Mathematical Biosciences and Engineering

MBE, 16(6): 7375–7383.
DOI: 10.3934/mbe.2019368
Received: 22 March 2019
Accepted: 15 July 2019
Published: 09 August 2019

http://www.aimspress.com/journal/MBE

Research article

High expression of agrin is associated with tumor progression and poor prognosis in hepatocellular carcinoma

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Abstract: The heparan sulfate proteoglycan agrin is known to accumulate in the context of hepatocellular carcinoma (HCC). Agrin is important for neoangiogenesis in HCC tissues, and is incorporated into newly formed vasculature, but exactly how agrin contributes to the pathology of HCC remains to be fully defined. We therefore examined the clinical relevance of agrin as it pertains to HCC progression and prognosis using tissue sections from a total of 313 HCC patients. We found that agrin expression was detectable in more HCC samples (25.4% vs. 77.1%; $P < 0.05$) compared to normal tissue controls. Agrin expression was notably linked to tumor size ($P = 0.041$) and metastasis ($P = 0.034$). The recurrence free survival rate of agrin-positive HCC patients was considerably lower than that of agrin-negative patients ($P = 0.001$). We further confirmed HCC survival to be independently correlated with tumor size, metastasis, microvascular invasion and edmondson Grade via a Cox regression analysis. Upregulation of Agrin may play a crucial role in HCC progression. Together our results suggest that Agrin has the potential to be used as a prognostic indicator in predicting HCC patient outcomes.

Keywords: agrin; hepatocellular carcinoma; progression; prognosis

1. Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths globally [1]. As treatments have advanced, patient outcomes for this disease have improved, with standard therapeutic avenues now including combinations of chemotherapy, radiotherapy, surgical resection,

and liver transplantation [2]. Despite these advances, no reliable HCC cure is available, even though extensive efforts have been made to understand the mechanisms governing the progression of this disease and to thereby develop novel therapeutics. Even so, the prognosis remains poor for many patients, with high rates of disease recurrence. Patients with HCC still show a poor prognosis and a high recurrence rate. Cell surface proteins and the signaling pathways that they engage in HCC cells are incompletely defined, with most therapies currently focused on the tyrosine kinase receptors [3–5]. It is therefore important that a broader examination of cell surface proteins relevant to HCC be developed in order to improve targeted therapeutic efforts, with the identification of reliable sensitive markers of HCC malignancy being paramount. In the present study, we propose the IHC-based detection of agrin, a heparan sulfate proteoglycan, to be one such promising means of identifying HCC tissues [6].

Agrin is a proteoglycan most commonly detected in the liver vasculature and in the basement membrane of the bile ducts, making it a marker of both such tissue types [7–9]. Levels of Agrin are very low in normal liver tissue samples, but the protein does accumulate in patients suffering from liver cirrhosis as they undergo ductal reactions and neovascularization, and it also accumulates in HCC tissues as these tumors similarly require angiogenesis in order to survive [10,11]. Previous reports have detected agrin both in the context of HCC and in cholangiocellular carcinomas [10,12] with IHC-efforts being an effective means of identifying primary and metastatic liver tumors.

Herein, we used IHC to measure the expression of Agrin in HCC and control tissue samples, using a large number of patient samples with the goal of better understanding how agrin is associated with HCC pathology and progression.

2. Materials and methods

2.1. HCC patients in tissue microarray

The present study was approved by the Ethics Committee of The Second Hospital of Nanjing (Nanjing, Jiangsu, China). All patients provided written informed consent. The expression levels of agrin were evaluated by immunohistochemical staining of tissue microarrays (TMAs) (Shanghai Biochip Co.,Ltd., Shanghai, People's Republic of China). The TMAs containing a total of 626 formalin-fixed, paraffin-embedded archival samples from a total of 313 HCC patients from the Chinese Han population, in addition to 313 corresponding controls derived from adjacent normal tissue samples.

The patient cohort consisted of 255 males and 58 females, with a median age of 58 years (range, 25–89 years) at the time of surgery. All patients had follow-up records for >5 years. The survival time was calculated from the date of surgery to the follow-up deadline or mortality.

2.2. Immunohistochemical staining

Immunohistochemical staining was performed by the standard method. Briefly, 5μm sections from the TMAs were baked at 70℃ for 2h. Then, the sections were de-paraffinized in xylene, rehydrated using a gradient of ethanol concentrations, boiled in 1 mM TE buffer with a high pressure cooker for 3 min to retrieve antigen, blocked with 3% hydrogen peroxide for 15 min to inhibit endogenous peroxidase activity and incubated with 10% goat non-immune serum for 20 min to reduce

background non-specific staining. After that, the sections were incubated with the anti-Agrin (1:400 dilution) at 4 ℃ overnight, then incubated with biotin-labele secondary antibody (Invitrogen, Carlsbad, CA) at room temperature for 15 min, followed by incubation with HRP-conjugated streptavidin (Invitrogen, Carlsbad, CA) at room temperature for 15 min. Then, Color development was performed with DAB Substrate Kit (Dako, Glostrup, Denmark). Finally, the sections were counterstained with hematoxylin, dehydrated, cleared and mounted.

2.3. Evaluation of the immunohistochemical staining

The immunohistochemical stainings of mertk were scored by two pathologists independently, based on the intensity and the proportion of positively stained cells. Staining intensity was evaluated with a four-tiered grading system: 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellow brown), and 3 (strong staining, brown). The percentage of positive cells were scored as follows: 0 for no cell stained, 1 for 1%–25% of cells stained, 2 for 26%–50% of cells stained, 3 for 51%–75% of cells stained and 4 for more than 75% of cells stained. Scores for intensity and percentage of positive cells were multiplied. Scores ≤ 6 was used to define tumors with low mertk expression and scores > 6 with high AGRN expression.

2.4. Statistical analysis

Data are shown as means ± SD. For comparison between 2 groups, significant differences were determined using the Student t test. For comparison of more than 2 groups, statistical significance was determined with a one-way ANOVA followed by a Bonferroni multiple-group comparison test. $P < 0.05$ was considered significant.

3. Results

3.1. Agrin expression is elevated in HCC tissue samples

Immunohistochemical (IHC) methods were used to assess the expression of Agrin in HCC tissue. Three pathologists independently evaluated HCC tissue microarrays under 40 ×(Figure 1A, C, E, G) and 200 ×magnification (Figure 1B, D, F, H).

Agrin showed positive staining in HCC cells in which cell surface and cytoplasmic Agrin staining was observed. Agrin was highly expressed in 210 (77.1%) of the 313 patients with HCC, which was significantly higher than the expression observed in the adjacent normal tissues (25.4%, 80/313, $P = 0.001$). Upon dividing samples into high expression (> 6 points) and low expression groups (0–5 points), Agrin expression in HCC tissues had an average of 7.32 ± 1.89 points, compared to 3.62 ± 1.76 points in adjacent healthy tissue ($P < 0.05$). Taken together, these data demonstrate elevated Agrin expression in HCC tumors.

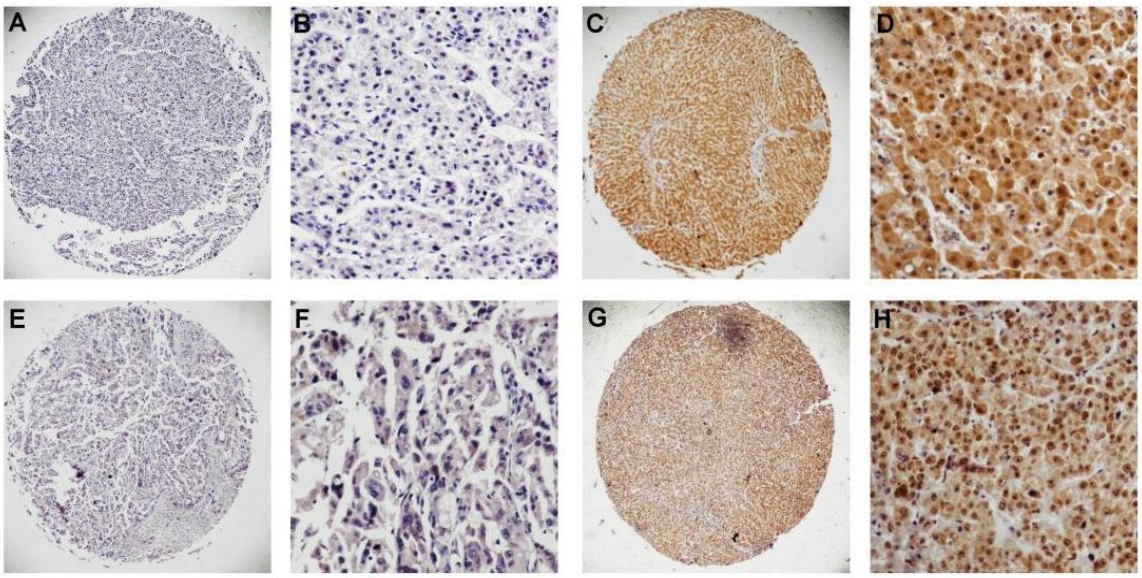

Figure 1. Agrin staining in HCC tissues. (A) Low expression of Agrin in normal tissues (Score = 2, Magnification ×40); (B) Low expression of Agrin in normal tissues (Score = 2, Magnification ×200); (C) High expression of Agrin in normal tissue (Score = 12, Magnification ×40); (D) High expression of Agrin in normal tissues (Score = 12, Magnification ×200); (E) Low expression of Agrin in HCC tissue (score = 4, Magnification ×40); (F) Low expression of Agrin in HCC tissue (score = 4, Magnification ×200); (G) High expression of Agrin in HCC tissue (score = 16, Magnification ×40); (H) High expression of Agrin in HCC tissue (score = 16, Magnification ×200).

3.2. Agrin association with HCC clinic opathological features

The patient cohort consisted of 255 males and 58 females, with a median age of 58 years (range 25–89 years) at the time of surgery. We further compared observed expression of Agrin to available clinicopathological parameters for HCC samples, revealed that cytoplasmic agrin staining did not correlate significantly with gender, age, microvascular invasion, HBs antigen, Cirrhosis and AFP but was significantly associated with tumor size (P = 0.041) and metastasis (P = 0.034) (Table 1). In all, 87.5% (28/32) of HCC patients with tumor metastasis were detected with high expression of Agrin, which was higher than that without metastasis (53%, 149/281, $\chi 2$ = 0.577, P = 0.014). 72.5% (95/131) of HCC patients with tumor size (≥5) were detected with high expression of Agrin, which was higher than that without metastasis (50%, 91/182, $\chi 2$ = 0.048, P = 0.021).

3.3. Agrin expression is associated with decreased survival time

Patients with agrin-positive tumor tissue samples had an average survival time of 37.728 ± 3.708 months, which was significantly below that of agrin-negative patients (64.961 ± 2.506 months, P = 0.001). A Kaplan-Meier survival curve confirmed a significant link between agrin expression and overall survival (Figure 2).

Table 1. Expression of AGRN in hepatocellular carcinoma tissues.

Clinical parameters	number	AGRN expression		$\chi 2$	*P* value
		Low	high		
Age (years)				0.708	0.426
<55	122	57	65		
≥55	191	80	111		
Gender				0.032	0.884
Male	255	166	89		
Female	58	26	32		
Size				0.048	0.021*
<5	182	91	91		
≥5	131	36	95		
Tumour number				0.297	0.373
Single	257	116	141		
multiple	56	21	35		
Edmondson Grade				0.958	1.000
I+II	194	83	111		
III	119	51	68		
Metastasis				0.577	0.014*
M0	281	132	149		
M1	32	4	28		
Microvascular invasion				0.286	0.295
Absence	156	68	88		
Presence	157	77	80		
HBs antigen				0.093	0.114
Negtive	68	24	44		
Positive	245	112	133		
Cirrhosis				0.0.342	0.396
Negtive	144	69	74		
Positive	169	108	143		
AFP				0.697	0.704
<20	164	74	90		
≥20	149	64	85		

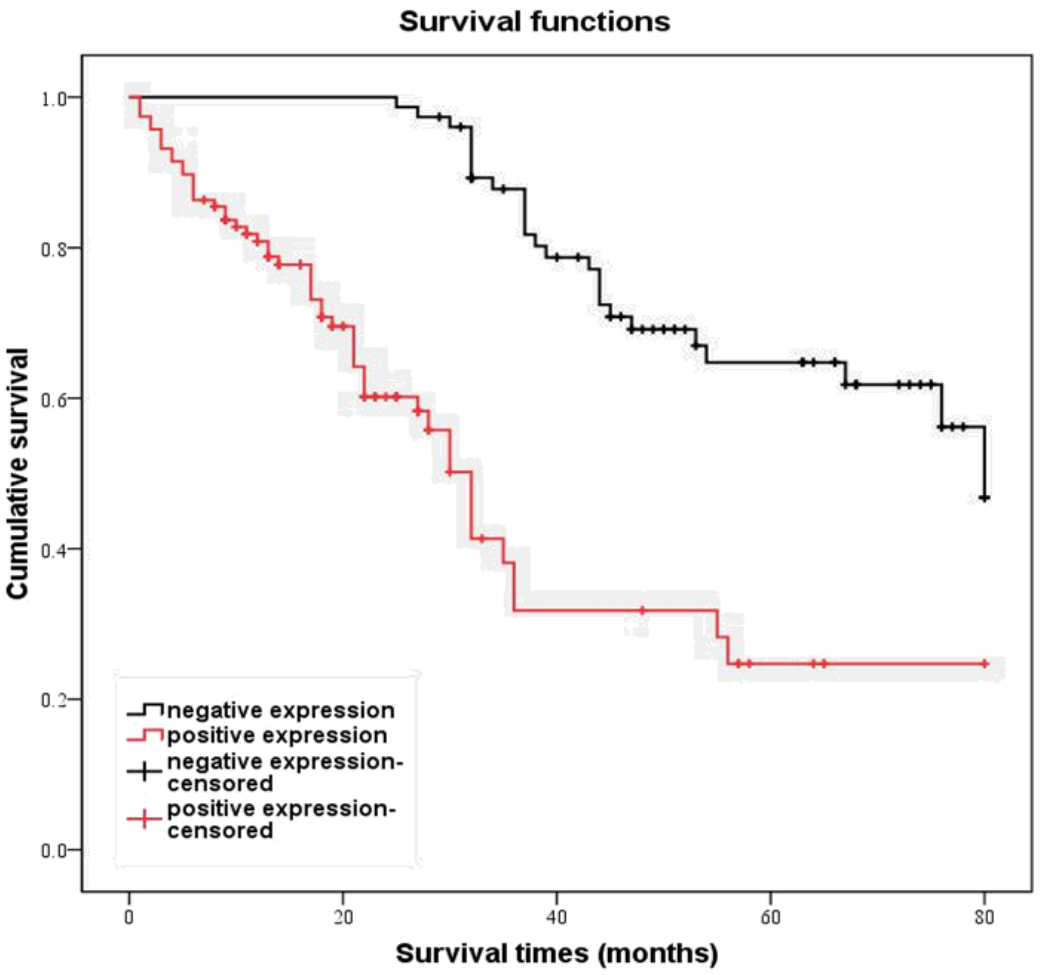

Figure 2. Relationship between Agrin expression and survival time.

We also used a Cox regression analysis to identify potential prognostic factors. We identified a significant correlation between agrin positivity and Tumour size ($P = 0.011$), Metastasis ($P < 0.001$), Microvascular invasion ($P = 0.011$), and Edmondson Grade ($P < 0.001$). There were no correlations between Agrin expression and gender ($P > 0.05$), age ($P > 0.05$), Cirrhosis ($P > 0.05$), or tumour number ($P > 0.05$). A subsequent multivariate regression analysis confirmed a significant correlation between agrin expression and Tumour size ($P = 0.025$) and Edmondson Grade ($P = 0.021$). There were no correlations between Agrin expression and other prognostic factors for HCC patients (Table 2).

4. Discussion

There is known to be a link between the extracellular matrix (ECM) and tumor progression, with complex interactions between tumor cells and the ECM controlling the progress of many aspects of tumor progression [13,14]. Proteoglycans (PGs), which comprise a substantial portion of the ECM, are known to be significantly differentially regulated in the context of tumor progression, and as such they are associated with processes giving rise to malignant tumors, contributing to altered invasion, adhesion, survival, and growth of cancerous cells [15–17]. Among the main heparan sulfate PGs (HSPG), identified in basement membrane, are Agrin and perlecan adre heparan sulfate PGs that are

expressed in basement membrane regions, and are overexpressed in different types of cancer including HCC, as well as in breast and prostate cancers [18–19]. Agrin is heavily post-translationally modified with heparan sulfate (HS) and chondroitin sulfate (CS) glycosaminoglycans (GAGs), substantially increasing the size of this protein relative to its predicted molecular weight [8]. Despite being detected in HCC and other cancer tissues, exactly what role it plays in these tumor tissues is uncertain, as is the full mechanisms governing its function in normal tissues.

Table 2. Univariate and multivariate Cox-regression analysis of the clinicopathological parameters in liver cancer patients.

Parameters	Univariate analysis				Multivariate analysis			
	Coefficient	HR	95.0% CI for HR	P	Coefficient	HR	95.0% CI for HR	P
Gender (Male/Female)	0.447	1.564	0.888–2.754	0.122	1.026	2.79	0.061–127.572	0.599
age(< 55 years/ ≧ 55 years)	0.008	1.008	0.592–1.717	0.977	−0.262	0.769	0.032–18.447	0.871
Tumour size(<50mm/≥50 mm)	0.645	1.906	1.16–3.131	0.011*	0.334	1.396	0.042–46.716	0.025*
Tumour number (Single/multiple)	0.321	1.378	0.735–2.584	0.317	1.515	4.549	0.238–86.79	0.314
Edmondson Grade (I+II/III)	0.943	2.568	1.685–3.914	0.000*	6.898	9.121	2.887–3.396	0.021*
Metastasis (M0/M1)	1.457	4.293	2.219–8.305	0.000*	−2.322	0.098	0.001–12.442	0.347
Microvascular invasion(−/+)	0.722	2.058	1.18–3.589	0.011*	1.959	0.33	0.137–366.4	0.33
HBV (−/+)	−0.027	0.947	0.539–1.76	0.93	0.802	2.23	0.065–76.753	0.657
Cirrhosis (−/+)	0.072	1.075	0.639–1.808	0.785	0.406	1.5	0.059–38.918	0.807
AFP(<50 ug/L /≥50 ug/L)	1.279	3.593	1.854–6.963	0.243	−4.955	0.054	0.001–1.097	0.054

In neuromuscular junctions, agrin has been shown to be critical for accurate postsynaptic organization, such that mice with mutate dagrin proteins die shortly after birth [9–11]. While its role in this context is at least partially understood, how agrin normally functions in other parts of the body remains uncertain, even though it is observed in tissues including liver vascular tissue and bile ducts, but is absent from normal liver lobules [7]. Benign tumors of the liver have also been found to be agrin

negative [6], which liver tissue suffering from chronic disease or HCC stains as agrin positive, particularly in basement membrane and tumor tissues [7].

How agrin contributes to cancer progression remains uncertain, but HCC cell lines have been shown to secrete this protein at high levels, and this was confirmed via a proteomic approach revealing it to be a particularly enriched plasma membrane protein in HCC cell line surface samples. Agrin overexpression can lead to increased activation of its corresponding neuronal receptor-Low-Density Lipoprotein (LDL)-receptor related protein 4 Lrp4/muscle-specific tyrosine kinase MuSK-leading to the generation of an agrin–Lrp4/MuSK signaling complex. This complex is believed to be able to sense and respond to the local ECM, driving the activation of key kinases in HCC cells that enable tumor growth and proliferation.

Agrin has also been found to be secreted by human hepatic stellate cells in response to stimulation with platelet-derived growth factor (PDGF). This secretion can trigger the epithelial-mesenchymal transition within HCC cells, potentially further increasing the deleterious nature of a given tumor [22]. Inhibiting the PDGF receptor can both disrupt agrin-mediate oncogenesis, and other associated processes such as liver inflammation and fibrosis.

In the present study, we assessed agrin expression in HCC tissue samples, revealing it to be expressed at higher levels in HCC tissues in a manner useful for predicting HCC patient prognosis. The increases in agrin expression we observed were associated with both tumor size and staging, consistent with the abovementioned reports regarding a role for agrin in tumor development and progression. These findings thus further cement a potentially important role for agrin in HCC progression, and suggest it may be a valuable prognostic marker, although further investigation of the underlying molecular mechanisms is warranted.

5. Conclusion

In summary, we have found that Agrin can be used as a diagnostic tool when screening tissue sections for the presence of HCC, and its overexpression in HCC tumors represents a possible therapeutic target to reduce HCC cell metastasis and migration. Future exploration of effective agrin inhibitors and their assessment as HCC therapies may aid future clinical HCC diagnosis and treatment.

Conflict of interest

The authors declare that they have no competing interests.

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