Network analysis of genes associated with esophageal squamous cell carcinoma progression

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ABSTRACT

This study aims to identify possible genes associated with esophageal squamous cell carcinoma (ESCC) by bioinformatics tool and further explore the function of immunoglobulin heavy chain variable family 4 gene (IGHV4)-28 in the ESCC progression.

The ESCC-related genes in Cancer Genome Atlas (TCGA) database were analyzed by bioinformatics tools, which finally identified IGHV4-28. The expression levels of IGHV4-28 in TE-4 and EC9706 cells were detected by quantitative reverse transcription-PCR (qRT-PCR). Then oe-IGHV4-28 or sh-IGHV4-28 was transfected into TE-4 and EC9706 cells to verify the effect on cell proliferation, migration, invasion, and apoptosis rate. In vivo, a nude mouse model of ESCC was developed, whereby the tumor volume and weight were calculated to evaluate the impact of IGHV4-8 on tumor growth. Bioinformatics analysis using TCGA database showed that IGHV4-28, IGLV6-57, and KPRP were all associated with ESCC progression. Kaplan-Meier (KM) analysis showed overexpression of IGHV4-28 is substantially associated with the survival rate of patients with ESCC. IGHV4-28 was highly expressed in TE-4 and EC9706 cell lines and overexpression of IGHV4-28 enhanced cell proliferation, invasion, and migration, as well as decreased apoptosis rate. Moreover, nude mice transplanted with IGHV4-28-silencing TE-4 cells showed restrained tumor weight and volume.

In summary, IGHV4-28 was increasingly expressed in ESCC and may serve as a therapeutic target in the treatment of ESCC.

Significance of this study

What is already known about this subject?

⇒ Esophageal cancer (EC) is one of the most lethal carcinomas worldwide and the most common and invasive malignant tumors among gastrointestinal malignancies.

⇒ Of the two main histological types, esophageal squamous cell carcinoma (ESCC) is globally predominant, especially in Asia where it accounts for 90% of all EC cases, while esophageal adenocarcinoma (EAC), the incidence of which has promptly increased, is the most common subtype in Western countries.

What are the new findings?

⇒ Immunoglobulin heavy chain variable family 4 gene (IGHV4)-28 was increasingly expressed in ESCC.

⇒ Knockdown of IGHV4-28 promoted cell proliferation, migration, and invasion of ESCC cells.

⇒ Silencing of IGHV4-28 inhibited transplantation tumor growth in vivo.

How might these results change the focus of research or clinical practice?

⇒ The potential of IGHV4-28 in evaluating prognosis and in treatment of ESCC; however, more data are required to validate the result of this study and the mechanism is also worth validation.

INTRODUCTION

Esophageal cancer (EC) is one of the most lethal carcinomas worldwide and the most common and invasive malignant tumors among gastrointestinal malignancies.[1] Of the two main histological types, esophageal squamous cell carcinoma (ESCC) is globally predominant, especially in Asia where it accounts for 90% of all EC cases, while esophageal adenocarcinoma (EAC), the incidence of which has promptly increased, is the most common subtype in Western countries.[2 3] There are multiple factors affecting the occurrence of EC, such as ethnicity, genetic factors, lifestyle (including smoking and alcohol abuse) and so on.[4 5] Unfortunately, early diagnosis of this disease is not easy as the clinical symptoms are not obvious and most of the cases are diagnosed at the advanced stage.[6] As symptoms such as difficulty and pain in swallowing, loss of body weight, and heartburn are noticed, this disease is mostly diagnosed in the late stage when lymph node metastasis (LNM) is likely presented.[7] Despite advances in surgical treatment and multimodality therapies, patients with late clinical presentation of EC have poor prognosis.[8] Therefore, it is of clinical significance to improve the understanding about the molecular determinants of ESCC development for shaping new diagnostic approaches and developing new therapeutic modalities.

In our study, we analyzed the complicated clinical characteristics and cancer genomics related to EC in Cancer Genome Atlas (TCGA) database to screen differentially expressed genes (DEGs) of EC and LNM. Then enrichment

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Table 1 The primer sequences used for qRT-PCR

Name of primer	Sequences (5′–3′)
IGHV4-28-F	GCTCACATGGGAAATACTTTCT
IGHV4-28-R	CAGTCCTGGGCCCGACTCCT
GAPDH-F	CACCCACTCCTCCACCTTTG
GAPDH-R	CCACCACCCTGTTGCTGTAG

F, forward; IGHV4, immunoglobulin heavy chain variable family 4 gene; qRT-PCR, quantitative reverse transcription-PCR; R, reverse.

analyses of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed to analyze the enrichment and distribution of those screened DEGs. In addition, based on follow-up data in TCGA, we localized 29 DEGs associated with the relapse of EC. Further Cox regression analysis identified three genes (immunoglobulin heavy chain variable family 4 gene (IGHV4)-28, IGLV6-57, and KPRP) are associated with the relapse of EC. IGLV6-57 is an immune gene that has been proved to have a certain prognostic potential in colorectal cancer[9] and early stage lung cancer.[10] KPRP mutation has been reported in pretreatment samples from patients with triple-negative breast cancer who had pathological complete response following neoadjuvant chemotherapy.[11] In addition, the decreased expression of KPRP in atopic dermatitis is associated with barrier dysfunction.[12] IGHV4-28, a protein coding gene of the immunoglobulin heavy chain variable (IGHV) family 4 gene, also known as VH, was reported to be increasingly expressed in patients with primary immune thrombocytopenia in a previous study.[13] However, IGHV4-28 is rarely reported for its implication in carcinoma among these three genes. Therefore, this study aims to explore the possible function of IGHV4-28 in ESCC progression.

MATERIALS AND METHODS

Bioinformatics analysis

RNAseq data of ESCC samples in TCGA database (n=80) and of normal esophageal mucosa in GTEx database (n=269) were downloaded to analyze the DEGs and LNM-related genes in ESCC. The protein-protein interaction (PPI) network of the DEGs was mapped using String database (https://stringdb.org/cgi/input.pl). The hub genes in EC tumorigenesis and LNM were identified by Cytoscape plugin cytoHubba. R software was used for enrichment analyses of GO and KEGG.

Cell culture

Two human ESCC cell lines (TE-4 and EC9706) and a normal esophageal epithelial cell line (Het-1A) were purchased from BeNa Culture Collection (Beijing, China). Cells were cultured under 37°C and 5% CO_2 in Dulbecco's modified eagle medium (DMEM; Gibco, Grand Island, New York, USA) in which 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Wilmington, Delaware, USA) and antibiotics (Gibco, Grand Island, New York, USA) were added.

Cell transfection

Plasmid with overexpression of IGHV4-28 (oe-IGHV4-28), plasmid with knockdown of IGHV4-28 (sh-IGHV4-28), and their corresponding negative controls (oe-NC and sh-NC) were purchased from GenePharma (Shanghai, China). Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) based on the instructions herein.

Quantitative reverse transcription-PCR

TRIZOL was used for total RNA extraction from tissues and cells. The RNA was subjected to reverse transcription using RT kit (Takara, Tokyo, Japan) strictly in accordance with the instructions. Biosystems 7300 real-time PCR system (ABI, Foster City, California, USA) was used for qRT-PCR with SYBR Green Mix (Takara). Three duplicates were set for each reaction of PCR. Data analysis was determined using $2^{-\Delta\Delta Ct}$ method[14] based on the following formula: ΔΔCt=-experimental group (Ct target gene−Ct internal control)−control group (Ct target gene−Ct internal control). The relative expression of target genes was normalized to GAPDH. The primer sequences are listed in table 1.

Cell counting kit 8 (CCK-8) assay

After cell transfection, cells were seeded in a 96-well plate and each group had three duplicate wells. Then the cells in each well were added with 10 µL CCK-8 reagent and cultured for 0, 24, 48, and 72 hours in a CO_2 incubator. The optical density at the wavelength of 450 nm was measured by a Model 680 microplate reader (Bio-Rad, Hercules, California, USA).

Transwell assay

A total of 5×10^4 cells suspended in serum-free DMEM were cultured in transwell chambers which were coated with matrixgel (BD Biosciences, Bedford, Massachusetts, USA). Then 600 µL of culture medium containing 10% FBS was added into the basolateral chamber. After 48 hours incubation, the cells passed through the membrane were fixed in 100% methanol and stained with 0.1% crystal violet. The non-invaded cells above the membrane were removed with cotton swabs. The fixed cells were observed using a microscope (Olympus, Tokyo, Japan) under five random selected fields with the magnification of 200×.

Scratch test

Transfected cells were seeded onto 6-well plates and when cell confluence reached 90%, a pipette tip was used for cell scratch test. The cells were washed once in serum-free medium, and then observed under a low-power phase contrast microscope (Olympus) and photographed. Cells were cultured in serum-free medium at 37°C and 5% CO_2 for 24 hours, and then photographed again. Image-Pro Plus software was used to measure the distance of cell migration. The migration ability of cells in each group was accordingly calculated.

Flow cytometry

Cell apoptosis rate was determined using Annexin V-fluorescein isothiocyanate (FITC) apoptosis kit (Beyotime, Shanghai). Cells were disassociated with pancreatin and centrifuged before resuspension with binding buffer. After that, cells were added with Annexin V-FITC and propidium iodide for incubation for 15 min. The cell apoptosis rate

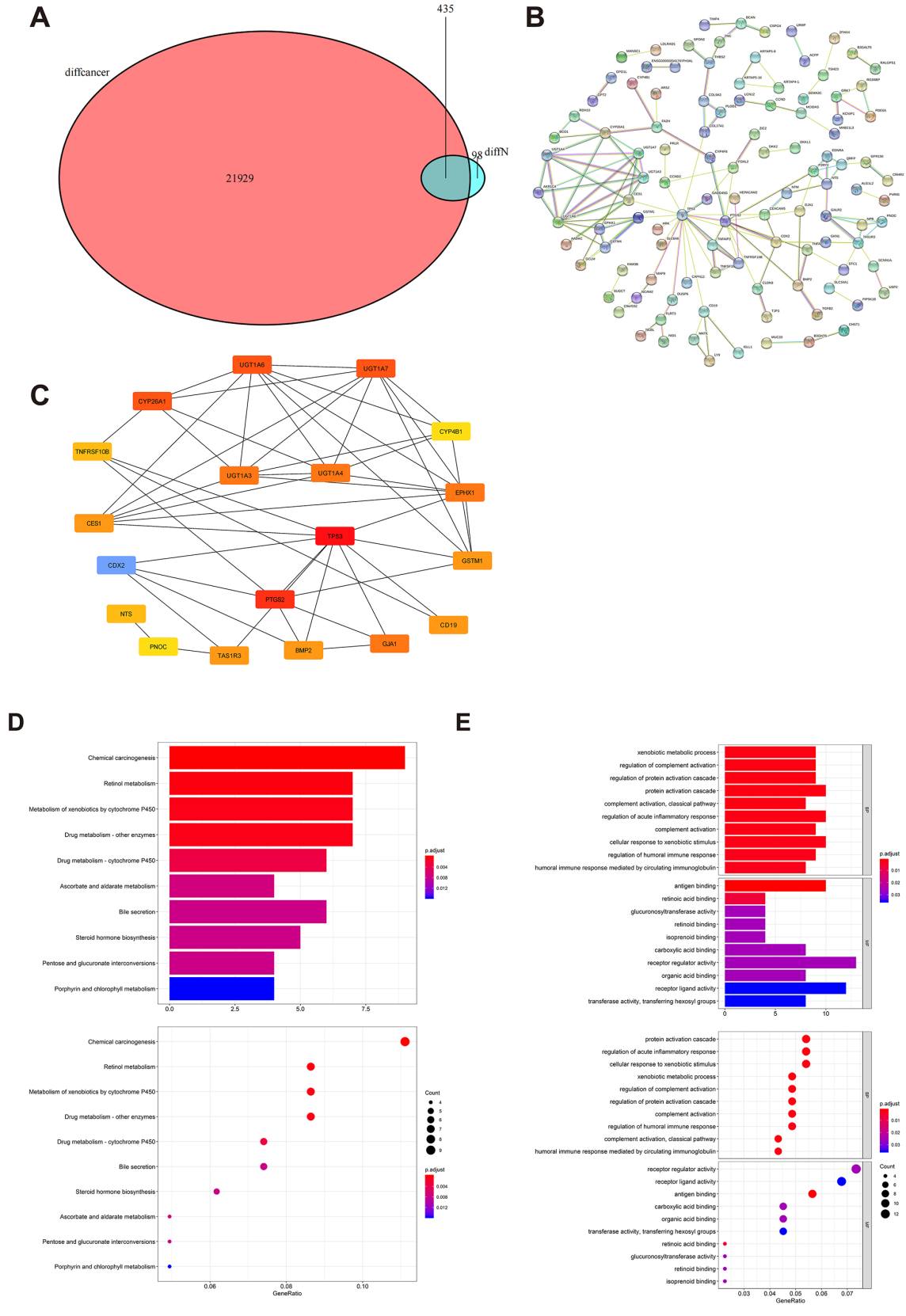

Figure 1 DEGs were screened and subjected to functional analysis using TCGA RNAseq data in EC. (A)The Venn diagram of DEGs related to both ESCC and LNM. (B)The PPI network of the screened 435 genes was mapped using String software. (C)cytoHubba analyzed the hub genes. (D)The GO functional enrichment analysis of these 435 genes. (E)The KEGG signaling pathway enrichment analysis of these 435 DEGs. DEGs, differentially expressed genes; EC, esophageal cancer; ESCC, esophageal squamous cell carcinoma; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; LNM, lymph node metastasis; PPI, protein-protein interaction; TCGA, the cancer genome atlas.

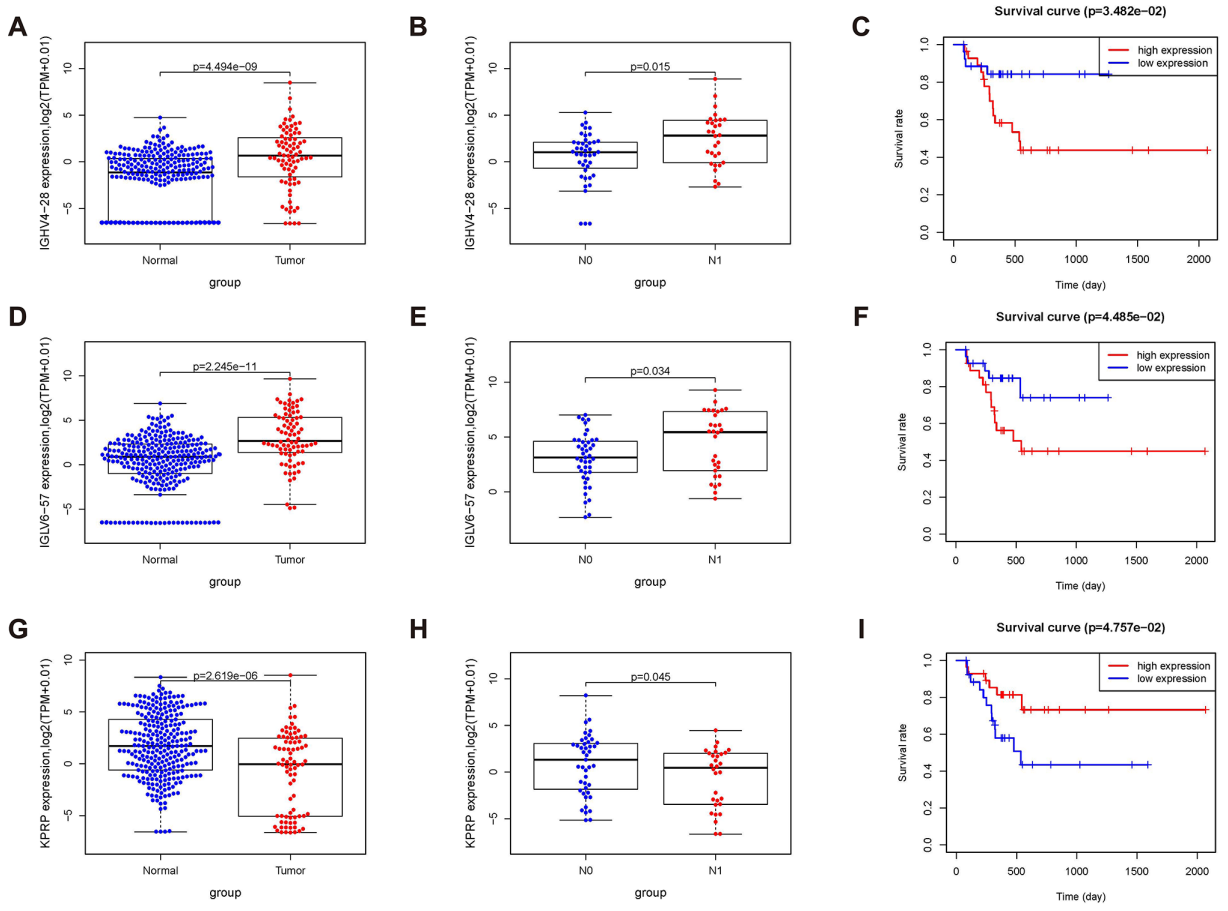

Figure 2 Three DEGs were associated with the relapse of ESCC. (A)IGHV4-28 expression was increased in ESCC tissues relative to normal tissues. (B)The expression of IGHV4-28 in ESCC samples was elevated compared with the samples without LNM. (C)KM survival curve showed that the survival rate of patients with ESCC with high IGHV4-28 expression was decreased than those with low IGHV4-28 expression (p=0.015). (D)IGLV6-57 expression was upregulated in ESCC samples versus normal tissues. (E)Increased IGLV6-57 expression was found in ESCC samples with LNM compared with the samples without LNM. (F)KM survival curve revealed that the survival rate of patients with high IGLV6-57 expression was reduced than those with low IGLV6-57 expression (p=0.034). (G)KPRP was decreasingly expressed ESCC tissues relative to normal tissues. (H)ESCC tissues with LNM had lower KPRP expression than those without LNM. (I)KM survival curve indicated that patients with low KPRP expression had poor survival rate than those with high KPRP expression (p=0.045). DEGs, differentially expressed genes; ESCC, esophageal squamous cell carcinoma; IGHV4, immunoglobulin heavy chain variable family 4 gene; KM, Kaplan-Meier; LNM, lymph node metastasis.

was determined using a flow cytometer. Each experiment was conducted in triplicate.

Subcutaneous transplantation tumor in nude mouse

Male BALB/c nude mice (n=15, 4~6 weeks old, weighting 16±2 g) available from Shanghai experimental animal central of Chinese Academy of Science were housed in specific pathogen-free environment under the conditions of (25°C–28°C), 50% humidity, and 12 hours/12 hours light/dark cycle. The mice were allowed free to access food and water, and the padding was replaced every 3 days under a sterile condition. The animal experiments were conducted based on the rules and regulations of animal experiments issued by the Second Xiangya Hospital of Central South University. The mice were randomly divided into TE-4, sh-IGHV4-28, and sh-NC groups. The mice were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg), and the skin of the right front limb was disinfected with iodine. Mice in TE-4 group were injected with 0.2 mL TE-4 cell suspension (1×10^6 cells) and mice in sh-IGHV4-28 group and sh-NC group were injected with equal volume of TE-4 cells transfected with sh-IGHV4-28 or sh-NC. The needle was withdrawn slowly to prevent the cell suspension from flowing out. Five weeks later, the nude mice were sacrificed by cervical dislocation to isolate the subcutaneous tumors. The tumors were weighted and the tumor volume was determined. Tumor volume=1/2×length×width^2.

Statistical analysis

Data were analyzed using GraphPad prism 7 software and expressed as mean±SD ($\bar{x}\pm s$). The screened DEGs were identified by Wilcoxon test. The association of DEGs with relapse of EC was analyzed using Kaplan-Meier (KM) survival analysis and Cox regression analysis. Two-group comparison was done by t-test, and multigroup comparison was performed by one-way analysis of variance, with Tukey's multiple comparisons test for post hoc analysis. P value of <0.05 was considered to have statistical significance.

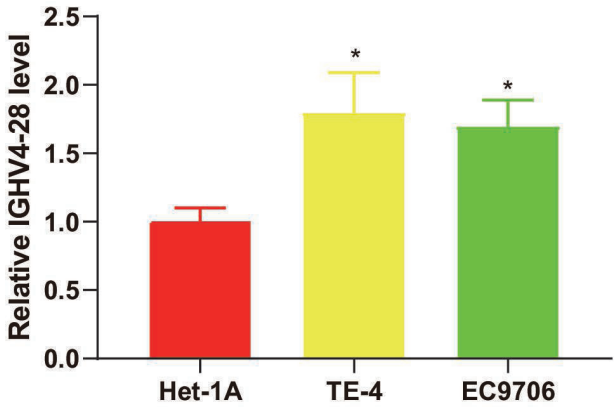

Figure 3 Elevated expression of IGHV4-28 was found in ESCC cell lines. The expression of IGHV4-28 in normal esophageal epithelial cells (Het-1A) and ESCC cell lines (TE-4 and EC9706) were determined by qRT-PCR. *P<0.01, when compared with Het-1A group. ESCC, esophageal squamous cell carcinoma; IGHV4, immunoglobulin heavy chain variable family 4 gene; qRT-PCR, quantitative reverse transcription-PCR.

RESULTS

Screening and function analysis of DEGs in EC

An integrated analysis on 269 cases of normal esophageal mucosa in GTEx database and 80 cases of ESCC samples in TCGA database showed there were 22364 DEGs with a >1.5-fold change (p<0.05). Then the results of Wilcoxon test on 43 cases without LNM and 30 cases with LNM identified 535 genes with a >1.5-fold change. After comparing the two sets of DEGs, 435 DEGs are found to have crucial effects on ESCC tumorigenesis and LNM (figure 1A). The PPI network of those 435 genes and the hub genes were analyzed, which confirmed the important implication of those 435 genes in ESCC initiation and LNM (figure 1B,C). The GO functional annotation showed that those genes were enriched in biological process, including complement activation and cascade activation (figure 1D). KEGG analysis showed those 435 genes were enriched in 10 pathways, such as chemical carcinogenic signal pathways (figure 1E).

Association between DEGs with the relapse of ESCC

KM method was used to analyze the association of those 435 DEGs with the relapse of ESCC based on the follow-up data from TCGA database. A total of 29 genes were identified to be associated with the relapse of ESCC and 3 genes (IGHV4-28, IGLV6-57, and KPRP) were closely associated with the relapse of ESCC based on the results of one-way Cox regression analysis. The expression levels of these three genes in normal esophageal mucosa tissues and ESCC tissues with or without LNM were measured. The results of bioinformatics analysis showed that IGHV4-28 was highly expressed in ESCC tissues compared with that in normal esophageal mucosa tissues or ESCC tissues without LNM (figure 2A,B). KM survival analysis curve showed the survival rate of patients with high IGHV4-28 expression was lowered than those with low IGHV4-28 expression (figure 2C, p=0.015). The results of expression profile analysis and KM survival analysis of IGLV6-57 were similar to those of IGHV4-28, which indicated that IGHV4-28 and IGLV6-57 were risk factors for ESCC. Intriguingly, the results of expression profile analysis and KM survival analysis of KPRP showed opposite trends. Specifically, KPRP expression was decreased in ESCC tissues relative to normal esophageal mucosa tissues or ESCC tissues without LNM (figure 2G,H). Patients with low KPRP expression had decreased survival rate than those with low KPRP expression (figure 2I, p=0.045), suggesting KPRP as a tumor suppressor in ESCC progression. Due to decreased survival rate in patients with higher IGHV4-28 expression, and rare in vivo and in vitro data with regard to the role of IGHV4-28 in ESCC, IGHV4-28 was selected in this study to explore its effect in ESCC both in vivo and in vitro.

Elevated expression of IGHV4-28 in ESCC cell lines

Elevated expression of IGHV4-28 in ESCC was found in TCGA database. qRT-PCR was used to measure the expression level of IGHV4-28 in ESCC cell lines (TE-4 and EC9706), which showed that IGHV4-28 expression was elevated in TE-4 and EC9706 cells relative to Het-1A cells (figure 3). Collectively, IGHV4-28 was increasingly expressed in ESCC cell lines.

Regulation of overexpression or knockdown of IGHV4-28 on ESCC cell proliferation, invasion, migration and apoptosis

TE-4 and EC9706 cells were transfected with oe-IGHV4-28 or sh-IGHV4-28, and the efficiency of oe-IGHV4-28 and sh-IGHV4-28 was verified by qRT-PCR. Specifically, compared with the control, the expression of IGHV4-2 was elevated in oe-IGHV4-28 group and suppressed in sh-IGHV4-28 group (figure 4A,B). Results of CCK-8, scratch, transwell, and flow cytometry assays showed that TE-4 and EC9706 cells had elevated proliferation, migration, and invasion abilities (figure 4C–H), and decreased apoptosis rate (figure 4I,J) in oe-IGHV4-28 group, while cells in sh-IGHV4-28 group had suppressed proliferation, migration, and invasion abilities (figure 4C–H) and enhanced apoptosis rate (figure 4I,J). Those data showed that overexpression of IGHV4-28 in vitro can increase the proliferation, migration, and invasion abilities, and suppress apoptosis of ESCC cells, while IGHV4-28 knockdown could restrain these malignant properties of ESCC cells.

Silencing of IGHV4-28 suppresses transplantation tumor growth

To evaluate the effect of IGHV4-28 on tumor growth in vivo, we injected TE-4 cells transfected with sh-IGHV4-28 or sh-NC into nude mice. The results of qRT-PCR showed that the expression of IGHV4-28 in sh-IGHV4-28 group was substantially decreased compared with sh-NC group (figure 5A). Moreover, compared with sh-NC group, the tumor volume and weight in sh-IGHV4-28 group were decreased (figure 5B,C), indicating that silencing of IGHV4-28 can suppress tumor growth.

DISCUSSION

LNM is a significant sign of cancer spread and a marker for poor clinical outcomes in numerous cancers, including EC.[15] Metastasis is the process that cancer cells invaded into surrounded tissues.[7] In patients with EC receiving

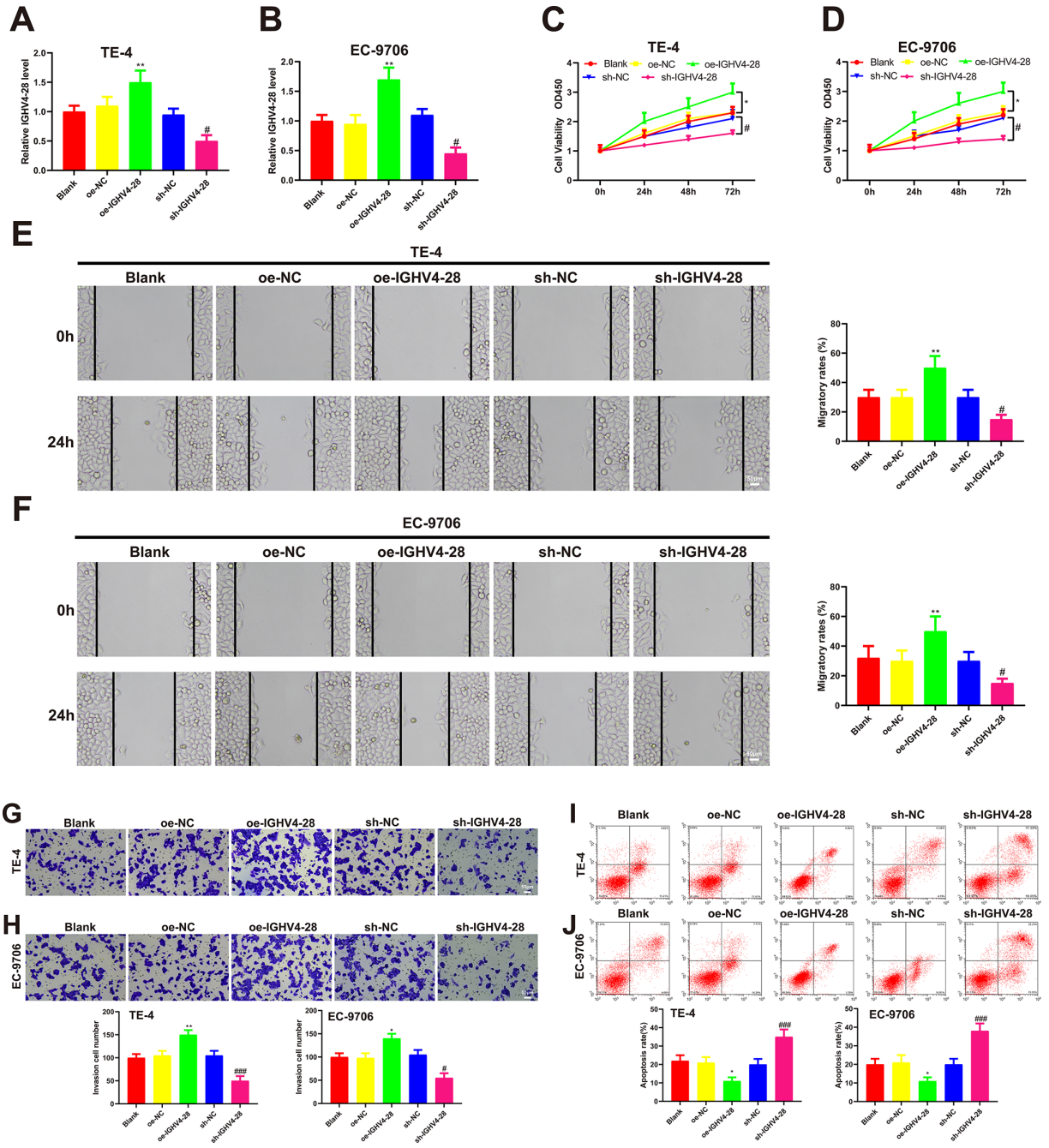

Figure 4 Overexpression or knockdown of IGHV4-28 affected proliferation, migration, invasion, and apoptosis of ESCC cells. After TE-4 and EC9706 cells were transfected with oe-IGHV4-28 or sh-IGHV4-28, (A–B)qRT-PCR was used to detect IGHV4-28 expression; (C–D)cell proliferation was measured by CCK-8 assay; (E–F)cell migration was tested by scratch test; (G–H)cell invasion was assessed by transwell assay; (I–J)cell apoptosis was evaluated by flow cytometry. *P<0.05, **p<0.01, when compared with oe-NC group; #p<0.05, ###p<0.001, when compared with sh-NC group. ESCC, esophageal squamous cell carcinoma; IGHV4, immunoglobulin heavy chain variable family 4 gene; qRT-PCR, quantitative reverse transcription-PCR.

neoadjuvant chemoradiation therapy, LNM is one of the crucial factors associated with overall survival.[16] Regardless of the metastatic sites, LMN is an independent risk factor for poor prognosis in patients with ESCC.[17–19] Therefore, in this study, we searched the dataset in TCGA database for DEGs in EC and the overlapped genes with LNM associated genes were identified. We finally localized three DEGs in EC that are associated with the LNM and relapse of EC, among which IGHV4-28 and IGLV6-57 are risk factors for EC initiation and progression, while KPRP might be a tumor suppressor in EC progression.

First, 435 DEGs were shown to have certain relationships with the LNM and the relapse of EC. GO function analysis showed that those DEGs were enriched in biological process, including xenobiotic metabolic process (GO: 0006805), regulation of complement activation (GO:

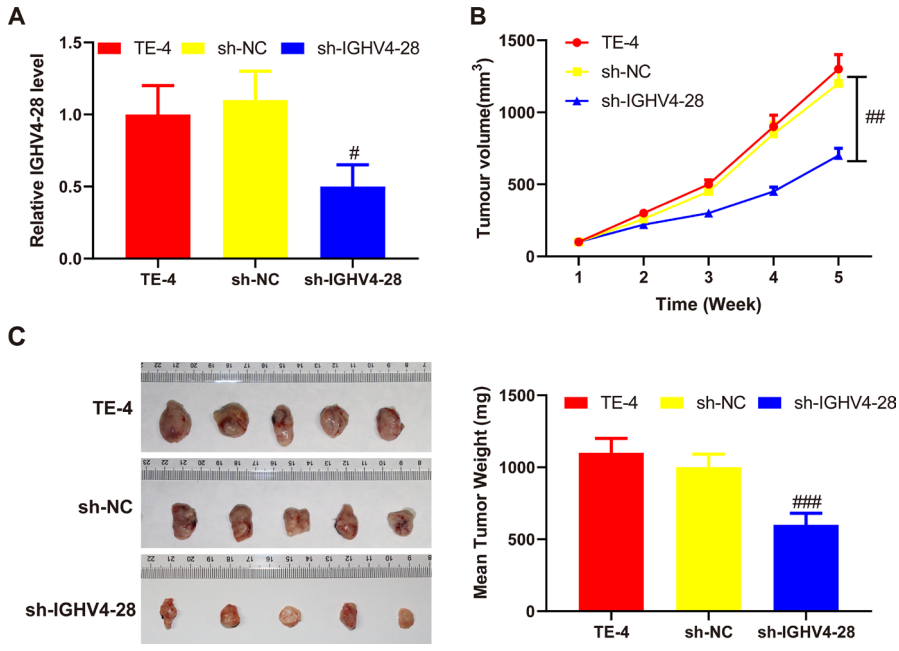

Figure 5 Inhibition of IGHV4-28 could suppress the tumor growth. After nude mice were injected TE-4 cells with sh-IGHV4-28 or sh-NC transfection, (A)the expression of IGHV4-28 was measured by qRT-PCR; (B)the changes in growth curve of tumor in nude mice were shown; (C)the volume and weight of tumor were measured. $^{\#}P<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, when compared with sh-NC group. IGHV4, immunoglobulin heavy chain variable family 4 gene; qRT-PCR, quantitative reverse transcription-PCR.

0030449), regulation of protein activation cascade (GO: 2000257), and protein activation cascade (GO: 0072376). Also, those DEGs were enriched in molecular function, including antigen binding (GO: 0003823), retinoic acid binding (GO: 0001972), and glucuronosyltransferase activity (GO: 0015020). Further KEGG analysis showed that those DEGs were associated with 10 signal pathways, such as chemical carcinogenesis (hsa05204), retinol metabolism (hsa00830), metabolism of xenobiotics by cytochrome P450 (hsa00980), and drug metabolism (hsa00983, hsa00982). The enrichment of chemical carcinogenesis suggested the implication of xenobiotics (such as alcohol and tobacco) in the initiation of EC and LNM.[20]

Based on follow-up data in TCGA dataset, we found that IGHV4-28, IGLV6-57, and KPRP genes were closely associated with the relapse of EC. IGHV4-28 and IGLV6-57 were highly expressed in patients with EC, whose elevated expressions are associated with decreased survival rate, while KPRP was found to be decreasingly expressed in EC tissues and may act as an antitumor gene in EC progression. IGHV4-28, IGLV6-57, and KPRP are all of predictive value for prognosis of patients with EC. Considering the decreased survival rate in patients with higher IGHV4-28 expression and limited data for IGHV4-28 in current database, we selected IGHV4-2 for our further experiments on ESCC cell lines. Detection of IGHV4-28 expression level in ESCC cell lines showed that IGHV4-28 was increasingly expressed in TE-4 and EC9706 cell lines, which was consistent with the expression pattern found in TCGA database. Then IGHV4-28 suppression or overexpression was achieved in TE-4 and EC9706 cell lines to observe its effect on cell biological functions. Overexpression of IGHV4-28 increased the cell proliferation, invasion, and migration of TE-4 and EC9706 cell lines, while IGHV4-28 suppression led to decreased cell proliferation, invasion, and migration. As we mentioned above that less information can be found regarding the implication of IGHV4-28 in carcinomas, a prior study demonstrated that IGHV4-28 was upregulated in patients with primary immune thrombocytopenia, an organ-specific autoimmune disorder.[13] The genes of IGHV4 family, IGHV4-61 and IGHV4-34, seems more likely to be documented in autoimmune disorders, including rheumatic fever, rheumatic heart disease,[21] chronic lymphocytic leukemia, and hairy cell leukemia-variant.[22] This study showed that IGHV4-28 was implicated in the proliferation, invasion, and migration of ESCC cells. A consistent study showed that through gene expression network analysis, IGHV4-31 was found to be highly associated with familial pancreatic cancer.[23] In our study, in vivo experiment further supported the regulatory role of IGHV4-28 in ESCC progression.

It is worth noticing that there are some limitations to the present study. First, although our study identified three genes associated with the relapse of EC, more validation is needed considering that the data extracted from TCGA are retrospective. Second, we only explored the function of IGHV4-28 on ESCC progression and the possible mechanism also remains to be determined and can be served as one of the directions for our future researches.

In conclusion, our study identified the possible DEGs related to EC initiation and LNM and screened three genes related to EC prognosis. Among these three genes, IGHV4-28 was found to regulate cell proliferation, migration, and invasion of ESCC cells and mediate transplantation tumor growth in vivo, suggesting the potential of IGHV4-28 in evaluating prognosis and in treatment of ESCC. However, more data are required to validate

the result of this study and the mechanism is also worth validation.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval The experimental design was approved by the ethical committee of the Second Xiangya Hospital of Central South University. This study does not involve human participants.

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Data availability statement The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

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