APOBEC3B: A Potential Factor Suppressing Growth of Human Hepatocellular Carcinoma Cells

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Abstract. *Background: To realize the role of apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B (APOBEC3B) in hepatocellular carcinoma (HCC) occurrence, mRNAs of APOBEC3B from tumor and non-tumor tissues of patients with hepatectomy were isolated and in vitro studies were designed. Materials and Methods: Seventy-two tumor and non-tumor tissue samples, as well as clinical data, were collected from HCC patients during hepatectomy. The mRNA of APOBEC3B was assessed by real-time polymerase chain reaction. The viability of pLV-APOBEC3B-transfected Hep 3B cells was then determined. Cell growth of pLV-APOBEC3B-transfected Hep 3B cells was evaluated by in vitro migration assay. Results: The real-time polymerase chain reaction results indicated a higher expression of APOBEC3B mRNA in tumor tissues than in non-tumor tissues of patients with HBsAg$^+$ HCC. The expression of APOBEC3B in tumor or non-tumor tissue was not found to be a risk factor of recurrence in patients with HCC. The cell viability assay results indicated the growth-inhibitory effects of APOBEC3B on Hep 3B cells. The cell migration results indicated that APOBEC3B inhibits wound healing in Hep 3B cells. Conclusion: Based on these observations, we infer that APOBEC3B is a potential factor contributing to suppression of tumor growth in HCC.*

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Chronic hepatitis virus infection, virus mutation, and host immunity are critical factors in the development of hepatocellular carcinoma (HCC) (1). Among these factors, chronic hepatitis B virus (HBV) infection is the most crucial risk factor for HCC worldwide. HBV contributes to more than 50% of HCC cases globally, and 70%-80% of HCC cases in regions where HBV is highly endemic. More than 350 million people are infected with chronic HBV and are at high risk of developing liver cirrhosis and HCC (2).

HCC is the second leading cause of death from cancer in Taiwan. Taiwan has the highest prevalence of chronic HBV infection in the Asia-Pacific region (3). Before the national HBV vaccination program was implemented in 1984, 15%-20% of the general population was chronically infected with HBV (4). Therefore, investigating the underlying mechanisms of HCC and potential therapies is critical because of the high incidence and mortality of this disease. HCC is a fatal disease, and surgical resection and orthopic liver transplantation are the only curative treatments. However, a previous study reported that up to 70% of patients with HCC experience relapse within five years of curative resection (5). Therefore, the high rate of recurrence is a major obstacle to improving prognosis.

APOBEC3 (A3) is a member of the mammalian apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like (APOBEC) family, and exhibits deoxycytidine deaminase activity to protect against viral infection, which has been broadly studied in the past decade (6, 7). The role of A3 proteins in the inhibition of viral infection was first demonstrated using cases of HIV-1. Previous research has indicated the potential role of A3 in the oncogenesis of HCC. Baumert *et al*. reported that APOBEC3C is packaged into viral particles, which enables it to edit newly-synthesized HBV DNA, indicating that APOBEC3C contributes to an innate anti-HBV host response (8). Xu *et al*. indicated that A3-

mediated HBx mutants, particularly *C*-terminally truncated mutants, cause an increase in function that enhances the colony-forming activity and proliferative capacity of neoplasia (9). However, Zhang *et al*. provided evidence of an association between APOBEC3G gene deletion and susceptibility to persistent HBV infection and HCC (10). In our previous study, we reported that A3G exerts tumor-suppressive effects in human HCC (11). Although A3 proteins potentially affect development of HCC, they have also been reported as potential mechanisms in B-cell lymphoma, non-Hodgkin's lymphoma, colorectal cancer, and breast cancer (12-15); thus, the mechanism by which A3 proteins are involved in carcinogenesis remains unclear.

HBV can integrate into the genome of infected hepatocytes and promote hepatocarcinogenesis through sustained inflammatory damage. Chronic inflammation drives a maladaptive reparative reaction and stimulates liver cell death and regeneration, which are associated with the eventual development of dysplastic nodules and cancer (16). The high rate of recurrence is a major obstacle to improving prognosis in patients with HCC. To elucidate the risk factors in HCC occurrence, we identified the risk factors of HCC associated with sex, HBV carrier state, vascular invasion, and APOBEC3B expression based on whether recurrence was observed. In addition, we constructed a pLV-APOBEC3B vector and transfected it into Hep 3B cells. The results indicated that APOBEC3B is a potential factor contributing to suppression of tumor growth in HCC.

Materials and Methods

Clinical data collection. This study was approved by the Institutional Review Board Committee of E-DA Hospital, Taiwan (EMRP-098-106). Seventy-two HCC patients underwent hepatectomy at the E-DA Hospital, Taiwan. Vascular invasion was confirmed based on the results of pathological features, and recurrence was confirmed using echo-computed tomography (CT) and data recorded approximately 18±7.67 months after hepatectomy.

RNA isolation and quantification of mRNA. Seventy-two hepatectomy specimens were collected immediately after surgery and stored at −80˚C until use. The RNA was extracted using a TRIzol reagent (Invitrogen, Carlsbad, California, USA), and then reverse-transcribed using random primers and ABI high-capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, California, USA) at 42˚C for 60 min, according to the manufacturer's instructions. Quantitative analysis of *APOBEC3B* cDNA was performed using real-time polymerase chain reaction (PCR) and TaqMan gene expression assays (Applied Biosystems). The TaqMan probe used for analyzing APOBEC3B was Hs00358981_m1 (NM_004900.3). The target cDNA was normalized to the endogenous RNA levels of the housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase. High cycling threshold values indicated low RNA expression levels.

Table I. *Expression of APOBEC3B mRNA in tumor and nontumor tissues obtained from patients with HCC during hepatectomy.*

		APOBEC3B		
	N	Normal tissue	Tumor tissue	*p*-Value
$HBsAg^+$	44	8.81±1.66	7.68±2.52	0.005
$HBsAg^-$	28	9.01±2.10	9.06±2.93	0.941
All HCC	72	8.89±1.83	8.22±2.75	0.047

N: Number of analyzed specimens. $HBsAg^+$ indicates surface antigen positivity.

Construction of the pLV-APOBEC3B plasmid. The APOBEC3B cDNA molecule and sequence (accession number NM_004900) were a gift of Harold C. Smith, Ph.D. (University of Rochester Medical Center, School of Medicine and Dentistry). To construct the APOBEC3B–pLKO AS2.puro (pLV-APOBEC3B) vector, a restriction cutting site of *Nhe*I at the 5' end and AscI at the 3' end was created using PCR amplification. Subsequently, the amplified APOBEC3B fragment was digested using a *Nhe*I/*Asc*I restriction enzyme and then cloned into the pLKO AS2.puro to generate pLV-APOBEC3B.

Cell culture and transfection. The human HCC cell line Hep 3B (BCRC 60434) was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan, ROC). This cell line was obtained from the tissue of an 8-year-old male. The cells were epithelial in morphology and evidenced an integrated HBV genome. The cells were cultured in 24-well plates at 37˚C in 5% CO_2 in a humidified environment by using a minimal amount of essential Eagle's medium containing 2 mM L-glutamine and Earle's Balanced Salt Solution. The solution was adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10% fetal bovine serum (Hyclone, Logan, UT, USA). The Hep 3B cells in each well were transfected with 2 μg of pLV-APOBEC3B by using lipofectamine (Invitrogen), according to the manufacturer's instructions.

Immunofluorescence. After transfecting the pLV-APOBEC3B, the cells were washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde in PBS for 5 min at room temperature, and then blocked using a blocking buffer (5% bovine serum albumin and 0.5% Triton X-100 in PBS) at 37˚C for 45 min. After washing the cells twice with PBS, they were probed with rabbit anti-APOBEC3B (1:500; Abgent, San Diego, California, USA). A goat anti-rabbit antibody was used to react with the primary antibody. The cell nucleus was stained using 4,6-diamidino-2-phenylindole (DAPI, 2 mg/ml; Sigma-Aldrich, St. Louis, MO USA), and immunofluorescent cell images were captured using a Nikon Eclipse 80i microscope (Tokyo, Japan).

Cell viability assay. Cell survival was analyzed using the CellTiter 96TM nonradioactive cell proliferation MTS assay (Promega, Madison, Wisconsin, USA). After recombinant DNAs were transfected into the Hep 3B cells for 48-72 h, 15 μl of MTS reagent were added to each well and plates incubated at 37˚C for 4 h to

Table II. *Factors predicting recurrence after hepatectomy.*

	Odds ratio	Standard error	z	95% CI	*p*-Value
Sex	0.434	0.321	−1.13	0.102-1.848	0.259
HBsAg	0.530	0.438	−0.77	0.105-2.676	0.442
VI	28.899	24.268	4.01	5.573-149.860	<0.0001
T_APOBEC3B	1.130	0.161	0.85	0.854-1.494	0.393
N_APOBEC3B	1.448	0.300	1.78	0.964-2.174	0.074
_cons	0.003	0.007	−2.29	0.000-0.427	0.022

Sex represents the sex of the sample, which 1 is male and 0 female; HBsAg is surface antigen, which 1 is positive and 0 negative; VI is the dummy variable of vascular invasion, which 1 represent the one with vascular invasion and 0 is the one without vascular invasion; T_APOBEC3B represents the mean number of threshold cycles for APOBEC3B in the tumor tissue and N_APOBEC3B in nontumor tissue which increased the cycle indicating the low APOBEC3B mRNA expression; _cons is the constant of the model. CI is the confidence interval.

allow purple formazan crystals to form. Subsequently, 100 μl of solubilization/stop solution were added to each well, and the reaction mixture was incubated in the dark for 60 min at room temperature. The developed color density was measured spectrophotometrically at 490 nm by using a microplate reader. All assessments were performed in triplicate.

In vitro migration/wounding assay. The Hep 3B cells were seeded into 24-well plates and allowed to reach 70%-80% confluence prior to transfection. After transfecting the pLV-APOBEC3B into Hep 3B cells for 24 h, the cell monolayer was scraped using a fine needle to create a wound, and then allowed to continue growing in an incubator with 5% CO_2. The migration distances were observed at 3, 24, 48, and 72 h, and the percentage wound-closure was measured using ImageJ Free Software, Version 1.47v (NIH, Bethesda, Maryland, USA).

Statistical analysis. In the present study, clinical data are presented as the mean±standard deviation (SD). A paired *t*-test was performed to compare the differences in APOBEC3B expression between the tumor and nontumor tissues. Student's *t*-test was performed to compare the differences between the patients with $HBsAg^+$ and $HBsAg^-$ HCC. Logistic regression was performed using Stata software (College Station, Texas, USA).

In vitro data are presented as the mean±the standard error of the mean (SEM) derived from triplicate measurements. Results with a *p*-value of less than 0.05 were considered statistically significant. The statistical analyses and *t*-tests were performed using Sigmaplot, Version 10.0 (Systat software Inc., Chicago, Illinois, USA).

Results

Hepatectomy tumor tissues exhibit high APOBEC3B mRNA expression. The real-time PCR results indicated that the mean number of threshold cycles for APOBEC3B in the non-tumor tissues was significantly ($p=0.047$) greater than that in tumor tissues (Table I), *i.e.* APOBEC3B expression in tumor was higher. On further analysis, APOBEC3B was more highly expressed in tumor tissues than non-tumor tissues ($p=0.005$) in patients with $HBsAg^+$ HCC, which was not the case for patients with $HBsAg^-$ HCC .

APOBEC3B might not be a risk factor for HCC recurrence. We performed logistic regression (logit model) to predict the multirisk factors of HCC recurrence. Table II shows that sex and HBV carrier state are not risk factors associated with recurrence in patients with HCC. However, increased risk for patients with portal vein invasion [odds ratio (OR)=28.899; 95% confidence interval (CI)=5.57-149.86; $p<0.0001$) is a highly significant factor associated with recurrence. These results indicate that in patients with portal vein invasion, recurrence is 27.89-times more likely than in patients without portal vein invasion. Moreover, an increase in threshold cycle of APOBEC3B in tumor tissue which indicates low APOBEC3B expression (OR=1.13; 95% CI=0.854-1.494; $p=0.393$) was not significant, these results indicate that the expression of APOBEC3B in tumor tissues is not associated with recurrence of HCC.

Recombinant APOBEC3B induces cytotoxicity in Hep 3B cells. To elucidate the role of APOBEC3B in HCC, we constructed a pLV-APOBEC3B vector and transfected it into the Hep 3B cell line. The immunofluorescence results revealed the localization of overexpressed APOBEC3B in the nucleus (Figure 1). Because the *in vivo* data indicated that APOBEC3B mRNA expression was higher in tumor tissue than in nontumor tissue, we harvested the cells following the transient transfection of pLV-APOBEC3B for 72 h, and then performed MTS assay. The results indicated the cytotoxicity of overexpression of APOBEC3B to Hep 3B cells (Figure 2A). Hep 3B cell viability was 88% and 48% after pLV and pLV-APOBEC3B transfection, respectively. The difference in viability between the pLV-APOBEC3B-transfected and pLV-transfected Hep 3B cells was statistically significant ($p<0.05$). These results indicate that recombinant APOBEC3B exhibits cytotoxic activity, and that it might inhibit Hep 3B cell growth.

Recombinant APOBEC3B inhibits Hep 3B cell migration. The migration/wounding assay results revealed that the Hep

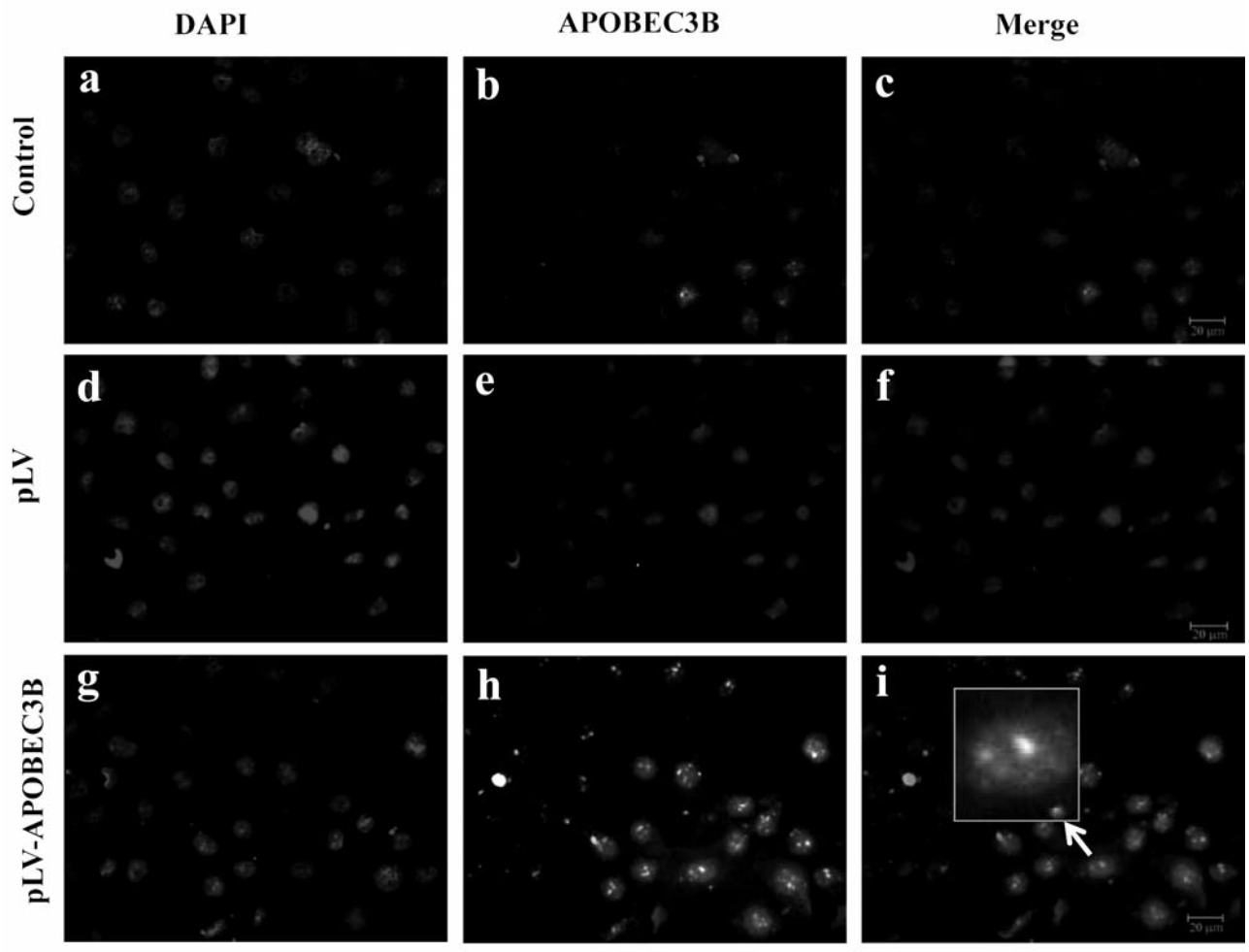

Figure 1. *Immunofluorescent staining of APOBEC3B overexpressed in Hep 3B cells. Panels a-c show the control cells. Panels d-f show the pLV-transfected cells. Panels g-i show the pLV-APOBEC3B-transfected cells. DAPI-counterstained cells are blue. Cells probed with anti-APOBEC3B exhibit green fluorescence. The arrow indicates a magnified pLV-APOBEC3B-transfected cell.*

3B cells migrated toward the wound area (Figure 3A). Figure 3B shows the wound-closure percentage in cultured Hep 3B cells. The migratory capacity was reduced in Hep 3B cells overexpressing APOBEC3B, indicating that APOBEC3B inhibits Hep 3B cell migration.

Discussion

The study results indicate that APOBEC3B might play a tumor-suppressive role in HBV-associated HCC. The real-time PCR results revealed that the threshold cycle value for APOBEC3B was higher in nontumor tissues (8.81±1.66) than in tumor tissues (7.68±2.52; Table I). The higher threshold cycle value of the nontumor tissues indicates that additional PCR cycles were necessary to approach the levels

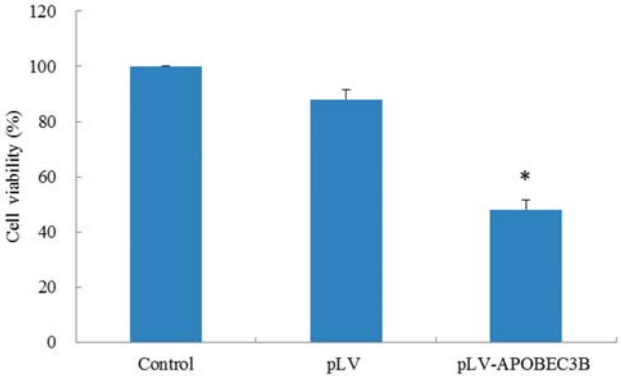

Figure 2. *Viability of Hep 3B cells. Hep 3B cells were transfected with pLV-APOBEC3B for 72 h. Data are presented as the mean±SEM (based on triplicate measurements). *p<0.05.*

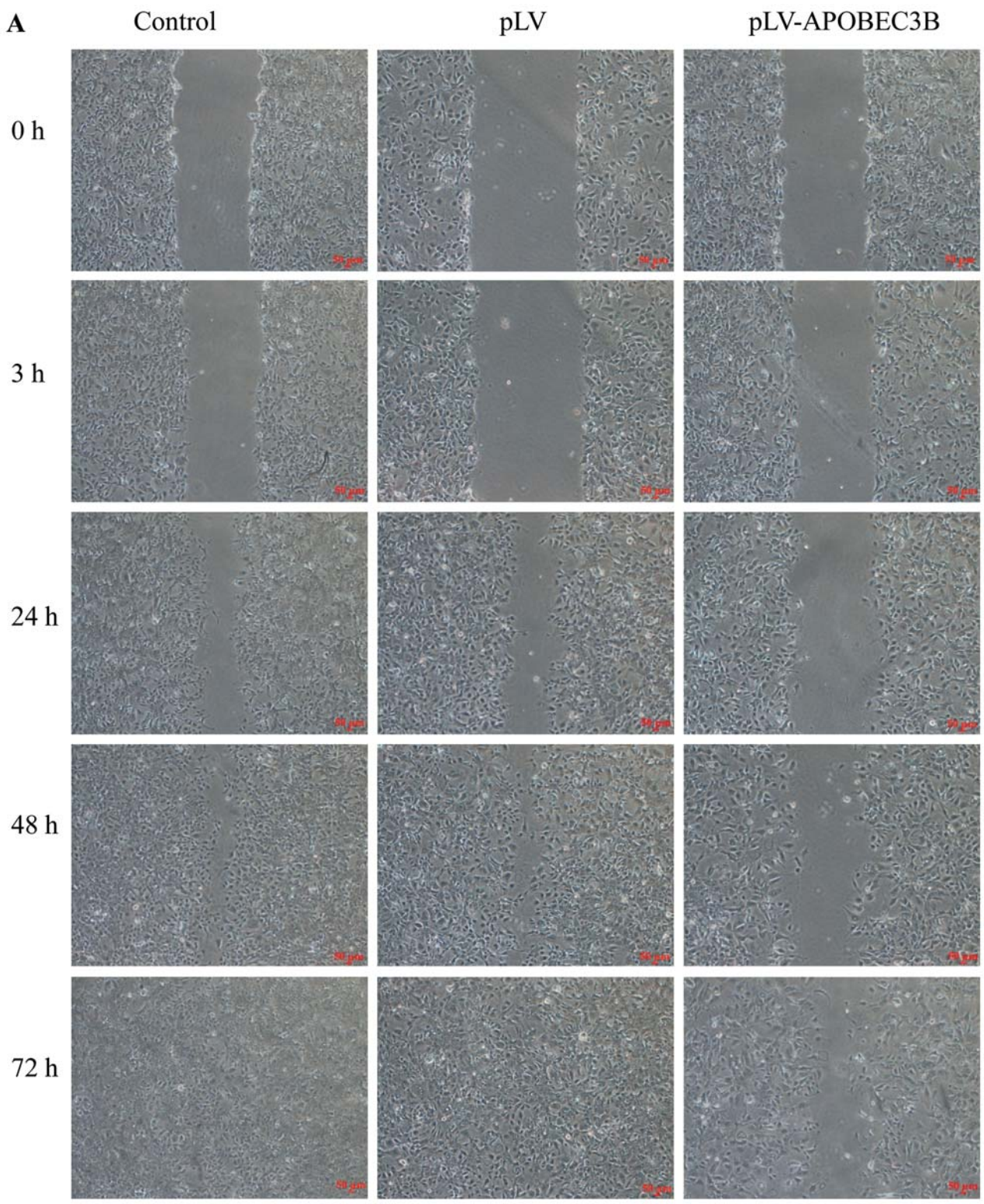

Figure 3. *Continued*

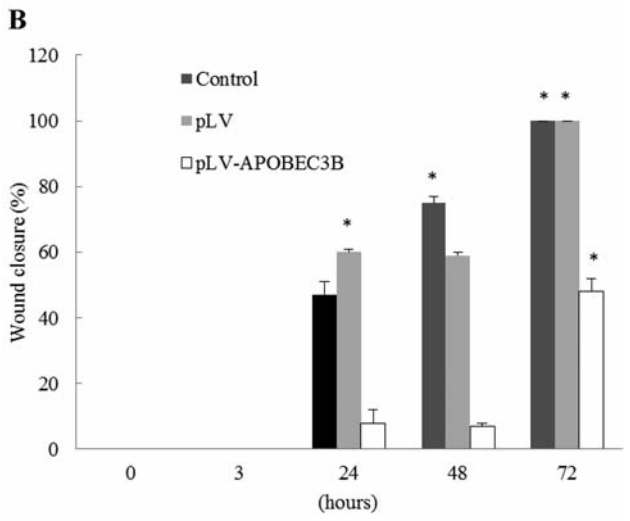

Figure 3. *Migration of pLV-APOBEC3B-transfected Hep 3B cells. A: A wound scratch was made in monolayers of cultured pLV-transfected and pLV-APOBEC3B-transfected Hep 3B cells. Wound closure was recorded at 3, 24, 48, and 72 h. B: The wound closure ratio was estimated using ImageJ. Data are presented as the mean±SEM (based on triplicate measurements). *p<0.05 and **p<0.01.*

at which low APOBEC3B mRNA expression occurred. Therefore, the results indicate that in tissue resected from patients with HCC those with $HBsAg^+$ express high levels of APOBEC3B mRNA in tumor tissue, and low levels in non-tumor tissue. We previously reported that APOBEC3G was highly expressed in non-tumor hepatectomy tissues (11). However, the results of this study indicate that APOBEC3B was highly expressed in patients with HBV-infected and in all tumor hepatectomy tissues (Table I). Further research is necessary to identify the correlation between A3 genes and HCC progression. This study demonstrated that hepatectomy tumor tissues exhibit high *APOBEC3B* mRNA expression.

In Asia and Africa, chronic HBV infection is a predominant risk factor for those with HCC. The high recurrence rate is a major obstacle to improving HCC prognosis. The role of microenvironments in tumor initiation and the progression of HCC are critical. Previous studies have indicated that the status of non-tumor tissue plays a crucial role in predicting tumor recurrence (5, 16). Tissue proximal to the tumor is relatively crucial because the tumor tissue is surgically removed. Based on the length of time following HCC treatment, tumor recurrence is classified as either early or late. Typically, early recurrence occurs within two years of HCC treatment, and it is primarily attributed to the intrahepatic dissemination of metastatic HCC cells (17). Moreover, tumor vascular invasion and tumor stage can predict the early recurrence of HCC (18). In the present study, patients with vascular invasion were found to be at a significantly high risk of recurrence (Table II, $p<0.0001$), indicating that vascular invasion is among the most critical factors predicting recurrence in patients with HCC. Furthermore, the results suggesting APOBEC3B is not a risk factor of recurrence following hepatectomy during the early stage of HCC.

HCC is a devastating disease, and it is among the most fatal types of cancer worldwide. Treatment options are limited, primarily because of the inefficiency of existing anticancer chemotherapeutic drugs against HCC. Therefore, the need for developing more effective therapeutic tools and novel strategies for treating HCC is urgent. Based on our clinical findings, we constructed a pLV-APOBEC3B vector and transfected it into Hep 3B cell lines, which was evidence of an integrated HBV genome. The results revealed that overexpression of pLV-APOBEC3B produces cytotoxic activity, indicating that APOBEC3B might inhibit Hep 3B cell growth (Figure 2). Moreover, the migration results indicated that overexpression of APOBEC3B *via* pLV-APOBEC3B inhibits Hep 3B cell migration, indicating the tumor-suppressive effects of APOBEC3B on hepatoma cells (Figure 3). A3 enzymes have been shown to inhibit the replication of a diverse set of retroviruses and retrotransposons and established the innate immunity of host (19). Moreover, Burns *et al*. reported that APOBEC3B hypermutates in the genomic DNA of breast cancer cell lines, suggesting that the inhibition of APOBEC3B might reduce the rate of tumor evolution and stabilize the targets of current therapeutics (15). Therefore, APOBEC3B could potentially represent a novel therapeutic target in carcinogenesis. Our clinical results (Table I) show that APOBEC3B expression is significantly higher in tumor tissues than in non-tumor tissues ($p=0.005$) in patients with HBV-associated HCC. Previous research has indicated that the complex interactions among viral factors, host immunity, and environmental determinants may influence HCC recurrence and patient survival (20). Whether high APOBEC3B expression in tumor tissue is an immune response of patients against HBV infection remains unclear; therefore, further study on this topic is necessary.

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