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Original Article

Correlation of APOBEC3 in tumor tissues with clinico-pathological features and survival from hepatocellular carcinoma after curative hepatectomy

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Abstract: Objective: This study aimed to evaluate the relationships between members of APOBEC3 in tumor tissues and hepatocellular carcinoma (HCC) aggressiveness and prognosis. Methods: Using the expression profile GSE36376 from Gene Expression Omnibus (GEO), we compared APOBEC3 expression between tumor and non-tumor tissues, and correlated this with clinico-pathological features and outcomes of HCC patients. Results: A3B, A3D, A3F and A3H were overexpressed in HCC tumor tissues compared to non-tumor tissues (all $P \leq 0.001$). Cox regression shown that A3G was negatively associated with overall survival of HCC patients (HR=2.277, 95% CI=1.324-3.915, $P=0.033$), in contrast, A3C level in tumor tissues might play a positive role in HCC overall survival (HR=0.364, 95% CI=0.182-0.727, $P=0.004$). Interestingly, A3F contributed to a poor disease-free survival of HCC (HR=3.383, 95% CI=1.249-9.715, $P=0.017$), while A3H may be a positive factor associated with HCC disease-free survival (HR=0.25, 95% CI=0.098-0.636, $P=0.004$). Cirrhosis, tumor size and intrahepatic metastasis were associated with HCC poor disease-free survival (HR=1.838, 95% CI=1.308-2.583, $P<0.001$; HR= 1.095, 95% CI=1.042-1.15, $P<0.001$ and HR=3.669, 95% CI=2.447-5.5, $P<0.001$; respectively). Logistic regression analysis indicated that up-regulation of A3F in tumor tissues promoted HCC vascular invasion, intrahepatic metastasis and AFP elevation (all $P<0.05$). In contrast, A3H might decrease these risks (all $P<0.05$). Conclusions: APOBEC3G and APOBEC3F might be risk factors for HCC development and survival, while APOBEC3C and APOBEC3H should play positive roles in HCC aggressiveness and prognosis. Further investigation for APOBEC3 mechanisms are needed in the future.

Keywords: APOBEC3, APOBEC3F, APOBEC3H, APOBEC3G, APOBEC3C, liver cancer, hepatocellular carcinoma, survival, prognosis

Introduction

Among APOBEC family, APOBEC3 proteins play an important role in innate cellular immunity inhibiting retroviral infection, hepatitis B virus propagation, and the retrotransposition of endogenous elements. Members of this gene family like A3B, A3D, A3F, and A3G contain two conserved cytidine deaminase domains, instead of one in A3A, A3C, and A3H [1-4]. APOBEC3 proteins defend against retroviruses by deaminating cytosine residues to uracil, resulting in hypermutation and degradation of the viral genome. Moreover, the APOBEC3 family has been subjected to strong and continuing selective pressures at the amino acid level [5, 6], which are associated with an increased risk of HCC [7, 8].

The worldwide incidence of HCC has increased; presently it constitutes the fifth most common cancer, representing around 5% of all cancers [9]. HBV replication and genome mutations were both contributed to HCC development. Previous reports have reported gene expression patterns from the tumor tissues that can predict the recurrence of HCC, and these gene signatures were shown to predict HCC recurrence [10, 11]. Recent studies indicate that a subclass of APOBEC cytidine deaminases may induce mutation clusters in human tumors. Two studies [12, 13] independently reached the

same conclusion that the A3B mutation signature is specifically enriched in six types of cancers, including those of the cervix, bladder, lung, head and neck, and breast, leading to a conclusion that A3B catalyzed genomic uracil lesions are responsible for a large proportion of both dispersed and clustered mutations in multiple distinct cancers. In another side, APOBEC3-mediated HBx mutants, especially the C-terminally truncated mutants, cause a gain of function that enhances the colony forming ability and proliferative capacity of neoplastic cells. Some of the APOBEC3 deaminases play a role in the carcinogenesis of HCC through the generation of HBx mutants, providing preneoplastic and neoplastic hepatocytes with a selective clonal growth advantage [14].

Considering APOBEC3 proteins' critical roles in viral genome hypermutation and degradation and HCC carcinogenesis, a further analysis clarifying relationships between APOBEC3 and HCC prognosis is urgently needed. This study sets out to define relationships between APOBEC3 and outcomes in HCC patients after curative hepatectomy, in the hope that these data may provide novel biomarker candidates as well as useful insights into the pathogenesis and progression of HCC.

Methods

Ethics statement

Informed consent was obtained from each patient included in the study, and this study was approved by the institutional review board of Samsung Medical Center, Seoul, Korea, as reported by Lim HY et al [15].

Patients

240 tumor tissues containing no necrosis or hemorrhage were available from primary HCC patients who were treated with surgical resection or liver transplantation at Samsung Medical Center, Seoul, Korea, from July 2000 to May 2006. None of the patients received preoperative chemotherapy.

Source of data

Tumor tissues of HCC patients after curative hepatectomy in this study were profiled using Illumina HumanHT-12 V4.0 expression beadchip (Illumina Inc., San Diego, CA). The expression data was retrieved from Gene Expression Omnibus (GSE36376, http://www.ncbi.nlm.nih.gov/geo/) [15]. We restricted our search to genes within the APOBEC3 family, and seven APOBEC3s were included in our analysis.

End points

The overall survival was defined as time from surgery to the date of death or last follow-up. The disease-free survival was defined as time from surgery to the date of tumor recurrence or death. The censoring time was defined as the final documented date of no evidence of tumor recurrence by imaging.

Clinico-pathological features of HCC patients including vascular invasion, major portal vein invasion, intrahepatic metastasis, multicentric occurrence, American Joint Committee on Cancer (AJCC) stage, and nontumor liver pathology were all considered. As presented by Lim HY et al [15], vascular invasion was considered present when a neoplastic cell group was surrounded by at least one or more endothelial cells or the tunica media of the vessel. Intrahepatic metastasis and multicentric occurrence were defined according to the criteria of the Liver Cancer Study Group of Japan. Patient serum α-fetoprotein levels were evaluated and three phase dynamic computed tomography scans were performed at least once every 3 months after surgery until December 31, 2010. When tumor recurrence was suspected, precise diagnostic imaging was performed by magnetic resonance imaging.

Statistical analysis

Parametric data were expressed as mean ± standard deviation (SD) or median values. The Kolmogorov-Smirnov test was used as a test of normality. Student's *t*-test was used to compare means for normally distributed continuous data, Mann-Whitney U-test was used for non-normally distributed continuous data, and the Chi-squared test or Fisher test was used for categorical variables. Factors associated with the outcomes and clinic-pathological features were assessed by univariate analysis and multivariate analysis separately using Cox and logistic regression. Only covariates significantly associated with outcomes at univariate analysis (two-sided P value<0.10) are shown and included in the multivariate model. Results

Table 1. Baseline characteristics of hepatocellular carcinoma patients after curative hepatectomy

Characteristics	Total (n=240)	Survival group (n=135)	*P* value$^{\Delta}$	Recurrence group (n=150)	*P* value$^{\blacktriangle}$
Male, n (%)	199 (82.9)	110 (81.5)	0.503	121 (80.7)	0.232
Age, median (IQR), years	53 (45-61)	53 (45-60)	0.698	52 (44-60)	0.031
Etiology, n (%)			0.302		0.002
HBV infection	186 (77.5)	108 (80.0)		126 (84.0)	
HCV infection	20 (8.3)	8 (5.9)		12 (8.0)	
Other	34 (14.2)	19 (14.1)		12 (8.0)	
Tumor size, median (IQR), cm	3.7 (2.5-6.15)	3.2 (2.2-5.0)	<0.001	3.95 (2.7-7.05)	0.029
Liver histology of non-tumor			0.352		0.01
Cirrhosis	115 (47.9)	57 (42.2)		84 (56.0)	
Chronic active hepatitis	58 (24.2)	36 (26.7)		37 (24.7)	
Chronic persistent hepatitis	36 (15.0)	23 (17.0)		20 (13.3)	
Reactive hepatitis	11 (4.6)	5 (3.7)		5 (3.3)	
Alcoholic hepatitis	11 (4.6)	7 (5.2)		3 (2.0)	
Vascular invasion, n (%)	133 (55.4)	60 (44.4)	<0.001	96 (64.0)	0.001
Major portal vein invasion, n (%)	9 (3.8)	1 (0.7)	0.011	8 (5.3)	0.159
Intrahepatic metastasis, n (%)	55 (22.9)	13 (9.6)	<0.001	50 (33.3)	<0.001
Multicentric occurrence, n (%)	13 (5.4)	7 (5.2)	0.857	8 (5.3)	0.941
Direct invasion of adjacent organ, n (%)	5 (2.1)	2 (1.5)	0.656	4 (2.7)	0.653
AJCC stage, n (%)			<0.001		<0.001
I	102 (42.5)	73 (54.1)		52 (34.7)	
II	100 (41.7)	53 (39.3)		64 (42.7)	
III	33 (13.8)	7 (5.2)		30 (20.0)	
IV	5 (2.1)	2 (1.5)		4 (2.7)	
BCLC stage, n (%)			<0.001		0.012
A	139 (57.9)	96 (71.1)		76 (50.7)	
B	91 (37.9)	37 (27.4)		66 (44.0)	
C	10 (4.2)	2 (1.5)		8 (5.3)	
Alpha-fetoprotein >200 ng/ml, n (%)	87 (36.3)	45 (33.3)	0.287	63 (42.0)	0.017
BMI, mean ± SD, kg/m^2	24.2±2.8	24.6±2.9	0.009	24.0±2.7	0.234

$^{\Delta}$compared to death patients; $^{\blacktriangle}$compared to patient without recurrence.

Table 2. APOBEC3 expression levels between tumor tissues and non-tumor tissues of HCC patients (unit)

APOBEC3	Tumor tissues	Non-tumor tissues	*P* value
A3A, mean ± SD	6.3±0.17	6.3±0.15	0.813
A3B, median (IQR)	5.95 (5.74-6.21)	5.95 (5.64-5.85)	<0.001
A3C, median (IQR)	6.93 (6.84-7.14)	6.91 (6.83-7.04)	0.081
A3D, median (IQR)	6.34 (6.23-6.46)	6.29 (6.2-6.37)	0.001
A3F, median (IQR)	6.72 (6.63-6.87)	6.65 (6.58-6.72)	<0.001
A3G, median (IQR)	6.66 (6.53-6.83)	6.66 (6.54-6.81)	0.508
A3H, median (IQR)	5.93 (5.83-6.05)	5.88 (5.81-5.97)	<0.001

were reported as hazard ratios (HR) or odd ratios (OR) with 95% confidence intervals (CI). The Kaplan-Meier method was used to compare overall survival between different groups, and the log-rank test was used to estimate the difference in survival. Statistical analyses were performed using PASW Statistics software version 18.0 from SPSS Inc. (Chicago, IL, USA). All statistical tests were two-tailed, and differences with P <0.05 were considered statistically significant.

Results

Patient characteristics

Among 240 HCC, Survival patients had smaller tumor size (P<0.001), lower risk of vascular invasion (P<0.001), major portal vein invasion (P=0.011), intrahepatic metastasis (P<0.001) and high level of body mass index (BMI,

Table 3. Cox regression analysis of risk factors associated with overall survival of HCC patients

Covariates	Univariate analysis, HR (95% CI)	*P* value	Multivariate Analysis, HR (95% CI)	*P* value
A3B, per increase of 1 unit	1.508 (1.061-2.141)	0.022		
A3C, per increase of 1 unit	0.398 (0.205-0.773)	0.007	0.364 (0.182-0.727)	0.004
A3D, per increase of 1 unit	0.411 (0.214-0.788)	0.007		
A3F, per increase of 1 unit	0.403 (0.157-1.036)	0.059		
A3G, per increase of 1 unit	2.077 (1.241-3.476)	0.005	2.277 (1.324-3.915)	0.003
Gender (male vs. female)	1.557 (0.989-2.452)	0.056		

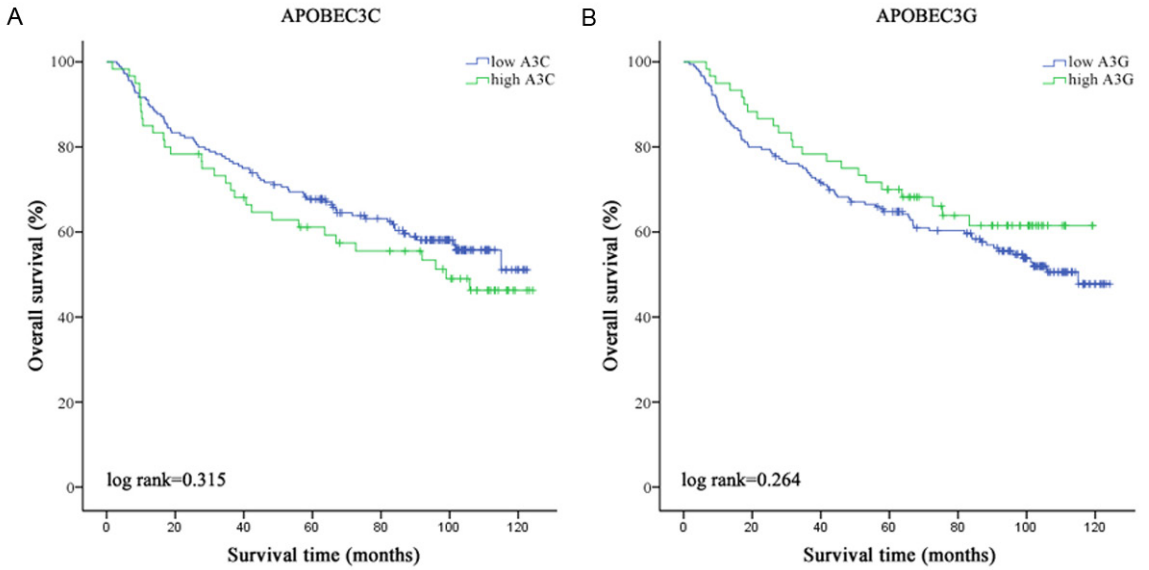

Figure 1. Analysis of overall survival of HCC patients. Overall survival analysis of (A) A3C and (B) A3G by Kaplan-Meier survival method.

P=0.009). On the other side, HCC recurrence patients were younger (*P*=0.031) and more frequently infected by HBV (84%). Moreover, they also had bigger tumor size (*P*=0.029), more frequently coexisted with cirrhosis (*P*=0.01), vascular invasion (*P*<0.001), intrahepatic metastasis (*P*<0.001). And, more HCC patients had increased AFP over 200 ng/ml compared to those without recurrence (*P*=0.017). The AJCC stage and BCLC stage distribution were also presented in **Table 1**.

APOBEC3 expression levels

As shown in **Table 2**, A3B, A3D, A3F and A3H were overexpressed in HCC tumor tissues compared to non-tumor tissues (all *P*≤0.001). No difference was found in A3A, A3C and A3G levels between tumor tissues and non-tumor tissues of HCC patients.

Factors associated with HCC overall survival

All characteristics and APOBEC3 included in the analysis are summarized in **Table 3**. Univariate analysis showed that A3B, A3C, A3D, A3F and A3G expression levels and patient gender were all factors associated with overall survival in HCC patients (all *P*<0.10). When these factors were evaluated by a multivariate model using forward selection, A3G was negatively associated with overall survival of HCC patients (HR=2.277, 95% CI=1.324-3.915, *P*=0.033), in contrast, A3C level in tumor tissues might play a positive role in HCC overall survival (HR=0.364, 95% CI=0.182-0.727, *P*=0.004).

Despite a significant correlation between A3 (including A3C and A3G) expression and HCC overall survival both in univariate and multivariate regression analyses, the extent of the asso-

Table 4. Cox regression analysis of risk factors associated with disease free survival of HCC patients

Covariates	Univariate analysis, HR (95% CI)	*P* value	Multivariate Analysis, HR (95% CI)	*P* value
A3F, per increase of 1 unit	2.168 (0.868-5.417)	0.098	3.383 (1.249-9.715)	0.017
A3G, per increase of 1 unit	0.455 (0.237-0.874)	0.018		
A3H, per increase of 1 unit	0.273 (0.106-0.704)	0.007	0.25 (0.098-0.636)	0.004
HBV infection (yes vs. no)	1.782 (1.159-2.739)	0.008		
Cirrhosis (yes vs. no)	1.691 (1.224-2.336)	0.001	1.838 (1.308-2.583)	<0.001
Age, per increase of 1 year	0.981 (0.966-0.996)	0.016		
Tumor size, per increase of 1 cm	1.129 (1.082-1.177)	<0.001	1.095 (1.042-1.15)	<0.001
AJCC stage, per increase 1 stage	1.944 (1.601-2.36)	<0.001		
BCLC stage, per increase 1 stage	1.907 (1.467-2.479)	<0.001		
Vascular invasion (yes vs. no)	2.324 (1.662-3.251)	<0.001		
Intrahepatic metastasis (yes vs. no)	5.059 (3.528-7.254)	<0.001	3.669 (2.447-5.5)	<0.001
AFP (>200 ng/ml vs. <200 ng/ml)	1.66 (1.198-2.298)	0.002		

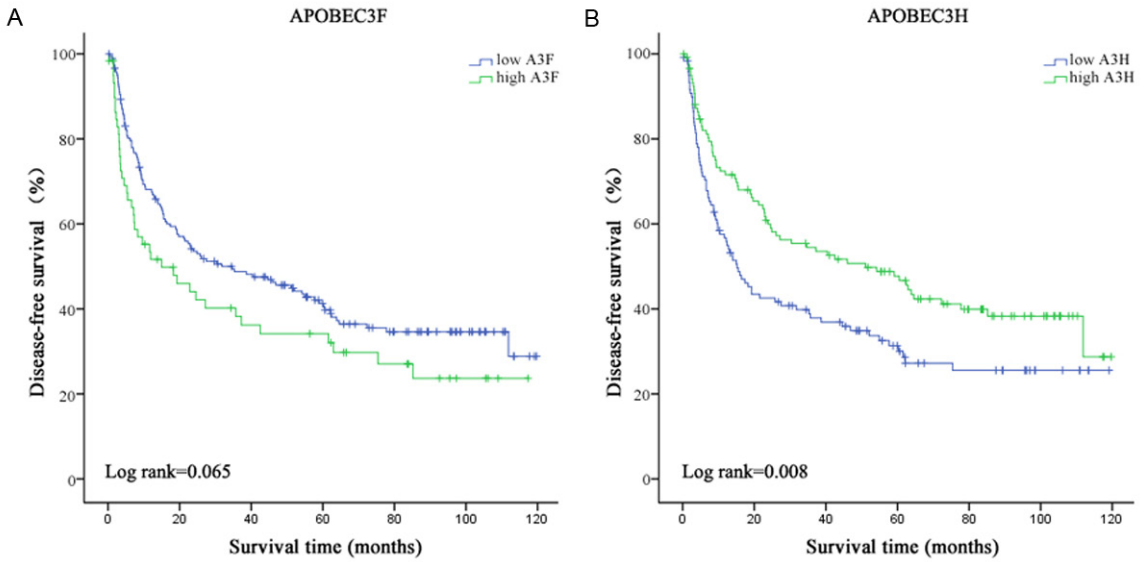

Figure 2. Analysis of disease-free survival of HCC patients. Disease-free survival analysis of (A) A3F and (B) A3H by Kaplan-Meier survival method.

ciation by Kaplan-Meier event analysis with log rank method is relatively small (both $P>0.05$, **Figure 1**).

Factors associated with HCC disease-free survival

Table 4 summarizes results from univariate and multivariate regression analyses. HBV infection, cirrhosis, age, tumor size, AJCC stage, BCLC stage, vascular invasion, intrahepatic metastasis, AFP and APOBEC3s, including A3F, A3G and A3H, were all associated with disease-free survival of HCC (all $P<0.10$). Furthermore, cirrhosis, tumor size and intrahepatic metastasis were clinico-pathological features associated with HCC poor disease-free survival (HR=1.838, 95% CI=1.308-2.583, $P<0.001$; HR= 1.095, 9% CI=1.042-1.15, $P<0.001$ and HR=3.669, 95% CI=2.447-5.5, $P<0.001$; respectively). Interestingly, A3F contributed to a poor disease-free survival of HCC (HR=3.383, 95% CI=1.249-9.715, $P=0.017$), while A3H may be a positive factor associated with HCC disease-free survival (HR=0.25, 95% CI=0.098-0.636, $P=0.004$).

We performed a Kaplan-Meier event analysis grouping by A3F and A3H median expression identified to be significantly associated with

Table 5. Relationship between APOBEC3 and HCC clinico-pathological characteristics by logistic regression analysis

APOBEC3	Vascular invasion			
	Univariate analysis, OR (95% CI)	*P* value	Multivariate Analysis, OR (95% CI)	*P* value
A3F, per increase of 1 unit	3.776 (0.818-17.435)	0.089	5.379 (1.111-26.039)	0.037
A3H, per increase of 1 unit	0.263 (0.069-1.009)	0.051	0.197 (0.049-0.798)	0.023
APOBEC3	Intrahepatic metastasis			
	Univariate analysis, OR (95% CI)	*P* value	Multivariate Analysis, OR (95% CI)	*P* value
A3F, per increase of 1 unit	4.263 (0.758-23.989)	0.1	6.741 (1.111-40.885)	0.038
A3H, per increase of 1 unit	0.14 (0.022-0.885)	0.037	0.101 (0.015-0.66)	0.017
APOBEC3	AFP over 200 ng/ml			
	Univariate analysis, OR (95% CI)	*P* value	Multivariate Analysis, OR (95% CI)	*P* value
A3B, per increase of 1 unit	0.473 (0.246-0.911)	0.025	0.53 (0.277-1.014)	0.055
A3F, per increase of 1 unit	5.897 (1.233-28.191)	0.026	7.685 (1.483-39.823)	0.015
A3H, per increase of 1 unit	0.226 (0.05-1.015)	0.052	0.166 (0.034-0.81)	0.026

disease-free survival presented above. As shown in **Figure 2**, A3F in tumor tissues might be a potential risk factor for HCC disease-free survival (P=0.065, **Figure 2A**), while A3H should play a protective role in HCC disease-free survival (P=0.008, **Figure 2B**).

Relationship between APOBEC3 and HCC clinico-pathological features

APOBEC3 in tumor tissues were related to vascular invasion, intrahepatic metastasis and AFP level over 200 ng/ml, which was shown in **Table 5**. A3F and A3H expression were all associated with HCC clinico-pathological features mentioned above in univariate analysis model (all P<0.10). When all APOBEC3 members were evaluated by a multivariate model using forward selection, overexpression of A3F was negatively related with the vascular invasion, intrahepatic metastasis and AFP level over 200 ng/ml (HR=5.379, 95% CI=1.111-26.039, P=0.037; HR=6.741, 95% CI=1.111-40.885, P=0.038 and HR=7.685, 95% CI=1.483-39.823, P=0.015, respectively). In contrast, A3H plays a positive role in the HCC clinico-pathological features above as shown in **Table 5**.

Discussion

It is postulated that APOBEC-mediated mutagenesis of viral DNA may result in an increase in viral mutation load to a level that exceeds the threshold for viral viability [16, 17]. Accordingly, induction of APOBEC family members in certain human tumors with an existing high mutation load may similarly increase mutation numbers to a point that exceeds tumor viability [18, 19]. Given this hypothesis, it is of great importance to evaluate the relationship between APOBEC3 proteins and clinico-pathological features and prognosis of HCC patients.

As a member of the mammalian apolipoprotein B mRNA-editing enzyme catalytic polypeptide like family, A3G exhibits deoxycytidine deaminase activity to protect against viral infection [20, 21]. However, a report by Noguchi et al [22] revealed that overexpression of A3G reduced the number of replicative intermediate of HBV and increased hypermutated genomes, suggesting that A3G might have a dual effect on HBV: induction of hypermutation and reduction of virus synthesis. Furthermore, Ding et al [23] elucidate that A3G promotes liver metastasis in an orthotopic mouse model of colorectal cancer and predicts human hepatic metastasis. In our study, no A3G expression difference was found between HCC tumor tissues and non-tumor tissues, but, up-regulation of A3G in tumor tissues should be a risk factor contributing to poor overall survival of HCC. To our surprise, in contrast to the results obtained for A3G, A3C showed positive effect on HCC overall survival. To our known, the A3C protein produces hypermutated DNA molecules at high frequency in transfected HepG2 cells and the Huh7 hepatoma cell line [24]. Inducing mutations at a frequency several-fold lower than A3G, A3C is still capable of inhibiting viral infection and blocking viral DNA persistence in the target cells [25]. Unfortunately, Kaplan-Meier analysis did not show significant difference on HCC overall survival base on the median

expression of both A3G and A3C in this study. Thus, whether A3G and A3C are truly associated with HCC overall survival still need further investigated. It has been somewhat unclear as to the precise mechanisms of A3G and A3C in HCC overall survival.

A report by Ying et al [26] revealed that interferon-α significantly up-regulated A3F in the HepG2 cell lines but less significantly in Huh7 cells at the same time point. In HIV infection, A3F is often coexpressed [27]. Also, A3F and A3G expressions are coordinately regulated in multiple liver cell lines, primary hepatocytes, and macrophages [26]. On the other hand, A3F mostly generates G-to-A mutations in HBV genome [16], which is an increased risk for persistent HBV infection and HCC development. Our analysis showed that A3F overexpression in tumor tissues is a risk factor for HCC disease-free survival, consistent with our previous result of A3G in HCC overall survival. On the contrary, A3H should be a protective factor in HCC disease-free survival. Kaplan-Meier test also indicated that A3H might more valuable than A3F in HCC disease-free survival. Similar to A3F and A3G, A3H had strong antiviral activities, including retroviruses and retrotransposons. Previous studies [5, 24] demonstrated that A3H mRNA has been detected in several human tissues, like peripheral blood mononuclear cells, liver. But, few data was available for A3H induced HBV genome mutations and HCC carcinogenesis. Compared to rhesus macaque, human A3H shows little activity against retroviruses and LINE-1 elements [5, 28, 29]. The lack of antiviral activity of human A3H correlates with its low steady-state expression at the protein level although mRNA levels [5]. However, our result showed that A3H was well expressed in HCC tumor tissues. Taken previous research together [30], A3H is very active against exogenous and endogenous retroviruses and the prolific primate non-LTR retroelement, LINE-1 based on its up-regulated expression. In this case, we assumed that antiviral activities of A3H in tumor tissues played more valuable role than genome mutations. Further investigation is essential for this point.

Consistent with previous reports [9, 31, 32], our analysis also indicated that clinico-pathological characteristics including cirrhosis, tumor size and intrahepatic metastasis contributed to poor disease-free survival of HCC. Therefore, we performed a further analysis on relationships between APOBEC3 proteins and HCC clinico-pathological features. In our logistic regression analysis, up-regulation of A3F in tumor tissues promoted HCC vascular invasion, intrahepatic metastasis and AFP elevation. In contrast, A3H might decrease the risk of vascular invasion, intrahepatic metastasis and AFP elevation in HCC patients. The precise mechanisms of A3F and A3H in HCC clinico-pathological features needed future research.

Taken together, APOBEC3 members played dual roles in HCC clinico-pathological characteristics and survival. Although this study based on national data bank, no direct first-hand data were available, our results presented clues about APOBEC3 in HCC carcinogenesis and tumor progression. Further research should be considered for APOBEC3 mechanisms in this population.

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Disclosure of conflict of interest

None.

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