Abstract. Esophageal squamous cell carcinoma (ESCC) is a common type of esophageal cancer and is prevalent worldwide. Understanding the mechanisms underlying its formation and the search for more effective therapeutic strategies are critical due to the occurrence of chemotherapeutic drug resistance. The aim of the present study was to determine the functional relevance and therapeutic potential of carbohydrate sulfotransferase 15 (CHST15) in ESCC. CHST15 levels were measured in different ESCC cell lines and evaluated in ESCC tissues using tissue chip immunohistochemistry. Cell growth and apoptosis assays, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, and clonogenic assays were conducted using TE-1 cells and lenti-shCHST15 virus constructs were used to investigate the function of CHST15 in cell proliferation and apoptosis. mRNA microarray analysis was performed to determine the underlying mechanism of CHST15 regulation in TE-1 cell proliferation and apoptosis. The results showed that knockdown of CHST15 inhibited TE-1 cell growth and proliferation, but induced cell apoptosis. CHST15 was more frequently detected in ESCC tissue compared with that in normal esophageal tissue. Microarray data analysis indicated that the inhibition of cell proliferation and activation of cell apoptosis in CHST15-knockdown cells may be caused by altered CHST15/ILKAP/CCND1 and CHST15/RABL6/PMAIP1 signaling axes, respectively.

Introduction

Esophageal squamous cell carcinoma (ESCC) is a predominant type of esophageal cancer (EC) that is among the leading causes of cancer-related death worldwide (1). China has a very high incidence of ESCC, with more than 100 cases per 100,000 population annually. Studies have shown that certain factors are associated with increased risk of ESCC, such as alcohol and tobacco consumption, genetic mutation, diet and nutrition deficiency (2,3).

Similar to other types of cancer, surgery, chemotherapy, radiotherapy, or a combination of these methods are the main treatments for patients with EC. However, with the increasing incidence of esophageal cancer and the poor 5-year survival rate, more research is needed to investigate the underlying mechanisms underlying EC, to determine how to prevent esophageal cancer development, and to discover more effective treatment strategies for patients with EC.

Studies have identified many prognostic markers related to cell proliferation, cell apoptosis, and metastasis in regards to ESCC, e.g., epidermal growth factor receptor (EGFR) has been reported to be associated with the clinical outcome of many types of cancer, including ESCC. EGFR overexpression is positive in most patients with EC (4). In addition, the level of phosphorylated mammalian target or rapamycin (mTOR), which has an important role in intracellular metabolic and anabolic processes, is associated with the poor prognosis of patients with EC (5). B-cell CLL/Lymphoma 2 (BCL2) family proteins, which are regulators of programmed cell death, such as Fas cell surface death receptor (FAS), BCL2 associated X, apoptosis regulator (BAX) and, especially,
BCL2 like 1 (BCL-X) were reported to contribute to ESCC progression (6). Octamer-binding protein 4 (OCT4) and SRY-box 2 (SOX2) also have a high prevalence in ESCC (7). SRY-box 2 (SOX2) also have a high prevalence in ESCC (7). In most cases, ESCC formation and progression is a complex result of multiple factors, for example, genetic alterations and risk factors of lifestyle. Very recently, Yokoyama et al reported that heavy smoking and drinking substantially accelerate the remodeling process of the esophageal epithelium via numerous driver-mutated clones in ESCC development (11). Overall, ESCC is a heterogeneous disease with variable outcomes. However, there are no widely accepted biomarkers for ESCC progression (20 -23). For example, Nishimura et al evaluated the safety and efficacy of a double-stranded RNA oligonucleotide that specifically represses CHST15 for use in patients with pancreatic cancer. The results showed that CHST15 reduction could predict tumor progression and overall survival (20). Ito et al indicated significant associations between CHST15 overexpression and disease-free survival and overall survival of patients with pancreatic ductal adenocarcinoma (21).

In the present study, we investigated the correlation between CHST15 expression and proliferation or apoptosis or both in esophageal cancer cells. We further performed gene chip microarray analysis to elucidate the underlying molecular mechanisms in the regulation of esophageal tumor formation or progression by CHST15.

Materials and methods

Construction of a recombinant lentiviral vector. The target sequence (ACACATCACAACAGGAT) from human CHST15 mRNA (NM_015892) was selected for the knockdown experiment. The sequence of the control short hairpin RNA (shRNA) was TTCTCAGACGTGTCGAT. The CHST15 shRNA and control shRNA oligonucleotides were designed as stem-loop structures and inserted into vector lenti-GV115-EGFP (GeneChem, Shanghai, China) at the Age1/EcoRI sites. Recombinant lentiviruses were produced by co-transfection of the shRNA vector and helper vectors (pHelper1.0 and pHelper2.0) into 293T cells. The medium was replaced with fresh medium 6 h post-transfection. After 48 h post-transfection, cell debris was removed from conditioned medium by centrifugation at 4,000 x g and 4°C for 10 min. The conditioned medium was then filtered through a 0.45-µm pore-size filter and centrifuged at 4°C and 100,000 x g for 2 h. Lentiviruses particles were resuspended in fresh medium and stored at -80°C. The lentivirus titer was calculated using a fluorescence titering assay.

Cell culture and recombinant lentivirus infection. Human ESCC cell lines TE-1, Eca-109, and EC9706 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in CM2-1 medium comprised of 90% Roswell Park Memorial Institute (RPMI)-1640 (Corning, Inc.), 10% fetal bovine serum (FBS; Ausbian) and 1% penicillin-streptomycin (Beyotime Institute of Biotechnology) at 37°C in a 5% CO₂ incubator. Cells were subjected to mycoplasma testing before experiments. TE-1 cells were plated on 6-well plates and infected with lenti-shCtrl or lenti-shCHST15 at a multiplicity of infection (MOI) of 10. At 72 h post-infection, the cells were observed under a fluorescence microscope (magnification, x200) and harvested to determine knockdown efficiency using quantitative RT-PCR or for other purposes.

Target validation of lenti-shCHST15 in 293T cells and western blotting. 293T cells were transfected with the CHST15 plasmid (GV143-hCHST15) using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) and then infected with lenti-shCtrl or lenti-shCHST15, respectively. Briefly, 293T cells were plated on 24-well plates and transfected with 0.5 µg of the constructs and 1 µl of Lipofectamine 2000 when cells reached 80-90% confluence. After 36-48 h of transfection, the cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), collected by scraping, and lysed using Radioimmunoprecipitation assay (RIPA) buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) supplemented with 1% protease inhibitor cocktail on ice for 10-15 min. Cells were further lysed by sonication at 200 W for four times, for 5 sec each time, with a 2-sec interval between pulses. Supernatants were collected after centrifugation at 12,000 x g, at 4°C for 15 min. Protein concentrations were determined using the bicinchoninic acid assay (BCA assay). An amount of 20 µg protein of each sample were loaded onto 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% non-fat milk at room temperature for 1 h. The blots were then incubated with anti-Flag antibodies (dilution 1:2,000, cat. no. F1804; Sigma-Aldrich; Merck KGaA) or anti-β-GAPDH antibody (dilution 1:2,000; cat. no. sc-32233; Santa Cruz Biotechnology, Inc.) overnight at 4°C followed by incubation with horse-radish peroxidase (HRP)-conjugated secondary antibodies (dilution 1:2,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Blots were visualized using the enhanced chemiluminescent detection method (GE Healthcare).

RNA extraction and cDNA synthesis. Cells were collected and resuspended in 1 ml TRIzol reagent (cat. no. 3101-100; Pufei). RNA was precipitated using isopropanol and dissolved in RNa-free water. The RNA concentration was measured using a NanoDrop 2000 instrument (Thermo Fisher Scientific, Inc.). cDNA for each sample was obtained via a reverse transcriptions reaction using a Promega M-MLV kit (cat. no. M1705; Promega). All the above steps were performed according to the manufacturer's instruction.
Quantitative real-time reverse transcription PCR (RT-qPCR). The qPCR reaction was set up by mixing primers, SYBR TAQ, and cDNA at the proportion according to the manufacturer’s instructions (cat. no. DRR041B, SYBR Master Mixture; Takara). The primer sequences for GAPDH were: 5’-TGACTT CAACACGCAGACACCA-3’ (forward) and 5’-CACCCCTGTT GCTGTAGCCAA AA-3’ (reverse). The primer sequences for CHST15 were: 5’-AACACCACCGACCCCTAC-3’ (forward) and 5’-TGATGGCGGAGAACTTGA-3’ (reverse); the product sizes for GAPDH and CHST15 were 121 and 232 bp, respectively. The qPCR reactions were performed utilizing the Mx3000P qPCR System (Agilent) and at 95°C for 15 sec; followed by 45 cycles at 95°C for 5 sec and 60°C for 30 sec. To compare mRNA levels between different samples, the 2−ΔΔCq method (24) was employed to analyze the data.

Cell growth assay. TE-1 cells infected with lent-shCtrl or lent-shCHST15 were plated at 800 cells/well onto a 96-well plate and cultured at 37°C in a 5% CO₂ incubator. Cells with enhanced green fluorescent protein (EGFP) fluorescence in each well were counted daily using a Celigo imaging cytometer (Nexcelon) for 5 days. A cell growth curve was drawn (based on cell numbers) by plotting the numbers of fluorescent-positive cells and time-points. For each cell type, the cell proliferation rates were calculated by dividing the cell number at each time-point by the cell number at day 1.

Cell apoptosis assay. Cell apoptosis was assessed using an Annexin V Apoptosis Detection Kit APC (cat. no. 88-8007; eBiosience). TE-1 cells were seeded on a 6-well plate and infected with lent-shCtrl or lent-shCHST15. Four days later, the cells were trypsinized and resuspended in fresh complete medium. The cells were washed with pre-cooled D-Hanks and 1X binding buffer. The cells were suspended in 1X binding buffer, stained with Annexin V-APC, and analyzed using a flow cytometer (Guava easyCyte HT; Millipore). All samples were examined in triplicate.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell viability after CHST15 knockdown was measured using an MTT assay. Cells were seeded onto 96-well plates at 2,000 cells/well. At each time point, 20 μl of 5 mg/ml MTT was added to each well and incubated with the cells for 4 h. Thereafter, the medium was completely removed and 100 μl of dimethyl sulfoxide (DMSO) was added into each well. After brief shaking, the absorbance at 570 nm of each well was obtained using the Affymetrix Fluidics Station 450 system, washing, the absorbance at 570 nm of each well was obtained using the Affymetrix Fluidics Station 450 system, and staining were performed on the chip using a GeneChip hybridization wash and stain kit. The array was processed with the Affymetrix GeneChip Fluidics Station 450 system, after which they were imaged using an Affymetrix Genechip Scanner 3000 7G for subsequent generation of cell intensity (CEL) files. The raw data were evaluated for quality control using signal histogram analysis, relative signal box plot, Pearson’s correlation analysis, and principal component analysis. Qualified data were then used for further analysis. After data cleaning, normalized probe sets were subjected to variance analysis, Bayesian P-value computation, and Benjamini-Hochberg false discovery rate (FDR) for multiple testing correction. Differentially expressed genes based on the comparison of CHST15 knockdown (KD) and control cells were selected if |fold change|>1.5 and the FDR was <0.05.

Gene Ontology and pathway analysis of differentially expressed genes were conducted using Ingenuity Pathway Analysis (IPA) software (www.qiagen.com/ingenuity, Qiagen)
and Databases for Annotation, Visualization, and Integrated Discovery (https://david.ncifcrf.gov/), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (https://www.genome.jp/kegg/pathway.html).

Gene Set Enrichment Analysis (GSEA) was performed using GSEA software v2.2.1 from the Broad Institute (25) to detect if a series of pre-defined biological processes or gene sets were enriched in the gene rank derived from differentially expressed genes between CHST15-KD and control cells. Gene sets were judged as significantly enriched if P<0.05 and the false discovery rates (FDR) was <0.25 in GSEA.

**Results**

**CHST15 expression in ESCC cell lines and CHST15 knockdown in TE-1 cells.** We examined CHST15 expression in three different ESCC cell lines: TE-1, Eca-109, and EC9706. Real-time PCR was performed and the ΔCq value (CHST15-GAPDH) of each cell line was calculated for fold change analysis. The data suggested that CHST15 is expressed in these cells, among which TE-1 has higher CHST15 expression than Eca-109 and EC9706 (Fig. 1). To investigate the function of CHST15 in TE-1 cells, a lentivirus CHST15 shRNA construct was designed and produced. CHST15-knockdown (KD) experiments were performed in TE-1 cells. First, we conducted target validation of lenti-CHST15 shRNA (shCHST15). 293T cells overexpressing CHST15 were infected with shCHST15. Western blot analysis showed a significantly decreased CHST15 protein level, which confirmed the targeting of CHST15 by lenti-shCHST15 (Fig. 2A). After 72 h of lenti-shCHST15 infection, CHST15 mRNA expression was significantly reduced by 78% (Fig. 2B) in the shCHST15 group compared with that in the Lenti-shCtrl control cells. Fig. 2C shows the TE-1 cells infected with Lenti-shCHST15 and Lenti-shCtrl constructs, respectively.

**Identification of CHST15 as a critical gene regulating cell proliferation and apoptosis of TE-1 cells.** Three days after shCHST15 infection of TE-1 cells, cell proliferation rates were evaluated and compared between shCHST15 and shCtrl cells. Cells were counted and images were taken daily from both groups for 5 days. Compared with the substantial increase in cell numbers of the shCtrl cells, the shCHST15 KD cells exhibited a significantly reduced proliferation rate (Fig. 3A and B), which indicated that deprivation of CHST15 inhibited TE-1 cell proliferation. This result was further confirmed using MTT assays, which assessed the viability of shCHST15 KD and control cells at different time points (Fig. 3C).

In addition, we determined whether CHST15 KD would affect the cell colony formation capacity. As expected, the shCHST15 group formed fewer colonies compared with the shCtrl cells (12 vs. 140) at 10 days after plating the cells on 6-well plates (Fig. 4). These results revealed that CHST15 KD significantly inhibited the colony formation capacity of the TE-1 cells.

We speculated that cell apoptosis is also altered in the CHST15 KD cells. At day 4 after infection, shCHST15 and shCtrl cells were stained with Annexin V-APC and analyzed by fluorescence activated cell sorting. The data revealed that the percentage of apoptotic cells was significantly higher in the shCHST15 infected cells (10%) compared with that in the shCtrl cells (2%) (Fig. 5). Taken together, the results suggested that CHST15 has a dual role of promoting proliferation and inhibiting apoptosis in TE-1 cells.

**Significant CHST15 overexpression in esophageal squamous cell carcinoma of patients.** These *in vitro* data prompted us to hypothesize that CHST15 may also be expressed in esophageal squamous cell carcinoma and play a possible role in carcinoma formation and progression *in vivo*. To determine the clinical significance of CHST15 in ESCC samples, *in situ* evaluation of CHST15 was conducted using an esophageal squamous cell carcinoma tissue chip which included 20 tissue sections from carcinoma specimens and 4 sections from adjacent normal tissue. Patients and sample information are shown in Table I. Immunohistochemistry for CHST15 in this tissue array is presented in Fig. 6A and B. The immunostaining signal of CHST15 was found only in the cytoplasm, but not in the cell membrane or nuclei. The CHST15 expression level in each tumor tissue specimen was evaluated by a score based on the percentage of immunopositivity and immunointensity. Statistical analysis showed that esophageal squamous cell carcinoma presented a 3.5-fold higher CHST15 level than that noted in the adjacent tissues. The relative CHST15 mRNA expression level in ESCC samples was calculated by retrieving data sets of 185 ESCC samples and 13 adjacent normal samples from the TCGA database (http://cancergenome.nih.gov), which showed that ESCC samples had higher CHST15 expression (Fig. 6C). Overall, these data indicated that CHST15 is overexpressed in esophageal squamous cell carcinoma and may have an important role in ESCC formation or progression.
Analysis of the mRNA profiles of CHST15 KD and control TE-1 cells. To understand the underlying molecular mechanism of the function of CHST15 in TE-1 cell proliferation and apoptosis, as well as its possible role in ESCC formation and proliferation, we performed genome-wide mRNA microarray analysis to compare the mRNA profiles of the shCHST15 and shCtrl TE-1 group cells. CHST15 KD cells were prepared by lentivirus-shCHST15 infection. Real-time PCR was used to detect the CHST15 knockdown efficiency. The CHST15 mRNA level was reduced by 58.3% compared with that in the control cells. A gene microarray experiment was conducted using shCHST15 and shCtrl cells with three biological repeats. Only qualified RNA samples (1.7 < A260/A280 < 2.2, RNA Integrity Number ≥ 7.0) were processed by hybridization to the GeneChip Primeview human gene array. We evaluated the raw data from the microarray by signal histogram analysis, relative signal box plot analysis, Pearson's correlation analysis, and principal component analysis. All of these analyses suggested that the microarray data were suitable for next-step analysis. After data filtering and cleaning, 554 differentially expressed gene transcripts were identified in this gene microarray, among which 188 genes were upregulated and 366 genes were downregulated in the shCHST15 group compared with the shCtrl control group (Fig. 7A and B).

To identify pathways related to transcriptome changes, we categorized these genes by their associated canonical signaling pathways using Ingenuity Pathway Analysis (IPA) software. Enrichment analysis of differentially expressed genes revealed that the integrin-linked kinase (ILK) and transforming growth factor-β (TGF-β) signaling pathways were inhibited in the CHST15 KD cells, whereas, checkpoint kinase (CHK) proteins in the cell cycle checkpoint control and p53 signaling pathways were activated in the CHST15 KD cells (Fig. 7C). We found that genes encoding proteins involved in the ILK signaling network, such as integrin subunit β6 (ITGB6), phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2β (PIK3C2B), protein tyrosine kinase 2 (PTK2), fermitin family member 2 (FERTM2), and cyclin D1 (CCND1), were downregulated in the shCHST15 TE-1 cells according to the dataset.

We also performed a GSEA analysis using the microarray dataset to gain a further insight into the biological processes that CHST15 may be involved in. This analysis was performed to enrich gene sets from differentially expressed genes between CHST15 KD cells and control cells that share common biological function, chromosomal location, and regulation. GSEA revealed that genes associated with cell growth, proliferation, blood vessel morphogenesis, and tissue development were markedly enriched in the dataset of differentially expressed genes (Fig. 8), suggesting that CHST15 may be involved in

Table I. Characteristics of the patients with ESCC (N=20).

| Characteristics          | Data   |
|--------------------------|--------|
| Age (mean ± SD) in years | 56.6±7.0 |
| Sex, n (%)               |        |
| Female                   | 10 (50) |
| Male                     | 10 (50) |
| Tumor grade, n (%)       |        |
| G1                       | 8 (40)  |
| G2                       | 10 (50) |
| GX                       | 2 (10)  |

GX, undetermined grade; ESCC, esophageal squamous cell carcinoma.
these biological processes, which are also features of cancer progression.

Differently expressed genes (DEGs) identified in the CHST15 knockdown cells were analyzed according to several functional network criteria within IPA. We generated a network with 22 DEGs related to cell proliferation and apoptosis processes (Table II), showing interactions with CHST15. This network diagram indicated a molecular mechanism by which CHST15 regulates the proliferation and apoptosis of TE-1 cells (Fig. 9).

**Discussion**

Esophageal squamous cell carcinoma (ESCC), a common type of esophageal cancer, is considered a serious malignancy, with a low 5-year overall patient survival rate. Despite the current availability of multiple treatment strategies, the survival rate for ESCC has not significantly improved as many patients present with local advanced disease at the time of diagnosis (3,26). Determining the molecular mechanism underlying ESCC development would be beneficial to identify new diagnostic approaches and therapeutic targets.

In the present study, we showed that carbohydrate sulfotransferase 15 (CHST15) is highly expressed in ESCC cell lines and ESCC tissues. We designed lenti-shCHST15 virus constructs and performed CHST15 knockdown experiments on TE-1 cells. Silencing of CHST15 inhibited TE-1 cell proliferation and promoted TE-1 cell apoptosis, suggesting that CHST15 contributes to the pathogenesis of ESCC. Tissue array immunostaining and bioinformatic analysis of TCGA data sets of ESCC and adjacent normal tissues both showed that CHST15 is overexpressed in ESCC samples, indicating that CHST15 may play an essential role in mediating the tumorigenicity of ESCC cells.

To gain a deeper insight into the molecular function of CHST15 in ESCC cells, a gene microarray assay was conducted to compare the mRNA profiles of CHST15-knockdown cells and control cells. Subsequent Gene Ontology (GO) and KEGG enrichment analysis of the identified differentially expressed genes (DEGs) indicated that CHST15 may be involved in integrin-linked kinase (ILK) and p53 signaling, which regulate cell proliferation and cell apoptosis, respectively. ILKs are important regulators of integrin-mediated signaling. The main function of ILK is to connect integrins to the cytoskeleton. ILK

| Gene symbol | Fold change | Location     | Family          |
|-------------|-------------|--------------|-----------------|
| APCDD1      | -2.249056649| Plasma membrane| Other           |
| BMP2        | 2.552303689 | Extracellular space| Growth factor  |
| BTG2        | -1.801089658| Nucleus      | Transcription regulator |
| CCND1       | -1.574172163| Nucleus      | Transcription regulator |
| CHST15      | -2.578621457| Plasma membrane| Enzyme          |
| CLU         | -1.726946433| Cytoplasm    | Other           |
| CREM        | 1.573881221 | Nucleus      | Transcription regulator |
| EEF1A1      | -1.600578387| Cytoplasm    | Translation regulator |
| HSPA5       | -1.588890827| Cytoplasm    | Enzyme          |
| LEPR        | 1.966722394 | Plasma membrane| Transmembrane receptor |
| MIR17HG     | 1.509101665 | Other        | Other           |
| NF1         | 1.510434197 | Cytoplasm    | Other           |
| PLAU        | 1.906482559 | Extracellular space| Peptidase  |
| PMAIP1      | 1.714217298 | Cytoplasm    | Other           |
| PTHLH       | 2.256082774 | Extracellular space| Other  |
| PTK2        | -1.699664502| Cytoplasm    | Kinase          |
| SAE1        | -1.840426328| Cytoplasm    | Enzyme          |
| TGFA        | -1.540818218| Extracellular space| Growth factor  |
| TIMP3       | -1.590628214| Extracellular space| Other  |
| TPM1        | 1.923481561 | Cytoplasm    | Other           |
| USO1        | -2.010936566| Cytoplasm    | Transporter     |
| WNT5A       | -1.526294473| Extracellular space| Cytokine   |

CHST15, carbohydrate sulfotransferase 15; APCDD1, APC down-regulated 1; BMP2, bone morphogenetic protein 2; BTG2, BTG anti-proliferation factor 2; CCND1, cyclin D1; CLU, cluster; CREM, cAMP responsive element modulator; EEF1A1, eukaryotic translation elongation factor 1 α 1; HSPA5, heat shock protein family A (Hsp70) member 5; LEPR, leptin receptor; MIR17HG, miR-17-92a-1 cluster host gene; NF1, neurofibromin 1; PLAU, plasminogen activator, urokinase; PMAIP1, phorbol-12-myristate-13-acetate-induced protein 1; PTHLH, parathyroid hormone like hormone; PTK2, protein tyrosine kinase 2; SAE1, SUMO1 activating enzyme subunit 1; TGFA, transforming growth factor-α; TIMP3, TIMP metalloproteinase inhibitor 3; TPM1, tropomyosin 1; USO1, USO1 vesicle transport factor; WNT5A, Wnt family member 5A.
recruits other adaptor molecules into a large complex to regulate actin dynamics and integrin function (27). Overexpression or activation of ILK leads to increased tumor cell proliferation, motility and invasion; thus, ILK may be a promising therapeutic target in many types of cancer (28,29). Downregulated ILK signaling in CHST15 knockout cells may contribute to the inhibition of cell proliferation. P53 is a well-known tumor suppressor that can inhibit cancer progression by provoking cell growth arrest, by enabling DNA repair, or by advancing cellular death programs (30). In this study, the increased apoptosis of TE-1 cells may be attributable to activated p53 signaling induced by CHST15 knockdown. The results of GSEA also suggested that CHST15 is significantly associated with cell growth and proliferation processes.

By analyzing DEGs and mapping using IPA software, we identified two possible signaling axes,
CHST15/ILK associated serine/threonine phosphatase (ILKAP)/CCND1 and CHST15/RAB, member RAS oncogene family like 6 (RABL6)/phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1), which regulate ESCC.
Figure 7. Differentially expressed genes and pathways in CHST15-knockdown (KD) TE-1 cells. (A) Volcano plot of differentially expressed genes. Red dots represent differentially expressed genes, which were selected with |fold change| >1.5 and false discovery rate (FDR) <0.05. (B) Hierarchical clustering heatmap of differentially expressed genes. Rows represent individual genes (188 upregulated genes and 366 downregulated genes in CHST15-KD cells). Each column shows the gene expression level of each sample from the shCHST15 group and the shCtrl group. (C) Canonical pathways differentially regulated in CHST15-knockdown TE-1 cells were generated by gene enrichment analysis and IPA software. All signaling pathways were ranked by -log10 (P-value). An orange bar indicates an activated pathway. Purple bars represent inhibited pathways. A darker color shade reflects a higher degree of activation or inhibition. CHST15, carbohydrate sulfotransferase 15; IPA, Ingenuity Pathway Analysis.

Figure 8. Identification of CHST15-associated biological processes by GSEA. The analysis showed that genes related to cell growth, cell proliferation, cell development, and blood vessel morphogenesis were significantly enriched in the dataset of differentially expressed genes. CHST15, carbohydrate sulfotransferase 15; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis.
cell proliferation and apoptosis, respectively. CHST15 is reported to interact with RABL6 (31). RABL6 is a small GTPase belonging to the Ras superfamily, which mainly relays signals from receptors at the cell plasma membrane and modulates many cellular signaling pathways that regulate cell proliferation, differentiation and survival (32). Tang et al demonstrated that knockdown of RABL6 upregulated retinoblastoma 1 (Rb) expression and thus downregulated Rb inhibitory downstream targets, such as cyclin A2, cyclin D1, c-Myc, and cyclin-dependent kinase 2 (33). RABL6 is also a binding partner of PMAIP1, which is a pro-apoptotic member of the BCL2 protein family, but only contains a BH3 domain. PMAIP1 expression was found to activate the mitochondrial apoptotic cascade and induce increased oxidative stress and calcium release, resulting in the activation of apoptosis signal-regulating kinase 1 (ASK1) and its downstream effectors JUN N-terminal kinase (JNK) and mitogen-activated protein kinase 14 (MAPK14, also known as p38) (34). PMAIP1 was also found to regulate p53-induced apoptosis (35). According to the microarray dataset, PMAIP1 expression was increased in the CHST15-knockdown TE-1 cells (Table II). Therefore, CHST15 may promote TE-1 cell apoptosis by interacting with RABL6 and affecting PMAIP1 expression and their subsequent downstream molecules.

CHST15 was also indicated to interact with ILKAP (31), which is a serine/threonine protein phosphatase associated with ILK. It selectively inhibits the ILK-mediated glycogen synthase kinase 3β (GSK3β) signaling pathway and further regulates the Wnt signaling pathway by modulating GSK3β phosphorylation. ILK-mediated inhibition of GSK3β was found to induce the expression of cyclin D1 (36,37). Cyclin D1 (encoded by CCND1) plays a central role in the regulation of proliferation, linking the extracellular signaling environment to cell cycle progression (38). In the CHST15-knockdown TE-1 cells, reduced cyclin D1 levels might be the main reason leading to the inhibition of cell proliferation through the CHST15/ILKAP/CCND1 signaling axis. These observations and predictions provide valuable clues for future detailed
investigation of possible CHSR15-associated intracellular signaling pathways.

In summary, the present study investigated the role of CHST15 in cell growth and apoptosis of ESCC and demonstrated the clinical implication of CHST15 in ESCC. CHST15 could be a promising diagnostic marker or therapeutic target for this disease.

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Availability of data and materials

The microarray dataset in this study is not publicly available since we are using this dataset for further studies. However, it is still available from the corresponding author upon reasonable request.

Authors’ contributions

LW and NB designed the study and analyzed the data. XW conducted the majority of experiments and wrote the manuscript. QF, DC and ZZ were involved in construction of the recombinant lentiviral vector. WW and LD conducted the cell growth and apoptosis assays and collected the data. XW, KX, XX, and GC conducted the gene chip microarray experiment and bioinformatic analyses. TZ and XW carried out the tissue chip immunohistochemistry experiment. All authors read and approved the manuscript and agree to be accountable for all aspects of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The use of human tissue specimen was approved by the Ethics Committee of National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College. The reference number is 19/140-1924. Twenty patients signed consent forms prior to participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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