Frequency of *CDK2* , *CCNE1* Copy Number Variation in Acral Melanoma and Implications for CDK Inhibitor in Targeted Therapy

Xiaowen Wu
Department of Renal Cancer and Melanoma
Junya Yan
Department of Oncology
Jiayi Yu
Department of Radiation Oncology
Jinyu Yu
Department of Renal Cancer and Melanoma
Zhiyuan Cheng
Department of Renal Cancer and Melanoma
Ting Yin
Department of Renal Cancer and Melanoma
Qian Guo
Department of Renal Cancer and Melanoma
Yihong Xie
Department of Renal Cancer and Melanoma
Yan Kong (k-yan08@163.com)
Peking University Cancer Hosptial Institute
Jun Guo
Department of Renal Cancer and Melanoma

Research

Keywords: acral melanoma, targeted therapy, CDK2, CCNE1, copy number variation

Posted Date: February 12th, 2020

DOI: https://doi.org/10.21203/rs.2.23330/v1

Abstract

Background: Acral melanoma have a high frequency of cell cycle-related gene copy number variation. However, the status and clinical significance of CNVs of CDK 2 and CCNE1 have not been fully elucidated.

Methods: A total of 490 acral melanoma samples were examined for CNVs of CDK 2 and CCNE1 using QuantiGenePlex DNA Assay. Correlations of CDK2 and CCNE1 CNVs to clinicopathologic features and prognosis of acral melanoma were evaluated.The sensitivity of cell lines and cell-derived xenograft (CDX) containing CCNE1 CNVs to CDK inhibitor AT7519,Dinaciclib and proteasome inhibitor Bortezomib were also analyzed.

Results: Among the 490 samples,140 cases, 139 cases and 39 cases respectively showed CDK2 gain (28.5%), CCNE1 gain (28.3%) and CDK2 gain plus CCNE1 gain (8.0%).The median progression-free survival (PFS) time for acral patients with CCNE1 gain was significantly shorter than that for patients without CCNE1 gain (17.0 versus 27.0 months; P =0.002). Furthermore, CCNE1 gain was an independent prognostic factor for patients receiving chemotherapy. The pan-CDK inhibitor AT7519 could inhibit the cell proliferation, induce apoptosis and cause cell cycle arrest in G2 phase of acral melanoma cells and inhibit the tumor growth of CDX with CCNE1 gain. Dinaciclib and Bortezomib showed CCNE1 copy number independent inhibitory effects on the proliferation of melanoma cells.

Conclusions: CDK2 and CCNE1 copy number variations were frequent in acral melanoma and CCNE1 gain may be a useful biomarker to predict the outcome of receiving chemotherapy in patients with acral melanoma. In addition, our study provides a basis for the use of CDK inhibitor in the treatment of acral melanoma. Keywords: acral melanoma, targeted therapy, CDK2 , CCNE1 , copy number variation

Background

In Asians, acral melanomas accounts for 41.8–58% of melanomas and is the most common subtype[1 – 5].Acral melanomas showed a different genomic landscape from cutaneous melanoma, with a lower mutations burden ,a higher frequency genomic instability and widespread copy number changes[6, 7].Cell-cycle aberrations were significantly higher in acral melanomas and were signifificantly associated with poor melanoma-specifific survival[6 – 8].

Cyclin E1 plays a role in cell cycle progression[9 – 11], inhibition of apoptosis[12], DNA transcription[13], replication[14], and DNA repair[13]. Post-translational regulation of cyclin E1 is involved in multiple proteasomal degradation pathways, including BTB-Cullin3-Rbx1[15] and SCF^{Fbw7}[16] pathways. According to TCGA database, the expression of CCNE1 and CDK2 in cutaneous melanoma tumor tissue are much higher than those in normal tissues [17].The gain frequency of CCNE1 and CDK2 in cutaneous melanomas is about 16.3% and 12.5% respectively[17].However, there is still a lack of data on the frequency of CCNE1 and CDK2 copy number variation in large samples from acral melanoma.

CCNE1 gain is related to poor survival in ovarian cancer, endometrioid endometrial carcinomas and breast cancer[18 – 21]. CCNE1-amplified ovarian cancer were insensitive to chemotherapy containing cisplatin[22] or CDK4/6 inhibitor PD0332991[23].Unfortunately,there are no drugs specifically for target CCNE1. But CDK inhibitor and proteasome inhibitor bortezomib may be effective therapeutic drugs for tumors carrying CCNE1 amplification[22, 24].Based on the above-mentioned factors,CCNE1 and CDK2 copy number variation may be a potential therapeutic target.While in melanoma,clinical trials and preclinical studies targeted CCNE1 or CDK2 gain have not yet been carried out.

Identifying predictive biomarkers and patient subsets which are most likely to benefit from certain inhibitors is important for the clinical development of new drugs.In this study,we investigated the CNV of CCNE1 and CDK2 in 490 acral melanoma patients, analyzed the correlation with clinical prognosis and evaluate the sensitivity to CDK inhibitor AT7519,Dinaciclib and proteasome inhibitor bortezomib.Our study indicates that CCNE1 gain may be a potential biomarker for predicting the treatment's effect in acral melanoma and AT7519 may selectively target acral melanoma patients with CCNE1 gain.

Methods

Patients and Tissue Samples

This study involved samples from 490 acral melanoma patients, hospitalized at the Peking Cancer Hospital & Institute during January 2015 and June 2018. All patients provided written informed consent for the tissues samples.Samples were tested by hematoxylin and eosin (H&E) staining and immunohistochemistry to confirm the diagnosis of melanoma. Clinical data, including age, sex, TNM (tumor-node-metastases) stage, thickness (Breslow), ulceration, treatment and survival status were collected. The duration of progression-free survival (PFS) were calculated from the beginning of any treatment to the progression of the disease.The overall survival (OS) were the interval from pathologic diagnosis of melanoma to death or to the last follow-up time.This study was approved by the Medical Ethics Committee of the Peking Cancer Hospital & Institute and was conducted according to the Declaration of Helsinki Principles.

Copy Number Variation Detection by QuantiGenePlex DNA Assay

Tissue homogenates were prepared according to 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).For each assay well, 40 µl homogenate was denatured with 2.5 M NaOH (final concentration 0.18 M) in the presence of DNA Probe. Neutralized tissue homogenate was transferred to each well of the Hybridization Plate containing Working Bead Mix. All the samples were performed in three duplicates. Hybridization Plate was sealed and incubated at 54 °C ± 1 °C in shaking incubator (600 rpm) for 18 to 22 hours. Unbound samples were washed away using the Bio-plex pro II wash station (Bio-Rad, Hercules, CA). Then the beads were sequentially hybridized with DNA Pre-Amplifier, DNA Amplifier, Label Probe and SAPE. Fluorescence intensities were measured by the Bio-plex 100 system (Bio-Rad) and the mean fluorescence intensities of the three duplicates of each sample were calculated.

For each test sample, normalized signal was divided by the reference DNA sample (G1521, Promega, Madison, WI) for CCNE1 and CDK2, and the values were multiplied by the known copy number of each gene in the reference genome. When the relative copy number is greater than or equal to 3.0, the copy number of CCNE1 or CDK2 is determined to be gain. When the relative copy number is less than 2.0, the copy number of gene is determined to be lost.

Treatment

Among the 490 acral melanoma patients, 251 patients had high-dose IFNα-2b treatment and 82 patients had chemotherapy containing cisplatin (DTIC 250 mg/m2, d1-5, DDP 40 mg, d1-3, Endostar Injection 7.5 mg/m2, d1-14, q4w).The treatment continued until the occurrence of progressive disease, unacceptable adverse events, death, or withdrawal of consent for any other reasons.

Effects of inhibitors on proliferation ,apoptosis and cell cycle

The WM2664 and A375 cell were purchased from ATCC (American Type Culture Collection) and were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS,Hyclone). The WCL-M3 acral melanoma cell lines were derived from patients. The cells were cultured in serum-free stem cell medium supplemented with growth factors.

CDK inhibitor AT7519, Dinaciclib and proteasome inhibitor Bortezomib were purchased from Selleck Chemicals (Houston, TX). After treatment with various concentrations of inhibitors or DMSO for 48 h and 72 h, proliferation of the cells was evaluated using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the instructions.

Cell cycle of cells at 24 h after exposure to inhibitor or DMSO were evaluated using PI staining. Apoptosis of cells at 24 h after exposure to inhibitor or DMSO were evaluated using Apoptosis Detection Kit (DOJINDO, Shanghai, China) according to the instructions. Analysis was performed on a FACS Calibur flow cytometer (BD Biosciences).

Cell Xenograft Models

The cells were cultured in vitro as describing above.After digestion, washed twice with PBS, 5 *10^6 cells were inoculated into a NOD/SCID (Nonobese Diabetic/Severe Combined Immunodeficiency) mouse(Female, 6–8 weeks, 18–22 g).When the tumor size of CDX models reached approximately 200 mm3, mice were randomized to treatment (CDK inhibitor AT7519 ,12 mg/kg in saline solution)or control (saline solution) groups (n = 4 per group).Tumor sizes were measured every 3 days and tumor volume used the formula:volume = 1/2*length* $width^2$. Percentage of tumor volume on day0 (V / V_0%) was used as a standard to measure the rate of tumor growth.The mice received intraperitoneal injection for 14 days, after which the mice were sacrificed and the tumors were fixed in 10% formalin for histologic,HE and immunohistological analysis. Care of experimental animals was in accordance with animal ethics.

Statistical Analysis

Statistical analyses were performed using SPSS 20.0 software. All statistical analyses were two-sided, and $P < 0.05$ was considered as statistically significant. The statistical significance between the outcomes of PFS and OS were calculated using Kaplan-Meier method.Cox proportional-hazards regression analysis was conducted to estimate the hazard ratio (HR).

Results

Aberrations of CDK2 and CCNE1 in acral melanoma

Among the 490 acral melanoma patients, the overall frequency of patients containing any CDK2 or CCNE1 copy number variation (CNV) (≥ 1 CNV) was 49.0% (240/490).140 cases (28.5%) contained CDK2 gain and 139 cases (28.3%) contained CCNE1 gain.Moreover, 39 cases (8.0%) contained concurrent CDK2 gain plus CCNE1 gain.Most of the copy number of samples with CDK2 or CCNE1 gain was about 3–4 copies (Table 1).We also analyzed the correlation between CDK2 and CCNE1 copy number variations in acral melanoma and there was not significantly different (P = 0.874, Supplementary Table S1).

Table 1
Copy number variations of CDK2 and CCNE1 in acral melanoma.

CNV status	n (%)
≥ 1 CNV	
Overall	240 (49.0)
CDK2 gain	140 (28.5)
3–4 copies	129 (26.3)
5–8 copies	10 (2.0)
> 8 copies	1 (0.2)
CCNE1 gain	139 (28.3)
3–4 copies	133 (27.1)
5–8 copies	4 (0.8)
> 8 copies	2 (0.4)
2 CNVs	
CDK2 gain + CCNE1 gain gain	39 (8.0)

Since targeted therapy of melanoma has been explored, we also analyzed the mutation frequency of genes that have been confirmed as promising targets in acral melanoma samples. In the samples containing either CDK2 or CCNE1 CNV (≥ 1 CNV), 12.2%, 19.6%, 14.8% and 4.8% of them also contained mutations in BRAF,KIT ,NRAS or PDGFRA respectively.CDK2 and CCNE1 CNVs and targeted gene mutations pattern were showed in Fig. 1.

Correlation of CDK2 and CCNE1 aberrations to clinicopathological features

In our cohort, the age (< 60 years or ≥ 60 years), gender, ulceration, thickness were all not significantly different between patients with or without CDK2 gain, CCNE1 gain or CDK2 gain plus CCNE1 gain (Table 2). Among the patients with CDK2 gain, the proportion of patients with stage III- IV was higher than those without CDK2 gain (P = 0.010; Table 2).While the TNM stage in patients with or without CCNE1 gain or CDK2 gain plus CCNE1 gain were not significantly different (Table 2).

Table 2
Correlation of CCNE1 and CDK2 aberrations to clinicopathologic features of acral melanoma.

Clinicopathologic factor	CDK2 aberration			CCNE1 aberration			CDK2 gain plus CCNE1 gain		
	Gain	Normal	P value	Gain	Normal	P value	Positive	Negative	P value
Total	140 (28.5)	350 (71.5)		139 (28.3)	351 (71.6)		39 (8.0)	451 (92.0)	
Age (years)			0.513			0.176			0.452
< 60	86 (61.4)	226 (64.6)		95 (68.3)	217 (61.8)		27 (69.2)	285 (63.2)	
≥ 60	54 (38.6)	124 (35.4)		44 (31.7)	134 (38.2)		12 (30.8)	166 (36.8)	
Gender n (%)			0.122			0.652			0.269
Male	82 (58.6)	178 (50.9)		76 (54.7)	184 (52.4)		24 (61.5)	236 (52.3)	
Female	58 (41.4)	172 (49.1)		63 (45.3)	167 (47.6)		15 (38.5)	215 (47.7)	
Ulceration n (%)			0.694			0.041			0.510
Yes	94 (70.7)	234 (68.8)		100 (76.3)	228 (66.7)		26 (74.3)	302 (68.9)	
No	39 (29.3)	106 (31.2)		31 (23.7)	114 (33.3)		9 (25.7)	136 (31.1)	
Thickness (mm)			0.295			0.560			0.240
< 1 mm	4 (3.1)	12 (3.6)		10 (7.8)	6 (1.8)		3 (9.1)	13 (3.0)	
1–2 mm	25 (19.2)	58 (17.2)		19 (14.8)	64 (18.8)		4 (12.1)	79 (18.2)	
2–4 mm	45 (34.6)	128 (37.9)		41 (32.0)	132 (38.8)		11 (33.3)	162 (37.2)	
> 4 mm	56 (43.1)	140 (41.4)		58 (45.3)	138 (40.6)		15 (45.5)	181 (41.6)	
Stages n (%)			0.010			0.798			0.284
	13 (9.4)	39 (11.3)		14 (10.9)	37 (10.6)		4 (10.5)	48 (10.7)	
	32 (23.0)	119 (34.4)		45 (32.8)	106 (30.5)		7 (18.4)	144 (32.2)	
	34 (24.5)	90 (26.0)		37 (27.0)	87 (25.0)		13 (34.2)	111 (24.8)	
	60 (43.2)	98 (28.3)		40 (29.2)	118 (33.9)		14 (36.8)	144 (32.2)	
TILs score			0.371			0.520			0.732
1	7 (28.0)	30 (44.1)		9 (34.6)	28 (41.8)		2 (40.0)	35 (39.8)	
2	10 (40.0)	21 (30.9)		11 (42.3)	20 (29.9)		1 (20.0)	30 (34.1)	
3	8 (32.0)	17 (25.0)		6 (23.1)	19 (28.4)		2 (40.0)	23 (26.1)	
Mitotic rate			0.159			0.144			1.000
≤ 4	7 (28.0)	30 (44.1)		7 (26.9)	30 (44.8)		2 (40.0)	35 (39.8)	
> 4	18 (72.0)	38 (55.9)		19 (73.1)	37 (55.2)		3 (60.0)	53 (60.2)	
OS(months)			0.906			0.343			0.035
Median (95% CI)	61.0 (55.0,67.0)	71.0 (45.6,96.4)		76.7 (63.4,90.1)	77.4 (68.6,86.2)		78.9 (70.1,87.7)	75.9 (67.8,84.1)	
PFS(months)			0.466			0.002			0.066
Median (95% CI)	20 .0 (12.7,27.3)	26.0 (23.2,28.8)		17.0 (11.6,22.4)	27.0 (23.2,30.8)		15.0 (9.3,20.7)	26.0 (23.0,29.0)	

According to the NCCN guidelines Version 1.2019,mitotic rate and the degree of tumor-infiltrating lymphocytes (TILs) are important clinicopathological features in melanoma.In our cohorts,the TILs and mitotic rate distribution for acral melanoma patients with or without any CNVs for CDK2, CCNE1 or its combinations were all not significantly different (Table 2).

Impact of CDK2 and CCNE1 CNVs on clinical outcomes

The OS and PFS are important clinical endpoints. The OS for patients with CDK2 gain or CCNE1 gain were comparable to patients without such aberrations (Table 2; Fig. 2A and 2B).

But patients with CCNE1 gain had a significantly shorter median PFS time (median PFS = 17.0 months) than patients without CCNE1 gain (median PFS = 27.0 months,P = 0.002; Table 2;Fig. 2E).While PFS were not significantly different between patients with or without CDK2 gain or CDK2 gain plus CCNE1 gain (Table 2; Fig. 2D and 2F).We also compared the OS and PFS in patients with CDK2 gain plus CCNE1 gain with CDK2 gain alone as well as CCNE1 gain alone. Patients with CDK2 gain plus CCNE1 gain had a shorter median PFS time (median PFS = 15.0 months) than patients with CCNE1 gain alone (median PFS = 17.0 months) and patients with CDK2 gain alone (median PFS = 25.0 months) (Fig.S1).

In this cohort ,only TNM stage (HR 2.31; 95%CI: 1.46–3.64;P < 0.001) and NRAS mutation (HR 2.39; 95%CI: 1.52–3.77; P < 0.001) are independent prognostic factors for acral melanoma patients OS .TNM stage (HR 2.39, 95%CI: 1.77–3.22; P < 0.001) and CCNE1 gain (HR 1.62, 95%CI:1.21–2.17; P = 0.001) are independent prognostic factors for acral melanoma patients PFS. Interestingly,the NRAS Q61 site mutation was an independent prognostic factor for acral melanoma patients OS and PFS, but BRAF V600 site mutation was not significance for melanoma patients OS or PFS (Table 3).

Table 3
Univariate and multivariate analysis of prognostic factors for OS and PFS of patients with acral melanoma.

Variable	OS								PFS					
	Univariate			Multivariate					Univariate			Multivariate		
HR	95% CI*	P value	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value			
Age (< 60 years/ ≥60 years)	1.26	0.86–1.84	0.239						1.01	0.77–1.33	0.929			
Gender (female/male)	0.93	0.64–1.35	0.713						1.08	0.84–1.40	0.540			
Ulceration (no/yes)	0.72	0.48–1.07	0.114						0.65	0.47–0.82	0.002	0.81	0.61–1.09	0.165
Thickness (≤ 2 mm/>2 mm)	0.60	0.41–1.02	0.063						0.56	0.42–0.77	< 0.001	1.00	0.70–1.44	0.993
TNM stage (III + IV/I + II)	2.04	1.30–2.78	0.001		2.31	1.46–3.64	< 0.001		2.86	2.27–3.85	< 0.001	2.39	1.77–3.22	< 0.001
CDK2 CN (gain/normal)	1.02	0.69–1.51	0.906						1.12	0.86–1.47	0.466			
CCNE1 CN (gain/normal)	1.25	0.80–1.91	0.343						1.52	1.19–2.08	0.002	1.62	1.21–2.17	0.001
CDK2 gain plus CCNE1 gain	3.19	1.06–4.12	0.035						1.54	1.02–2.86	0.066			
NRAS mutations (no/yes)	0.42	0.16–0.54	< 0.001		2.39	1.52–3.77	< 0.001		0.74	0.44–1.11	0.136			
NRAS Q61 mutations (no/yes)	0.42	0.15–0.56	0.0003						0.54	0.26–0.73	0.002	0.66	0.51–0.85	0.001
BRAF mutations (no/yes)	0.85	0.72–1.90	0.538						0.77	0.49–1.12	0.162			
BRAF V600E mutations (no/yes)	1.19	0.69–2.01	0.553						0.83	0.54–1.21	0.316			
KIT mutations (no/yes)	0.67	0.38–1.04	0.074						0.79	0.52–1.10	0.156			
PDGFRA mutations (no/yes)	0.79	0.21–2.76	0.686						0.67	0.24–1.50	0.284			
*95% CI for HR.														

Effect ofCDK2 and CCNE1 CNVs on Response to chemotherapy

Among the 490 acral melanoma patients, 82 patients received a standard chemotherapy containing cisplatin(DTIC, DDP, and Endostar combined chemotherapy) as a first-line treatment.

Patients with CDK2 gain had a similar median PFS compared to patients without CDK2 gain (median PFS: 7.0 versus 8.2 months,P = 0.507, Fig. 3A).While patients with CCNE1 gain experienced a shorter median PFS than patients without CCNE1 gain (median PFS: 4.8 versus 7.4 months,P = 0.006, Fig. 3B).In this research cohort,CCNE1 gain was the an independent prognostic factor for acral melanoma patients PFS after a standard chemotherapy containing cisplatin (HR 0.43, 95% CI:0.23–0.81; P = 0.009; Table S2).

Sensitivity of Melanoma Cells to Multiple Inhibitors

The WM2664,WCL-M3, A375 malanoma cells were evaluated for the efficacy of CDK inhibitor AT7519, Dinaciclib and proteasome inhibitor Bortezomib by determining cell proliferation in vitro. The WM2664(with CCNE1 gain) and A375 (with CCNE1 normal) are CUTANEOUS melanoma cells ,WCL-M3(with CCNE1 gain) is acral melanoma cell.

The pan-CDK inhibitor AT7519 significantly inhibited the proliferation of WM2664 (with CCNE1 gain) and WCL-M3(with CCNE1 gain) (Fig. 4). AT7519 had a weak inhibitory effect on the proliferation of cells without CCNE1 gain. Similar results were observed in vivo experiments(Fig. 5). Dinaciclib showed comparable inhibitory efficiency on WM2664 (with CCNE1 gain),WCL-M3 (with CCNE1 gain) and A375 (with CCNE1 normal) .Bortezomib showed comparable inhibitory efficiency on WM2664,WCL-M3, A375.Apoptosis and cycle detection experiments showed that AT7519 and Bortezomib can cause different degrees of cell apoptosis and increased proportion of S phase in the cell cycle, regardless of whether the cell carries CCNE1 or CDK2 copy number variations.Compared with the control group, there was no significant difference in the proportion of apoptosis and cell cycle of the four kinds of cells treated with Dinaciclib affter 24 hours.The results of cell cycle and apoptosis are shown in the Supplemental Figure S2-3.

Discussion

Acral melanoma arises on the hands and feet in people, which accounts for a much higher proportion of melanomas in Asians than in Caucasians.[1 – 5] Acral melanoma genomes showed greater proportions of copy number variation than in cutaneous melanomas[6].

CDK2 and CCNE1 are important molecules related to cell functions.Cyclin E1 (encoded by CCNE1) - CDK2 complex controls G1 to S transition in cell-cycle[9].The complex makes the cells to initiate DNA synthesis by phosphorylation of its downstream substrates such as Rb, CDC6, NPAT and P107, so that the cells are irreversibly involved in the S phase[10, 11, 25]. Cyclin E1 –CDK2 complex abnormal is associated with chromosomal instability[20]. CCNE1 also an important role in inhibition of apoptosis[12], DNA transcription[13], replication[14], and DNA repair[13],mediating asymmetric cell division, specifying cell fate and in driving stem cell self-renewal[26, 27] .Post-translational regulation of cyclin E1 is involved in multiple proteasomal degradation pathways, including BTB-Cullin3-Rbx1[15] and SCF^{Fbw7}[16] pathways.This study investigated the frequency of CDK2 and CCNE1 CNVs in 490 cases acral melanoma,analyzed the association of CDK2 and CCNE1 CNVs with clinical prognosis and evaluated the sensitivity of cells to multiple inhibitors.

In this study,140 cases, 139 cases and 39 cases showed CDK2 gain (28.5%), CCNE1 gain (28.3%) and CDK2 gain plus CCNE1 gain (8.0%), respectively. Most of the copy number of samples with CDK2 or CCNE1 gain was about 3 to 4 copies.There was no correlation between CDK2 and CCNE1 copy number variations.In cutaneous melanomas,the frequency of CDK2 gain and CCNE1 gain were about 12.5% and 16.3%,respectively [17].The proportion of CDK2 and CCNE1 gain in acral melanoma were higher than that reported in cutaneous melanomas.

Prior research have shown CCNE1 gain significantly correlated with shorter DFS(disease-free survival) and reduce OS in ovarian cancer,endometrioid endometrial carcinomas and breast cancer[18 – 21].By analyzing the TCGA data in cutaneous melanomas [17], patients with CDK2 gain had a shorter median OS time than patients without CDK2 gain. Compared with patients without CCNE1 gain,patients with CCNE1 gain had a comparable OS but a shorter median DFS time.In our study, CCNE1 gain is the independent prognostic factor for acral melanoma patients PFS. Patients with CCNE1 gain had a significantly shorter PFS time than patients without CCNE1 gain. But CDK2 gain or CCNE1 gain were not independent prognostic factors for acral melanoma patients OS.The results difference between cutaneous melanomas and acral melanomas may be caused by subtype differences.

For advanced acral melanoma patients,targeted therapy, immunotherapy and chemotherapy are the main options.By now,chemotherapy containing cisplatin is still one of the effective options for the treatment of patients with advanced acral melanoma[28].In our study,82 patients receiving the DTIC/DDP/Endostar combined chemotherapy. Patients harboring CCNE1 gain had a significantly shorter PFS than those without CCNE1 CNVs after receiving chemotherapy.In other studies, CCNE1 gain was associated with primary or acquired drug resistance in breast cancer, ovarian cancer and pancreatic cancer[23, 29 – 31]. In addition, CCNE1 gain and over expression were associated with resistance to CDK4/6 therapies in breast cancer [32].But the reasons underlying treatment failure are not entirely clear. There was a view that cells have an intact high-fifidelity system for repairing the double stranded DNA breaks which were sustained after platinum exposure[33].Enhanced self-renewal due to CCNE1 copy number variation may also be one of the reasons for the mechanism for treatment failure[29].These findings suggested that CCNE1 gain is an important prognostic factor for acral melanoma and may be a potential biomarker for new treatments.

Given that acral melanoma with CCNE1 gain has a shorter PFS after receiving chemotherapy, new therapies are needed to improve the prognosis of these patients.Although there are no drugs specifically for target CCNE1, preclinical and clinical studies on CCNE-related geneshave been conducted in a variety of tumors.A Phase II clinical trials(NCT02656849) aim to test the effectiveness of pan-CDK inhibitor Roniciclib (BAY1000394) in treating cancer with MCL1,MYC or CCNE1 gain[34].Another Phase II clinical trial (NCT02873975) is studying the CHK1 Inhibitor LY2606368 in tumor patients with replicative stress, including CCNE1 gain[35].Dinaciclib is a highly potent inhibitor of CDK1, CDK2, CDK5 and CDK9 that has entered Phase III clinical studies.Dinaciclib can inhibit cell cycle progression in more than 100 tumor cell lines and induce tumor regression in mouse models[36].In the study of ovarian cancer, CDK inhibitor dinaciclib combined AKT inhibitors may selectively target patients with CCNE1-amplified HGSC[37]. In an estrogen receptor-positive breast cancer study, combined targeting of both CDK4/6 and PI3K may be the

treatment of patients bearing a CCNE1 gain[30]. Recent research has found that the proteasome inhibitor bortezomib can DISRUPTS THE CELL CYCLE, INDUCES APOPTOSIS, inhibit DNA repair mechanisms, including homologous recombination[33, 38]. CCNE1-amplified ovarian cancer cells showed specific sensitivity to the proteasome inhibitor bortezomib[39].CDK inhibitor AT7519 (developed by Astex), a pyrazole 3-carboxyamide compound, could inhibit CDK1,CDK2,CDK4, CDK6, CDK9[33]. In our previous research, we found that pan-CDK inhibitor AT7519 can inhibit the cell viability of acral melanoma cells and the tumor growth of PDX containing cell cycle aberrations.In the Phase II clinical trial enrolling patients with multiple myeloma, more than one-third of patients achieved PR(partial response) after receiving AT7519 combined Bortezomib treatment(NCT01183949).In this study, we evaluated the sensitivity of cell lines and cell-derived xenografts (CDX) to pan-CDK inhibitor AT7519,Dinaciclib and proteasome inhibitor Bortezomib.

In this study,we found that pan-CDK inhibitor AT7519 could significantly inhibited the proliferation of WM2664 (with CCNE1 gain) and WCL-M3(with CCNE1 gain) in vivo and in vitro.In vitro results also found that AT7519 can cause different degrees of cell apoptosis and increased proportion of S and G2 phase in the cell cycle, regardless of whether the cell carries CCNE1 or CDK2 copy number variations.Pan-CDK inhibitor Dinaciclib showed comparable inhibitory efficiency on WM2664,WCL-M3,A375.Proteasome inhibitor Bortezomib showed comparable inhibitory efficiency on WM2664 WCL-M3 and A375.These data suggest that melanoma cells carrying CCNE1 CNVs may be sensitive to the pan-CDK inhibitor AT7519.But more experimental evidence is needed to prove this point.Dinaciclib and Bortezomib had good inhibitory effect on melanoma cell proliferation, although we did not observe a CCNE1 CNV-dependent specificity.This may be due to Dinaciclib has a strong inhibitory effect on CDK1, CDK5 and CDK9,in addition to CDK1.Molecular markers that can predict the efficacy of Dinaciclib and Bortezomib need to be verified by further research.

In this study,we found that CDK2 and CCNE1 copy number variations in acral melanoma were frequent and showed greater proportions of CNVs than in cutaneous melanomas.CCNE1 gain may be a potential biomarker for predicting the effect of treatment in acral melanoma.In addition, this study also made a preliminary exploration for the use of CDK inhibitors and proteasome inhibitor in the treatment of acral melanoma.Pan-CDK inhibitor AT7519 can inhibit growth of acral melanoma cells carrying CCNE1 CNVs in vivo and in vitro,providing an experimental basis for clinical applications.

Conclusions

In conclusion,CDK2 and CCNE1 copy number variations in acral melanoma were frequent.CCNE1 gain may be a potential biomarker for predicting the effect of treatment in acral melanoma.In addition, this study provides a basis for the use of CDK inhibitor in the treatment of acral melanoma.

Abbreviations

CDK:cyclin-dependent kinases,CNV：copy number variation,TNM :tumor-node-metastases stage,PFS:progression-free survival,OS:overall survival ,TIL:tumor infiltrating lymphocyte, FFPE:Formalin-Fixed Paraffin-Embedded Tissues,DTIC: Dacarbazin, DDP:cisplatin, TCGA:Cancer Genome Atlas, ATCC:American Type Culture Collection ,NOD/SCID:Nonobese Diabetic/Severe Combined Immunodeficiency,CDX:cell-derived xenograft .

Declarations

Ethics approval and consent to participate: This study was approved by the Medical Ethics Committee of the Peking Cancer Hospital & Institute and was conducted according to the Declaration of Helsinki Principles.

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests: The authors declare that they have no competing interests.

Funding: This work was supported by Beijing Natural Science Foundation (7194244),China Postdoctoral Science Foundation funded Project (2019T120026,2019M650389),Natural Science Foundation of China (81902789,81672696, 81772912), Fostering Young Scholars of Peking University Health Science Center, Clinical Medicine Plus X - Young Scholars Project (Peking University).

Authors' contributions:

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

Collection and assembly of data: Drs. Jun Guo, Yan Kong, Xiaowen Wu,Junya Yan,Jiayi Yu,Jinyu Yu,Zhiyuan Cheng,Ting Yi ,Qian Guo,Yihong Xie.

Data analysis and interpretation: Drs. Jun Guo, Yan Kong, Xiaowen Wu,Junya Yan,Jiayi Yu.

Manuscript writing: Drs. Xiaowen Wu,Junya Yan

Final approval of manuscript: All authors.

Acknowledgements: We appreciate Drs. Zhongwu Li and Aiping Lu in the Department of Pathology of Peking University Cancer Hospital and Institute for help in pathologic analysis of tissue samples.

References

1. Bai X, Kong Y, Chi Z, Sheng X, Cui C, Wang X, Mao L, Tang B, Li S, Lian B, et al. MAPK Pathway and TERT Promoter Gene Mutation Pattern and Its Prognostic Value in Melanoma Patients: A Retrospective Study of 2,793 Cases. Clin Cancer Res. 2017;23:6120-7.
2. Ishihara K, Saida T, Otsuka F, Yamazaki N. Statistical profiles of malignant melanoma and other skin cancers in Japan: 2007 update. Int J Clin Oncol. 2008;13:33-41.
3. Chang JW, Yeh KY, Wang CH, Yang TS, Chiang HF, Wei FC, Kuo TT, Yang CH. Malignant melanoma in Taiwan: a prognostic study of 181 cases. Melanoma Res. 2004;14:537-41.
4. Luk NM, Ho LC, Choi CL, Wong KH, Yu KH, Yeung WK. Clinicopathological features and prognostic factors of cutaneous melanoma among Hong Kong Chinese. Clin Exp Dermatol. 2004;29:600-4.
5. Chi Z, Li S, Sheng X, Si L, Cui C, Han M, Guo J. Clinical presentation, histology, and prognoses of malignant melanoma in ethnic Chinese: a study of 522 consecutive cases. BMC Cancer. 2011;11:85.
6. Hayward NK, Wilmott JS, Waddell N, Johansson PA, Field MA, Nones K, Patch AM, Kakavand H, Alexandrov LB, Burke H, et al. Whole-genome landscapes of major melanoma subtypes. Nature. 2017;545:175-80.
7. Kong Y, Sheng X, Wu X, Yan J, Ma M, Yu J, Si L, Chi Z, Cui C, Dai J, et al. Frequent Genetic Aberrations in the CDK4 Pathway in Acral Melanoma Indicate the Potential for CDK4/6 Inhibitors in Targeted Therapy. Clin Cancer Res. 2017;23:6946-57.
8. Sheen YS, Tan KT, Tse KP, Liao YH, Lin MH, Chen JS, Liau JY, Tseng YJ, Lee CH, Hong CH, et al. Genetic alterations in primary melanoma in Taiwan. Br J Dermatol. 2019.
9. Caldon CE, Musgrove EA. Distinct and redundant functions of cyclin E1 and cyclin E2 in development and cancer. Cell Div. 2010;5:2.
10. Dulić V, Lees E, Reed SI. Association of human cyclin E with a periodic G1-S phase protein kinase. Science. 1992;257:1958-61.
11. Pagano M, Pepperkok R, Lukas J, Baldin V, Ansorge W, Bartek J, Draetta G. Regulation of the cell cycle by the cdk2 protein kinase in cultured human fibroblasts. J Cell Biol. 1993;121:101-11.
12. Siu KT, Rosner MR, Minella AC. An integrated view of cyclin E function and regulation. Cell Cycle. 2012;11:57-64.
13. DeGregori J, Kowalik T, Nevins JR. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. Mol Cell Biol. 1995;15:4215-24.
14. Zhao J, Kennedy BK, Lawrence BD, Barbie DA, Matera AG, Fletcher JA, Harlow E. NPAT links cyclin E-Cdk2 to the regulation of replication-dependent histone gene transcription. Genes Dev. 2000;14:2283-97.
15. Singer JD, Gurian-West M, Clurman B, Roberts JM. Cullin-3 targets cyclin E for ubiquitination and controls S phase in mammalian cells. Genes Dev. 1999;13:2375-87.
16. Koepp DM, Schaefer LK, Ye X, Keyomarsi K, Chu C, Harper JW, Elledge SJ. Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. Science. 2001;294:173-7.
17. The Cancer Genome Atlas (TCGA).https://cancergenome.nih.gov/ .
18. Nakayama N, Nakayama K, Shamima Y, Ishikawa M, Katagiri A, Iida K, Miyazaki K. Gene amplification CCNE1 is related to poor survival and potential therapeutic target in ovarian cancer. Cancer. 2010;116:2621-34.
19. Nakayama K, Rahman MT, Rahman M, Nakamura K, Ishikawa M, Katagiri H, Sato E, Ishibashi T, Iida K, Ishikawa N, et al. CCNE1 amplification is associated with aggressive potential in endometrioid endometrial carcinomas. Int J Oncol. 2016;48:506-16.
20. Kuhn E, Wang TL, Doberstein K, Bahadirli-Talbott A, Ayhan A, Sehdev AS, Drapkin R, Kurman RJ, IeM S. CCNE1 amplification and centrosome number abnormality in serous tubal intraepithelial carcinoma: further evidence supporting its role as a precursor of ovarian high-grade serous carcinoma. Mod Pathol. 2016;29:1254-61.
21. Han JY, Wang H, Xie YT, Li Y, Zheng LY, Ruan Y, Song AP, Tian XX, Fang WG. Association of germline variation in CCNE1 and CDK2 with breast cancer risk, progression and survival among Chinese Han women. PLoS One. 2012;7:e49296.
22. Wiedemeyer WR, Beach JA, Karlan BY. Reversing Platinum Resistance in High-Grade Serous Ovarian Carcinoma: Targeting BRCA and the Homologous Recombination System. Front Oncol. 2014;4:34.
23. Taylor-Harding B, Aspuria PJ, Agadjanian H, Cheon DJ, Mizuno T, Greenberg D, Allen JR, Spurka L, Funari V, Spiteri E, et al. Cyclin E1 and RTK/RAS signaling drive CDK inhibitor resistance via activation of E2F and ETS. Oncotarget. 2015;6:696-714.

24. Yan J, Yu J, Wu X, Xu T, Yu H, Dai J, Ma M, Tang H, Xu L, Chi Z, et al. Increased AURKA Gene Copy Number Correlates with Poor Prognosis and Predicts the Efficacy of High-dose Interferon Therapy in Acral Melanoma. J Cancer. 2018;9:1267-76.
25. Tetsu O, McCormick F. Proliferation of cancer cells despite CDK2 inhibition. Cancer Cell. 2003;3:233-45.
26. Chia W, Prokopenko SN. Cyclin E at the centre of an identity crisis. Nat Cell Biol. 2005;7:3-5.
27. Chia W, Somers WG, Wang H. Drosophila neuroblast asymmetric divisions: cell cycle regulators, asymmetric protein localization, and tumorigenesis. J Cell Biol. 2008;180:267-72.
28. Cui C, Mao L, Chi Z, Si L, Sheng X, Kong Y, Li S, Lian B, Gu K, Tao M, et al. A phase II, randomized, double-blind, placebo-controlled multicenter trial of Endostar in patients with metastatic melanoma. Mol Ther. 2013;21:1456-63.
29. Etemadmoghadam D, deFazio A, Beroukhim R, Mermel C, George J, Getz G, Tothill R, Okamoto A, Raeder MB, Harnett P, et al. Integrated genome-wide DNA copy number and expression analysis identifies distinct mechanisms of primary chemoresistance in ovarian carcinomas. Clin Cancer Res. 2009;15:1417-27.
30. Herrera-Abreu MT, Palafox M, Asghar U, Rivas MA, Cutts RJ, Garcia-Murillas I, Pearson A, Guzman M, Rodriguez O, Grueso J, et al. Early Adaptation and Acquired Resistance to CDK4/6 Inhibition in Estrogen Receptor-Positive Breast Cancer. Cancer Res. 2016;76:2301-13.
31. Franco J, Witkiewicz AK, Knudsen ES. CDK4/6 inhibitors have potent activity in combination with pathway selective therapeutic agents in models of pancreatic cancer. Oncotarget. 2014;5:6512-25.
32. O'Leary B, Cutts RJ, Liu Y, Hrebien S, Huang X, Fenwick K, Andre F, Loibl S, Loi S, Garcia-Murillas I, et al. The genetic landscape and clonal evolution of breast cancer resistance to palbociclib plus fulvestrant in the PALOMA-3 trial. Cancer Discov. 2018;8:1390-403.
33. Asghar U, Witkiewicz AK, Turner NC, Knudsen ES. The history and future of targeting cyclin-dependent kinases in cancer therapy. Nat Rev Drug Discov. 2015;14:130-46.
34. BAY 1000394 for MCL-1-, MYC-, and CCNE1-Amplified Tumors. https://www.clinicaltrials. gov .
35. A Study of LY2606368 (Prexasertib) in Patients With Solid Tumors With Replicative Stress or Homologous Repair Deficiency.https://www.clinicaltrials.gov .
36. Parry D, Guzi T, Shanahan F, Davis N, Prabhavalkar D, Wiswell D, Seghezzi W, Paruch K, Dwyer MP, Doll R, et al. Dinaciclib (SCH 727965), a novel and potent cyclin-dependent kinase inhibitor. Mol Cancer Ther. 2010;9:2344-53.
37. Au-Yeung G, Lang F, Azar WJ, Mitchell C, Jarman KE, Lackovic K, Aziz D, Cullinane C, Pearson RB, Mileshkin L, et al. Selective Targeting of Cyclin E1-Amplified High-Grade Serous Ovarian Cancer by Cyclin-Dependent Kinase 2 and AKT Inhibition. Clin Cancer Res. 2017;23:1862-74.
38. Kanska J, Zakhour M, Taylor-Harding B, Karlan BY, Wiedemeyer WR. Cyclin E as a potential therapeutic target in high grade serous ovarian cancer. Gynecol Oncol. 2016;143:152-8.
39. Etemadmoghadam D, Weir BA, Au-Yeung G, Alsop K, Mitchell G, George J, Davis S, D'Andrea AD, Simpson K, Hahn WC, et al. Synthetic lethality between CCNE1 amplification and loss of BRCA1. Proc Natl Acad Sci U S A. 2013;110:19489-94.

Figures

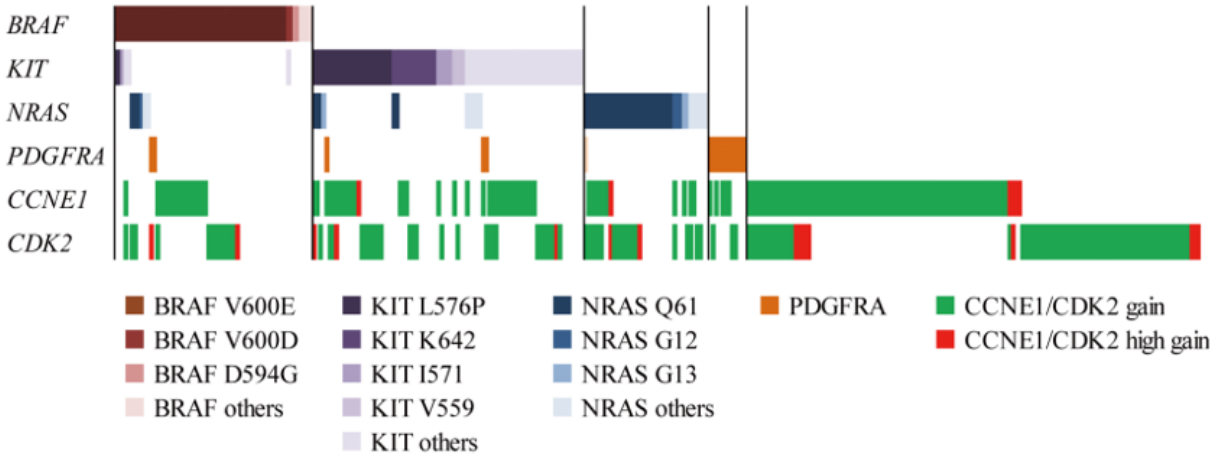

Figure 2

CDK2, CCNE1 CNVs and targeted gene mutation pattern of acral melanoma(n=490).Patients harboring at least one gene mutation of BRAF,KIT,NRAS and PDGFRA and CDK2, CCNE1 CNVs are presented.

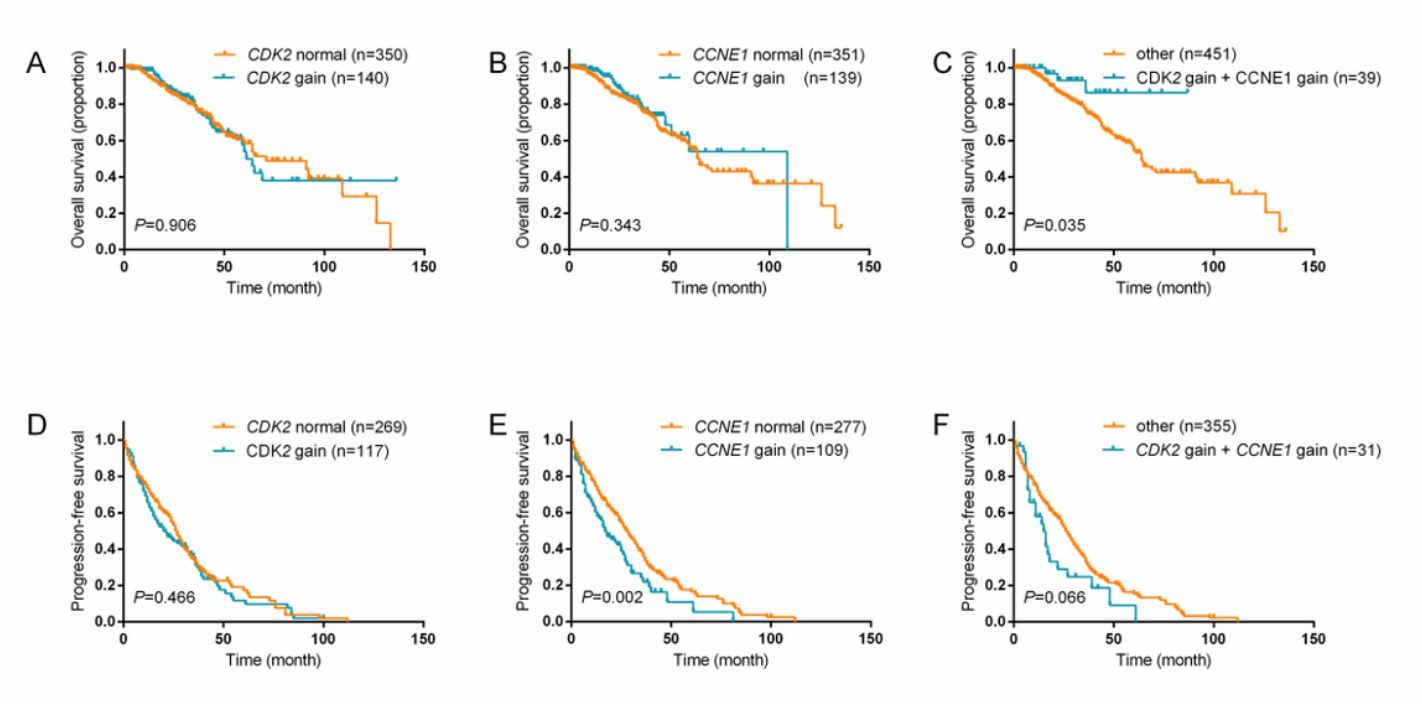

Figure 4

OS and PFS of acral melanoma patients in relation to CDK2 and CCNE1 CNVs. Fig.2A-2C showed OS of acral melanoma patients in relation to CDK2 and CCNE1 CNVs.Fig.2D-2F showed PFS of acral melanoma patients in relation to CDK2 and CCNE1 CNVs.

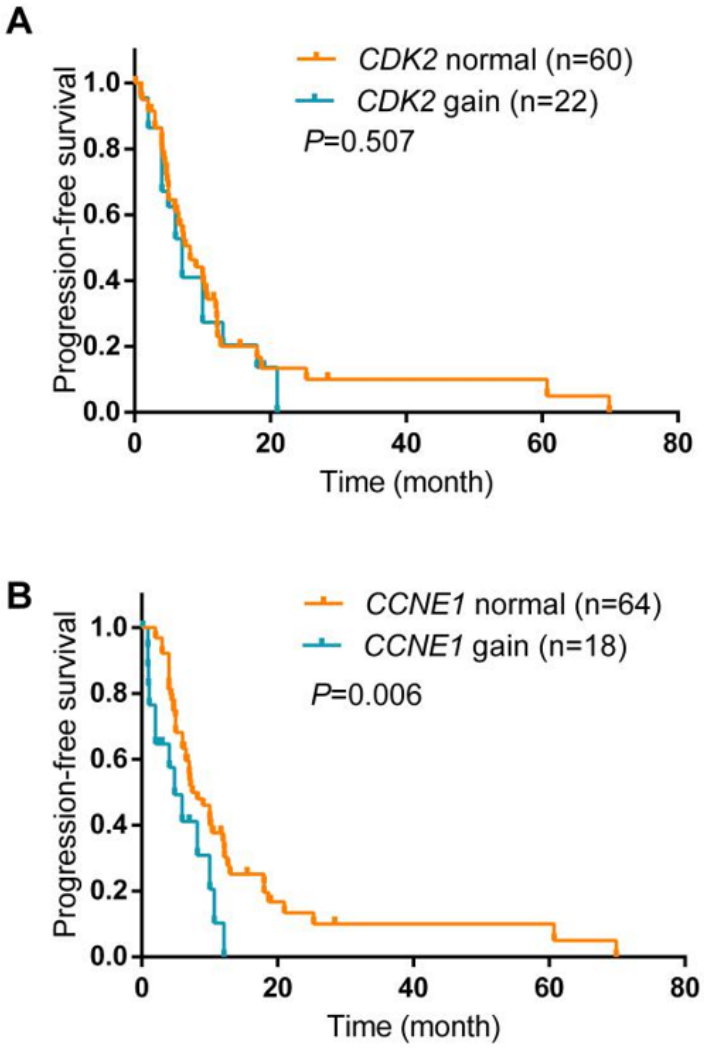

Figure 5

Effect of CDK2 and CCNE1 CNVs on Response to DTIC, DDP, and Endostar combined chemotherapy.Fig.3A showed the correlation between CDK2 copy number and PFS of melanoma patients received DTIC, DDP, and Endostar combined chemotherapy.Fig.3B showed the correlation between CCNE1 copy number and PFS of melanoma patients received chemotherapy.

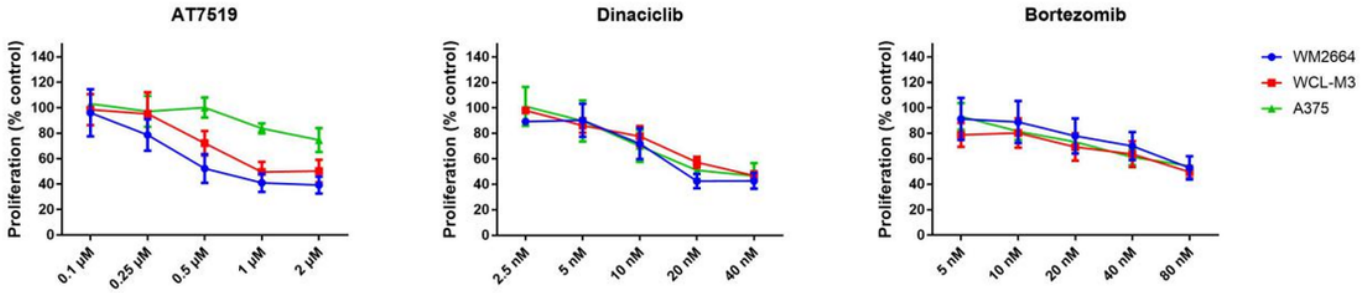

Figure 8

Sensitivity of melanoma cells to CDK inhibitor AT7519,Dinaciclib and proteasome inhibitor Bortezomib.Melanoma cells were treated with DMSO or indicated concentrations of inhibitors for 48 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 was determined by using one-way ANOVA (as compared to control group). The

WM2664 cell (with CCNE1 gain) and A375 cell (with CCNE1 normal) are cutaneous melanoma cells ,WCL-M3 cell (with CCNE1 gain) is acral melanoma cell.

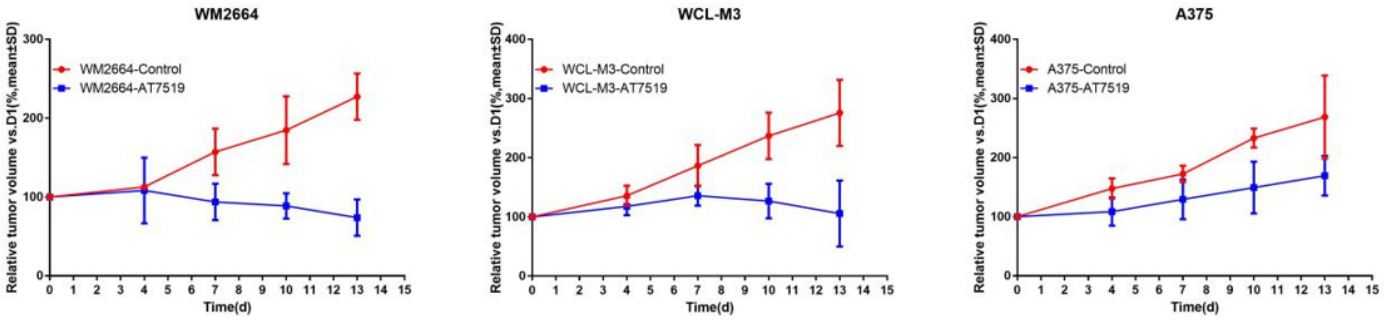

Figure 10

Sensitivity of CDX (cell-line-derived xenograf) to CDK inhibitor AT7519.Mice were randomized to treatment (CDK inhibitor AT7519 ,12 mg/kg in saline solution or control (saline solution) groups .The mice received intraperitoneal injection for 14 days.Tumor sizes were measured every 3 days and tumor volume used the formula:volume=1/2*length* width2.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTablesandFigures.docx
- SupplementaryTablesandFigures.docx