motion”. To examine whether TEAD4 regulates these cancer-related gene set, we measured the expression levels in TEAD4-knockdown cells. Except for FUT8, the other six genes were downregulated by a TEAD4 knockdown (Figure 5E). Moreover, among these genes, the ADM expression level was significantly correlated with the TEAD4 expression in 311 GC tissues (Figure 5F; r = 0.21, P = 0.000051). Therefore, ChIP-seq and microarray analysis revealed the oncogenic characteristics of TEAD4 and this factor’s novel target genes that are involved in the proliferation and migration of GC cells.

Integration of TEAD4-binding genes and TEAD4-coregulated genes reveals a novel target, FSCN1

Although microarray analysis detects differential gene expression on a genome-wide scale, this technique can result in false negatives.

Fig. 5. ChIP-seq and microarray analysis reveal the functional importance of TEAD4 in cancer. (A) Enrichment of a TEAD-binding motif in TEAD4 ChIP-peaks. (B) Confirmation of the validity of the ChIP-seq experiment using the promoters of CTGF and CYR61. (C) The intersection of TEAD4-binding genes derived from MKN28 and SNU216 cells. (D) Integration of 876 common TEAD4-binding genes from ChIP-seq with 1017 TEAD4-regulated genes from microarray analysis in SNU216 cells. Gene ontology was analyzed for 87 overlapped genes using DAVID. (E) The expression levels of genes involved in the categories ‘Positive regulation of cell proliferation’ and ‘Cell motion’ were measured by comparing SNU216 TEAD4-knockdown cells to the NSC. (F) The expression correlation between TEAD4 and ADM in 311 GC tissues.
To search for important targets of TEAD4 excluded from the microarray analysis, we calculated the expression correlation between TEAD4 and other genes using 311 GC samples from GENT. This analysis revealed 928 genes highly correlated ($r \geq 0.3$) with TEAD4 expression in GC tissues. The lists were then integrated with the 876 TEAD4-binding genes, generating 62 candidate targets of TEAD4 (Figure 6A and Supplementary Table S5, available at Carcinogenesis Online).

Among these 62 candidates, the most highly enriched gene as determined by TEAD4 ChIP was FSCN1 (Figure 6B), which is the top 23rd ranked gene among the total ChIP-seq data. The FSCN1 expression level showed a high correlation with TEAD4 expression in 311 GC tissues (Figure 6C; $r = 0.46$, $P = 2.83 \times 10^{-4}$). Notably, FSCN1 was previously listed as one of the 57 conserved YAP1 signatures (9). Collectively, these findings raised the possibility that FSCN1 may be regulated by TEAD4-YAP1. Indeed, the knockdown of either TEAD4 or YAP1 significantly decreased the expression level of FSCN1 in AGS cells, which have a high transfection efficiency (Figure 6D), indicating the contribution of both TEAD4 and YAP1 to FSCN1 expression. Furthermore, the knockdown of FSCN1 expression resulted in decreased cell proliferation (Figure 6E) in addition to the functional role of FSCN1 in the migration of GC cells as described previously (24). Therefore, the integration of both the ChIP-seq data and the expression correlation data obtained from GENT successfully revealed a novel TEAD4 target, FSCN1, involved in the proliferation and migration of GC cells.

**Discussion**

Here, we described a novel linkage between Hippo pathway-related genes and GC. Among the 14 different tissue types investigated using MENT, only GC tissues exhibited concurrent DNA hypomethylation in nine Hippo pathway-related genes, indicating that the DNA hypomethylation may be a potential GC-specific epigenetic signature. Moreover, the DNAm patterns of the Hippo pathway-related genes were highly correlated with each other, demonstrating that those genes may be coregulated at the epigenetic level.

Focusing on the TEAD4, we identified three types of novel perturbations of TEAD4 in GC (Figure 6F). First, at the epigenetic level, the TEAD4 promoter has significant DNA hypomethylation in GC. Because the DNA hypomethylation at the TEAD4 promoter was significantly correlated with a larger tumor size, a higher grade tumor and worse disease survival, the epigenetic alteration can act as an important GC marker with prognostic and clinical applications. Second, at the transcriptional level, TEAD4 was found to be overexpressed in GC. Consistent with our observation, TEAD4 is the third most highly overexpressed gene in GC according to the Oncomine database. Because the DNAm level of TEAD4 was inversely correlated with the TEAD4 expression level, the epigenetic perturbation may partially lead to the transcriptional activation of TEAD4 in GC. In contrast to TEAD4, the promoter region of other TEADs, TEAD1 and TEAD2, do not show significant differences in the level of methylation and expression between normal and GC (Supplementary Figure S7, available at Carcinogenesis Online). Third, at the posttranslational level, TEAD4 was frequently observed in the nuclei of GC tissues, accompanied by YAP1. Thus, we can speculate that TEAD4 and YAP1 translocate together into the nuclei of GC cells and subsequently form a complex to synergistically regulate target genes that are related to cancer phenotypes.

To identify TEAD4-regulated genes, we performed ChIP-seq and microarray analysis in two GC cell lines, MKN28 and SNU216. Remarkably, these genome-wide approaches directly connected the functional relevance of TEAD4 to cancer. The overlapped gene set that was derived from the ChIP-seq and the microarray analysis

![Fig. 6. ChIP-seq and GENT reveals a novel TEAD4 target, FSCN1, in GC.](https://academic.oup.com/carcin/article-abstract/35/5/1020/272338)
revealed significant cancer-related categories, including cell proliferation and migration. As the result, we identified seven novel TEAD4 targets, **ANG**, **ADM**, **EDN2**, **OSR2**, **ARD5B**, **CALD1** and **FSCN1**, involved in proliferation and migration (24–30). Previously, these TEAD4 targets were reported to be associated with cancer as follows. In GC, **ANG**, a neovascularization factor that coordinates with **VEGF**, was overexpressed and it promoted the cell proliferation (25). **ADM** was highly expressed in colon cancer, and its expression was correlated with cancer stage and survival rate (26). EDNs, including **EDN2**, are a group of small peptides involved in several malignancies through paracrine/autocrine actions (27). **OSR2** plays roles in proliferation and development by regulating genes involved in cell cycle and proliferation. The **ARID** proteins are implicated in cell growth, differentiation and development (30). **FSCN1**, an actin bundling protein, promoted cell motility and metastasis, and the **FSCN1** knockdown inhibited those phenotypes in GC (24). In the future, detailed studies are required to demonstrate how TEAD4 regulates these targets and these targets contribute to GC development.

In conclusion, we uncovered the importance of Hippo pathway-related genes in GC using the integrative analysis. In particular, the dysregulation of **TEAD4** at the epigenetic, transcriptional and post-translational levels may contribute to GC development.

**Supplementary material**

Supplementary Tables S1–S5 and Figures S1–S7 can be found at http://carcin.oxfordjournals.org/

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**References**


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