Analysis of *mTOR* Gene Aberrations in Melanoma Patients and Evaluation of Their Sensitivity to PI3K-AKT-mTOR Pathway Inhibitors

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STATEMENT OF TRANSLATIONAL RELEVANCE

Targeted therapy has made success in melanoma treatment. However, these validated targets have not benefited all melanoma patients, leaving more than 50% of melanoma patients without proper targets for targeted therapy. Mammalian target of rapamycin (mTOR) has been the key targets for cancer treatment. Unfortunately, clinical trials using mTOR inhibitors have not reached significant improvements of survivals in advanced melanoma patients. We set out to examine the gene aberrations of *mTOR* in 412 melanoma patients, and found that 43 (10.4%) of them were identified to contain nonsynonymous *mTOR* mutations. *mTOR* nonsynonymous mutations are frequent in acral and mucosal melanomas (11.0% and 14.3%, respectively). Gain-of-function mutations of *mTOR* exist, and are responsive to targeted therapy inhibitors. Our study has greatly improved the understanding of *mTOR* nonsynonymous mutation status in melanoma, and indicates that selection of patients bearing these active *mTOR* mutations may be a better strategy to conduct future clinical trials.

ABSTRACT

Purpose: Mammalian target of rapamycin (mTOR) is a validated target in cancer. It remains to be determined whether melanoma patients bearing *mTOR* mutation could be selected for treatment with PI3K-AKT-mTOR pathway inhibitors.

Experimental Design: 412 melanoma samples were included. Gene aberrations in all exons of *mTOR* were detected by Sanger sequencing and confirmed by using Agilent's SureSelect Target Enrichment System. HEK293T cells stably expressing mTOR mutants were constructed by using Transcription Activator-like Effector Nucleases technique. Function of mTOR mutants and *in vitro* sensitivity of gain-of-function mTOR mutations to PI3K-AKT-mTOR pathway inhibitors were analyzed.

Results: The overall incidence of somatic nonsynonymous mutations of *mTOR* was 10.4% (43/412). *mTOR* nonsynonymous mutations were relatively more frequent in acral (11.0%) and mucosal (14.3%) melanomas than in CSD (6.7%) and Non-CSD (3.4%) melanomas. Of the 43 cases with *mTOR* mutations, 41 different mutations were detected, affecting 25 different exons. The median survival time for melanoma patients with *mTOR* nonsynonymous mutation was significantly shorter than that for patients without *mTOR* nonsynonymous mutation ($P = 0.028$). Transient expression of mTOR mutants in HEK293T cells strongly activated the mTOR-p70S6K pathway. In HEK293T cells with stable expression of H1968Y or P2213S mTOR mutants, LY294002 and AZD5363 showed higher potency than Temsirolimus or BYL719 in

inhibiting the PI3K-AKT-mTOR pathway and cell proliferation.

Conclusions: *mTOR* nonsynonymous mutations are frequent in melanoma patients. *mTOR* nonsynonymous mutation may predict a worse prognosis of melanoma. Clinical trials with PI3K-AKT-mTOR pathway inhibitors may be beneficial for melanoma patients with specific mTOR mutations.

INTRODUCTION

Malignant melanoma is one of the most aggressive cancers with extremely poor prognosis, and the median survival time for patients with stage IV disease is about 6 to 8 months (1-3). Individualized targeted therapy has achieved successes in melanoma treatment. Among these targets, BRAF and C-KIT are the most attractive (4-6). BRAF and C-KIT inhibitors have been recommended as the first line of therapy by National Comprehensive Cancer Network in Melanoma Treatment Guidelines (2013 edition) for advanced melanoma patients with corresponding genetic mutations (7). The frequency of *BRAF* and *CKIT* mutation in Chinese melanoma patients is about 25.5% and 10.8% (8, 9), respectively, thus leaving more than 50% of Chinese melanoma patients without validated targets for targeted therapy.

Mammalian target of rapamycin (mTOR) is a serine and threonine protein kinase and plays crucial roles in transcriptional regulation, initiation of protein synthesis, ribosome biogenesis, metabolism and apoptosis etc. after being activated by various factors (10-12). mTOR signaling pathway has been the key targets for cancer treatment (13-15). Inhibitors for mTOR, including rapamycin and the derivatives RAD001, CCI-779 and AP23573, usually bind FKBP12, inhibit tumor growth and even induce apoptosis (16-19). Unfortunately, clinical trials using mTOR inhibitors in melanoma patients are not successful. A phase II clinical trial using RAD001 in 24 cases of metastatic melanoma showed that the median

progression-free survival (PFS) was about 3 months (20). Another phase II clinical trial using RAD001 in combination with temozolomide in 48 patients with advanced stage-IV unresectable melanomas showed that the median PFS was only 2.4 months (21). Genetic selection of specific target may be useful for the establishment of therapeutic strategy for advanced melanoma patients. The two clinical trials using mTOR inhibitors have not screened the genetic *mTOR* nonsynonymous mutations (termed as mutation in this study if not specified) in the melanoma patients (20, 21). Therefore, it remains to be determined whether melanoma patients bearing certain genetic mutations in *mTOR* will respond better to mTOR inhibitors.

Genetic aberrations of *mTOR* have not been extensively investigated. Data from the TCGA and COSMIC (until Sep. 2015) suggested that the frequency of *mTOR* nonsynonymous mutations in all the cancers is about 1.86% to 3.00%, and it is about 3.61% to 7.19% in cutaneous melanomas (22, 23). These databases mainly include the data derived from Caucasian melanoma patients, which had not been validated in larger cohorts of melanoma samples (24-26). More importantly, large-scale screening of *mTOR* aberrations in clinical samples has not been reported.

We collected 412 melanoma tissue samples from melanoma patients, and examined all samples for aberrations in all the 58 exons of *mTOR* gene. This study represents the first systematic analysis of somatic *mTOR* mutations in melanoma patients. Kinase activities of these mutated mTOR as well as their sensitivity to PI3K-AKT-mTOR pathway inhibitors were evaluated.

MATERIALS AND METHODS

Patients and tissue samples

This study involved samples from primary lesions of 412 melanoma patients, hospitalized during January 2007 and January 2013 at the Peking Cancer Hospital & Institute. These samples were analyzed by hematoxylin and eosin staining and by immunohistochemistry to confirm the diagnosis of melanoma. Clinical data, including age, sex, TNM (tumor-node-metastases) stage, thickness (Breslow), ulceration and survival (follow-up persisted until December 2011, or until the missing of follow-up or death of patients) were collected. This study was approved by the Medical Ethics Committee of the Beijing Cancer Hospital & Institute and was conducted according to the Declaration of Helsinki Principles.

DNA preparation and mutation screening

Genomic DNA was extracted from formalin-fixed, paraffin-embedded sections using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). To detect DNA sequence aberrations, we amplified exons 1 to 58 of the *mTOR* gene by PCR in at least two separate preparations of genomic DNA. The primer sequences are listed in **Supplementary Table S1**. We purified PCR products with QIAquick (Qiagen), and directly sequenced them using Big Dye Terminator sequencing chemistry on an ABI3130 automated sequencer (Applied Biosystems, Foster City, CA). All

aberrations were confirmed by repeated bidirectional sequencing on the ABI sequencer. To exclude single nucleotide polymorphisms, all the nonsynonymous mutations were tested in DNA derived from peripheral blood mononuclear cells.

To further confirm the detected nonsynonymous mutations, we used Agilent's SureSelect Target Enrichment System (Santa Clara, CA). Capture of the target regions was performed with reagents from a custom design HaloPlex Target Enrichment kit (Agilent), following the manufacturer's protocol. Briefly, genomic DNA was digested in eight different restriction reactions. Restricted fragments were hybridized to probes whose ends are complementary to the target fragments. During hybridization, fragments were circularized, and sequencing motifs including index sequences were incorporated. Target DNA was captured by using streptavidin beads and ligation of circularized fragments. Finally, captured target libraries were amplified by PCR. Paired-end sequencing (100 bp reads) of all samples was performed on a HiSeq2500 instrument (Illumina, San Diego, CA). TruSeq PE Cluster Kit V3 (Illumina) was used to generate the clusters and TruSeq SBS Kit V3 (Illumina) was used for sequencing. Image analysis and base calling were performed using the Illumina RTA software. Sequence reads were trimmed to remove Illumina adapter sequences and aligned to the human reference genome (version hg19). The variants were called by Agilent SureCall software. All of the targeted enrichment, sequencing and data analysis were performed at Shanghai Biotechnology Corporation (Shanghai, China).

Immunohistochemistry

Immunohistochemical (IHC) analyses were performed using antibodies against phospho-AKT (Ser473), phospho-mTOR (Ser2448), phospho-S6RP (Ser235/236) and phospho-4EBP1 (Thr37/46) (Cell Signaling Technology, Beverly, MA) as described (4, 8). The staining score for each sample, counting the intensity and density of the staining, was graded as 0, 1, 2, and 3 ("0" as negative, and "3" as the strongest; or "0" as negative, and "1", "2" and "3" as positive) by three pathologists independently (typical staining was shown in **Supplementary Fig. S1**), without the knowledge of *mTOR* mutation status of these patients.

Plasmid construction

A mammalian expression vector (pCMV6) containing the human wild-type *mTOR* cDNA with Flag tag at the C-terminal was obtained from Sino Biologicol, Inc. (Beijing, China). All the *mTOR* mutants were generated by site-directed mutagenesis. S2215Y mutant of mTOR was used as a positive gain-of-function control (27).

Authentication and culture of HEK293T cells

Human embryonic kidney 293 (HEK293) transformed by expression of the large T antigen from SV40 virus that inactivates retinoblastoma protein (HEK293T) is a cell line derived from HEK293 cells, and was obtained from Cell Bank of Chinese

Academy of Sciences (Shanghai, China) in Dec. 2014. The cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (heat-inactivated; Gibco of Thermofisher, Waltham, MA). The cells have been authenticated by the cell bank by examining the expression of large T antigen and the competence to replicate vectors carrying the SV40 region of replication. Additionally, the cells also were authenticated to show characteristics of HEK293 cells, including epithelial phenotype, hypotriploidy of chromosome, and the typical chromosome markers (such as der(1)t(1;15) (q42; q13), der(19)t(3;19)(q12; q13), der(12)t(8;12) (q22; p13) and paired der(1) and M8 (or Xq^+)).

Immunoprecipitation and Western blotting

HEK293T cells were transiently transfected with 4 μg plasmid DNA of Flag-tagged wild type or indicated mutants of mTOR using 10 μl Neofect DNA transfection reagent (Neofect Biotech Co., Beijing, China) as instructed. After a 36h incubation, cells were harvested and lysed. Supernatants were collected by centrifugation. A small portion of the cell lysate was saved as input performed with anti-Flag antibody (Sigma, St Louis, MO), and the remaining cell lysate was incubated with 15 μl M2-Flag agarose beads (Sigma) at 4℃ for 1h on a rotator. Beads were washed 5 times and the bound protein complexes were solubilized in 1.25 × SDS loading buffer and subjected to Western blotting. Western blotting analysis of protein complexes was performed with anti-p70 S6 kinase (p70S6K) antibody and

anti-phospho-p70S6K (Thr421/Ser424) antibody (Epitomics, Burlingame, CA).

Transcription activator-like effector nucleases

Heterozygous or homozygous HEK293T cells stably expressing mTOR mutants were constructed from a single base substitution method mediated by TALEN (transcription activator-like effector nucleases) and ssODN (single stranded oligodeoxynucleotide). Three plasmids containing TALEN-L, TALEN-R and Single-base mutation ssODN were constructed (Sidansai Biotechnology Co., Shanghai, China) and co-transfected into HEK293T cell lines using FuGene HD transfection reagent (Roche). HEK293T cells with single-base mutation were selected by puromycin and verified by Sanger sequencing.

mTOR inhibitors and proliferation assays

LY294002 (#S1105), AZD5363 (#S8019) and Temsirolimus (#S1044) were purchased from Selleck Chemicals (Houston, TX). BYL719 was applied from Novartis (Basel, Switzerland). All inhibitors were dissolved at 10 mM in dimethylsulfoxide (DMSO) as stock solutions and stored at −80℃. All inhibitors were diluted in DMEM supplemented with 0.1% fetal bovine serum (FBS) before each experiment, and corresponding concentration of DMSO in DMEM supplemented with 0.1% FBS was used as a vehicle control. After treatment of indicated HEK293T cells for 24h with indicated concentrations of inhibitors,

proliferation of the cells was evaluated by CCK-8 method (Dojindo Molecular Technologies Inc., Shanghai, China) according to the manufacturer's instruction. To assess the activation of mTOR-associated signaling mediators, we analyzed the corresponding cells by Western blotting using antibodies against phospho-AKT (Ser473), phospho-AKT (Thr308), phospho-mTOR (Ser2448), phospho-S6RP (Ser235/236) and phospho-4EBP1 (Thr37/46) (Cell Signaling Technology).

Statistical analyses

Statistical analyses were performed using SPSS 16.0 software. Continuous data such as age and thickness were described using means ± SD for normally distributed data. The correlations between mutational status and clinical parameters were evaluated by Chi-square test or Fisher's exact test. Kaplan-Meier estimates of time-to-event overall survival (OS) were calculated. Log-rank tests were used to estimate the statistical significance between the time-dependent outcomes of OS. Cox proportional-hazards regression analysis was conducted to estimate the Hazard Ratio (HR) for OS. All statistical analyses were two sided, and $P < 0.05$ was considered as statistically significant.

RESULTS

mTOR gene aberrations in melanoma

Of the 412 melanoma samples analyzed, 43 (10.4%) of them were identified to contain nonsynonymous *mTOR* mutations. The mutation frequencies of *mTOR* in acral, mucosal, melanomas on skin with chronic sun-induced damage (CSD), melanomas on skin without chronic sun-induced damage (non-CSD) melanoma and unknown primary subtypes were 11.0%, 14.3%, 6.7%, 3.4% and 11.1%, respectively (**Table 1**). The nonsynonymous mutation frequency of *mTOR* in acral and mucosal melanoma (38/315, 12.1%) tends to be higher than that in other melanoma (5/97, 5.2%) (P = 0.052). Surprisingly, synonymous aberrations were detected in all the melanomas (**Supplementary Table S2**), which may indicate that the exon regions of *mTOR* were rather unstable and varied at high frequency. Since these synonymous aberrations were unrelated to the analysis of sensitivity to PI3K-AKT-mTOR inhibitors, though most of them had not been deposited in COSMIC database, they were not further evaluated in this study.

Of the 43 cases with *mTOR* mutations, 41 different mutations were detected. Point mutation resulting in single amino acid substitutions was the most common type (40/41) of *mTOR* mutation (as illustrated in **Fig. 1A**, using the Hotspotter applications (28)). There were 3 cases with mutations in 2 separate exons, 2 cases with in-frame deletions in exon 39 (amino acids 1830-1833). *mTOR*

nonsynonymous mutations did not cluster defined hotspots. However, 6 recurrent mTOR mutations were found: P1128L (2 cases), V1275A (2 cases), C1303R (2 cases), A1836T (2 cases), G1914A (2 cases) and 5490-5501 base pair deletion (TGCCGCCACCAC, 2 cases) (**Fig. 1B** and **Supplementary Table S2**).

We detected nonsynonymous *mTOR* mutations in 25 of 58 exons, which affected the Huntingtin, Elongation factor 3, A subunit of protein phosphatase 2A and TOR1 domain (HEAT, 20 cases), the FRAP, ATM and TRRAP domain (FAT, 15 cases), the FKBP12-rapamycin binding domain (FRB, 3 cases), the kinase domain (kinase, 4 cases), or both the HEAT and the FAT domain (1 case) (**Fig. 1C**).

To ensure the accuracy of the above results, 8 *mTOR* nonsynonymous mutation samples with the fresh tissues available were confirmed utilizing Agilent's Sureselect Target Enrichment System (**Supplementary Table S3**), which convincingly verified the reliability of the above mutations detected by DNA sequencing of PCR products.

To exclude the possibility that the detected aberrations were due to polymorphisms, we extracted DNA from the peripheral blood mononuclear cells from all the 43 patients harboring *mTOR* nonsynonymous mutations, and examined the mutation status of *mTOR* in the corresponding exon. No mutations in the corresponding exons of *mTOR* were detected, indicating that the 43 mutations detected by us were indeed somatic mutations.

Furthermore, we analyzed the relationship between *mTOR* nonsynonymous

mutations with the mutations in *CKIT*, *BRAF*, *NRAS* and *PDGFRA*. Among the 43 cases with *mTOR* mutation, 2 cases were found to bear C-KIT mutation (N822K and V559A, respectively), 7 cases with $BRAF^{V600E}$ mutation, 4 cases with NRAS mutation (1 case of Q61L, 1 case of G12C, 1 case of G12D and 1 case of G13D), and 1 case with $PDGFRA^{N656D}$ mutation (**Supplementary Table S4**). These data indicate that *mTOR* mutations may be not mutually exclusive to the other genetic mutations of validated targets. Further analysis of the frequency of *CKIT*, *BRAF* and *NRAS* mutation in patients of our cohort showed that mutation frequency of these genes did not significantly differ between patients with or without *mTOR* mutation (**Table 2**). Therefore *mTOR* mutation may be unrelated to the other mutations such as *CKIT*, *BRAF* and *NRAS* mutation.

Correlation of mTOR mutation to clinicopathologic features

In our cohort, the mean age, gender, average thickness, ulceration rate, primary sites and clinical stages of melanomas or patients were all not significantly different between melanomas or patients with or without *mTOR* nonsynonymous mutations (**Table 2**). These data indicate that *mTOR* mutation may be unrelated to these clinical features of melanomas.

Then we analyzed the prognostic significance of *mTOR* nonsynonymous mutation for OS. The survival data were collected for patients (n = 392) who were diagnosed as primary melanoma or melanoma of unknown primary site

(**Supplementary Table S2**). The median follow-up period was 31.0 (range: 3.0-300.0) months. We found that the median survival time for patients with *mTOR* mutations (43.1 months, 95% CI: 34.8-51.8 months) was significantly shorter than that for patients without nonsynonymous *mTOR* mutations (81.3 months, 95% CI: 60.2-102.4 months; $P = 0.028$; **Supplementary Fig. S2**). In univariate Cox analysis, we found that the hazard ratio (HR) for patients bearing *mTOR* nonsynonymous mutation was 0.645 (95% CI: 0.43-0.96; $P = 0.031$). Therefore, *mTOR* mutation, together with thickness, ulceration status and TNM stage, may be of prognostic significance for melanoma patients, and melanoma patients with *mTOR* mutations may show higher risk of death (**Table 3**). For multivariate Cox regression assay, we excluded the variate thickness since the data for 118 patients (most of them had mucosal melanoma or melanoma with the primary site unknown). We found that patients with *mTOR* mutations may tend to ($P = 0.08$) have a higher risk of death than patients without *mTOR* mutations while TNM stage and ulceration may definitely serve as independent prognostic factor for OS (**Table 3**).

Since mTOR activation may activate the downstream substrates eukaryotic initiation factor 4E (eIF4E) binding protein-1 (4EBP1) and p70 ribosomal protein S6 kinase 1 (p70S6K, which in turn phosphorylate the 40S ribosomal protein S6 (S6RP)) (15). We examined the activation of these mTOR-associated molecules, including phospho-AKT, phospho-mTOR, phospho-S6RP and phospho-4EBP1 by immunohistochemistry (**Supplementary Fig. S1**). We found that the positive rate of

phospho-AKT, phospho-mTOR and phospho-4EBP1 was not significantly different between melanomas bearing nonsynonymous *mTOR* mutations and those without nonsynonymous *mTOR* mutation. However, the positive rate for phospho-S6RP was significantly higher in melanomas bearing *mTOR* mutations than that in patients without nonsynonymous *mTOR* mutation (P = 0.004; **Supplementary Table S5**). Taken together the previous report that phospho-S6RP is positive in the majority of melanomas (29), these data indicate that *mTOR* mutation may mainly affect the p70S6K-S6RP signaling pathway in melanoma.

We further analyzed the correlation between activation of mTOR-associated molecules and OS. We found that the survival times for patients negative or positive for phospho-AKT, phospho-mTOR, phospho-S6RP or phospho-4EBP1 were not significantly different (data not shown), indicating that activation of these mTOR-associated molecules may not predict prognosis in melanoma.

Functional analysis of mTOR mutations

To investigate whether the somatic nonsynonymous mutations of *mTOR* could activate mTOR/p70S6K pathway, 7 nonsynonymous mutants were overexpressed in HEK293T cells. Among the 7 *mTOR* mutations functionally examined, P1128L, V1275A, C1303R, T1830_T1834 [del] and G1914A were repeated mutations (2 cases for each mutation) while P2213S may affect the ATP pocket of kinase domain (30). H1968Y was initially regarded as repeated mutation, which, however, was

confirmed to be non-repeated after the functional assays in HEK293T cells in a later validation sequencing experiment. But H1968Y was shown in this study due to its gain-of-function mutation. The phosphorylation levels of endogenous p70S6K were analyzed by Western blotting following immunoprecipitations. Because S2215Y was previously demonstrated to be an activating mutation identified in large intestine adenocarcinoma (27), it was used as the positive control. Strong phosphorylation of p70S6K was observed in H1968Y and P2213S mutants, whereas the other mutations showed weak to medium phosphorylation of p70S6K (**Fig. 2A and 2B**).

To further analyze the effects of mTOR mutants on downstream signaling pathway, we stably expressed mTOR mutants (C1303R and G1914A as non-functional mutation, and H1968Y and P2213S as functional mutation, and S2215Y as positive control) in HEK293T cells by TALEN. However, only H1968Y, P2213S and S2215Y cell clones were finally established. Function of mTOR mutants in these cells were analyzed by Western blotting. We found that the expression levels of mTOR (mutants) were equivalent between the established cells and wild type HEK293T cells (data not shown), indicating for the advantages of TALEN technique. But we found that phospho-p70S6K, phospho-4EBP1 and phospho-AKT were strongly detected in HEK293T cells with heterozygous or homozygous expression of H1968Y or P2213S mTOR mutants (**Fig. 2C-2L**). Therefore, H1968Y and P2213S mutations may be gain-of-function mutations.

Sensitivity of mTOR active mutations to PI3K-AKT-mTOR pathway inhibitors

To analyze the sensitivity of the gain-of-function mTOR mutations to PI3K-AKT-mTOR pathway inhibitors, HEK293T cells stably expressing H1968Y or P2213S mTOR mutants were treated with indicated inhibitors as described previously (31-34). The phosphorylation status of p70S6K, 4EBP1 and AKT was examined by Western blot under indicated drug concentrations. Phosphorylation levels of p70S6K, 4EBP1 and AKT induced by H1968Y, P2213S or S2215Y mutants were more significantly decreased by exposing to AZD5363 and LY294002 than BYL719 and Temsirolimus (**Fig. 3A and 3B**). For AZD5363, expression of phospho-AKT was more remarkably inhibited than phosho-p70S6K and phospho-4EBP1; For LY294002, levels of phospho-4EBP1 were more dramatically inhibited than p-p70S6K and phospho-AKT.

To confirm the effects of inhibitors on cells stably expressing active mTOR mutants, cell proliferation assays were performed. Consistent with the results of western blotting, proliferations of HEK293T cells stably expressing H1968Y, P2213S and S2215Y mTOR mutants were more significantly inhibited by AZD5363 and LY294002 than BYL719 and Temsirolimus (**Fig. 3C-3J and Supplementary Table S6**). However, it should be noted that BYL719 and Temsirolimus did inhibit the cell proliferation at different concentrations, suggesting that these drugs were active but affected cell proliferation to similar extent between different strains of HEK293T cells. We also tested the other inhibitors, including Wortmannin,

MK-2206 2HCl, everolimus and rapamycin, but found that they could not significantly affect the proliferation of HEK293T cells (data not shown). Therefore, melanoma patients bearing H1968Y, P2213S and S2215Y mTOR mutations may be sensitive to certain but not all PI3K-AKT-mTOR pathway inhibitors.

DISCUSSION

Although PI3K-AKT-mTOR signaling pathway is crucial during tumorigenesis and tumor progression (35-37) and inhibitors targeting this pathway have been clinically trialed in a diversity of cancers (38-40), *mTOR* gene aberrations in cancer have not been extensively investigated. Because *mTOR* spans 58 exons and mTOR protein is composed of 2,549 amino acids (41), it is difficult to analyze the genetic aberrations of *mTOR* and the functional consequences of *mTOR* nonsynonymous mutations. Currently the gene aberration data of *mTOR* mainly come from genomic sequencing or exome sequencing of cancer samples. However, these data have not been validated. More importantly, due to the difference of predominant melanoma subtypes between Caucasians (mainly cutaneous melanomas) and Asians (mainly acral and mucosal melanomas) (42-45), *mTOR* aberrations in acral and mucosal melanomas remain largely unknown. Our data have thus greatly filled this gap. The data from TCGA and COSMIC databases have collected 278 and 857 cases of cutaneous melanomas, and show a *mTOR* nonsynonymous mutation frequency about 7.19% and 3.61%, respectively (22, 23). In our cohort, the nonsynonymous *mTOR*

mutation frequency in CSD plus non-CSD melanomas is about 4.5% (4/88), which is similar to the TCGA and COSMIC data. This disparity may be due to the difference in melanoma subtypes. It should be noted that the frequency of *mTOR* mutations in acral and mucosal melanomas is about 12.1%, which is significantly higher than that in cutaneous melanomas. Therefore our study is of great significance for patients with acral or mucosal melanomas.

Thirty-five new types of nonsynonymous mutation have been identified in our study. The other six mutations have been reported by TCGA or COSMIC databases, with R311C, G716D, T1834_T1837del and R1811H identified in cancer samples, and with A1836T, A1134V and R1811H identified in tumor cell lines (22, 23, 46). We note that there are 12 cases of the detected 43 cases with *mTOR* nonsynonymous mutation showing signature of ultraviolet damage (C to T alteration), and two of them (C5902T for H1968Y, and C6637T for P2213S) were found to be gain-of-function *mTOR* mutation, indicating that ultraviolet damage may somehow be related to functional mutation of *mTOR*. However, this correlation may need to be supported by more data, such as analyzing the functional activity of all mTOR aberrations (identified in COSMIC and our study) with C to T nucleotide alteration. The newly-identified nonsynonymous mutations mainly affect the HEAT domain (45.7%, 16/35) and FAT domain (34.3%, 12/35) of mTOR. In HEK293T cells stably expressing H1968Y, the levels of phospho-p70S6K, phospho-4EBP1 and phospho-AKT were relatively high, which may indicate the importance of FAT

domain in regulating mTOR activity. With the advances of structural insights into mTOR and the advantages of TALEN, the effects of *mTOR* nonsynonymous mutations on regulation and activity of mTOR would be fully addressed in future studies. Since the nonsynonymous mutation of *mTOR* is rather variable and only 6 of them can be repeatedly detected, the relation of *mTOR* mutation to the activation of downstream signaling components in clinical samples could not be established at present. In our study, *mTOR* mutation could be concurrently detected in samples bearing *BRAF*, *NRAS* or *CKIT* mutation, which may raise the concern whether *mTOR* mutation may be related to clinical resistance to BRAF or MEK inhibitors. One patient containing mTOR-A215S and BRAF-V600E mutation and treated with vemurafenib achieved partial response in our center, indicating that *mTOR* mutation may not affect response to these inhibitors. More studies are required to further clarify this concern.

Inhibitors targeting PI3K-AKT-mTOR signaling pathway have been clinically trialed in a panel of cancers. It may be expected that these inhibitors may also be beneficial for melanoma patients. However, phase II clinical trials in advanced melanomas have shown limited effects of mTOR inhibitors (20, 21). Our study detected two new gain-of-function mutations of mTOR, that is, H1968Y and P2213S, in addition to S2215Y (27). Our study suggests that these two mutations lead to activation of downstream signaling mediators, and they are sensitive to PI3K (LY294002 but not Wortmannin and BYL719) and AKT inhibitors (AZD5363 but

not MK-2206 2HCl), but not to mTOR inhibitors (everolimus, temsirolimus and rapamycin). It has been indicated by previous studies that mTOR inhibitors mainly affect the mTOR-Rapor complex but not the mTOR-Rictor complex while the latter directly phosphorylates AKT on Ser473 and activates AKT together with PDK1 (47). Therefore the PI3K-AKT inhibitors may be more potent in inhibiting mTOR mutations and cell proliferation than the mTOR inhibitors. Further studies are required to functionally evaluate the other mTOR nonsynonymous mutations in melanomas and screen sensitive drugs for these mTOR mutants. However, our study at least identified two mTOR mutants that are sensitive to PI3K-AKT-mTOR pathway inhibitors, and melanoma patients bearing these two mutations may be candidates for targeted therapy using PI3K-AKT-mTOR pathway inhibitors.

Screening the patient population for specific biomarkers is useful to select proper patients for targeted therapy. Our study suggests that *mTOR* nonsynonymous mutation in melanoma patients is rather frequent (10.3%) and mTOR may thus be a potential target for targeted therapy in Asian melanoma patients. Since only a portion of patients are sensitive to PI3K-AKT-mTOR inhibitors, it should be cautious to select candidates for clinical trials.

Authors' Contributions

Conception and design: Drs. Jun Guo and Yan kong.

Provision of study materials or patients: Drs. Jun Guo, Yan kong and Lu Si.

Collection and assembly of data: Drs. Jun Guo, Yan kong, Lu Si, Yiqian Li, Xiaowen Wu, Xiaowei Xu, Jie Dai, Huan Tang, Meng Ma, Zhihong Chi, Xinan Sheng and Chuanliang Cui.

Data analysis and interpretation: Drs. Jun Guo, Yan kong, Lu Si and Yiqian Li.

Manuscript writing: Drs. Jun Guo, Yan kong and Lu Si.

Final approval of manuscript: All authors.

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Table 1. *mTOR* mutations in melanoma

Melanoma subtypes	No. Cases	No. cases with mutation (%)
Acral melanoma	210	23 (11.0)
Mucosal melanoma	105	15 (14.3)
CSD	30	2 (6.7)
Non-CSD	58	2 (3.4)
Unknown primary	9	1 (11.1)
Total	412	43 (10.4)

Abbreviations: CSD, melanomas on skin with chronic sun-induced damage; non-CSD, melanomas on skin without chronic sun-induced damage.

Table 2. Correlation of *mTOR* nonsynonymous mutation to clinicopathologic features of melanomas

Clinicopathologic feature	*mTOR* mutation (%)*	Wild type *mTOR* (%)†	*P* value‡
Gender (Female)	21/43 (51.2)	169/369 (43.6)	0.705
Age (median, year)	54	53	/
Thickness(mm)	5.38 ± 3.98	4.78 ± 3.91	0.592
Ulceration rate	19/40 (47.5)	129/347 (37.2)	0.203
Primary site			
Acral	23/43 (53.5)	187/369 (50.7)	0.727
Mucosal	15/43 (34.9)	90/369 (24.4)	0.135
Acral + Mucosal	38/43 (88.4)	277/369 (75.1)	0.122
Non-CSD	2/43 (4.7)	56/369 (15.2)	0.156
CSD	2/43 (4.7)	28/369 (7.6)	0.396
Non-CSD + CSD	4/43 (9.3)	84/369 (22.8)	0.092
Unknown primary	1/43 (2.3)	8/369 (2.2)	0.389
TNM stage			
I	2/43 (4.7)	21/369 (5.7)	0.779
II	25/43 (58.1)	157/369 (42.5)	0.151
III	9/43 (20.9)	102/369 (27.6)	0.348
IV	7/43 (16.2)	89/369 (24.1)	0.250
Other mutations			
BRAF	7/42 (14.3)	78/265 (29.4)	0.105
CKIT	2/42 (4.8)	24/215 (11.2)	0.328
NRAS	4/42 (9.5)	29/246 (11.8)	0.870

Abbreviation: TNM, tumor-node-metastases.

* The rates of *mTOR* mutation of samples with indicated clinicopathological features were calculated as No. samples showing indicated clinicopathological features to that of 43 (or data available) cases showing *mTOR* mutations.

† The rates of wild type *mTOR* of samples with indicated clinicopathological features were calculated as No. samples showing indicated clinicopathological features to that of 369 cases (data available) showing wild type *mTOR*.

‡ For evaluation of age and thickness, the unpaired t tests were used. For evaluation of gender, ulceration, stages and subtypes, the chi-square tests were used.

Table 3. Cox regression analysis of *mTOR* mutation and clinicopathological factors with overall survival

Factors	**Group**	**HR (95% CI)**	***P* value**
Univariate analysis			
Age (n = 392)	> 60 *vs* ≤60	0.941 (0.685, 1.292)	0.707
Thickness (n = 294)	Overall		0.001
	< 1 mm	0.284 (0.137, 0.589)	0.001
	1-2 mm	0.549 (0.310, 0.970)	0.039
	2-4 mm	0.653 (0.445, 0.959)	0.03
	>4 mm	1	
TNM stage (n = 392)	Overall		< 0.001
	I	0.176 (0.064, 0.487)	0.001
	II	0.531 (0.376, 0.752)	< 0.001
	III	0.718 (0.485, 1.063)	0.098
	IV	1	
Ulceration (n = 377)	Ulceration *vs* non-ulceration	1.708 (1.274, 2.289)	< 0.001
mTOR mutation			
All subtypes	Mut *vs* WT	1.553 (1.045, 2.310)	0.031
Acral (n = 210)	Mut *vs* WT	1.051 (0.568, 1.947)	0.873
Mucosal (n = 105)	Mut *vs* WT	1.784 (0.946, 3.366)	0.074
Acral + Mucosal (n = 315)	Mut *vs* WT	1.454 (0.944, 2.241)	0.090
Cutaneous (n = 307)	Mut *vs* WT	1.284 (0.766, 2.154)	0.343
Multivariate analysis			
mTOR mutation	Mut *vs* WT	1.456 (0.956, 2.217)	0.08
TNM stage			< 0.001

	I	0.200 (0.072, 0.558)	0.002
	II	0.486 (0.337, 0.701)	< 0.001
	III	0.738 (0.492, 1.107)	0.142
	IV		
Ulceration	Ulceration *vs* non-ulceration	1.749 (1.300, 2.353)	< 0.001

Abbreviation: TNM, tumor-node-metastases; Mut, mTOR nonsynonymous mutation; WT, without nonsynonymous mutation; HR, hazard ratio; CI, confidence interval.

FIGURE LEGENDS

Figure 1. Illustration of the detected *mTOR* nonsynonymous mutations. (A) Hotspotter application illustration of *mTOR* mutation sites. (B) Distribution of mTOR mutations according to mTOR domains. The enclosed circles indicate for synonymous mutations, with the yellow circles for non-repetitive mutations and the red circles for repetitive mutations. The six repetitive nonsynonymous mutations were indicated with "**". (C) Distribution of mTOR mutations in relation to exons and mTOR domains. Each triangle indicates for one episode of non-synonymous mutation. HEAT, Huntingtin, Elongation factor 3, A subunit of protein phosphatase 2A and TOR1 domain; FAT, FRAP, ATM and TRRAP domain; FRB, FKBP12-rapamycin binding domain; FATC, FAT domain at C-terminus.

Figure 2. Effects of mTOR mutations on p70S6K and downstream signaling pathway. (A and B) HEK293T cells were transiently transfected with indicated vectors (1, wild type mTOR; 2, P1128L; 3, V1275A; 4, C1303R; 5, T1830_T1834 [del]; 6, H1968Y; 7, G1914A; 8, P2213S; 9, S2215Y). Expression of Flag-tagged mTOR in whole cell lysate (WCL) was examined by Western blotting using anti-Flag Ab (A). Otherwise, endogenous p70S6K was immunoprecipitated (IP) and then examined with indicated Abs by Western blotting (IB). Results in (A) were quantified by measuring the relative intensity of phospho-p70S6K bands to corresponding total p70S6K bands (B, presented as mean ± SD of 3 scans). (C-L) HEK293T cells stably expressing mTOR mutants (H1968Y, P2213S and S2215Y) were constructed by TALEN. After nutrient starvation, wild type (WT, for HEK293T cells not expressing mutants but wild type *mTOR* after TALEN edition and selection) or mutated HEK293T cells (heterozygous, C-G; or homozygous, H-L) were lysed, and the activation of indicated molecules were

examined by Western blotting. The relative phosphorylation levels of signaling mediators were quantified by measuring the relative intensity of phosphorylated bands to corresponding total bands (D-G, I-L; presented as mean ± SD of 3 scans).

Figure 3. Sensitivity of gain-of-function mTOR mutations to PI3K-AKT-mTOR inhibitors. HEK293T cells stably expressing mTOR mutants (H1968Y, P2213S and S2215Y) were constructed by TALEN. After nutrient starvation, wild type (WT, for HEK293T cells not expressing mutants but wild type *mTOR* after TALEN edition and selection) or mutated HEK293T cells (heterozygous, A, C-F; or homozygous, B, G-J) were treated with indicated inhibitors or vehicle. The activation of indicated molecules was examined by Western blotting (A and B). The proliferation of HEK293T cells was evaluated by CCK-8 method (C-J), and the results were presented as mean ± SD of 3 independent experiments.

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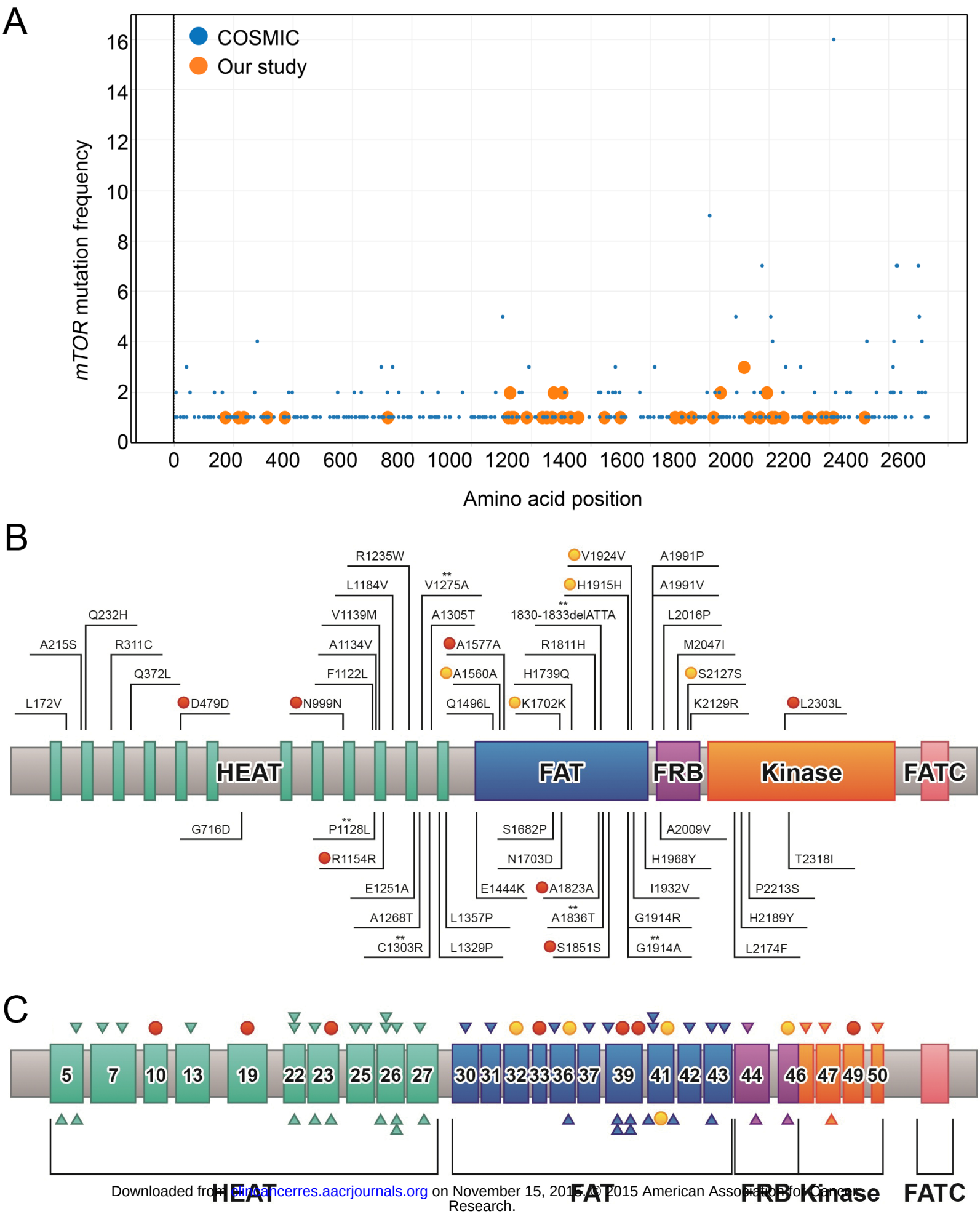
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.



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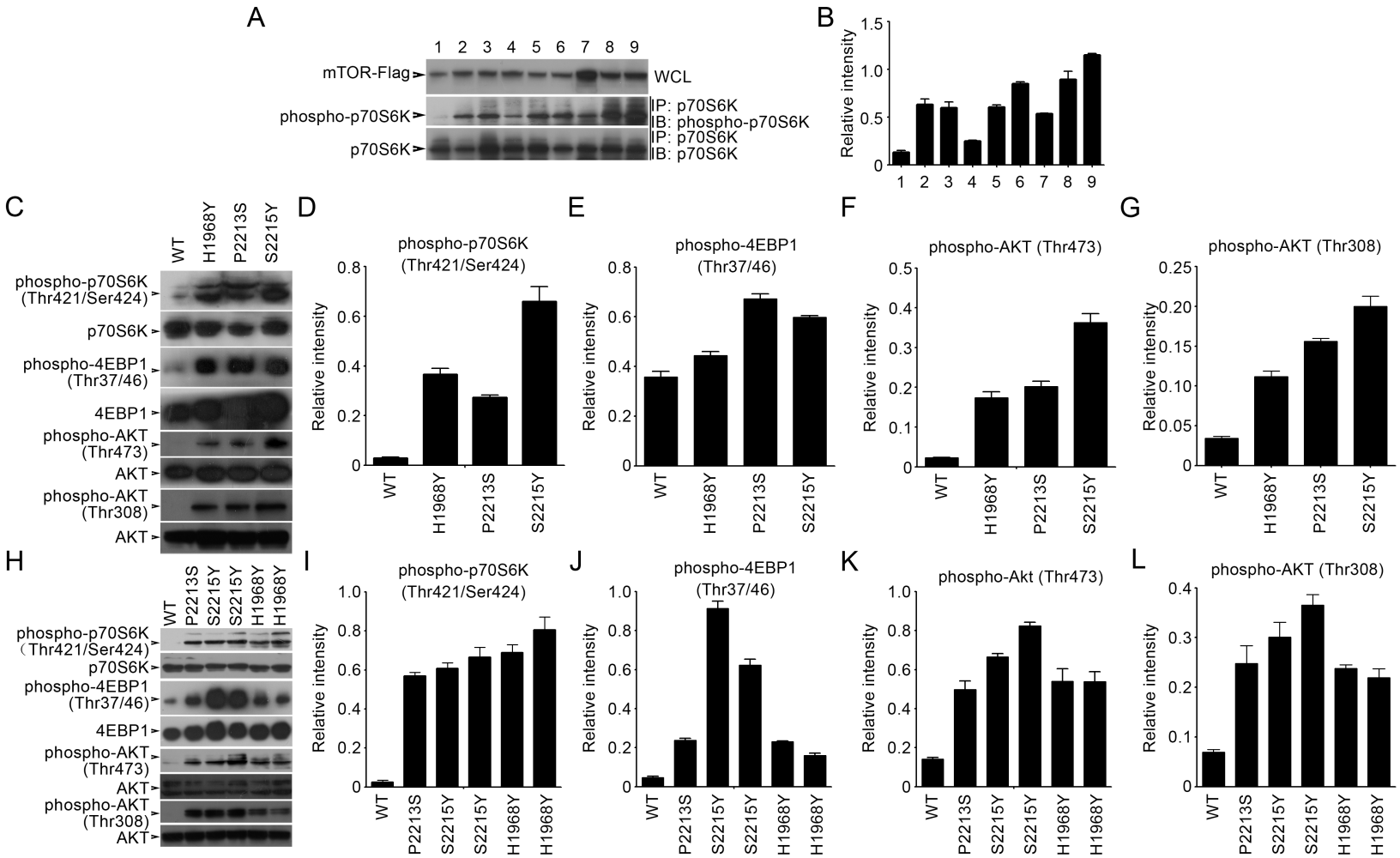
Kong et al., Figure 2



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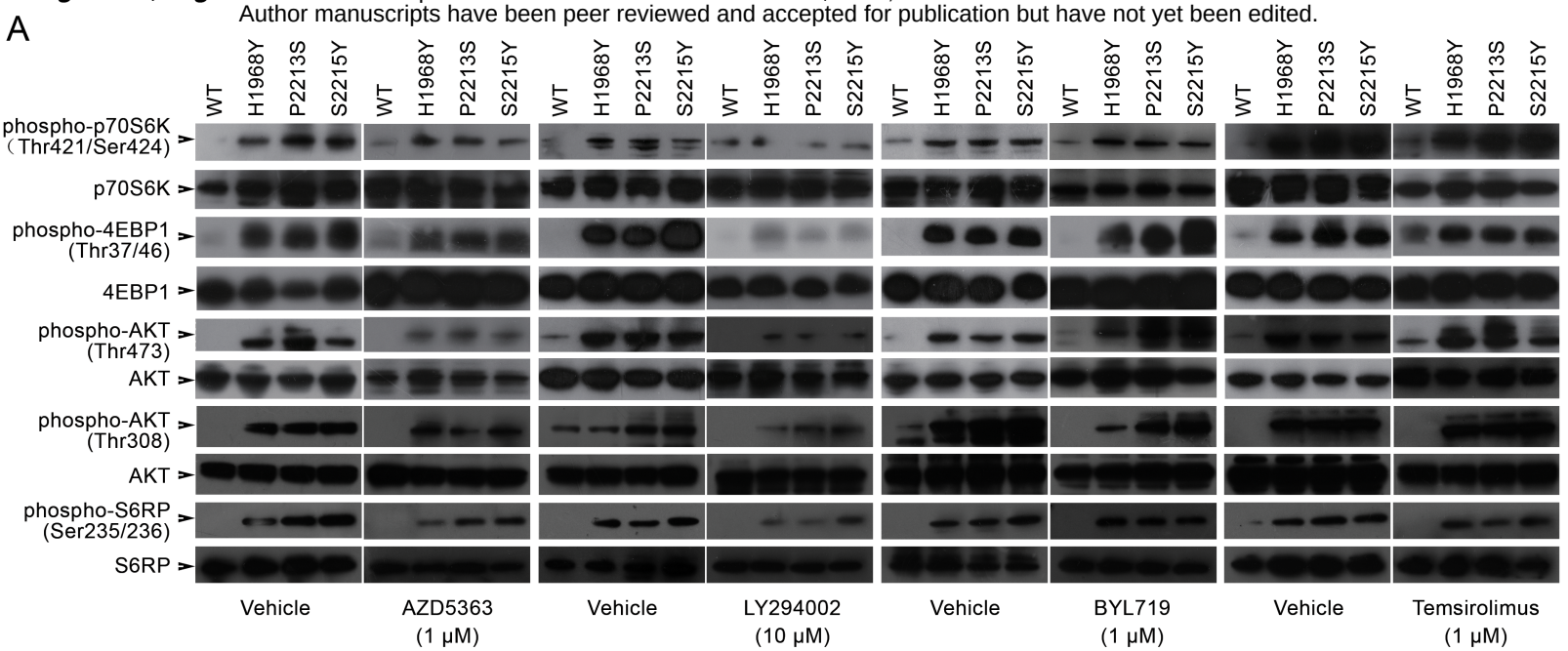

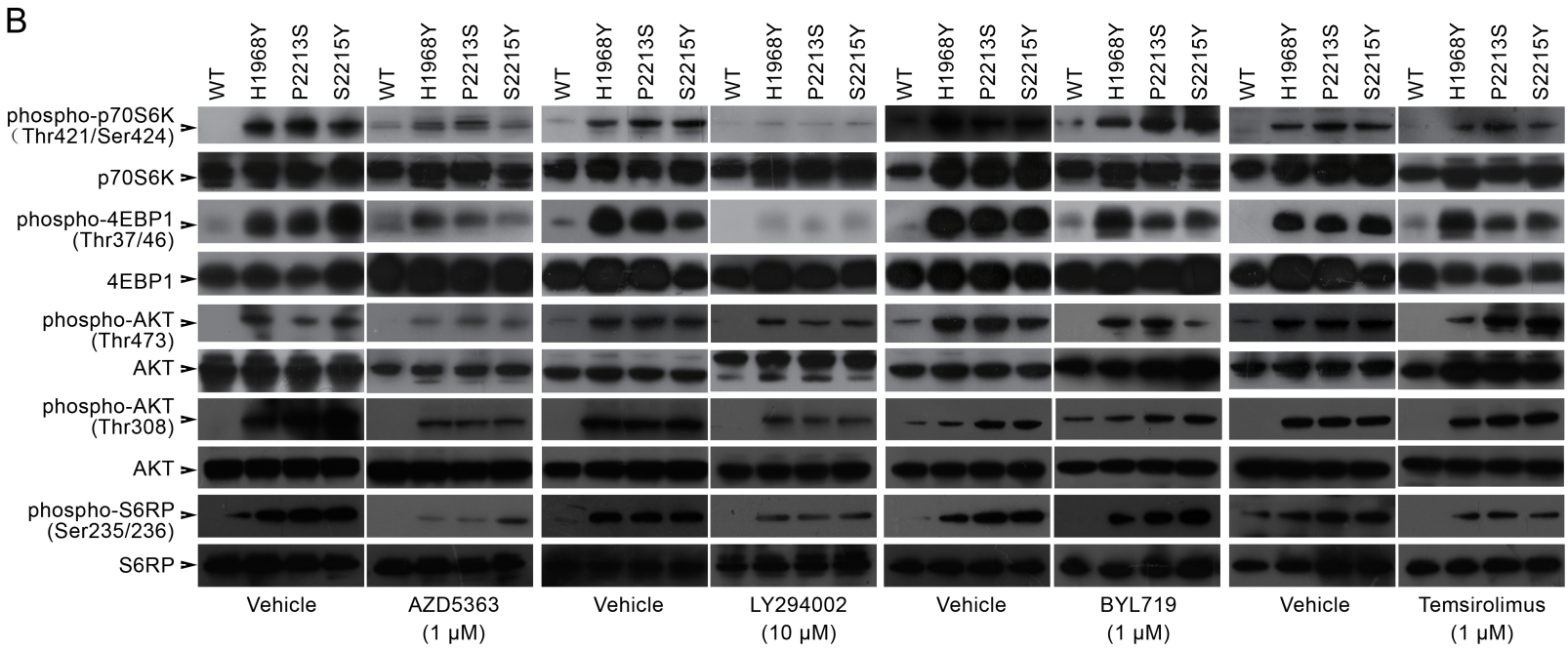

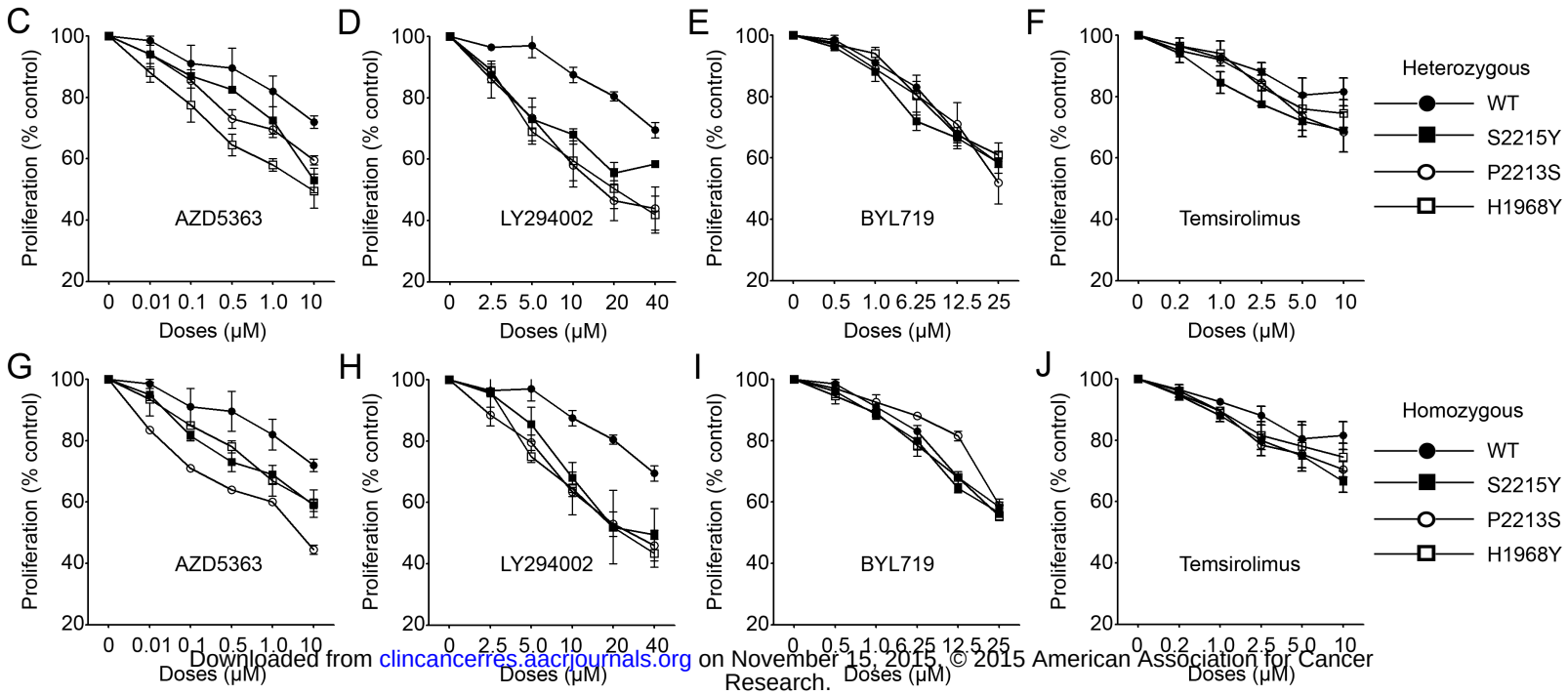

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Analysis of mTOR Gene Aberrations in Melanoma Patients and Evaluation of Their Sensitivity to PI3K-AKT-mTOR Pathway Inhibitors

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