Introduction

Neuritin, also known as candidate plasticity-related gene 15, is a novel neurotrophin discovered by Nedivi et al. (1). It is an activity-induced glycosylphosphatidylinositol-anchored axonal protein, mainly expressed in the brain (2,3). Neuritin has been proven to have a profound effect in the nervous system by promoting neurite outgrowth and dendritic growth, regulating synaptic plasticity, promoting synaptic maturation and neuronal migration, regenerating peripheral nerves and spinal axons, and regulating proliferative neuron apoptosis (3-13). However, information on the functions of neuritin in other organs is scarce.

Numerous studies have revealed that neuritin is also expressed in the liver, lungs, and skeletal muscle (4,14,15), suggesting a possible role in non-nervous system organs. Furthermore, neuritin overexpression has been reported in some tumour cells and in relation to the promotion of tumour angiogenesis (16). Neuritin was also highly upregulated in hypoxic cells (17-19) and was shown to inhibit cell apoptosis (7,11,20). Overall, these studies suggested that neuritin may not function exclusively as a neurotrophin in the nervous system, and may also play crucial roles in tumourigenesis.

Cervical cancer, endometrial cancer, oesophageal cancer, and lung squamous cell carcinoma (LUSC) are the most common cancers affecting people worldwide (21-24). More effective methods of detecting early stage cancers
are urgently required to improve their diagnosis and treatment. In this study, we initially investigated neuritin immunoreactivity in lung, cervical, endometrial, and oesophageal cancers. Based on these results, we further examined neuritin protein and gene expression in LUSC tissues by immunohistochemistry (IHC) and reverse transcription-polymerase chain reaction (RT-PCR), and evaluated the relationships between neuritin expression and clinical features in patients with LUSC.

Methods

Patients and tissue specimens

We obtained tissues from 30 patients with LUSC, five patients with cervical cancer, five with endometrial cancer, and five with oesophageal cancer for investigation of neuritin expression. All patients had histologically confirmed cancer with no history of any other cancers, and all patients underwent resection at the First Affiliated Hospital of Shihezi University School of Medicine (Shehezi, Xinjiang, China). None of the patients had received any preoperative anticancer treatment. Data on clinicopathological parameters, including sex, age, tumour stage, lymph node metastasis, and histological grade were obtained from hospital and surgical records. The present study was approved by the Human Ethics Committee of the First Affiliated Hospital of Shihezi University School of Medicine (No. 2007-025-01), and written informed consent was obtained from all patients according to the committee's regulations.

IHC

IHC was conducted using paraffin-embedded tumour tissues, and matched normal tissues obtained from the autopsy files of the Department of Pathology, the First Affiliated Hospital of Shihezi University School of Medicine. IHC staining was performed using a ChemMate™ Dako EnVision™ Detection Kit (GK500705, Dako, Glostrup, Denmark) according to the manufacturer’s instructions. The specimens were cut into 4-µm sections, unfolded on the surface of water at 25 °C, and then laid on polysine adhesion slides overnight at 60 °C. The slides were deparaffinized with dimethylbenzene and rehydrated through a graded series of ethanols (100%, 95%, 90%, 80%, and 70%). After washing three times with phosphate-buffered saline (PBS), the slides were boiled in antigen retrieval buffer (0.01 M sodium citrate-hydrochloric acid, pH 6.0) for 10–15 min in a microwave oven at 95–97 °C. Endogenous peroxidase was blocked by incubation with 3% peroxide for 10 min followed by three additional rinses in PBS for 5 min each. Nonspecific binding was blocked by incubation with bovine serum albumin. The sections were then incubated with primary polyclonal rabbit anti-neuritin (FL-142) antibody (sc-25651, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight, followed by PBS, and subsequently incubated with ChemMate™ EnVision™/HRP (Dako) at room temperature for 30 min. After further washing in PBS, the sections were developed using 3,3′-diaminobenzidine, washed under running tap water, lightly counterstained with haematoxylin, dehydrated, and mounted with coverslips. Negative control experiments were conducted by replacing the primary antibody with PBS, and cerebellum tissue was used as a positive control. All the slides were reviewed by experienced pathologists. Neuritin immunostaining was scored by multiplying the intensity score (0= negative, 1= weak, 2= moderate, and 3= strong) by the percentage of positively stained cells (0=0%; 1=1–33.3%; 2=33–66.6%; and 3=66.6–100%). Neuritin expression was considered positive when the IHC score was ≥2.

RNA extraction and RT-PCR

RNA was isolated from fresh tissue samples obtained from patients in the First Affiliated Hospital of Shihezi University School of Medicine within 30 min after resection, and immediately stored in liquid nitrogen until use. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. RNA integrity was verified by 1.5% agarose gel electrophoresis and the quality and concentration of the RNA at 260 and 280 nm were determined using a UV spectrophotometer. The concentration was validated using the formula RNA (µg/mL) = OD_{260} ×40 µg/mL × dilution ratio. Total RNA (1 µg) was reverse-transcribed in a 25-µL reaction volume (containing 12 µL RNase-free dH2O, 5 µL One Step RNA PCR Buffer, 5 mM MgCl₂, 1 mM dNTP mixture, 0.4 µM P1, 0.4 µM P2, 0.8 U/µL RNase inhibitor, 0.1 U/µL AMV RTase XL, and 0.1 U/µL AMV-Optimized Taq) using a One Step RNA PCR Kit (AMV) (RRO24A, TaKaRa, Ohtsu, Shiga, Japan) according to the manufacturer’s instructions. The reverse transcription reactions were performed in one cycle of 50 °C for 30 min and 94 °C for 120 s, followed by PCR for 30 cycles of 94 °C
for 30 s, 64 °C for 30 s, and 72 °C for 90 s. Neuritin gene amplification was carried out using the reverse primer P1 (5’-CCGGAATTCATGGGACTTAGTTGAACGGCAG ATAT-3’) and forward primer P2 (5’-CGCGGATCCGAAGGAAAGCCAGGTCGCTAAAGCT-3’). Neuritin expression levels were normalized to values for the endogenous reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analysis

Statistical analysis was performed using the SPSS statistical software package (SPSS version 17.0; SPSS, Chicago, IL, USA). Differences between groups were analysed using Fisher’s exact test and the χ² test. A P value of <0.05 was regarded as statistically significant.

Results

Neuritin protein expression profiles in tumour tissues and corresponding normal tissues

Neuritin protein expression was detected in human cervical cancer, endometrial cancer, oesophageal cancer, and LUSC tissues and in their corresponding normal tissues (Figure 1). Five samples were obtained from each specimen. Immunoreactivity for neuritin was located in the cytoplasm. Cervical cancer, endometrial cancer, and LUSC tissue specimens showed moderate or strong immunostaining for neuritin, whereas oesophageal cancer tissues showed relatively weaker expression. Neuritin staining was weakly positive in normal cervical, endometrial, and oesophageal tissues but negative in normal lung tissue. Neuritin expression thus clearly differed between normal lung tissue and LUSC tissue.

Patient characteristics

Specimens from 30 patients with LUSC were subjected to IHC analysis of neuritin expression. The relationships between neuritin protein expression and clinicopathologic parameters are shown in Table 1. The database on which these analyses were based was created by the International Association for the Study of Lung Cancer, to inform revisions to lung cancer guidelines in the seventh edition of the Union for International Cancer Control TNM Classification of Malignant Tumor staging manual. The 30 patients included 21 men, and the male-to-female ratio was 7:3. The median age was 56 years (range, 37–72 years) and 14 patients (46.7%) were >60 years old. Eighteen patients (60.0%) had advanced clinical TNM stage (stage III–IV) disease.

Association between neuritin protein expression and clinical TNM stage in human LUSC

Neuritin protein expression was determined by IHC in 30 human LUSC tissues and 20 normal lung tissue samples. Representative images are shown in Figure 2. Among the 30 squamous cell carcinoma tissue specimens, five (16.7%) were scored as 0 to 1+, 14 (46.7%) as 2+ to 4+,
and 11 (36.7%) as 6+ to 9+. Among the 20 normal lung tissue specimens, 15 (75.0%) were scored as 0 to 1+ and only five (25.0%) were scored as 2+. Accordingly, 25 of the 30 squamous cell carcinoma tissue specimens (83.3%) were positive for neuritin expression compared with five of the 20 normal lung tissue specimens (25.0%), with all the other specimens testing negative for neuritin expression (Figure 2). The neuritin-positivity ratio was significantly higher among squamous cell carcinoma tissues compared with normal lung tissues (P<0.001). The positivity ratio was also significantly lower in patients with higher clinical TNM stage (I–IV, P=0.049), and in patients with distant metastasis of LUSC compared with those without (P=0.023). However, there was no association between neuritin positivity and patient age, sex, local lymph node metastasis, or histological grade (Table 1).

**Neuritin gene expression in human LUSC and normal lung tissues by RT-PCR**

We confirmed the difference in neuritin expression between LUSC tissues and normal lung tissues by RT-PCR. A DNA fragment of the expected size for neuritin (446 bp) was amplified by agarose gel electrophoresis of DNA from LUSC specimens, but not from normal lung tissue specimens (Figure 3).

**Discussion**

Neuritin is a neurotrophic factor with multiple roles in neural development and plasticity (9,12,25-28). However, some recent reports have also shown an association between neuritin and cancers, including upregulation of neuritin in Kaposi’s sarcoma, astrocytoma, and glioma cell lines, and in gastric cancer tissues and melanoma patients (20,29-31). These findings suggest that neuritin may be related to tumour development.

We initially examined the previously unreported expression of neuritin in common human cancers, including cervical, endometrial, and oesophageal cancer, and in LUSC. Expression levels of neuritin were higher in carcinoma tissues than in the corresponding normal tissues, with a particularly marked difference in between LUSC and normal lung tissues. We therefore detected neuritin protein and mRNA expression in 30 LUSC tissues and 20 normal lung tissues. We therefore speculated that neuritin may participate in the development of LUSC.

We also investigated the clinical value of neuritin in LUSC by analysing its expression in relation to various clinical features in patients with LUSC. Neuritin positivity was significantly lower in patients with higher grade tumours, and in patients with distant metastasis, suggesting that high expression levels of neuritin may be negatively correlated with the degree of malignant differentiation and
The current results showed that neuritin expression levels were higher in LUSC than in normal lung tissue, but were negatively correlated with the degree of malignant differentiation and distant metastasis. However, these apparently contradictory findings were supported by other studies. Overexpression of neuritin significantly increased microvascular density (16,17,20), and injection of NIH3T3 cells transfected with neuritin into the abdomen of nude mice resulted in the formation of abdominal tumours (25). However, neuritin also induced mesenchymal stem cells to differentiate into neuron-like cells (32), and ectopic expression of neuritin in NIH3T3 cells resulted in changes in cell morphology and increased protuberance (25).

The present study had some limitations. We only used samples from lung squamous carcinoma, and not other types of lung cancer. In addition, our results need to be validated in a larger and more diverse patient population.

**Figure 2** Neuritin protein expression levels in LUSC tissues and normal lung tissues using IHC analysis. Representative IHC images of LUSC tissues and corresponding normal lung tissues using anti-neuritin antibody (1:50 dilution). Scale bar, 100 µm. (A) Positive neuritin immunoreactivity was mainly localized in the cytoplasm in LUSC tissue. (B) Neuritin expression in normal lung tissue. (C, D) Neuritin-positivity ratios in LUSC tissues and normal lung tissues ($\chi^2=17.014$, $P<0.001$).

**Figure 3** RT-PCR analysis of neuritin expression in human LUSC tissues and corresponding normal lung tissues. Lane 1: positive control; lanes 2 and 3: LUSC tissues; lane 4: normal lung tissues. Neuritin expression was normalized to GAPDH.
validated in a larger sample size in future.

Lung cancer is one of the most common causes of death worldwide (24), and the diagnosis and prognosis of LUSC, as the most common type of lung cancer, is thus of great significance. The current results suggested that neuritin may have clinical value as a novel diagnostic and prognostic marker in patients with LUSC.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The present study was approved by the Human Ethics Committee of the First Affiliated Hospital of Shihezi University School of Medicine (No. 2007-025-01), and written informed consent was obtained from all patients according to the committee's regulations.

References

18. Han Y, Chen X, Shi F, et al. CPG15, a new factor upregulated after ischemic brain injury, contributes to neuronal network re-establishment after glutamate-


