Supplementary Materials

TSC1/2 mutations define a molecular subset of HCC with aggressive behavior and treatment implication

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Supplementary Materials and Methods

1. Patient sample cohorts

The demographic data are listed in Supplementary Table 1. The patients' ages ranged from 24 to 74 years (mean = 52.5 years) for the targeted-seq cohort and 24 to 71 years (mean = 46.4 years) for the WES cohort. Seventy-one were male patients and 24 were female for the targeted-seq cohort; Ten patients were male and 6 were female for the WES cohort

2. DNA and RNA extraction, library construction and next-generation sequencing (NGS)

Total DNA was isolated from the paired HCCs and their corresponding NTL tissues using method described previously [1]. Total RNA was extracted from HCC cell lines using Trizol reagent. Library preparation (DNA or RNA), exome capture and custom target enrichment were performed by Illumina TruSeq DNA and RNA Library Preparation and Exome Enrichment Kits and NimbleGen EZ Developer Kit, respectively, according to manufacturer's protocol. Target enriched libraries were then PCR amplified and sequenced using Illumina HiSeq2000 system. Paired-end reads of 101 b.p. were generated. Library preparation and NGS for targeted-seq and WTS were performed by Centre for Genomic Sciences, The University of Hong Kong, while those for WES were done by Axeq (Seoul, Korea). Read alignments of HCCs and their corresponding NTLs were then analyzed using VarScan (version 2.3.4) [2] to identify somatic mutations (single nucleotide variations [SNVs] and indels). VarScan made use of both HCC and their corresponding NTL data simultaneously and utilized heuristic and statistical algorithms to detect sequence variants and takes read depth, base quality, variant allele frequency and statistical significance into account. It classified variants by somatic status, and somatic sequence variants classified with high confidence were extracted for subsequent investigation. Read alignments of data for targeted-seq on HCCs and WTS on HCC cell lines were also analyzed using VarScan to

detect sequence variants, with default parameters.

Sequence variants were assigned to corresponding genes by genomic coordinates and annotated with genetic function, amino acid alteration and deleterious outcome prediction by dbNSFP [3] using ANNOVAR [4].

*3. **Bioinformatics analysis***

Genome and transcriptomic sequencing reads were mapped to the reference human genome (hg19) using Burrows-Wheeler Aligner (BWA) (version 0.7.5a) [5] and TopHat (version 2.0.6). PCR duplicate reads were removed by PICARD (version 1.105). Alignments were refined by local realignment around insertions/deletions (indels) and base-quality score recalibration by Genome Analysis Toolkit (GATK) (version 2.8-1) [6]. To exclude potential sequence polymorphisms e.g. SNPs and irrelevant polymorphic sequences, three *in silico* filtering steps were performed as follow to exclude 1) all the potentially normal sequence variants documented in 1000 Genomes (Version 2014Oct_ALL); 2) relatively common sequence variants with frequency ≥0.001 in Exome Sequencing Project (ESP6500_ALL) and dbSNP (Build 138) and 3) variants without deleterious outcome predicted by any tools of dbNSFP (Version 2.3) [3].

*4. **Coding region (CDS) screening by targeted-seq for genes in mammalian target of rapamycin (mTOR) signaling pathway***

In order to find out the mutation landscape and identify the key mutant genes for the mTOR signaling pathway, 81 mTOR pathway-related genes were selected based on KEGG pathway definition and literature search, and the CDS of them was screened for mutations by targeted-seq using a cohort of 95 cases. Assay design was provided by NimbleGen, following manufacturer's protocol.

5. ***Sanger sequencing confirmation on mutated genes in mammalian target of rapamycin (mTOR) signaling pathway***

Sequencing primers were designed to confirm somatic mutations identified on genes in mTOR signaling pathway and were listed in Supplementary Table 5. We amplified genomic intervals containing the mutations by PCR. Products were sequenced using BigDye Terminator Cycle Sequencing Kit (Life Technologies) and detected on ABI 3730xl sequencers. Confirmation was performed on pairs of HCC and corresponding NTL genomic DNA samples to verify the somatic mutations detected by NGS strategies.

6. Immunohistochemical staining for TSC1, TSC2 and phospho-S6 in human HCC samples

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded (FFPE) sections, as described [7], using anti-TSC1 (#6935, 1:250), anti-TSC2 (#4308, 1:250), anti-phospho-S6 Ser235/6 (#4858, 1:25) and anti-phospho-S6 Ser240/4 (#5364, 1:250) rabbit monoclonal antibodies (Cell Signaling Technology, Beverly, CA).

7. ***Examination of the mutational status of TSC1 and TSC2 in HCC cell lines***

Complementary to the mutation screening in human HCCs, *TSC1* and *TSC2* were screened for mutations in 7 HCC cell lines (BEL7402, HepG2, Hep3B, Huh7, MHCC97L, PLC and SMMC) using WTS. Non-synonymous coding and splice site mutations were extracted. Coding region (CDS) of *TSC1* and *TSC2* were examined for similar mutations by Sanger sequencing in H2P and H2M cell lines.

8. Colony Formation Assay

HCC cells were seeded onto 24-well plates at a density of 2.5×10^4 cells per well and subjected to Rapamycin treatment for 4 days at 1, 10 and 100 nM. The cells were fixed with 4% formaldehyde and visualized by crystal violet staining.

9. Western blotting detection for TSC1 and TSC2

Cell culture in 12-well format was washed once with PBS and subjected to the direct SDS-lysis with 60ul standard Laemmli sample buffer with DTT. The whole cell lysate were boiled for 10 minutes. One-tenth of the cell lysate was subjected to 10% SDS-PAGE analysis. Immunoblotting was performed with the use of anti-TSC1 antibody (1:1000 Cell Signaling Technology, Beverly, CA), anti-TSC2 antibody (1:1000 Abcam, Cambridge, MA, USA) in 4% BSA/TBST. Anti-β-actin antibody (1:5000, Sigma, St. Louis, MO) in 4% milk/TBST was used as normalization control.

10. HCC PDTX model

To dissociate PDTX HCC tumor, tumor was removed from mice and diced into pieces of $1mm^3$ size in ice cold DMEM/F-12 medium with a surgical blade. The diced tumor was homogenized twice in C tube using the human tumor program in a gentleMACS dissociator (Miltenyi Biotec, Auburn, CA) with the presence of DNAase and Liberase (Roche), followed by 37^{o}C incubation in water bath for 5 minutes. The homogenization steps were repeated for two times to ensure complete tumor dissociation. The dissociated samples were subjected to pass through a sterile 100uM nylon cell strainer to remove any unwanted debris. The tumor cells were collected and separated from the blood cells from the flow-through by centrifugation at 2,700rpm for 3 minutes. The cell viability of the dissociated tumor cells was assessed by trypan blue staining using a hemocytometer. To ensure sufficient dissociated cells for subsequent subcutaneous injection, at least 10 tumors from the same PDTX being grown

for a duration of 4-6 weeks in five mice were harvested for tumor dissociation at the same time. At least 1×10^6 viable, unsorted tumor cells were re-suspended in 100ul 1:1 DMEM/F-12 Matrigel mix (v/v) and subcutaneously injected into 4-6 weeks aged, immune-deficient *BALB/c nude* mice followed by a period of 2-4 weeks incubation. When the diameter of the tumor reached 5mm, Rapamycin (LC laboratories, Woburn, MA) was given by daily intraperitoneal injection at the indicated concentrations for 2-4 weeks, depending on the proliferation rate with respect to the vehicle-treated tumor. Sterile 5% Tween80 (Anatrace, Maumee, OH) / 5% PEG400 (USB-Affymetrix, Inc., USA) in water was used as the injection vehicle. The body weight and the size of the tumor were monitored and measured throughout the treatment. The tumor mass (g) of the harvested tumor was measured at the end-point of the experiment. The tumor volume in mm^3 was calculated by the following formula: 1/2 x Length (mm) × Width (mm) × Depth (mm). Animal experiment was approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR 4035-16), Li Ka Shing Faculty of Medicine, University of Hong Kong.

11. Generation of stable Rheb knockdown HCC cells by shRNA

pLK0.1-Puro based lentiviral shRNA expression vector carrying specific Rheb targeting sequence (shRheb#25: TTATGTTGGTTGGGAATAAGA and shRheb#99: CCTATTATGTTGGTTGGGAAT) and non-targeted control (shNTC) were transfected into 293FT packaging cells to produce viral containing supernatant for subsequent HCC cells viral transduction. SMMC and PLC cells were seed on 6-well plate at a density of 8×10^4 cells per well and incubated with lentiviral supernatant of shNTC, shRheb#25 and shRheb#99 for 24 hours in the presence of 8ug/ml Polybrene (Sigma, St. Louis, MO). The transduced cells were then re-plated on 10cm plate and selected in the presence of puromycin at 0.5ug/ml (SMMC) and 0.9ug/ml (PLC) for 4 Days. To confirm the Rheb knockdown efficiency in SMMC cells,

cell lysates were prepared and subjected to immunoblotting against Rheb specific antibody (1:1000, Cell Signaling Technology).

12. ***Statistical Analysis***

Contingency table was tested using Fisher's exact test. Quantitative comparison between groups was done by Student *t* test. All tests were computed as 2-tailed and a *P* value < 0.05 was considered statistical significant.

Supplementary Figure Legends

Supplementary Figure 1. Examination of *TSC1* and *2* mutations using TCGA data. (A) mTOR pathway-related mutant genes in TCGA HCC dataset. (B) Frequencies of *TSC1* and *2* mutations in TCGA cancer panel.

Supplementary Figure 2. Sanger sequencing confirmation of the two additional *TSC2* and *TSC1* mutations identified by whole exome sequencing in the WES cohort.

Supplementary Figure 3. PCR amplification of specific mutant gene fragments from genomic DNA of HCC samples (T) and their corresponding non-tumorous livers (NT) for Sanger sequencing confirmation. (A) *TSC2* mutants and (B) *TSC1* mutants identified in the Targeted-seq cohort.

Supplementary Figure 4. Schematic diagram illustrating the mechanism behind the overlapping peaks observed in direct Sanger sequencing in HCC cases carrying both wildtype and mutant *TSC2* with internal deletion. (A) Case 265T and 354T carried AGG deletion at 4025-4027 nucleotide position in TSC2 Exon34, which resulted in a frameshift when compared to the wildtype *TSC2*. (B) Case 311T carried AGA deletion at 4653-4655 nucleotide position in *TSC2* Exon28, which also resulted in a frameshift when compared to the wildtype *TSC2*.

Supplementary Figure 5. IHC staining in additional HCC cases showing the reduction of TSC2 expression in the tumors with mTOR pathway activation. (A) HCC tumor carrying non-sense TSC2 mutation (TSC2 Q1377X, Case 533T). (B) HCC tumor carrying non-synonymous TSC2 mutation (TSC2 T1071R, Case 235T). NT: Non-tumorous liver.

Supplementary Figure 6. Stable expression of shRNA against Rheb (restoration of TSC2 mimic) drastically suppressed proliferation of *TSC2* mutant-carrying PLC cells. (A) Brightfield images showing the number of shRNA expressing puromycin resistant cells obtained 4 days after selection. Non-viral transduced cells subjected to puromycin treatment served as a negative control. (B) Table summarizing the shRheb stable cells obtained after puromycin selection. (C) The efficiency of Rheb knockdown in SMMC shclone#25 and #99 was confirmed by western blotting using Rheb specific antibodies. (D) The mutational status of *TSC* complex would predict the Rheb dependency as well as the Rapamycin sensitivity in HCC cells.

Supplementary References

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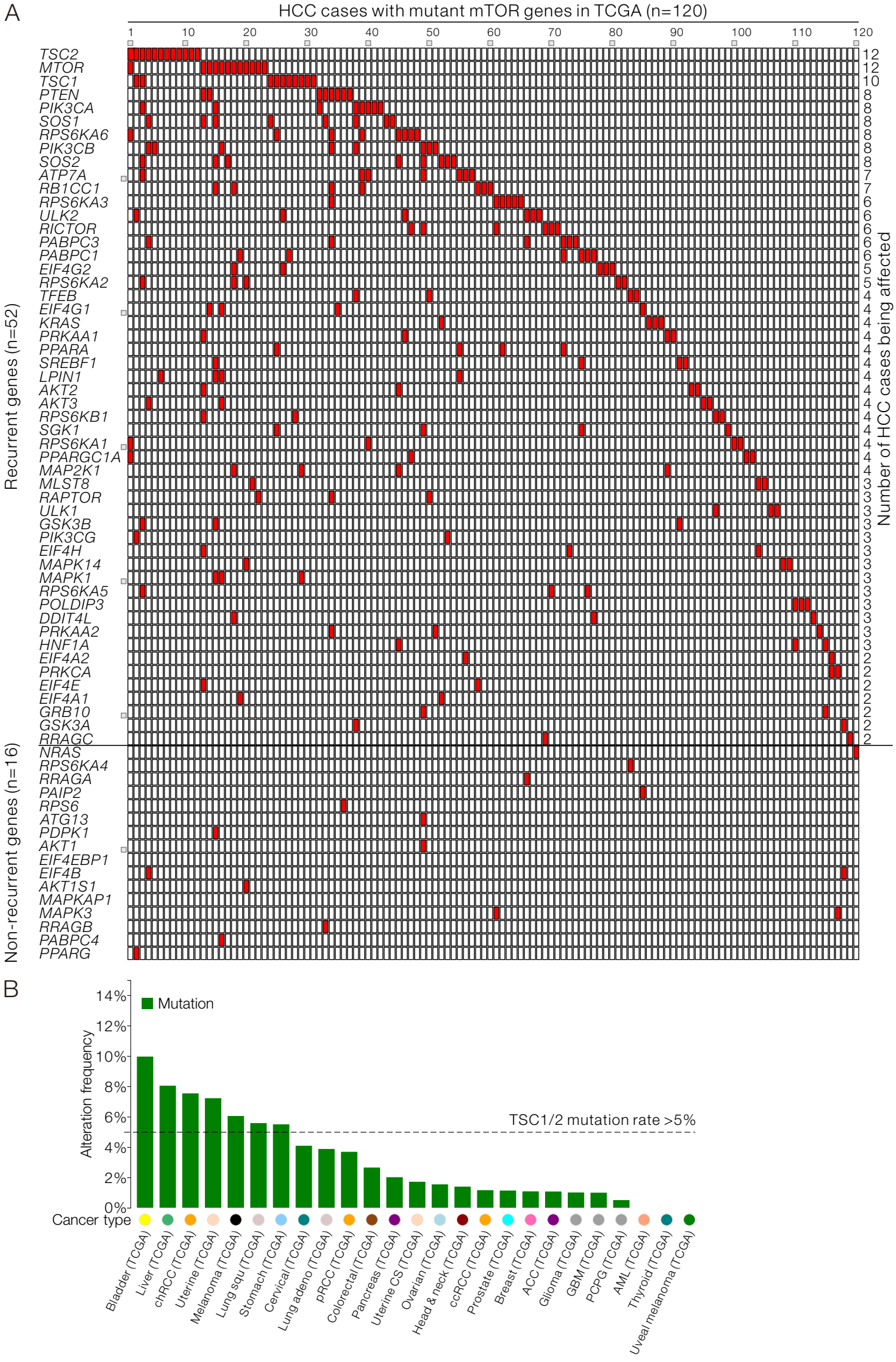
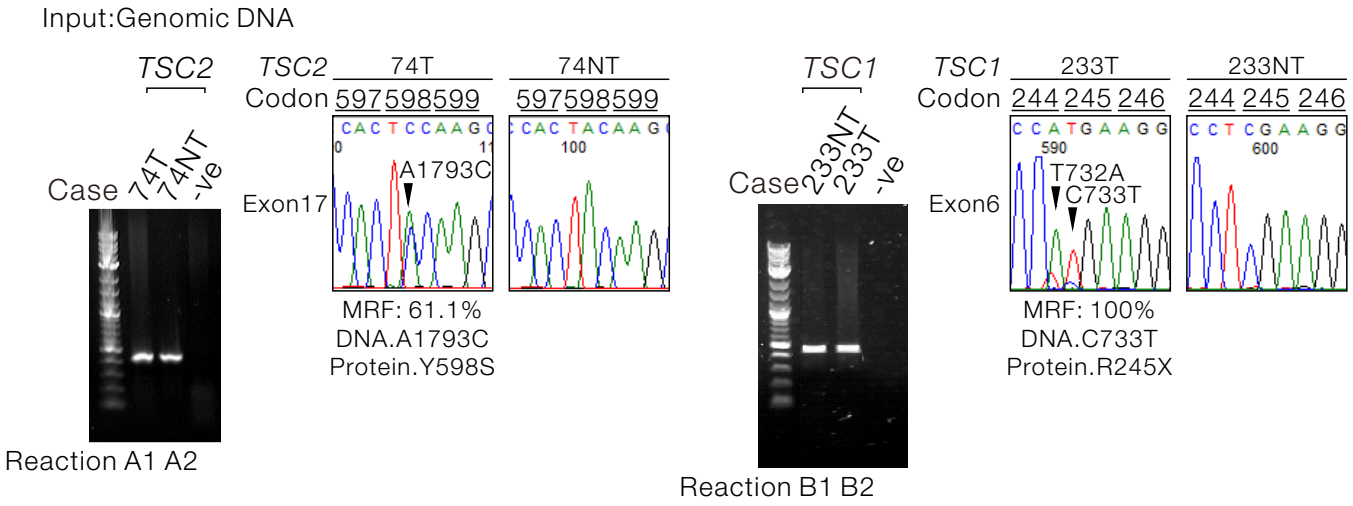
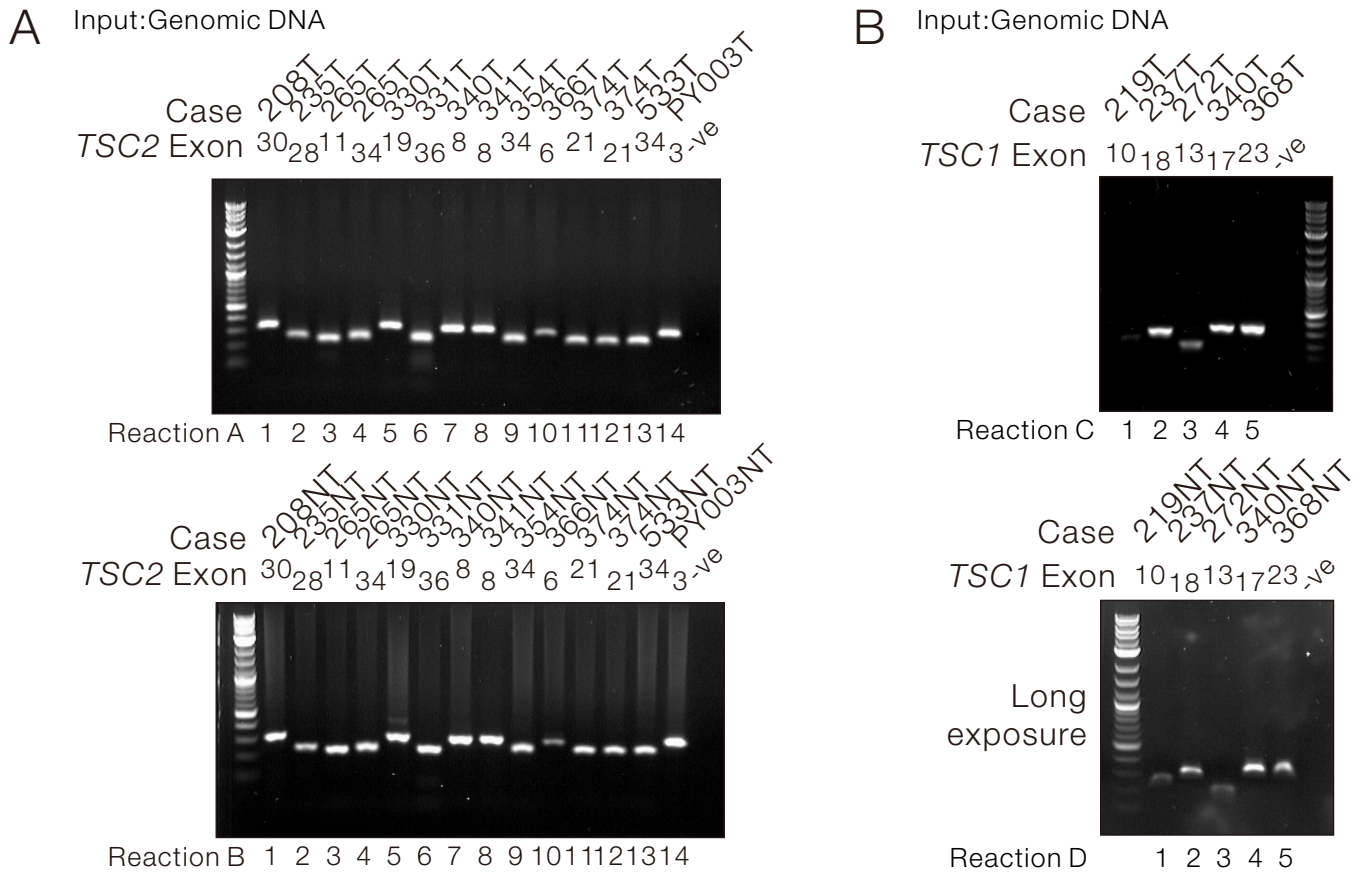
Supplementary Figure 1



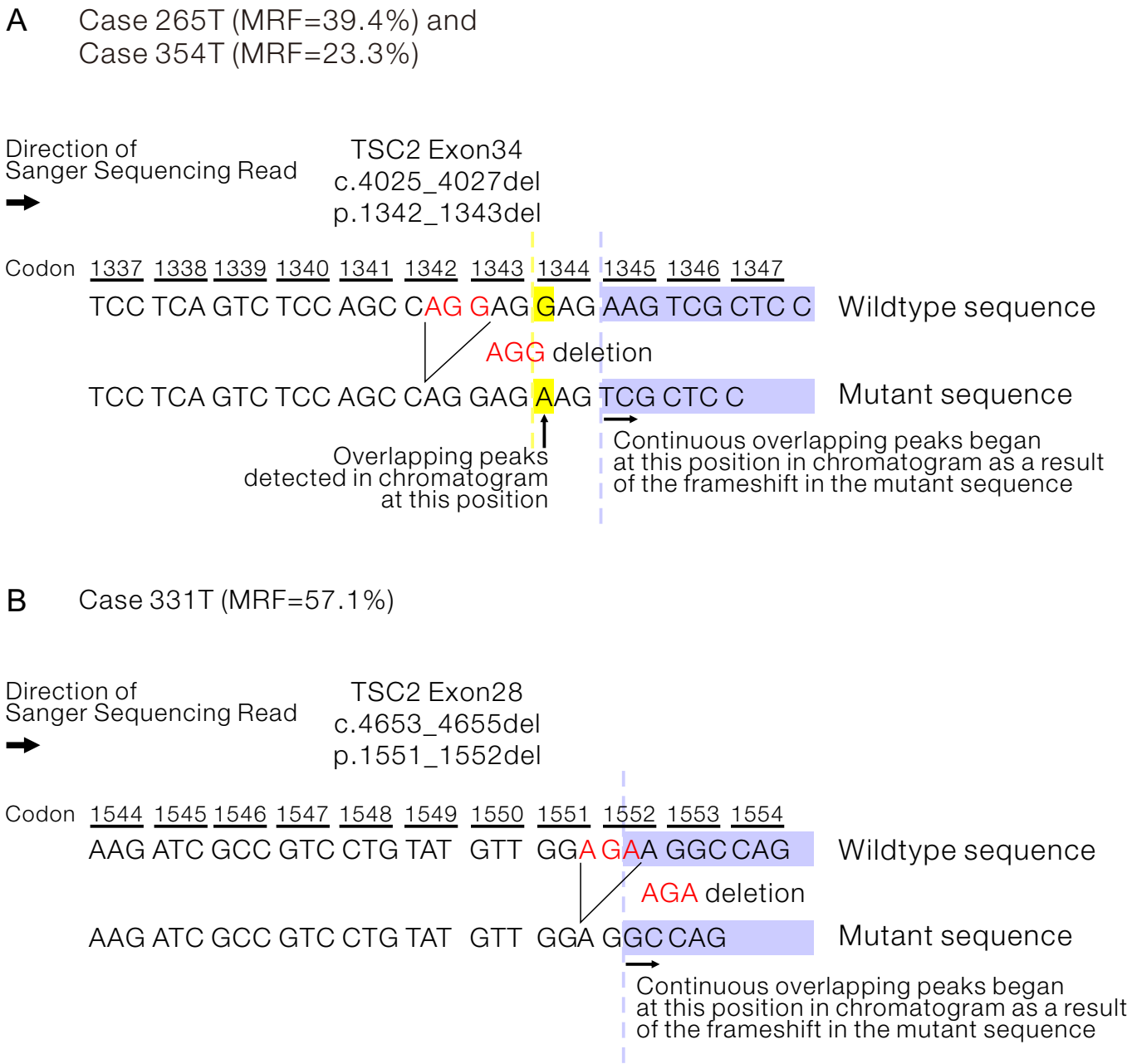
Supplementary Figure 2



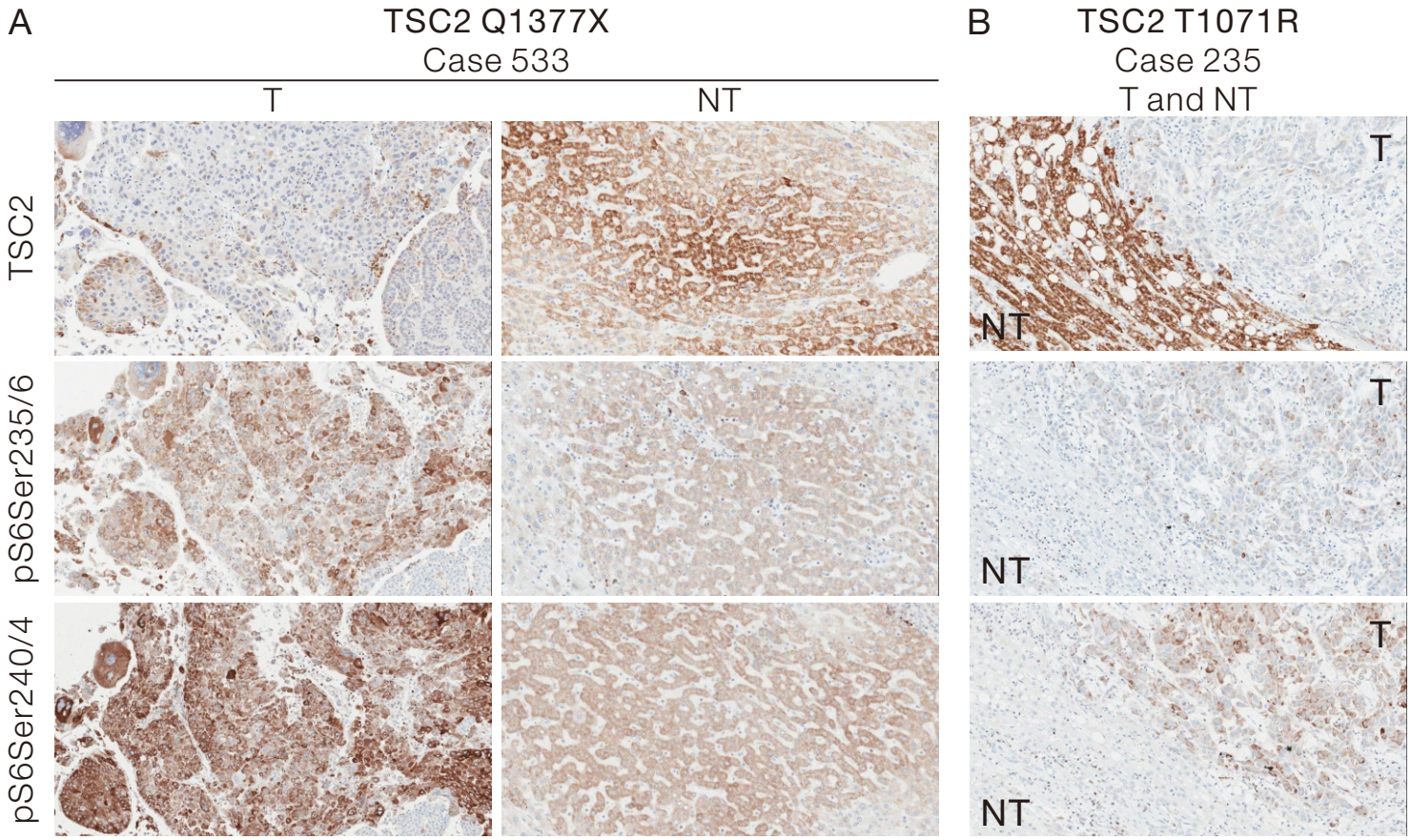
Supplementary Figure 3



Supplementary Figure 4

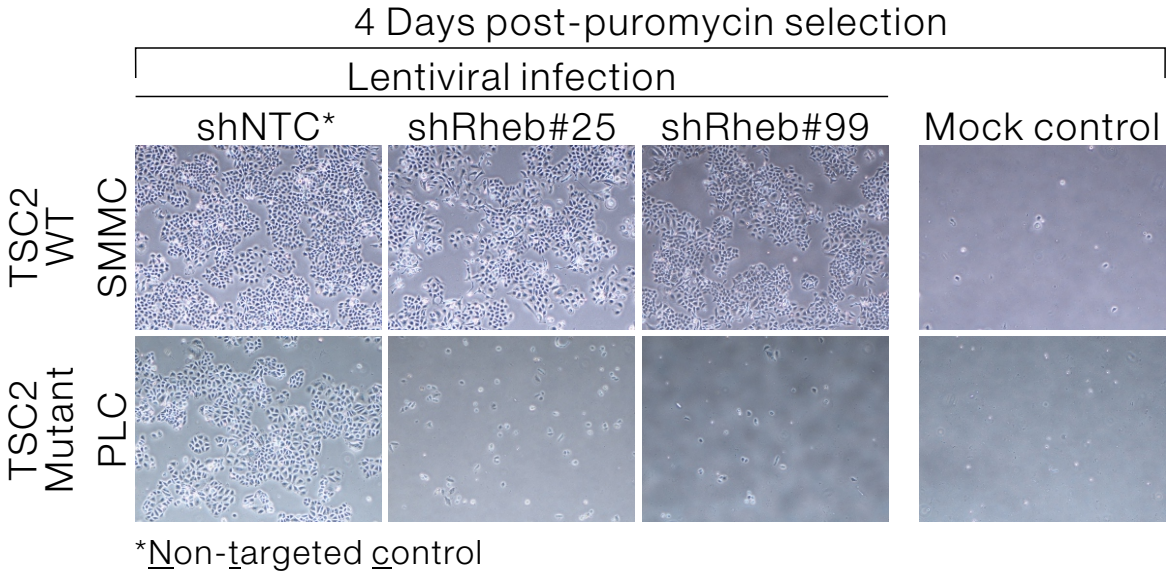


Supplementary Figure 5



Supplementary Figure 6

A

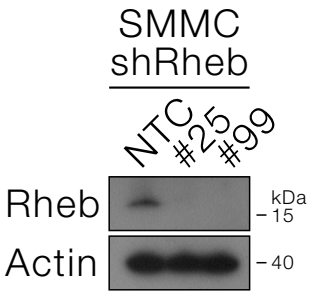


*Non-targeted control

B Generation of puromycin resistant shRheb stable cells

	shNTC	shRheb#25	shRheb#99
TSC2 WT SMMC	Yes	Yes	Yes
TSC2 Mutant PLC	Yes	No	No

C Western blot



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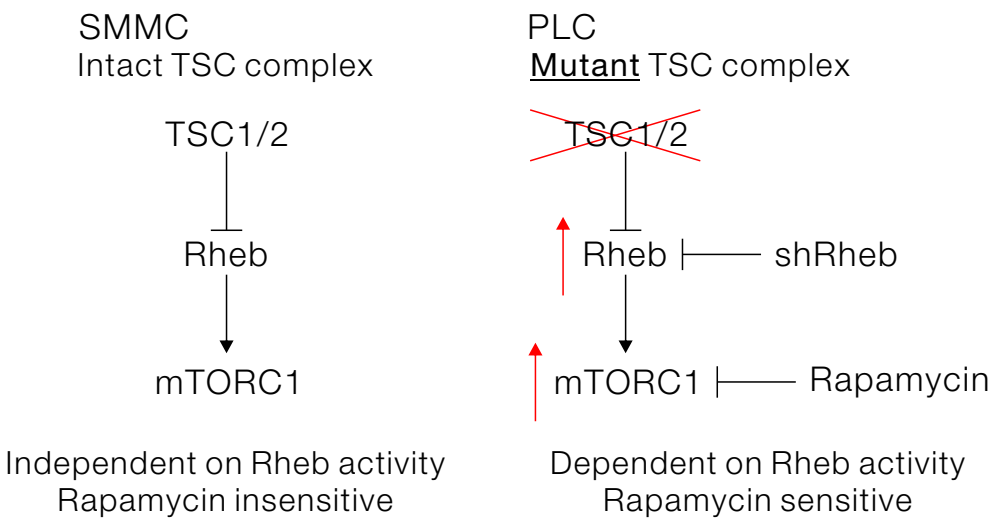

Supplementary Table 1

Demographic data of the patients in the sample cohorts

Parameters	Sample cohort			
	Targeted-seq (n = 95)		WES (n = 16)	
Gender				
Male	71	(74.7%)	10	(62.5%)
Female	24	(25.3%)	6	(37.5%)
Mean age (range)	52.5	(24-74)	46.4	(24-71)
Average tumor size (range, in cm)	7.8	(2-27)	7.8	(1-18)
Background liver disease				
Normal	1	(1.1%)	2	(12.5%)
Chronic hepatitis	42	(44.2%)	5	(31.3%)
Cirrhosis	52	(54.7%)	9	(56.3%)
Liver invasion				
Yes	32	(33.7%)	5	(31.3%)
No	58	(61.1%)	11	(68.8%)
Tumor microsatellite formation				
Yes	51	(53.7%)	7	(43.8%)
No	41	(43.2%)	9	(56.3%)
Tumor encapsulation				
Yes	31	(32.6%)	7	(43.8%)
No	62	(65.3%)	8	(50%)
Venous invasion				
Yes	54	(56.8%)	8	(50%)
No	41	(43.2%)	8	(50%)
Cellular differentiation				
Edmondson grade I-II	35	(36.8%)	6	(37.5%)
Edmondson grade III-IV	60	(63.2%)	10	(62.5%)
TNM staging				
I-II	35	(36.8%)	7	(43.8%)
III-IV	60	(63.2%)	9	(56.3%)

Supplementary Table 2

List of 81 mTOR pathway-related genes

AKT1	PDCD4
AKT1S1	PDPK1
AKT2	PIK3CA
AKT3	PIK3CB
ATG13	PIK3CG
ATP7A	POLDIP3
DDIT4	PPARA
DDIT4L	PPARG
DEPTOR	PPARGC1A
EIF4A1	PRKAA1
EIF4A2	PRKAA2
EIF4B	PRKCA
EIF4E	PRR5
EIF4EBP1	PTEN
EIF4G1	RB1CC1
EIF4G2	RHEB
EIF4H	RICTOR
FKBP1A	RPS6
GRB10	RPS6KA1
GRB2	RPS6KA2
GSK3A	RPS6KA3
GSK3B	RPS6KA4
HIF1A	RPS6KA5
HRAS	RPS6KA6
IRS1	RPS6KB1
KRAS	RPTOR
LAMTOR1	RRAGA
LPIN1	RRAGB
MAP2K1	RRAGC
MAPK1	RRAGD
MAPK14	SGK1
MAPK3	SOS1
MAPKAP1	SOS2
MLST8	SREBF1
MTOR	STK11
NRAS	TFEB
PABPC1	TSC1
PABPC3	TSC2
PABPC4	ULK1
PAIP1	ULK2
PAIP2	

Supplementary Table 3

Summary of sequencing coverage on targeted-seq

Target size (bases) = 7,189,881	**# of on-target bases**	**On-target coverage (fold)**
104T	1,057,460,773	147.1
106T	1,104,749,719	153.7
200T	1,090,198,693	151.6
202T	1,126,325,055	156.7
203T	1,106,868,289	153.9
206T	1,182,342,058	164.4
208T	1,172,456,768	163.1
211T	1,240,909,028	172.6
214T	1,121,770,033	156.0
216T	1,000,621,815	139.2
217T	1,066,796,971	148.4
218T	999,588,418	139.0
219T	997,220,384	138.7
220T	1,077,355,527	149.8
221T	1,079,559,605	150.1
222T	1,065,753,064	148.2
225T	1,021,923,369	142.1
232T	978,589,971	136.1
235T	1,048,056,271	145.8
236T	1,176,716,745	163.7
237T	1,047,504,602	145.7
238T	1,092,248,775	151.9
239T	1,110,787,921	154.5
240T	1,086,687,423	151.1
244T	1,073,061,585	149.2
246T	1,099,436,650	152.9
248T	1,177,440,956	163.8
249T	1,139,260,009	158.5
250T	1,016,757,870	141.4
253T	1,020,449,217	141.9
255T	1,124,081,348	156.3
256T	1,081,201,421	150.4
257T	1,104,019,987	153.6

258T	1,065,077,622	148.1
259T	1,081,646,569	150.4
261T	1,024,775,247	142.5
265T	1,078,639,848	150.0
266T	1,045,141,385	145.4
269T	1,142,517,128	158.9
270T	1,175,326,979	163.5
271T	1,327,206,044	184.6
272T	1,200,486,419	167.0
297T	1,273,271,461	177.1
301T	1,417,083,876	197.1
302T	1,218,667,703	169.5
303T	1,151,192,513	160.1
305T	1,204,282,088	167.5
311T	1,105,199,798	153.7
312T	1,038,857,169	144.5
316T	977,119,463	135.9
326T	1,027,652,067	142.9
329T	1,003,752,997	139.6
330T	1,140,639,204	158.6
331T	1,031,922,231	143.5
334T	1,016,112,289	141.3
335T	1,029,206,521	143.1
336T	992,523,151	138.0
340T	1,085,164,623	150.9
341T	991,738,403	137.9
342T	1,128,056,851	156.9
343T	1,155,316,247	160.7
346T	1,016,521,219	141.4
348T	1,091,123,016	151.8
350T	1,280,197,796	178.1
351T	1,081,874,381	150.5
352T	1,210,162,705	168.3
354T	1,134,830,011	157.8
356T	1,138,368,033	158.3
361T	1,154,592,134	160.6
362T	1,142,323,069	158.9
363T	1,056,011,340	146.9

364T	1,049,555,575	146.0
365T	1,077,812,051	149.9
366T	979,487,849	136.2
367T	1,091,104,870	151.8
368T	1,100,781,875	153.1
370T	1,063,422,461	147.9
372T	1,180,054,021	164.1
373T	1,140,040,632	158.6
374T	1,088,166,496	151.3
376T	1,144,312,194	159.2
377T	1,159,247,209	161.2
378T	1,097,494,842	152.6
379T	1,256,255,503	174.7
528T	1,127,149,154	156.8
533T	1,206,178,923	167.8
541T	1,113,054,750	154.8
555T	1,194,899,542	166.2
570T	1,203,634,750	167.4
589T	1,025,161,419	142.6
89T	1,084,894,765	150.9
95T	1,159,123,294	161.2
PY003T	1,212,016,182	168.6
QE022T	1,128,179,044	156.9
QM007T	1,159,981,971	161.3
	Mean	**154.3**

Supplementary Table 4

Summary of sequencing coverage on WES

Exome size (bases) = 62,085,286	**# of on-target bases**	**On-target coverage (fold)**
74NT	2,031,897,016	32.7
74T	2,178,992,952	35.1
251NT	3,133,474,018	50.5
251T	2,051,619,346	33.0
254NT	2,429,346,837	39.1
254T	1,778,169,960	28.6
308NT	2,814,903,309	45.3
308T	1,780,598,821	28.7
315NT	2,220,563,651	35.8
315T	1,906,774,393	30.7
383NT	2,215,235,838	35.7
383T	2,074,175,142	33.4
84NT	2,070,195,337	33.3
84T	2,472,701,279	39.8
105NT	3,638,732,506	58.6
105T	4,717,055,256	76.0
107NT	3,464,698,327	55.8
107T	3,627,611,395	58.4
133NT	1,967,457,650	31.7
133T	2,508,876,928	40.4
151NT	2,686,184,039	43.3
151T	2,178,664,486	35.1
228NT	3,397,280,592	54.7
228T	2,818,288,111	45.4
233NT	3,255,493,788	52.4
233T	2,539,196,032	40.9
149NT	2,781,697,840	44.8
149T	3,650,920,765	58.8
229NT	4,252,615,373	68.5
229T	3,583,214,645	57.7
234NT	3,892,739,437	62.7
234T	3,843,638,664	61.9
	Mean	**45.3**

Supplementary Table 5

Primers used for target gene product amplification from genomic DNA for Sanger Sequencing

Reaction index	Target gene/ HCC Case	PCR primer	Primer sequence (5'>3')	Product size (bp)
A1, A2	*TSC2*/ 74T/NT	Forward	GACTGCGTTTTCACCTCCTG	415
		Reverse	GTCCTTTTCTCTGCCCCAAC	
B1, B2	*TSC1*/ 233T/NT	Forward	GCTGTATGAGTGCTTCCAAG	486
		Reverse	TTCTTCCTCGCCACTCCAAT	
C1, D1	*TSC2*/ 208T/NT	Forward	CGGGGGGAGCATTCAGCTTGA	324
		Reverse	AAGATGGCGCTCAGGCCAG	
C2, D2	*TSC2*/ 235T/NT	Forward	ACGGGCAAGCTGGGTTTC	245
		Reverse	CTCGGGACAGCCGGTGTC	
C3, D3	*TSC2*/ 265T/NT	Forward	TGCTGGCCGGGCTCGTGT	225
		Reverse	GGCCGAGCCTGCCTGTCTG	
C4, C9, C13, D4, D9, D13	*TSC2*/ 265T/NT,354T/NT, 533T/NT	Forward	TGCCTGCTGACAGGGGTTC	240
		Reverse	CAGGGTCCCCGAGGATGTC	
C5, D5	*TSC2*/ 330T/NT	Forward	GTGGCGCTGTTTGCATGTC	322
		Reverse	GGTGGCAGCTCCCTGTCC	
C6, D6	*TSC2*/ 331T/NT	Forward	GGGGCTCAGGCAGGGCTCT	233
		Reverse	CCCGGCAGTGTTGAGCTCTG	
C7, C8, D7, D8	*TSC2*/ 340T/NT, 341T/NT	Forward	CCATGGCAGCGGGGAGAGGT	305
		Reverse	GGCCCTTGTCCCATCCATGT	
C10, D10	*TSC2*/ 366T/NT	Forward	GGAGGTGAGTGGGAGATGTAG	292
		Reverse	GGCGTGAGCCATTGTG	
C11, C12, D11, D12	*TSC2*/ 374T/NT	Forward	CCTCTGGCTACCCCGTGAC	236
		Reverse	GGCCCCATCAAGCTCGC	
C14, D14	*TSC2*/ PY003T/NT	Forward	TGGAAAATGCAGTGGGAGTCTTTA	296
		Reverse	GCAAACCAGATCATCGGCAGTCAG	
E1, F1	*TSC1*/ 219T/NT	Forward	CACACTAACCCCCTGTGTTC	238
		Reverse	TTCCCAACCACATACTAAATCT	
E2, F2	*TSC1*/ 237T/NT	Forward	CCTGTGTTGGAAGACAGCTAA	303
		Reverse	ACTGCTCTCCGGCATTCTC	
E3, F3	*TSC1*/ 272T/NT	Forward	CAACATTTTTCGTCTTGTGA	170
		Reverse	ACATATAACCCAATTAGAAGAGG	
E4, F4	*TSC1*/ 340T/NT	Forward	GGCTTGATTGAACCATCTGTA	312
		Reverse	CTCGGCTGCTGTGCTTTAT	
E5, F5	*TSC1*/ 368T/NT	Forward	CCTCCGAATGTGGACAGTC	298
		Reverse	CAGACGCTTCTCCCATAGTC	

The reaction index was served as a unique tag to help the identification of the corresponding DNA gel photo in Supplementary Figure 2 and 3.