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Research article

Radiotherapy prognosis-associated gene GCNT3 promotes the proliferation, migration and invasion of lung adenocarcinoma cells

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ABSTRACT

Lung cancer is a life-threatening malignant tumour that is prevalent worldwide. Here, the GCNT3 gene in lung adenocarcinoma was studied via public databases, and cytology and molecular biology experiments were performed to further explore the role of this gene in lung adenocarcinoma. In this study, abnormally high GCNT3 expression levels were observed in tumour tissues compared with normal tissues at both the mRNA and protein levels. In the pancancer analysis, abnormal GCNT3 expression was observed in many tumour types. Moreover, the survival analysis revealed that among patients receiving radiotherapy, those with high GCNT3 expression levels had a worse prognosis. Cell and molecular biology experiments showed that the proliferation, migration and invasion capabilities of the A549 cell line were decreased after knockdown of GCNT3, and epithelial-mesenchymal transformation was significantly inhibited. In subsequent studies, we found that the sensitivity of cells to radiotherapy was enhanced after GCNT3 knockdown. Overall, our findings reveal that GCNT3 is an important factor affecting the radiotherapy sensitivity of lung adenocarcinoma, and GCNT3 inhibition deserves further study as a radiotherapy sensitising strategy.

1. Introduction

Lung cancer is one of the most common malignant tumours that not only causes the most deaths among malignant tumours but also places a heavy burden on health resources worldwide. Non-small-cell lung cancer (NSCLC), which accounts for the vast majority of lung cancers, is mainly composed of squamous cell carcinoma (LUSC) and adenocarcinoma (LUAD). With the development of early lung cancer screening, an increasing number of lung adenocarcinomas are identified in the early stage of the disease, and a variety of targeted therapies and immunotherapy methods provide a wealth of therapeutic approaches for these patients. However, the prognosis of lung cancer patients remains poor; for example, the 5-year survival rate is still under 20% [1].

Glucosaminyl (N-acetyl) transferase 3 (GCNT3) belongs to the glycosyltransferase enzyme family, and recent studies have shown that GCNT3 plays different roles in a variety of cancers. In colorectal and epithelial ovarian cancer, GCNT3 overexpression is associated with better clinical outcomes and responses to initial therapy [2]. Juanjuan Liu et al have found that miR-BART1-5p directly targeted GCNT3, which regulated the proliferation and migration of gastric cancer, what's more, E-cadherin, N-cadherin, vimentin, and p-ERK were found to be downstream molecules of the miR-BART1-5p/GCNT3 pathway [3]. Li Q et al observed that downregulation of GCNT3 in NSCLC decreases cell proliferation, migration and invasion, which indicates that GCNT3 may play a cancer-promoting role in NSCLC [4]. In our study, public databases were used to explore the differential expression of GCNT3 and its relationship with patient prognosis, and cytological and molecular biology experiments were performed to explore the relevant mechanisms.

2. Materials and methods

2.1. Public database analysis

The GCNT3 expression level of LUAD patients in TCGA database was obtained from the UALCAN Browser [5] (http://ualcan.path.uab.edu/analysis.html), and patients were divided into different groups according to sex, race, TNM stage and age. Moreover, GCNT3 protein expression levels and pancancer expression status were also obtained from the UALCAN browser. The RNA level of GCNT3 in different types of tumours

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in the GEO database was analysed via the Oncomine browser [6] (https://www.oncomine.org/resource/main.html). GCNT3 expression and survival data from TCGA dataset were obtained from the XENA browser [7] (https://xenabrowser.net/).

2.2. Clinical sample validation

Clinical samples, including LUAD cancer and normal tissues, were collected from 20 patients who underwent surgical resection at the Department of Thoracic Surgery of Xuanwu Hospital, Capital Medical University. After surgical resection, the samples were washed with PBS and immediately stored at −80 °C. All of the cancer tissues and normal tissues were confirmed by pathologists in the subsequent pathological examination.

Samples were washed with PBS again before use and then cut into small pieces. T-PER™ Tissue Protein Extraction Reagent (78510) with ROCHE cOmplete™ Protease Inhibitor (ROCHE 04693116001) was used to extract tissue proteins with an ultrasonic cell lyser, and the total protein concentration was determined by Pierce™ Coomassie Plus (Bradford) Assay Reagent (Thermo 23238). Total tissue RNA was extracted using the TRIzol™ Plus RNA Purification Kit (Thermo 12183555). All sample extraction procedures above were performed on ice.

2.3. Immunoblotting (western blot)

Electrophoresis of the samples was performed using 12% SDS–PAGE with a 120 V Bio–Rad PowerPac™ HC electrophoresis apparatus, and the samples were transferred from the gel to Immobilon®-P PVDF membrance (Millipore IPVH00010) with a Bio-Rad Trans-Blot® SD Semi-Dry Transfer Cell. BSA (5% in TBST) (Merck v900933) was used to block the membrane. The membrane was then incubated with the primary antibody for 16 h and subsequently incubated with the secondary antibody for 2 h. Pierce™ Fast Western Blot Kit (ECL Substrate) (Thermo 35055) was used to visualise the sample bands using a GE ImageQuant LAS 500 chemiluminescence CCD camera.

2.4. RT–qPCR

The concentrations of total RNA were determined by a NanoDrop™ One Microvolume UV–Vis Spectrophotometer. Two-step RT–qPCR was performed, cDNA was reverse transcribed with a High-Capacity RNA-to-cDNA™ Kit in an Eppendorf Mastercycler nexus X2 thermal cycler. The 0.5 μg cDNA template synthesised above was used for each PCR, and the amplification procedure was performed with innuMIX™ qPCR MasterMix Sygreen (Analytik Jena 845-AS-1300200) in Analytik Jena qTower 2.2. The real-time SYBR green signal was captured to evaluate the cDNA level. The primers for GCNT3 were synthesised by Sangon Biotech (Shanghai) Co., Ltd, the sequence of primers was as follows:

TACTTGTGACCTGCCCTTTAC (forward).
GTTTCCCTTCAGCACCTACA (reverse).

2.5. Cell culture and transfection

The A549 cell line was obtained from the Chinese Academy of Medical Sciences. After resuscitation, all the cells were cultured in DMEM (Gibco 12491015) containing 10% foetal bovine serum (Gibco 10100147) in a 37 °C incubator with 5% CO_2. Cell passaging was performed when the cellular fusion rate was nearly 60%–70%. Penicillin and streptomycin (Gibco 15140122) were added to the cell culture medium to prevent cell contamination, and PCR was performed to detect mycoplasma contamination.

Small interfering RNA (siRNA) was designed and synthesised by Sangon Biotech (Shanghai) Co., Ltd. SiRNA transfection was carried out when the fusion degree of the cells reached 60%–70%. Lipofectamine® RNAiMAX (Invitrogen 13778150) was used to transfect siRNA into cells. RNA was extracted 48 h after transfection, and protein was extracted 72 h after transfection. The siRNA sequences used in this study were as follows: GCUACUGCGAGCUGUGUAUTT (5′-3′; GCNT3) and UUCUCCGAACGUGUCACGUTT (5′-3′; negative control (NC)).

2.6. Cytological tests

Cytological assays were carried out with normal and GCNT3-knockdown A549 cell lines. Colony formation and MTT assays were used to evaluate cell proliferation. Colonies were stained with crystal violet in the colony formation assay. In the MTT assay, formazan was dissolved in DMSO, and the OD values were obtained by spectrophotometry at 492 nm. A wound-healing assay was performed to evaluate cell migration. Transwell assays were conducted to evaluate cell invasion, and 40 μl Matrigel® matrix (Corning 354234) was added to each Transwell chamber. All cytological experiments were carried out on an ultraclean platform.

2.7. Cell irradiation

After cell adhesion, 6 MeV electron accelerator radiation was used to treat the cells, and the dose rate was 2 Gy/min. The total dose was 1 Gy, and a cell colony count assay was performed 72 h after irradiation.

2.8. Statistical analysis

Statistical analyses were performed using SPSS 23.0. A t-test was used for normally distributed measurement data, while the rank-sum test was used for count data and non-normally distributed measurement data. The expression level was grouped into different groups by the cut-off value obtained by the area under the curve (AUC) method in MedCalc 20.0. Kaplan–Meier analysis was performed to evaluate the overall survival difference between different groups. The WB bands were quantified by ImageJ, and all bar graphs were obtained from SigmaPlot 12.5 software.

3. Results

3.1. Abnormally high GCNT3 expression was observed in lung adenocarcinoma tissues via a public database

Compared with normal tissues, increased GCNT3 mRNA levels were observed in cancer tissues in TCGA-LUAD database (Figure 1A). In the subgroup analysis, abnormally high GCNT3 expression levels were observed in cancer tissues in most age groups, all sex groups, all race groups and all TNM stage groups (Figure 1C–F). Moreover, elevated GCNT3 expression levels were observed in the CPTAC database at the protein expression level, which provided good verification of TCGA database results (Figure 1B).

3.2. Abnormal GCNT3 expression was observed in various kinds of tumours

Abnormal GCNT3 expression was observed in many kinds of tumours in the pancancer analysis. GCNT3 was highly expressed in some tumours, such as cholangiocarcinoma, lung squamous carcinoma, pancreatic adenocarcinoma, and uterine corpus endometrial carcinoma. However, in some other tumours, such as colon adenocarcinoma, head and neck squamous cell carcinoma, renal clear cell carcinoma, and rectum adenocarcinoma, GCNT3 expression levels were suppressed compared with those in normal tissues (Figure 2A–C).

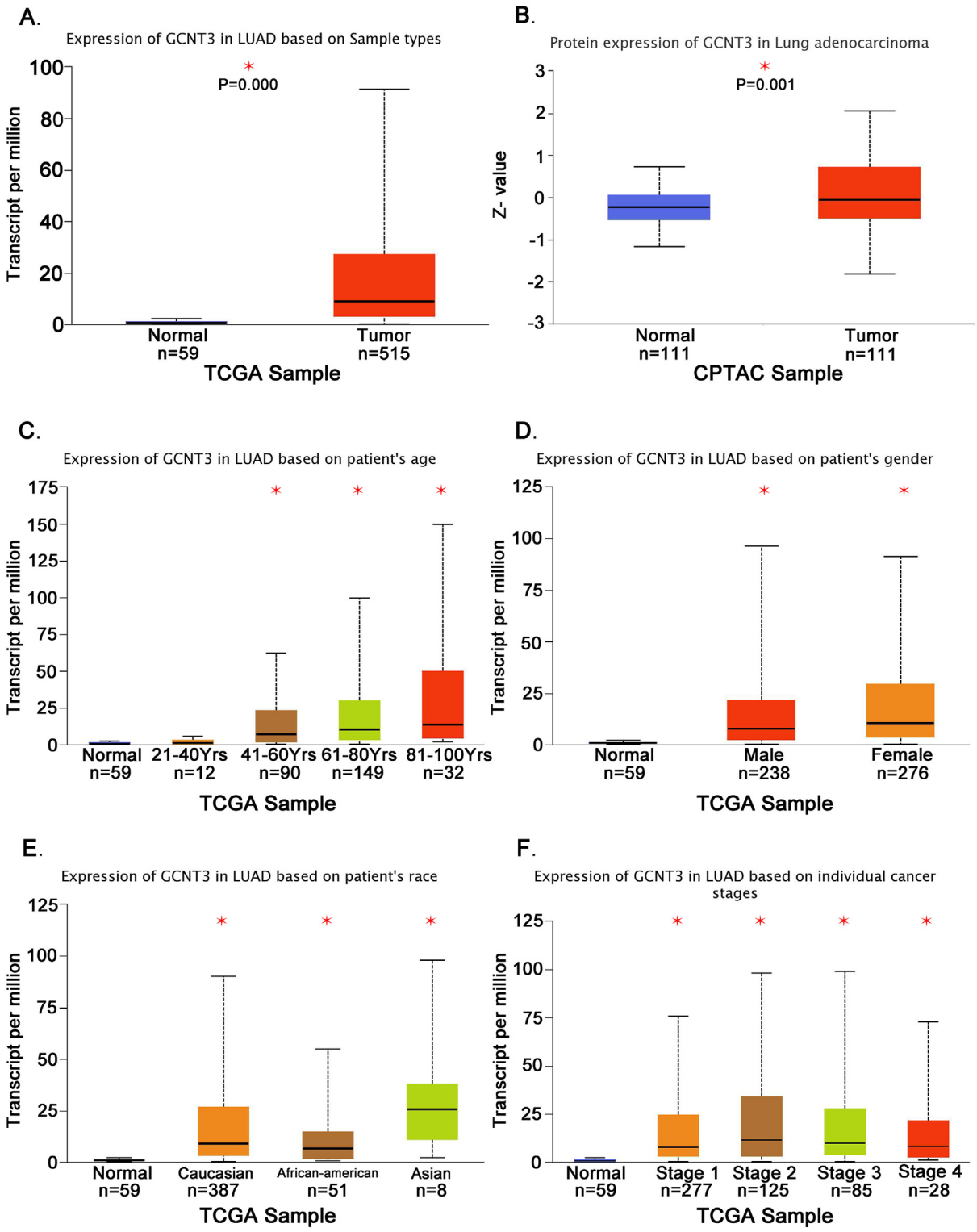

Figure 1. GCNT3 expression in public database. A: Expression of GCNT3 in TCGA-LUAD database. B: Protein expression of GCNT3 in CPTAC-LUAD database. C–F: Expression of GCNT3 based on different ages, genders, races and cancer stages in TCGA-LUAD database.

3.3. Among patients receiving radiotherapy, those with high GCNT3 levels had worse prognoses

The patients in TCGA-LUAD database were divided into a high expression group and a low expression group by ROC curve analysis based on GCNT3 expression levels. Kaplan–Meier survival analysis showed that there was no significant difference in overall survival between these two groups (Figure 2D). However, in the subgroup of patients who received radiotherapy, patients with high GCNT3 expression had significantly worse OS outcomes (Figure 2E), which was not observed in patients who did not receive radiotherapy (Figure 2F).

3.4. GCNT3 was highly expressed in clinical tumour samples

A total of 20 patients with lung adenocarcinoma were included in our study, and the clinical characteristic data are shown in Table 1. Similar

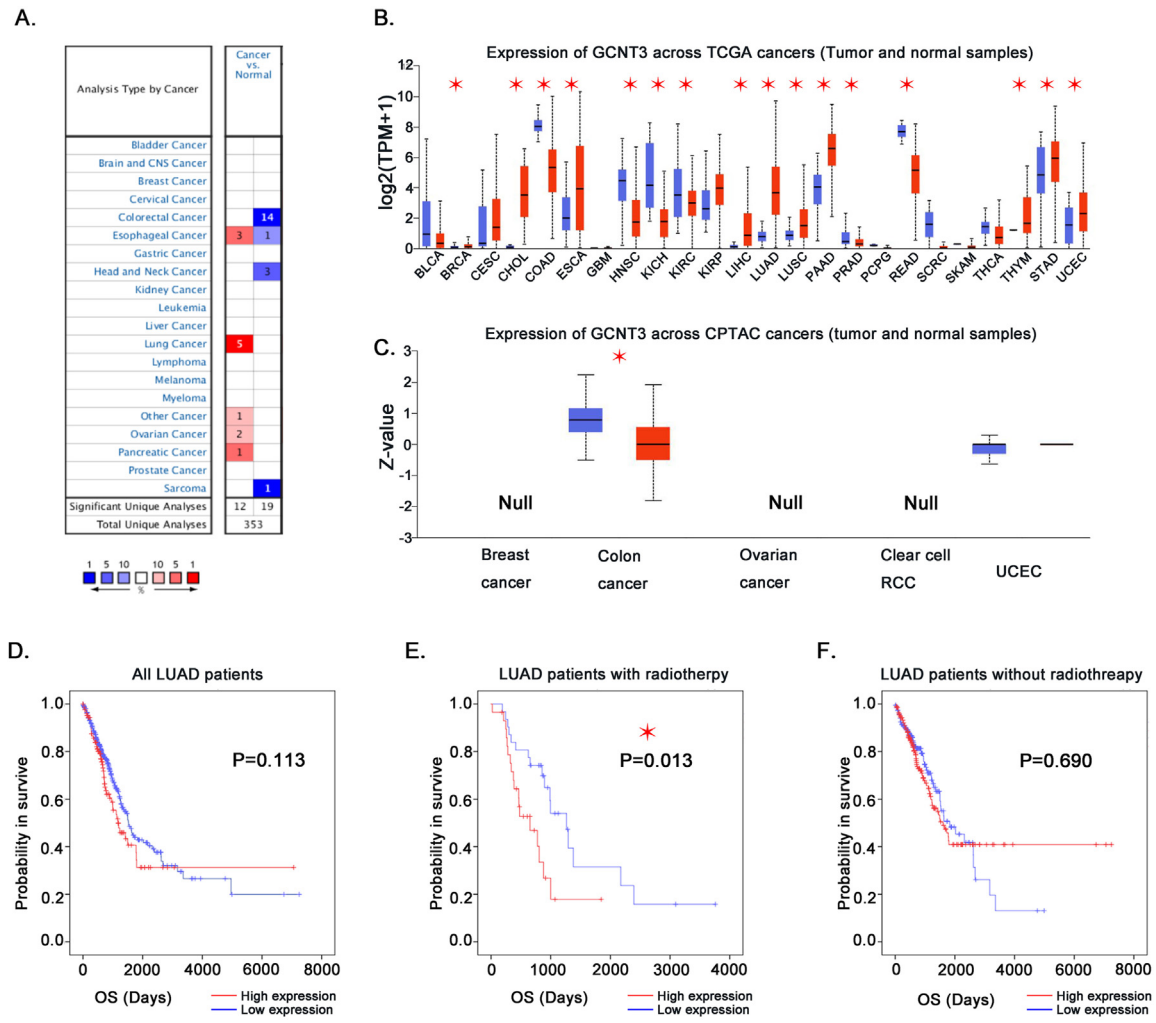

Figure 2. Pan-cancer analysis and survival analysis in lung adenocarcinoma patients. A–B: Pan-cancer analysis of GCNT3 expression in TCGA and GEO database. C: Pan-cancer analysis of GCNT3 expression in CPTAC database. D–F: Survival analysis in different GCNT3 expression groups in all patients, patients with radiotherapy and patients without radiotherapy.

Table 1. Clinical characteristic data.

Parameters		Number of Cases
Gender	Female	12
	Male	8
Age	≤60	8
	>60	12
TNM Stage	I	17
	II	2
	III	1
	IV	0

results were observed in our clinical verification: RT–PCR showed that compared with normal tissues (Figure 3C), the mRNA level was sharply increased in cancer tissues, and WB results showed significantly high expression of GCNT3 in cancer tissues (Figure 3A and B).

3.5. Downregulation of GCNT3 significantly inhibited the proliferation ability of the A549 cell line

A549 cells were selected to explore the effect of GCNT3 on the proliferation of lung adenocarcinoma, and siRNA was synthesised to interfere with GCNT3 expression. The results showed that compared with the normal A549 cell line, cells with GCNT3 knocked down had substantially decreased proliferation ability after siRNA transfection, both in the colony formation assay and MTT assay (Figure 4A and B).

3.6. Inhibition of GCNT3 expression significantly inhibited the migration and invasion abilities of the A549 cell line

Wound-healing and Transwell assays were performed on in siRNA-transfected cells and normal A549 cells to investigate the effect of GCNT3 on the migration and invasion abilities of lung adenocarcinoma cells. The results showed that after transfection, the wound healing rate was significantly slower (Figure 4C and D), and the number of GCNT3-knockdown cells passing through the Transwell membrane was significantly lower compared with the number of normal A549 cells (Figure 4E and F). The results indicated that migration and invasion were significantly inhibited when GCNT3 was knocked down.

3.7. The EMT process was affected after the inhibition of GCNT3 expression

The expression levels of EMT-related molecules were measured by immunoblotting to explore the role of GCNT3 in tumorigenesis and development. The results showed that the expression level of E-cadherin was sharply increased, while that of N-cadherin and vimentin was

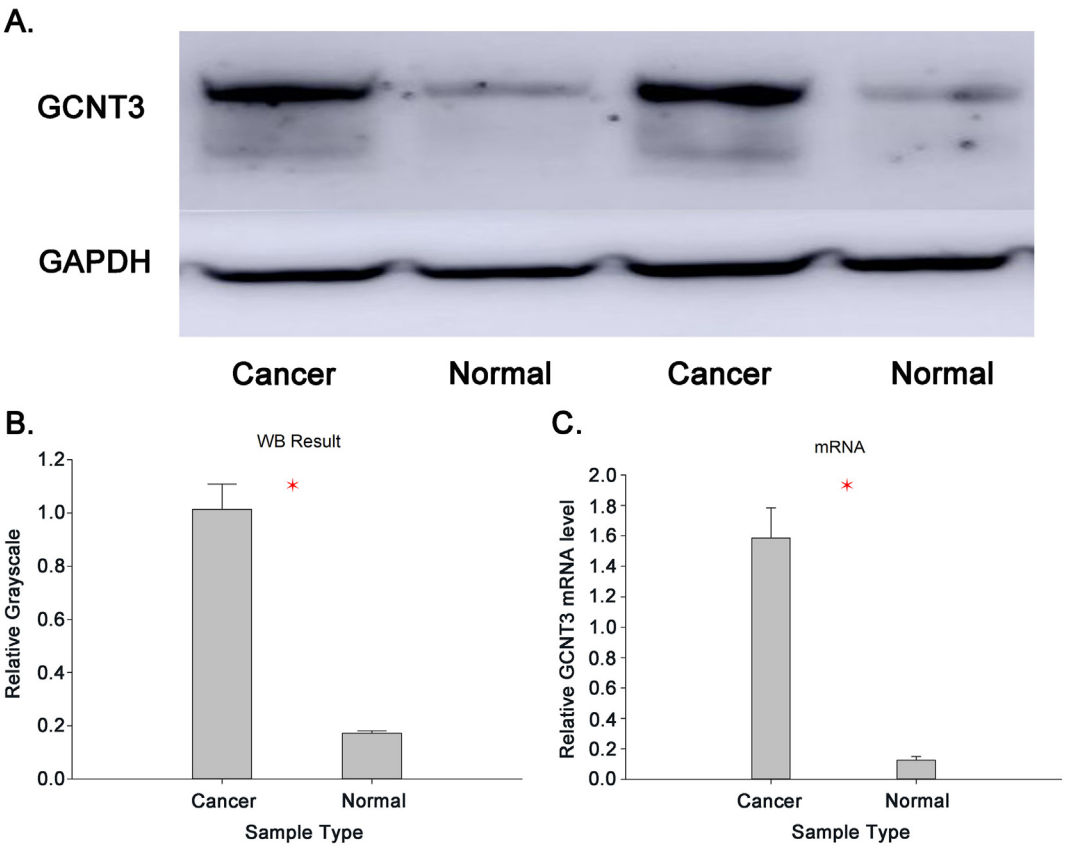

Figure 3. Clinical sample validation of GCNT3 expression. A–B: Protein expression of GCNT3 measured by western blot. C: MRNA level of GCNT3 measured by qRT-PCT.

decreased after siRNA transfection. The results indicated that the EMT process was blocked when GCNT3 expression was knocked down (Figure 5A and B).

3.8. Inhibition of GCNT3 expression significantly increased the sensitivity of A549 cells to radiotherapy

After the same time of cell culture, compared with normal A549 cell, the proliferation of cells receiving irradiation was reduced, and the proliferation of GCNT3 knocked down cell without irradiation was also reduced. Compared with these cells, seldom cell could be observed in GCNT3 knocked down cell after irradiation, which indicated that the proliferation ability of this group was significantly damaged (Figure 5C). Similar results were observed through the OD value curve of MTT assay (Figure 5D).

4. Discussion

The management of lung adenocarcinoma patients should follow the principle of individualised treatment. For patients with advanced lung adenocarcinoma, surgical resection can no longer provide sufficient survival benefits, and driver gene mutation-oriented targeted therapy, immune checkpoint inhibitor therapy, chemotherapy and radiotherapy become the mainstream treatment methods to control tumour progression [8]. However, some patients show low sensitivity to radiation, and it is difficult to obtain survival benefits from radiotherapy [9]. Therefore, it is critical to explore new mechanisms and targets for radiotherapy resistance in lung adenocarcinoma [10]. In our study, the role of the GCNT3 gene in tumorigenesis, tumour development and radiation resistance was explored.

Public databases were used to evaluate the expression levels in different tissues and the survival status of LUAD patients. The results showed a clear abnormal increase in GCNT3 expression levels in tumour tissues compared with those in normal tissues. The pancancer analysis revealed that the GCNT3 expression status varied significantly among different tumour types. In some malignancies, GCNT3 expression levels were significantly higher than that in normal tissues, while in other kinds of tumours, the expression levels of GCNT3 were significantly decreased. GCNT3 might play different roles in different kinds of tumours, such as in castration-resistant prostate cancer, Daiki Yamamoto et al have observed that cell proliferation was significantly decreased by GCNT3 knockdown while cell migration of these cells was significantly increased [11]. Sumardika et al have found that GCNT3 is overexpressed in highly metastatic melanomas. Silencing and functional inhibition of GCNT3 greatly suppressed migration and invasion of melanoma cells [12]. In lung adenocarcinoma, abnormally high expression of GCNT3 indicated that GCNT3 might play a promoting role in lung cancer cells, which was explored in subsequent cytological experiments.

In order to verify the expression of GCNT3 in the Asian population, clinical samples were collected from surgical resection, and WB experiment was performed. Result, revealed that GCNT3 expression levels in lung adenocarcinoma tissues were significantly higher than that those in normal tissues. This result indicates that high expression of GCNT3 is present in the Asian population, in accordance the results available in the public database. All the tumour and normal tissues were collected from surgical resection, the TNM stages of these patients were before IIIA, thus the relationship between the efficacy of postoperative adjuvant therapy and GCNT3 is still unclear, what's more, due to the short study time, there were no statistical data on the overall survival of the clinical

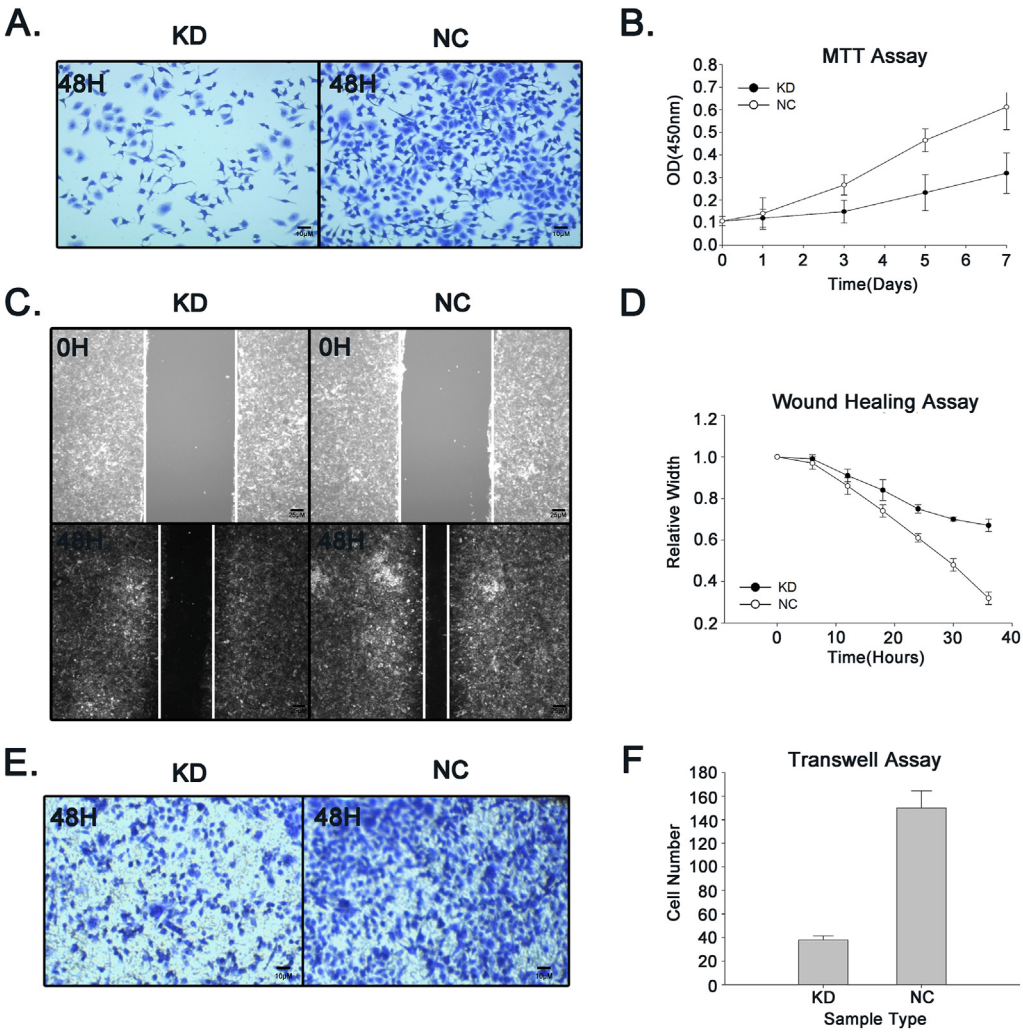

Figure 4. Inhibition of GCNT3 expression significantly inhibited the proliferation, migration and invasion abilities of the A549 cell line. A–B: Colony formation assay and MTT assay showed the proliferation of the A549 cell line. C–D: Wound-healing assay showed the migration of the A549 cell line. E–F: Transwell assay showed the invasion of the A549 cell line.

patients, which will be explored in the future study with larger sample size and more advanced cancer cases.

SiRNA was transfected to knock down the expression level of GCNT3 in a lung adenocarcinoma cell line to verify the promoting effect of GCNT3 on lung adenocarcinoma. The results showed a great promoting effect of GCNT3 on the proliferation, migration and invasion capabilities of cancer cells. However, the exact regulatory pathway of GCNT3 on tumour cells is still unclear nowadays, integrated transcriptomic and proteomic analyses reveal that GCNT3 is linked to cellular cycle, mitosis and proliferation, response to drugs and metabolism pathways in some kinds of tumours [2]. In our study, the relationship between GCNT3 and EMT process was studied. It is known that the EMT process is a central factor affecting the biological behaviour of tumour cells [13]. Our results revealed a significant inhibitory effect on the EMT process after GCNT3 knockdown, indicating that GCNT3 may regulate the proliferation, invasion and migration capabilities of LUAD cells by participating in the EMT process and affecting the occurrence and development of lung adenocarcinoma. Qian et al have studied the microRNA upstream of GCNT3, the results showed that miR-302b-3p can reduce the expression of GCNT3, thereby inhibiting the proliferation, migration and invasion of tumour cells. In our study, similar results was observed through siRNA interference, and similar effect of EMT process was verified [4].

Radiotherapy is an effective treatment for unresectable lung cancer [14] that plays an important role in the clinical management of lung cancer patients. However, not all patients can benefit from radiotherapy because of cancer cell resistance [15]. In our study, significant OS outcome differences were observed between different GCNT3 expression groups after radiotherapy. Compared with the low GCNT3 expression group, patients with high GCNT3 expression had shorter OS times. A 2 Gy/min radiation exposure was performed in GCNT3-knockdown and normal A549 cell lines to explore the role of GCNT3 in tumour radiotherapy. The results showed that GCNT3 knockdown promoted sensitivity to radiation exposure, which decreased the proliferation ability of the cells after exposure. This result provides us with the following new idea in radiotherapy: GCNT3, as a potential biomarker, can predict the curative effect of radiotherapy in lung adenocarcinoma patients. Moreover, GCNT3 is a potential therapeutic target, and GCNT3 inhibitors could improve tumour sensitivity to radiotherapy. A novel small molecule GCNT3 inhibitor, talniflumate, was recently reported to disrupt mucin biosynthesis and malignant cellular behaviours in pancreatic cancer [16]; however, its role in lung adenocarcinoma was unclear. This molecule has great potential for preventing radiotherapy resistance and tumour progression and deserves to be further investigated in the future.

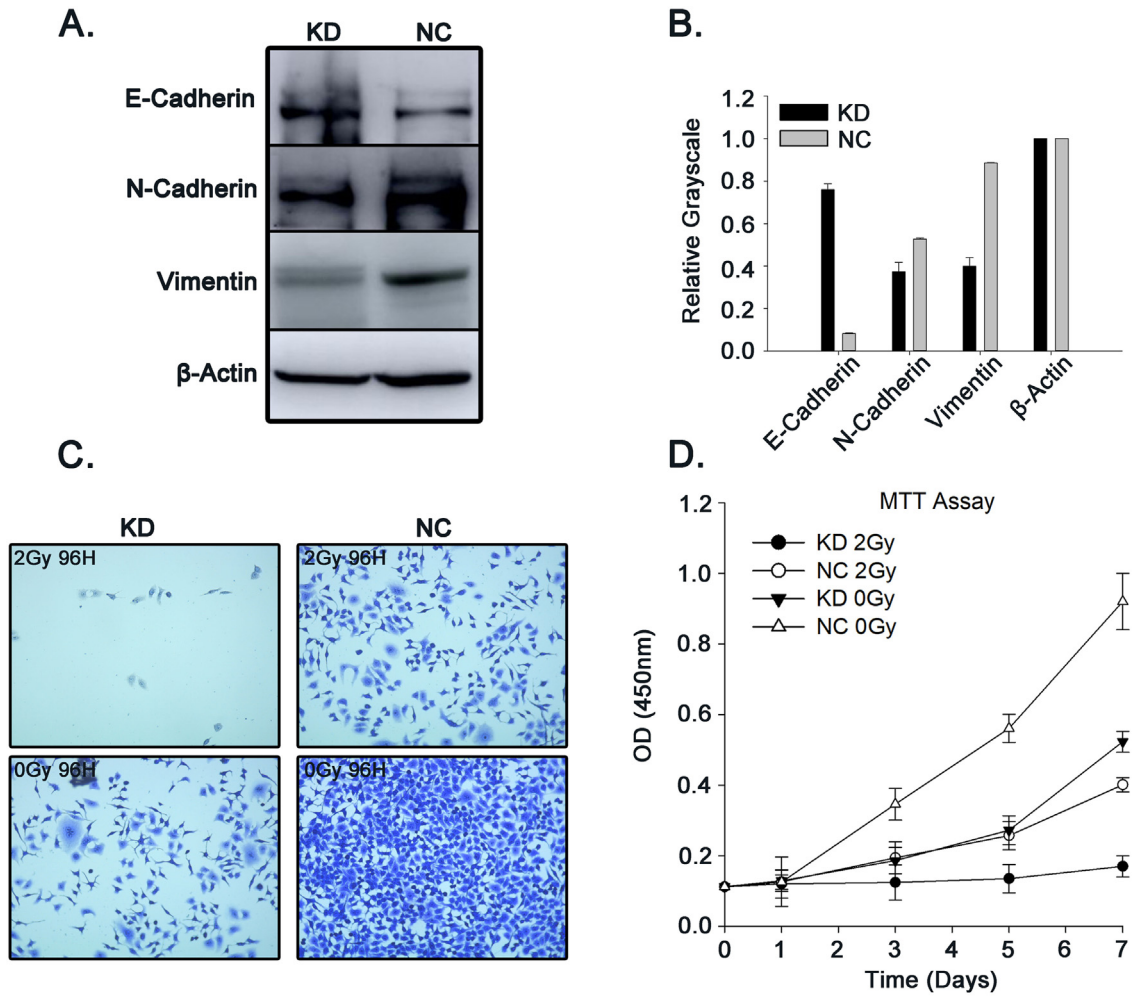

Figure 5. The EMT process was regulated after the inhibition of GCNT3 expression, and the inhibition of GCNT3 expression significantly increased the sensitivity of A549 cells to radiotherapy. A–B: the EMT process related protein measured by western blot. C: cell status of GCNT3 knocked down and normal A549 cell with or without 2Gy irradiation. D: MTT assay of GCNT3 knocked down and normal A549 cell with or without 2Gy irradiation.

Declarations

Author contribution statement

Teng Zhao: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Xin Zhao: Performed the experiments; Analyzed and interpreted the data.

Kun Qian, Kejian Shi: Analyzed and interpreted the data.

Yanfei Gu: Conceived and designed the experiments.

Yi Zhang: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Results in this study are based upon data generated by the TCGA Research Network: https://www.cancer.gov/tcga.

The data used in this study can be acquired for free via:

1. UCSC XENA browser (https://xenabrowser.net/)
2. The Human Protein Atlas database (https://www.proteinatlas.org/)
3. UALCAN browser (http://ualcan.path.uab.edu/analysis.html)

Declaration of interest's statement

The authors declare no competing interests.

Additional information

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