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

Yan Kong1, Lu Si1, Yiqian Li1, Xiaowen Wu1, Xiaowei Xu2, Jié Dai1, Huan Tang1, Meng Ma1, Zhihong Chi1, Xian Sheng1, Chuanliang Cui1, and Jun Guo1

Abstract

Purpose: 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: A total of 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 nuclease 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 chronic sun-induced damage (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. Clin Cancer Res; 1–10. ©2015 AACR.

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.

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 1.5 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, tumor-node-metastases (TNM) stage, thickness (Breslow), ulceration and survival (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). 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). 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. 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). 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.

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) 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 Biological, Inc. 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 in December 2014. The cells were cultured in Dulbecco’s modified Eagle Medium containing 10% fetal bovine serum (PBS; heat-inactivated; Gibco of Thermofisher). 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. In addition, 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.) as instructed. After a 36-hour 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), and the remaining cell lysate was incubated with 15 μL M2-Flag agarose beads (Sigma) at 4°C for 1 hour on a rotator. Beads were washed five 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).

Transcription activator-like effector nucleases
Heterozygous or homozygous HEK293T cells stably expressing mTOR mutants were constructed from a single-base substitution method mediated by transcription activator-like effector nucleases (TALEN) and single-stranded oligodeoxynucleotide (ssODN). Three plasmids containing TALEN-L, TALEN-R, and single-base mutation ssODN were constructed (Sidansai Biotechnology Co.) and cotransfected 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. BYL719 was applied from Novartis. All inhibitors were dissolved at 10 mmol/L in dimethylsulfoxide (DMSO) as stock solutions and stored at −80°C. All inhibitors were diluted in DMEM supplemented with 0.1% 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 24 hours with indicated concentrations of inhibitors, proliferation of the cells was evaluated by CCK-8 method (Dojindo Laboratories). HEK293T cells were subjected to Western blotting. Western blotting analysis of protein was performed 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 mean ± SD for normally distributed data. The correlations between mutational status and clinical parameters were evaluated by χ² test or Fisher 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 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 are rather unstable and varied at high frequency. Because these synonymous aberrations were unrelated to the analysis of sensitivity to PI3K-AKT–mTOR inhibitors, although 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 application; 28). There were three cases with mutations in two separate exons, two cases with in-frame deletions in exon 39 (amino acids 1830–1833). mTOR nonsynonymous mutations did not cluster defined hotspots. However, six recurrent mTOR mutations were found: P1128L (two cases), V1275A (two cases), C1303R (two cases), A1836T (two cases), G1914A (two cases), and 5490 to 5501 base pair deletion (TGCCGCCACCAC, two 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, eight mTOR non-synonymous 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 PDGFRα. Among the 43 cases with mTOR mutation,
Two cases were found to bear C-KIT mutation (N822K and V559A, respectively), seven cases with BRAFV600E mutation, four cases with NRAS mutation (one case of Q61R, one case of G12C, one case of G12D, and one case of G13D), and one case with PDGFRAN656D 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).

Table 1. mTOR mutations in melanoma

<table>
<thead>
<tr>
<th>Melanoma subtypes</th>
<th>Number of cases</th>
<th>Number of cases with mutation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acral melanoma</td>
<td>210</td>
<td>23 (11.0)</td>
</tr>
<tr>
<td>Mucosal melanoma</td>
<td>105</td>
<td>15 (14.3)</td>
</tr>
<tr>
<td>CSD</td>
<td>30</td>
<td>2 (6.7)</td>
</tr>
<tr>
<td>Non-CSD</td>
<td>58</td>
<td>2 (3.4)</td>
</tr>
<tr>
<td>Unknown primary</td>
<td>9</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td>Total</td>
<td>412</td>
<td>43 (10.4)</td>
</tr>
</tbody>
</table>

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

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 nonrepetitive mutations and the red circles for repetitive mutations. The six repetitive nonsynonymous mutations were indicated with "//C3/C3." C, distribution of mTOR mutations in relation to exons and mTOR domains. Each triangle indicates for one episode of nonsynonymous mutation. HEAT, Huntington, 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.
Table 2. Correlation of mTOR nonsynonymous mutation to clinicopathologic features of melanomas

<table>
<thead>
<tr>
<th>Clinicopathologic feature</th>
<th>mTOR mutation (%)</th>
<th>Wild type mTOR (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (female)</td>
<td>22/43 (51.2)</td>
<td>169/369 (45.8)</td>
<td>0.705</td>
</tr>
<tr>
<td>Age (median, year)</td>
<td>54</td>
<td>53</td>
<td>-</td>
</tr>
<tr>
<td>Thickness (mm)</td>
<td>5.38 ± 5.98</td>
<td>4.78 ± 3.91</td>
<td>0.592</td>
</tr>
<tr>
<td>Ulceration rate</td>
<td>19/40 (47.5)</td>
<td>129/347 (37.2)</td>
<td>0.203</td>
</tr>
<tr>
<td>Primary site</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acral</td>
<td>23/43 (53.5)</td>
<td>187/369 (50.7)</td>
<td>0.727</td>
</tr>
<tr>
<td>Mucosal</td>
<td>15/43 (34.9)</td>
<td>90/369 (24.4)</td>
<td>0.135</td>
</tr>
<tr>
<td>Acral + mucosal</td>
<td>38/43 (88.4)</td>
<td>277/369 (75.1)</td>
<td>0.122</td>
</tr>
<tr>
<td>Non-CSD</td>
<td>2/43 (4.7)</td>
<td>56/369 (15.2)</td>
<td>0.156</td>
</tr>
<tr>
<td>CSD</td>
<td>2/43 (4.7)</td>
<td>28/369 (7.6)</td>
<td>0.396</td>
</tr>
<tr>
<td>Non-CSD + CSD</td>
<td>4/43 (9.3)</td>
<td>84/369 (22.8)</td>
<td>0.092</td>
</tr>
<tr>
<td>Unknown primary</td>
<td>1/43 (2.3)</td>
<td>8/369 (2.2)</td>
<td>0.389</td>
</tr>
<tr>
<td>TNNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2/43 (4.7)</td>
<td>21/369 (5.7)</td>
<td>0.779</td>
</tr>
<tr>
<td>II</td>
<td>25/43 (58.1)</td>
<td>157/369 (42.5)</td>
<td>0.151</td>
</tr>
<tr>
<td>III</td>
<td>9/43 (20.9)</td>
<td>102/369 (27.6)</td>
<td>0.348</td>
</tr>
<tr>
<td>IV</td>
<td>7/43 (16.2)</td>
<td>89/369 (24.1)</td>
<td>0.250</td>
</tr>
<tr>
<td>Other mutations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAF</td>
<td>7/42 (14.3)</td>
<td>78/265 (29.4)</td>
<td>0.105</td>
</tr>
<tr>
<td>CKIT</td>
<td>2/42 (4.8)</td>
<td>24/215 (11.2)</td>
<td>0.328</td>
</tr>
<tr>
<td>NRAS</td>
<td>4/42 (9.5)</td>
<td>29/246 (11.8)</td>
<td>0.870</td>
</tr>
</tbody>
</table>

*The rates of mTOR mutation of samples with indicated clinicopathologic features were calculated as number of samples showing indicated clinicopathologic features to that of 43 (or data available) cases showing mTOR mutations.

**The rates of wild-type mTOR of samples with indicated clinicopathologic features were calculated as number of samples showing indicated clinicopathologic 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 χ2 tests were used.

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 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 TNNM 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 have a higher risk of death than patients without mTOR mutations whereas TNNM stage and ulceration may definitely serve as independent prognostic factor for OS (Table 3).

Because 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]; ref. 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 (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, seven nonsynonymous mutants were overexpressed in HEK293T cells. Among the seven mTOR mutations functionally examined, P1128L, V1275A, C1303R, T1830_T1834 [del], and G1914A were repeated mutations (two cases for each mutation) whereas P2213S may affect the ATP pocket of kinase domain (30). H1968Y was initially regarded as repeated mutation, which, however, was confirmed to be nonrepeated 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 B).

To further analyze the effects of mTOR mutations on downstream signaling pathway, we stably expressed mTOR mutants (C1303R and G1914A as nonfunctional mutation, and H1968Y and P2213S as functional mutation, and S2215Y as positive control) in HEK293T cells by TALEN. However, only H1968Y, P2213S,
Table 3. Cox regression analysis of mTOR mutation and clinicopathologic factors with overall survival

<table>
<thead>
<tr>
<th>Factors</th>
<th>Group</th>
<th>HR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univariate analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (n = 392)</td>
<td>60 vs. 60</td>
<td>0.941 (0.685–1.292)</td>
<td>0.707</td>
</tr>
<tr>
<td>Thickness (n = 294)</td>
<td>Overall</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;1 mm</td>
<td>0.284 (0.137–0.589)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>1–2 mm</td>
<td>0.549 (0.310–0.970)</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>2–4 mm</td>
<td>0.653 (0.445–0.959)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>&gt;4 mm</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TNM stage (n = 392)</td>
<td>Overall</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>0.176 (0.064–0.487)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0.531 (0.376–0.752)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>0.718 (0.485–1.063)</td>
<td>0.098</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ulceration (n = 377)</td>
<td>Ulceration vs. nonulceration</td>
<td>1.708 (1.274–2.289)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>mTOR mutation</td>
<td>All subtypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mut vs. WT</td>
<td>1.553 (1.045–2.350)</td>
<td>0.031</td>
</tr>
<tr>
<td>Acral (n = 210)</td>
<td>Mut vs. WT</td>
<td>1.051 (0.568–1.947)</td>
<td>0.873</td>
</tr>
<tr>
<td>Mucosal (n = 105)</td>
<td>Mut vs. WT</td>
<td>1.784 (0.946–3.366)</td>
<td>0.074</td>
</tr>
<tr>
<td>Acral + Mucosal (n = 315)</td>
<td>Mut vs. WT</td>
<td>1.454 (0.944–2.241)</td>
<td>0.090</td>
</tr>
<tr>
<td>Cutaneous (n = 307)</td>
<td>Mut vs. WT</td>
<td>1.284 (0.766–2.154)</td>
<td>0.343</td>
</tr>
<tr>
<td>Multivariate analysis</td>
<td>mTOR mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mut vs. WT</td>
<td>1.456 (0.956–2.217)</td>
<td>0.08</td>
</tr>
<tr>
<td>TNM stage</td>
<td>I</td>
<td>0.200 (0.072–0.558)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0.486 (0.337–0.701)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>0.738 (0.492–1.107)</td>
<td>0.142</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ulceration</td>
<td>Ulceration vs. nonulceration</td>
<td>1.749 (1.300–2.353)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; Mut, mTOR nonsynonymous mutation; WT, without nonsynonymous mutation.

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-4E BP1, and phospho-AKT were strongly detected in HEK293T cells with heterozygous or homozygous expression of H1968Y or P2213S mTOR mutants (Fig. 2C–L). 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, 4E BP1, and AKT was examined by Western blot under indicated drug concentrations. Phosphorylation levels of p70S6K, 4E BP1, 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 B). For AZD5363, expression of phospho-AKT was more remarkably inhibited than phospho-p70S6K and phospho-4E BP1. For LY294002, levels of phospho-4E BP1 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–J 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; refs. 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

---

Kong et al.

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-4E BP1, and phospho-AKT were strongly detected in HEK293T cells with heterozygous or homozygous expression of H1968Y or P2213S mTOR mutants (Fig. 2C–L). 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, 4E BP1, and AKT was examined by Western blot under indicated drug concentrations. Phosphorylation levels of p70S6K, 4E BP1, 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 B). For AZD5363, expression of phospho-AKT was more remarkably inhibited than phospho-p70S6K and phospho-4E BP1. For LY294002, levels of phospho-4E BP1 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–J 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; refs. 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 \textit{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 \textit{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 \textit{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 \textit{mTOR} mutation, indicating that ultraviolet damage may somehow be related to functional mutation of \textit{mTOR}. However, this correlation may need to be supported by more data, such as analyzing the functional activity of all \textit{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 \textit{mTOR}. In HEK293T cells stably expressing \textit{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 \textit{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 three scans).

The newly-identified nonsynonymous mutations mainly affect the HEAT domain (45.7%, 16/35) and FAT domain (34.3%, 12/35) of \textit{mTOR}. In HEK293T cells stably expressing \textit{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 \textit{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 three scans).

**Figure 2.**
Effects of \textit{mTOR} mutations on p70S6K and downstream signaling pathway. A and B, HEK293T cells were transiently transfected with indicated vectors (1, wild-type \textit{mTOR}; 2, P1128L; 3, V1275A; 4, C1303R; 5, T1830_T1834 [del]; 6, H1968Y; 7, G1914A; 8, P2213S; 9, S2215Y). Expression of Flag-tagged \textit{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 three scans). C–L, HEK293T cells stably expressing \textit{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 \textit{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 three 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 three independent experiments.
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, H1966Y and P22135, 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–Raptor complex whereas the latter directly phosphorylates AKT on Ser473 and activates AKT together with PI3K (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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Kong, X. Xu, J. Guo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Kong, L. Si, Y. Li, X. Wu, J. Dai, H. Tang, M. Ma, Z. Chi, X. Sheng, C. Cui, J. Guo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Kong, L. Si, Y. Li, J. Guo
Writing, review, and/or revision of the manuscript: Y. Kong, L. Si, J. Guo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Kong, J. Guo
Study supervision: Y. Kong, J. Guo

Acknowledgments
The authors thank the staff in the Department of Pathology of our hospital for help in collection and pathologic analysis of tissue samples.

Grant Support
This work was supported by grants from the Major State Basic Research Development Program of China (2013CB911004), National Natural Science Foundation of China (81172196, 81102068, 81272991, 81301984), Doctoral Fund of Ministry of Education of China (20110001120070, 20120001110048), New Century Excellent Talents in University (NCET-13-0007), Beijing Nova Program (Z121107002512042, xx2013027), Beijing Municipal Natural Science Foundation (7152033), Beijing Talents Fund (2014000021223ZK26), and Foundation of Peking University Cancer Hospital.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 10, 2015, revised September 23, 2015, accepted September 26, 2015; published OnlineFirst October 21, 2015.


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

Yan Kong, Lu Si, Yiqian Li, et al.

*Clin Cancer Res* Published OnlineFirst October 21, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-15-1110

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2015/10/21/1078-0432.CCR-15-1110.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.