Published in final edited form as:

Cancer Lett. 2007 June 18; 251(1): 28–35.

Clinical Significance of Serum Angiocidin Levels in Hepatocellular Carcinoma

Yamini Sabherwal, Vicki L. Rothman, Ronnie T.P. Poon^{*}, and George P. Tuszynski

Center for Neurovirology, Department of Neuroscience, Temple University, Philadelphia, PA 19122

^{} Center for Cancer Research and Department of Surgery, University of Hong Kong, Pokfulam, Hong Kong, People's Republic of China*

Abstract

Angiocidin, a tumor-secreted protein, was measured in serum of 27 healthy volunteers and 33 hepatocellular carcinoma (HCC) patients. Healthy controls either hepatitis B surface antigen (HBsAg) positive or negative showed undetectable levels. Patients had levels of angiocidin ranging from 15.09–195.73 pg/ml. Patients with stages III–IV had higher levels of angiocidin (97 ± 13 pg/ml, $n=17$) compared to those with stages I–II (63 ± 37 pg/ml, $n=16$), $p<0.043$. Patients with microsatellite tumor nodules had higher average levels (98 ± 55 pg/ml, $n=17$) compared to those without microsatellite nodules (51 ± 27 pg/ml, $n=20$), $p<0.032$. Our studies suggest that angiocidin predicts advanced stage and intra-hepatic metastasis.

Keywords

angiocidin; hepatocellular carcinoma; tumor markers; angiogenesis; metastasis

Introduction

Hepatocellular carcinoma (HCC) is a highly vascularized cancer of the liver [1]. Worldwide it is one of the most common causes of death from cancer with an estimated one million cases reported yearly. In Asia and sub-Sahara Africa the incidence has been reported as high as 1 in 1,000. At the time of diagnosis only about 25% of tumors are amenable to surgery and the rest are too advanced for resection. Surgery remains the major treatment option in early stage tumors providing a 5 year survival of 40–50%. Therefore early detection of HCC is critical for effective treatment.

Detection of blood markers for HCC seem a viable strategy for detection of early stage disease before the clinical signs of the disease become apparent and surgery is no longer an option. A commonly used serum marker for HCC is AFP, which is a protein normally made by immature liver cells in the fetus. At birth, infants have relatively high levels of AFP, which fall to normal adult levels by the first year of life. However, elevated serum AFP levels above 500 ng/ml are only observed in about 60% of HCC patients, while 40% of patients with HCC have normal AFP levels [2]. Therefore, a normal AFP level does not exclude HCC. In addition, elevated

Address correspondence to: Dr. George Tuszynski, Department of Neuroscience, Temple University, 1900 North 12th Street, Philadelphia, PA 19122 or email gpt@temple.edu.

Supported by grant R01 CA88931 to gpt from the National Institutes of Health

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

AFP levels are observed in patients with cirrhosis, indicating that AFP is not specific for HCC. Therefore, new serum and tumor markers are needed that are more prognostic for HCC and predictive of disease severity.

A potential new serum and tumor marker for HCC is angiocidin. Our laboratory has shown that angiocidin is over-expressed and secreted by many solid tumors [3]. We postulate that angiocidin acts as an endogenous inhibitor of angiogenesis and metastasis based on our *in vitro* and *in vivo* data showing that recombinant angiocidin inhibits growth and vascularization of Lewis lung carcinoma in the mouse model [3,4]. Angiocidin, or the CSVTCG specific receptor [5], was originally isolated as a thrombospondin-1 (TSP-1) binding protein. Its high affinity for TSP-1, a platelet secreted protein, may play a role in modulating the angiogenic activity of TSP-1. In a recent study showing the immunolocalization of TSP-1 in 60 HCC patients, it was shown that the CSVTCG- specific receptor or angiocidin was over-expressed in the TSP-1 positive cases [6]. Positive staining for angiocidin was found in the tumor cells of 57 (95%) of the 60 cases studied and in the stromal cells of 53 (88%) of the patients. Tumor tissue showed more intense staining than the surrounding non-tumor tissue. These results indicate that angiocidin plays a role in HCC tumor progression and that angiocidin is a tumor marker in HCC. Therefore, we decided to determine if angiocidin was present in the blood of HCC patients and whether angiocidin blood levels correlated with the presence and stage of HCC. In this study, we developed a sensitive ELISA assay to quantitate angiocidin levels in biological fluids and discovered that elevated angiocidin levels in the serum of HCC patients correlated with the advanced stage and metastatic status of HCC.

Material and Methods

Antibodies and reagents

All chemicals were reagent grade and unless specified otherwise obtained from Sigma-Aldrich, Saint Louis, MO. Tissue culture media and serum were purchased from Fisher Scientific, Pittsburgh, PA. Recombinant angiocidin was purified as previously described [3]. Monoclonal and polyclonal antibodies against angiocidin were prepared from purified recombinant protein (Covance, Denver PA). Purified rabbit anti-angiocidin IgG (5 mg each) was biotinylated with the EZ Link Sulfo-NHS Biotinylation kit purchased from Pierce Chemical Co., Rockford, IL. Final protein solutions were dialyzed against PBS and stored at a concentration of 1 mg/ml at -20°C until needed.

Patient Samples

Thirty-three HCC patients were recruited in this study. The study protocol was approved by the Research Ethics Committee of the University of Hong Kong and the Internal Review Board of Temple University and informed consent was obtained from all participants. There were 28 males and 5 females with a mean age of 55 years. Twenty-nine of the 33 patients were positive for hepatitis B surface antigen (HBsAg). In addition, 17 normal healthy subjects expressing no HBsAg with a mean age of 35 and 10 healthy HBsAg carriers participated in the study as controls. Blood samples were taken and serum was prepared and stored at -70°C until needed.

ELISA Analysis for Angiocidin

Serum levels of angiocidin were measured using a capture ELISA. Briefly, 1 μg of mouse monoclonal anti-angiocidin IgG in 100 μl of phosphate-buffered saline (PBS, pH 7.4) was incubated at 4°C with shaking overnight per well of a 96 well microtiter plate (Nunc MaxiSorpTM, Fisher Scientific, Pittsburgh, PA). Each well was then blocked for 30 min at room temperature with 200 μl of 1% BSA in PBS, aspirated and washed with 200 μl of PBS. Each well was then incubated for 1.5 hours with 100 μl of various concentrations of angiocidin (30–500 pg/ml) or serum in high salt TBST (Tris buffered saline, pH 7.4, containing 0.05% Tween

20 and 0.50 M NaCl) at room temperature with shaking. Wells were aspirated and washed three times with 250 μ l of TBST. Each well was then incubated with 100 μ l of a 1 to 8000 dilution of a stock 1.4 mg/ml solution in PBS of biotinylated rabbit polyclonal anti-angiocidin IgG prepared according to the instructions provided with the EZ Link Sulfo-NHS Biotinylation kit obtained from Pierce Chemical Co., Rockford, IL. Wells were then washed under the same conditions followed by the addition of 100 μ l of a 1 to 25,000 dilution of a 1 mg/ml TBST solution of streptavidin coupled horseradish peroxidase (HRP) (Pierce Chemical Co, Rockford, IL.). Wells were aspirated, washed three times and developed for 3–15 minutes with 100 μ l of 1-step Ultra TMB (3,3',5,5'-tetramethylbenzidine) obtained from Pierce Chemical Co., Rockford, IL. Color development was stopped by the addition of 50 μ l of 0.50 M sulfuric acid. The plate was read at 450 nm with an ELISA reader. Serum levels of angiocidin were extrapolated from the standard curve drawn by linear regression using GraphPad Prism 3.03 software (San Diego, CA).

Assay performance statistics were evaluated by performing inter- and intra-assay variation and recovery analysis performed as previously described [7]. Briefly, for the intra and inter precision analysis four standard curves were performed either on the same day or on four successive days, respectively. The standard deviation in the absorbance reading for a given concentration of angiocidin (350 pg/ml) was calculated. Recovery analysis was performed by comparing standard curves generated in TBST with those where the diluent was either 1:10 and 1:5 normal serum.

Western Blot Analysis

Proteins were analyzed on Pharmacia Phast gel system (GE Healthcare, Piscataway, N.J.) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gels, and the gel electroblotted onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked for an hour with 5% non-fat dry milk in TBST, treated with 1 μ g/ml biotinylated rabbit anti-angiocidin IgG in TBST overnight at 4° C with shaking. After three washes in TBST, membranes were incubated with 0.01 μ g/ml streptavidin-HRP in TBST for one hour at room temperature. Blots were developed using the chemiluminescence (ECL) system (Amersham, Arlington Heights, IL).

Tissue Culture

Human hepatocellular carcinoma cell line (Hep3B) was grown DMEM medium (Sigma Chemical Co) supplemented with 10% fetal bovine serum (FBS), 50 U/ml of penicillin, 50 μ g/ml of streptomycin and 50 μ g/ml of gentamicin sulfate and kept in 5% CO₂ at 37°C.

To determine the levels of secreted angiocidin, 10⁶ Hep3B cells were seeded in a 75 cm² flask and grown for 72 hours in 10 ml of media. After 72 hours, media was removed and centrifuged for 20 min at 3,000 $\times$ g and angiocidin levels in the supernatant measured by ELISA.

Statistical Analysis

Mann-Whitney U and Wilcoxon rank tests were used to evaluate differences between unpaired and paired observations, respectively. Statistical significance was taken as P<0.05. All statistical analyses were performed using the SPSS 11.0 statistical software package (SPSS Inc., Chicago, IL).

Results

A capture ELISA measures picogram/ml levels of angiocidin

We developed a sensitive capture ELISA for the measurement of angiocidin levels in biological fluids. A schematic of the assay is shown in Fig. 1. A mouse monoclonal antibody against

angiocidin is adsorbed on the surface of microtiter wells and serves as the capture antibody. This antibody predominantly recognizes a single 55 kDa band in most tumor cell extracts including HCC (Fig. 2). Higher molecular forms of angiocidin are also detected in whole cell extracts of our HCC tumor cell line (Fig 2 lane 3 in panel marked angiocidin blot) as well as other tumor lines (unpublished results). In serum of HCC patients angiocidin was undetectable by Western blot analysis (Fig. 2 lane 3, 4 in panel marked angiocidin blot). However, in our ELISA assay system low levels of antigen can be readily detected. The captured antigen is bound by a biotinylated rabbit anti-angiocidin antibody and the signal from the final antigen-antibody complex is amplified by coupling to streptavidin HRP and its substrate TMB producing a blue color. The reaction is stopped with 50 μ l of 0.5 M sulfuric acid and the yellow color read at 450 nm on an ELISA reader. The absorbance readings generated by this assay as a function of angiocidin concentration are linear in the range of 15 to 500 pg/ml (Fig. 3) and maximum absorbances generally range from 1 to 1.5 absorbance units. Background absorbances in the absence of angiocidin range from 0.150 to zero when the biotinylated second antibody is sufficiently titrated out.

Serums from normal healthy individuals generally contain angiocidin levels below the assay detection limit of 10 pg/ml of angiocidin. When normal serum diluted 1:5 and 1:10 with high salt TBST is used as the diluent for angiocidin and compared to high salt TBST alone, angiocidin is captured and recovered 84% in the 1:5 serum and 100% in the 1:10 serum. (Table 1). Therefore, serum diluted 1:10 can be assayed with 100% recovery.

The intra-assay variability is generally quite low when multiple standard curves are generated in the same day using the same plate containing immobilized capture antibody (Table 2). Significantly more inter-assay variability is observed on successive days on freshly incubated plates (Table 2).

Angiocidin is secreted by a HCC cell line

When we cultured an HCC cell line, Hep3B, significant angiocidin was detected in the conditioned media after 48 hours (mean of 35 pg/ml or 22 ng/ 10^6 cells, Fig. 4). These results indicate that HCC cells can secrete angiocidin.

Angiocidin serum levels in HCC correlate with disease progression

Using our capture ELISA, we analyzed angiocidin levels in the serum of 33 HCC patients as well as 17 normal healthy controls and 10 HBsAg carriers without cancer. We found that angiocidin levels in both control groups ranged below the detection limit of 10 pg/ml angiocidin while the HCC patient group expressed serum angiocidin levels in the range of 15.09–195.73 pg/ml with a mean of 80.34 pg/ml (Fig. 4).

In order to investigate the clinical significance of serum angiocidin levels in HCC progression, serum angiocidin levels were compared between patient groups expressing various disease related parameters. These are summarized in Table 3 and include patient groups with the following: HBsAg status, cirrhosis, serum AFP, tumor size, TNM staging, encapsulation, venous invasion, and microsatellite tumor nodule. We only found statistically higher levels of angiocidin in the serum of patients who had microsatellite nodules in their tumors and whose tumors were stage III and IV. Near statistical significance, $p < 0.065$, was also observed in the serum levels of angiocidin for patients that expressed HBsAg as compared to those with no hepatitis B surface antigen HBsAg in their serum. Statistical significance may have been achieved if more hepatitis B negative patients (only 4 negative patients were tested) were tested. Interestingly, no correlation was observed in patients expressing high serum AFP levels with those expressing high angiocidin levels. These results suggest that angiocidin could

complement the prognostic value of AFP especially in predicting severity of the stage and metastatic status of HCC patients.

Discussion

In 1993 we isolated a protein from lung carcinoma by peptide affinity chromatography using a highly hydrophobic sequence from the type 1 repeat domain of thrombospondin-1, a matrix protein over-expressed in tumor stroma [5]. The immunohistochemical localization of this protein showed selective expression on invasive cancer cells and capillary endothelial cells [8–10]. These results suggested that the protein could function in mechanisms of metastasis and angiogenesis.

Two years ago, we cloned the full-length cDNA for this protein and expressed the protein in bacteria. The recombinant protein, referred to as angiocidin, had potent anti-angiogenic and anti-tumor activity [3]. The recombinant angiocidin induced endothelial apoptosis when added to growth cultures of endothelial cells in full media or when cells were plated on angiocidin adsorbed on tissue culture plastic. Antibodies prepared against the recombinant angiocidin showed over-expression of angiocidin in hepatocellular carcinoma [6], prostate, lung, bladder, and melanoma cancers [3]. Angiocidin showed both matrix and cytoplasmic distribution. A Blast search of the full-length cDNA of angiocidin revealed that the protein sequence had a high degree of homology with S5a [11] and antisecretory factor [12] but differed from them by three additional amino acids in its carboxyl terminus (G₂₅₅ER₂₅₇). Since it is not clear how many genes encode for angiocidin and its homologues, we have elected to call our recombinant protein angiocidin, based on its observed functional activity in inhibiting angiogenesis.

A major focus of our research is to discover how angiocidin blocks tumor progression and angiogenesis despite the fact that tumor cells and tumor vasculature over-express angiocidin and its homologues. If angiocidin is a tumor suppressive protein, then why do tumor cells over-express this protein and its homologues and why should our recombinant protein inhibit tumor growth? One explanation is that angiocidin and its homologues function as endogenous inhibitors of angiogenesis and tumor growth in much the same way as has been shown for the tumor inhibitor angiostatin by Folkman and colleagues [13]. These inhibitors circulate and bind to tumors at distant sites and inhibit their growth and vascularization. Consistent with this concept, we have detected high picogram per ml levels of angiocidin in the sera of patients with prostate, breast, and HCC while the sera of healthy individuals contained levels undetectable by our ELISA assay which is sensitive to low picogram per ml levels [14–16]. In this study we describe the ELISA assay as well as the results showing that serum levels of angiocidin correlate with disease progression in HCC. Furthermore our results suggest that circulating blood levels of angiocidin may arise from angiocidin secreted by the tumor.

We developed a sensitive capture ELISA for the measurement of angiocidin levels in biological fluids. The ELISA uses a mouse monoclonal antibody as a capture antibody and a biotinylated rabbit polyclonal antibody as the detector (Fig. 1). Serum in dilutions as low as 1:10 did not significantly interfere with the assay since we could recover 100% of exogenously added angiocidin (Table 1). In the range 15–500 pg/ml the ELISA was highly reproducible and the standard curve was linear (Fig. 3). The quantitative lower limit of the assay was 10 pg/ml. The intra- and inter-assay variations ranged from 1.4% to 18.9%, respectively (Table 2). More variability (18.9%) was observed when the assay was performed on successive days. We don't know the reason for this but it may be related to subtle differences in binding activity each time the capture antibody is adsorbed onto the plastic.

Our antibodies recognize a major band at 55 kDa polypeptide and slightly higher bands in HCC cell extracts (Fig. 2). The major 55 kDa protein in HCC extracts is similar in size to recombinant

angiocidin. The higher bands in the total cell extracts may represent partially reduced forms of angiocidin protein since angiocidin contains four cysteine residues capable of forming disulfide bonds. We found no reactivity of our antibodies with serum samples from either normal patients or cancer patients as seen by Western blot analysis. Angiocidin in cancer patients' sera was present in too low a concentration to detect by Western blot analysis (Fig. 2).

Using our ELISA assay, we have been able to detect angiocidin in tumor-conditioned media, and serum of cancer patients (Table 2). A major conclusion of these studies is that serum from normal healthy volunteers or those non-cancer HBsAg carriers had angiocidin levels below the assay detection limits of 10 pg/ml. In contrast HCC patients had elevated levels of angiocidin in a broad range with a mean value of 80.34 pg/ml (Table 3, Fig. 4). We found a statistically significant difference in angiocidin serum levels in patients with stages III–IV of HCC (97 ± 13 pg/ml, $n=17$) compared to those with stages I–II (63 ± 37 pg/ml, $n=16$), $p<0.043$. Although the means of these groups showed a significant difference, there was a considerable overlap in the angiocidin levels between the groups. More patient samples are needed before we can confidently conclude that angiocidin levels are predictive of disease stage in HCC. A significantly better correlation was found in microsatellite tumor nodule status among the HCC patients. A microsatellite nodule indicates intrahepatic metastasis [17]. In our patient group we found a much higher statistical correlation with less overlap in the angiocidin serum levels of patients with microsatellite tumor nodules (98 ± 55 pg/ml, $n=17$) compared to those with no microsatellite nodules (51 ± 27 pg/ml, $n=20$), $p<0.032$. However, no statistically significant correlation was found in patients showing evidence of venous invasion as compared to those with no venous invasion. The significant correlation of serum angiocidin levels with the presence of microsatellite nodules is highly significant because even in early HCC lesions, intrahepatic metastasis occurs in 10% of cases, and identification of these cases is important for decision on treatment options that include a number of minimally invasive locoregional therapies such as thermal ablation and cryotherapy, which may not be effective in the presence of microscopic metastasis. Our data support a further large-scale study to evaluate the prognostic significance of serum angiocidin levels on tumor recurrence and long-term survival in HCC patients.

The origin of serum angiocidin is yet unknown. The most likely source of secreted angiocidin is tumor cells. We have shown by immunohistochemical staining studies that angiocidin is over-expressed in HCC [6] as well as other tumor types such as breast [8], colon [10], head and neck [9], prostate and melanoma [3]. Additionally, in HCC there is enhanced gene expression of antiseecretory factor [18], a highly homologous protein that lacks three amino acids contained in the carboxyl terminus of angiocidin [3]. Our assay would not differentiate between such isoforms of angiocidin. *In vitro*, we have shown that an HCC cell line, Hep3B, secretes angiocidin into the conditioned media as measured by ELISA. We also have data that other cell types such as platelets and monocytes secrete angiocidin (data not shown). Therefore, the increased serum levels of angiocidin in HCC patients could arise from a variety of sources with the most likely source being tumor tissue.

In summary our data suggest that serum levels of angiocidin may have prognostic value for the detection and prognosis of HCC. High serum values may not only indicate the presence of HCC but also predict stage and metastatic state of the disease. We feel that serum angiocidin values together with information of other blood markers of HCC may prompt a more cautious response for clinical decision making and providing better and curable treatment options for HCC patients worldwide.

References

1. Varela M, Sala M, Llovet JM, Bruix J. Treatment of hepatocellular carcinoma: is there an optimal strategy? *Cancer Treat Rev* 2003;29:99–104. [PubMed: 12670452]
2. Zhou L, Liu J, Luo F. Serum tumor markers for detection of hepatocellular carcinoma. *World J Gastroenterol* 2006;12:1175–1181. [PubMed: 16534867]
3. Zhou J, Rothman VL, Sargiannidou I, Dimitrov S, Qiu C, Smith E, Sheffield J, Sharma M, Tuszynski GP. Cloning and characterization of angiocidin, a tumor cell binding protein for thrombospondin-1. *J Cell Biochem* 2004;92:125–146. [PubMed: 15095410]
4. Sabherwal Y, Rothman VL, Dimitrov S, L'Heureux D, Marcinkiewicz C, Sharma M, Tuszynski GP. Integrin $\alpha 2\beta 1$ mediates the anti-angiogenic and anti-tumor activities of angiocidin, a novel tumor-associated protein. *Exp Cell Res* 2006;312:2443–2453. [PubMed: 16762342]
5. Tuszynski GP, Rothman VL, Papale M, Hamilton BK, Eyal J. Identification and characterization of a tumor cell receptor for CSVTCG, a thrombospondin adhesive domain. *J Cell Biol* 1993;120:513–521. [PubMed: 8421063]
6. Poon RT, Chung KK, Cheung ST, Lau CP, Tong SW, Leung KL, Yu WC, Tuszynski GP, Fan ST. Clinical significance of thrombospondin 1 expression in hepatocellular carcinoma. *Clin Cancer Res* 2004;10:4150–4157. [PubMed: 15217952]
7. Tahara T, Usuki K, Sato H, Ohashi H, Morita H, Tsumura H, Matsumoto A, Miyazaki H, Urabe A, Kato T. A sensitive sandwich ELISA for measuring thrombopoietin in human serum: serum thrombopoietin levels in healthy volunteers and in patients with haemopoietic disorders. *Br J Haematol* 1996;93:783–788. [PubMed: 8703803]
8. Tuszynski GP, Nicosia RF. Localization of thrombospondin and its cysteine-serine-valine-threonine-cysteine-glycine-specific receptor in human breast carcinoma. *Lab Invest* 1994;70:228–233. [PubMed: 7511188]
9. Arnoletti JP, Albo D, Jhala N, Granick MS, Solomon MP, Atkinson B, Rothman VL, Tuszynski GP. Computer-assisted image analysis of tumor sections for a new thrombospondin receptor. *Am J Surg* 1994;168:433–436. [PubMed: 7526719]
10. Wakiyama T, Shinohara T, Shirakusa T, John AS, Tuszynski GP. The localization of thrombospondin-1 (TSP-1), cysteine-serine-valine-threonine-cysteine-glycine (CSVTCG) TSP receptor, and matrix metalloproteinase-9 (MMP-9) in colorectal cancer. *Histol Histopathol* 2001;16:345–351. [PubMed: 11332689]
11. Young P, Deveraux Q, Beal RE, Pickart CM, Rechsteiner M. Characterization of two polyubiquitin binding sites in the 26 S protease subunit 5a. *J Biol Chem* 1998;273:5461–5467. [PubMed: 9488668]
12. Nilsson LA, Lange S, Lonnroth I. Induction of anti-secretory factor in mice by the nonintestinal parasite *Schistosoma mansoni*. *J Parasitol* 1992;78:1055–1058. [PubMed: 1491298]
13. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 1997;88:277–285. [PubMed: 9008168]
14. Sabherwal Y, Rothman V, Albo D, Tuszynski G. Angiocidin, an endogenous inhibitor of angiogenesis, is increased in the sera of cancer patients. *Proceedings of the American Association for Cancer Research* 2004;45:1143.
15. Sabherwal Y, Rothman VL, Tuszynski GP. Sensitive capture ELISA for measuring angiocidin in human serum: serum angiocidin levels in healthy volunteers and in prostate cancer patients. *Proceedings of the American Association for Cancer Research* 2005;46:743.
16. Sabherwal Y, Rothman VL, Poon RT, Tuszynski GP. Clinical significance of serum angiocidin levels in hepatocellular carcinoma. *Proceedings of the American Association for Cancer Research* 2005;46:1319.
17. Okusaka T, Okada S, Ueno H, Ikeda M, Shimada K, Yamamoto J, Kosuge T, Yamasaki S, Fukushima N, Sakamoto M. Satellite lesions in patients with small hepatocellular carcinoma with reference to clinicopathologic features. *Cancer* 2002;95:1931–1937. [PubMed: 12404287]
18. Tanaka K, Kondoh N, Shuda M, Matsubara O, Imazeki N, Ryo A, Wakatsuki T, Hada A, Goseki N, Igari T, Hatsuse K, Aihara T, Horiuchi S, Yamamoto N, Yamamoto M. Enhanced expression of

mRNAs of antisecretory factor-1, gp96, DAD1 and CDC34 in human hepatocellular carcinomas. *Biochim Biophys Acta* 2001;1536:1–12. [PubMed: 11335099]

Fig 1.

Angiocidin capture ELISA. Schematic representation of angiocidin capture ELISA. Angiocidin is captured by an immobilized monoclonal antibody and the bound angiocidin detected with a biotinylated second anti-angiocidin antibody as described in MATERIALS AND METHODS. Antibody antigen complex is detected with streptavidin HRP followed by development with the HRP substrate, TMB.

Fig 2.

Western blot analysis of HCC patient sera. Samples were separated by SDS-PAGE and blotted with anti-angiocidin antibody as described in MATERIALS AND METHODS. Lane 1, molecular weight standards; Lane 2, 1 µg purified recombinant angiocidin; Lane 3, 10 µg of HepB3; lane 4, 5 µg of serum from a normal volunteer; lane 5, 5 µg of serum from an HCC patient.

Fig 3. Standard curve for angiocidin ELISA. Angiocidin levels in the range of 10–300 pg/ml showed a linear dose-response in the ELISA. Data points are the mean of three determinations and the error bars represent the standard error of the mean. The data points were fitted to a straight line and the correlation coefficient was 0.97. The data was analyzed using GraphPad Prism version 3.02.

Fig 4.

Scatter plot of serum angiocidin levels in normal healthy volunteers, normal hepatitis B surface antigen (HBsAg) carriers, HCC patients, and the 48 hour conditioned media of the hepatocellular carcinoma cell line, Hep3B. Angiocidin levels were measured by ELISA as described in MATERIAL AND METHODS. 17 normal healthy volunteers, 10 HBsAg carriers and 33 HCC patients were studied. The data was analyzed using GraphPad Prism version 3.02.

Table 1

Recovery of angiocidin in normal serum

Angiocidin Sample (ng)	O.D. (450 nm)	% Recovery
High salt TBST diluent	0.358	100
1.25	0.580	100
2.5	0.829	100
5		
1:5 serum diluent	0.285	80
1.25	0.494	85 Av. 84
2.5	0.720	87
5		
1:10 serum diluent	0.361	100.8
1.25	0.583	100.5
2.5	0.840	101
5		

Table 2

Intra- and inter-assay ELISA variability					
	n	Mean/OD	Mean	S.D.	Std. Error of Mean
Intra-assay	1	0.959	0.98025	0.01455	0.007273
	2	0.985			
	3	0.985			
	4	0.992			
Inter-assay	1	0.678	0.87575	0.1894	0.09472
	2	0.915			
	3	1.12			
	4	0.79			

Table 3

Serum angiocidin levels in patients with HCC

VARIABLE	PARAMETER	N = 33	ANGIOCIDIN ($\mu\text{g/ml}$)	P value
Age	Mean=55	N = 19	0.086 $\pm$ 0.051	0.048
	<55 years	N = 14	0.074 $\pm$ 0.046	
	$\geq$ 55 years			
Sex	Male	N = 28	0.080 $\pm$ 0.050	0.89
	Female	N = 5	0.084 $\pm$ 0.050	
Hepatitis B surface Antigen	Negative	N = 4	0.058 $\pm$ 0.018	0.065
	Positive	n = 29	0.085 $\pm$ 0.052	
Cirrhosis	Absent	N = 15	0.084 $\pm$ 0.052	0.764
	Present	N = 18	0.079 $\pm$ 0.048	
Serum AFP level	<20 ng/ml	N = 12	0.068 $\pm$ 0.040	0.205
	$\geq$ 20 ng/ml	N = 21	0.089 $\pm$ 0.053	
Tumor Size	<5 cm	N = 16	0.086 $\pm$ 0.047	0.611
	$\geq$ 5 cm	N = 17	0.077 $\pm$ 0.052	
TNM staging	I–II	N = 16	0.063 $\pm$ 0.037	0.043*
	III–IV	N = 17	0.097 $\pm$ 0.013	
Encapsulation	Absent	N = 14	0.068 $\pm$ 0.044	0.76
	Present	N = 9	0.074 $\pm$ 0.044	
Venous Invasion	Absent	N = 20	0.071 $\pm$ 0.039	0.379
	Present	N = 13	0.087 $\pm$ 0.053	
Microsatellite Tumor Nodule	Absent	N = 20	0.051 $\pm$ 0.027	0.032*
	Present	N = 13	0.098 $\pm$ 0.055	

* significance, $p < 0.05$