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Original paper

# Clinical significance of nuclear localisation of agrin in lung adenocarcinoma

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Agrin has recently been identified as a novel oncogene that is overexpressed in several types of human cancers. However, its role in lung cancer has not yet been investigated. The purpose of the current study was to investigate agrin protein expression in lung cancer and evaluate its clinicopathological and prognostic significance. In this study, A total of 86 lung adenocarcinoma samples paired with adjacent non-tumour tissue samples and eight lung adenocarcinoma non-paired samples were selected for immunohistochemical staining for agrin. Strong staining of agrin in nuclei of lung adenocarcinoma tissues was observed, but not in the nuclei of normal lung tissues ($p < 0.001$). Consistent with staining in lung adenocarcinoma tissues, the nuclei staining of agrin was also detected in lung cancer cell lines by immunofluorescence. This is the first report demonstrating that agrin is highly expressed in nuclei of lung adenocarcinoma tissues and that it is strongly correlated with lymph node metastasis ($p = 0.002$), clinical stage ($p = 0.024$), and poor differentiation ($p = 0.022$). Agrin-positive nuclear staining of lung adenocarcinoma cells could be used to identify greatly increased risk of metastasis in patients after surgery, which might serve as a valuable prognostic marker.

**Key words:** agrin, lung adenocarcinoma, immunohistochemistry, lymph node metastasis, differentiation.

## Introduction

Lung cancer is one of the most commonly diagnosed malignancies and the leading cause of cancer death in both sexes combined worldwide [1]. According to a report by Bray *et al.* in 2018, the incidence and mortality rates of lung cancer remain elevated [2]. However, available regimens for lung cancer include surgery, radiotherapy, and chemotherapy, and the combination of these regimens has slightly improved the overall survival [3]. Non-small cell lung cancer (NSCLC) comprises about 85% of all diagnosed lung cancer, and lung adenocarcinoma (LAC) is the most common subtype of NSCLC [4]. The remarkably heterogeneous nature of LAC has become more apparent over the last decade [5]. The development of LAC is a combination of multifactorial, multistage, and multiple genetic alteration processes [6]. Hence, novel diagnostic markers and pathways that may be targeted for developing new LAC therapies are critically needed.

Agrin, a member the heterogeneous family of heparan sulphate proteoglycans, is characterised by its role in the development of the neuromuscular junction during embryogenesis [7, 8, 9, 10]. Recent studies have shown that agrin is aberrantly overexpressed in human cancer tissues and highly associated with poor clinical prognosis, including hepatocellular carcinoma, colorectal cancer, and oral cancer [11, 12, 13]. Subsequent studies show that the high expression of agrin in oral squamous cell carcinoma is important for its role on cell migration, adhesion, and

cisplatin cell resistance [14]. Moreover, in hepatocellular carcinoma, Chakraborty et al. showed that agrin enhances cellular proliferation, migration and oncogenic signalling in a manner dependent on LRP4 and MuSK [15]. The further study recently published in "Cell Reports" reveals that agrin acts as a mechanotransduction signal to promote liver cancer development by activation of YAP [16]. However, thus far, the expression of agrin and its role in lung cancer remain largely unknown.

Here, we aim to investigate agrin expression in 94 primary operable lung adenocarcinoma cases, and evaluate its clinicopathological significance and prognostic impact in LAC.

## Material and methods

### Sample selection

Data from 94 patients who underwent surgical resection for lung adenocarcinoma were obtained in consultation with the surgeon and pathologist at the Huzhou Central Hospital (Zhejiang, China) between July 2004 and June 2009. All the patients' tissues with a histopathological diagnosis of lung adenocarcinoma according to the 2004 World Health Organisation classification were used in this study, including acinar, papillary, bronchioloalveolar carcinoma, or solid with mucin or mixed types of these patterns. The seventh edition of the TNM Classification of the Union for International Cancer Control.

Complete baseline and follow-up data were available for all the 94 patients, after excluding patients with missing information those with follow-up loss and those who received any chemotherapy or radiation treatment prior to the surgery. All pathologic specimens were independently reviewed by two pathologists. The detailed information of lung cancer patients is listed in Table I. This study was approved by the Ethics Committee of Huzhou Central Hospital, and all participants provided informed consent.

### Immunofluorescence staining

NSCLC cell lines (H1299, H1975, and H520) were grown on coverslips to 70-80% confluence. Cells were then washed with PBS, fixed with 3% paraformaldehyde for 10 min, and permeabilised with buffer containing 0.5% Triton X-100 for 5 min. Cells were incubated with rabbit anti-agrin antibody (Abcam; 1:1000) for 20 min at room temperature. After three five-minute washes with PBS, secondary antibody was added at room temperature for 20 min. Cells were then stained with DAPI to visualise nuclear DNA. Images were captured with use of a fluorescence microscope (DM3000; LEICA).

### Tissue microarray block construction and immunohistochemistry

The tissue microarray (TMA) was generated and consisted of 94 lung adenocarcinoma tissues. All tissues were confirmed by reviewing haematoxylin and eosin (HE)-stained slides. One representative tissue core, 2 mm in diameter, was carefully chosen from each formalin-fixed, paraffin-embedded archival block and subsequently re-arranged into recipient paraffin blocks. The sections of tissue microarrays (4 $\mu$m) were deparaffinised using xylene and rehydrated through graded alcohols. TMA blocks were heated in a high-pressure cooker with 10 mmol/l of citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked by treating with 0.3% $H_2O_2$ in methanol for 30 min at room temperature. Afterwards, TMA sections were treated with a rabbit polyclonal antibody targeting agrin (Abcam; 1:5000) in a humidified chamber at 4°C overnight. The sections were washed in PBS, incubated with secondary antibodies, and then incubated with Vectastain ABC reagent for 30 min.

The slides were assessed by two pathologists who were blinded to the patients' clinicopathologic information. Based on the staining intensity and staining extensity of positive cells, a semi-quantitative counting method was used to score agrin expression as follows: for staining extensity (0 = 9% or less of cells stained positive; 1 = 10-25%; 2 = 26-50%; 3 = 51-80%; and 4 = 81% or more); the staining intensity was also evaluated and graded from 1 to 3 (0 = negative, 1 = weak, 2 = moderate, and 3 = strong). The final score was calculated by adding the strongest intensity score and the total extensity score (maximum value of 6). We defined a sample as high agrin expression when the sample had a combined score for staining intensity and percentage of positive cells greater than 3, and a sample was low agrin expression if the combined score was less than 4.

### Statistical analysis

All comparisons were analysed using SPSS version 18.0 (IBM, Chicago, IL, USA), and differences were considered to be statistically significant at $p < 0.05$. Using Pearson's $\chi^2$ test, associations between agrin expression level and the patient's clinicopathological parameters were assessed.

## Results

To determine the prognostic significance of agrin expression in human lung adenocarcinoma, we analysed the agrin protein levels in lung adenocarcinoma specimens and paired adjacent normal tissue using a large tissue microarray. Positively stained cells displayed yellow, buffy, and brown granules in extracellular

**Table I.** Association of agrin expression in nuclei with clinicopathological parameters in 94 lung adenocarcinomas specimens

| Variables | Case | Agrin protein expression level | | p-value |
|---|---|---|---|---|
| | | Low (n = 41)[a] | High (n = 53)[a] | |
| Age (year) | | | | |
| ≤65 | 61.00 | 24 | 37 | 0.256 |
| >65 | 33.00 | 17 | 16 | |
| Gender | | | | |
| Male | 51.00 | 19 | 32 | 0.176 |
| Female | 43.00 | 22 | 21 | |
| Tumor size (cm) | | | | |
| ≤ 3 | 34.00 | 15 | 19 | 0.941 |
| >3 | 60.00 | 26 | 34 | |
| Differentiation | | | | |
| Well | 28.00 | 18.00 | 10.00 | **0.022** |
| Moderate | 23.00 | 9.00 | 14.00 | |
| Poor | 38.00 | 11.00 | 27.00 | |
| TNM stage | | | | |
| I/II | 51.00 | 27.00 | 24.00 | **0.024** |
| III/IV | 38.00 | 11.00 | 27.00 | |
| T stage | | | | |
| T1 | 19.00 | 9 | 10 | 0.341 |
| T2 | 52.00 | 25 | 27 | |
| T3/4 | 23.00 | 7 | 16 | |
| LN metastasis | | | | |
| Negative | 39.00 | 23 | 16 | **0.002** |
| Positive | 62.00 | 17.00 | 35.00 | |

[a] *In each case, the intensity (0 = negative, 1 = weak, 2 = moderate and 3 = strong) and extensity (0 = 9% or less of cells stained positive; 1 = 10-25%; 2 = 26-50%; 3 = 51-80%; and 4 = 81% or more) of tumor nucleus staining were evaluated. The final score was calculated by adding up the strongest intensity score and the total extensity score. (maximum value of 6). We defined a sample as high agrin expression when the sample had a combined score for staining intensity and percentage of positive cells greater to 3, and a sample low agrin expression if the combined score was less than 4.*

matrix (ECM), cytoplasm, and nuclei (Fig. 1). In normal tissue, positive agrin staining was predominantly localised to the cytoplasm and ECM and a few small parts in nuclei (6.97%, 6/86). The rate of positive agrin expression was significantly higher in nuclei of lung adenocarcinoma tissues (86.1%, 81/94) than that in paired normal tissues ($p < 0.001$, Fig. 3A). Consistent with staining in lung adenocarcinoma tissues, the nuclei staining of agrin was also detected in H1299, H1975, and H520 cell lines by immunofluorescence (Fig. 2).

To evaluate the role of agrin in lung adenocarcinoma progression, the clinicopathological features of patients and the correlation with agrin expression was analysed, as seen in Table I. In total, 94 patients (51 men and 43 women; mean age = 62.1 years, median age = 64 years) with diagnosed primary lung adenocarcinoma were included in this study; 34 patients with small tumour size and 60 patients with large tumour size. Fifty-one cases had well or moderately differentiated tumour, and 38 had poorly differentiated. At the time of diagnosis, there were 62 patients showing signs of lymph node metastasis. Statistical analysis indicated that high agrin expression in nuclei of lung adenocarcinoma patients' tissues was significantly associated with lymph node metastasis ($p = 0.002$, Fig. 3B), clinical stage ($p = 0.024$, Fig. 3C), and poor differentiation ($p = 0.022$, Fig. 3D), but it was not related to gender, age, smoking status, histological type, or tumour size ($p > 0.05$, Table I). The rate of strongly positive Agrin expression was also higher in the lung adenocarcinoma patients with LN metastases (68.6%, 35/51) than in those without metastases (31.3%, 16/51; $p = 0.002$; Table I and Fig. 3B). Moreover, the rate of strongly positive agrin expression in nuclei was significantly higher

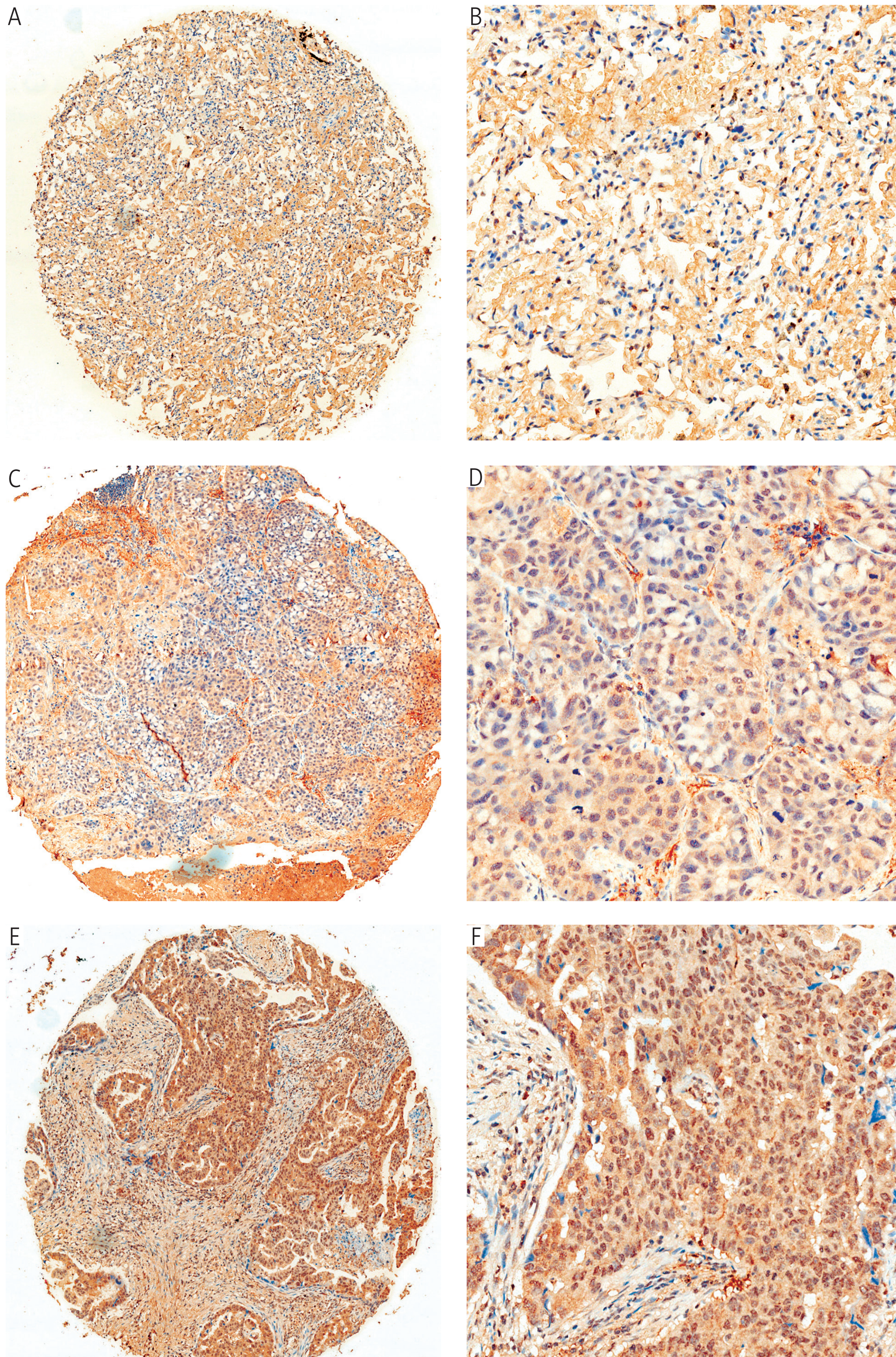


**Fig. 1.** IHC staining for agrin protein expression in lung tissues. Representative microphotographs of agrin immunohistochemical staining in lung tissues. Positive staining of agrin is shown in brown. (A and B) The normal lung tissues were negative for agrin protein in nuclei. (C and D) The lung adenocarcinomas were weakly positive for agrin in nuclei. (E and F) The agrin protein exhibited strongly positive staining in the nuclei of lung adenocarcinomas (B, D, F, and H), indicating higher magnification of the selected area in (A, C, E, and F), respectively (original magnification, ×50; ×200)

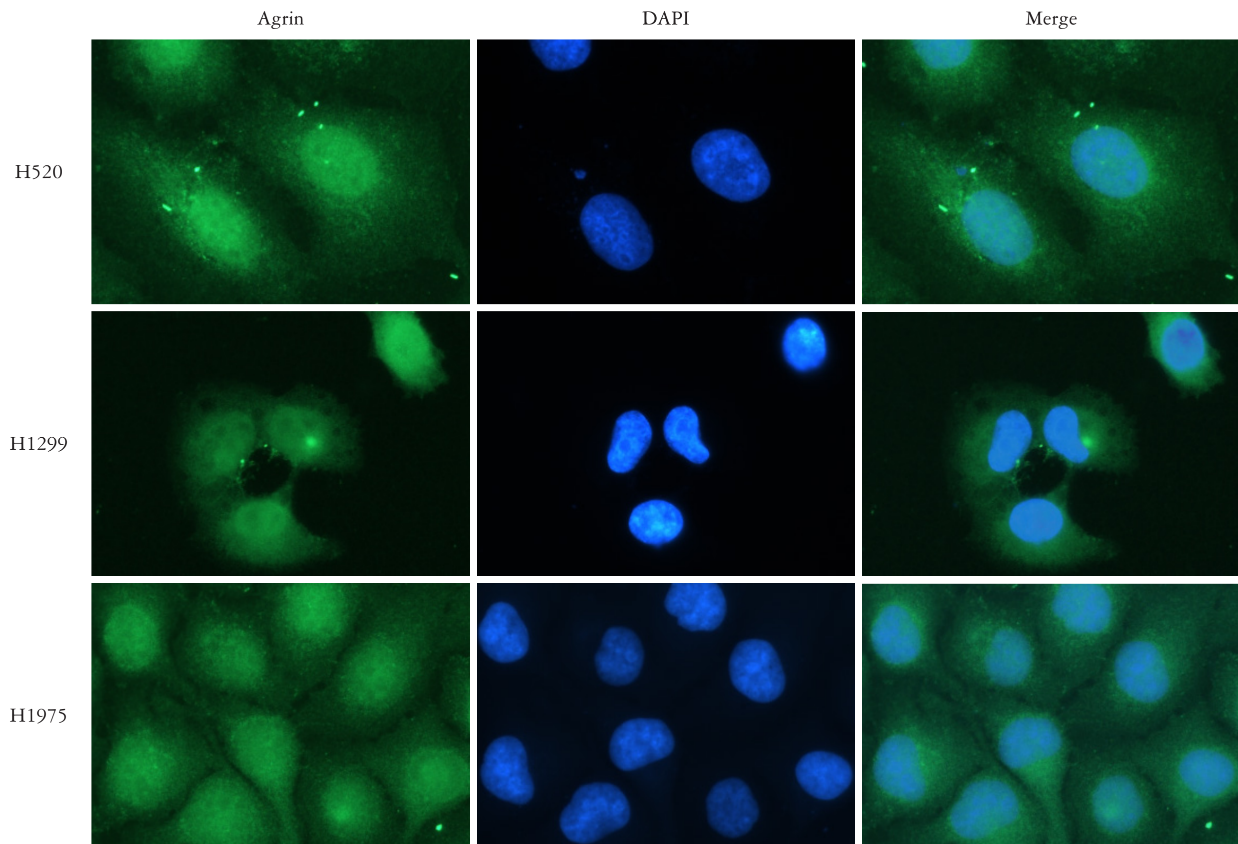


**Fig. 2.** Immunofluorescence staining for the agrin protein in lung cancer cells. The agrin protein was located in the cytoplasm and nuclei of the A549, H1299, and H1975 cells (green indicates agrin staining, and blue indicates DAPI)

in the poorly differentiated lung adenocarcinoma patients (52.9%, 27/51) than in the moderately differentiated (27.4%, 14/51) and well differentiated adenocarcinoma patients (19.6%, 10/51; p = 0.022; Table I and Fig. 3D).

## Discussion

In addition to the neuromuscular junction role, recently studies have shown that the expression of agrin is dysregulated in cancer cells and is involved in carcinogenesis [11, 12, 13]. In this study, the expression of agrin was assessed in 86 normal tissue samples and 94 lung adenocarcinoma tissues. Strong staining of agrin in nuclei of lung adenocarcinoma tissues was observed, but not in nuclei of normal lung tissues. Consistent with staining in lung adenocarcinoma tissues, the nuclei staining of agrin was also detected in lung cancer cell lines by immunofluorescence. This is the first report demonstrating that agrin is highly expressed in nuclei of lung adenocarcinoma tissues and that it is strongly correlated with lymph node metastasis, clinical stage, and poor differentiation. Agrin-positive nuclear staining of lung adenocarcinoma cells could be used to identify greatly increased risk of metastasis in patients after surgery, which might serve as a valuable prognostic marker.

Recent studies indicate that agrin is a target of neurotrypsin and several metalloproteinases [17], generating protein subfragments that can have diverse regulatory activities. The C-terminal soluble fragment of agrin is one of its processing products released from the basement membrane, which has been demonstrated to be a promising new biomarker in colorectal cancer [18]. César Rivera *et al.* also found ~72 kDa agrin processing products in oral cancer cell secretomes, but not in normal or immortalised cells [14]. The results of these studies serve as a basis for greater understanding of the role of agrin in developing oncogenic signals expressed as a membrane protein or a secreted proteoglycan in ECM.

However, these soluble fragment of agrin may form complexes with cell membrane receptors, such as Lrp4-MuSKcomplex in HCC cells [15], which could enter cancer cells to amplify oncogenic signals via endocytosis. Such endocytic events make agrin exert their functions not only on the cell surface but also within the cell [19]. The phenomenon of nuclear translocation of growth hormone and growth hormone receptor complex, which have been reported in a number of cancers [20, 21, 22, 23], may be a good

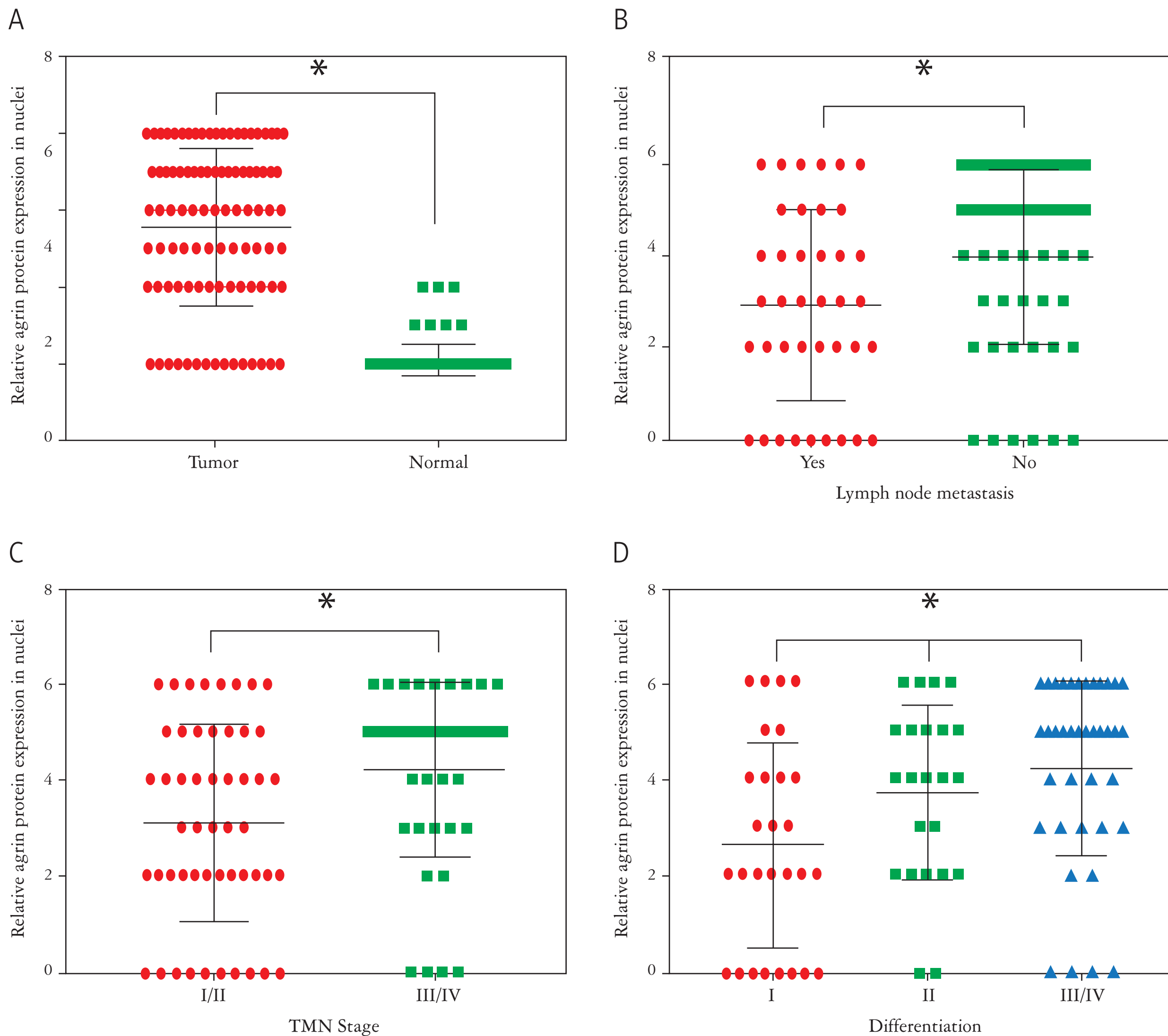


**Fig. 3.** Relative agrin expression in nuclei of lung adenocarcinoma tissue and its clinical significance (A). Immunohistochemical expression of agrin in LUAD tumours (n = 94) and paired adjacent normal tissue (n = 86). Agrin expression was significantly higher in patients with lymph node metastasis (B), in an advanced clinical stage (C), and poor differentiation (D)

example [24, 25]. The increased nuclear localisation of GHR is strongly correlated with high proliferative status of cancer cells and is sufficient to induce tumourigenesis and tumour progression. There are also many studies showing that several cytokines, growth factors, and their receptors become nuclear-localised, which is considered to be necessary for them to function fully [26]. Here we showed that the nuclear localisation of agrin is strongly correlated with lymph node metastasis, clinical stage, and poor differentiation in LAC. This nuclear localisation of agrin may be important for its role in developing oncogenic signals in LAC, which needs to be identified in future work.

In conclusion, the nuclear expression of agrin correlates with tumour lymph node metastasis, clinical stage, and differentiation, reflects aggressive tumour features, and could be an important biomarker and therapeutic target.

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*The authors declare no conflict of interest.*

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