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Original Research

AEBP1 promotes papillary thyroid cancer progression by activating BMP4 signaling

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ABSTRACT

Papillary thyroid cancer (PTC) is the most prevalent endocrine cancer worldwide. Approximately 30 % of PTC patients will progress into the advanced or metastatic stage and have a relatively poor prognosis. It is well known that epithelial-mesenchymal transition (EMT) plays a pivotal role in thyroid cancer metastasis, resistance to therapy, and recurrence. Clarifying the molecular mechanisms of EMT in PTC progression will help develop the targeted therapy of PTC. The aberrant expression of some transcription factors (TFs) participated in many pathological processes of cancers including EMT. In this study, by performing bioinformatics analysis, adipocyte enhancer-binding protein 1 (AEBP1) was screened as a pivotal TF that promoted EMT and tumor progression in PTC. *In vitro* experiments indicated that knockout of AEBP1 can inhibit the growth and invasion of PTC cells and reduce the expression of EMT markers including N-cadherin, TWIST1, and ZEB2. In the xenograft model, knockout of AEBP1 inhibited the growth and lung metastasis of PTC cells. By performing RNA-sequencing, dual-luciferase reporter assay, and chromatin immunoprecipitation assay, Bone morphogenetic protein 4 (BMP4) was identified as a downstream target of AEBP1. Over-expression of BMP4 can rescue the inhibitory effects of AEBP1 knockout on the growth, invasion, and EMT phenotype of PTC cells. In conclusion, these findings demonstrated that AEBP1 plays a critical role in PTC progression by regulating BMP4 expression and the AEBP1-BMP4 axis may present novel therapeutic targets for PTC treatment.

Introduction

Thyroid cancer is the most common type of endocrine cancer in women and there is an increased incidence and upward trend of mortality rate of thyroid cancer from 2000 to 2016 in China [1]. Papillary thyroid cancer (PTC) accounts for 85 %–90 % of thyroid cancer [2]. Although a majority of PTC patients have a favorable prognosis, there are still some patients who die from recurrence and distant metastasis. Genetic alterations such as $BRAF^{V600E}$, and RET/PTC are reported to be associated with the proliferation and metastasis of PTC [3] and several drugs which inhibit their protein kinases activity can help stabilize the disease of PTC patients [4]. Besides, aberrant expression of genes in cancer may be involved in the progression of tumors, and drugs targeting the genes aberrantly expressed in PTC may also have the capability of blocking the progression of PTC. Therefore, it is significant to screen and validate the pivotal genes in PTC progression.

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Adipocyte enhancer-binding protein 1 (AEBP1), as a transcription factor (TF) [5] aberrantly expressed in many types of tumors [6], is associated with the aggressive features and tumor progression of many cancers such as glioblastoma (GBM) [7], gastric cancer (GC) [8], colorectal cancer (CRC) [9,10], and breast cancer (BC) [11]. Notably, several studies indicated that elevated AEBP1 expression was associated with epithelial-mesenchymal transition (EMT) in cancers. EMT is a cell biological process in which tumor cells lose their epithelial features and acquire mesenchymal features, which can promote tumor metastasis, resistance to therapy, and recurrence [12]. AEBP1 expression was found to be positively correlated with EMT scores in 329 colon adenocarcinoma (COAD) patients [13] and upregulation of AEBP1 was found to promote the tumor growth and metastasis of GC and COAD by regulating the expression of EMT-related genes [8,10]. In PTC, the dysregulated network of TFs is reported to be associated with the initiation of EMT and contributes to tumor recurrence and metastasis [14]. However, the role of AEBP1 in PTC progression remains elusive.

Herein, we carried out bioinformatics analysis and a series of *in vitro* and *in vivo* experiments to determine whether AEBP1 promotes PTC progression. We found that AEBP1 expression was higher in PTC samples compared to normal thyroid tissues and PTC patients with high expression of AEBP1 have worse prognosis. *In vitro*, AEBP1 promotes PTC cellsgrowth, migration, and invasion. Mechanistically, AEBP1 regulates the expression of bone morphogenetic protein 4 (BMP4) and promotes the EMT process in PTC. Our findings suggest that AEBP1 may be a crucial target for the treatment of PTC.

Materials and methods

Bioinformatics analysis

The mRNA expression data and clinical information of GDC TCGA Thyroid Cancer (THCA) (TCGA-THCA) cohort were downloaded from UCSC Xena (https://xenabrowser.net) [15]. The data from GSE60542 [16], GSE33630 [17,18], and GSE153659 [19] cohorts was downloaded from Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/). The signature profiles of proliferation, invasion, metastasis, and EMT were obtained from CancerSEA database (http://biocc.hrbmu.edu.cn/CancerSEA/) [20]. The single-sample gene set enrichment analysis (ssGSEA) algorithm [21] was used to calculate the scores of samples in TCGA-THCA cohort. To screen genes correlated with progression of PTC, weighted gene co-expression network analysis (WGCNA) was performed based on FPKM data of 2570 genes, which were composed of TFs, protein kinases, and epifactors.

Cell culture

The human PTC cell lines (BCPAP and TPC-1) were purchased from Procell Life Science (Wuhan, China) with STR profiles. All cells were cultured in DMEM medium (Gibco) supplemented with 10 % fetal bovine serum (FBS) (Gibco) and 1 % penicillin/streptomycin (Gibco) at 37 °C with 5 % of CO_2.

Plasmids and lentivirus production

The AEBP1 cDNA (CCDS5476.1) was cloned into the pCDH plasmid and the BMP4 cDNA (CCDS9715.1) was cloned into pLENTI plasmid for genes over-expression. The plasmids used for CRISPR–Cas9-mediated knockout (KO) of AEBP1 were purchased from Tsingke company (Beijing, China). The plasmids were transfected into 293 T cells along with the psPAX2 and pMD2.G plasmids for viral packaging. The supernatants of 293T cells were collected to infect PTC cells and experienced puromycin selection. The sequences of single guide RNA (sgRNA) are caccgGCGCCGAATGTACTCAACTG (sgAEBP1#1) and caccgGCCACTCCAGGTATCCACG (sgAEBP1#2).

Cell growth assay

PTC cells were cultured in 96-well plates with DMEM containing 10 % FBS and 1 % penicillin/streptomycin (1500 cells/well, three wells per group). Then, equal amounts of the CCK-8 reagent (C0048XL, Beyotime, China) were added into each well at different time and the viability of cells was examined using the absorbance at 450 nm.

Colony forming assay

PTC cells were seeded in 6-well plates with DMEM containing 10 % FBS and 1 % penicillin/streptomycin (300 cells/well, three wells per group). Three weeks after cells were seeded, the numbers of colonies were counted after cells were fixed with 10 % formalin and stained with 0.1 % crystal.

Cell cycle assay

PTC cells were seeded in 24-well plates with DMEM containing 10 % FBS and 1 % penicillin/streptomycin for 24 h, Then, cells were digested with trypsin and suspended into single cell, and fixed with 70 % ethanol at 4°C overnight. PBS with 0.2 mg/mL RNase A was used to re-suspended and washed these cells and wash buffer with 50 μg/mL PI (C1052, Beyotime, China) for staining cells for 30 min. Next, the percentages of cells of G0/G1, S, and G2/M were measured by Beckman Cytoflex.

Cell adhesion assay

PTC cells (3×10^4 cells/well, three wells per group) were cultured in 96-well plates coated with fibronectin for 2 h. Then, the cells were fixed with 10 % formalin, stained with crystal violet and subsequently the cells were washed with ddH2O and incubated with 100 μl 33 % acetic acid. The absorbance detected at 560 nm was used to evaluate the cell adhesion.

Transwell assay

For transwell assays, chambers (24-well, 8 μm pore, Corning) with Matrigel (BD Science, USA) were utilized for measuring cell invasion and chambers without Matrigel were utilized for measuring cell migration. PTC cells were seeded in the upper chambers with 200 μL serum-free DMEM (5×10^4 cells/well, 3 wells per group). 500 μL of DMEM containing 10 % FBS was added into the lower chambers. After cells were incubated at 37°C for 24 h, the cells in the upper surface of the chamber were removed and the cells in the lower surface of the chamber were fixed with 4 % paraformaldehyde and stained with 0.1 % crystal violet for counting.

Western blot

Total protein was lysed with RIPA buffer (KeyGEN, Biotech), and the protein concentration was determined using a BCA Protein Assay Kit (ZJ101, EpiZyme). Total protein was separated by SDS–PAGE gel electrophoresis and transferred onto PVDF membrane (Millipore, USA). Membranes were blocked in 5 % skim milk for 1 h and incubated overnight with primary antibodies at 4°C. Then, membranes were incubated with secondary antibody and visualized. The primary antibodies used were anti-AEBP1 (1: 1000, sc-271374, Santa Zruz, USA), anti-GAPDH (1: 10000, AC003, Abclonal, China), anti-BMP4 (1: 2000, ab124715, Abcam, UK), anti-E-cad (1: 2000, 14472, CST, USA), anti-N-cad (1: 1000, 13116, CST, USA), anti-vimentin (1: 1000, 3932, CST, USA), anti-TWIST1 (1: 1000, 90445, CST, USA), anti-ZEB2 (1: 1000, ab223688, Abcam, UK), anti-Smad1/5/9 (1: 1000, ab300164, Abcam, UK), and anti-p-Smad1/5/9 (1: 1000, AP0850, Abclonal).

RNA-sequencing

Total RNA from the BCPAP cells carrying sgAEBP1 or NC were extracted using Trizol reagent (Invitrogen). RNA purification, reverse transcription, library construction and sequencing were performed by Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China) according to the manufacturer's instructions (Illumina, San Diego, CA). In this study, count data of mRNA expression was extracted to perform differential expression analysis with DESeq2 method. The pearson correlation analysis was performed with transcripts per million (TPM) data.

Chromatin immunoprecipitation (ChIP) and real-time PCR (RT-PCR)

ChIP assay was performed using a Pierce Magnetic ChIP Kit (CST, USA) according to the instructions. 1 % formaldehyde was added to PTC cells for cross-linking. Then, the lysates of cells were experienced digestion with MNase and sonication for obtaining DNA fragments. After immunoprecipitation with an anti-AEBP1 antibody (1:100, sc-271374, Santa Zruz, USA), samples were treated with Proteinase K and recovered with a DNA clean-up column. RT-PCR was performed with Hieff UNICON® Power qPCR SYBR Green Master Mix (YEASEN, China) on a QuantStudio Dx instrument (Applied Biosystem, USA) according to the instructions. The sequences of primers are F1: CACACAGTTCTGCACACAGC and R1: AGGAGCTGTTGGATCGGTTT (Primer#1), F2: GGGGTCTACCTCAGGGTCAT and R2: GCAGAACTGTGTGCCAGAGA (Primer#2), and F3: TAGCTCCCTCACAGCTCCAT and R3: AGGTGGAATGTGGTGGCTTT (Primer#3).

Dual-luciferase reporter assay

The promoter sequences of BMP4 was cloned into the pGL3-basic vector. Then, pGL3-basic, Renilla plasmid, and pCDH plasmids with an NC sequence or the AEBP1 cDNA were co-transfected into 293 T cells. Firefly, and Renilla luminescence were measured by the Dual-Glo® Luciferase Assay System (Promega, USA) 48 hours after transfection.

Immunohistochemistry (IHC)

PTC samples, along with paired adjacent normal thyroid specimens, were collected from the Affiliated Hospital of Jining Medical University for tissue microarray (TMA) construction. IHC staining of AEBP1 (1: 100, ab254973, Abcam, UK) and BMP4 (1: 200, ab124715, Abcam, UK) using TMA were performed following a standard IHC protocol as described previously [22]. The IHC-score (0–12) was obtained by multiplying the score of staining intensity with the score of positive cell frequency [23]. The score of staining intensity were defined as: 0 = negative; 1 = weak; 2 = moderate; and 3 = strong. The score of positive cell frequency was defined as: <5 % = 0; 5 %-25 % = 1; 26 %–50 % = 2; 51 %-75 % = 3; >75 % = 4.

Xenograft model

All animal procedures and protocols in our study were in accordance with the Institute of Biophysics, Chinese Academy of Science's Policy on Care and Use of Laboratory Animals. Female BALB/c nude mice (5 weeks old) were purchased from the HFK BIOSCIENCE (Beijing). 5×10^6 BCPAP cells with sgAEBP1 or control vector cells in 200 ul PBS were subcutaneously injected into the right flank of mice (6 mice/group). Tumor volumes were measured every 7 days and calculated according to the formula as follows: volume = 0.5 × tumor length × width^2. Tumor weights were obtained after dissecting the tumor tissues from euthanized mice. The tumor tissues were fixed and dissected for IHC staining with anti-AEBP1 (1: 100, ab254973, Abcam, UK), anti-BMP4 (1: 200, ab124715, Abcam, UK), anti-E-Cad (1: 1000, 14472, CST, USA), and anti-N-cad (1: 200, 13116, CST, USA). For the nude mouse model of pulmonary metastasis assay, PTC cells (1×10^6) were suspended in 200 μL PBS and injected into mice via the tail vein (5 mice/group). Four weeks after injection, the mice were euthanized, and the lung of dead mice was excised and fixed in formalin. Paraffin-embedded lungs were systematically sectioned and stained with hematoxylin and eosin (H&E) staining, and images were captured by iViewer software.

Statistical analysis

All data in this study were analyzed by GraphPad Prism and R software. Student's t-test was used to compare differences between two groups. The log-rank tests were performed after Kaplan–Meier survival analysis to assess patient's prognosis. $P < 0.05$ was considered statistically significant.

Results

AEBP1 was screened out and considered to be associated with progression and EMT in PTC

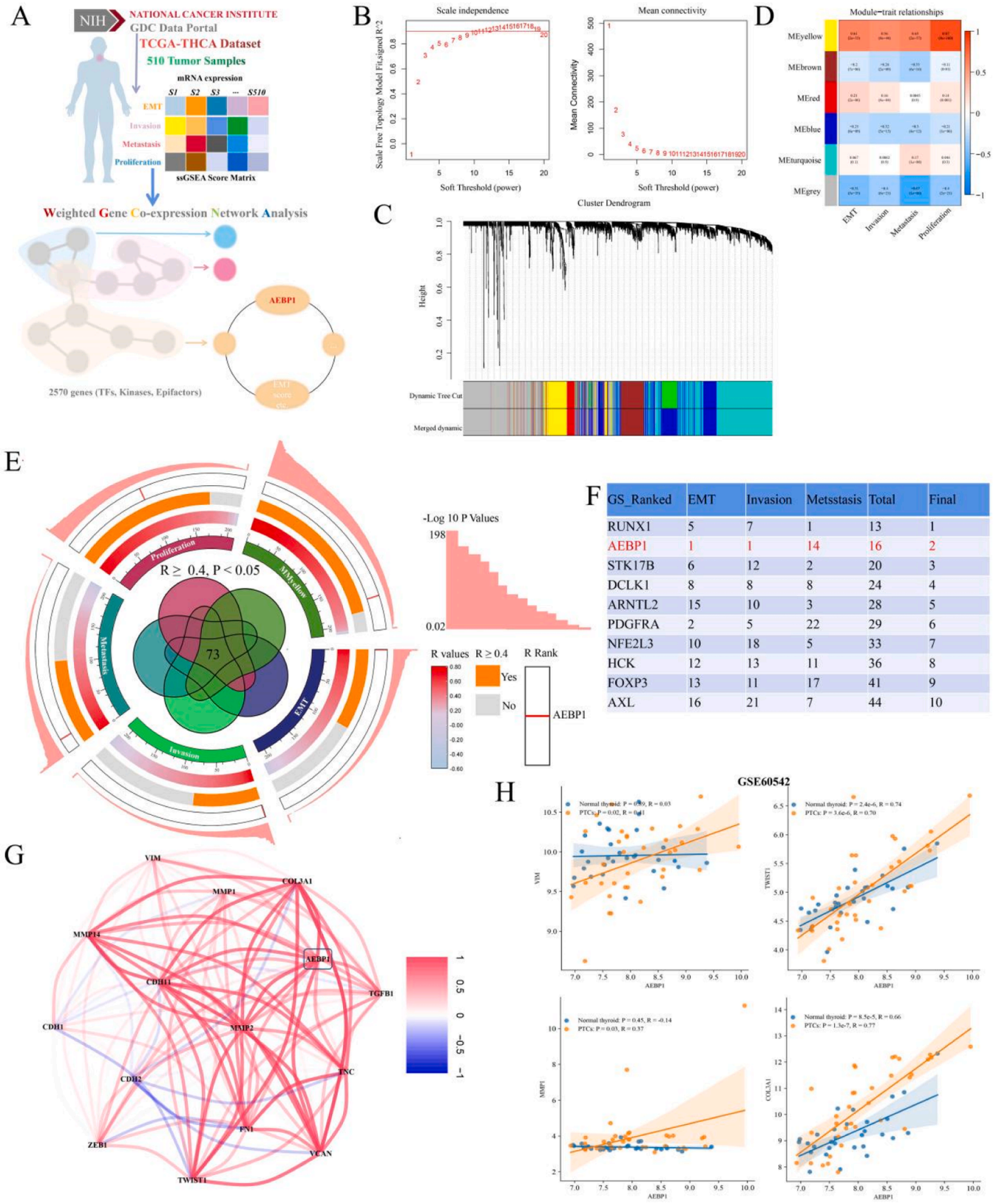
To identify pivotal genes in the progression of PTC, we used transcriptome data of TFs, kinase, and epifactors from TCGA-THCA to perform WGCNA. Then, proliferation, invasion, metastasis, and EMT score of samples in TCGA-THCA were calculated using ssGSEA method. Through pearson correlation analysis, core genes in the module identified by WGCNA which was associated with proliferation, invasion, metastasis, and EMT in PTC were screened out (Fig. 1A). In WGCNA, 10 was selected as the soft threshold power (Fig. 1B) and 6 co-expression modules were clustered (Fig. 1C). The yellow module had the strongest positive correlation with proliferation, invasion, metastasis, and EMT score (Fig. 1D). Total 73 genes in yellow module were significantly associated with proliferation, invasion, metastasis, and EMT scores with $P < 0.05$ and correlation coefficient (R) ≥ 0.4 (Fig. 1E). Among which, AEBP1 had the strongest positive correlation with invasion and EMT scores (Fig. 1F). We further found that AEBP1 had the strong positive correlation with EMT markers such as TWIST 1, VIM, MMP1, and COL3A1 both in TCGA-THCA (Fig. 1G) and GSE60542 (Fig. 1H) datasets. These results from bioinformatics analysis suggested that AEBP1 may be involved in PTC EMT and progression.

AEBP1 overexpression is associated with advanced TNM stage and poor prognosis in PTC

Firstly, the expression of AEBP1 in thyroid cancer samples and normal thyroid samples was examined using multiple cohorts including TCGA, GSE33630, and GSE153659, and we found that AEBP1 had higher expression in the PTC samples (Fig. 2A and B). Moreover, ATC had higher expression of AEBP1 than PTC (Fig. 2C), and PTC samples with more advanced TNM stage had higher AEBP1 expression in the TCGA cohort (Fig. 2D–F). We then validated the expression of AEBP1 at the protein level in PTC patients with metastatic disease (N1) compared to those that have primarily local disease (N0) via IHC staining (Fig. 2G and H). PTCs with more advanced T stage had higher IHC-scores (Fig. 2I). Besides, high expression of AEBP1 indicated worse overall survival (OS), disease specific survival (DSS), progression free interval (PFI), and disease free interval (DFI) in PTC patients (Fig. 2J). These results suggest that AEBP1 may be associated with the progression of PTC.

AEBP1 promotes the growth and migration of PTC cells in vitro

To explore the role of AEBP1 in PTC, AEBP1 was knocked out in BCPAP cells and overexpressed in TPC-1 cells (Fig. 3A). Results of CCK8 assays showed that knock out of AEBP1 decreased the growth rate of BCPAP cells (Fig. 3B). We also found that knock out of AEBP1 inhibited the colony formation of BCPAP cells (Fig. 3C). Flow cytometry was



GS_Ranked	EMT	Invasion	Metsstasis	Total	Final
RUNX1	5	7	1	13	1
AEBP1	1	1	14	16	2
STK17B	6	12	2	20	3
DCLK1	8	8	8	24	4
ARNTL2	15	10	3	28	5
PDGFRA	2	5	22	29	6
NFE2L3	10	18	5	33	7
HCK	12	13	11	36	8
FOXP3	13	11	17	41	9
AXL	16	21	7	44	10

Fig. 1. AEBP1 was screened as a gene associated with proliferation, invasion, migration, and EMT in PTC by bioinformatics analysis. A. Flow diagram showed the illustration of bioinformatics analysis. B. Selected soft-thresholding powers. The left panel showed the scale-free fit index and soft-thresholding power. The right panel showed the mean connectivity and the soft-thresholding power. C. Clustering dendrogram of genes, six modules were screened. D. Yellow module was the most significant positively correlated with proliferation, migration, invasion and EMT. E. Circos plot showed that 73 genes were screened for further analysis. F. AEBP1 was screened as for further analysis. G. AEBP1 expression is significant associated with EMT related genes expression in PTC sample in TCGA-THCA cohort. H. AEBP1 expression is significant associated with expressions of vimentin, TWIST1, MMP1, COL3A1 in PTC samples in GSE60542 cohort.

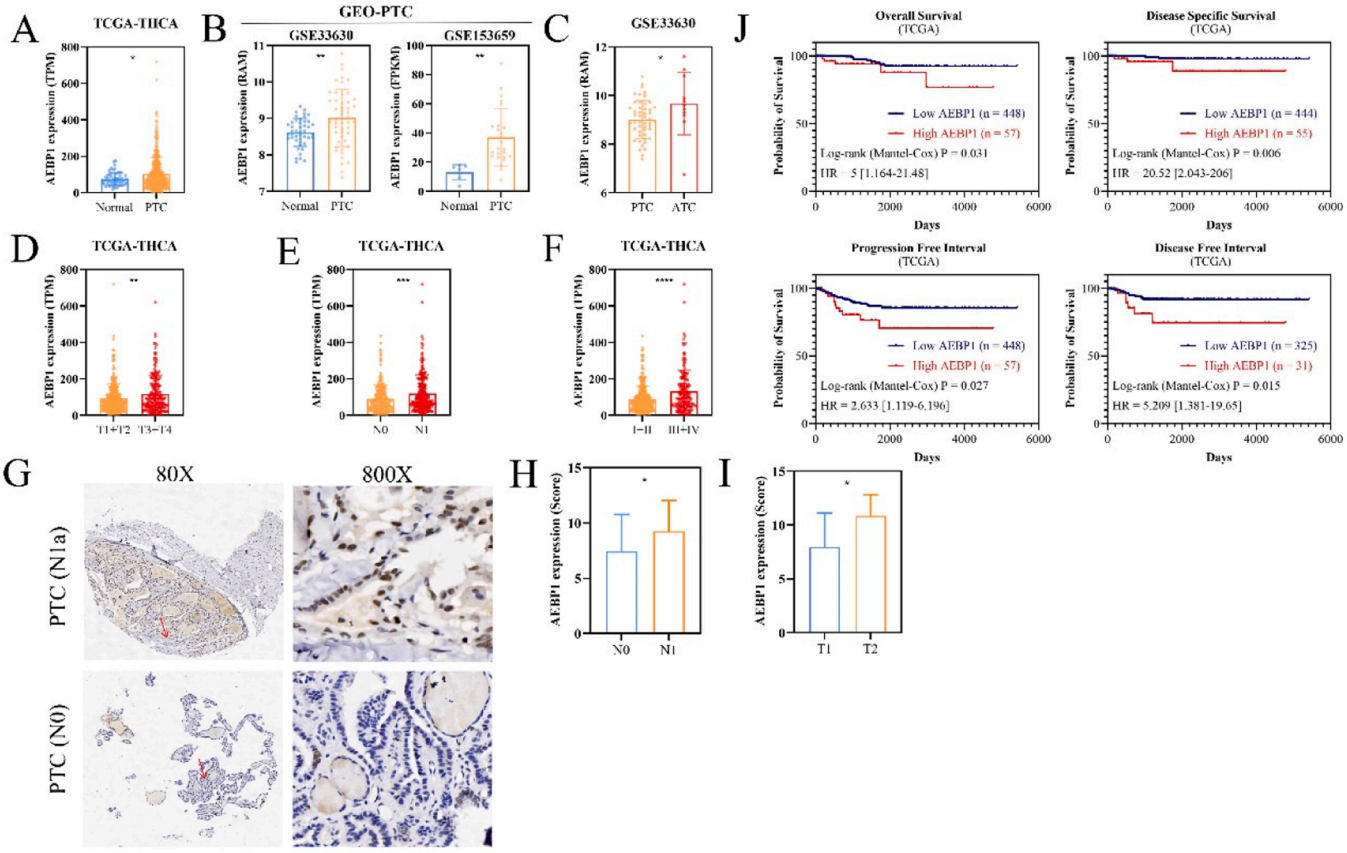


Fig. 2. Higher expression of AEBP1 is detected in PTC samples with aggressive phenotype and poor prognosis. A. PTC samples have higher expression of AEBP1 than normal thyroid samples in TCGA-THCA cohort. B. PTC samples have higher expression of AEBP1 than normal thyroid samples in both GSE33630 and GSE153659 cohorts. C. ATC samples have higher expression of AEBP1 than PTC samples in GSE33630 cohort. D. T3/4 stage PTC samples have higher expression of AEBP1 than T1/2 stage PTC samples in TCGA-THCA cohort. E. N1 stage PTC samples have higher expression of AEBP1 than N0 stage PTC samples in TCGA-THCA cohort. F. III/IV stage PTC samples have higher expression of AEBP1 than I/II stage PTC samples in TCGA-THCA cohort. G. IHC staining of AEBP1 in PTC samples with metastatic disease (N1) compared to those that have primarily local disease (N0). H. N1 stage PTC samples have higher IHC-scores of AEBP1 than N0 stage PTC samples. I. T2 stage PTC samples have higher IHC-scores of AEBP1 than T1 stage PTC samples. J. Patients with high AEBP1 PTC had poor prognosis than patients with low AEBP1 PTC. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

conducted to evaluate the effect of AEBP1 on cell cycle, and knock out of AEBP1 was found to increase the percentage of cells in G0/G1 phase, while the percentages of cells in S or G2/M phase were reduced (Fig. 3D). Moreover, we found that AEBP1 knock-out increased the cell adhesion of BCPAP cells (Fig. 3E). Results from Transwell showed that knock out of AEBP1 significantly inhibited the migration and invasion abilities of BCPAP cells (Fig. 3F). Besides, we also found that overexpressed AEBP1 reduced cell adhesion and increased the growth rate, colony formation ability, the percentages of cells in S or G2/M phase, migration and invasion abilities of TPC-1 cells (Fig. 3G–K). These results indicate that AEBP1 promotes the growth and migration of PTC cells *in vitro*.

AEBP1 regulates the expression of EMT related proteins in PTC cells

Three paired AEBP1 knockout BCPAP cells and parental BCPAP cells were experienced RNA-sequencing analysis, and we screened out 387 up-regulated and 853 down-regulated mRNAs in AEBP1 knockout BCPAP cells (Fig. 4A). The down-regulated mRNAs were experienced pathway enrichment analysis, and we found that the epithelial mesenchymal transition (HALLMARK) pathway was enriched (Fig. 4B). The expression of mesenchymal markers and EMT related TFs was found to be decreased in AEBP1 knockout BCPAP cells, while the expression of epithelial markers was increased (Fig. 4C). Western blot analysis validated that knock out of AEBP1 decreased the expression of N-cad, Vimentin, TWIST1 and ZEB2 in BCPAP cells, while increased the expression of E-cad (Fig. 4D). Moreover, we also found that overexpressed AEBP1 increased the expression of N-cad, Vimentin, TWIST1 and ZEB2 in TPC-1 cells, while decreased the expression of E-cad (Fig. 4E). These results indicate that AEBP1 may participate in the EMT process of PTC cells.

AEBP1 promotes the transcription of BMP4 in vitro

TGF-beta signaling pathway played a critical role in EMT process and was enriched when down-regulated genes in AEBP1 knockout BCPAP cells were used as input for both KEGG and HALLMARK pathway enrichment analysis (Fig. 4B). Among the 10 down-regulated genes in TGF-beta signaling pathway (Fig. 5A), BMP4 expression was significantly correlation with AEBP1 expression in both TCGA cohort (Fig. 5B) and our patients (Fig. 5C). The PTC patients with high AEBP1 expression (IHC-scores >=6) had higher IHC-scores of BMP4 than that with low AEBP1 expression (Fig. 5D). Dual-luciferase reporter assay showed that overexpressed AEBP1 increased the transcription of BMP4 (Fig. 5E). According to previous study [5], GAAAT was identified as a genomic DNA binding motif of AEBP1, and three specific pairs of primers were designed for targeting the potential binding regions within the promoter of *BMP4* in CHIP-PCR assay (Fig. 5F). The results found that AEBP1 could bind to the three regions (Fig. 5 G). Moreover, western blot analysis showed that overexpressed AEBP1 elevated the expression of

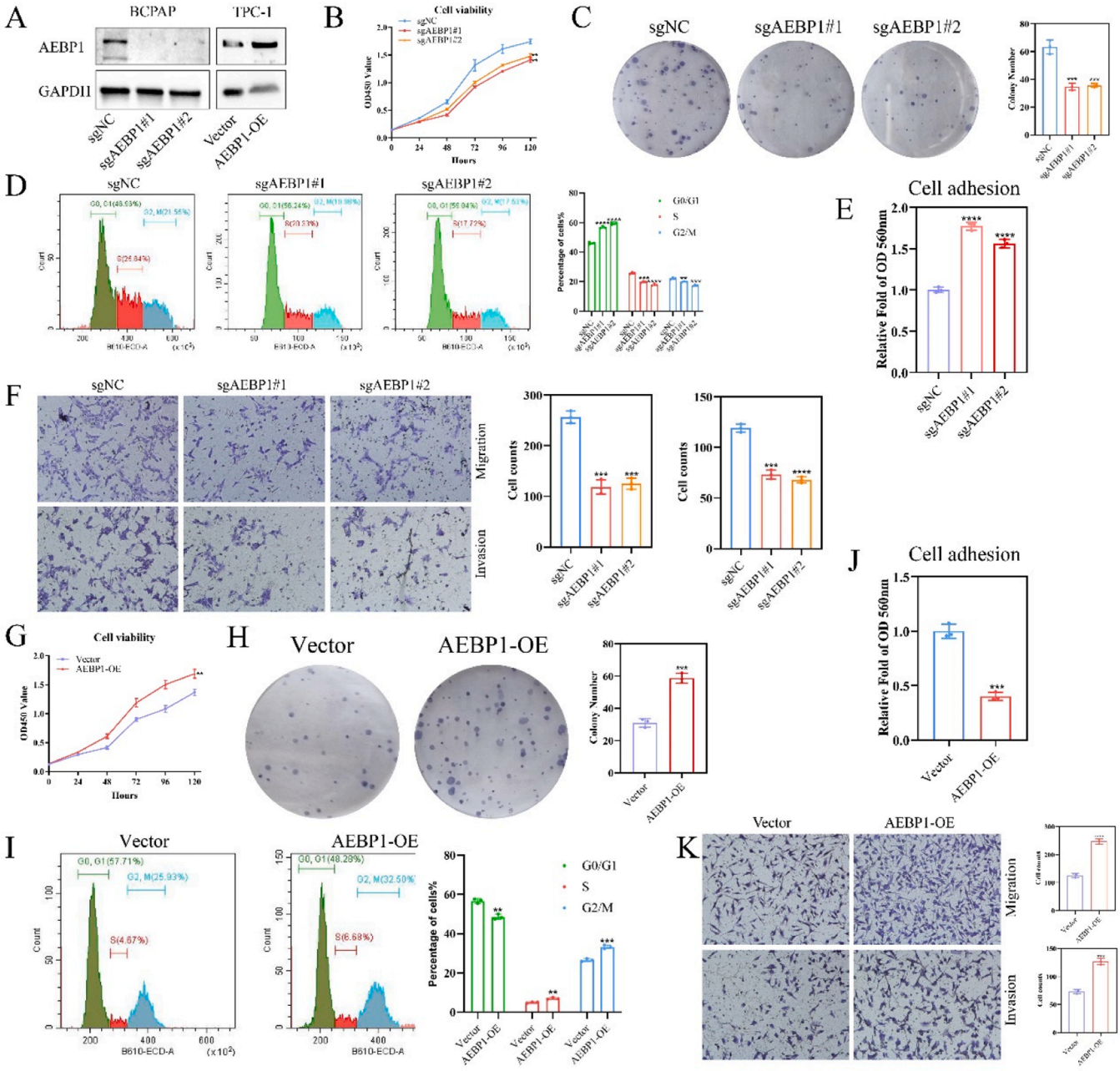


Fig. 3. AEBP1 promotes the growth and migration of PTC cells *in vitro*. A. Western blot showed BCPAP cells with AEBP1 was knocked out and TPC-1 cells with AEBP1 overexpression. B. CCK8 assays showed that AEBP1 knockout inhibited the growth of BCPAP cells. C. AEBP1 knockout inhibited the colony formation of BCPAP cells. D. AEBP1 knockout decreased the percentage of BCPAP cells in S and G2/M phase. E. AEBP1 knockout increased the cell adhesion of BCPAP cells. F. AEBP1 knockout inhibited the migration and invasion of BCPAP cells. G. CCK8 assays showed that AEBP1 overexpression promoted the growth of TPC-1 cells. H. AEBP1 overexpression promoted the colony formation of TPC-1 cells. I. AEBP1 overexpression increased the percentage of TPC-1 cells in S and G2/M phase. J. AEBP1 overexpression reduced the cell adhesion of TPC-1 cells. K. AEBP1 overexpression promoted the migration and invasion of TPC-1 cells. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

BMP4 and p-smad1/5/9 in TPC-1 cells (Figure H), while knocked out of AEBP1 reduced the expression of BMP4 and p-smad1/5/9 in BCPAP cells (Figure I). These results suggest that AEBP1 may participate in the activation of BMP4 signaling.

Overexpressed BMP4 rescues inhibitory phenotype induced by AEBP1 knockout in BCPAP cells

To investigate the biological role of BMP4 in PTC cells, we overexpressed BMP4 in BCPAP cells (BMP4-OE) and AEBP1 knockout BCPAP cells (sgAEBP1+BMP4-OE). We found that overexpressed BMP4 promoted the cell growth, colony formation, migration and invasion of BCPAP cells (Fig. 6A–E). Moreover, BMP4 overexpression could rescue the inhibitory effects of AEBP1 knockout on the cell growth, colony formation, migration and invasion of BCPAP cells (Fig. 6A–E). Besides, we observed that overexpressed BMP4 elevated the expression of TWIST1, ZEB2, and N-cad, while decreased the expression of E-cad, and rescued these proteins' expression changes caused by AEBP1 knockout in BCPAP cells (Fig. 6F). These results indicate that AEBP1 can promote PTC cells proliferation, migration, invasion and EMT via activating BMP4 signaling.

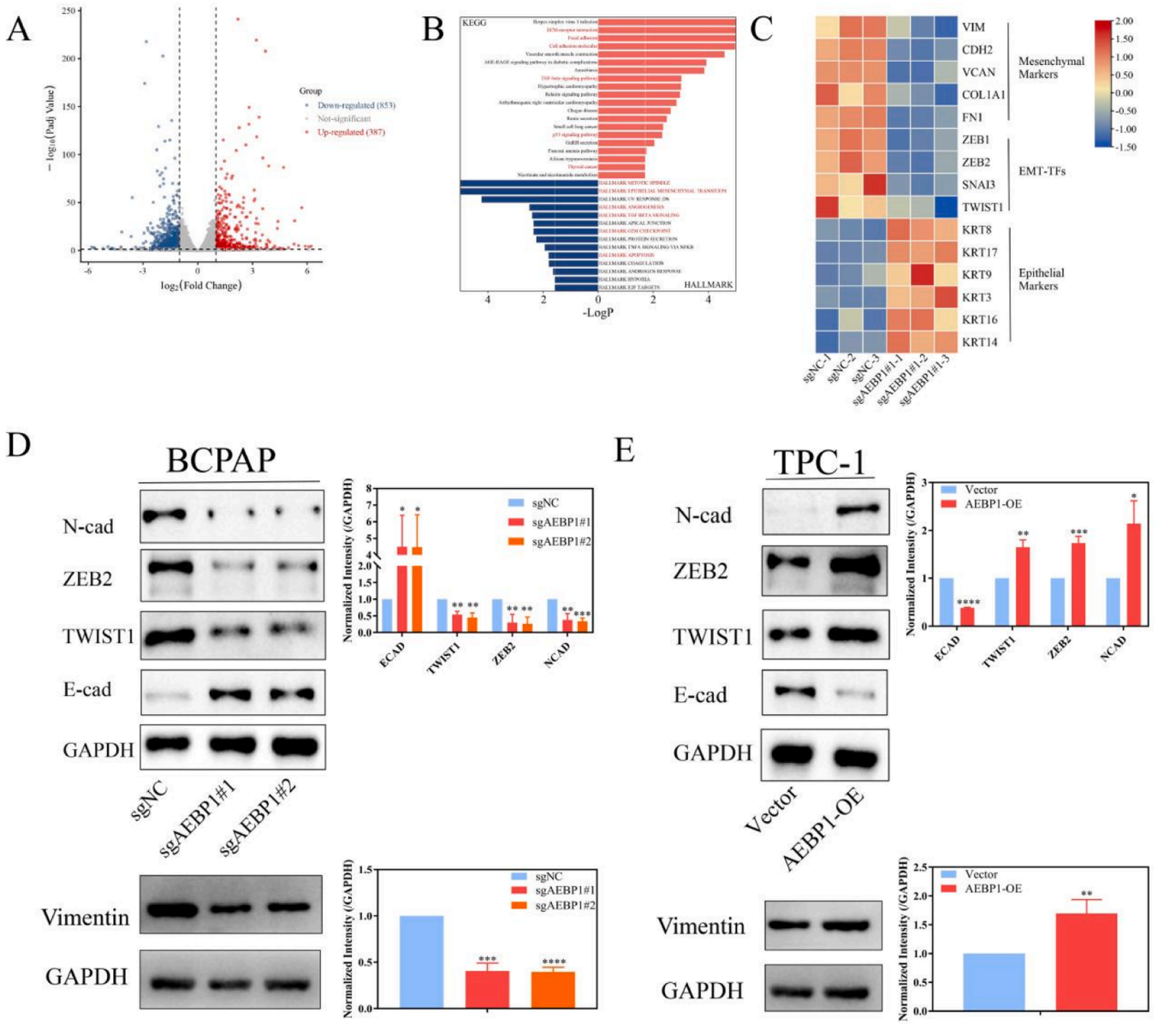


Fig. 4. AEBP1 promotes EMT of PTC cells *in vitro*. A. Volcano plot showed differentially expressed genes between BCPAP cells and BCPAP cells with AEBP1 knockout. B. Pathway enrichment analysis showed that EMT pathway was enriched. C. Heatmap showed that mesenchymal genes and EMT related transcription factors were down-regulated in BCPAP cells with AEBP1 knockout, while epithelial genes were up-regulated. D. Western blot showed that AEBP1 knockout reduced the expression of Vimentin, TWIST1, ZEB2, and N-cad, while elevated the expression of E-cad in BCPAP cells. E. Western blot showed that AEBP1 overexpression elevated the expression of Vimentin, TWIST1, ZEB2, and N-cad, while reduced the expression of E-cad in TPC-1 cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

AEBP1 knockout inhibits the proliferation and lung metastasis of PTC cells in vivo

We investigated the effects of AEBP1 knockout on tumorigenesis and metastatic ability of BCPAP cells in mice. We observed that the level of tumor volume (Fig. 7A and B) and tumor weight (Fig. 7C) in AEBP1 knockout (sgAEBP1) group were lower than that in the NC (sgNC) group. The expression of AEBP1, BMP4, E-cad, and N-cad was examined in tumor sections by IHC staining, and we found that AEBP1 knockout decreased the expression of BMP4 and N-cad, while increased the expression of E-cad (Fig. 7D). Moreover, our results showed that the number of lung metastatic nodules in AEBP1 knockout group was less than that in the NC group (Fig. 7E and F). These findings demonstrated that AEBP1 knockout inhibited the proliferation and lung metastasis of PTC cells *in vivo*.

Discussion

Recurrence and distant metastasis are the main causes of cancer related death in PTC. Although patients with PTC usually have a favorable outcome, with a 5-year survival rate of over 97 % [24], up to 30 % of patients diagnosed at the early stage will relapse into the locally advanced or distant metastatic stage [25–28] and approximately two-thirds of the metastatic tumors will become refractory to radioactive iodine, with a 10-year survival rate of only 10 % [28–30]. In this clinical setting, it is significant to screen more novel therapeutic targets and investigate the potential molecular mechanisms regarding progression of PTC, which would be beneficial to develop effective targeted therapies and improve the overall prognosis for patients with PTC.

Dysregulation of transcription factors has been shown to contribute to the development and progression of many types of cancers [31]. In PTC, as a classical transcription factor, HIF-1α induced by hypoxia stress

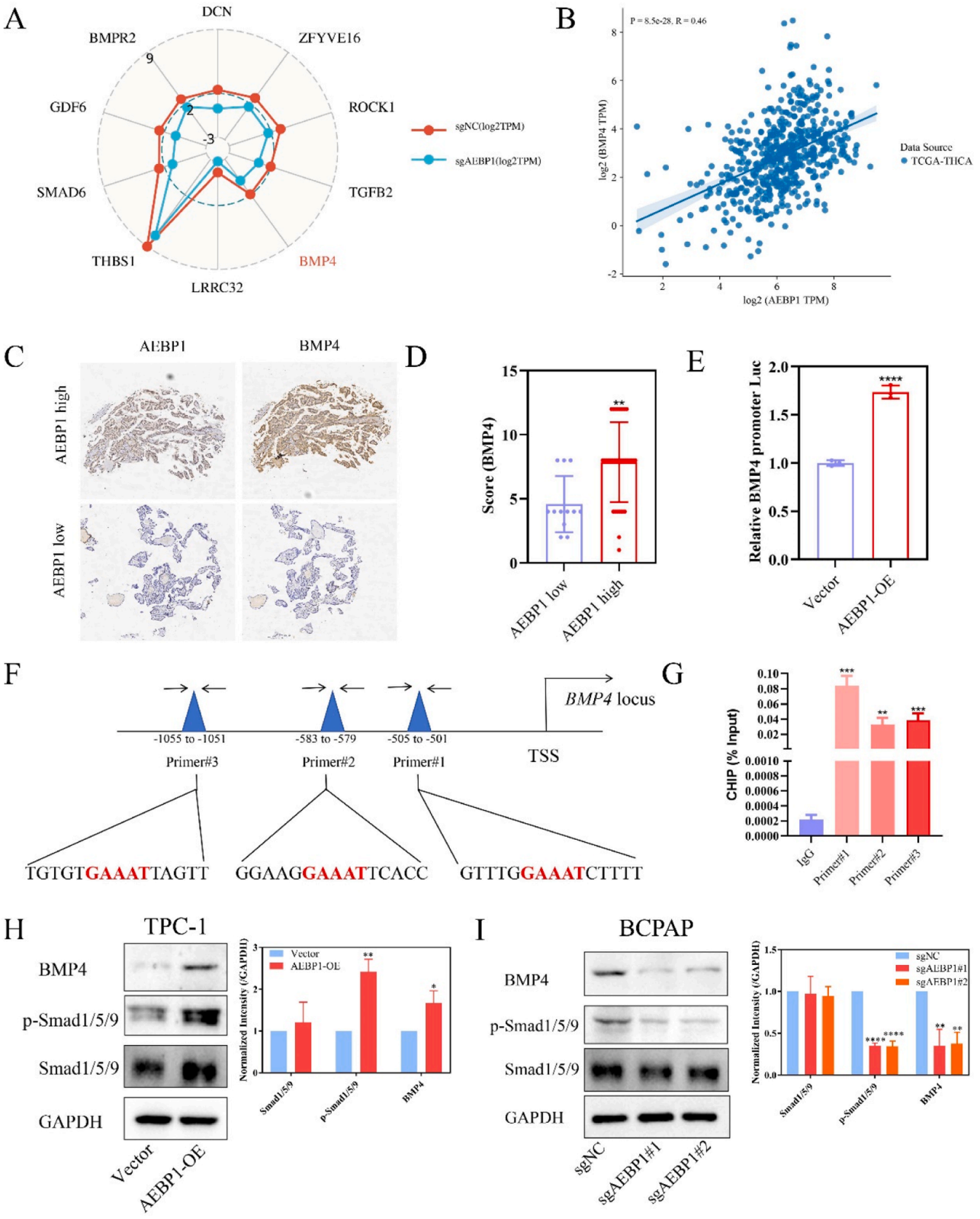


Fig. 5. AEBP1 promotes the transcription of BMP4. A. RNA sequencing data showed that BMP4 expression was down-regulated by AEBP1 knockout in BCPAP cells. B. BMP4 expression was significant positively correlated with AEBP1 expression in TCGA-THCA cohort. C. IHC staining of BMP4 in AEBP1 high and AEBP1 low PTC samples. D. AEBP1 high PTC samples had higher IHC-scores of BMP4 than AEBP1 low PTC samples. E. Luciferase report assay showed that AEBP1 overexpression elevated the BMP4 transcription activity. F. Illustration of three potential binding sites of AEBP1 in BMP4 promoter. G. CHIP-qPCR assay showed that AEBP1 could bind to BMP4 promoter. H. Western blot showed that AEBP1 overexpression elevated the expression of BMP4 and p-smad1/5/9 in TPC-1 cells. I. Western blot showed that AEBP1 knockout reduced the expression of BMP4 and p-smad1/5/9 in BCPAP cells. $^{*}P < 0.05$,$^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

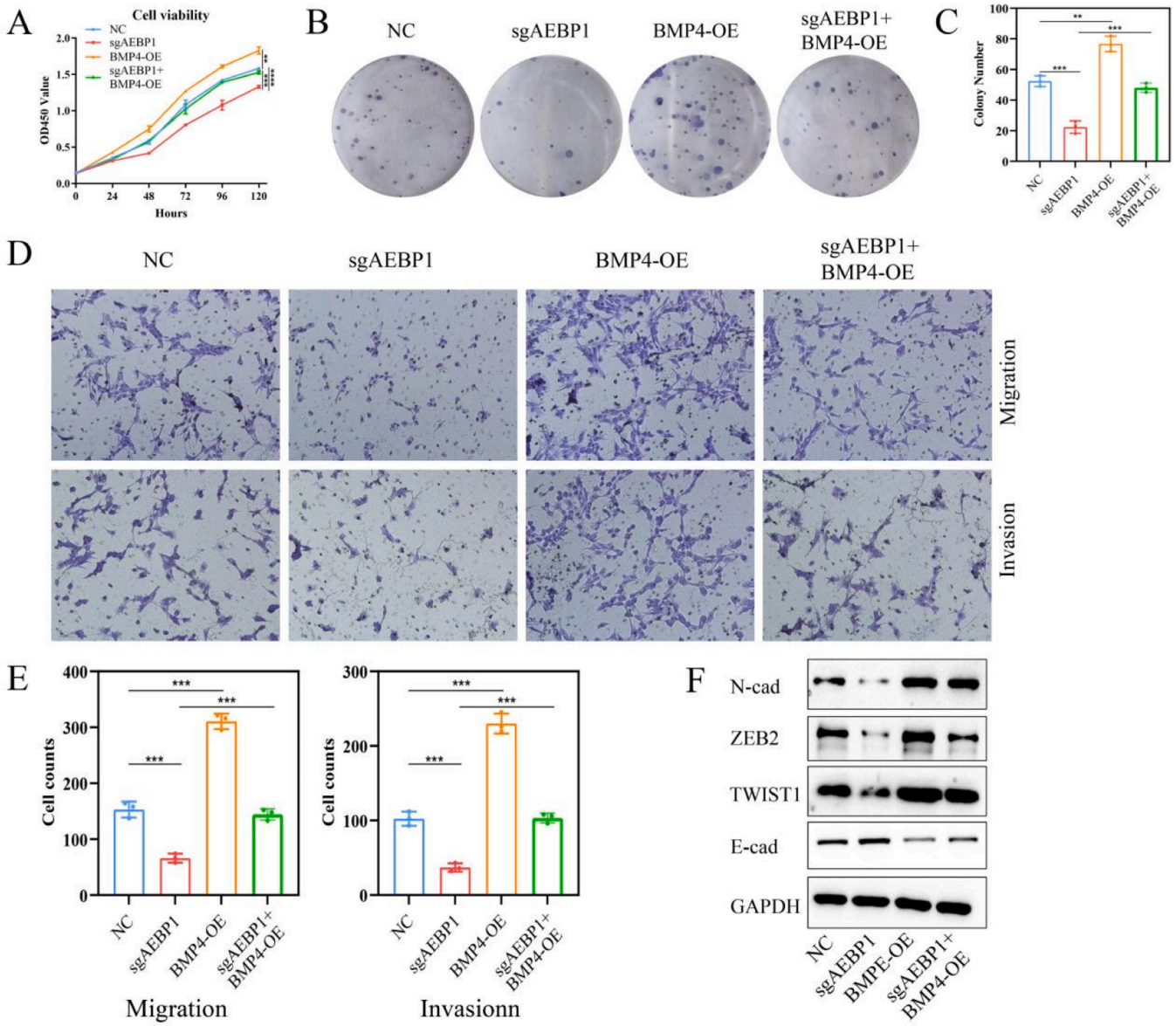


Fig. 6. BMP4 overexpression rescues the effects of AEBP1 knockout on BCPAP cells. A. CCK8 assays showed that BMP4 overexpression rescued the inhibitory effects of AEBP1 knockout on the cell growth of BCPAP cells. B and C. BMP4 overexpression rescued the inhibitory effects of AEBP1 knockout on the colony formation of BCPAP cells. D and E. BMP4 overexpression rescued the inhibitory effects of AEBP1 knockout on the migration and invasion of BCPAP cells. F. Western blot showed that BMP4 overexpression rescued the expressions change of TWIST1, ZEB2, N-cad and E-cad which induced by AEBP1 knockout in BCPAP cells. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

is reported to promote tumor progression by regulating the expression of TERT [32]. RUNX2 is found to be associated with tumor invasion and metastasis of PTC by regulating the transcription of EMT-related genes [33]. In our study, WGCNA was carried out using transcriptome data from TCGA-THCA cohort, and AEBP1 was screened as a pivotal transcription factor that correlated with proliferation, metastasis, and EMT of PTC.

AEBP1 is a transcription factor involved in the progression of many types of cancers. Several studies have reported that AEBP1 was highly expressed and correlated with the proliferation and prognosis of GBM [34,35]. In CRC, AEBP1 silencing inhibited the proliferation, migration, and invasion of tumor cells *in vitro* and decreased the number of metastatic lung nodules in a xenograft mouse model [10]. According to previous studies, AEBP1 also promoted the progression of GC, bladder cancer, and melanoma by regulating the growth, metastasis, and response to therapy of tumor cells [8,36,37]. There is still a lack of reports on the correlation of AEBP1 with PTC progression. Our study first revealed that AEBP1 promoted growth, migration, and invasion of BCPAP and TPC-1 cells *in vitro* and attenuated tumorigenesis and lung metastasis of BCPAP cells *in vivo*. Studies have shown that EMT promoted many types of cancer metastasis and progression, including PTC [14,38,39]. In our study, we found that AEBP1 could enhance the expression of EMT-related proteins such as TWIST1, N-cadherin, and ZEB2 in PTC cells, indicating that AEBP1 plays an important role in the EMT process of PTC cells which is concordant with our bioinformatics analysis.

Mechanistically, BMP4 was identified as a downstream target of AEBP1 and over-expression of BMP4 could rescue the inhibitory effects of AEBP1 knockout on proliferation, migration, and invasion of PTC cells. In addition, the expressions of EMT makers such as TWIST1, N-cadherin, and ZEB2 were restored by BMP4 overexpression in AEBP1 knockout BCPAP cells. BMP4 is a member of BMP family and induces EMT process in cancer [40]. Previous studies showed that BMP4 was abnormally expressed and promoted tumor metastasis by inducing EMT

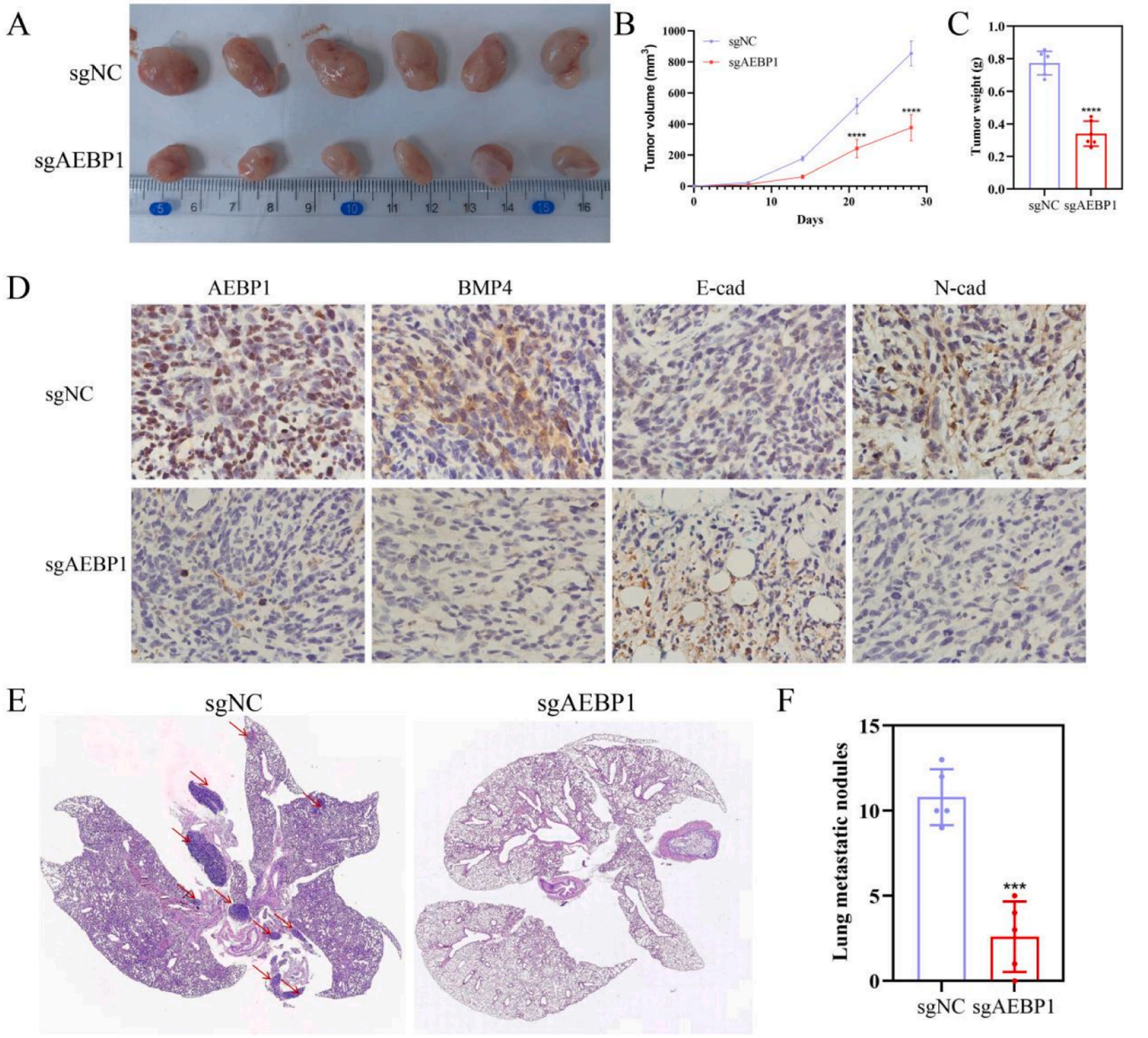


Fig. 7. Knock out of AEBP1 inhibits the proliferation and lung metastasis of PTC *in vivo*. A. Equal amounts of BCPAP sgNC or sgAEBP1 cells were subcutaneously injected into the right flank of the nude mice and tumor volumes and weights were measured 4 weeks after injection. B. Tumor volumes were reduced in sgAEBP1 group compared to sgNC group. C. Tumor weights were reduced in sgAEBP1 group compared to sgNC group. D The expressions of AEBP1, BMP4, N-cad, E-cad were examined in tumor sections by IHC staining, and knock out of AEBP1 was found to decrease the expressions of BMP4 and N-cad, while increase the expression of E-cad. E. Equal amounts of BCPAP sgNC or sgAEBP1 cells were injected into nude mice via the tail vein, the lung of mice was excised and fixed in formalin 4 weeks after injection and paraffin-embedded lungs were systematically sectioned and stained with hematoxylin and eosin (H&E) staining. F. Knock out of AEBP1 was found to decrease the lung metastasis of BCPAP cells in nude mice. $^{***}P < 0.001$, $^{****}P < 0.0001$.

in many types of solid tumors, such as prostate cancer, GC, esophageal squamous cell carcinoma, and hepatocellular carcinoma [41–44]. Xiaomei Meng et al. reported that BMP4 expression was positively correlated with tumor size, capsular invasion, and TNM stage of PTC [45]. Qinyi Zhou and colleagues have reported that activation of BMP4/7 signaling promoted thyroid cancer proliferation via regulating iron homeostasis [46]. Our study indicated that BMP4 played a critical role in the EMT process and PTC progression induced by AEBP1. Thus, BMP4 may represent a potential therapeutic target for PTC patients with high expression of AEBP1.

In conclusion, our study showed that AEBP1 promoted the growth, migration, invasion, metastasis, and EMT of PTC cells. AEBP1 can regulate the transcription of BMP4, and BMP4 overexpression can rescue the effects of AEBP1 knockout on PTC cells. The AEBP1-BMP4 axis may present novel therapeutic targets for PTC treatment.

Availability of data and materials

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human: HRA005597) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa-human [47,48].

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Ethics approval and consent to participate

The studies involving human specimens were reviewed and approved by The Ethics Committee of the Ethics Committee of Affiliated Hospital of Jining Medical University (approval number:2021-08-C015). All the participants provided written informed consent. All animal procedures and protocols implemented in this study were in accordance with the Institute of Biophysics, Chinese Academy of Science's Policy on Care and Use of Laboratory Animals.

CRediT authorship contribution statement

Gaoda Ju: Data curation, Writing – original draft, Investigation. **Tao Xing:** Data curation, Investigation. **Miaomiao Xu:** Data curation, Investigation. **Xin Zhang:** . **Yuqing Sun:** Data curation. **Zhuanzhuan Mu:** Data curation. **Di Sun:** Data curation. **Sen Miao:** Methodology. **Li Li:** Data curation. **Jun Liang:** Conceptualization, Writing – review & editing. **Yansong Lin:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] R. Zheng, S. Zhang, H. Zeng, et al., Cancer incidence and mortality in China, J. Natl. Cancer Cent. 2 (1) (2016) 1–9, 2022/03/01/2022.
[2] S.N. Silva, Special issue: genetic perspectives in thyroid cancer, Genes 12 (2) (2021) (Basel).
[3] Y.C. Henderson, T.D. Shellenberger, M.D. Williams, et al., High rate of BRAF and RET/PTC dual mutations associated with recurrent papillary thyroid carcinoma, Clin. Cancer Res. 15 (2) (2009) 485–491.
[4] P. Fallahi, S.M. Ferrari, M.R. Galdiero, et al., Molecular targets of tyrosine kinase inhibitors in thyroid cancer, Semin. Cancer Biol. 79 (2022) 180–196.
[5] J. Ladha, S. Sinha, V. Bhat, S. Donakonda, S.M.R. Rao, Identification of genomic targets of transcription factor AEBP1 and its role in survival of glioma cells, Mol. Cancer Res. 10 (8) (2012) 1039–1051.
[6] A.F. Majdalawieh, M. Massri, H.S. Ro, AEBP1 is a novel oncogene: mechanisms of action and signaling pathways, J. Oncol. 2020 (2020) 8097872.
[7] S. Sinha, A. Renganathan, P.B. Nagendra, V. Bhat, B.S. Mathew, M.R.S. Rao, AEBP1 down regulation induced cell death pathway depends on PTEN status of glioma cells, Sci. Rep. 9 (1) (2019) 14577.
[8] J.Y. Liu, L. Jiang, J.J. Liu, et al., AEBP1 promotes epithelial-mesenchymal transition of gastric cancer cells by activating the NF-κB pathway and predicts poor outcome of the patients, Sci. Rep. 8 (1) (2018) 11955.
[9] A. Yorozu, E. Yamamoto, T. Niinuma, et al., Upregulation of adipocyte enhancer-binding protein 1 in endothelial cells promotes tumor angiogenesis in colorectal cancer, Cancer Sci. 111 (5) (2020) 1631–1644.
[10] Y. Xing, Z. Zhang, F. Chi, et al., AEBP1, a prognostic indicator, promotes colon adenocarcinoma cell growth and metastasis through the NF-κB pathway, Mol. Carcinoges. 58 (10) (2019) 1795–1808.
[11] J. Li, Y. Ruan, C. Zheng, et al., AEBP1 contributes to breast cancer progression by facilitating cell proliferation, migration, invasion, and blocking apoptosis, Discov. Med. 35 (174) (2023) 45–56.
[12] C.L. Chaffer, B.P. San Juan, E. Lim, R.A. Weinberg, EMT, cell plasticity and metastasis, Cancer Metastasis Rev. 35 (4) (2016) 645–654.
[13] D. Li, Z. Liu, X. Ding, Z. Qin, AEBP1 is one of the epithelial-mesenchymal transition regulatory genes in colon adenocarcinoma, Biomed Res. Int. 2021 (2021) 3108933.
[14] H. Shakib, S. Rajabi, M.H. Dehghan, F.J. Mashayekhi, N. Safari-Alighiarloo, M. Hedayati, Epithelial-to-mesenchymal transition in thyroid cancer: a comprehensive review, Endocrine 66 (3) (2019) 435–455.
[15] M.J. Goldman, B. Craft, M. Hastie, et al., Visualizing and interpreting cancer genomics data via the Xena platform, Nat. Biotechnol. 38 (6) (2020) 675–678.
[16] M. Tarabichi, M. Saiselet, C. Trésallet, et al., Revisiting the transcriptional analysis of primary tumours and associated nodal metastases with enhanced biological and statistical controls: application to thyroid cancer, Br. J. Cancer 112 (10) (2015) 1665–1674.
[17] G. Tomás, M. Tarabichi, D. Gacquer, et al., A general method to derive robust organ-specific gene expression-based differentiation indices: application to thyroid cancer diagnostic, Oncogene 31 (41) (2012) 4490–4498.
[18] G. Dom, M. Tarabichi, K. Unger, et al., A gene expression signature distinguishes normal tissues of sporadic and radiation-induced papillary thyroid carcinomas, Br. J. Cancer 107 (6) (2012).
[19] F. Yang, M. Lian, H. Ma, et al., Identification of key genes associated with papillary thyroid microcarcinoma characteristics by integrating transcriptome sequencing and weighted gene co-expression network analysis, Gene 811 (2022) 146086.
[20] H. Yuan, M. Yan, G. Zhang, et al., CancerSEA: a cancer single-cell state atlas, Nucleic Acids Res. 47 (D1) (2019) D900–D908.
[21] G. Bindea, B. Mlecnik, M. Tosolini, et al., Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer, Immunity 39 (4) (2013) 782–795.
[22] D. Kaemmerer, L. Peter, A. Lupp, et al., Comparing of IRS and Her2 as immunohistochemical scoring schemes in gastroenteropancreatic neuroendocrine tumors, Int. J. Clin. Exp. Pathol. 5 (3) (2012) 187–194.
[23] K.W. Min, S.W. Chae, D.H. Kim, et al., Fascin expression predicts an aggressive clinical course in patients with advanced breast cancer, Oncol. Lett. 10 (1) (2015) 121–130.
[24] D.F. Schneider, H. Chen, New developments in the diagnosis and treatment of thyroid cancer, CA Cancer J. Clin. 63 (6) (2013) 374–394.
[25] H.M. Zhang, Z.Y. Li, Z.T. Dai, et al., Interaction of MRPL9 and GGCT promotes cell proliferation and migration by activating the MAPK/ERK pathway in papillary thyroid cancer, Int. J. Mol. Sci. 23 (19) (2022).
[26] Y. Ito, A. Miyauchi, M. Kihara, M. Fukushima, T. Higashiyama, A. Miya, Overall survival of papillary thyroid carcinoma patients: a single-institution long-term follow-up of 5897 patients, World J Surg 42 (3) (2018) 615–622.
[27] I.J. Nixon, R. Simo, K. Newbold, et al., Management of invasive differentiated thyroid cancer, Thyroid 26 (9) (2016) 1156–1166.
[28] J. Capdevila, J.C. Galofré, E. Grande, et al., Consensus on the management of advanced radioactive iodine-refractory differentiated thyroid cancer on behalf of the Spanish Society of endocrinology thyroid cancer working group (GTSEEN) and Spanish rare cancer working group (GETHI), Clin. Transl. Oncol. 19 (3) (2017) 279–287.
[29] J.A. Vallejo Casas, M. Sambo, C. López López, et al., Initial clinical and treatment patterns of advanced differentiated thyroid cancer: ERUDIT study, Eur. Thyroid J. 11 (5) (2022).
[30] C. Durante, N. Haddy, E. Baudin, et al., Long-term outcome of 444 patients with distant metastases from papillary and follicular thyroid carcinoma: benefits and limits of radioiodine therapy, J. Clin. Endocrinol. Metab. 91 (8) (2006) 2892–2899.
[31] Y. Li, A.S. Azmi, RM. Mohammad, Deregulated transcription factors and poor clinical outcomes in cancer patients, Semin. Cancer Biol. 86 (Pt 3) (2022) 122–134.
[32] H. Song, X. Chen, Q. Jiao, et al., HIF-1α-mediated telomerase reverse transcriptase activation inducing autophagy through mammalian target of rapamycin promotes papillary thyroid carcinoma progression during hypoxia stress, Thyroid 31 (2) (2021) 233–246.
[33] D.F. Niu, T. Kondo, T. Nakazawa, et al., Transcription factor Runx2 is a regulator of epithelial-mesenchymal transition and invasion in thyroid carcinomas, Lab. Invest. 92 (8) (2012) 1181–1190.
[34] S.P. Reddy, R. Britto, K. Vinnakota, et al., Novel glioblastoma markers with diagnostic and prognostic value identified through transcriptome analysis, Clin. Cancer Res. 14 (10) (2008) 2978–2987.
[35] L. Cheng, X. Shao, Q. Wang, X. Jiang, Y. Dai, S. Chen, Adipocyte enhancer binding protein 1 (AEBP1) knockdown suppresses human glioma cell proliferation, invasion and induces early apoptosis, Pathol. Res. Pract. 216 (2) (2020) 152790.
[36] Y. Di, D. Chen, W. Yu, L. Yan, Bladder cancer stage-associated hub genes revealed by WGCNA co-expression network analysis, Hereditas 156 (2019) 7.
[37] W. Hu, L. Jin, C.C. Jiang, et al., AEBP1 upregulation confers acquired resistance to BRAF (V600E) inhibition in melanoma, Cell Death Dis. 4 (11) (2013) e914.
[38] S. Heerboth, G. Housman, M. Leary, et al., EMT and tumor metastasis, Clin. Transl. Med. 4 (2015) 6.
[39] A.D. Rhim, E.T. Mirek, N.M. Aiello, et al., EMT and dissemination precede pancreatic tumor formation, Cell 148 (1-2) (2012) 349–361.
[40] A. Serrao, L.M. Jenkins, A.A. Chumanevich, et al., Mediator kinase CDK8/CDK19 drives YAP1-dependent BMP4-induced EMT in cancer, Oncogene 37 (35) (2018) 4792–4808.
[41] F. Xu, X. Shangguan, J. Pan, et al., HOXD13 suppresses prostate cancer metastasis and BMP4-induced epithelial-mesenchymal transition by inhibiting SMAD1, Int. J. Cancer 148 (12) (2021) 3060–3070.
[42] G. Deng, Y. Chen, C. Guo, et al., BMP4 promotes the metastasis of gastric cancer by inducing epithelial-mesenchymal transition via ID1, J. Cell Sci. 133 (11) (2020).
[43] J. Zhang, A. Luo, F. Huang, T. Gong, Z. Liu, SERPINE2 promotes esophageal squamous cell carcinoma metastasis by activating BMP4, Cancer Lett. 469 (2020) 390–398.

[44] S. Zeng, Y. Zhang, J. Ma, et al., BMP4 promotes metastasis of hepatocellular carcinoma by an induction of epithelial-mesenchymal transition via upregulating ID2, Cancer Lett. 390 (2017) 67–76.
[45] X. Meng, P. Zhu, N. Li, et al., Expression of BMP-4 in papillary thyroid carcinoma and its correlation with tumor invasion and progression, Pathol. Res. Pract. 213 (4) (2017) 359–363.
[46] Q. Zhou, J. Chen, J. Feng, J. Wang, E4BP4 promotes thyroid cancer proliferation by modulating iron homeostasis through repression of hepcidin, Cell Death Dis. 9 (10) (2018) 987.
[47] T. Chen, X. Chen, S. Zhang, et al., The genome sequence archive family: toward explosive data growth and diverse data types, Genom. Proteom. Bioinform. 19 (4) (2021) 578–583.
[48] Database resources of the national genomics data center, China national center for bioinformation in 2022, Nucl. Acids Res. 50 (D1) (2022) D27–D38.