The Significance of Agrin and Heparan Sulfate Degrading Enzymes in Liver Tumors

Doctoral (Ph.D.) Theses

Áron Somorácz MD

Semmelweis University
Doctoral School of Pathology

Tutor: Péter Tátrai, PhD

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Introduction

Differential diagnosis of liver tumors is a challenging field of pathology, with many difficulties still remaining to be resolved. About 85-90% of primary liver cancers is hepatocellular carcinoma (HCC) that is the fifth most common malignancy all over the world, and ranks third in tumor-related mortality. Intrahepatic cholangiocellular carcinoma (CCC) is encountered much less frequently; however, the global incidence of this rare tumor is increasing. Since liver is often affected by metastatic cancer, metastases are more common than primary cancers. The origin of a large subset of these secondary liver carcinomas is the gastrointestinal tract, with the majority being colorectal cancers.

Distinguishing a well-differentiated HCC from a hepatocellular adenoma is among the most problematic tasks in the differential diagnosis of hepatic neoplasms. Because of the increasing incidence of liver adenomas due to the common use of oral contraceptives and anabolic steroids, this difficulty emerges more often than before. Based on genetic analysis, a subset of HCAs has the potential for malignant transformation, which emphasizes the importance of detection of incidentally occurring malignant foci in a benign tumor.

About 90% of HCCs develop in the cirrhotic liver, arising from a nodule with dysplastic foci. According to the degree of dysplasia, these nodules, conspicuous due to their larger size, are classified as low-grade (LGDN), and high-grade (HGDN) dysplastic nodules. Small HCC (sHCC) represents the earliest malignant lesion that is, by definition, smaller than 2 cm in diameter. Demonstration of malignant foci in the cirrhotic tissue, as well as their discrimination from dysplastic but not yet malignant nodules, is of utmost importance. However, it often poses difficulties, since no clear-cut border exists between HGDNs and sHCCs.

Differential diagnosis of CCCs and metastatic adenocarcinomas can be similarly dubious. Especially, metastases from ductal pancreatic cancer can mimic CCCs to the limit of indistinguishability. Moreover, the immunophenotype of these tumors shows a remarkable overlap in the routinely used markers.

The hypothesis that agrin could serve as an immunohistochemical marker for the detection of malignant hepatocellular lesions was based on the observation made by
Tátrai et al., namely that agrin is accumulated selectively in HCC microvessels. Whereas the dense microvascular network of HCCs showed an intense immunostaining, no reaction could be detected associated to sinusoids in healthy liver tissue or to capillarized microvessels in the cirrhotic liver. Later, Batmunkh et al. demonstrated that agrin is also highly expressed in CCCs where strong positivity of tumorous basement membranes could be observed.

Agrin is a heparan sulfate proteoglycan (HSPG) that was first discovered in neuromuscular junctions as the organizer of acetylcholine receptor aggregation. Furthermore, the important role of agrin in synapse formation in central nervous system, as well as in the maintenance of blood-brain barrier was also confirmed. In addition, its accumulation in alveolar and glomerular basement membranes was described. Some functions of agrin can be attributed to the core protein, while others to the heparan sulfate (HS) side chains. One isoform of the core protein has a laminin binding site on its N-terminus and integrin target sequence on its C-terminus, which enable it to connect epithelial cells to their basement membrane. HS side chains can bind various growth factors (e.g. bFGF, VEGF) and influence their signaling pathways. This effect can be activating when HS functions as a co-receptor. On the other hand, the sequestration of growth factors can also inhibit access to their receptors. Based on these properties of HSPGs, we aimed to examine the links between the accumulation of agrin in liver cancer and its potential role in hepatocarcinogenesis.

When investigating HSPGs we cannot ignore their modifying enzymes that via alteration of their structure vastly influence ligand binding capacity, and thus the activation of affected signaling pathways. Sulfatase-1 (SULF1) and Sulfatase-2 (SULF2) are extracellular endosulfatases that can remove 6-O-sulfate groups from the amino sugar of HS disaccharide units. Sulfatases can act either as tumor suppressors or as oncogenes, which phenomenon is not surprising considering the dual behavior of HS. With regard to HCC., downregulation of SULF1 expression could be detected in 30% of tumors, whereas SULF2 overexpression could be demonstrated in the majority of the cancers at mRNA level according to gene expression data available in the literature.

Heparanase more drastically modifies HS structure by hydrolyzing glycosidic bonds within the polysaccharide chain. The role of heparanase seems to be verified in
multiple steps of tumor progression, including neoangiogenesis and metastasis. The majority of studies discussing heparanase expression in HCC reports an overexpression in 45-50% of tumors; however, controversial results also exist.
Aims

Building on our previous knowledge about agrin expression in the healthy and diseased liver, we aimed to expound the following issues:

1. The applicability of agrin immunohistochemistry in the differential diagnosis of hepatocellular lesions through qualitative and quantitative evaluation of immunoreactions.

2. Investigation of agrin expression in the most common metastatic adenocarcinomas of the liver at both mRNA and protein level.

3. The diagnostic utility of agrin immunohistochemistry in distinguishing primary liver cancers from metastatic tumors.

   Based on our data accumulated on the cellular origin of agrin in liver cancers we decided to use a cholangiocellular carcinoma-derived cell line with confirmed high expression of agrin for *in vitro* experiments. In these we aimed to reveal the possible role of agrin in cholangiocellular carcinogenesis. Our questions were the following:

4. The effect of agrin silencing by siRNA on the proliferation and migration of MzCha2, a cholangiocellular carcinoma-derived cell line.

   An additional direction of our research was to determine the importance of heparan sulfate degrading enzymes in primary liver cancers. These enzymes can deeply influence the biological processes controlled by all HSPGs including agrin. In our initial experiments we investigated:

5. Expression of sulfatase-1, sulfatase-2, and heparanase in HCCs and CCCs at both mRNA and protein level.
Methods

- Agrin, CD34, and cytokeratin-7 immunohistochemistry to investigate blood vessel- and biliary tract-derived agrin expression in hepatocellular lesions

- Agrin immunohistochemistry on CCCs, as well as on metastases from colorectal and ductal pancreatic cancer

- Semiquantitative (hepatocellular lesions), and qualitative (CCCs and metastatic adenocarcinomas) evaluation of agrin immunoreactions to devise a diagnostic algorithm

- Quantitative evaluation of agrin immunoreactions by digital morphometry

- Total RNA isolation followed by real-time RT-PCR to determine agrin mRNA expression in healthy liver samples, HCCs, CCCs, and liver metastases from colorectal cancer

- Agrin silencing by siRNA in MzCha2 cholangiocellular carcinoma-derived cell line

- Examining the effect of agrin silencing on cell proliferation and migration by sulforhodamine B test and Boyden chamber

- Investigation of sulfatase-1, sulfatase-2, and heparanase expression in healthy liver samples, HCCs, and CCCs by real-time RT-PCR and Western blot analysis

- Detection of sulfatase-2 by immunofluorescence, as well as of heparanase by immunohistochemistry in control liver samples, HCCs, and CCCs
Results

1. The applicability of agrin immunohistochemistry in the differential diagnosis of hepatocellular lesions through qualitative and quantitative evaluation of the immunoreactions

Agrin immunohistochemistry was performed on 25 HCC, 8 sHCC, 30 HCA, 10 FNH, 25 cirrhosis, 8 large regenerative nodule (LRN), 23 LGDN, and 7 HGDN samples. The parenchyma of cirrhotic nodules, LRNs, and FNHs was mostly devoid of agrin. Based on cytokeratin-7 immunostaining, the occasionally observed scattered positivity was associated to proliferating bile ducts that participated in liver regeneration and invaded the periphery of nodules. LGDNs containing mild dysplasia showed the same picture. As a contrast, heterogeneity in agrin immunoreaction was observed in HGDNs. Some of them showed no remarkable staining, whereas strong reaction could be detected in a subset of HGDNs that was in parallel with the extent of dysplasia.

Agrin positivity was demonstrated only in the immediate periarterial zones in typical HCAs without dysplasia, and the parenchyma was overwhelmingly agrin-negative. On the other hand, larger foci were stained in dysplastic adenomas. In HCCs and sHCCs, ubiquitous and strong agrin immunostaining was seen to be localized to the microvessels.

CD34 immunohistochemistry, used for the visualization of vascular structures in the lesions, was positive to a varying extent even in cirrhotic nodules. This staining was more pronounced and extended in dysplastic nodules. Ubiquitous reaction was often detected in HCAs and uniformly seen in HCCs and sHCCs. Based on these observations, CD34 immunostaining alone is not specific enough to differentiate between hepatocellular lesions, even though it detects malignancy with a considerably high sensitivity.

In semiquantitative analysis, immunoreactions were graded between 0 and 4+ according to the extent of immunopositive areas. Applying this evaluation system, agrin immunohistochemistry could detect malignant hepatocellular lesions with a sensitivity of 94% and a specificity of 88%. The latter was improved to 93% by adding the
criterion that only lesions with 4+ grade CD34 immunoreaction were accepted as malignant.

Glypican-3, widely considered as a standard marker of HCC for years, was also immunostained in a selection of representative samples. While some HCCs were strongly and diffusely labeled, negative or dubious reactions also occurred. The significance of single-cell glypican-3 positivity in adenomas is also unknown and may mislead the pathologist.

2. Investigation of agrin expression in the most common metastatic adenocarcinomas of the liver at both mRNA and protein level

Agrin immunohistochemistry was carried out on 20 metastatic tumors from colorectal cancers (CRCm), as well as on 18 metastases from pancreatic ductal carcinomas (PDCm). No microvessel-associated agrin positivity could be detected in CRCm samples, although CD34 immunostaining was present. On the other hand, positive reactions were observed in basement membranes surrounding the tumorous pseudoglandules. The staining was absent from areas lacking glandule-like structures. A similar tendency was encountered in PDCm samples, with the difference that the majority of these tumors showed a solid growing pattern without tumorous pseudoglandules, resulting in a weaker overall agrin positivity. Our question was whether agrin was missing due to a lack of basement membranes in poorly differentiated tumor areas without glandule forming tendency. This hypothesis was rejected, since laminin immunohistochemistry demonstrated the maintenance of basement membrane remnants even around poorly organized cell nests. Agrin immunostaining was also performed on 16 CCC samples. Unlike in metastatic adenocarcinomas, diffuse basement membrane-associated immunoreaction was detected even in poorly differentiated Grade III CCCs, despite no obvious glandule forming tendency. According to morphometric analysis, agrin is significantly overexpressed in CCCs at protein level as compared with the investigated metastatic cancers.

Agrin mRNA expression was analyzed in HCCs, CCCs, CRCm cases, and control healthy liver tissue samples. Significantly higher expression rates were measured in all tumor types as compared with the control liver samples. The highest
value was measured in the CCC group, while HCC and CRCm expressed agrin mRNA at lower rates. Intriguingly, HCC and CRCm did not significantly differ at the mRNA level.

3. The diagnostic utility of agrin immunohistochemistry in distinguishing primary liver cancers from metastatic tumors.

By qualitative evaluation of agrin immunoreactions, cases were either classified as complete / ubiquitous (C/U) or incomplete / missing (I/M). Reactions were referred to the I/M category when the largest agrin-negative area exceeded one field of view using 10x objective magnification, otherwise samples were noted as C/U. An attempt was made to find a correlation between C/U versus I/M categories, and primary versus metastatic origin of the tumors. Based on a comparison with expert histopathological diagnoses, agrin immunohistochemistry could detect CCCs with a sensitivity of 81%, whereas it had a specificity of 82% in distinguishing between primary and metastatic origin.

4. The effect of agrin silencing by siRNA on the cell proliferation and migration of MzCha2, a cholangiocellular carcinoma-derived cell line

Application of agrin-targeting siRNA resulted in an average decrease of 47% in agrin mRNA expression. The effect was also confirmed at protein level by immunofluorescent labeling.

Using medium containing 20% FCS, agrin silencing increased cell proliferation rate by 31% as compared with the control cell line. This phenomenon could not be demonstrated when applying media with lower FCS concentrations (1%, 10%).

According to migration experiments performed in Boyden chamber, agrin silencing did not influence substantially the migration of MzCha2 cells.
5. Expression of sulfatase-1, sulfatase-2, and heparanase in HCCs and CCCs at both mRNA and protein level

The highest SULF1 mRNA expression was measured in CCCs. The mean expression value was also found to be elevated in the HCC group as compared with the control samples; however, the individual cases showed substantial deviation. No significant difference was demonstrated in SULF2 mRNA expression between the sample groups. Heparanase overexpression was seen in CCCs.

A 180 kDa and 110 kDa protein was detected in CCCs with SULF1 antibody by Western blot analysis. Only the smaller product was found in HCCs, whereas no immunoreaction was observed in control samples. SULF2 antibody revealed a 60 kDa protein in CCCs, and an 80 kDa product in HCCs and control livers. The 50 kDa subunit of heparanase was demonstrated in HCCs, while specific immunoreaction was seen at 20 kDa in CCCs.

According to immunofluorescent detection, SULF2 is produced by the tumor cells in HCCs, whereas stromal cells are the suspected source in CCCs. Based on SULF2/CD34 double immunofluorescence reactions, tumor cells surrounding microvessels were selectively labeled by SULF2 in HCCs, which fosters the hypothesis that SULF2 might play a role in neoangiogenesis. A strong cytoplasmic staining was seen in HCCs by heparanase immunohistochemistry, which was less intense in CCCs. Heparanase reaction was conspicuously inhomogeneous in the peritumoral liver tissue, as far as hepatocytes in the invasion zone were strongly positive while distant areas showed only a faint reaction.
Conclusions

1. We proved that agrin immunohistochemistry provides a useful aid in the differential diagnosis of hepatocellular lesions, especially when supplemented with CD34 immunostaining. The evaluation system we devised is easy to acquire, which might facilitate its application in routine work. Thus, there is strong evidence to support the application of agrin and CD34 as complementary markers next to glypican-3, and addition of these markers enhances reliability and sensitivity of histopathologic diagnosis.

2. We demonstrated that agrin expression of the investigated metastatic adenocarcinomas was significantly lower at both mRNA (CRCm) and protein (CRCm, PDCm) level as compared with primary liver cancers. If agrin still appeared, it was associated to tumorous basement membranes, similar to CCC.

3. We provide evidence that agrin is a useful marker in distinguishing between CCCs and liver metastases from colorectal and ductal pancreatic cancer; however, sensitivity and specificity are not as convincing as what we experienced in the discrimination of benign versus malignant hepatocellular lesions. It is also worthy of note that based on agrin immunohistochemistry alone, HCCs can be unequivocally discriminated from classical adenocarcinomas in which no microvessel-associated agrin positivity could be observed.

4. According to in vitro experiments with MzCha2 cell line, agrin produced by cholangiocellular carcinoma-derived cells has a negative effect on cell proliferation, whereas it does not seem to be involved in the control of cell migration.

5. HCCs are heterogenous with regard to the expression of heparan sulfate degrading enzymes. The correlation between expression rates and tumor behavior needs further investigation, as literature data are controversial. The production of sulfatases was dominant in CCCs, while high heparanase mRNA expression was also detected.
List of publications

In the topic of the doctoral thesis:

(equal contributor: Tárai P)
IF (2009): 2,961

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