TP53 and RET may serve as biomarkers of prognostic evaluation and targeted therapy in hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) is the most common malignancy of the liver. Genomic analysis is conducted to identify genetic alterations in driver genes which are all druggable targets for cancer therapy. In the present study, we performed an exome sequencing of 45 driver genes in 100 paired samples from HCC patients including tumors and matched adjacent normal tissues using Illumina HiSeq 2000 platform. Non-synonymous mutations were ascertained using the iPLEX MassARRAY system and Sanger sequencing. Clinicopathological relevance with genetic variations was assessed using SPSS software. The prognostic analyses of patients with gene mutation status were summarized using Kaplan-Meier curves. Sixty-one non-synonymous somatic mutations were identified in 43% of the HCC patients. The most frequent mutations were: *TP53* (20%), *RET* (6%), *PLCE1* (5%), *PTEN* (4%) and *VEGFR2* (3%). Patients with mutations in TP53 had a lower overall survival (OS) (P=0.002) than those without mutations. Recurrent mutations in the *Ret* proto-oncogene (*RET*) were associated with poor outcomes for both disease-free survival (DFS) (P=0.028) and OS (P=0.001) in HCC patients. The mutational status of sorafenib-targeted genes were associated with decreased DFS (P=0.039), and decreased OS (P=0.15) without statistical significance. Mutual exclusion of *TP53* and *RET* mutations were observed in the present study. In conclusion, patients with *TP53* mutations, *RET* mutations and sorafenib-targeted gene mutations were demonstrated to be associated with poor HCC prognosis, which suggests that both *TP53* and *RET* may serve as biomarkers of prognostic evaluation and targeted therapy in HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the most common malignancy of the liver. It is the fifth most common cancer in men and the seventh amongst women worldwide (1,2). There is a high incidence of HCC in China due to hepatitis B virus (HBV) infection (3). More than 80% of HCC tumors are inoperable with poor prognosis, and only 10-20% of HCC patients undergo curative treatments (4,5). Results from clinical trials show a lack of survival benefits following HCC treatment with chemotherapeutic agents and conventional drugs. Thus, effective and well-tolerated treatment strategies for advanced HCC are urgently needed (6).

Sorafenib, an oral multikinase inhibitor of *BRAF*, *RAF1*, *FLT3*, *KIT*, *VEGFR* and *PDGFR*, has been approved for the treatment of advanced HCC. The Sorafenib HCC Assessment Randomized Protocol trial and the Asia-Pacific trial demonstrated that sorafenib improves the survival of patients with advanced HCC (7,8). Since this major development in HCC treatment, a focus has been shifted to identify novel agents that target driver genes and key molecular pathways in hepatocarcinogenesis (9). Their findings include components of the RAS/RAF/mitogen-extracellular activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK),

and phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathways that regulate cell proliferation, apoptosis, and protein synthesis; receptor tyrosine kinases (RTKs), including the epidermal growth factor receptor (*EGFR*), *KIT*, *FLT3* and *RET*, which transmit growth factor signals to downstream intracellular pathways; and proangiogenic factors that bind VEGFR and PDGFR which induce angiogenic signaling via the RAS/RAF/MEK/ERK, PI3K/AKT/mTOR and Wnt signal transduction pathways (6,10-12). A thorough understanding of the mutations of genes associated with molecular-targeted therapy is needed to screen compounds and antibodies effective in HCC treatment.

Targeted sequencing allows accurate analysis of multiple cancer genes (13-15). However, mutational profiling of driver genes in Chinese patients with HCC has not been reported, to date. In the present study, we detected multiple mutations in 45 genes in 100 patients with HCC using next-generation targeted sequencing. These genes were categorized according to the following biological processes or signaling pathways: RTKs, angiogenesis, RAS/RAF/MEK/ERK and PI3K/AKT/mTOR. In particular, we identified numerous novel somatic mutations in the driver genes and further found that patients with *TP53*, *RET* and sorafenib-targeted gene mutations, were associated with poor HCC prognosis.

Materials and methods

Patients. We analyzed 100 patients who underwent HCC resection between November 2009 and December 2011. Patients were subjected to pathological assessment in order to establish histological diagnosis and tumor cellularity. Only patients with a pathological diagnosis of HCC and tumor nuclei ≥80% of the total cellular nuclei were included. The present study was approved by the Ethics Committee of The First Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, China). Signed informed consent forms were obtained from patients before their participation in the present study.

Exon capture array and deep sequencing. A customized NimbleGen HD 2.1 Array was constructed using the SeqCap v2 software. Target sequence capturing was performed using the SeqCap EZ Reagent kit. The captured DNA was randomly fragmented into an average size of 200-300 bp, and both ends of the fragments were ligated with adaptors that bind to different index primers. The enriched DNA fragments were eluted from the array and amplified by ligation-mediated PCR. On average, we sequenced the target exon regions of each sample to a mean depth of 75x using the Illumina HiSeq 2000 platform.

Genome mapping and mutation detection. Mapping and Assembly with Quality software (http://maq.sourceforge.net) was used to align the sequence reads to the referenced human genome (hg19). The parameters used for the alignment were as follows: i) maximum distance between sequences, 300; ii) maximum allowed sum of qualities for 2-paired reads, 70; and iii) number of mismatches in the first 24 bp of mismatches. Single nucleotide variations (SNVs) of high quality were obtained using the following filtering parameters: i) SNVs with depth ≥5; ii) consensus quality ≥30; iii) 3-bp flanking quality ≥40; iv) highest mapping quality ≥30; and v) SNVs with variant depth ≥8. We defined the variant depth as 8 based on the results of a previous study (13). The high quality SNVs were filtered using the dbSNP (v.132) and 1K Genome databases to define the mutations (16).

MassARRAY and Sanger sequencing validation. Non-synonymous mutations were validated using the iPLEX MassARRAY system (Sequenom Inc., San Diego, CA, USA) and Sanger sequencing in HCC tumors and paired peritumoural liver tissues to discriminate somatic and germline mutations. Both the PCR and MassEXTEND® primers for each mutation were *in silico* designed using the MassARRAY Assay design 4.0 software. Multiplex PCR was performed using the GeneAmp PCR System 9700 Dual 384-Well Sample Block Module (Applied Biosystems, Foster City, CA, USA), followed by dephosphorylation, single-base extension, and desalting. The MassARRAY Nanodispenser RS1000 was used to spot reactions with the 384 SpectroCHIP, which was loaded into a MALDI-TOF mass spectrometer. Genotype calls by MassARRAY Type 4.0 were confirmed by examining the spectra for each assay and sample. Mutations not detected by the iPLEX MassARRAY were reconfirmed by Sanger sequencing.

Immunohistochemistry and tissue microarray. In patients with multinodular tumors, samples were obtained from the largest tumor. Rabbit anti-human monoclonal Ret antibody (EPR2871, 1:500; Abcam, Cambridge, MA, USA) was used to detect the protein expression of RET. The intensity of RET was calculated based on mean area of positive staining. Tissues were incubated with primary rabbit anti-human monoclonal Ret antibody (EPR2871, 1:500; Abcam), then, treated with biotinylated goat anti-rabbit secondary antibodies. Antibodies were visualized using diaminobenzidine hydrogen peroxidase as the chromogen, and slides were counterstained with 0.5% hematoxylin. In addition, we analyzed another 90 independent samples to elucidate RET protein expression using tissue microarray. Matched 90 pairs of primary HCC samples and peritumoral liver tissues were used to prepare tissue microarray (Shanghai Biochip Co., Ltd., Shanghai, China) as previously described (17). The intensity of RET was classified into high expression and low expression based on the mean area of positive staining. High expression was defined as ≥40% staining of a tumor section, and low expression as <40%.

Statistical analysis. Statistical analyses were performed using SPSS software (version 21.0; SPSS, Inc., Chicago, IL, USA). Age, gender, tumor stage, number, size and grade, HBV infection, and α-fetoprotein (AFP) were the covariates of clinical characteristics included in the model. Chi-square and Fisher's exact tests were applied to compare the frequencies between genetic and clinical variables. The prognosis analyses of patients with gene mutation status were summarized using Kaplan-Meier curves. Univariate disease-free survival (DFS) and overall survival (OS) analyses were carried out using log-rank tests, and multivariate analyses were conducted using Cox's proportional hazards model.

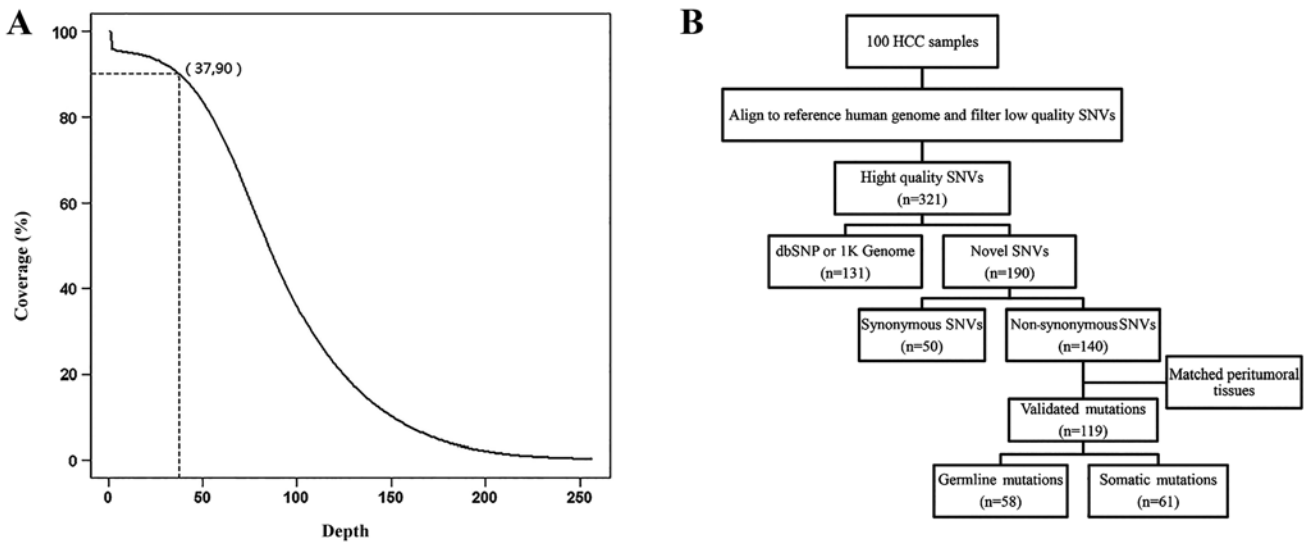

Figure 1. Overall cumulative coverage and flow-chart of the data analysis and mutation detection of genes in HCC patients. (A) A read coverage of 90% of the targeted exons was achieved as the sequencing depth was 37x. (B) In the cohort of 100 HCC patients, 321 SNVs were detected by quality filters. After excluding single nucleotide polymorphisms in dbSNP and 1K Genome, 190 mutations remained of which 119 corresponded to non-synonymous mutations. After validation, 60 somatic mutations and 58 germline mutations were finally obtained. HCC, hepatocellular carcinoma; SNVs, single nucleotide variations.

Postoperative mortality was assessed, with deaths unrelated to tumor recurrence considered censored observations at the time of death. $P<0.05$ was considered to indicate a statistically significant result.

Results

Genomic alteration landscape in HCC by whole-exome sequencing. Whole exons of 45 genes were sequenced in 100 patients with HCC using array-based sequence capture and Illumina HiSeq 2000 sequencing. Among them, 15 genes were associated with RTKs, 8 with angiogenesis, 13 with the RAS/RAF/MEK/ERK pathway, and 9 with the PI3K/AKT/mTOR pathway. For each sample, we generated an average of 33.75 Mb bases, 97.9% of which were well-mapped to the human genome. The average sequencing depth was 75x (Table I). We achieved a read coverage of 90% of the targeted exons at the sequencing depth of 37x (Fig. 1A). These results indicate high quality targeted sequencing for mutation analysis.

SNV identification, validation and annotation. A computational pipeline was developed to discover novel SNVs (Fig. 1B). A total of 321 exonic SNVs were identified in the 100 HCC patients. Approximately 40.8% (131/321) of the SNVs were present after the data were filtered using the dbSNP and 1K Genome databases. In most SNVs identified we observed genetic polymorphisms, which confirmed our sequencing data. The remaining 190 novel SNVs included 50 synonymous and 140 non-synonymous SNVs. In a random validation of 40 SNVs with MassARRAY and Sanger sequencing, the confirmation rate was 90%. Furthermore, non-synonymous SNVs were validated in the original HCC and paired peritumoural liver tissues using MassARRAY and Sanger sequencing. We validated 119 mutations, of which 60 were somatic mutations and 58 were germline mutations. In the somatic mutations, 34 were novel mutations and 27 were recorded in COSMIC, a public database of somatically acquired mutations in cancer (18).

Table I. Summary of sequencing coverage of the 100 HCC samples.

Category	Mean of 100 HCC samples
Total reads	393,273.06
Total bases	35,394,575.4
Average read length (bp)	90
Mappable reads	385,309.01
Mappable bases	34,677,810.9
Mappable base rates (%)	97.90299419
Average sequencing depth	75x
Mean coverage over target gene	75x

HCC, hepatocellular carcinoma.

The annotations of somatic mutations observed in the present study, are summarized in Table II. The list of functional domains (identified using the NCBI database) which harbor the mutations and molecular-targeted agents of the mutated genes were identified using the DrugBank database (Table II) (19). A few of the mutations were observed in the protein kinase domains, particularly the tyrosine kinase motifs of the target genes, which play a key role in the signaling pathways that contribute to carcinogenesis.

Mutation frequency of each gene distributed in 4 biological categories. A total of 60 somatic mutations occurred within 45 genes, with an average mutation frequency of 0.62/affected individual. The number of somatic mutations ranged from 0 to 3 in the HCC patients. Fig. 2 shows the complete somatic mutation frequency of each gene in the 4 categories including

Table II. List of non-synonymous somatic mutations and annotation of functional domains and molecular-targeted agents.

Biological classification	Gene	Nucleotide (genomic)	Amino acid change	Mutation type	No. of samples	Mutation in domain	Molecular-targeted agents
RTKs	ERBB1 (EGFR)	g.chr7:55225446A>T	E.11:H433L	M	1	Approximate	Cetuximab, trastuzumab, lidocaine, gefitinib, erlotinib, lapatinib, panitumumab, vandetanib, afatinib
	ERBB2	g.chr17:37866722A>T	E.6:T297S	M	1		Trastuzumab, lapatinib, ado-trastuzumab emtansine, pertuzumab, afatinib
	ERBB3	g.chr12:56478815A>T	E.3:M91L	M	1	Recep_L_domain	
	ERBB3	g.chr12:56495385A>G	E.28:D1192G	M	1		
	FGFR2	g.chr10:123260441A>T	E.10:L488Q	M	1		Palifermin, thalidomide, regorafenib, ponatinib
	C-FMS (CSF1R)	g.chr5:149449842A>T	E.8:W408R	M	1		Imatinib, sunitinib
	FLT3	g.chr13:28608267A>G	E.14:Y597H	M	1		Sorafenib, sunitinib, ponatinib
	NTRK2	g.chr9:87285754G>A	E.1:A31T	M	1	Sig_peptide	Amitriptyline
	RET	g.chr10:43595961A>T	E.2:D43V	M	1		Sorafenib, cabozantinib,
	RET	g.chr10:43595967C>T	E.2:A45V	M	1		regorafenib, ponatinib
	RET	g.chr10:43607598G>A	E.8:R525Q	M	1		
	RET	g.chr10:43608385T>G	E.9:I578S	M	1		
	RET	g.chr10:43612043G>T	E.12:K716N	M	1		
	RET	g.chr10:43615096C>A	E.14:S837Y	M	1	PTKc_RET, Pkinase_Tyr	
Angiogenesis	VEGFR1 (FLT1)	g.chr13:28893583A>T	E.24:L1088[a]	N	1	PTKc_VEGFR, Pkinase_Tyr	Sorafenib, sunitinib, pazopanib, axitinib, regorafenib
	VEGFR2 (KDR)	g.chr4:55979579T>C	E.7:S290G	M	1	V-set, Ig1_VEGFR	Sorafenib, sunitinib, pazopanib,
	VEGFR2 (KDR)	g.chr4:55974048A>T	E.10:I423N	M	1		axitinib, cabozantinib,
	VEGFR2 (KDR)	g.chr4:55955069T>C	E.26:H1159R	M	1	Pkinase_Tyr	regorafenib, ponatinib
	VEGFR3 (FLT4)	g.chr5:180048252G>T	E.14:A674D	M	1		Sorafenib, sunitinib, pazopanib, axitinib, cabozantinib, regorafenib, ponatinib
	PDGFRA	g.chr4:55133573C>A	E.5:R293S	M	1	Ig1_PDGFR-αβ	Becaplermin, imatinib, sunitinib, pazopanib, regorafenib, ponatinib
	PDGFRB	g.chr5:149515249T>G	E.2:D78A	M	1		Becaplermin, sorafenib, imatinib, dasatinib, sunitinib, pazopanib, regorafenib
	TIE1	g.chr1:43779006G>A	E.13:A113T	M	1	Interdomain contacts, FN3	

Table II. Continued.

Biological classification	Gene	Nucleotide (genomic)	Amino acid change	Mutation type	No. of samples	Mutation in domain	Molecular-targeted agents
RAS/RAF/MEK/ERK pathway	RASSF1	g.chr3:50369548T>A	E.3:Q62L	M	1		
	MAP2	g.chr2:210574644A>T	E.7:E281V	M	1		Estramustine, paclitaxel, docetaxel
	MAP2	g.chr2:210594945G>A	E.11:A502T	M	1		
	PLCE1	g.chr10:96005789A>T	E.7:Q836L	M	1		
	PLCE1	g.chr10:96006158A>G	E.7:Q959R	M	1		
	PLCE1	g.chr10:96022320A>T	E.13:Q1295L	M	1		
	PLCE1	g.chr10:96053334G>T	E.22:G1702V	M	1	Required for activation by RHOA, RHOB	
	PLCE1	g.chr10:96058211T>A	E.23:I1748N	M	1	Required for activation by RHOA, RHOB	
	SHC1	g.chr1:154938476C>T	E.10:G116R	M	1		
PI3K/PTEN/AKT/mTOR pathway	TP53	g.chr17:7579355A>G	E.4:L111P	M	2	P53 DNA-binding domain	
	TP53	g.chr17:7578542G>A	E.5:L130F	M	1	P53 DNA-binding domain	
	TP53	g.chr17:7578535T>A	E.5:K132M	M	1	P53 DNA-binding domain	
	TP53	g.chr17:7578515T>C	E.5:K139E	M	1	P53 DNA-binding domain	
	TP53	g.chr17:7578457C>A	E.5:R158L	M	1		
	TP53	g.chr17:7578436T>G	E.5:Q165P	M	1		
	TP53	g.chr17:7578394T>A	E.5:H179L	M	1		
	TP53	g.chr17:7578226T>A	E.6:D208V	M	1	P53_tetramer	
	TP53	g.chr17:7578206T>C	E.6:S215G	M	1	P53_tetramer	
	TP53	g.chr17:7578177C>A	E.6:E224D	M	1	P53_tetramer	
	TP53	g.chr17:7577586A>T	E.7:I232N	M	1		
	TP53	g.chr17:7577523G>A	E.7:T253I	M	1		
	TP53	g.chr17:7577130A>C	E.8:F270V	M	1		
	TP53	g.chr17:7577114C>A	E.8:C275F	M	1		
	TP53	g.chr17:7577105G>C	E.8:P278R	M	1		
	TP53	g.chr17:7577098T>A	E.8:R280S	M	1		
	TP53	g.chr17:7577097C>G	E.8:D281H	M	1		
	TP53	g.chr17:7577095G>C	E.8:D281E	M	1		
	TP53	g.chr17:7577082C>T	E.8:E286K	M	1		
	TP53	g.chr17:7574006A>C	E.10:F341V	M	1		
	TP53	g.chr17:7574003G>A	E.10:R342[a]	N	1		

Table II. Continued.

Biological classification	Gene	Nucleotide (genomic)	Amino acid change	Mutation type	No. of samples	Mutation in domain	Molecular-targeted agents
	PIK3CA	g.chr3:178917513A>T	E.2:M130L	M	1		
	PIK3CA	g.chr3:178947074G>T	E.17:G837V	M	1	PI3Kc_IA_α, PI3Kc	
	PTEN	g.chr10:89692781C>A	E.5:P89T	M	1	PTPc	
	PTEN	g.chr10:89692948A>T	E.5:K144N	M	1	PTPc	
	PTEN	g.chr10:89711875G>T	E.6:G165[a]	N	1	PTPc	
	PTEN	g.chr10:89717630C>T	E.7:Q219[a]	N	1	PTEN_C2	
	MTOR	g.chr1:11298522T>A	E.11:T647S	M	1	HEAT 2	Pimecrolimus, sirolimus, everolimus, temsirolimus
	MTOR	g.chr1:11288742C>A	E.18:D1005Y	M	1	HEAT 4, DUF3385	
	STK11	g.chr19:1221966C>T	E.7:P294L	M	1	S_TKc, PKc	

Coordinates refer to the human reference genome hg19 release [Genome Reference Consortium Human Build 37 (GRCh37), Feb. 2009]. Domain information was obtained from the NCBI database. Information concerning molecular-targeted agents was obtained from the DrugBank database. [a]Stop codon. E, exon; M, missense; N, nonsense.

RTKs, angiogenesis, the RAS/RAF/MEK/ERK and the PI3K/AKT/mTOR pathways.

There were 14 somatic mutations in the RTK genes (Fig. 2), with 4 mutations identified in the *EGFR* family, including 1 in *EGFR*, 1 in *ERBB2* and 2 in *ERBB3*. *RET* was found with a recurrent somatic mutation of 6% in HCC, which was validated using MassARRAY and Sanger sequencing. The RET protein is composed of an extracellular ligand-binding domain, a hydrophobic transmembrane domain and a cytoplasmic part with a protein tyrosine kinase domain (TK domain) (20). Immunohistochemical staining of HCC with *RET* mutation further revealed a significant (Fig. 3A, the left 4 panels) or slight increase (Fig. 3A, the right 2 panels) in the expression of RET in tumor tissues compared with peritumoral tissues. We also performed a tissue microarray study of a cohort containing another 90 HCC patients. The protein expression tendency revealed that RET protein levels were higher in HCC tissues than in paired peritumoral liver tissues using Student's t-test (P=0.012, Fig. 3B and C). The group with the high expression of RET included 28.9% (26/90) of the patients. F*GFR2*, *CSF1R*, *FLT3* and *NTRK2* had one mutation each, while there was no mutation for *ERBB4*, *FGFR1*, *FGFR3*, *FGFR4*, *IGF1R*, *MET* and *KIT*.

Eight somatic mutations were identified within genes associated with angiogenesis (Fig. 2). Of these mutations, 5 were identified in members of the VEGFR family, including one mutation in *VEGFR1* (*FLT1*), 3 mutations in *VEGFR2* (*KDR*), and 1 mutation in *VEGFR3* (*FLT4*). *PDGFRA*, *PDGFRB* and *TIE1* had one mutation each, while no mutation was detected in *PDGFRL* and *TEK* (*TIE2*).

In the RAS/RAF/MEK/ERK pathway, 9 somatic mutations were detected (Fig. 2). Somatic mutations occurred mostly in *PLCE1* (5%). Another recurrent mutated gene was *MAP2* (2%), and *RASSF1* and *SHC*1 exhibited one somatic mutation each. No somatic mutation was observed in *ERK*, *RAF1*, *ARAF*, *BRAF*, *NRAS*, *MEK1*, *MEK2*, *CXCR4* and *KRAS*.

Twenty-nine somatic mutations were detected within 9 genes associated with the PI3K/AKT/mTOR pathway, including recurrent mutations in *TP53* (20%), *PTEN* (4%), *mTOR* (2%), *PIK3CA* (2%), and a single mutation in *STK11* (Fig. 2). *TP53* was the highest mutated gene. No somatic mutation was observed in *BAD*, *PDK1*, *AKT1* and *RPS6KB1*.

Analysis of clinical characterization and prognosis. The clinicopathological characteristics of patients, including age, gender, tumor stage (American Joint Committee on Cancer; ver. 7), number, size, grade, serological HBV concentration and presence of the tumor marker AFP are summarized in Table III. The median follow-up of cases was 31.6 months (range, 1.8-48.1). A total of 39% of the patients died, with a median OS of 41.5 months and a 3-year OS of 64.0% as estimated by Kaplan-Meier analysis. During follow-up, 62 cases with recurrence were identified for a median DFS of 21.2 months. Correlation analysis of the clinical characteristics was based on our data. AFP-positive patients demonstrated a higher rate of *RET* mutations compared to those who were AFP-negative (11.1 vs. 0%; P=0.039), without correlation to other genes.

The univariate analysis of DFS indicated that the significant predictors of DFS were the somatic mutation status of

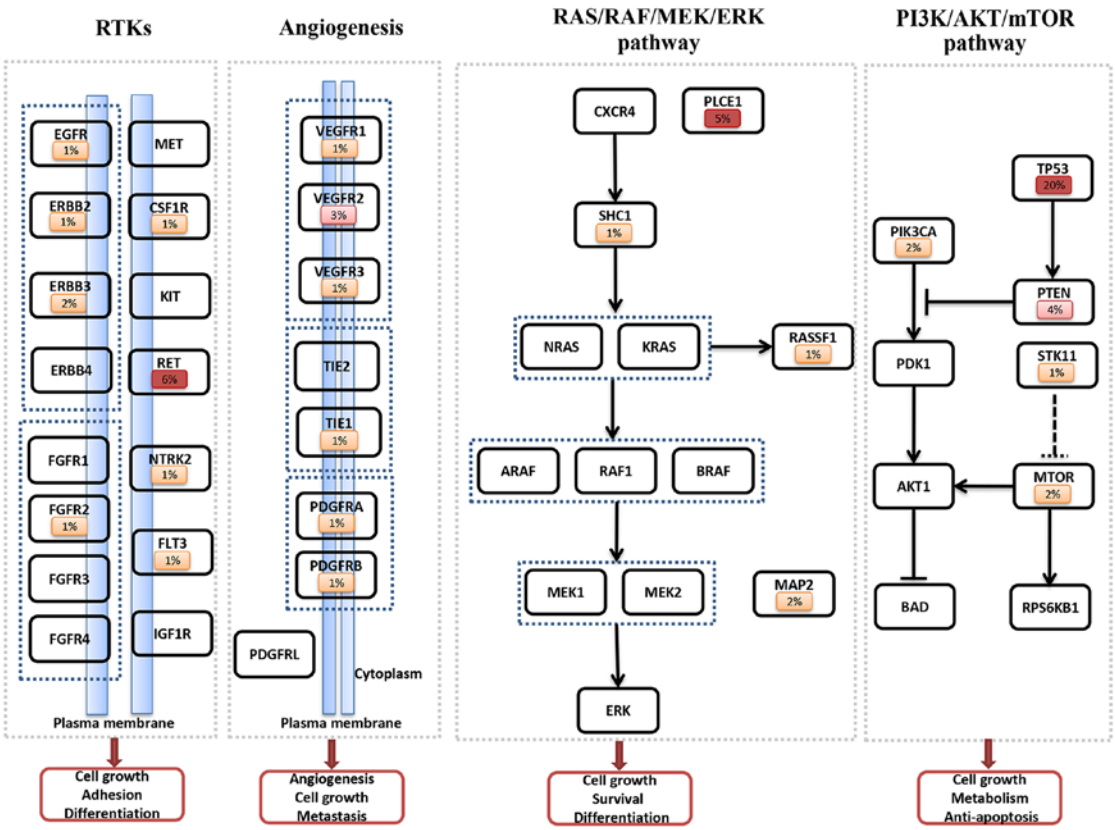

Figure 2. Mutation frequencies in 4 categories of molecular-targeted therapy-related genes. A total of 45 genes were classified into 4 categories, including RTKs, angiogenesis, the RAS/RAF/MEK/ERK and the PI3K/AKT/mTOR pathways. Genes are indicated in pink to red. The darkness of the color is positively correlated with the percentage of tumors with genetic alterations. The mutation frequency of each gene in 100 tumors is indicated. RTKs, receptor tyrosine kinases;MEK, mitogen-extracellular activated protein kinase; PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; ERK, extracellular signal-regulated kinase.

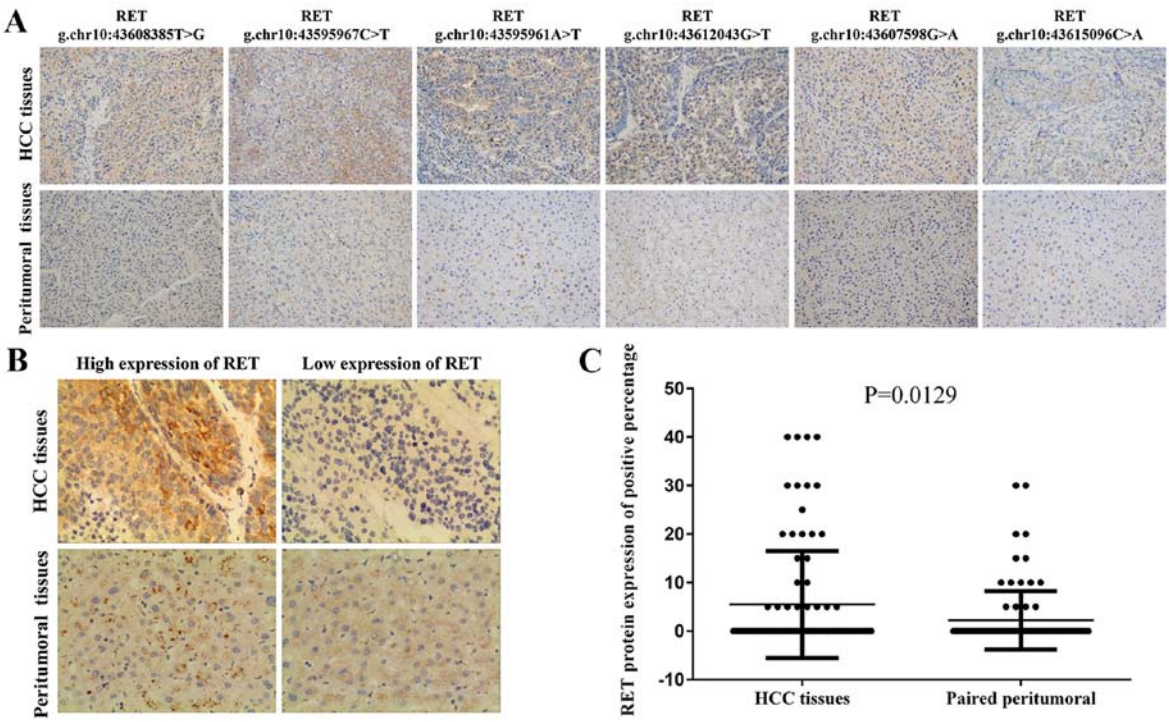

Figure 3. Expression of RET in HCC and peritumoral tissues with mutations in RET. (A) Immunohistochemical staining of HCC with *RET* mutation revealed significant (left 4 panels) or slight increase (right 2 panels) in the expression of RET in tumor tissues (upper panels) compared with peritumoral tissues (lower panels). (B) Representative expression of RET in HCC and paired peritumoral tissues using tissue microarray (magnification, x200). High expression is defined as ≥40% staining of the tumor section, and low expression as <40%. (C) Statistical results of RET expression in HCC and paired peritumoral tissues using Student's t-test (P=0.0129). HCC, hepatocellular carcinoma.

Table III. Patient characteristics.

Factors	Total no. of patients (N=100)
Age, years	
Median	55
Standard deviation	11
Gender	
Male	84
Female	16
HBV-DNA	
Positive	64
Negative	36
Stage	
I	65
II	23
IIIA	7
IIIB	4
IIIC	1
AFP (ng/ml)	
Positive >20	60
Negative ≤20	40
Tumor grade (differentiation)	
Well	6
Moderately	44
Poorly	50
Tumor number	
Solitary	90
Multifocal	10
Tumor size (cm)	
Median	5
Standard deviation	3

The clinical staging of the tumors was according to the TNM classification system of the AJCC (edition 7). TNM, tumor-node-metastasis; AJCC, American Joint Committee on Cancer; HBV, hepatitis B virus; AFP, α-fetoprotein.

RET (P=0.028), tumor size (P<0.001), tumor stage (P<0.001), and tumor marker AFP concentration (P=0.030) (Table IV and Fig. 4A). The somatic mutation status of *TP53* was associated with decreased DFS without statistical significance (Table IV and Fig. 4C). Meanwhile, the univariate analysis of OS suggested that the somatic mutation status of *RET* (P=0.001) and *TP53* (P=0.002), tumor size (P=0.002), tumor stage (P=0.009) and AFP concentration(P=0.007) were associated with the OS obtained from the follow-up (Table IV, Fig. 4B and D). Furthermore, the mutation status of sorafenib-target genes were associated with decreased DFS (P=0.039) and decreased OS (P=0.15) without statistical significance, which suggest poor prognosis in these patients (Fig. 4E and F).

The conditional multivariable analysis of DFS revealed that the somatic mutation status of *RET* (hazard ratio (HR)=3.592; 95% confidence interval (CI), 1.331-9.693; P=0.012), age of patients (HR=1.029; 95% CI, 1.003-1.055; P=0.027), tumor size (HR=1.090; 95% CI, 0.999-1.188; P=0.053), tumor stage (HR=1.624; 95% CI, 1.254-1.101; P<0.001), and tumor marker AFP concentration (HR=1.000; 95% CI, 1.000-1.000; P=0.034) (Table IV) were significant predictors of DFS. Conditional multivariable survival analysis demonstrated that the independent predictors of OS were the somatic mutation status of *TP53* (HR=4.101; 95% CI, 1.941-8.668; P<0.001), *RET* (HR=4.270; 95% CI, 1.511-12.066; P=0.006), and tumor size (HR=1.145; 95% CI, 1.042-1.258; P=0.005) (Table IV). In addition, mutual exclusion of *TP53* and *RET* mutations was observed in the present study (Fig. 4G). These results suggest that both *TP53* and *RET* are significant biomarkers in the prognosis of HCC.

Discussion

To date, sorafenib, an oral multi-kinase inhibitor of *BRAF*, *RAF1*, *FLT3*, *KIT*, *VEGFR* and *PDGFR*, is the main clinical treatment used in advanced HCC. With the development of findings in new target driver genes and molecular-targeted therapies in HCC, RTKs, angiogenesis, RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways have been reported to be involved in hepatocarcinogenesis (6,10-12). However, mutational profiling of these driver genes in Chinese patients with HCC has not been reported, to date. In the present study, targeted deep sequencing was used to conduct the simultaneous analysis of 45 driver genes in 100 patients with HCC, which were categorized according to the following biological processes or signaling pathways: RTKs, angiogenesis, RAS/RAF/MEK/ERK and PI3K/AKT/mTOR. To the best of our knowledge, this is the first comprehensive analysis of driver genes in Chinese patients with HCC.

In the present study, 61 non-synonymous somatic mutations were identified in 43% of the HCC patients. Among members of RTKs, we found somatic mutations in the EGFR family, of which *FLT3*, *C-FMS* and *FGFR2* are the targets of multikinase inhibitors, including sorafenib, sunitinib and regorafenib, which exhibit effective results in HCC (21). Somatic mutations were also observed in angiogenesis-associated genes including *VEGFR1*, *VEGFR2*, *VEGFR3*, *PDGFRA* and *PDGFRB*, particularly non-synonymous mutations of L1088* (*VEGFR1*) and H1159R (*VEGFR2*) located in the catalytic domain of TK. Sorafenib targets both VEGFR2 and VEGFR3 with encouraging outcomes in advanced HCC (7,22). The dual inhibition of VEGF and PDGF signaling demonstrated marked anti-angiogenic effects *in vivo* (23). Linifanib (ABT-869) is a novel selective inhibitor of VEGF and PDGF RTK families in a phase III clinical trial for HCC treatment (24). Recurrent mutations in *PLCE1* were also observed, since the RAS/RAF/MEK/ERK pathway participates in HCC growth and progression (6). Moreover, several studies have reported that the genetic variations in *PLCE1* are associated with esophageal squamous cell carcinoma and gastric adenocarcinoma (25,26). Thus, *PLCE1* may be further studied for HCC treatment. In addition, mutations of *PIK3CA*, *PTEN* and *mTOR* in the PI3K/AKT/mTOR signaling pathway were observed. *PIK3CA* mutations have been reported to sensitize cancer cells to mTOR inhibitor everolimus (27). In the present study, in our findings, somatic mutation of G837V was identified in the catalytic

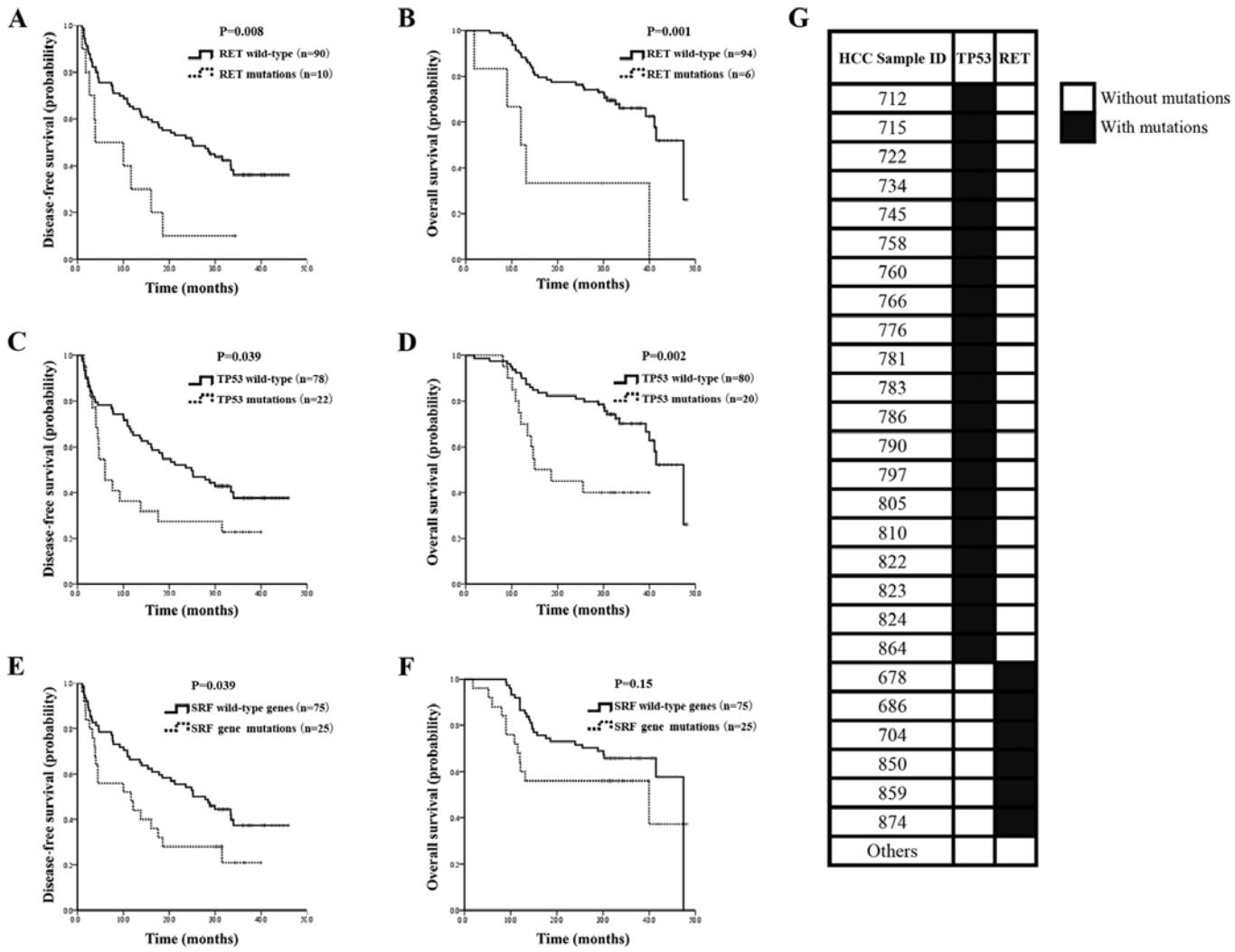

Figure 4. Kaplan-Meier survival estimates according to any mutations in *TP53*, *RET* and sorafenib-target genes, and mutation status of *TP53* and *RET* in HCC patients. (A) Data are shown for the DFS of patients with and without mutations in *RET* (median DFS, 3.700 vs. 24.833 months, respectively, P=0.028). (B) Data are shown for the OS of patients with and without mutations in *RET* (median OS, 12.000 vs. 47.433 months, respectively, P=0.001). (C) Data are shown for the DFS of patients with and without mutations in *TP53* (median DFS, 24.172 vs. 24.833 months, respectively, P=0.133). (D) Data are shown for the OS of patients with and without mutations in *TP53* (median OS, 14.967 vs. 47.433 months, respectively, P=0.002). (E) Data are shown for the DFS (median DFS, 11.667 vs. 27.833 months, respectively, P=0.039) of patients with and without mutations in sorafenib-target genes. (F) Data are shown for the OS (median OS, 39.967 vs. 47.433 months, respectively, P=0.15) of patients with and without mutations in sorafenib-target genes. (G) Mutations of *TP53 and RET* are mutually exclusive. Black indicates patients with mutations and white indicates patients without mutations. SRF, sorafenib; HCC, hepatocellular carcinoma; DFS, disease-free survival; OS, overall survival.

subunit of *PIK3CA*, and both G165* and Q219* mutations of *PTEN* were truncating mutations which destroyed the function of PTEN. *PTEN* (G165*, Q219*) mutations may serve as molecular markers for mTOR inhibitor-targeted therapy.

As shown in Fig. 2, the most frequent mutations were: *TP53* (20%), *RET* (6%), *PLCE1* (5%), *PTEN* (4%) and *VEGFR2* (3%). Genome-wide sequencing analyses have revealed many mutant genes, such as *TP53* and β-catenin (*CTNNB1*) in HCC (28,29). The recurrent mutations of *TP53* (20%) identified in the present study were similar with earlier studies, which confirmed the reliability of our sequencing data. Further prognostic analysis which revealed that patients with mutations in *TP53* had lower overall survival (OS) than those without mutations was consistent with earlier studies (28,30). Dysregulation of *RET* activity is an important contributor to several human types of cancer including thyroid, lung, breast and pancreatic tumors (31-34), suggesting that *RET* is an important target for therapeutic intervention in many diseases (35). Genetic aberrations, including rearrangement, germline and somatic activation mutations, are responsible for a fraction of papillary thyroid carcinoma, medullary thyroid carcinoma, and a small subset of non-small cell lung cancer (7,32,36-38). In addition, the prognostic study further revealed that HCC patients with *RET* somatic mutations had poorer DFS and OS compared with wild-type patients in the present study. In particular, the mutual exclusion of *TP53* and *RET* mutations were observed. All aforementioned results indicated that both *TP53* and *RET* are significant biomarkers in the prognosis of HCC.

With the development of genome technology and lower costs for sequencing, the simultaneous analysis of genetic variation of the set of driver genes in tumor tissue after resection or biopsy is available. Presently, personalized medicine is emerging with genomic technology applied in clinical oncology. In addition, finding subpopulations of

Table IV. Predictors of disease-free and overall survival.

	Univariate analysis				Multivariate analysis			
	Disease-free survival		Overall survival		Disease-free survival		Overall survival	
Factor	Median DFS (months) (95% CI)	P-value	Median OS (months) (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
TP53		0.133		0.002	1.843 (0.982-3.459)	0.057	4.101 (1.941-8.668)	<0.001
No mutation	24.833 (14.899-34.767)		47.433 (40.041-54.826)					
Mutation	24.172 (0-11.314)		14.967 (6.347-23.586)					
RET		0.028		0.001	3.592 (1.331-9.693)	0.012	4.270 (1.511-12.066)	0.006
No mutation	24.833 (15.402-34.265)		47.433 (40.063-54.803)					
Mutation	3.700 (1.219-6.181)		12.000 (7.039-16.961)					
Age (years)		0.075		<0.001	1.029 (1.003-1.055)	0.027		
Tumor number		0.176		0.550				
Solitary	24.833 (13.773-35.894)		41.467 (37.317-45.616)					
Multifocal	10.900 (0-25.362)		41.100 (17.999-64.201)					
Tumor size		<0.001		0.002	1.090 (0.999-1.188)	0.053	1.145 (1.042-1.258)	0.005
HBV-DNA		0.184		0.711				
Negative	31.467 (20.117-42.816)		NA					
Positive	17.567 (10.319-24.815)		41.467 (37.465-45.469)					
Tumor grade (differentiated)		0.237		0.196				
Well	NA		NA					
Moderately	25.133 (12.471-37.796)		NA					
Poorly	13.600 (5.669-21.531)		NA					
Gender		0.901		0.892				
Male	21.200 (12.373-30.027)		47.433 (38.739-56.128)					
Female	18.567 (0-51.495)		41.100 (25.428-56.772)					
Stage		<0.001		0.009	1.624 (1.254-2.101)	<0.001		
I	33.367 (22.994-43.740)		NA					
II	13.767 (0-45.259)		NA					
IIIA	4.000 (1.605-6.395)		NA					
IIIB	1.400 (1.302-1.498)		NA					
IIIC	NA		NA					

Table IV. Continued.

Factor	Univariate analysis				Multivariate analysis			
	Disease-free survival		Overall survival		Disease-free survival		Overall survival	
	Median DFS (months) (95% CI)	P-value	Median OS (months) (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
AFP (ng/ml)		0.030		0.007	1.000 (1.000-1.000)	0.034		
Positive >20	33.433 (26.512-40.355)		39.967 (30.592-49.342)					
Negative ≤20	13.767 (8.037-19.496)		NA					

The clinical staging of the tumors was according to the tumor-node-metastasis (TNM) classification system of the American Joint Committee on Cancer (AJCC) (edition 7). Multivariate analysis, Cox proportional hazards regression model. NA, not applicable; AFP, α-fetoprotein; HR, hazard ratio; 95% CI, 95% confidence interval.

patients who may benefit from molecular-targeted therapy is important. Identification of specific genetic variations in individual patients may serve as a guide to develop effective drugs used in the treatment of HCC patients. In the present study, we conducted the mutation profiling of driver genes and clinical prognostic analysis, which indicated that *TP53* and *RET* mutations may serve as biomarkers for targeted therapy in HCC. However, the present study has certain limitations. Firstly, we only analyzed a small group of patients, and a perspective analysis of frequent mutations may be carried out in a large-scale study of patients with HCC to confirm our results. Secondly, numerous genetic mutations were identified; however, not all mutations were functional, and may serve as passenger mutations. Thus, functional analysis of mutations is needed to further illustrate the carcinogenic mechanism in HCC. Although, further studies are needed to guide molecular-targeted therapy in HCC, in the present study we identified *TP53* and *RET* mutations to be suitable markers for prognostic evaluation and targeted therapy in HCC.

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