

Anaplastic lymphoma kinase protein expression predicts micrometastases and prognosis for patients with hepatocellular carcinoma

JIANHUA LIU¹, HAOSHENG JIN², HONGXIA TIAN³, GUODA LIAN⁴, SHAOJIE CHEN⁴,
JIAYU LI⁴, XUCHAO ZHANG³ and DONG MA¹

¹Department of Oncology, Cancer Center; ²Department of Hepatobiliary Surgery, Guangdong General Hospital; ³Medical Research Center, Lung Cancer Institute, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, Guangdong 510180; ⁴Department of Gastroenterology, The Second Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong 510080, P.R. China

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Abstract. The present study aimed to investigate anaplastic lymphoma kinase (*ALK*) status in hepatocellular carcinoma (HCC) and to evaluate whether abnormalities in expression were associated with patient prognosis. *ALK* status was investigated using immunohistochemistry (IHC), reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and fluorescence *in situ* hybridization (FISH) assays in 342 HCC patients. In addition, rapid amplification of complementary DNA ends-coupled PCR sequencing was performed, in order to confirm the presence of *ALK* abnormalities in patients exhibiting *ALK* messenger RNA (mRNA) overexpression. The correlation between *ALK* expression and the clinicopathological features and prognosis of the HCC patients was statistically analyzed. The results of the present study revealed overexpression of *ALK* protein and mRNA; furthermore, *ALK* gene copy number gains were observed via IHC (44.7%; 153/342), RT-qPCR (47.4%; 162/342) and FISH (32.7%; 112/342) analyses, although *ALK* rearrangement or mutation was not demonstrated in the results of any of these assays. *ALK* protein expression levels were significantly associated with hepatitis C virus (HCV) status ($P < 0.001$) and the presence of micrometastases ($P = 0.011$). Within the entire patient cohort, *ALK* expression was associated with poor progression-free survival (PFS; $P = 0.041$). Subsequent analysis in patient subgroups that demonstrated hepatitis B surface antigen positivity, HCV negativity, stage III-IV disease, recurrence and

micrometastasis positivity revealed that overall survival (OS) and PFS were significantly reduced in those patients exhibiting *ALK* expression compared with those patients who were negative for *ALK* expression. Multivariate analysis revealed that *ALK* expression was an independent risk factor for OS ($P = 0.042$) and PFS ($P = 0.033$), particularly for patients with stage III-IV tumors. Thus, *ALK* may serve as a novel indicator for the metastatic behavior and prognosis of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed malignancy and the third most common cause of cancer-associated mortality globally (1). Previous evidence has suggested the existence of marked geographic variation, with a high prevalence of HCC in East Asian countries, particularly China (2). Although significant advances have been made with regard to surgical resection, ablation and chemotherapy (3), the prognosis of HCC remains unfavorable. As our understanding of the molecular mechanisms underlying the initiation and progression of HCC increases, targeted therapy has become a promising alternative to currently used treatments, representing a landmark in drug development and a significant step towards the development of personalized medicine for the treatment of HCC (4). However, the efficacy of existing targeted drugs has plateaued in recent years due to increasing drug resistance (5). Therefore, the discovery of novel molecular targets is urgently required for the development of more effective and efficient therapeutic agents.

In general, receptor tyrosine kinases (RTKs), which are crucial for signal transduction, are dysregulated in a diverse range of tumor types (6). The anaplastic lymphoma kinase (*ALK*) gene, a proto-oncogene that encodes a transmembrane RTK, was initially identified in anaplastic large cell lymphomas carrying an abnormal t(2;5)(p23;q35) translocation (7). Subsequently, its fusion variants were additionally identified in inflammatory myofibroblastic tumors (8) and diffuse large B-cell lymphomas (9). The human *ALK* gene has a significant role in brain and neuronal development during

Correspondence to: Dr Dong Ma, Department of Oncology, Cancer Center, Guangdong General Hospital, Guangdong Academy of Medical Sciences, 123 Huifu Road West, Guangzhou, Guangdong 510180, P.R. China
E-mail: madong8005@126.com

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embryogenesis, however, is downregulated in adults (10). It is well-known that genetic abnormalities involving *ALK* include translocation (also known as 'rearrangement'), amplification and mutation, as well as *ALK* overexpression (11,12). DNA amplification and point mutations were originally identified in neuroblastoma, indicating that the *ALK* gene may possess high carcinogenic and neoplastic potential (13). In addition, *ALK* fusion oncogenes, resulting from chromosomal translocations, proved to be the most commonly observed *ALK* aberrations in cancer, and were able to induce constant *ALK* kinase activity via their own constitutive self-association, as well as acting as an oncogenic addiction pathway (14). A number of specific small-molecule *ALK* inhibitors are capable of efficiently suppressing such activity (15).

Echinoderm microtubule-associated protein-like 4 (EML4)-*ALK* fusion variant was initially reported in 2007, as an oncological driver in non-small cell lung carcinoma (NSCLC) (16), and this led to a focus on the development of novel targeted agents for cancer diagnosis and therapy (11,15). Subsequently, the success of crizotinib (PF-02341066), a targeted agent against EML4-*ALK*, in the treatment of advanced *ALK*-rearranged NSCLCs, led to an interest in the significance of *ALK* status in various other epithelial malignancies, and represented the beginning of a novel era of the targeting of *ALK* abnormalities for therapeutic purposes in solid tumors (17). Aberrant *ALK* genes have been reported in various solid tumors (often in addition to hematological malignancies), including esophageal (18), breast (19), colorectal (20) and renal carcinoma (21), indicating an association between *ALK* abnormalities and human cancer development. However, little research has been conducted to evaluate the abnormalities and clinical significance of the *ALK* gene in HCC.

In order to improve our current knowledge, the present study comprehensively detected *ALK* gene status in a large HCC cohort, and investigated whether *ALK* abnormalities are associated with patient clinicopathological features and prognosis.

Materials and methods

Clinical specimens and follow-up. A total of 342 HCC tumor specimens and corresponding normal non-cancerous tissues were obtained from patients who had undergone surgical resection at Guangdong General Hospital (Guangzhou, China), between June 2005 and October 2010. For the performance of immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) assays, sections of the resected malignant tissues were fixed using 10% formalin and subsequently embedded in paraffin, followed by longitudinal slicing into 4- μ m thick serial sections. The remaining HCC tissues were frozen using liquid nitrogen and stored at -80°C, until required for intensive investigation by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and rapid amplification of complementary DNA (cDNA) ends (RACE)-coupled PCR sequencing. All patients had not received any anticancer therapy prior to surgery. Micrometastases were defined in accordance with the criteria proposed by Hu *et al* (22). Disease staging was based on the tumor-node-metastasis staging system of the Union

for International Cancer Control (23). Clinical history was extracted from patient medical records. All patients received post-operative follow-up by telephone every 3 months for the initial 2 years of the study, and subsequently every 6 months until mortality or the study endpoint was reached. Overall survival (OS) and progression-free survival (PFS) were measured from the date of surgery until mortality/censoring or local recurrence/distant metastasis/censoring, respectively. The survival analysis utilized in the present study was designed to compare OS and PFS among patients. Each participant signed written informed consent for the use of their resected tissues and personal information for research purposes, and approval was obtained from the Ethics Committee of Guangdong General Hospital (Guangzhou, China).

ALK IHC analysis. ALK IHC staining was performed as previously described (24). In brief, unstained slides were successively submerged in xylene (Guangzhou Yikang Biological Science Technology Co., Ltd., Guangzhou, China), graded alcohol series and tap water for deparaffinization. Following deparaffinization, the intrinsic peroxidase activity of samples was inhibited using 3% hydrogen peroxide. Antigen retrieval was performed by microwave heating at 95°C, following submersion of the sections in ethylenediaminetetraacetic acid buffer (pH 8.0; Shanghai Xibao Biological Technology Ltd., Shanghai, China). The samples were incubated with rabbit monoclonal anti-human ALK [D5F3[®]; Cell Signaling Technology, Inc., Danvers, MA, USA; 1:50 dilution in antibody diluent (Dako, Glostrup, Denmark)] at 4°C overnight; non-specific protein binding sites were blocked with 5% goat serum (Shanghai Xibao Biological Technology Ltd.). Subsequent to being washed, immunoreaction was visualized using biotinylated goat anti-rabbit IgG secondary polyclonal antibodies (dilution, 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h, and thereafter, streptavidin-horse-radish-peroxidase complex (Shanghai Xibao Biological Technology, Ltd.) and 3-amino-9-ethyl carbazole (Shanghai Xibao Biological Technology, Ltd.) were added. Finally, all samples were counterstained using hematoxylin (Shanghai Xibao Biological Technology, Ltd.), dehydrated and sealed with cover slips for microscopic examination (CX31; Olympus Corporation, Tokyo, Japan). Two experienced investigators, who were blinded to the patient clinicopathological profiles, confirmed the immunostaining levels separately. The scoring scheme utilized to assess staining was as follows: 0, absent staining; 1+, weak cytoplasmic staining; 2+, moderate and smooth cytoplasmic staining, and 3+, strong and granular cytoplasmic staining in $\geq 10\%$ of tumor cells (24).

ALK FISH analysis. FISH, a gold standard for confirming gene status, was applied to 342 unstained HCC samples with the Vysis *ALK* Break Apart FISH Probe kit (Abbott Laboratories, Chicago, IL, USA) according to standard FISH protocols. Following deparaffinization, dehydration and treatment with citric acid, the tumor sections were washed with phosphate-buffered saline, followed by dehydration in alcohol and air-drying at 37°C. Subsequently, the slides were added to 5 μ l of diluted *ALK* probe and denatured. The probe was hybridized and the slides were washed. The reagent was comprised of two DNA probes labeled with Spectrum Orange

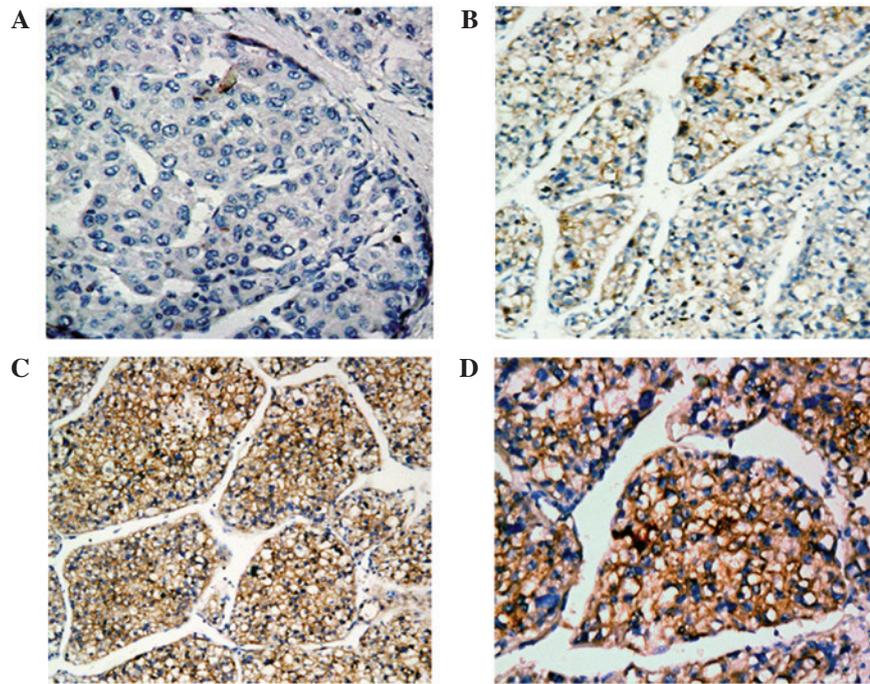


Figure 1. ALK protein expression visualized using immunohistochemistry in hepatocellular carcinoma samples. (A) Negative ALK staining in tumor cells (magnification, x200). (B) Faint (C) and strong cytoplasmic staining in tumor nests (magnification, x100). (D) Strong cytoplasmic staining in the majority of tumor cells (magnification, x200). ALK, anaplastic lymphoma kinase.

(red) and Spectrum Green (green), which were able to test various genetic rearrangements of *ALK* at 2p23, by hybridizing and breakpoint flanking. FISH signals were evaluated under a fluorescence microscope (IX72; Olympus Corporation) using an oil immersion objective. Two pathologists independently analyzed all FISH results. *ALK* translocation positivity was defined as the separation of red and green signals, or a single red signal, in at least 15% of analyzed cells. However, since the criteria for gain of *ALK* copy number have not been established, the present study adopted the previously published cut-off values for colorectal cancer in the current analysis (25). Briefly, the positivity for gain of *ALK* gene copy number was considered to be 3-5 copies of *ALK* per cell in $\geq 10\%$ of all detected cells, and amplification was regarded to be ≥ 6 copies of *ALK* per cell in $\geq 10\%$ of all detected cells.

ALK RT-qPCR analysis. Total RNA was isolated from matching pairs of frozen HCC tissue blocks with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocols. Each tissue was trimmed at low temperature to protect the RNA from degradation. Prior to amplification, the purity and concentration of the extracted RNA were assessed using gel electrophoresis and absorbance at A260/A280. Reverse transcription for cDNA was subsequently conducted with a cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific). cDNA was synthesized using 2.5 μg of total RNA as template and 1 mmol/l oligo(dT) primer in 25 μl of a solution that included 200 units of M-MLV reverse transcriptase. Subsequently, 5 μl of cDNA was applied as the template in a 20- μl reaction for RT-qPCR analysis with SYBR® GreenER™ qPCR SuperMix (Invitrogen; Thermo Fisher Scientific) and the ABI 7500 cyclor (Applied Biosystems; Thermo Fisher Scientific) under

the following thermal cycling conditions: 95°C for 2 min, 40 cycles of 95°C for 15 sec and a final extension at 72°C for 5 min. The remaining generated cDNA was stored at -20°C for further RACE-coupled PCR sequencing. β -actin served as the internal control. The primers were purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China) and synthesized as follows: Sense, 5'-CCTGGAGCTGGTCATTACGA-3' and antisense, 5'-TGGTTTGTGAAGGAGCCATT-3'. The melting curve of each sample was analyzed in order to guarantee the product specification. As stated in a previous study (26), RT-qPCR analysis for *ALK* was defined as positive when the specimen Cq value was < 30 .

RACE-coupled PCR sequencing analysis. Cases with RT-qPCR positivity were selected for RACE-coupled PCR sequencing analysis. Stored cDNA was purified using a High Pure PCR Product Purification kit (Roche Diagnostics, Indianapolis, IN, USA). Subsequently, reactions mixing purified cDNA were initiated by incubation at boiling temperature followed by incubation on ice. The following procedure was performed for targeted capture of rearranged sequences using previously published protocols (11). Following two successive runs of PCR, amplification of desired cDNA segments spanning exon 20 of the *ALK* gene and upstream sequences, which may possess the transcript sequences of *ALK* fusion partners, was achieved. The primers used were selected according to the study by Zhang *et al* (11), and were obtained from Shanghai GeneChem Co., Ltd. The first and second pairs of primers used were as follows: Sense, 5'-CGCGTCCACTAG TACGGGGGGGGGG-3' and antisense, 5'-GGCACCTCC TCGACGTCAGTATG-3'; and sense, 5'-GCGCACGGC TCCACTAGT-3' and antisense, 5'-ACCAGGAAACAGCTA TGACCGGTCTTGCCAGCAAAGCAGTAG-3', respectively.

Following purification and labeling of the PCR products with M13 sequencing primer, 5'-GAAACAGCTATGACC-3', the amplified fragments were sequenced using the Genetic Analyzer 3730xl (Applied Biosystems; Thermo Fisher Scientific). The resulting files were aligned to the *ALK* reference sequence (National Center for Biotechnology Information accession number, NM_004304.3) in order to determine whether *ALK* mutations or fusions were present.

Statistical analysis. The χ^2 test was performed in order to estimate the association between *ALK* protein expression and clinicopathological features. Kaplan-Meier survival curves were plotted with significance calculated using log-rank statistics. Independent prognostic markers of OS and PFS were assessed using univariate and multivariate proportional Cox models. $P < 0.05$ was considered to indicate a statistically significant difference. All data analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA).

Results

***ALK* protein is detectable using IHC.** *ALK* protein expression was investigated using IHC staining in all HCC biopsies (Fig. 1). In the normal non-cancerous (confirmed by histopathological examination) biopsied samples adjacent to the tumor samples, cytoplasmic *ALK* staining was absent or poor, and occurred in very few cells (Fig. 1A). Of the 342 enrolled patients, 153 exhibited various degrees of brown cytoplasmic staining in the majority of tumor cells (Fig. 1B-D).

***ALK* status detection via FISH.** No HCC specimens demonstrated any evidence of rearrangement or amplification in the *ALK* locus using a break-apart FISH assay. However, in 112/342 images, gain of *ALK* gene copy number was observed, which was regarded as indicating FISH-positivity (Fig. 2A), and the remaining samples were regarded to be FISH-negative (Fig. 2B). Among the 112 FISH-positive patients, 108 were additionally positive for *ALK* protein expression identified via IHC.

***ALK* status detection via RT-qPCR and RACE-coupled PCR sequencing assays.** In order to compare the levels of *ALK* messenger RNA (mRNA) in 342 HCC specimens with that of adjacent non-cancerous tissues, RT-qPCR was performed. The results of the present study revealed that *ALK* mRNA was significantly increased in 162 HCC samples. Notably, of the 162 cases exhibiting RT-qPCR positivity, 139 were IHC-positive and 101 were additionally FISH-positive (Table I). Fig. 3A illustrates nine representative cases. Furthermore, *ALK* status was detected in 162 cases that were RT-qPCR positive using RACE-coupled PCR sequencing. Following alignment of the gene sequence with the *ALK* reference sequence based on a previous study (11), no mutations or fusions with potential partners were detected (Fig. 3B).

Certain clinicopathological features are associated with *ALK* protein overexpression. The present study evaluated the association between *ALK* gene expression and clinicopathological features of HCC patients (Table II). All analyses were performed according to IHC outcomes, as *ALK* protein was more stable than DNA and RNA, which assured comparability

Table I. Concordance among IHC, FISH and RT-qPCR results for anaplastic lymphoma kinase expression in 342 hepatocellular carcinoma patients.

RT-qPCR	FISH	IHC-positive (n=153)	IHC-negative (n=189)
Positive	Positive	101	2
Positive	Negative	38	21
Negative	Positive	7	2
Negative	Negative	7	164

IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

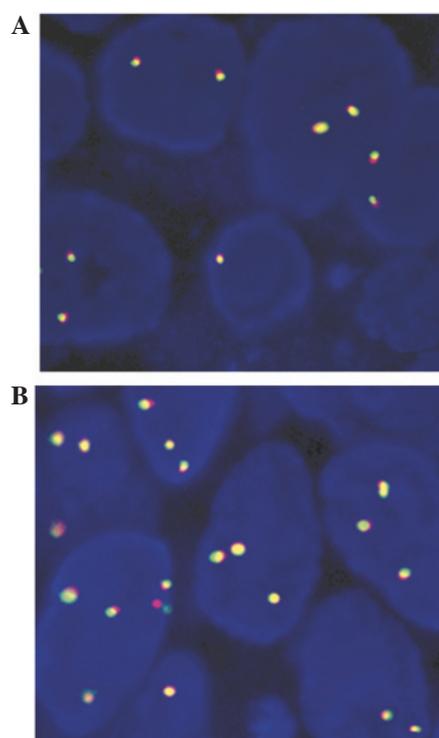


Figure 2. Fluorescence *in situ* hybridization results in representative tumor tissue samples from hepatocellular carcinoma patients. (A) Gain of *ALK* gene copy number positivity. (B) Gain of *ALK* gene copy number negativity. *ALK*, anaplastic lymphoma kinase. Magnification, x1,000.

among histological specimens. The results of the present study indicated that *ALK* protein expression was highly correlated with hepatitis C virus (HCV) status ($P < 0.001$) and micrometastases ($P = 0.011$), however, it was not significantly associated with patient age, gender, hepatitis B surface antigen (HBsAg), α -fetoprotein (AFP), cirrhosis, tumor size, tumor multiplicity, clinical stage, vascular invasion, Child-Pugh classification, recurrence and lymph node metastasis ($P > 0.05$).

Survival analysis. By the cutoff day on June 1, 2013, 68.4% (234/342) of the enrolled patients, all of whom exhibited recurrence or uncontrolled HCC, had succumbed. The median follow-up duration was 36.0 months (range, 0.6-79 months),

Table II. Correlation between clinicopathological features and negative (n=189) and positive (n=153) anaplastic lymphoma kinase (ALK) protein expression in hepatocellular carcinoma patients.

Clinicopathological feature	n	ALK protein		P-value
		Negative, n (%)	Positive, n (%)	
Age at diagnosis, years				
<60	265	153 (57.7)	112 (42.3)	0.088
≥60	77	36 (46.8)	41 (53.2)	
Gender				
Male	270	145 (53.7)	126 (46.7)	0.165
Female	72	45 (62.5)	27 (37.5)	
Hepatitis B surface antigen				
Negative	81	45 (55.6)	36 (44.4)	0.952
Positive	261	144 (55.2)	117 (44.8)	
Hepatitis C virus				
Negative	324	189 (58.3)	135 (41.7)	<0.001
Positive	18	0 (0.0)	18 (100.0)	
α-fetoprotein, ng/ml				
<20	169	90 (53.3)	79 (46.7)	0.460
≥20	173	99 (57.2)	74 (42.8)	
Cirrhosis				
Absent	155	90 (58.1)	65 (41.9)	0.343
Present	187	99 (52.9)	88 (47.1)	
Tumor size, cm				
<5	189	108 (57.1)	81 (42.9)	0.437
≥5	153	81 (52.9)	72 (47.1)	
Tumor multiplicity				
Single	276	151 (54.7)	125 (45.3)	0.674
Multiple	66	38 (57.6)	28 (42.4)	
Clinical stage				
I-II	163	90 (55.2)	73 (44.8)	0.986
III-IV	179	99 (55.3)	80 (44.7)	
Vascular invasion				
No	210	117 (55.7)	93 (44.3)	0.832
Yes	132	72 (54.5)	60 (45.5)	
Child-Pugh score				
A	316	177 (56.0)	139 (44.0)	0.331
B	26	12 (46.2)	14 (53.8)	
Recurrence				
No	250	144 (57.6)	106 (42.4)	0.152
Yes	92	45 (48.9)	47 (51.1)	
Lymph node metastasis				
No	288	153 (53.1)	135 (46.9)	0.066
Yes	54	36 (66.7)	18 (33.3)	
Micrometastases				
No	297	172 (57.9)	125 (42.1)	0.011
Yes	45	17 (37.8)	28 (62.2)	

and 13 (3.8%) cases were lost during the follow-up period. The potential effect of the *ALK* gene on survival was assessed. In the total patient cohort, it was observed that OS did not

significantly differ between patients with positive or negative *ALK* expression ($\chi^2=3.238$; $P=0.072$; Fig. 4A). However, PFS for patients with positive *ALK* expression was significantly poorer

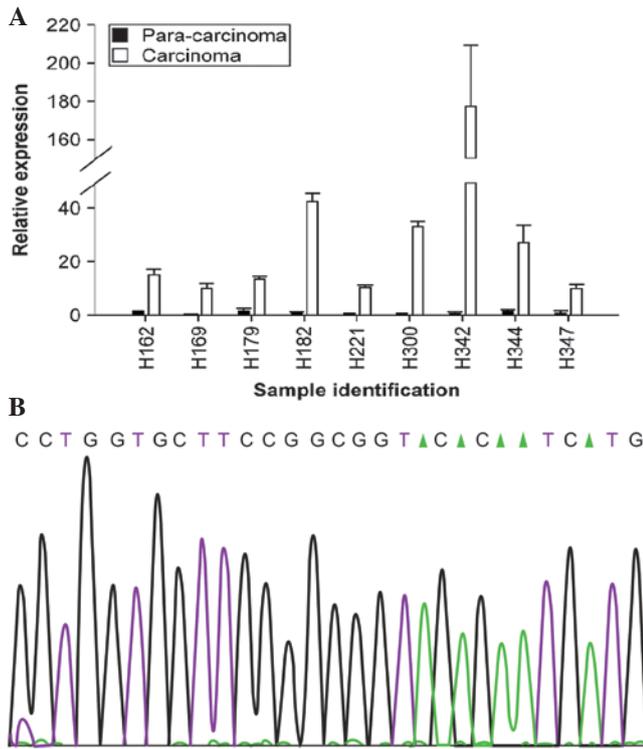


Figure 3. Detection of ALK status using reverse transcription-quantitative PCR and rapid amplification of complementary DNA ends-coupled PCR sequencing. (A) Results of transcriptional profiling revealing messenger RNA expression levels of the *ALK* gene in 9 representative pairs of para-carcinoma and carcinoma tissues, from patients exhibiting hepatocellular carcinoma. (B) Representative sequencing chromatogram demonstrating no presence of fusions or mutations in the *ALK* gene. ALK, anaplastic lymphoma kinase; PCR, polymerase chain reaction.

compared with that of patients with negative *ALK* expression ($\chi^2=4.187$; $P=0.041$; Fig. 4B). Following additional stratification of 342 HCC patients, OS and PFS were found to be markedly reduced in patients exhibiting *ALK* expression compared with patients without *ALK* expression, in subgroups that were HBsAg positive ($P=0.026$ vs. 0.015; Fig. 5A-B), HCV negative ($P=0.043$ vs. 0.021; Fig. 5C-D), stage III-IV ($P=0.002$ vs. 0.001; Fig. 5E-F), recurrence positive ($P=0.029$ vs. 0.034; Fig. 6A-B) and micrometastasis positive ($P=0.039$ vs. 0.036; Fig. 6C-D). However, in the HBsAg-negative ($P=0.863$ vs. 0.869), HCV-positive (no comparison analysis), stage I-II ($P=0.895$ vs. 0.825), recurrence-negative ($P=0.375$ vs. 0.267) and micrometastasis-negative ($P=0.184$ vs. 0.152) subgroups, *ALK* expression exerted no impact on OS or PFS. Furthermore, *ALK* expression was not associated with survival stratified by age, gender, AFP, cirrhosis, tumor size, tumor multiplicity, vascular invasion, Child-Pugh classification and lymph node metastasis.

In addition, Cox proportional hazards model was applied in order to reveal the independent impacts of the following features on OS and PFS: Age, gender, HBsAg, HCV, AFP, cirrhosis, tumor size, tumor multiplicity, clinical stage, vascular invasion, recurrence, lymph node metastasis, micrometastases, Child-Pugh classification and *ALK* protein expression. The results of univariate and multivariate analyses indicated that *ALK* protein expression exerted a significant prognostic effect on PFS in HCC patients (hazard ratio, 1.365; 95% confidence interval, 1.029-1.810; $P=0.031$; Table III), although it was not

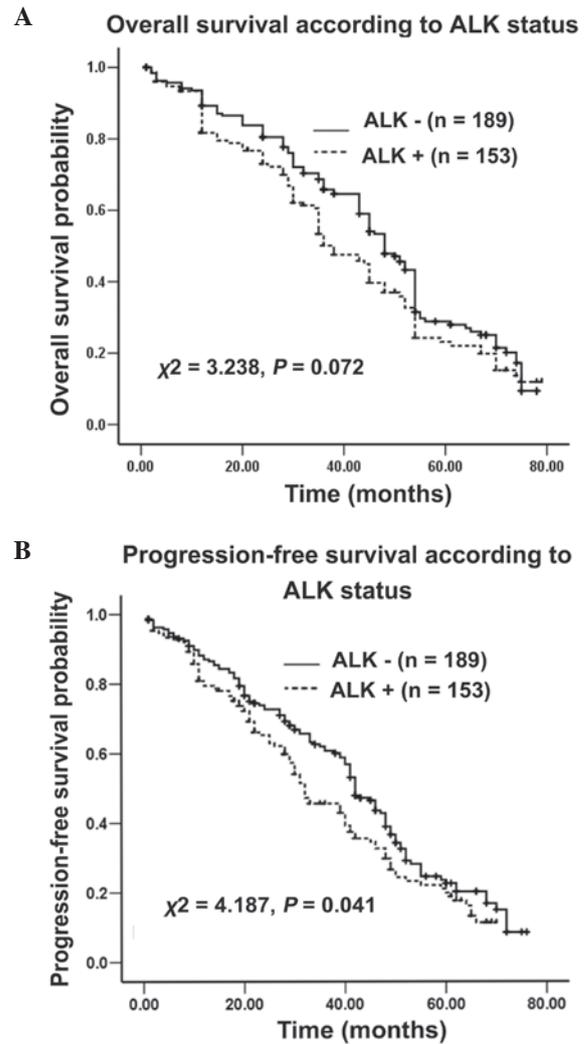


Figure 4. Kaplan-Meier survival curves of hepatocellular carcinoma patients. (A) Overall survival (ALK-negative vs. ALK-positive); (B) Progression-free survival (ALK-negative vs. ALK-positive). ALK, anaplastic lymphoma kinase.

an independent prognosticator for OS (hazard ratio, 1.290; 95% confidence interval, 0.973-1.709; $P=0.076$). *ALK* protein expression may be an independent risk factor for OS ($P=0.041$ vs. 0.042) and PFS ($P=0.029$ vs. 0.033; Table IV), particularly for stage III-IV patients.

Discussion

To the best of our knowledge, the results of the present study demonstrated the first evidence that *ALK* expression was increased at a transcriptional level, and additionally at a translational level, in human HCC samples compared with adjacent normal tissue samples. *ALK* rearrangement was not observed in any of the examined samples. The expression of *ALK* protein was significantly correlated with the aggressiveness and prognosis of primary HCC.

HCC is a pathologically and clinically heterogeneous neoplasm, exhibiting high malignancy and a consequent poor prognosis due to its aggressive features (27). Despite the complexity of hepatocarcinogenesis, the discovery of additional prognostic predictors and therapeutic targets for HCC has attracted particular interest. As a result, a number

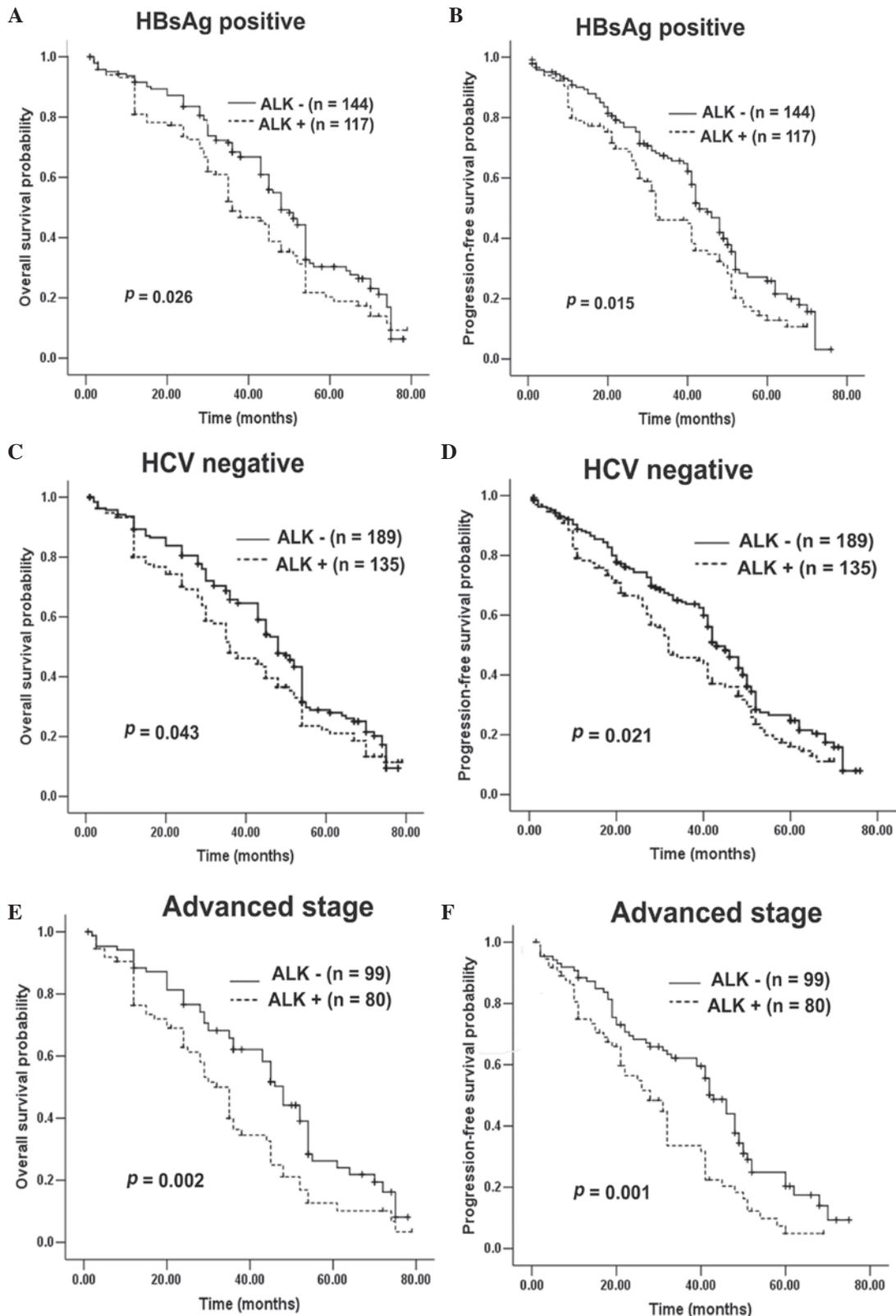


Figure 5. Kaplan-Meier survival curves of certain subgroups stratified according to clinicopathological features. (A) OS and (B) PFS (ALK-negative vs. ALK-positive) of patients exhibiting HBV infection. (C) OS and (D) PFS (ALK-negative vs. ALK-positive) of patients without HCV infection. (E) OS and (F) PFS (ALK-negative vs. ALK-positive) of patients exhibiting advanced stage (III-IV) tumors. OS, overall survival; PFS, progression-free survival; ALK, anaplastic lymphoma kinase; HBV, hepatitis B virus; HCV, hepatitis C virus; HBsAg, hepatitis B surface antigen.

of genes, including epidermal growth factor receptor, transforming growth factor β and *c-MET*, have been identified as molecular targets of HCC (28). Recently, much attention has

been paid to the *ALK* gene, a member of the RTK family, the dysregulation of which is associated with abnormal development and malignant transformation in human cancer (29).

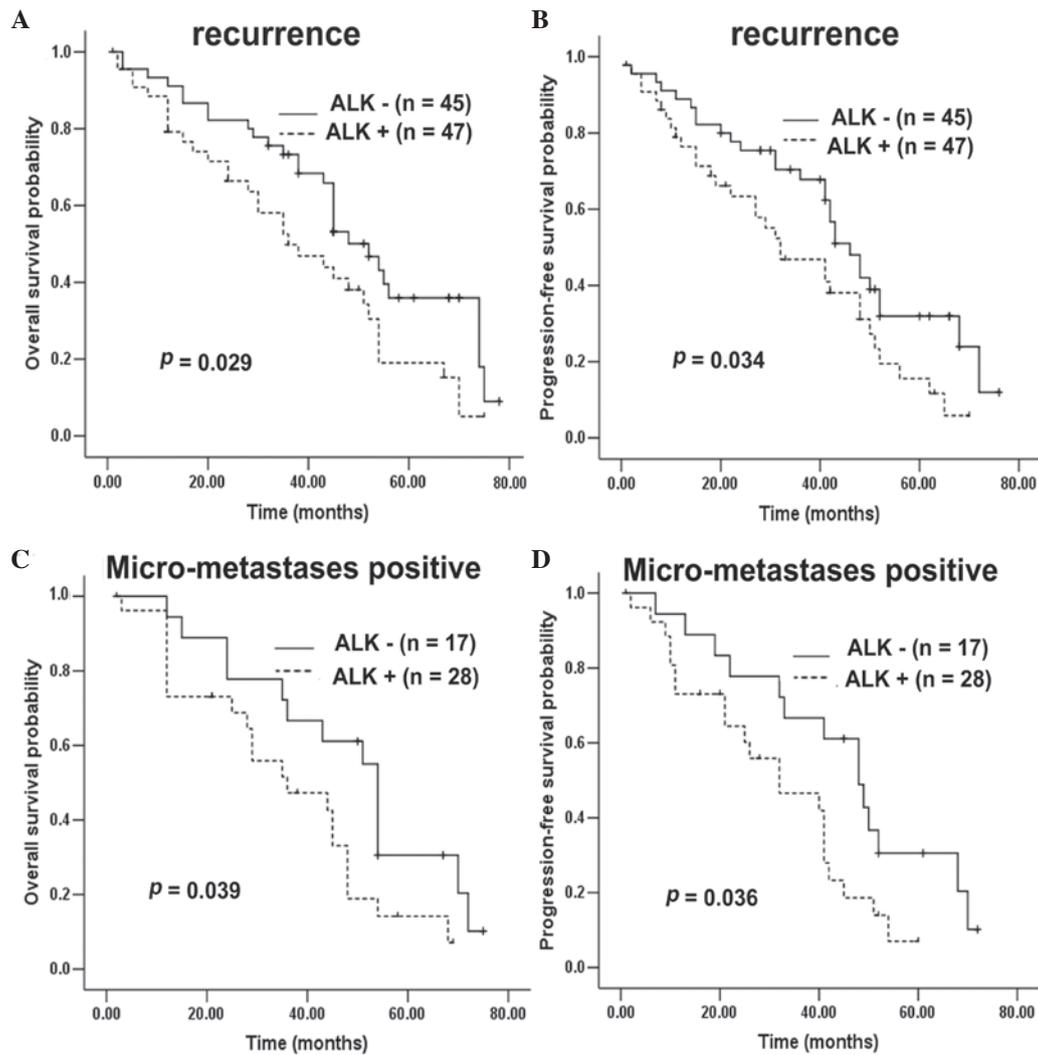


Figure 6. Kaplan-Meier survival curves of subgroups stratified according to certain clinicopathological features. (A) OS and (B) PFS (ALK-negative vs. ALK-positive) of patients with recurrence. (C) OS and (D) PFS (ALK-negative vs. ALK-positive) of patients exhibiting micrometastases. OS, overall survival; PFS, progression-free survival; ALK, anaplastic lymphoma kinase.

However, to the best of our knowledge, the clinical value of *ALK* abnormalities in human HCC has not previously been comprehensively evaluated.

In the present study, *ALK* status was investigated in a large cohort of HCC patients. IHC, RT-qPCR and FISH analyses revealed that increased expression of *ALK* protein and mRNA, as well as *ALK* gene copy number gain, occurred in HCC, with the rates of expression observed in patients being 44.7% (153/342), 47.4% (162/342) and 32.7% (112/342), respectively. Notably, there was concordance in the assessment of *ALK* expression levels among IHC, RT-qPCR and FISH detection methods, demonstrating that *ALK* gene upregulation was present at post-transcriptional and transcriptional levels. Meanwhile, the results of the present study revealed that RT-qPCR possessed a higher sensitivity (90.8%) and that FISH had increased specificity (97.9%) compared with IHC staining, which demonstrated similar results to those of a previously published study (30). However, it is notable that *ALK* overexpression or *ALK* gene copy number gain is not indicative of *ALK* rearrangement. To the best of our knowledge, in patients exhibiting NSCLC,

copy number gain and amplification of the *ALK* gene are reportedly highly expressed, while *ALK* translocations are rare (13). Similar results have also been described in esophageal carcinoma (18). In the present study, no rearrangement, amplification or mutation of the *ALK* locus was identified using FISH and RACE-coupled PCR sequencing assays. A recent retrospective study (12) reported that *ALK* gene copy number gain was common in HCC investigated with FISH analysis, whereas *ALK* rearrangement was not observed. These previous results supported the results of the present study, which indicated that the *ALK* gene was upregulated in HCC, and that this may be a potential biomarker for HCC.

The present study additionally characterized the association between *ALK* protein and the clinicopathological features of HCC. The results of the present study demonstrated that *ALK* protein overexpression possessed significant correlation with HCV status and micrometastases. Briefly, *ALK* may be a valuable indicator for the identification of subsets of HCC cases with HCV infection and increased invasive tendency. Various experimental model systems have proved that *ALK* gene overexpression contributes to cell migration

Table III. Univariate and multivariate analyses of progression-free survival in 342 hepatocellular carcinoma patients.

A, Univariate analysis			
Parameter	Variable	Hazard ratio (95% confidence interval)	P-value
Age at diagnosis, years	<60 vs. ≥60	1.012 (0.747-1.372)	0.938
Gender	Male vs. female	0.880 (0.635-1.219)	0.443
Hepatitis B surface antigen	Positive vs. negative	1.068 (0.787-1.449)	0.673
Hepatitis C virus	Positive vs. negative	0.816 (0.433-1.538)	0.529
α-fetoprotein, ng/ml	<20 vs. ≥20	0.932 (0.721-1.205)	0.591
Cirrhosis	Absent vs. present	1.026 (0.791-1.330)	0.849
Tumor size, cm	<5 vs. ≥5	1.143 (0.883-1.478)	0.310
Tumor multiplicity	Single vs. multiple	1.050 (0.763-1.447)	0.763
Clinical stage	I-II vs. III-IV	1.228 (0.949-1.589)	0.118
Vascular invasion	No vs. yes	1.113 (0.854-1.450)	0.430
Recurrence	No vs. yes	1.002 (0.749-1.340)	0.990
Lymph node metastasis	No vs. yes	1.099 (0.779-1.550)	0.591
Micrometastases	No vs. yes	1.397 (0.974-2.004)	0.069
Anaplastic lymphoma kinase protein	Positive vs. negative	1.304 (1.005-1.691)	0.045
Child-Pugh classification	A vs. B	6.220 (3.764-10.278)	<0.001
B, Multivariate analysis			
Parameter	Variable	Hazard ratio (95% confidence interval)	P-value
Anaplastic lymphoma kinase protein	Positive vs. negative	1.365 (1.029-1.810)	0.031
Child-Pugh classification	A vs. B	7.198 (4.261-12.159)	<0.001

Table IV. Univariate and multivariate analyses of overall survival for 179 stage III-IV hepatocellular carcinoma patients.

A, Univariate analysis			
Parameter	Variable	Hazard ratio (95% confidence interval)	P-value
Age at diagnosis, years	<60 vs. ≥60	1.026 (0.700-1.505)	0.894
Gender	Male vs. female	0.958 (0.625-1.499)	0.883
Hepatitis B surface antigen	Positive vs. negative	1.062 (0.708-1.593)	0.770
Hepatitis C virus	Positive vs. negative	0.885 (0.389-2.014)	0.771
α-fetoprotein, ng/ml	<20 vs. ≥20	0.875 (0.648-1.240)	0.453
Cirrhosis	Absent vs. present	1.137 (0.804-1.609)	0.468
Tumor size, cm	<5 vs. ≥5	1.095 (0.569-1.407)	0.631
Tumor multiplicity	Single vs. multiple	1.264 (0.725-2.203)	0.408
Vascular invasion	No vs. yes	1.104 (0.609-1.224)	0.410
Recurrence	No vs. yes	1.091 (0.744-1.599)	0.656
Lymph node metastasis	No vs. yes	1.081 (0.636-1.361)	0.711
Micrometastases	No vs. yes	1.230 (0.832-1.816)	0.299
Anaplastic lymphoma kinase protein	Positive vs. negative	1.416 (0.999-2.006)	0.041
Child-Pugh classification	A vs. B	6.898 (3.593-13.240)	<0.001
B, Multivariate analysis			
Parameter	Variable	Hazard ratio (95% confidence interval)	P-value
Anaplastic lymphoma kinase protein	Positive vs. negative	1.894 (1.023-3.507)	0.042
Child-Pugh classification	A vs. B	8.610 (4.246-17.786)	<0.001

and invasion (10,31,32), which are crucial steps in the metastatic cascade, and may lead to distant organ involvement and tumor aggressiveness (33). Furthermore, Shao *et al* (34) reported that ALK protein was overexpressed in HCC and may be involved in the progression of HCC tumors, although the patient cohort investigated in this previous study was small. Similarly, the results of the present study suggested a significant impact of ALK overexpression on the promotion of the development and metastasis of HCC.

Activation of proto-oncogenes and inactivation of tumor suppressors contributes to the occurrence and progression of malignancies. Specifically, the ALK oncogene is activated by its endogenous ligands, midkine and pleiotrophin, which serve as mitogenic and angiogenic factors in cancer (10). Given that the activated ALK gene induces an intricate system of downstream signaling cascades, including phosphoinositide 3-kinase/protein kinase B and mitogen-activated protein kinase pathways, it is possible that the ALK gene may mediate cellular proliferation, motility and apoptosis, and thereby possess a significant role in the promotion of tumorigenesis and development *in vitro* and *in vivo* (35). Recently, Hasan *et al* (33) illustrated that MYC proteins that targeted key proliferative pathways in the cancer development process, and thus contributed to tumor growth and metastasis in neuroblastoma, regulated ALK expression. Notably, Di Paolo *et al* (36) reported that the RNA interference-based knockdown of ALK, independent of fusion gene status, resulted in the downregulation of proliferation and the upregulation of apoptosis in neuroblastoma tumor cells, thereby ultimately inhibiting tumor growth and prolonging survival *in vivo*. Therefore, these previous results confirm that ALK has a significant role in the progression and metastasis of HCC.

In addition, ALK overexpression has been documented as possessing an unfavorable prognosis in multiple human cancers, including neuroblastoma (36), breast carcinoma (37) and basal cell carcinoma (38). Similarly, the present study revealed that ALK protein was significantly correlated with poor PFS in the entire study cohort, particularly for patients with HBsAg positivity, HCV negativity, advanced stage tumors, recurrence or micrometastases. Notably, there were certain differences between the results of the present study and the previous findings of Jia *et al* (12), which demonstrated that ALK gene copy number gain predicted the survival of patients exhibiting HBsAg negativity, however, did not impact on the survival of patients with HBsAg positivity. The primary reasons for the disagreement between these sets of results may be due to the genetic backgrounds and research methods utilized (protein versus gene copy number). The results of the current study concluded that the prognosis of HCC patients exhibiting ALK overexpression tended to be poorer.

Due to the retrospective nature of the present study, a number of patients and their clinical information were lost to follow-up, particularly those patients who were not hospitalized following surgery, which may have led to bias in the results. Accordingly, additional experiments *in vitro* and *in vivo* are required to confirm the results of the present study. In addition, it remains to be elucidated whether HCC patients exhibiting ALK overexpression may benefit from

treatment with ALK inhibitors. Further research focusing on these issues is required.

In conclusion, ALK may serve as a valuable predictor of micrometastases and poor survival. Therefore, radiological diagnosis in combination with ALK detection may assist with the prognostic evaluation of novel HCC cases, as optimal individualized therapeutic strategies remain to be devised.

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