

Reduced expression of ASS is closely related to clinicopathological features and post-resectional survival of hepatocellular carcinoma

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Abstract. Argininosuccinate synthetase (ASS) has previously been proven to be reductively expressed in hepatocellular carcinoma (HCC) and various types of HCC cell lines. Arginine, the product of ASS, has been used as a target in HCC by recombinant human arginase or arginine deiminase, which is now in the phase II clinical trial stage. This study aimed to present the levels of ASS expression in HCCs and its correlation with clinicopathological features and prognosis of HCC patients. Immunohistochemical detection of ASS was performed on samples from 71 patients with HCC. Positive staining was found in 21 HCCs, with a score of 2, as well as in normal liver tissues. Reduced ASS staining was found in 70.4% (50/71) of HCC tissues, including 21 with a score of 0 and 29 with a score of 1. The staining score in cancer tissues was significantly associated with gender, background liver, histopathological differentiation, recurrence, TNM staging and portal vein invasion ($P<0.05$), but not with age, viral status, tumor size and serum α -fetoprotein level. Patients with a high ASS expression had significantly poorer overall and disease-free survival ($P<0.001$ and $P<0.001$, respectively). These data showed that ASS was reductively or negatively expressed in a large portion of HCC, and that ASS levels in HCCs correlated inversely with prognosis. In conclusion, a high expression of ASS may be a novel marker of poor prognosis of patients presenting with HCC.

Introduction

Hepatocellular carcinoma (HCC), one of the most common fatal malignancies in many countries in Asia and Africa, is the

leading cause of cancer mortality in China and its incidence is also increasing in Western countries (1). HCC is complex in etiology and pathogenesis (2). Risk factors include aflatoxin, alcohol, hepatitis B virus, hepatitis C virus and cirrhosis. Different risk factors lead to different molecular pathways, making the pathogenesis of HCC more complex to interpret (3,4). Surgical resection provides an opportunity for cure, but the outcome of surgical HCC patients remains grave mainly due to frequent tumor recurrence (5-7). Other therapeutic strategies have a similar shortcoming. Although HCC prognosis depends on various elements, the biological characteristics of HCC are important. Thus, in order to aid in the prediction of prognosis or the selection of a therapeutic target, more potential markers should be explored.

Arginine is an indispensable amino acid to children, but one which is semi-essential to adults (8). Since 1930, arginine has been known to influence the growth of transplantable mice tumors (9). Argininosuccinate synthetase (ASS) is a key enzyme in the synthesis of arginine from citrulline and is present in all tissues and culture cells studied. This metabolic pathway allows cells to synthesize arginine from citrulline, making this amino acid non-essential for the growth of humans and most mammalian cells. It is well known that the highest ASS activity is found in the liver, where the enzyme is involved in the urea cycle and in the elimination of ammonia (10). Published studies showed HCC to be auxotrophic for arginine due to the lack of expression of ASS (11-14). However, the relationship between reduced levels of ASS expression in HCC tissues and its clinicopathological significance remains unclear.

The present study aimed to determine the relationship between the reduced level of ASS expression in HCC tissues by immunohistochemical detection (IHC) and the clinicopathological features and post-resectional survival. Our results suggested that the ASS staining score can be used as an independent marker for the prognosis of patients presenting with HCC.

Materials and methods

Patients. Tissue samples were obtained from 71 patients with HCC who underwent surgical resection in the Union Hospital

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Table I. Relationship between ASS expression and clinicopathological features of HCC.

Variables	Patients	ASS expression			P-value ^a
		Low	Middle	High	
All cases	71	21	29	21	
Gender					0.020
Male	59	14	28	17	
Female	12	7	1	4	
Age (years)					0.787
<60	47	14	18	15	
≥60	24	7	11	6	
Background liver					0.047
Normal liver	3	1	2	0	
Chronic liver	21	10	9	2	
Cirrhosis	47	10	18	19	
Viral status					0.237
Hepatitis virus B	51	15	20	16	
Hepatitis virus C	12	3	6	3	
Both hepatitis virus B and C	2	0	0	2	
Non-B, Non-C	6	3	3	0	
Tumor size					0.630
<5 cm	38	13	15	10	
≥5 cm	33	8	14	11	
Portal vein invasion					<0.001
No	52	21	21	10	
Yes	19	0	8	11	
Histopathological differentiation					0.008
Well-differentiated	21	11	6	4	
Moderately differentiated	26	8	13	5	
Poorly differentiated	24	2	10	12	
Serum AFP level					0.301
<25 ng/ml	35	13	14	8	
≥25 ng/ml	36	8	15	13	
TNM stage					<0.001
I-II	25	18	6	1	
III-IV	46	3	23	20	
Recurrence					0.015
No	17	9	7	1	
Yes	54	12	22	20	

ASS, arginosuccinate synthetase; HCC, hepatocellular carcinoma; AFP, α -fetoprotein. ^aChi-square test.

Affiliated to Tongji Medical College from 2000 to 2002. The patients included 59 males and 12 females, with a median age of 49 years (range 33-74). The clinicopathological features of the patients including gender, age, background liver, viral status, tumor size, portal vein invasion, histopathological differentiation, serum α -fetoprotein (AFP) level, TNM staging and recurrence of HCC are shown in Table I. The Institutional Ethics Committee approval for the project was obtained prior to the commencement of the study.

Immunohistochemical staining. ASS expression was detected immunohistochemically for paraffin-embedded specimens from 71 patients with HCC. Surgical specimens were formalin-fixed (10%), paraffin-embedded and sectioned at 4 μ m. For heat-induced epitope retrieval, deparaffinised sections were soaked in 10 mM citrate buffer (pH 6.0) and treated at 95°C for 30 min in a microwave oven. Immunohistochemical staining was performed using the avidin-biotin immunoperoxidase technique. Endogenous peroxidase activity was first

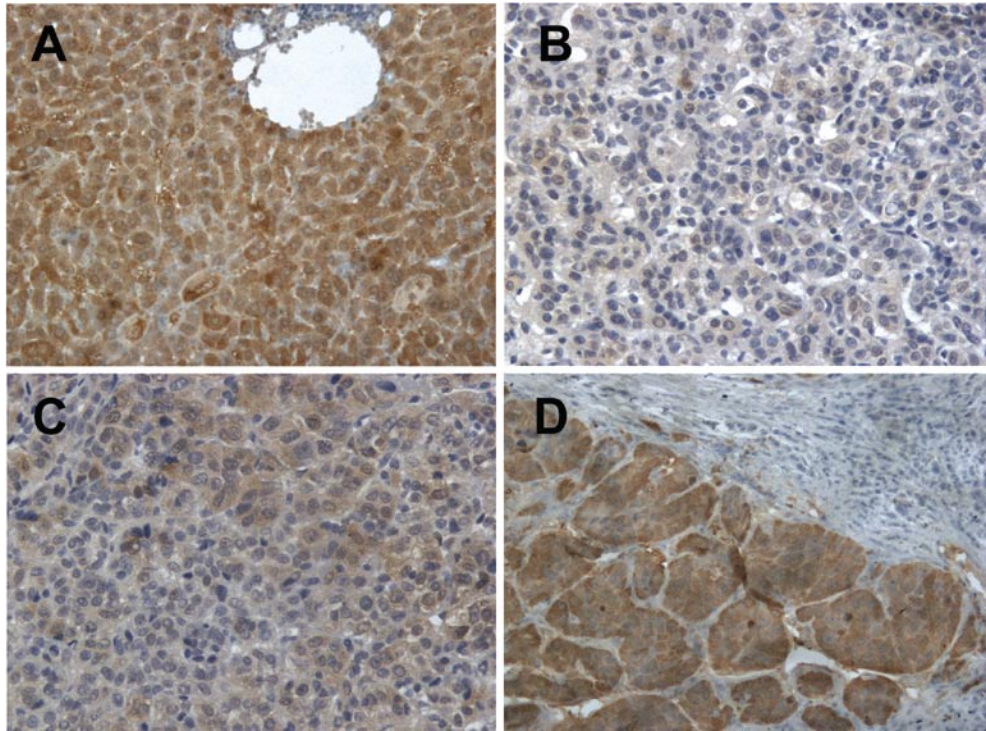


Figure 1. Representative staining pattern of ASS expression detected by IHC (positivity + intensity). (A) Normal liver tissue. (B, C and D) HCC tissues; B, score of 0+0; C, score of 1+1; D, score of 2+2. Original magnification x200.

blocked by incubation with 0.3% H_2O_2 in methanol for 15 min. Non-specific immunoglobulin binding was then blocked by incubation with 10% normal goat serum for 10 min. Sections were incubated at room temperature for 2 h with the anti-ASS monoclonal antibody (BD Pharmingen) at a 1:50 dilution. The sections were rinsed and incubated for 30 min with biotinylated second antibody. After washing, the sections were incubated for 30 min with horseradish peroxidase-conjugated streptavidin and treated with 3,3'-diaminobenzidine tetrahydrochloride in 0.01% H_2O_2 for 10 min. The slides were counterstained with Meyer's hematoxylin. As a negative control, the primary antibody was replaced with normal mouse IgG at an appropriate dilution.

Staining assessment and scoring. ASS expression levels were classified semi-quantitatively based on the total combined scores of the percentage of positively stained tumor cells together with the staining intensity. A tumor was scored as '0' if <5% of tumor cells were stained positive, '1' if 5-50% were stained positive and '2' if >50% of the cells were stained positive. The staining intensity was scored as '0' if no staining of cells occurred or if there was only weak staining, '1' if there was moderate staining and '2' in cases of strong staining. The final score of ASS expression was defined as 'low ASS expression' if the sum of the positivity score and the staining intensity score was 0-1, 'middle ASS expression' if the sum was 2 and 'high ASS expression' if the sum was 3-4. In each case, at least three different tumor areas were evaluated and the mean of the results was taken as the final expression score. The scoring procedure was carried out twice by two independent pathologists who had no knowledge of the clinical data and corresponding

hematoxylin and eosin slides. The concordance rate between the two primary pathologists was >95%. In cases of significant disagreement, the slides in question were reviewed again simultaneously by the original two pathologists, together with a third pathologist, at a multiheaded microscope, in order to resolve the divergence of opinion.

Statistical analysis. The Chi-square test was used to show differences of categorical variables. Patient survival and differences were determined by the Kaplan-Meier method and log-rank test. Cox regression (proportional hazard model) was adopted for the multivariate analysis of prognostic factors. Statistical software package SPSS 11.5 (SPSS Inc., Chicago, IL) was employed for the analyses. $P < 0.05$ was defined as statistically significant.

Results

Clinical profiles of the patients. Tissue samples were obtained from 71 patients. Twenty-one (29.6%) of the tissue samples were histopathologically well-differentiated, 26 (36.6%) were moderately differentiated and 24 (33.8%) were poorly differentiated. Portal vein invasion of the tumor cells occurred in 19 (26.8%) of the 71 patients, but not in the remaining 52 (73.2%) patients (Table I). Up to August 31, 2008 (the census date), 22 (31%) patients were alive, while 49 (69%) patients had succumbed to the disease. Follow-up ranged from 3 to 93 months (median 36 months).

ASS expression in HCC and its clinicopathological significance. A reduced ASS expression was detected in HCC

Table II. Univariate survival analysis of overall and disease-free survival in 71 patients with HCC.

Variables	No. of cases	OS			DFS		
		Mean \pm SE (months)	95% CI	P-value ^a	Mean \pm SE (months)	95% CI	P-value ^a
Gender				0.354			0.424
Male	59	41 \pm 5	(32-50)		34 \pm 5	(25-43)	
Female	12	44 \pm 8	(28-59)		40 \pm 8	(25-55)	
Age (years)				0.673			0.920
<60	47	42 \pm 5	(32-52)		37 \pm 5	(26-47)	
\geq 60	24	38 \pm 6	(27-50)		32 \pm 6	(20-44)	
Background liver				0.004			0.002
Normal liver	3	69 \pm 4	(62-77)		65 \pm 4	(56-74)	
Chronic liver	21	62 \pm 8	(46-78)		57 \pm 9	(40-74)	
Cirrhosis	47	30 \pm 4	(22-38)		23 \pm 4	(16-31)	
Viral status				0.120			0.273
Hepatitis virus B	51	39 \pm 5	(30-49)		33 \pm 5	(23-42)	
Hepatitis virus C	12	41 \pm 10	(20-61)		36 \pm 11	(15-58)	
Both hepatitis virus B and C	2	19 \pm 10	(0-37)		9 \pm 4	(2-15)	
Non-B, Non-C	6	64 \pm 9	(45-82)		55 \pm 10	(37-74)	
Tumor size				0.860			0.980
<5 cm	38	42 \pm 6	(31-53)		35 \pm 6	(24-46)	
\geq 5 cm	33	42 \pm 6	(30-54)		36 \pm 6	(23-48)	
Portal vein invasion				<0.001			<0.001
No	52	54 \pm 5	(44-64)		46 \pm 5	(36-56)	
Yes	19	12 \pm 1	(10-15)		7 \pm 1	(5-10)	
Histopathological differentiation				0.005			0.008
Well-differentiated	21	60 \pm 8	(44-75)		50 \pm 8	(35-65)	
Moderately differentiated	26	42 \pm 6	(29-54)		37 \pm 7	(24-50)	
Poorly differentiated	24	25 \pm 5	(15-36)		19 \pm 6	(8-30)	
Serum AFP level				0.516			0.397
<25 ng/ml	35	45 \pm 6	(33-57)		39 \pm 6	(27-51)	
\geq 25 ng/ml	36	38 \pm 5	(27-48)		31 \pm 6	(20-42)	
TNM stage				<0.001			<0.001
I-II	25	75 \pm 6	(63-87)		67 \pm 6	(54-79)	
III-IV	46	25 \pm 4	(18-32)		19 \pm 4	(12-26)	
ASS expression				<0.001			<0.001
Low	21	75 \pm 6	(64-86)		66 \pm 6	(54-77)	
Middle	29	35 \pm 6	(24-47)		31 \pm 6	(19-43)	
High	21	17 \pm 4	(10-25)		11 \pm 4	(3-19)	

ASS, arginosuccinate synthetase; HCC, hepatocellular carcinoma; OS, overall survival; DFS, disease-free survival; AFP, α -fetoprotein. ^aLog-rank test.

tissues obtained from 50 patients (70.4%) in accordance with the aforementioned criteria. Normal liver tissue as a control exhibited positive staining (Fig. 1A) with 21, 29 and 21 HCC samples exhibiting staining scores of 0, 1 and 2, respectively (Fig. 1B-D). The staining scores were significantly associated with gender, background liver, histopathological differentiation, recurrence, TNM staging and portal vein invasion of HCC (Table I; $P < 0.05$), but not with age, viral status, tumor size

and serum AFP level of the tumor antigen marker (Table I; $P > 0.05$).

Factors influencing overall or disease-free survival of HCC patients after tumor resection. Using the Kaplan-Meier method and log-rank test, HCC tissues with a higher staining score of ASS were correlated with a shorter overall or disease-free survival of patients (Table II, Fig. 2A and B; log-rank value 33.00

Table III. Multivariate survival analysis of overall and disease-free survival in 71 patients with HCC.

Variables	OS			DFS		
	RR	95% CI	P-value ^a	RR	95% CI	P-value ^a
TNM stage	2.246	(0.834-7.234)	0.103	2.398	(0.841-6.837)	0.102
Background liver	0.934	(0.479-1.823)	0.842	1.387	(0.768-2.507)	0.278
Portal vein invasion	3.052	(1.507-6.179)	0.002	2.258	(1.085-4.698)	0.029
Histopathological differentiation	1.401	(0.903-2.172)	0.004	1.469	(0.923-2.337)	0.105
ASS expression	2.139	(1.283-3.568)	0.004	1.880	(1.128-3.133)	0.015

ASS, arginosuccinate synthetase; HCC, hepatocellular carcinoma; OS, overall survival; DFS, disease-free survival; RR, relative risk; CI, confidence interval. ^aCox regression test.

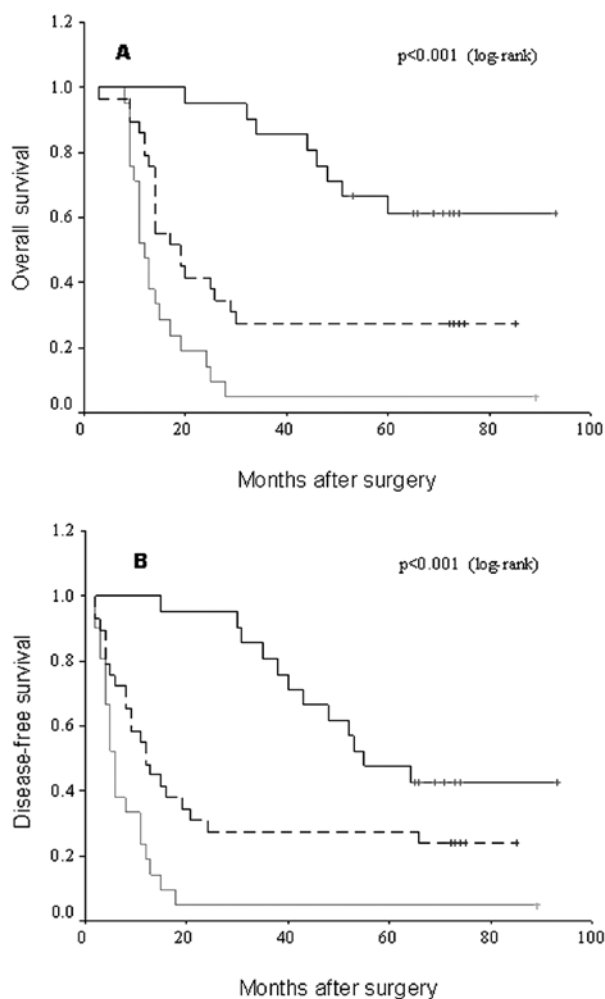


Figure 2. Comparison of survival of patients with HCC after resection based on staining scores of ASS. Score 0, black solid line; score 1, black dashed line; score 2, gray solid line. (A) Overall and (B) disease-free survival after resection.

and 27.72; $P<0.001$ and $P<0.001$, respectively). Survival benefits were found in patients with earlier TNM staging, a higher histopathological differentiation grade, absence of portal vein invasion and a better background liver for overall or disease-free survival (Table II; $P<0.05$), whereas other patients showed

no predictive values (Table II; $P>0.05$). The above-mentioned significant parameters were included in a multivariate Cox regression analysis which revealed that the ASS staining score (RR, 2.139; 95% CI, 1.283-3.568; $P=0.004$), portal vein invasion (RR, 3.052; 95% CI, 1.507-6.179; $P=0.002$) and histopathological differentiation (RR, 1.401; 95% CI, 0.903-2.172; $P=0.004$) were independent prognostic markers for overall survival of patients with HCC (Table III; $P<0.05$). On the other hand, the ASS staining score (RR, 1.880; 95% CI, 1.128-3.133; $P=0.015$) and portal vein invasion (RR, 2.258; 95% CI, 1.085-4.698; $P=0.029$) were independent prognostic markers for disease-free survival of patients with HCC (Table III; $P<0.05$).

Discussion

ASS is a key enzyme which converts citrulline to arginine. Tumors which usually express reduced ASS include hepatocellular carcinoma, melanoma, some mesotheliomas and some renal cell cancer types (11,14-19). To the best of our knowledge, this is the first study concerning ASS expression levels detected by IHC and its prognostic significance for HCC patients. Our results suggested that ASS is reductively expressed in a large portion (70.4%) of HCC tissues with a staining score of 0 or 1. Moreover, the magnitude of down-regulated expression correlated with better prognosis, especially for the staining score of 0. A reduced ASS expression predicts the same clinical value as early TNM staging, rare portal vein invasion and a reduced possibility of recurrence.

As previously reported, depletion of arginine causes substantial havoc to cells, in particular tumor cells (13). Due to their rapid proliferation, tumor cells require amino acids as nutrients. Specifically, arginine is the first amino acid that is exhausted and depleted faster than any other nutrient by normal cell metabolism, but exhibits low recycling efficiency (20). When arginine was depleted, favorable conditions were anticipated prior to the resumption of normal cell division. However, tumor cells, with their cell cycle aberrations, continued to progress with the cell cycle despite the absence of arginine, leading to a gross imbalance and cell death (20). Based on this fact, a strategy to degrade arginine was developed to cure ASS (-) tumors because ASS-expressed normal cells produce arginine from citrulline, whereas ASS (-) tumor

cells cannot. Several enzymes, including human arginase and arginine deiminase (ADI) which is a microbial enzyme from mycoplasma spp, are currently in phase II clinical trials (21-23). These enzymes have a high affinity to arginine and catalyze the degradation of arginine. It was reported that ADI activated anti-proliferation on a number of malignant cells *in vitro* and anti-tumor effects *in vivo* (15). The extent of ADI anti-proliferation depends on the endogenous activity of ASS, the rate-limiting enzyme involved in the recycling of citrulline into arginine. However, arginine depletion can induce ASS expression in certain melanoma cell lines which can lead to *in vitro* drug resistance (13).

A reduced expression of ASS in cancer cells and its higher expression in the normal liver cells of HCC patients suggested that only cancer cells are auxotrophic for arginine. Thus, ADI was able to exert its anti-tumor activity without any adverse effect on surrounding normal liver cells (16). ASS is the rate-limiting enzyme in the conversion of citrulline to arginine for ammonia detoxification through the urea cycle in the liver. A probable explanation for the loss of ASS activity in HCC is cellular dedifferentiation which may occur during tumorigenesis. Since dedifferentiation is a regressive process of cells to a primitive embryonic state, it may be related to a decrease of ASS expression in the neonatal level of liver. Thus, the level of ASS remains low during the fetal period in the liver (16,24). Another explanation involves the down-regulation of ASS gene expression at the transcriptional level by its promoter methylation (14).

HCC remains a troublesome malignancy with poor prognosis, due to its strong drug resistance to conventional cancer treatments, as well as its frequent metastasis and recurrence. In spite of extensive research and various therapies, a molecular marker needs to be found that can be used as a target for the treatment of HCC. The current study provided a novel candidate, ASS, as a molecular marker of both the biological behaviors and surgical outcome of HCC. Measurement by IHC of a reduced ASS expression was closely correlated with better prognosis and allowed for a prediction of post-resectional survival.

Taken together, ASS in cancer tissues was reductively expressed in numerous patients presenting with HCC. ASS had a direct correlation with cancer differentiation, TNM staging, portal vein invasion and recurrence, as well as the overall and disease-free survival of patients with HCC. Therefore, we concluded that ASS may be a novel marker for predicting prognosis of patients presenting with HCC, and suggest that ASS be used as a target for the treatment of HCC. In order to improve therapy for HCC, more studies need to be conducted in order for ASS expression or activity in tumor cells to be attenuated.

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