Frequent Genetic Aberrations in the CDK4 Pathway in Acral Melanoma indicate the potential for CDK4/6 Inhibitors in Targeted Therapy

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STATEMENT OF TRANSLATIONAL RELEVANCE

The distribution of melanoma subtypes is biased among populations. In Asian populations, acral melanoma (AM) comprises 47.5-65% of all melanomas. However, systemic therapy for metastatic AM has not been successfully established. It has been a dilemma to treat metastatic AM patients in clinic. Our study investigated genetic aberrations of CDK4 pathway in AM and evaluated the significance of using CDK4/6 inhibitors as targeted therapy of AM. The overall frequency of AMs that contain at least one aberration in *Cdk4*, *Ccnd1* and $P16^{INK4a}$ was 82.7%. The pan-CDK inhibitor AT7519 and selective CDK4/6 inhibitor PD0332991 could inhibit the cell viability of primary AM cells and the tumor growth of patient-derived xenografts (PDX) with *Cdk4* gain plus *Ccnd1* gain, *Cdk4* gain plus $P16^{INK4a}$ loss and *Ccnd1* gain plus $P16^{INK4a}$ loss in mice. Our study thus provides key evidence for the significance of CDK4/6 inhibitors in targeted therapy of AM.

ABSTRACT

Purpose: Effective therapies for the majority of metastatic acral melanoma (AM) patients has not been established. Thus, we investigated genetic aberrations of CDK4 pathway in AM and evaluate the efficacy of CDK4/6 inhibitors in targeted therapy of AM.

Experimental Design: A total of 514 primary AM samples were examined for the copy number variations (CNVs) of CDK4 pathway-related genes, including *Cdk4*, *Ccnd1* and $P16^{INK4a}$, by QuantiGenePlex DNA Assay. The sensitivity of established AM cell lines and patient-derived xenograft (PDX) containing typical CDK4 aberrations to CDK4/6 inhibitors was evaluated.

Results: Among the 514 samples, 203 cases, 137 cases and 310 cases respectively showed *Cdk4* gain (39.5%), C*cnd1* gain (26.7%) and $p16^{INK4a}$ loss (60.3%). The overall frequency of AMs that contain at least one aberration in *Cdk4*, *Ccnd1* and $P16^{INK4a}$ was 82.7%. The median overall survival time for AM patients with concurrent *Cdk4* gain with $P16^{INK4a}$ loss was significantly shorter than that for patients without such aberrations (P = .005). The pan-CDK inhibitor AT7519 and selective CDK4/6 inhibitor PD0332991 could inhibit the cell viability of AM cells and the tumor growth of PDX with *Cdk4* gain plus *Ccnd1* gain, *Cdk4* gain plus $P16^{INK4a}$ loss and *Ccnd1* gain plus $P16^{INK4a}$ loss.

Conclusions: Genetic aberration of CDK4 pathway is a frequent event in AM. AM cell lines and PDX containing CDK4 pathway aberrations are sensitive to CDK4/6 inhibitors. Our study provides evidence for the testing of CDK4/6 inhibitors in AM patients.

INTRODUCTION

Malignant melanoma is a cancer arising from melanocytes, and the incidence is rising globally (1, 2). According to clinical factors and molecular profiles, melanoma is subdivided into four subtypes: cutaneous melanoma with chronic sun-induced damage, cutaneous melanoma without chronic sun-induced damage, acral melanoma (AM) and mucosal melanoma (3, 4). In Caucasians, the major subtype of melanoma is non-acral cutaneous melanoma, and the prevalence of acral and mucosal melanoma is only about 5% and 1% respectively (5, 6). In asian populations the major subtypes of melanoma are acral and mucosal melanoma, which comprise more than 70% of all melanomas (7). Up to date, successful therapeutics for advanced or metastatic acral melanoma has not been established. Targeted therapies using inhibitors specific for BRAF and c-Kit and checkpoint immunotherapies have greatly improved the outcomes of metastatic melanomas (8-14). However, the overall frequency of *Braf* and *Kit* mutations is about 10-60% and 0-28% respectively in Caucasians (4, 15), leaving more than 30% of patients lacking of proper targeted therapy. More importantly, due to the subtype bias, there are more than 50% of Asian patients incapable of benefiting from BRAF and c-Kit targeted therapy, given that the overall mutation frequency of *Braf* and *Kit* in this population is approximately 25.5% and 10.8% respectively (16, 17). Therefore, new targets particularly for acral and mucosal melanomas are needed for Asian patients.

Cyclin-dependent kinases (CDK) are serine threonine kinases that drive cell-cycle progression and regulate cell proliferation (18). Dysregulation of CDKs plays a central role in tumorigenesis and tumor progression. The $P16^{INK4a}$ (encoded by *Cdkn2a*)–cyclin D (popularly CCND1, encoded by *Ccnd1*)–CDK4/6–retinoblastoma protein (Rb1) pathway, well known as CDK4 pathway, promotes G1 to S cell-cycle

transition, and is commonly dysregulated in most cancers (19). Gain or overexpression of CCND1, gain or active mutation of CDK4, and loss of $P16^{INK4a}$ are all common events in cutaneous melanoma development and progression (3, 20-22). The CDK4 pathway is associated with activating genomic alterations in 22-78% of cases in cutaneous melanoma (23) and CDK4 inhibitor PD0332991 (also known as Palbociclib) has demonstrated anti-tumor activity in NRAS mutant melanomas in a preclinical mouse model (24), indicating the CDK4 pathway as a potential therapeutic target. Recently, a number of highly selective CDK4/6 inhibitors, such as PD0332991, LEE011 (also known as Ribociclib) and LY2835219 (also known as Abemaciclib), have been developed and entered clinical trials (25-29). Although the final outcomes of these clinical trials have not been completely evaluated at present, targeted therapies using CDK4/6 inhibitors are still expected for melanoma patients.

AM is more aggressive than cutaneous melanoma, and patients with AM often show worse prognosis than those with melanomas at other sites (7). The frequency of *Braf* or *Kit* mutation in AM is only about 15.5% and 11.9% respectively (16, 17), leaving a majority of AM patients with no suitable targeted therapy. To deal with this dilemma, we set out to investigate the aberrations of CDK4 pathway in AM and tested the sensitivity of primary AM cell lines and patient-derived xenograft (PDX) models containing typical CDK4 pathway aberrations to CDK4/6 inhibitors. Our study indicates that CDK4 pathway aberration is frequent (more than 80%) in AM; and AM cells containing aberrations in CDK4 pathway are responsive to CDK4/6 inhibitors. Our study thus provides key evidence for the therapeutic potential of CDK4/6 inhibitors in targeted therapy of AM and facilitates the establishment of strategy for targeted therapy of AM in the future.

PATIENTS AND METHODS

Patients and tissue samples

This study involved samples from primary lesions of 514 AM patients, hospitalized from January 2007 until October 2015 at the Peking Cancer Hospital & Institute. Informed consent for use of material in medical research (including archiving materials, and establishment of cell lines and tumor models) was obtained from all participants that were planned to be enrolled in clinical trials. These samples were analyzed by hematoxylin and eosin (H&E) staining and by immunohistochemistry to confirm the diagnosis of AM. Tissue slices containing more than 70% of tumor cells were used for further study. Clinical data, including age, sex, TNM (tumor-node-metastases) stage, thickness (Breslow), ulceration and survival (follow-up persisted until December 2015, 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.

QuantiGenePlex DNA assay

Tissue homogenates were prepared according to the procedure recommended in the user manual of QuantiGene Sample Processing Kit for Formalin-Fixed, Paraffin-Embedded Tissues (FFPE; Panomics of Affymetrics, Santa Clara, CA). The branched DNA (bDNA) assay was performed according to the procedure described in the user manual of QuantiGenePlex DNA Assay (Panomics). The mean fluorescence intensities of the duplicates were calculated for all genes. The background values were subtracted from each probe set signal. Values of tested genes were normalized to the geometric means of *Rpph1*, *Rpp30* and *Rplp0*. For each test sample, normalized signal

was divided by the reference DNA sample (G1521, Promega, Madison, WI) for each test gene, and the values were multiplied by the known copy number (usually 2 copies) of each gene in the reference genome. Rounded values to nearest whole number was taken as the copy number for each gene in each sample.

DNA preparation and TaqMan copy number assays

Genomic DNA was extracted from FFPE sections using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). To validate the results of QuantiGenePlex DNA Assay, the copy numbers of *Cdk4*, *Ccnd1* and *P16^{INK4a}* were further quantified by TaqMan Copy Number Assays (Applied Biosystems of ThermoFisher, Waltham, MA). A TaqMan probe targeted on the *Rnasep* gene was used as a control. Quantitative real-time PCR was performed using the ABI 7500 FAST real-time PCR system (Applied Biosystems). Copy numbers were then determined by CopyCaller v2.0 software (Applied Biosystems) using the comparative Ct (ΔΔCt) method. When the relative copy number is greater than or equal to 3.0, the copy number of *Cdk4* or *Ccnd1* is determined to be gained. When the relative copy number is less than 2.0, the copy number of gene is determined to be lost.

Immunohistochemistry of protein expression

Immunohistochemistry analyses were performed using antibodies against CDK4 (dilution 1:100), p16^{INK4a} (dilution 1:100), CCND1 (dilution 1:1000), Ki67 (dilution 1:400) and phospho-Rb (Ser795) (Abcam, Cambridge, UK) as described (11, 17). 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, without the

177 knowledge of copy number variations of samples.

178

179 ***Cell lines and primary cell culture***

180 The SK-Mel-5 (Catalog no. HTB-70) and A2058 (Catalog no. CRL-11147) cell lines
181 were obtained from American Type Culture Collection and were cultured at 37°C in
182 Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen of ThermoFisher,
183 Waltham, MA) supplemented with penicillin and streptomycin (Invitrogen) and
184 containing 10% fetal bovine serum (HyClone of GE Healthcare, Logan, UT).

185 The AMC-1 to AMC-5 AM cell lines (**Supplementary Table S1**) were derived
186 from hospitalized patients. Approximately 1 cm^3 AM tissue from surgical specimen
187 was separated, and the tumor tissue was then cut into approximately 1 mm^3 fragments
188 and resuspended in 30 ml DMEM containing 50 x collagenase IV (Invitrogen) and 1 x
189 DNase (Takara, Kusatsu, Japan) at 37°C for 1 hour to prepare single cell suspensions.
190 The suspension was slowly layered onto 15 ml Histopaque (Sigma, St. Louis, MO),
191 and the interface cell fraction was collected after spinning. The cells were then
192 cultured in serum-free stem cell medium supplemented with growth factors. Half of
193 the medium was replenished on day 4 or once a week. Once the cells had reached
194 confluence, cells were dissociated into small clumps by collagenase IV and passaged
195 with one to three dilutions. The established cell lines were then analyzed for cell
196 viability.

197

198 ***Cell viability assays***

199 CDK4/6 inhibitors including LEE011 (#S7440), PD0332991 (#S1116), LY2835219
200 (#S7158) and pan-CDK inhibitor AT7519 (#S1524) were purchased from Selleck
201 Chemicals (Houston, TX). All inhibitors were dissolved at 10 mM in

dimethylsulfoxide (DMSO) as stock solutions. After treatment with various concentrations of inhibitors or DMSO for 24 hours, viability of the cells was evaluated using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the instructions. To assess the activity of CDK4 pathway inhibitors, we analyzed the corresponding cells by Western blotting using antibodies against Rb and phospho-Rb (Ser795) (Abcam).

Patient-derived xenograft model and treatment

Fragments of patient-derived AM tissues bearing typical CDK4 pathway aberrations were cut into fragments and then subcutaneously inoculated into a 5 week-old NOD/SCID (non-obese diabetic and severe combined immunodeficiency) female mouse (4-6 week-old, 18-22 gram-weight) to establish the PDX model. When the tumor size reached approximately 1 cm^3, the mice were sacrificed, and tumor tissues were separated and re-inoculated into new mice. 5 PDX models containing typical CDK4 pathway aberrations (**Supplementary Table S2**) were finally established.

When the tumor size reached approximately 200 mm^3, mice were randomized (treatment arm versus control arm) and treated with control buffer or CDK4 inhibitors (PD0332991 or AT7519). For PD0332991 treatment, mice received PD0332991 (50 mg/kg in pH 4.5 sodium lactate buffer) via oral gavage daily. For AT7519 treatment, mice received AT7519 (12 mg/kg in saline solution) via intraperitoneal injection daily. Tumor sizes were measured every 3 days and tumor volume calculated using the formula: volume=length*$width^2$/2. The treatment lasted for 14 days, after which the mice were sacrificed and the tumors were fixed in 10% formalin for histological and immunohistological analysis. The above experiments were repeated twice. All animal care and experimental procedures were carried out in accordance with the Animal

Care Ethics approved by the Medical Ethics Committee of the Beijing Cancer Hospital & Institute.

Statistical analysis

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 aberration 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 hazard proportion models were used to estimate the hazard ratios (HRs) and corresponding 95% interval confidences (CIs). All statistical analyses were two-sided, and $P < .05$ was considered as statistically significant.

RESULTS

Aberrations of Cdk4, Ccnd1 and P16^{INK4a} in AM

Among the 514 samples, 203 cases (39.5%), 137 cases (26.7%) and 310 cases (60.3%) respectively showed *Cdk4* gain, *Ccnd1* gain and *P16^{INK4a}* loss respectively (**Table 1**). Moreover, 35.2% of AMs contained more than two concurrent aberrations, and 8.6% of AMs contained three aberrations. The overall frequency of AM containing any copy number variation (CNV) (≥ 1 CNV) was 82.7%, with 89 cases harboring no CNV aberrations in these three genes. Additionally, 76 cases were found to harbor *Ccnd1* loss, and 4 cases were found to harbor *P16^{INK4a}* gain (**Supplementary Table S3**). We then stratified the CNVs of *Cdk4* and *Ccnd1* gain, and found that most of the copy number of samples with *Cdk4* or *Ccnd1* gain was about 3-4 copies (**Table 1**).

To confirm the above detected aberrations, we verified the CNVs by q-PCR and by using available DNA in 349 cases of AM samples. The frequency of *Cdk4* gain, *Ccnd1* gain and *P16*^{INK4a} was about 42.9%, 26.2% and 52.1% respectively (**Supplementary Table S4**), which was comparable to that detected by the QuantiGenePlex DNA Assay (**Table 1**). We also examined the protein levels of CDK4-related molecules by immunohistochemistry (typical staining of CDK4, CCND1, and P16^{INK4a} was shown in **Supplementary Fig. S1**). As summarized in **Supplementary Table S5**, the protein expression levels of CDK4, P16^{INK4a} and CCND1 were significantly changed between samples with normal gene copy numbers and samples with aberrated gene copy numbers. Furthermore, we examined the mutation status of *Cdk4*, *Ccnd1* and *P16*^{INK4a} by DNA sequencing of all exons after PCR amplification in randomly selected 200 cases of AM. No missense mutation of *Cdk4* or *Ccnd1* was detected, and the frequency of missense mutation of *P16*^{INK4a} was only about 6.9% [9 out of 130 assessable samples carrying non-germline mutations: 1 case of E33A (G98C) mutation, 1 case of G45D (G135A) mutation, 3 cases of N71K (C213G) mutation, 2 cases of D74A (A221C) mutation, 1 case of G101R (G301A) mutation, and 1 case of A109S (G325T) mutation]. These data indicate that the CNV aberrations, but not genetic mutations, of CDK4 pathway are prevalent in AM.

Since strategy for targeted therapy of melanoma has been explored, we also analyzed the mutation frequency of genes that have been confirmed as promising targets in AM samples whose genomic DNAs were available. In the samples containing at least one CDK4 pathway aberration, 9.8%, 14.6% and 15.4% of them also contained mutations in *Kit*, *Braf* or *Nras* respectively. These data indicate that CDK4/6 inhibitors may be combined with clinically validated inhibitors for these targets.

Correlation of CDK4 pathway aberrations to clinicopathological features

In our cohort, the mean age was not significantly different between patients with or without any CNVs for *Cdk4*, *Ccnd1*, $P16^{INK4a}$ or other indicated stochastic combinations (**Table 2 and Supplementary Table S6**). The gender distribution and ulceration rate for patients with any CNVs for *Cdk4* and $P16^{INK4a}$ or other indicated stochastic combinations were not significantly different (**Table 2 and Supplementary Table S6**). However, more males tended to harbor *Ccnd1* gain than females did; and patients with ulceration were more likely to contain *Ccnd1* aberrations (**Table 2**). The median thickness of samples with *Cdk4* gain was 5 mm (range: 0.2-30.0 mm), whereas that without *Cdk4* gain was 3 mm (range: 0.1-40.0 mm) ($P < 0.0001$; **Table 2**). Moreover, the median thickness of AM with any CDK4 pathway aberrations (≥ 1 CNV) was more than that of AM without any CDK4 pathway aberrations ($P < 0.0001$; **Table 2**). Among the patients with $P16^{INK4a}$ loss, the percentages of patients with stage I, II, III, and IV of AM were significantly different from those without $P16^{INK4a}$ loss ($P = 0.007$; **Table 2**). The percentages of patients with stage I-IV of AM were significantly different between patients with CDK4 pathway aberrations and those without any CDK4 pathway aberrations ($P = 0.018$; **Table 2**).

The OS of patients with $P16^{INK4a}$ loss ($P = 0.016$) or *Cdk4* gain ($P = 0.038$) was significantly shorter than those without $P16^{INK4a}$ loss or without *Cdk4* gain, respectively (**Table 2; Fig. 1A and 1B**). However, the OS for AM patients with or without *Ccnd1* aberrations were comparable (**Table 2; Fig. 1C**). The OS for patients with *Cdk4* gain plus $P16^{INK4a}$ loss ($P =0.005$) or with *Cdk4* gain plus *Ccnd1* loss ($P = 0.007$) was significantly shorter than patients without such aberrations (**Fig. 1D; Supplementary Table S6**). No other combinations showed an association with

patient survival (**Fig. 1E-1H**; **Supplementary Table S6)**. In univariate Cox analysis, the clinicopathologic factors, such as age, ulceration status, TNM stage, *Cdk4* gain, $P16^{INK4a}$ loss and *Cdk4* gain plus $P16^{INK4a}$ loss, may be of prognostic significance for melanoma patients; For multivariate Cox regression assay, the age, TNM stage and ulceration status are independent prognostic factors for OS (**Supplementary Table S7**).

Since mitotic rate and tumor-infiltrating lymphocytes are two important clinically relevant pathological features, we examined these two features in 107 cases of AM samples as described (30-33). When correlating mitotic rate or TILs to the CNV status of *Cdk4*, *Ccnd1* and $P16^{INK4a}$, we found that the CNVs of these three genes were not significantly different between samples with various mitotic rate or TILs (**Supplementary Table S8**).

Sensitivity of primary AM cells to CDK4/6 inhibitors

The primary AM cells lines (AMC-1 to AMC-5 with wild-type *c-Kit*; CDK4 pathway aberrations for these cells are listed in **Supplementary Table S1**) were evaluated for the efficacy of CDK4/6 inhibitors at previously described concentrations by determining cell viability *in vitro* (34-37). SK-Mel-5 (*Ccnd1* gain plus $P16^{INK4a}$ loss) and A2058 (CDK4 pathway normal) was respectively used as the positive and negative control (20). The pan-CDK inhibitor AT7519 significantly inhibited the cell viability of SK-Mel-5, AMC-1 (*Cdk4* gain) and AMC-3 (*Cdk4* gain plus $P16^{INK4a}$ loss) (**Fig. 2A**). LY2835219 could not significantly inhibit the viability of all 7 cell lines after 24h treatments (**Fig. 2B**). For PD0332991, SK-Mel-5 and AMC-3 cells were strikingly sensitive at a concentration higher than 1 μM, whereas other cell lines were resistant after 24h treatments (**Fig. 2C**). For LEE011, AMC-1 was sensitive at a

concentration of higher than 0.5 μM, whereas other 6 cell lines (including the positive control SK-Mel-5 cells) were resistant after 24h treatments (**Fig. 2D**). AT7519 and LEE011 showed comparable inhibitory efficiency on AMC-1; AT7519 and PD0332991 showed comparable inhibitory efficiency on AMC-3 and SK-Mel-5; Moreover, at lower concentration (less than 2 μM), AT7519 tended to show stronger inhibitory effect on AMC-1, AMC-3 and SK-Mel-5 **(Fig. 2)**. Similar effects were observed for these inhibitors when used at a single dose at both 24h and 48h after treatments (**Supplementary Fig. S2**). Moreover, when compared to the chemotherapy drug dacarbacine (DTIC), the pan-CDK inhibitor AT7519 tended to be more efficient than DTIC in SK-Mel-5, AMC-1 and AMC-3 cells (**Supplementary Fig. S2**). These data indicate that AM cells may be responsive to pan-CDK inhibitors despite that highly selective CDK4/6 inhibitors also elicit inhibitory effects to lesser extent.

When comparing the genotype of cell lines with the inhibitory effects of CDK4/6 inhibitors, we noted that the cell lines (SK-Mel-5, AMC-3, and to lesser extent AMC-1 and AMC-2), containing either *Cdk4* gain or *Ccnd1* gain, could be responsive to CDK4/6 inhibitors (AT7519, PD0332991 or LEE011) (**Fig. 2**). Meanwhile, the cell lines (A2058, AMC-4 and AMC-5), containing no CDK4 pathway aberrations as either *Cdk4* gain or *Ccnd1* gain, could not be inhibited by CDK4/6 inhibitors regarding cell viability (**Fig. 2**).

It was surprising to observe that all the CDK4/6 inhibitors did not work equally well in inhibiting cell viability (**Fig. 2**). So we examined the inactivation of Rb (phosphorylation of Rb) protein in the cell lines by Western blotting. As shown in **Supplementary Fig. S3**, we found that all four inhibitors were effective in inhibiting Rb phosphorylation in SK-Mel-5 while only AMC-1 and AMC-3 were responsive to AT7519 or PD0332991 regarding Rb inactivation, which could partially contribute to

the observed inhibitory effects for CDK4/6 inhibitors (in **Fig. 2**). These data indicate that Rb phosphorylation may be used as indicator for the efficiency of CDK4/6 inhibitors. However, why the four CDK4/6 inhibitors could not all cause dephosphorylation and activation of Rb protein may require further studies.

Sensitivity of PDX models to CDK4/6 inhibitors

To analyze the sensitivity of AM containing typical CDK4 pathway aberrations to CDK4/6 inhibitors, we tried to establish PDX models for all types of CDK4 pathway aberrations detected in our study. The success rate of PDX model was only about 25%, which was comparable to previous studies on cutaneous melanoma, uveal melanoma and head and neck cancer (38-40). Only 5 different PDX models were established (CDK4 pathway aberrations for these models are listed in **Supplementary Table S2**).

Since AT7519 and PD0332991 showed more robust inhibition of cell viability in vitro (**Fig. 2**), we treated the PDX models with AT7519 and PD0332991. As compared to the buffer-treated group, AT7519 and PD0332991 showed no inhibitory effect on tumor growth in PDX-017 model without CDK4 pathway aberrations (**Fig. 3A and 3B**). AT7519 and PD0332991 could significantly inhibit the growth of PDX-012 model with *Cdk4* gain plus $P16^{INK4a}$ loss (**Fig. 3C and 3D**), PDX-015 model with *Ccnd1* gain plus $P16^{INK4a}$ loss (**Fig. 3E and 3F**), and almost eliminate the tumor of PDX-001 model with *Cdk4* gain plus *Ccnd1* gain (**Fig. 3G and 3H**). Moreover, AT7519 but not PD0332991 could elicit inhibitory effects on tumor growth of PDX-006 models with *Cdk4* gain (**Fig. 3I and 3J**). The appearance of tumor nodules after the treatments was shown in **Supplementary Fig. S4**, showing the efficacy of CDK4/6 inhibitors in inhibiting AM tumor growth *in vivo*.

As further evidence, we examined the proliferation of AM cells in PDX models

after treatments by immunohistochemical staining of Ki-67 (**Fig. 4**). In consistent with the results of tumor volume changes (**Fig. 3; Supplementary Fig. S4**), we found that the number of Ki-67^{+} cells was significantly decreased after AT7519 and PD0332991 treatments in PDX models with *Cdk4* gain plus *P16^{INK4a}* loss (**Fig. 4C**), *Ccnd1* gain plus *P16^{INK4a}* loss (**Fig. 4D**), *Cdk4* gain plus *Ccnd1* gain (**Fig. 4E**) or *Cdk4* gain (**Fig. 4F**), but not in PDX model without CDK4 pathway aberrations (**Fig. 4B**). These data together indicate that the CDK4/6 inhibitors may be effective in inhibiting AM growth *in vivo*.

To correlate the inhibitory effects of AT7519 and PD0332991 on tumor growth to the status of Rb inactivation, we examined the phosphorylation of Rb (Ser795) in PDX sections after the treatments. Both inhibitors could significantly decrease the levels of phosphorylated Rb in tumor nodules derived from PDX models containing either *Cdk4* gain or *Ccnd1* gain (PDX-012, PDX-015, PDX-001 and PDX-006), but not those derived from PDX models containing no aberrations in CDK4 pathway (PDX-017; **Supplementary Fig. S5**). Together with the in vitro assays (**Supplementary Fig. S3**), it may be inferred that the status of Rb inactivation may be indicator for the inhibitory efficacy of CDK4/6 inhibitors.

DISCUSSION

AM accounts for almost 50% of all melanomas and is the most common subtype in Asian populations. However, it has been an intractable challenge to treat the AM patients at advanced stage. In the treatment guideline of National Comprehensive Cancer Network (NCCN) for melanoma of the United States (2016 Edition), vemurafenib plus cobimetinib as well as dabrafenib plus trametinib have been

recommended as first-line treatment for patients harboring $BRAF^{V600E}$ mutations. Imatinib have also been recommended as first-line treatments for patients with harboring *Kit* mutations. Yet the key point of targeted therapy of AM is that the incidence of genetic mutation for *Braf* and *Kit* in AM is low (4, 16, 17, 41). In the past 5 years, immune checkpoint therapy by blocking CTLA-4 and PD-1/PD-L1 has made great progresses in both acral melanoma and other melanoma subtypes (13, 14, 42, 43). Our study has greatly promoted the clinical understanding of AM by providing evidence that CDK4 pathway aberrations are rather frequent in AM and CDK4/6 inhibitors are effective in inhibiting growth of AM. Our study implicates that targeted therapy using CDK4/6 inhibitors may be an alternative choice for most of AM patients in addition to immune checkpoint therapy.

Currently, Palbociclib (PD0332991, Ibrance) from Pfizer, Ribociclib (LEE011) from Novartis and Abemaciclib (LY2835219) from Lily represent mainstream CDK4/6 selective inhibitors. On August 3, 2016, CDK4/6 inhibitor Ribociclib was appraised as therapeutic breakthrough by American FDA and was used in combination with letrozole as the first-line therapy of advanced and metastatic breast cancer that is positive for estrogen receptor (ER) and negative for HER2. A phase Ⅲ clinical trial suggested that most metastatic breast cancer patients could benefit from the combination therapy of Palbociclib and Fulvestrant (44). Lately, the results of a phase I clinical trial of CDK4/6 inhibitor abemaciclib was commented (45). However, previous studies have not clearly established the relationship between aberrations of CDK4 pathway in AM and the sensitivity of AM to CDK4/6 inhibitors. Young et al. found that 37 of 47 melanoma cell lines were sensitive to PD0332991, and put forward that $P16^{INK4a}$ loss indicated the sensitivity to PD0332991 while loss of Rb1 indicated PD0332991 resistance (20). Our study showed that AM cells with *$P16^{INK4a}$*

loss (AMC-4) were not sensitive to all the 4 screening inhibitors, indicating that AM cells may respond differentially to PD0332991 as compared to non-acral cutaneous melanomas. It was noted that the pan-CDK inhibitor AT7519 was more effective than the other selective inhibitors at lower concentrations, indicating that it may be necessary to synchronously inhibit other CDKs in addition to CDK4/6 to achieve maximum efficacy. The experiments in PDX models suggest that AT7519 is effective in inhibiting the *in vivo* growth of AM bearing *Cdk4* gain plus *Ccnd1* gain, *Cdk4* gain plus $P16^{INK4a}$ loss or only *Cdk4* gain. In contrast, the selective CDK4/6 inhibitor PD0332991 was effective in AM bearing *Cdk4* gain plus *Ccnd1* gain, and *Cdk4* gain plus $P16^{INK4a}$ loss. Therefore, AM patients harboring concurrent CDK4 pathway aberrations (concurrent two aberrations of *Cdk4* gain, $P16^{INK4a}$ loss or *Ccnd1* gain) may be potential populations suitable for CDK4/6 inhibitor treatment. However, due to the fact that individual genotypes were tested only in a single model, the efficacy of CDK4 inhibitors may need to be further evaluated by other independent systems.

There are limitations, unresolved concerns and potential perspectives in our study. As an initial screening assay of CDK4 pathway aberrations, only the CNVs of *Cdk4*, *Ccnd1* and $P16^{INK4a}$ in genome DNA have been determined in our study. The CNVs for other CDK-related genes (e.g. *Cdk2*, *Cdk6*, *Ccne*, *Rb* and *E2F* members etc.) and aberration of genes that are potentially amendable for CDK4 pathway blockade (e.g. *Tp53*, *Pten*, *Arid2* and *Rac1* etc.) have not been examined. At DNA level, the epigenetic aberrations of *Cdk4*, *Ccnd1* and $P16^{INK4a}$ have not been examined in our study. Moreover, the mRNA alterations of these genes are also unavailable at present due to technical limitations in fixed samples. Large scale sequencing of both DNA and RNA of acral melanoma samples in future may help to provide unabridged molecular profile for acral melanoma and may contribute to resolve the question why

AM cells do not respond equally and effectively to CDK4/6 inhibitors. Recently, a large, high-coverage whole-genome sequencing study of 183 cases of melanomas with differential subtypes (including 35 acral melanomas) is published, proving an excellent profile for genetic aberrations in AM (46). The data showed that acral and mucosal melanomas were dominated by structural changes and mutation signatures of unknown aetiology, and they also found that greater proportions of the acral and mucosal melanoma genomes showed copy number variation than in cutaneous melanomas. Despite the limitations in genetic profiles, our study may still help to the understanding of aberrations in CDK4 pathway in AM and may push forward the establishment of targeted therapy for AM. In our study, we note that the CDK4 pathway aberrations can be combined with aberrations in validated targets (such as *Kit*, *Braf* and *Nras*), indicating that CDK4/6 inhibitors may be used in combination with the validated drugs for acral melanoma treatments. Considering that immune checkpoint therapy has demonstrated efficacy in acral melanomas (42, 43) and that LEE001 has been combined with MEK inhibitor MEK162 for advanced or metastatic melanoma containing *Nras* mutation in undergoing multi-center, open-label phase Ib/II clinical trial (NCT01781527) (26), the therapeutics by combining CDK4/6 inhibitors with immune checkpoint therapy or MEK inhibitors may be expected for acral melanoma patients in the future.

Identification of new targets suitable for targeted therapy may be a promising strategy for rare and intractable cancers as AM. Our study suggests that CDK4 pathway aberrations in AM is rather frequent (82.7%) and thus a majority of AM patients may be suitable for targeted therapy of CDK4/6 inhibitors, which warrants clinical trials in the future.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The authors have no disclosures of potential conflicts of interests.

AUTHOR CONTRIBUTIONS

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

Provision of study materials or patients: Drs. Jun Guo, Yan Kong and Xinan Sheng.

Collection and assembly of data: Drs. Jun Guo, Yan Kong, Xinan Sheng, Xiaowen Wu, Junya Yan, Meng Ma, Jiayi Yu, Lu Si, Zhihong Chi, Chuanliang Cui, Jie Dai, Yiqian Li, Huan Yu, Tianxiao Xu, Huan Tang , Bixia Tang, Lili Mao, Bin Lian, Xuan Wang, Xieqiao Yan, Siming Li.

Data analysis and interpretation: Drs. Jun Guo, Yan Kong, Xinan Sheng, Xiaowen Wu.

Manuscript writing: Drs. Jun Guo, Yan Kong and Xinan Sheng, Xiaowen Wu.

Final approval of manuscript: All authors.

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Table 1. Copy number variations of genes related to CDK4 pathway and mutation status of therapeutic targets in acral melanoma

CDK4 aberrations	CNV status	Genetic mutation of therapeutic targets		
	(n = 514)	% (No. positive cases/No. examined cases)		
	n (%)	*Kit*	*Braf*	*Nras*
≥ 1 CNV				
Cdk4 gain	203 (39.5)	9.4 (19/202)	16.3 (33/202)	12.2 (23/188)
3-4 copies	144 (28.0)	6.3 (9/143)	16.8 (24/143)	12.8 (17/133)
5-8 copies	24 (4.7)	20.8 (5/24)	12.5 (3/24)	9.1 (2/22)
> 8 copies	35 (6.8)	14.3 (5/35)	17.1 (6/35)	12.1 (4/33)
Ccnd1 gain	137 (26.7)	9.8 (13/133)	15.2 (20/132)	14.4 (17/118)
3-4 copies	73 (14.2)	11.4 (8/70)	20 (14/70)	19.4 (12/62)
5-8 copies	39 (7.6)	5.3 (2/38)	8.1 (3/37)	11.4 (4/35)
> 8 copies	25 (4.9)	12.0 (3/25)	12.0 (3/25)	4.0 (1/25)
$P16^{INK4a}$ loss	310 (60.3)	9.4 (29/308)	15.6 (48/308)	16.7 (47/282)
Overall	425 (82.7)	9.8 (41/419)	14.6 (61/418)	15.4 (59/384)
≥ 2 CNVs				
Cdk4 gain plus*Ccnd1* gain	73 (14.2)	9.7 (7/72)	19.4 (14/72)	9.4 (6/64)
Cdk4 gain plus $P16^{INK4a}$loss	119 (23.2)	7.6 (9/119)	18.5 (22/119)	20.2 (24/119)
Ccnd1 gain plus $P16^{INK4a}$loss	77 (15.0)	10.4 (8/77)	16.9 (13/77)	17.9 (12/67)
Overall	181 (35.2)	8.9 (16/180)	17.2 (31/180)	13.3 (22/166)
3 CNVs				
Overall	44 (8.6)	9.1 (4/44)	20.5 (9/44)	15.8(6/38)

Abbreviations: CNV, copy number variation.

629 **Table 2**. Correlation of CDK4 pathway aberrations to clinicopathologic features of acral melanoma

Clinicopathologic factor	*Cdk4* aberration			*Ccnd1* aberration			
	Gain	Normal	*P* value[a]	Gain	Loss	Normal	*P* value[a]
Age (year)			0.257				0.676
Median (range)	55.6 ± 13.8	54.2 ± 13.6		55.5 ± 12.2	54.0 ± 13.1	54.6 ± 14.4	
Gender n (%)			0.864				0.012
Male	115 (56.7)	171 (55.9)		91 (66.4)	43 (56.6)	154 (51.2)	
Female	88 (43.3)	135 (44.1)		46 (33.6)	33 (43.4)	147 (48.8)	
Total	203 (39.9)	306 (60.1%)		137 (26.7)	76 (14.8)	301 (58.6)	
Ulceration n (%)			0.886				0.040
Yes	146 (73.0)	220 (73.6)		96 (71.1)	64 (85.3)	210 (71.4)	
No	54 (27.0)	80 (26.4)		39 (28.9)	11 (14.7)	84 (28.6)	
Thickness (mm)			< 0.0001				0.054
Median (range)	5.0 (0.2, 30.0)	3.0 (0.1, 40.0)		4.0 (0.1, 25.0)	4.0 (0.2, 15.0)	3.5 (0.1, 40.0)	
Stages n (%)			0.396				0.650
Ⅰ	19 (9.4)	35 (11.4)		14 (10.2)	3 (10.5)	37 (12.3)	
Ⅱ	107 (52.7)	138 (45.1)		67 (48.9)	42 (55.3)	140 (46.5)	
Ⅲ	51 (25.1)	91 (29.7)		42 (30.7)	18 (23.7)	83 (27.6)	
Ⅳ	26 (12.8)	42 (13.7)		14 (10.2)	13 (17.1)	41 (13.6)	
Survival (months)			0.038				0.213

Median (95% CI)	42.2 (35.8, 48.6)	54.1 (40.8, 67.4)		47 (35.4, 58.6)	41.9 (22.7, 61.1)	44.8 (35.6, 54.0)
Total n (%)	203 (39.9)	306 (60.1%)		137 (26.7)	76 (14.8)	301 (58.6)

Table 2 (continued)

Clinicopathologic factor	*P16*^{INK4a} aberration			Overall aberration (≥ 1 CNV)		
	Loss	Normal	*P* value[a]	Yes	No	*P* value[a]
Age (year)			0.625			0.361
Median (range)	55.0 ± 13.8	54.4 ± 13.4		55.0 ± 13.7	53.5 ± 13.3	
Gender n (%)			0.654			0.561
Male	172 (55.5)	115 (57.5)		245 (56.6)	43 (53.1)	
Female	138 (44.5)	85 (42.5)		188 (43.4)	38 (46.9)	
Ulceration n (%)			0.964			0.204
Yes	225 (73.5)	143 (73.7)		318 (74.5)	52 (67.5)	
No	81 (26.5)	51 (26.3)		109 (25.5)	25 (32.5)	
Thickness (mm)			0.061			< 0.001
Median (range)	4.0 (0.5, 30.0)	3.0 (0.1, 40.0)		4.0 (0.1, 30.0)	2.4 (0.1, 40.0)	
Stages n (%)			0.007			
Ⅰ	22 (7.1)	32 (16.0)		37 (8.5)	17 (21.0)	0.018
Ⅱ	149 (48.1)	97 (48.5)		212 (49.0)	37 (45.7)	
Ⅲ	96 (31.0)	46 (23.0)		125 (28.9)	18 (22.2)	
Ⅳ	43 (13.9)	25 (12.5)		59 (13.6)	9 (11.1)	

Survival (months)			0.016		0.124
Median (95% CI)	43.2 (39.7, 46.7)	63.7 (44.7, 82.7)		44.8 (37.5, 52.1)	46.7 (39.6, 53.8)
Total n (%)	310 (60.8)	200 (39.2)		433 (84.2)	81 (15.8)

[a] For evaluation of age, the two independent sample t-test or one-way ANOVA was used. For evaluation of gender, ulceration and stages, the Chi-square tests or Fisher's exact tests were used. For evaluation of thickness, Mann-Whitney U tests were used. For evaluation of OS time, Log-Rank tests were used.

FIGURE LEGENDS

Figure 1. Overall survival of acral melanoma patients in relation to CDK4 pathway aberrations. CNV, copy number variations.

Figure 2. Sensitivity of acral melanoma cells to CDK4/6 inhibitors. After nutrient starvation, primary acral melanoma cells (AMC-1 to AMC-5) and control melanoma cells (A2058 as negative control and SK-Mel-5 as positive control) were treated with indicated concentrations of inhibitors for 24 hours. The cell viability was evaluated by CCK-8 method, and the results were presented as mean ± SD of 3 independent experiments. The statistical significance of the growth curves (as compared to A2058 group) was evaluated by repeated measure variance analysis.

Figure 3. Sensitivity of PDX models containing CDK4 aberrations to CDK4/6 inhibitors in vivo. When the tumor size reached approximately 200 mm^3, mice (n = 4 per group) were treated with buffer control or inhibitors daily. Tumor volume was evaluated as % of the tumor volume on day 0 and presented as mean ± SD. The comparison of the growth curves was done with the repeated measure variance analysis. The data are representative of these independent experiments.

Figure 4. Proliferation index of acral melanoma cells from PDX models containing CDK4 aberrations after CDK4/6 inhibitors treatments. On day 14 of treatments, the tumor nodules were excised and examined by H&E staining and immuohistochemical staining (for Ki-67). The sections were evaluated under microscope, and typical staining was photographed (A), and the Ki-67^+ cells under 5 random fields were

counted. Bar = 50 μm. The results of Ki-67$^+$ cells (B-F) were presented as mean ± SE of three sections. ns, $P > .05$; *, $P < .05$; **, $P < .01$; ***, $P < .001$ (One-way ANOVA followed by Bonferroni multiple comparison).

Figure 1

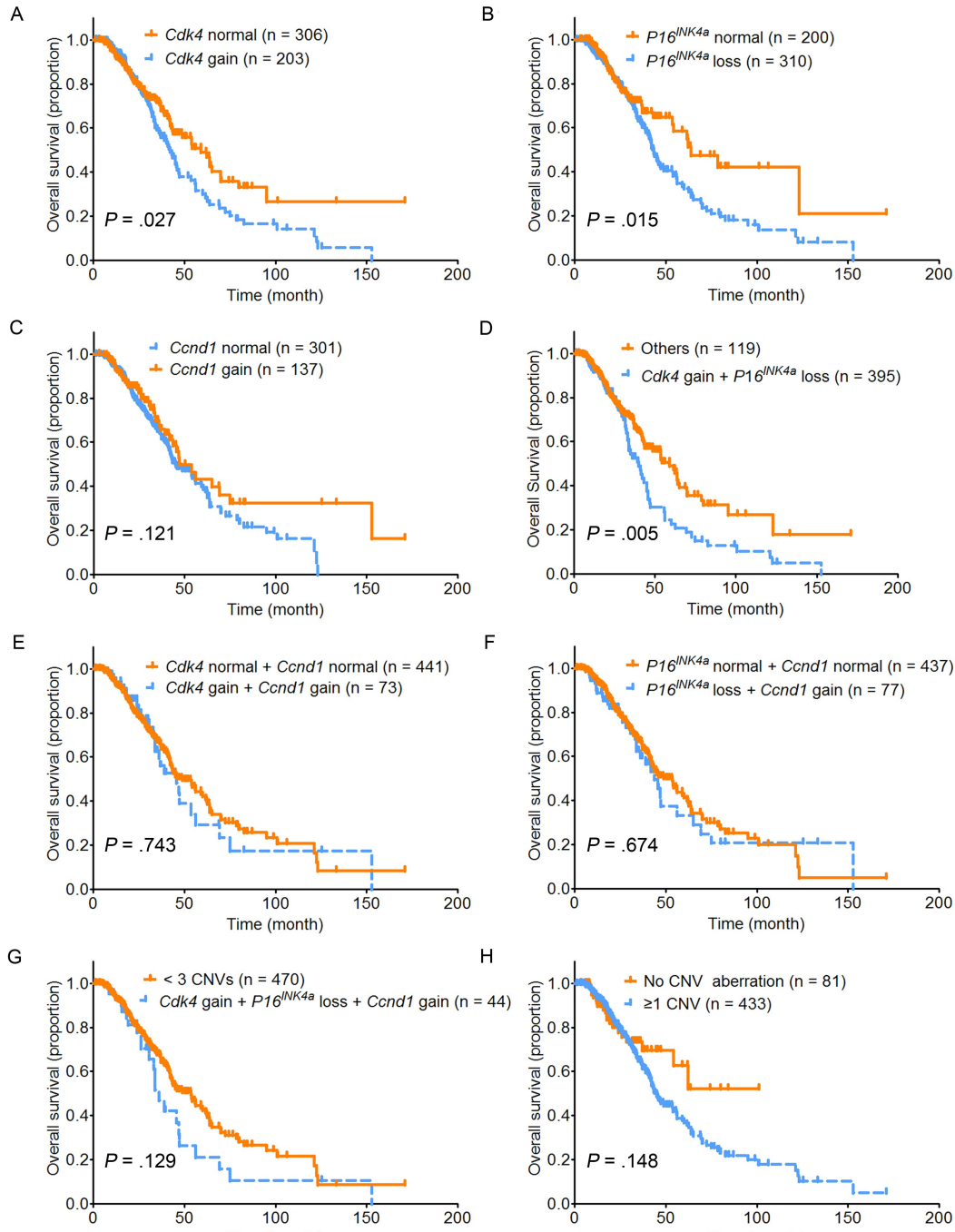
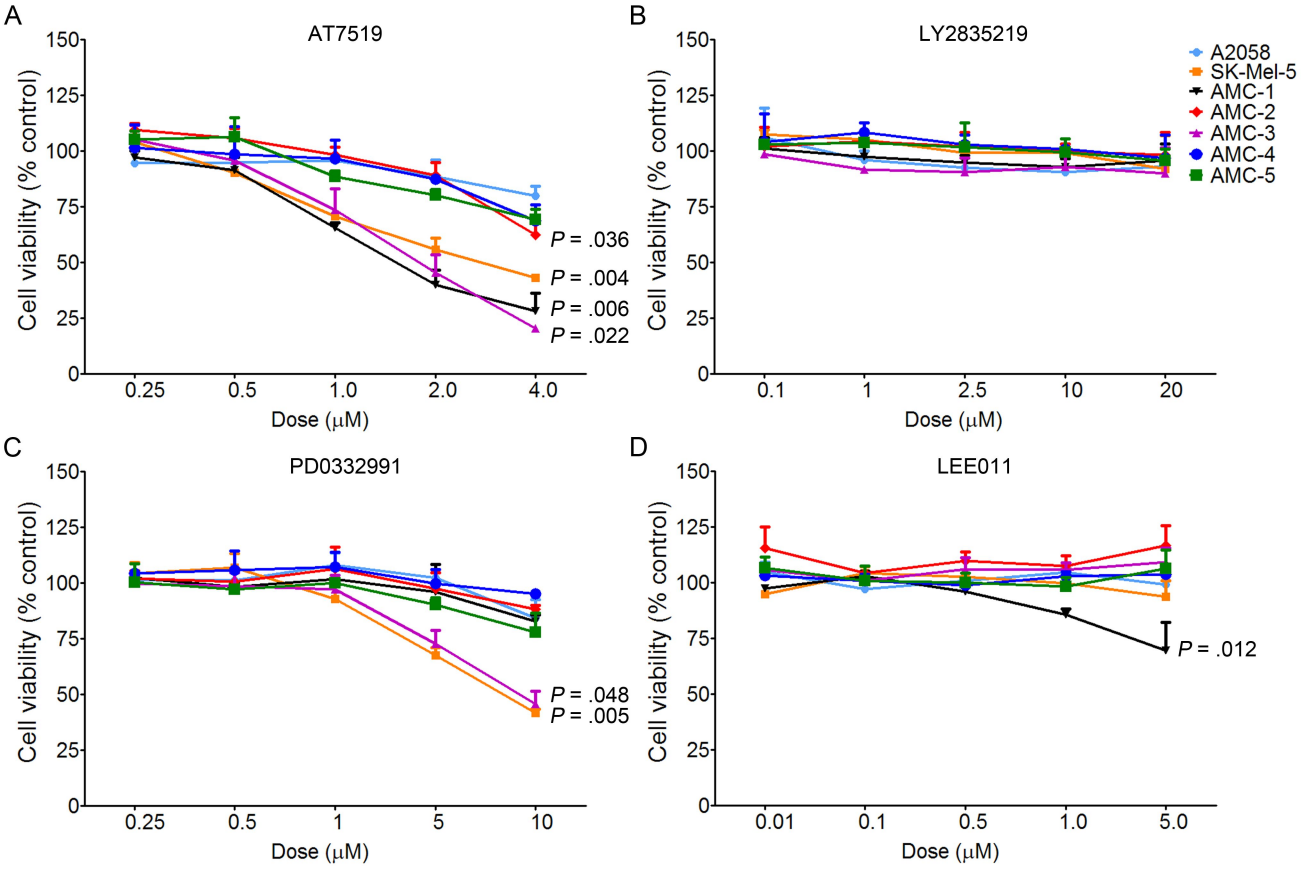


Figure 2



Cell line genotype:

A2058: CDK4 pathway normal; SK-Mel-5: *Ccnd1* gain plus $P16^{INK4a}$ loss; AMC-1: *Cdk4* gain; AMC-2: Ccnd1 gain; AMC-3: *Cdk4* gain plus $P16^{INK4a}$ loss; AMC-4: $P16^{INK4a}$ loss; AMC-5: CDK4 pathway normal.

Figure 3

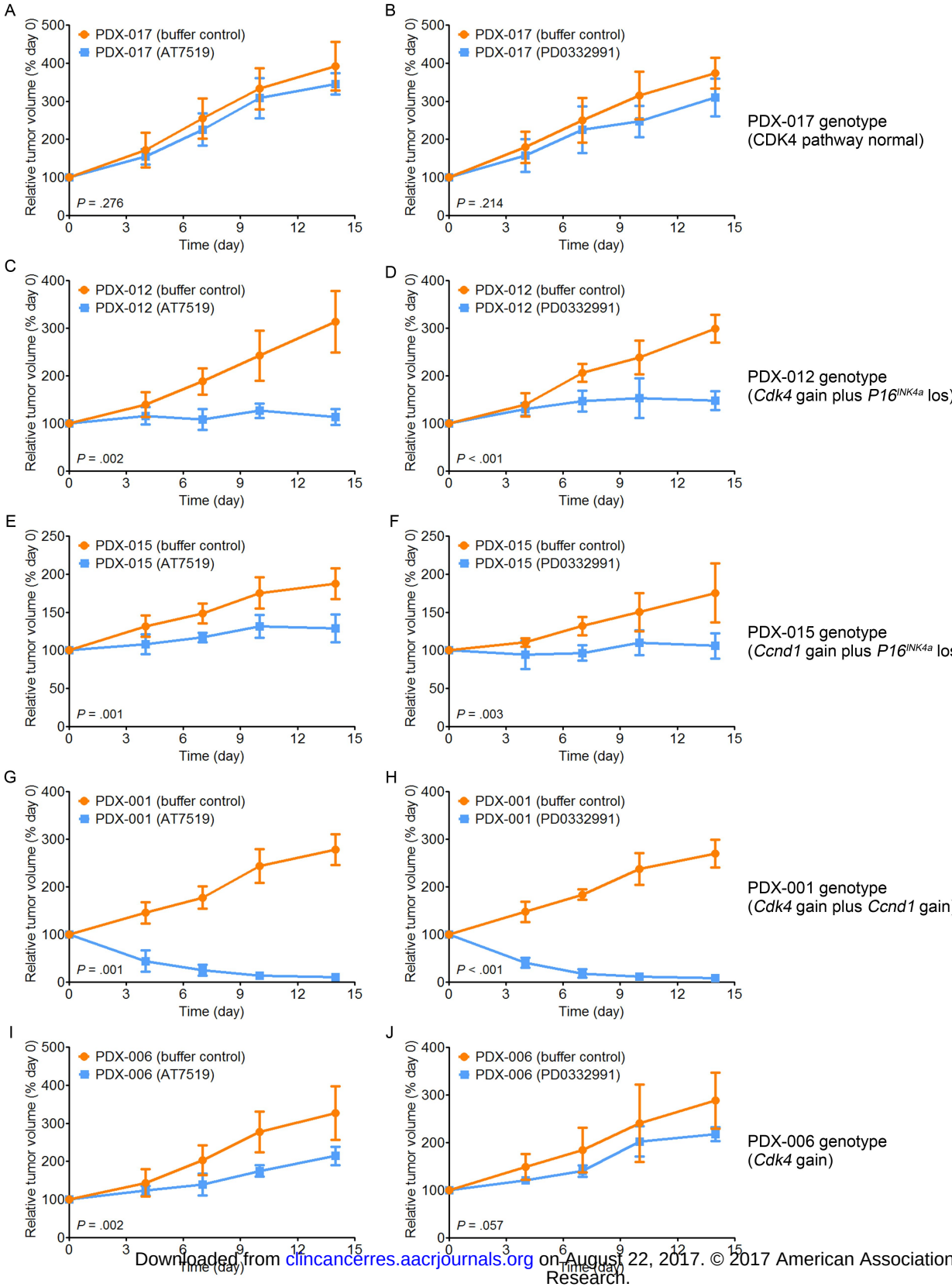
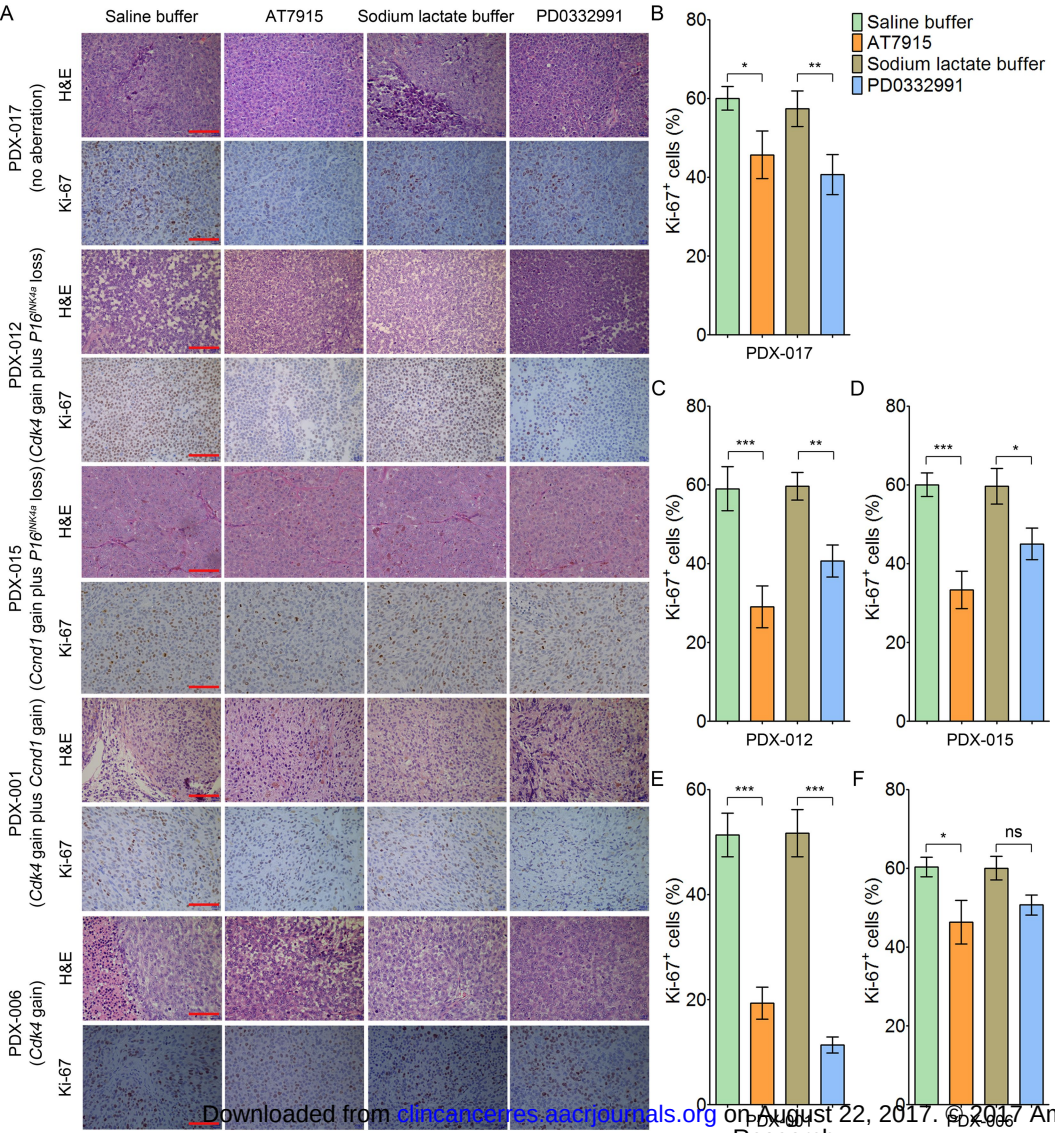


Figure 4



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Frequent Genetic Aberrations in the CDK4 Pathway in Acral Melanoma indicate the potential for CDK4/6 Inhibitors in Targeted Therapy

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