ORIGINAL ARTICLE

AFP-L3 in Chronic Liver Diseases with Persistent Elevation of Alpha-fetoprotein

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Background: Alpha-fetoprotein (AFP) is an important marker for hepatocellular carcinoma (HCC). However, persistent elevation of AFP is found in patients with chronic liver diseases. The value of AFP-L3, which is more specific than AFP, was examined in such patients.

Methods: We enrolled patients without image-detectable tumor, but with transient AFP value > 900 ng/mL (group A) or with persistent AFP value > 50 ng/mL for longer than 6 months (group B). Forty-one patients with HCC and AFP value > 50 ng/mL were included as the HCC control group (group C). AFP-L3 measurement was done by lectin-affinity electrophoresis coupled with antibody-affinity blotting. The study patients were followed with AFP, liver biochemistry and abdominal ultrasound at 3- to 6-month intervals. Additional studies were done when a tumor was suspected.

Results: One of 17 patients in group A and 13 of 39 patients in group B developed HCC within 2 years. When the cutoff value of AFP-L3 ratio was 15%, both the sensitivity and specificity were 71% for prediction of HCC during the next 2 years in all patients. Ninety percent of tumors larger than 5 cm had AFP-L3 > 15%, compared with only 60% for tumors smaller than 2 cm. Three patients in group A had AFP-L3 ratio > 17.5%. One patient developed HCC 10 months later; the other 2 patients were associated with hepatic failure.

Conclusion: AFP-L3 provides a clue in HCC detection in patients with persistent elevation of AFP. However, AFP-L3 could be highly elevated in severe hepatitis. [*J Chin Med Assoc* 2007;70(8):310–317]

Key Words: α-fetoprotein, chronic hepatitis, hepatocellular carcinoma, lectin-reactive α-fetoprotein

Introduction

Alpha-fetoprotein (AFP) and ultrasonography are 2 important noninvasive modalities for early detection of hepatocellular carcinoma (HCC).[1] Cirrhotic nodules are found frequently with increasing use of abdominal ultrasound.[2] AFP elevation is not uncommon in such cases because liver inflammation is generally coexistent in the cirrhotic liver.[3,4] AFP-L3 is a fucosylated species of AFP that is the product of α1-6 fucosyltransferase in the presence of GDP fucose.[5–7] This enzyme activity was higher in HCC tissues than in the surrounding non-tumor tissue.[8] Therefore, AFP-L3 is considered more specific than AFP in diagnosis of HCC.[9–11] It has been used for early detection of HCC[9–18] and evaluation for recurrent tumor after treatment.[19–25]

Transient elevation of AFP in patients with chronic liver disease is associated with hepatocyte regeneration in responding to liver necroinflammation.[26,27] Persistent elevation of AFP is found in some of these patients. Detection of HCC by ultrasound in patients with elevation of AFP may be difficult when cirrhotic nodule is present. We evaluated AFP-L3 in patients with persistent elevation of AFP to understand whether it may be of help in the diagnosis of HCC.

Methods

Patients

Patients who visited Chang Gung Memorial Hospital with chronic liver disease and elevated AFP by radioimmunoassay were reviewed from January 2002 to December 2002. Patients with persistent elevation of AFP > 50 ng/mL for longer than 6 months were included as study group B. Patients with single elevation

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of AFP > 900 ng/mL and transaminase level > 200 U/L were included as liver regeneration control (group A). Patients with HCC and AFP > 50 ng/mL were randomly selected as the disease control group (group C). We selected patients in group B and C with total AFP > 50 ng/mL because it is the lowest limit for detection of AFP-L3. Total AFP > 900 ng/mL is relatively rare in patients with chronic hepatitis and generally associated with severe liver damage. Therefore, total AFP > 900 ng/mL was used as a lower enrolment limit for patients in group A. Patients who were diagnosed with acute hepatitis as evidenced by positive anti-HBc IgM or anti-HAV IgM were excluded from this study.

All of the patients received liver biochemistry, AFP, viral markers and abdominal ultrasound studies. Serum AFP was measured by radioimmunometric assay (ELSA2-AFP; CIS Bio-International, Cedex, France). Hepatitis B surface antigen (HBsAg) and anti-hepatitis C virus antibody (anti-HCV) were measured by enzyme-linked immunosorbent assay (Abbott Ausria-II and Abbott HCV-ELISA III; Abbott Laboratories, Abbott Park, IL, USA). Residual sera were frozen in –20°C until AFP-L3 study. This study was approved by the ethics committee of Chang Gung Memorial Hospital.

Assay of serum AFP-L3

AFP-L3 studies were done by lectin-affinity electrophoresis coupled with antibody-affinity blotting (AFP Differential Kit, kindly supplied by Wako Chemicals, Osaka, Japan). Sera with AFP > 200 ng/mL were diluted to approximately 100 ng/mL using normal human serum provided in the kids. Each prepared sample (4 μL) was applied to wells on the lectin-agarose gel. Before electrophoresis, the electrophoretic chamber and electrode buffer were cooled to below 15°C. Electrophoresis was carried out at a constant voltage of 200 V ± 10% for 50 minutes. After electrophoresis, separated AFP bands were blotted on nitrocellulose membranes pre-coated with affinity-purified equine antibodies to human AFP at room temperature for 30 minutes at 37°C with 4 mL of rabbit AFP antibody solution. Then the membrane was exposed to affinity-purified goat rabbit immunoglobulin G antibodies labeled with horseradish peroxidase for 30 minutes at 37°C. For coloring reaction, 10 mL of the enzyme-substrate solution was added. The system was kept at room temperature for 30 minutes and then gently washed and dried after color developed. AFP bands were then measured by scanning with a Fluor-S Multi-Imager (Bio-Rad Laboratories, Hercules, CA, USA). The bands appearing after electrophoresis are shown in Figure 1. The results of AFP-L3 are expressed as percentage of AFP-L3 to total AFP.

Subsequent clinical evaluation

Liver biochemistry, AFP and abdominal ultrasound studies were done prospectively at 3- to 6-month intervals after enrolment. Patients who failed to follow the study protocol were actively reminded by telephone.

Diagnosis of liver cirrhosis was mainly based on ultrasound scoring system,[28] varices detected by endoscopy or histology study.

Computed tomography (CT), magnetic resonance imaging (MRI), angiography, cytology and/or histology were done when HCC was suspected.

Diagnosis of HCC was made mainly by histology or cytology studies. Long-term evaluation of tumor progression by imaging and AFP studies were used for those patients who could not undergo histology or cytology studies.

Statistical analysis

Data are expressed as mean ± standard deviation. Statistical significance was determined by using the Student's t test for continuous variables and the χ^2 test for the difference in proportions between groups. Scheffe's method was used for multiple comparisons. Receiver operating characteristic curves were created to establish the optimal cutoff values. A life table was used to compare the HCC-free survival between patients with AFP-L3 greater or lower than 15%. Statistical analysis was performed using SPSS version 11.5.0 (SPSS Inc., Chicago, IL, USA) for Windows. A p value less than 0.05 was considered statistically significant.

Results

The clinical characteristics of the 17 patients in group A, 39 patients in group B and 41 patients in group C are listed in Table 1. Patients in group A were the youngest, with the highest alanine aminotransferase level, highest HBsAg prevalence and lowest rate of liver cirrhosis among the 3 groups. The clinical characteristics of the patients and diagnostic modalities of HCC are listed in Table 2. The mean AFP-L3 ratio was highest in group C, followed by group B and finally group A.

HCC and AFP-L3 cutoff value

HCC was detected in 1 patient in group B 20 months after enrolment. His AFP-L3 was strongly positive at the time of study (Figure 1). Based on this finding, we evaluated the value of AFP-L3 in prediction of HCC within 2 years after enrolment. One patient in group A and 13 patients in group B developed HCC

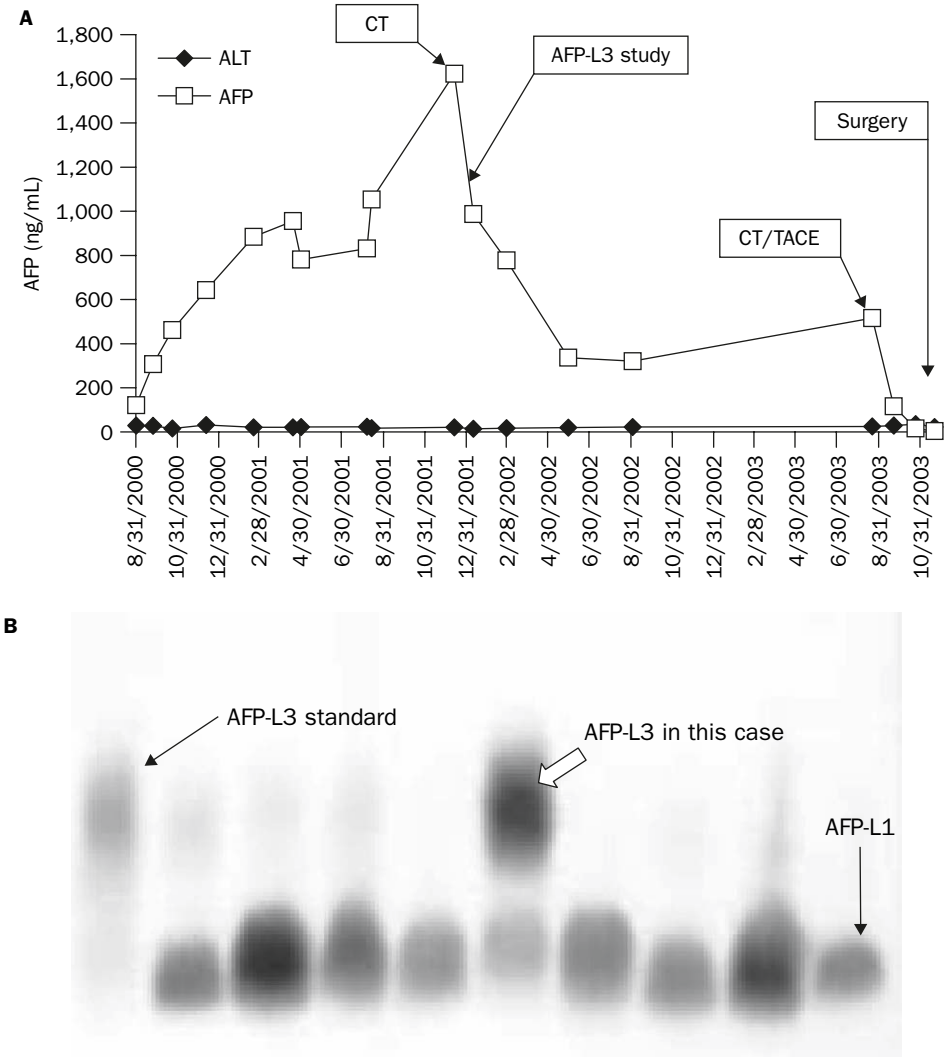

Figure 1. (A) A patient with persistent elevation of α-fetoprotein (AFP) without imaging-detectable hepatocellular carcinoma (HCC). An HBsAg carrier presented with persistently elevated total AFP level. Repeated ultrasound studies and a computed tomography (CT) scan were unable to locate the nodule. Three years later, a 2.5-cm nodule was found in the caudate lobe by imaging and histology studies. AFP dropped to normal after transhepatic arterial chemoembolization (TACE) and surgical excision. (B) A strong AFP-L3 (AFP-L3 to total AFP ratio, 70%; white arrow) was identified by lectin-affinity electrophoresis 20 months before the diagnosis of HCC.

within 2 years after AFP-L3 study. Additional HCC was detected in 2 patients in group A and 1 patient in group B during the third year.

In the total number of study patients, both sensitivity and specificity of AFP-L3 for prediction of HCC in the next 2 years were 71% if the cutoff value was 15% (Figure 2). The sensitivity decreased to 66.5% while the specificity increased to 82% if the cutoff ratio was 17.5%.

Similar results were found when group A was excluded. The sensitivity was 70% and specificity was 73% when the cutoff value of AFP-L3 ratio was 15%. The sensitivity decreased to 66.7% while the specificity increased to 88.5% if the cutoff ratio was 17.5%. When only group B was examined, the sensitivity was 53.8% and specificity was 72%.

When the cutoff value of AFP-L3 was 15%, the HCC prediction rate was 70.9% for the total number of study patients ($p=0.000$). For each group of patients, the HCC prediction rate was 100% in group A, 53.8% in group B ($p=0.098$) and 75.6% in group C (Table 3).

HCC-free survival analysis

We divided all the patients in group A and B into 2 groups according to AFP-L3 level at enrolment. By life table analysis, those patients with AFP-L3 ≥15% showed a lower HCC-free survival than those patients with AFP-L3 <15% ($p=0.072$; Figure 3).

Table 1. Demographic characteristics of study patients

	Group A (transient high AFP elevation)	Group B (persistent AFP elevation)	Group C (HCC)	Multiple comparison
Total case no.	17	39	41	
AFP at study	> 900 ng/mL	> 50 ng/mL	> 50 ng/mL	
M:F	15:2	22:17	30:11	A ≠ B = C
Age, yr	45.5 ± 15.1	56.7 ± 13.0	57.5 ± 13.8	A ≠ B = C
Duration of AFP elevation, yr (range)	–	3.6 ± 3.2 (0.5–15)	–	
Viral markers				
HBsAg(+)	15	11	19	
Anti-HCV(+)	1	19	16	
Dual infection	1	8	3	
NBNC	0	1	3	
AFP (ng/mL)	1,254.4 ± 354.4	151.4 ± 170.2	2,802.2 ± 7,493.3	C ≠ A ≠ B
ALT (mU/mL)	267.6 ± 226.4	129.7 ± 95.2	75.1 ± 67.9	A ≠ B ≠ C
Total bilirubin (mg/dL)	7.1 ± 9.2	4.1 ± 12.4	1.9 ± 2.2	A ≠ B ≠ C
Cirrhosis	5	31	40	A ≠ B = C

Table 2. Timing of detection and diagnostic modalities of hepatocellular carcinoma (HCC) in different groups

	Group A	Group B	Group C
Total case no.	17	39	41
HCC total cases/males	3/3	14/10	41/29
Time of HCC detection			
At enrolment	0	0	41
< 1 yr	1	8	–
1–2 yr	0	5	–
> 2 yr	2	1	–
Mean ± SD (mo)	33.0 ± 16.1	12.7 ± 8.0	–
Range	(9–42)	(4–25)	–
HCC-free follow-up duration, mo (range)*	22.2 ± 16.2 (1–42)	26.7 ± 13.3 (1–47)	–
CT/MRI study			
Before HCC detection	9	25	–
At HCC detection	3	14	39
Diagnosis of HCC			
Cytology	1	9	20
Liver biopsy	1	3	6
Longitudinal imaging studies	1	2	15
AFP-L3/total AFP ratio (%)†	14.2 ± 11.4	17.0 ± 13.9	32.4 ± 24.2

**Included mortalities with short follow-up period; †p < 0.001.*

Relation of tumor size with AFP-L3

AFP-L3 ≥ 15% was found in 57.1% of patients with HCC in group B when their tumors were undetectable. For tumors < 2 cm, 60% of them had AFP-L3 ≥ 15%; it was 81.3% for tumor size 2–5 cm and 90% for tumor > 5 cm. A significant correlation of AFP-L3 with tumor size was found ($p = 0.037$, χ^2 for trend).

Sequential AFP-L3 studies

Eleven patients in group B received a second AFP-L3 study when HCC was found. Six of them had AFP-L3

≥15% in both studies separated 6 to 35 months apart (mean, 16.7±11.8 months). In 2 of the 5 patients in whom initial AFP-L3 ratio <15%, it had become >15% in 5 and 28 months later (mean, 12.6±9.9 months), respectively.

A tail-like AFP antibody reactive variant was found during lectin-affinity electrophoresis in a 72-year-old woman with HCV antibody (Figure 4). Persistent elevation of AFP around 100 ng/mL was found for 2 years before the patient was enrolled into group B. The tail-like AFP variant protein migrated slower than AFP-L3 in lectin-affinity electrophoresis, which suggests a high affinity with lectin. Ultrasound-guided needle aspiration from the tumor showed dysplastic cells only. The tumors were small, multifoci and slow growing initially.

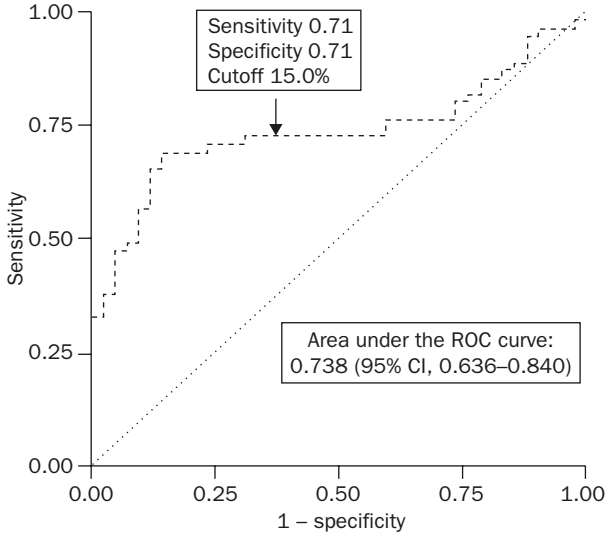

Figure 2. The receiver operating characteristic (ROC) curve for AFP-L3 in the total number of study patients.

The patient received repeated transhepatic arterial chemoembolization and local pure ethanol injections. One of the tumors had progressively enlarged to 6 cm when she died 5 years after the first AFP-L3 study.

Discussion

Different clinical characteristics were found between group A and B. HBV infection was the main etiology (94%) in group A, whereas HCV infection was the main etiology (70%) in group B. Liver cirrhosis was found in 75% of patients in group B and only 29% of patients in group A. This difference may be associated with different viral behaviors. HBV infection usually presents with intermittent acute exacerbation,[26] whereas HCV infection generally presents with mild persistent inflammation.[29–31]

Elevated AFP-L3 ratio ≥17.5% was found in 3 patients in Group A. A similar result was observed in patients with fulminant hepatitis who received liver transplantation.[32] Investigators found that AFP-L3 level correlated positively with weight of the removed liver. These data suggest that AFP-L3 is produced during regeneration and is not specific to HCC. Fortunately, the elevated AFP level in such situations is generally transient. We may differentiate regeneration from HCC easily by chronologic studies. If AFP level does not drop to normal level, further imaging and/or invasive studies are indicated.

In group B, 13 of the 39 patients developed HCC 2 years after enrolment, suggesting that this group of patients should be monitored closely. The sensitivity for HCC detection with AFP-L3 was 53.8% and the specificity was 70.0% when the AFP-L3 cutoff value

Table 3. AFP-L3 ratio at enrolment for prediction of hepatocellular carcinoma (HCC) and correlation with tumor size

Group	HCC	AFP-L3 <15%	AFP-L3 ≥15%	Total	*p*
A	Non-HCC	11 (68.7%)	5 (31.3%)	16	NS
	HCC	0 (0%)	1 (100%)	1	
B	Non-HCC	19 (73.1%)	7 (26.9%)	26	0.098
	HCC	6 (46.2%)	7 (53.8%)	13	
C	HCC	10 (24.4%)	31 (75.6%)	41	
Total	Non-HCC	30 (71.4%)	12 (28.6%)	42	0.000
	HCC	16 (29.1%)	39 (70.9%)	55	
Size of HCC at AFP-L3 study					
Undetectable	HCC	6 (42.9%)	8 (57.1%)	14	0.037
<2 cm	HCC	6 (40%)	9 (60%)	15	
2–5 cm	HCC	3 (18.7%)	13 (81.3%)	16	
>5 cm	HCC	1 (10%)	9 (90%)	10	

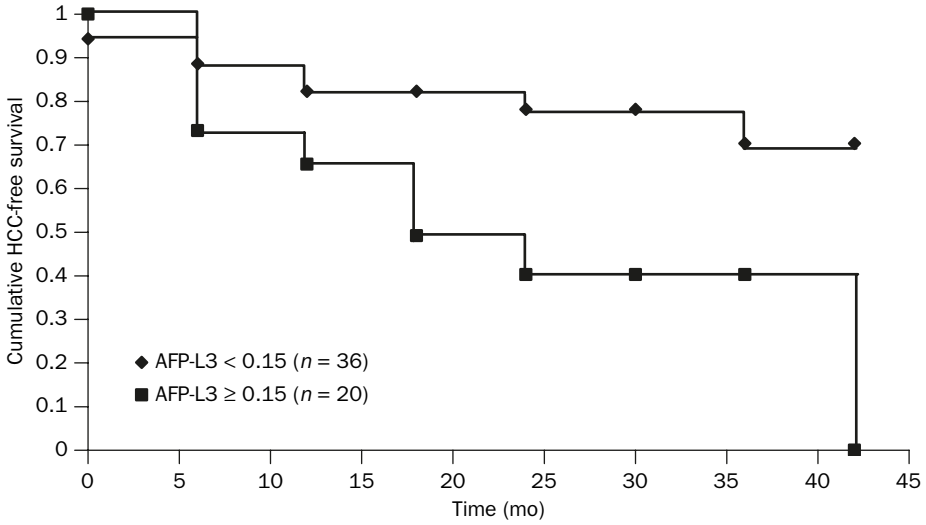

Figure 3. Hepatocellular carcinoma (HCC)-free survival analysis between patients with different AFP-L3 ratios in group A and B patients. Patients with AFP-L3 ratio ≥ 15% of total α-fetoprotein (AFP) developed more HCC and showed a lower HCC-free survival ($p = 0.072$).

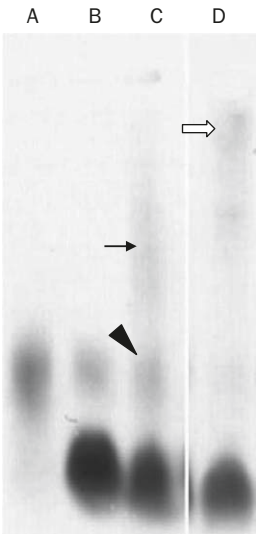

Figure 4. α-Fetoprotein (AFP) tailing phenomenon in a patient with hepatocellular carcinoma (HCC). A hepatitis C virus antibody-positive patient was found to have elevated AFP persistently for 2 years before the first AFP-L3 study. A faint AFP-L3 (Lane C, arrowhead) and an additional tail (arrow) were found. Computed tomography revealed liver cirrhosis without tumor at that time. The second AFP-L3 study was done 3 years later. The AFP antibody-reactive tail was still observed and migrated slower than in the first study (Lane D, white arrow). Aspiration cytology revealed dysplastic cells only, but both computed tomography and angiography studies showed multiple small hypervascular nodules in both lobes of the liver. Repeated pure ethanol ablation and transarterial chemoembolization were performed. The main tumor progressively enlarged to 6 cm and the patient died of hepatic failure 5 years after the first AFP-L3 study. Lane A is the standard loaded with 100 ng of AFP-L3. Lane B is of a patient with acute exacerbation (AFP-L3 ratio, 10%).

was 15%. The sensitivities of AFP-L3 from other reports were generally around 30% to 60%, while the specificity was around 70% to 90%.[9–25] Although the present study focused on patients with persistent elevation of AFP (group B), our results were similar to those of other reports. In patients with persistent elevation of AFP, the total AFP can no longer provide information for early detection. Our data suggest that about 60% of such patients may obtain reliable information for prediction of HCC.

The diagnosis of HCC is difficult in the early stage, even with histologic study. Taketa et al reported that AFP-L3 changed from negative to positive in 4.0 ± 4.9 months before HCC was detected by imaging study.[11] In this study, 8 of the 14 patients with HCC detected within 2 years had AFP-L3 ≥ 15% at 4 to 24 months before the imaging-detectable HCC. One such case is presented in Figure 1. The patient had high and persistent elevation of AFP 2 years before a 2.5-cm nodule in the caudate lobe was removed. The AFP-L3 ratio was 70% at 20 months before it was detected by imaging study. In group B, AFP-L3 elevation could be identified 12.4 months (range, 4–25 months) before the diagnosis of HCC.

A tail-like AFP antibody-reactive protein was found in a 72-year-old patient with HCV antibody. Persistent elevation of AFP around 100 ng/mL was found 2 years before she was enrolled into group B. This protein was not associated with poor preparation of sera, because 2 lectin-affinity electrophoreses separated by 36 months showed similar findings. The tumors were characterized by small, multifoci and slow growing nodules. This type

of AFP-L3 should be classified into positive test for HCC. More observations in this AFP variant are needed to disclose its significance.

In conclusion, AFP-L3 study provides a clue in HCC detection in patients with persistent elevation of AFP. However, AFP-L3 could be highly elevated in patients with severe liver damage.

Acknowledgments

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