Analysis of Mucosal Melanoma Whole-Genome Landscapes Reveals Clinically Relevant Genomic Aberrations

Rong Zhou1,2,3, Chaoji Shi2, Wenjie Tao1,2,3, Jiang Li4, Jing Wu1,2,3, Yong Han1,2,3, Guizhu Yang5, Ziyue Gu1,2,3, Shengming Xu1,2,3, Yujue Wang1,2,3, Lizhen Wang4, Yanan Wang1, Guoyu Zhou1, Chenping Zhang1,2,3, Zhiyuan Zhang1,2,3, and Shuyang Sun1,2,3

Abstract

Purpose: Unlike advances in the genomics-driven precision treatment of cutaneous melanomas, the current poor understanding of the molecular basis of mucosal melanomas (MM) has hindered such progress for MM patients. Thus, we sought to characterize the genomic landscape of MM to identify genomic alterations with prognostic and/or therapeutic implications.

Experimental Design: Whole-genome sequencing (WGS) was performed on 65 MM samples, including 63 paired tumor blood samples and 2 matched lymph node metastases, with a further droplet digital PCR-based validation study of an independent MM cohort (n = 80). Guided by these molecular insights, the FDA-approved CDK4/6 inhibitor palbociclib was tested in an MM patient-derived xenograft (PDX) trial.

Results: Besides the identification of well-recognized driver mutations of BRAF (3.1%), RAS family (6.2%), NF1 (7.8%), and KIT (23.1%) in MM, our study also found that (i) mutations and amplifications in the transmembrane nucleoporin gene POM121 (30.8%) defined a patient subgroup with higher tumor proliferation rates; (ii) enrichment of structural variations between chromosomes 5 and 12 defined a patient subgroup with significantly worse clinical outcomes; (iii) over 50% of the MM patients harbored recurrent focal amplification of several oncogenes (CDK4, MDM2, and AGAP2) at 12q13-15, and this co-occurred significantly with amplification of TERT at 5p15, which was verified in the validation cohort; (iv) the PDX trial demonstrated robust antitumor effects of palbociclib in MM harboring CDK4 amplification.

Conclusions: Our largest-to-date cohort WGS analysis of MMs defines the genomic landscape of this deadly cancer at unprecedented resolution and identifies genomic aberrations that could facilitate the delivery of precision cancer treatments. See related commentary by Shoushtari, p. 3473

Introduction

Melanomas are tumors that originate from melanocytes with varying anatomic distribution, clinical features, and biological behaviors (1, 2). Mucosal melanomas (MM) are one of the deadliest subtypes of melanoma; as most MMs metastasize and/or recur after resection (3, 4). MM has significantly worse prognosis compared with other melanoma subtypes, with 5-year survival rates ranging from only 20% to 25% (5–7). It is also notable that MMs occur more frequently among Asian populations than among Caucasian populations (8, 9).

In contrast to cutaneous melanoma (CM), for which ultraviolet radiation that causes a predominant C to T nucleotide transition signature has been established as the major risk factor, the molecular pathogenesis of MM remains elusive (10). CM is the best genomically characterized type of melanoma, and the treatment of CM has benefited enormously from significant clinical breakthroughs in the last decade that have revolutionized its molecular classification and targeted therapy (11). Recent The Cancer Genome Atlas (TCGA) research has advanced our understanding of CM by defining four molecular subtypes of CMs based on the presence of specific driver mutations—BRAF-mutant CM, RAS-mutant CM, NF1-mutant CM, and triple wild-type CM—and there are now FDA-approved molecular-subtype-specific inhibitor drugs, for example, the BRAF V600E/V600K mutation as an indicator for responsiveness to vemurafenib treatment (12–14). Compared with CMs with multiple definite driver oncogenes, the genomic underlying of MMs has not been fully characterized.

Several studies have utilized array-based comparative genomic hybridization or whole-exome sequencing (WES) and targeted sequencing to investigate the mutational spectra of MM (15–18). Furney and colleagues (2013) conducted the first whole-genome sequencing (WGS) analysis of 5 MM samples (16). More recently,
Translational Relevance

Mucosal melanoma (MM) is one of the deadliest subtypes of melanoma, and it occurs more frequently among Asians than among Caucasians. The lack of understanding about the molecular pathogenesis of MMs has hindered genomics-era precision medicine advances that could help patients with this disease. Here, in the largest-to-date cohort whole-genome sequencing (WGS) analysis of MMs, we confirmed previously characterized genomic aberrations and highlighted the prognostic and therapeutic significance of recurrent genomic aberrations such as POM121 mutation/amplification and large-scale genomic rearrangements between chromosomes 5 and 12. Additionally, the patient-derived xenograft trial demonstrated robust antitumor effects of the FDA-approved CDK4/6 inhibitor palbociclib in MMs harboring CDK4 amplification. To our knowledge, this is the first cohort of xenograft models in which interpatient genetic heterogeneity was represented and has been used for targeted drug evaluation in MM. Thus, our study demonstrates how the genomic insights of MMs can inform patient stratification and precision cancer treatments.

the largest-to-date WGS study \( n = 183 \), published in 2017) of various melanoma subtypes included 8 MMs (19). In general, these studies found that MMs lack common molecular drivers known for CM and also lack UV-light related mutation signatures. MMs have lower overall mutational burdens than CMs. It is notable that detailed profiling of the genomic landscape, especially the characterization of large-scale genomic rearrangements such as structural variations (SV; deletions, duplications, inversions, translocations, etc.), has been limited in these small-cohort studies. Except for the well-recognized BRAF-activating mutation in all melanoma subtypes, the only gene that has been associated with the targeted treatment of MM patients is KIT (20–22). Imatinib is currently the only targeted drug for KIT-mutant melanomas recommended in Clinical Practice Guidelines of the National Comprehensive Cancer Network. Although imatinib sometimes conferred early disease control, the cancers of most patients ultimately progressed (23). Thus, patients diagnosed with this particularly deadly form of melanoma have not yet fully benefited from genomics-era advances in precision oncology.

Here, seeking to further characterize the molecular pathogenesis of this deadly subtype of melanoma, we describe the largest-to-date MM cohort \( n = 65 \) that has been used for genomic landscape profiling, with validation of the recurrent genomic pattern in an independent MM cohort \( n = 80 \). Guided by the molecular insights, a patient-derived xenograft (PDX) trial is conducted to evaluate the therapeutic efficacy of the selected targeted agent in a population-based PDX cohort. Together, these data provided insights into the recurrent genomic aberrations associated with clinical relevance and demonstrates how these insights could inform triage of patients for effective precision cancer treatments.

Materials and Methods

Tumor samples

Eligible patients had a diagnosis of either primary or recurrent MM, and none of the patients was treated by chemotherapy or radiotherapy before the operation. Tumor samples were collected from gingiva \( n = 28 \), hard palate \( n = 13 \), multisites \( n = 11 \); hard palate/lip and gingiva, and other site \( n = 13 \). Fresh-frozen tumor samples and blood samples were mainly obtained from Ninth People’s Hospital in Shanghai with written informed consent from each patient and research ethics board approval in accordance with the Declaration of Helsinki. After being checked by the two different pathologists (J. Li and L. Wang), the tumor tissues or regional lymph nodes were immediately frozen in liquid nitrogen and stored at \(-80^\circ C\). The validation cohort included 80 archived formalin-fixed paraffin-embedded (FFPE) MM clinical samples (diagnosed between 2004 and 2013), collected from gingiva \( n = 36 \), hard palate \( n = 29 \), multisites \( n = 5 \); hard palate and gingiva, lip/gingiva and buccal, and other site \( n = 10 \). Detailed clinicopathologic information for patients in the WGS and validation cohort is provided in Supplementary Table S1.

WGS processing

WGS was performed on Illumina HiSeq X10 instruments at the WuXi NextCode (https://www.wuxinextcode.com) facility in Shanghai with 150 base pair (bp) pair-end libraries. The average depth was \( 69 \times \) in the tumor samples and \( 35 \times \) in the normal samples. Sequence data were aligned to the hg19 (GRCh37) genome with BWA (v0.7.12-r1044); this generated a sorted level binary file (RAM). Mark duplication was performed with Sention (ref. 24; version 201611.02) on each BAM file. The reads were then realigned to the hg19 (GRCh37) genome with Sention to better identify the InDel mutations.

Single-nucleotide variant calling and Sanger sequencing

All somatic mutations were detected with each of the paired tumor-normal samples using Sention with the TNHaplotyper algorithm. All high-confident variants were annotated with ANNOVAR (ref. 25; hg19, v1.2) for consequence prediction and variant effect prediction. Two methods, MutSigCV (ref. 26; v1.4) and OncodriveFML (27; ref. v2.0.2), were used to identify significant mutated genes among all high-confident variants, with a threshold of \( q < 0.1 \). Sanger sequencing was conducted as previously reported (19), the PCR products were sequenced on an ABI-3500Dx Genetic Analyzer (Applied Biosystems) and data were analyzed with Sequence analysis (v5.4) using hg19 (GRCh37) genome as reference.

Copy-number variant detection

All somatic copy-number variant (CNV) determining was carried out with CNV kit (ref. 28; v0.8.3). Whole genome was split into bins with window size of 5k bp against each chromosome and the \( \log_2 \) ratio was estimated for each bin. Several bins would join together to a larger segment with a significance threshold of \( 1 \times 10^{-5} \) and the copy number of segment would be estimated. Genes with copy number at least three were defined as gain and no more than one were defined as loss.

SV detection

SVs were identified using Manta (ref. 29; v1.1.1) software, which identified all of the structural variants based on split reads and spanning paired reads, and classified them into five kinds: translocation breakend (BND), deletion (DEL), tandem duplication (TDUP), insertion (INS), and inversion (INV). Depending on...
the structural variant type and the copy-number status in breakpoints (30), all variants were further classified into eight groups: deletions, duplications, tandem duplications, fold-back inversions, amplified inversions, inversions, intrachromosomal, or interchromosomal translocations.

**Commonly mutated genes and pathways**

A manually curated list of commonly mutated tumor suppressor genes and oncogenes was created and analyzed. The frequency of SVs, CNVs, and single-nucleotide variants (SNV) were calculated for each gene. All genes were overlaid onto pathways defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) gene sets from MSigDB v6.0 (http://software.broadinstitute.org/gsea/msigdb).

**Detection of breakpoint clustering**

Chromosomes containing highly significant clustering distributions of breakpoints were identified as recently described (31) with a stringent threshold of $P < 0.00001$. Similarly, chromosomes with high numbers of translocations were identified with a minimum threshold of 10 translocation breakpoints per chromosome (32). Chromosomes that contained all these characters were categorized as clustered interchromosomal translocation. Chromothripsis was detected using Shatterproof (ref. 33; v0.14) with the default settings. Chromosomes with a maximum final score above 0.517 were considered positive (34). Breakage–fusion–bridge (BBF) was detected based on the evidence of fold-back inversions and telomere loss and inversions as previously reported (35).

**IHC**

IHC was performed on FFPE tissue sections of tumor samples and PDX xenografts. Briefly, 4-μm-thick sections were cut and processed for IHC detection using antibodies against Ki67 (diluted 1: 100, #M7240, Dako) and CDK4 (diluted 1: 200, #108357, Abcam). Slides were developed with 3-amino-9-ethylcarbazole (ECL, #K3464, Dako) peroxidase reaction and counterstained with Mayer’s hematoxylin. CDK4 expression evaluation of the tissue sections using commercial probes (ZytoVision GmbH). The colocalization status of CDK4 and TERT was examined by commercial custom probes (Empire Genomics). All fluorescence in situ hybridization (FISH) experiment procedures were performed according to the manufacturer’s instructions. The signal number of CDK4, CEN12, TERT, and CEN5 was scored by counting a minimum of 100 non-overlapping nuclei per slide. The related ratio and the average copy number of CDK4 and TERT were then calculated. Amplification was defined by examining first the CDK4/CEN12 ratio followed by the average CDK4 copy number. Nonamplification was defined as a CDK4/CEN12 ratio $< 2.5$ with an average CDK4 copy number $< 5.0$ signals per nucleus, whereas amplification was defined as a CDK4/CEN12 ratio $< 2.5$ with an average CDK4 copy number $\geq 5.0$ signals per nucleus or a ratio $\geq 2.5$ or an uncountable sample due to clustering of green signals.

**Fluorescence in situ hybridization**

CDK4 and TERT amplification tests were performed on FFPE tissue sections using commercial probes (ZytoLight SPEC CDK4/CEN12 Dual Color Probe and TERT/CEN5 Dual Color Probe, ZytoVision GmbH). The colocalization status of CDK4 and TERT was examined by commercial custom probes (Empire Genomics). All fluorescence in situ hybridization (FISH) experiment procedures were performed according to the manufacturer’s instructions. The signal number of CDK4, CEN12, TERT, and CEN5 was scored by counting a minimum of 100 non-overlapping nuclei per slide. The related ratio and the average copy number of CDK4 and TERT were then calculated. Amplification was defined by examining first the CDK4/CEN12 ratio followed by the average CDK4 copy number. Nonamplification was defined as a CDK4/CEN12 ratio $< 2.5$ with an average CDK4 copy number $< 5.0$ signals per nucleus, whereas amplification was defined as a CDK4/CEN12 ratio $< 2.5$ with an average CDK4 copy number $\geq 5.0$ signals per nucleus or a ratio $\geq 2.5$ or an uncountable sample due to clustering of green signals.

**Laser capture microdissection and droplet digital PCR**

The FFPE tumor tissues were cut into at least six sections (8 μm thickness) and mounted on PEN Membrane Glass Slides (Applied Biosystems). Sections were stained with hematoxylin and eosin to distinguish normal or nonlesional mucosa from melanoma. Genomic DNA was isolated from collected specimens using a QIAamp DNA FFPE Tissue Kit (#56404, Qiagen). Droplet digital PCR (ddPCR) was performed on a QX200 ddPCR system (Bio-Rad). RPP30 was used as the reference gene and its copy number. In brief, 10 ng of genomic DNA isolated from microdissected FFPE samples or xenograft samples was added into each well. After PCR amplification, droplets were sequentially counted for CDK4 or TERT or AGAP2 or MDM2 (FAM-labeled) or RPP30 (VIC-labeled). Data analysis was performed using QuantaSoft software version 1.6.6 (Bio-Rad). To set up a threshold to distinguish samples with amplified target genes, Target/Reference ratio in melanoma and in nonlesional mucosa tissues adjacent to MMs was calculated.

**PDX trial**

PDX model was established as we previously reported (36), and xenograft from established PDX model was trimmed into 20–30 mm³ fragments for subcutaneous implantation in nude mice (Shanghai SLAC Laboratory Animal Co.). Xenograft-bearing mice with an average tumor volume 150 to 250 mm³ were randomized into the control arm and the treatment arm on the basis of tumor size, tumor growth rate, and mouse bodyweight and were treated with vehicle (sodium lactate buffer, 50 mmol/L, pH 4.0, orally, daily) or palbociclib (Pfizer, orally, daily) at a dose of 120 mg/kg, 90 mg/kg, or 60 mg/kg in PDX models in a 28-day or a 56-day long-term treatment schedule. Tumor size and bodyweight measurements for mice were taken twice weekly using, respectively, a digital caliper and electronic scale. The percentage of tumor growth inhibition (TGI) was defined as $100 \times [1 – (TV_{i_treated}/TV_{i_control})/TV_{f_control}/TV_{i_control})]$, where $TV_{i}$ is the average tumor volume at the end of study and $TV_{i}$ is the average tumor volume at the initiation of treatment. For the PDX trial, each PDX model xenograft was transplanted in three mice, and the first mouse to reach 150 to 250 mm³ was assigned to the treatment group, the second to the vehicle group, and the third mouse was excluded, utilizing the one mouse per patient per treatment group setup described previously (37, 38). Treatment response was assessed by monitoring changes in tumor volume of the treated mouse and comparing to the tumor volume of the vehicle-treated mouse (treated/control %) using the following formulas: $T/C(\%) = ([TV_{i_treated}–TV_{i_control}]/[TV_{f_control}–TV_{i_control}])$. The drug response criteria were as follows: progression if $T/C > 50\%$, suppression if $T/C = 50–0\%$, and regression if $T/C < 0\%$. Animal care and experiments were performed under the approval and supervision of the Institutional Animal Care and Use Committee (IACUC) of the Shanghai Jiao Tong University School of Medicine.

**Western blotting**

Freshly frozen xenograft tissue was lysed using 1% SDS solution (Beyotime), containing 1% phosphatase and proteinase inhibitor cocktail (Bimake). Approximately 200 mg of xenograft tissue was homogenized and lysed using 0.5 mL of lysis buffer. The protein concentration of individual samples was determined with BCA Protein Assay Kit (Beyotime). An equal quantity of protein was separated by 10% SDS-PAGE and transferred onto a transfer
membrane (Millipore) and was blocked for 1 hour with 5% nonfat dry milk followed by incubation with primary and secondary antibodies. The antibodies against the following proteins were used: Rb (#ab24, Abcam), GAPDH (#2118, CST), and phospho-Rb (S807/811, #8516, CST). The chemiluminescence signal was developed with ECL-plus reagent (Beyotime) and detected by autoradiography.

Data deposition
The raw sequence data have been deposited in the Genome Sequence Archive in BIG Data Center, Beijing Institute of Genomics (BIG), Chinese Academy of Sciences, under accession number HRA000004. The Web link for these publicly available data (with controlled access on reasonable request) is http://bigd.big.ac.cn/gsa-human/s/sdtAbxY4.

Results
Clinical samples and genomic landscape of MMs
Patients eligible for this study had a diagnosis of either primary or recurrent MM, and none of the patients were treated with chemotherapy or radiotherapy before operation. The fresh-frozen MM specimens and matched blood samples were mainly obtained from the Ninth People’s Hospital in Shanghai between 2014 and 2017 (Supplementary Table S1). We performed high-depth WGS on a total of 65 samples (including 63 matched tumor and blood samples and two lymph node metastases). Analysis of SNVs across all tumors revealed a low overall mutation burden, with a median of 4.53 mutations per megabase (range, 0.26–34.56 mutations per megabase; Fig. 1A, all the coding SNVs and InDels are provided in Supplementary Table S1). In contrast, we detected a total of 60,129 somatic SVs and 225,665 CNVs among all tumors which were highly variable across each individual (Fig. 1B and C), with an average of 925 SVs and 3,472 CNVs per individual (range, 20–4,302 and 132–15,221, respectively).

We next used MutSigCV and OncodriveFML (q < 0.1) to identify significantly mutated genes, and sorted MMs based on the well-characterized driver genes (BRAF V600/K601, RAS family, and NF1 loss-of-function mutations) for CM (Fig. 1D; Supplementary Table S2). We found that only 10 of 65 MMs featured any of these mutations with clinical relevance for MMs that was the only gene other than KIT supported in both our MutSigCV and OncodriveFML analyses of our genomic data. We found 6 previously unidentified somatic mutations in POM121 in these 10 MMs, and 4 of these mutations, including two identical insertions, were validated by Sanger sequencing in 5 MMs (Fig. 2A; Supplementary Fig. S2). Notably, 1 of these 10 MMs also featured POM121 amplification; a total of 11 of the 65 MMs POM121 amplification (Supplementary Fig. S3). We then evaluated the clinical relevance of the recurrent genomic alterations in POM121 and found that patients with POM121 mutation (rather than amplification) tended to have worse clinical outcomes (median overall survival 9.0 vs. 25.0 months, log-rank P = 0.098; Fig. 2B). Consistent with its pathophysiologic functions observed in prostate cancer, we found that MMs with POM121 mutation or amplification had significantly higher tumor proliferation rates than MMs without POM121 alteration; measured as Ki67 expression index (fraction of Ki67-positive tumor cells >20%; 17/20 samples, 90.5% vs. 22/43 samples; 64.8%; P = 0.012, Fisher exact test; Fig. 2C). Representative antibody staining for Ki67 is shown in Fig. 2D.

SV pattern identified in MMs
Our WGS data from the MMs allowed us to comprehensively catalog large-scale structural rearrangements that are not detectable using exome sequencing data and array-based comparative genomic hybridization. We found that interchromosomal translocations constitute more than half of all the SVs (54.1%, 32,560 of 60,129). Notably, interchromosomal translocations (17%) and intrachromosomal translocations (27%) between or within chromosome (chr) 5 and chr12 accounted for 44% of all the detected SVs (Supplementary Fig. S4A and S4B). Clusters of breakpoints, which indicate complex genomic rearrangements, were found to be abundant across the chromosomes of most MM samples, and a recurrent pattern of clustered interchromosomal translocations between chr5 and chr12 was also observed (Fig. 3A). This pattern of SVs was similar to a phenomenon initially reported for ovarian cancer wherein a large number of interchromosomal translocations occurred for chromosomes that have a high number of clustered breakpoints (32). Both chr5 and chr12 are very strongly enriched for this clustered interchromosomal translocation SV trend (Fig. 3A). Additionally, manual review of the cirros plots as well as visual inspection using the Integrative Genome Viewer and Sanger sequencing further validated these observed SVs between chr5 and chr12 in two representative MM samples (MM023 and MM065; Supplementary Fig. S5).

Our finding that MMs feature a highly rearranged genomic profile suggested that there may be genetic mechanisms that can induce complex genomic rearrangements that could be driving the oncogenic process for this disease. Pursuing this idea, we examined the distribution in MMs of various known oncogenic genetic events (e.g., BFB and chromothripsis; Supplementary Figs. S4C and 56; Supplementary Table S4). We observed that the distribution of BFBs was significantly correlated with the distribution of clustered interchromosomal translocations (Kendall τ = 0.270, P = 0.032; Fig. 3B).

Prognosis of MM subgroups with recurrent SV pattern
The aforementioned WGS study of multiple melanoma subtypes also detected BFBs in 17.8% (32 of 178) melanoma samples, whereas the possible clinical significance of BFBs was not discussed (19). We here evaluated the prognostic significance the
observed clustered interchromosomal translocations and found that patients with clustered interchromosomal translocations between chr5 and chr12 showed significantly worse clinical outcomes (median overall survival 9.0 vs. 28.0 months, log-rank \( P = 0.047 \); Fig. 3C). In light of our speculations about the underlying oncogenic genetic events, it is notable that BFBs, but not chromothripsis, were tightly associated with worse prognosis (median overall survival 9.0 vs. 34.0 months, log-rank \( P = 6.9 \times 10^{-4} \); Fig. 3D). No correlation was detected between \( POM121 \) mutation and clustered interchromosomal translocations between chr5 and chr12 (Kendall correlation analysis; data not shown).

Note that for two of the patients in MM cohort, we also sequenced matched lymph node metastases, and the same SV- and CNV-driven genomic profile was also observed in the metastases, showing strong concordance with the primary tumor.

Figure 1.
Genomic profiles of MM. Each column represents an individual tumor that underwent WGS; MMs were grouped into two groups based on the classification of CM proposed by the TCGA: BRAF mutated or RAS mutated, NF1 mutated, triple wild type. A–C, The top panel shows the mutation burden of each tumor, whereas the middle and bottom show, respectively, the number of CNVs and SVs. Gain (≥3 copies) is represented in orange; loss (≤1 copy) is represented in green. D, Published melanoma driver genes and significantly mutated genes (bold font) identified by MutSigCV (marked with an asterisk) or OncodriveFML (marked with an octothorpe) in MMs. E, Clinical covariates including sex, age, risk factors (drinking and smoking), and tumor sites.
Supplementary Fig. S7; Supplementary Table S5). Perhaps future work should compare differences in BFBs and/or other differences between CM and MM to help deepen our scientific understanding of how these types of oncogenic genetic events influence metastasis and morbidity.

**CNV landscape and recurrently amplified genes in MMs**

Previous studies have examined the CNV features of MMs by using array-based comparative genomic hybridization and WES based on relatively small sample sizes (<20 samples; refs. 15, 17, 40). Our WGS data from a relatively large patient cohort provided

**Figure 2.**
Recurrent POM121 mutations identified in MMs. A, Map of POM121 functional domains and the sites of amino acid changes found by WGS. B, Patients with POM121 mutation tend to have worse prognosis. C, Ki67 expression of patients according to the mutational status of POM121. The *P* value was calculated by the Fisher exact test. D, Representative images of different staining levels of Ki67 expression in MMs. Scale bars, 100 μm; H&E, hematoxylin and eosin.
the first opportunity to robustly assess the genome-wide CNV landscape of MM. In accordance with the aforementioned reports (17, 40), we found that amplification of the 12q13–15 locus—which contains the well-recognized oncogene CDK4—in more than 50% of MMs (33 of 65 samples), making it the most commonly altered genomic region among all the MMs we examined (i.e., this was the region with the highest median of log ratio of read depth value; Fig. 4A; Supplementary Table S6).

GISTIC analysis was performed to define a more detailed CNV profile of MM by determining regions of significant recurrent gain and loss. We identified recurrent CNVs in regions with well-recognized cancer driver genes, including gain of 1p12 (NOTCH2), 4q12 (KIT), 5p15 (TERT), 11q13 (CCND1), and 12q13–15 (CDK4, MDM2, and AGAP2) chromosomal regions, and loss of the 9p21 (CDKN2A/B) and 17q15 (TP53) chromosomal regions among the MMs (Fig. 4B; Supplementary Table S6). Other melanoma-associated driver genes such as MET, BRAF, PDGFRA, and KRAS were also found to be recurrently amplified among the MMs (Supplementary Fig. S3).

**Correlation between the SV and CNV patterns**

Of particular note, amplification of 12q13–15 (containing CDK4) and 5p15 (containing TERT), which were highly significantly co-associated (Kendall τ = 0.608, P = 1.15 × 10^{-6}), was found to be the most significant regions of amplification (Q = 1.10 × 10^{-34} and 5.59 × 10^{-21}, respectively; GISTIC analysis; Fig. 4B). Validation of CDK4 and TERT copy-number alteration by FISH supported that the MM patients did indeed
Figure 4.
Genomic landscape of CNV and significant regions of recurrent copy-number alteration in MMs. A, The landscape of CNV in MMs. The orange line and the red block indicate copy-number gain, and the green line and blue block indicate copy-number loss. B, GISTIC analysis was performed to determine significant regions of recurrent CNVs, and significant oncogenes in these regions were labeled. C, FISH analysis of the copy-number alteration of CDK4 (top) and TERT (bottom) amplification in representative MM samples with CDK4 or TERT amplification. Scale bars, 5 μm. D, Concordance analysis of CDK4 status defined by CNV detection from the WGS data or by FISH analysis in 35 MM samples with enough tumor samples. The two methods showed a high degree of agreement (McNemar test $P = 1.000$, $\kappa = 0.828$, $P = 9.226 \times 10^{-7}$).
Zhou et al.

exhibit amplification (Fig. 4C, Supplementary Fig. S8A). High levels of concordance were observed between FISH analysis and WGS results when defining the copy number of CDK4 in 35 of the 65 samples (McNemar test P = 1.000, χ² = 0.828, P = 9.226 × 10⁻²⁷; Fig. 4D). Additionally, IHC with an antibody against CDK4 further demonstrated apparently increased accumulation of CDK4 significantly correlated with the amplification of CDK4 in these 35 samples (McNemar test P = 0.375, χ² = 0.708, P = 2.100 × 10⁻³⁵; Supplementary Fig. S8B–S8D).

Notably, we observed a striking similarity between the SV and CNV patterns: both of the amplified 12q13–15 and 5p15 regions demonstrated a significantly high number of interchromosomal translocations between chr5 and chr12 (P = 5.561 × 10⁻¹⁰ and 7.780 × 10⁻¹⁰, respectively, Wilcoxon matched-pairs signed rank test; Supplementary Table S4), and patients with concurrent amplifications of 12q13–15 and 5p15 were overrepresented among the patients with poor prognosis (from Fig. 3C). FISH analysis also validated the colocalization and amplification of CDK4 and TERT from chr5 and chr12 in representative samples (Supplementary Fig. S9). It is notable that this situation in MM appears to fit with an intriguing previously proposed idea, which holds that some oncogenic genetic events such as BFB can both drive interchromosome translocation between chromosomes and simultaneously increase gene copy numbers on the translocated chromosomal segments (32, 35).

Characterization of the identified genomic pattern in an independent MM cohort

To further support this genomic pattern observed between chr5p15 and chr12q13–15 and validate the detected amplified genes within these two regions, we retrospectively collected a validation cohort of 80 MM FFPE samples and used laser capture microdissection to differentially sample MM versus nonlesional mucosa tissues (Fig. 5A). We used PCR probes targeting TERT, as a proxy covering 5p15, and used probes targeting CDK4, MDM2, and AGAP2 as proxies covering 12q13–15. An averaged copy-number ratio for these target genes was calculated for each of these genes relative to the reference gene RPP30, and a cutoff value of >3 standard deviations above the mean (compared with the PCR data from the nonlesional mucosa samples) was used to define samples as positive for the amplification of a given target gene in an MM sample.

Consistent with the genomic features we demonstrated in the WGS MM cohort, amplifications of TERT, CDK4, MDM2, and AGAP2 were found, respectively, in 65% (52 of 80), 78.75% (63 of 80), 50% (40 of 80), and 48.75% (39 of 80) of the melanoma tissue samples (Fig. 5B–F). Additionally, concurrent amplifications of TERT with one of these three genes (CDK4, MDM2, and AGAP2) located on chr12 were found in 55% (44 of 80), 38.75% (31 of 80), and 40% (32 of 80) of samples, respectively. Spearman correlation analysis indicated that the copy numbers of TERT that were significantly correlated with the copy numbers of each of the three genes located on chr12 (r = 0.380, P = 0.001 for TERT and CDK4; r = 0.339, P = 0.002 for TERT and MDM2; r = 0.477, P = 8.00 × 10⁻⁶ for TERT and AGAP2). Thus, this follow-up analysis of an independent MM cohort showing nonrandomly amplified oncogenes located on chr5p15 and chr12q13–15 apparently recapitulated the MM genomic features initially identified in our WGS–data–based discovery, suggesting potential functional relevance of these coamplified oncogenes.

Evaluation of palbociclib in a population-based PDX trial

Of these coamplified oncogenes, CDK4 is the most well-recognized therapeutic target, and we selected palbociclib, the first FDA-approved anti-CDK4/CDK6 inhibitor, for evaluation. A pilot experiment exploring the therapeutic efficacy of palbociclib in PDX-MM011 (a CDK4–amplified model; Supplementary Fig. S10A) demonstrated dose-dependent efficacy of palbociclib in 4 weeks of treatment, with TGI percentages ranging from 46.02% at a dose of 60 mg/kg to 76.08% at a dose of 90 mg/kg (Fig. 6A and B). Palbociclib administered at a 120 mg/kg dose was not well tolerated by the mice (bodyweight loss >15%). Palbociclib provided well-tolerated and sustained tumor suppression (TGI = 60.31% at the dose of 60 mg/kg, TGI = 87.05% at the dose of 90 mg/kg) throughout the 8 weeks of drug administration of the pilot experiment (Fig. 6A and B).

Next, to evaluate the therapeutic efficacy of palbociclib across a genetically heterogeneous MM population, we conducted a PDX trial with 24 PDX models established from 24 MM patients using one mouse per patient per treatment group (1 × 1 × 1) treatment format (37). ddPCR was applied to characterize the CDK4 and TERT copy numbers of each model: 14 of the models featured CDK4 amplification, 13 of the models featured TERT amplification, and concurrent amplification of CDK4 and TERT was observed in 9 models (Supplementary Fig. S10B). We used a palbociclib dose of 90 mg/kg for a total of 8 weeks, and the drug response was defined as progression, suppression, and regression. Among this MM PDX cohort, 37.5% (9/24) of the models, including 2 models as regression, 7 models as suppression, (PDX-MM014), or progression (PDX-MM002), were used for additional palbociclib efficacy validation with relatively large sample sizes in each treatment arm (n = 7–12 mice per treatment group). Treatment with a 90 mg/kg palbociclib dose for 4 weeks caused significant tumor inhibitions in PDX-MM081 (TGI = 104.37 %) and PDX-MM014 (TGI = 62.21%); palbociclib caused no significant effect in PDX-002 (TGI = 18.34%; Fig. 6E–G). Western blot analysis revealed decreased Rb phosphorylation in the xenografts treated with palbociclib when compared with samples treated with vehicle control in PDX models that were responsive to the treatment (Supplementary Fig. S10C). Thus, our research using a genetically annotated PDX cohort representing molecularly defined MM patient subgroups illustrated the potent therapeutic efficacy of palbociclib. Genotype and drug response correlation analysis (indicated by the PFS comparison between cases with or without CDK4 amplification), as well as the follow-up study using large sample size treatment arms, further demonstrate that MMs harboring CDK4 amplification are more likely to benefit from palbociclib treatment.
Discussion

The present study illustrates the power of how a large patient cohort collected mainly from one anatomic site/oral cavity—can be examined with modern genomics analyses that yielded scientific insights about the molecular pathogenesis of the disease in question and suggest candidate strategies for precision therapies which have a high probability of success. It bears mention that, owing to the location and morphology of most MMs (which exhibited a restricted flat lesion within the mucosal lining) and the danger of causing functional impairment of the affected organ, the collection of fresh tumor tissue that meets the quality and quantity needed for WGS is quite difficult and extremely time-consuming. In addition to the profound differences in the genomic feature of MM that we depicted when compared with CM, note that our unprecedentedly high-resolution analysis of the genomic landscape of MM also revealed particular significantly mutated gene, as well as the unique SVs and correlated CNVs profligies with prognostic and therapeutic relevance.

Specifically, we identified recurrent mutations of POM121 (n = 10), a previously unappreciated gene in melanoma, was the only significantly mutated gene in MM other than KIT supported by both our MutSigCV and OncodriveFML analysis methods. During the preparation of this article, Rodriguez-Bravo and colleagues first reported an oncogenic role for POM121 in enhancing in prostate cancer tumor aggressiveness through interaction with
importin β to promote the nuclear import of MYC, E2F1, AR, and GATA2 (41). Furthermore, they demonstrated that targeting a POM121–importin β axis using the importin β inhibitor impotazole decreases the growth of patient-derived prostate cancer xenografts. Our results contribute to the emerging evidence about the oncogenic role of POM121, as we found that patients with POM121 mutations tend to have worse prognosis and we observed that MMs with either POM121 mutation or POM121 amplification had significantly higher tumor proliferation rates. Clearly, further research will be necessary to determine the pathomechanistic role of POM121.

Considering that about 98% of the human genome is noncoding DNA and that somatic mutations in noncoding regulatory regions are known to be major drivers of carcinogenesis (42).
example, mutation in the TERT promoter that drives TERT overexpression occurs as an early tumorigenic event in >70% of CMs (43). Of note, we here detected 10 mutations (including two highly recurrent hotspot mutation sites in the TERT promoter: C228T and C250T) in 9 tumor samples (9 of 65, 13.8%) and further verified with targeted Sanger sequencing of the TERT promoter region in all 65 tumor samples (Supplementary Table S2). Telomere length estimation analysis using qMotif tool was also conducted (19), whereas the telomere length was not correlated with other driver genetic lesions, including TERT promoter mutation, TERT amplification, ATRX loss, TP53 loss, CDK4 amplification, POM121 mutation/amplification, KIT mutation, and chr5–12 clustered CTX (Mann–Whitney U test; data not shown). Additionally, we identified some candidate driver loci with significantly mutated sequences in noncoding regions and illustrated the mutation signature of MM as previously reported (19, 44), which could direct future hypothesis-driven research about the biological basis of this deadly melanoma (Supplementary Figs. S11 and S12; Supplementary Table S2).

Previous studies have reported that melanomas that arise from sun-protected tissues (e.g., MM, acral melanoma) have similar genomic features to MM, including a high frequency of CNVs and SVs (16, 19). Our WGS results further identified oncogenic genetic events resembling BFB in 24 of 65 MM samples, which are potentially involved in the recurrent localized genomic rearrangements between these amplified regions of chr8 and/or chr12. In acral melanomas, enriched clustered breakpoints indicative of localized genomic rearrangements were found to be more enriched in chr11, chr5, and chr17, whereas no such recurrent pattern was observed in CM (with significantly less SVs) or MM (possibly owing to the small sample size included in the study or owing to racial disparities; ref. 19). Thus, our characterization of the recurrent oncogenic pattern of MM supports that MM is rightly conceptualized as a subtype of melanoma distinct from CM and distinct from acral melanomas.

More recently, Ablain and colleagues used a zebrafish modeling approach and confirmed that SPRED1 loss (which was also observed in 24.6% samples in this study; Supplementary Fig. S3), especially in tumors driven by KIT mutation, is a driver alteration in MM (18). The MM zebrafish models, which can model different genotype combinations, significantly furthered our understanding of the mechanisms of MM development. In the present study, after our data-driven target definition and selection of palbociclib, a population-based PDX trial was conducted to test the efficacy of this FDA-approved CDK4/6 inhibitor. To our knowledge, this is the first cohort of MM PDX models in which interpatient genetic heterogeneity was represented and has been used for targeted drug evaluation. Our work offers a demonstration of how truly genome-scale precision medicine (here, at the SV level) can successfully inform therapeutic strategies, at least at the preclinical PDX model drug evaluation stage. We anticipate the launch of a novel genotype-driven precision clinical trial of palbociclib in MM in the very near future, and are also exploring the use of importin β inhibitors in treating MM.

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

Authors' Contributions
Conception and design: R. Zhou, Z. Zhang, S. Sun
Development of methodology: J. Li, L. Wang, Z. Zhang, S. Sun
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Han, G. Yang, Z. Gu, S. Xu, Y. Wang, L. Wang, Y. Wang, G. Zhou
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Zhou, W. Tao, J. Li, J. Wu
Writing, review, and/or revision of the manuscript: R. Zhou, C. Shi, W. Tao, J. Wu, C. Zhang, Z. Zhang, S. Sun
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Shi, W. Tao, J. Li, J. Wu, Z. Zhang, S. Sun
Study supervision: C. Zhang, Z. Zhang, S. Sun

Acknowledgments
The authors greatly thank Dr. Hao Chen and Jiaochun Shi (from WuXi NextCode, WuXi AppTec, Shanghai, China), and Dr. Feng Zhang (from Fudan University) for assistance with bioinformatics analyses. The authors greatly thank Dr. Bosong Wang (from Shanghai Jiao Tong University School of Medicine) and Dr. Yibiao Zhou (from Fudan University) for statistical analysis. The authors thank Shiqiang Wang (from the animal lab of Shanghai Ninth People’s Hospital of Shanghai Jiao Tong University School of Medicine) and Zhongbiao Xiao (from the animal lab of Shanghai Jiao Tong University School of Medicine) for animal studies. The authors greatly thank the Genome Sequence Archive (GSA) team of BIG Data Center for their help in data archiving. This work was funded by the National Key Research and Development Program of China (2017YFC0908500) and by grants from the National Natural Science Foundation of China (81572656 and 81202131), the China Postdoctoral Science Foundation (2013M531191), the Shanghai Postdoctoral Sustentation Fund, China (13R2141500), and the Shanghai Summit and Plateau Disciplines.

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 October 19, 2018; revised January 11, 2019; accepted February 14, 2019; published first February 19, 2019.


Analysis of Mucosal Melanoma Whole-Genome Landscapes Reveals Clinically Relevant Genomic Aberrations

Rong Zhou, Chaoji Shi, Wenjie Tao, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-18-3442

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2019/02/19/1078-0432.CCR-18-3442.DC1

Cited articles
This article cites 43 articles, 6 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/25/12/3548.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/25/12/3548.full#related-urls

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, use this link http://clincancerres.aacrjournals.org/content/25/12/3548.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.