# Genomic Heterogeneity and Branched Evolution of Early Stage Primary Acral Melanoma Shown by Multiregional Microdissection Sequencing



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Acral melanoma (AM) is an extremely aggressive subtype of melanoma that is prevalent in eastern Asia. AM exhibits high intertumoral and intratumoral heterogeneities with poor prognosis. To associate the genomic heterogeneities with phenotypic traits and efficacy of treatments, a method is needed to recover genomic information from limited samples with high specificity and sensitivity from early stage AM specimens. We performed laser capture microdissection to isolate single micro-tumor nests, containing only dozens of cells, from stained tissue slices and then applied multiple annealing and looping-based amplification cycles, a highly efficient whole-genome amplification method originally developed for single cells, to amplify the whole genome of each tumor nest for sequencing. We were able to accurately profile the landscape of copy number alterations and single nucleotide variations of every single micro-tumor nest and to quantitatively characterize the heterogeneities at different levels, between tumor and nevi, among patients, among different phenotypes within a same tumor, and among adjacent tumor cell clusters with identical phenotypic appearance. We have found that genomic heterogeneity exists extensively and that branched evolution happens in the early stage of AM development. We are able to build the phylogenetic tree among these phenotypically addressable cell clusters.

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#### **INTRODUCTION**

Acral melanoma (AM) is an aggressive subtype of melanoma that has generally poor prognosis and is common in nonwhites (Lv et al., 2016). Although there have been genomic studies of other subtypes of melanoma (Bastian, 2014; The Cancer Genome Atlas Network, 2015), the divergent clinical and epidemiological features of AM suggest its unique genomic background. First, AM occurs in regions of the extremities that are not frequently exposed to mutation-causing UV (Parkin et al., 2005; Pfeifer et al., 2005). Second, progression from existing nevi, a preexisting mutational field, into AM happens at very low frequency (Phan et al., 2007). Third, genetically, melanoma is a remarkably heterogeneous cancer class among all cancers (Curtin et al., 2005); AM has divergent mutational profiles compared with other melanoma subtypes and intrinsically high genomic heterogeneity (Hayward et al., 2017). Cancer heterogeneity is a complex phenomenon that manifests at all levels of the disease, from diverse mutations in the patient population (Alexandrov et al., 2013; Kandoth et al., 2013), to differing mutations found in multiple tumors in the same patient (McGranahan and Swanton, 2017; Murtaza et al., 2015), to subclonal genetic diversity in the same single tumor (Gerlinger et al., 2012; Jamal-Hanjani et al., 2017). Among them, intratumoral heterogeneity is difficult to detect and measure through conventional inspection of biopsy samples or pathology sections; therefore, it has been largely underestimated and neglected. Intratumoral heterogeneity is generated by the tumor's genetic instability, microenvironmental cues, or oncogenic signaling (Burrell et al., 2013) and, in turn, fosters tumor adaptation in a branched evolutionary process (Marusyk et al., 2012). Intratumoral heterogeneity, as well as the resulting tumor adaptation, results in drug resistance and ultimately treatment failure (Junttila and de Sauvage, 2013). In melanoma, studies have already shown that significant inter- and intratumoral heterogeneities may be associated with resistance to single small molecule inhibitor monotherapy (Kemper et al., 2015) and to

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Abbreviations: AM, acral melanoma; CLI, copy number alteration level index; CNA, copy number alteration; DOH, degree of heterogeneity; L, level; LCM, laser capture microdissection; MALBAC, multiple annealing and looping-based amplification cycles; Mb, mega base pair; SNV, single nucleotide variation; TCGA, The Cancer Genome Atlas

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immunotherapy (Menzies et al., 2014). Thus, in addition to understanding disease pathogenesis, characterizing the genomic heterogeneity of AM is also a critical step toward achieving personalized therapy.

Intratumoral heterogeneity is considerable at advanced stages of melanoma (Bastian, 2014; The Cancer Genome Atlas Network, 2015), but studies of early stage AM's heterogeneities are scarce because of challenges in analyzing limited numbers of biopsy samples. Recently, single cell genomic analysis has enabled deciphering of tumor heterogeneity at the finest resolution (Navin et al., 2011; Navin, 2014). However, many attempts are unable to link the genomic data with pathological data because of the loss of spatial information during tissue dissociation (Meacham and Morrison, 2013). This calls the integrated analyses of both physical traits and genomics to further decipher how these heterogeneities contribute to the tumorigenesis process and to help identif therapeutic targets (Viros et al., 2008).

We developed a workflow to retrieve and profile cells from multiple sites in a single micro-tumor nest. Specifically, we applied laser capture microdissection (LCM) to precisely dissect single tumor nests with high cellular purity, each yielding 20-50 cells; subsequently, we performed whole-genome amplification using multiple annealing and looping-based amplification cycles (MALBAC) (Zong et al., 2012). LCM ensured a higher purity of acquired tumor cells, even from early stage tissue samples. Using MALBAC ensured highly uniform and efficient amplification of whole genomes from samples that contained merely dozens of cells (Ni et al., 2013). To our knowledge, such a workflow has not been previously implemented for AM, and we have expanded our understanding of the complexity and evolution of primary AM by accurately profiling the landscape of copy number alterations (CNAs) and single nucleotide variations (SNVs). This quantitative assessment greatly advances our basic understanding of AM etiology and will facilitate the development of tools for early diagnosis, precision treatment, and prognosis for patients.

#### RESULTS

# Hierarchical heterogeneity of primary AM guides sample preparation

The process of microdissection on a single tumor nest is shown (Figure 1a-d). The heterogeneity of primary AM is extremely complex and should be surveyed from diverse perspectives. For this reason, we classified the heterogeneity into four categories (Figure 1e), ranked by the diversities in malignancy, patients, and phenotype of tissues. Level (L) 1 is the difference between pathologically normal tissue (nevus) and lesion (early stage primary AM). The genomic differences at this level not only provide possible clues for early diagnostics but also serve as a reference for comparing heterogeneities among tumor samples. L2, the most commonly mentioned intertumoral heterogeneity, is the genomic variation among patients. This intertumoral variation is mostly relevant in personalized treatment decisions. Both L3 and L4 are intratumoral heterogeneities, that is, the genomic variations within the same primary tumor. L3 focuses on the genomic variations among physical regions with distinct phenotypical differences; L4 describes variations among two microscopically adjacent cell clusters within the same tumor in one individual patient, even though they have identical gross and pathological appearance. L3 heterogeneity has recently been studied in various other cancers, with the goal of investigating their pathogenesis and evolution. However, L4 heterogeneity has rarely been studied because of experimental challenges.

To generate the data set that can display all aspects of the heterogeneity categories, we enrolled seven patients (Figure 1f). The patient information is listed in Supplementary Table S1 (online) and the images of all the tumor nest samples are available in Supplementary Table S2 (online). Each patient had at least one nevus and two adjacent tumor samples. For two patients (P1 and P3), we were able to identify multiple phenotypes (dark patch, regression, or nodular) from the same tumor. In total, we collected 41 microdissected samples (Figure 1f), among which 22 were microscale tumor cell clusters from three phenotypes, 10 were microscale samples from nevi, and 9 were from normal tissues and were used as controls. All samples were demographically and pathologically characterized before LCM. The tumor samples each got dissections with an average size of approximately 0.01 mm<sup>2</sup> and each containing approximately 20-50 cells. We collected two adjacent tumor cell clusters for each phenotype, allowing us to profile the L4 heterogeneity and also providing biological duplicates for assessing the L3 heterogeneity.

# L1 genomic variation: the distinguishable CNA patterns between nevi and melanoma

We found that CNA profiles of nevi are almost identical to those of normal skin tissues (see Supplementary Figures S1 and S2 online), indicating that in nevi, nearly all melanocytes' genomes remain intact and normal. In contrast, the CNA profiles of all tumors (see Supplementary Figure S3 online) significantly differ (P < 0.001, Student t test) from their normal tissue counterparts, indicating severe structural variations in melanoma. To quantify this difference, we calculated the CNA level index (CLI), which is the ratio of significant CNA regions in the whole genome (see the Supplementary Materials and Methods online). In general, higher CLI values reflect a more severe stage of disease (Figure 2a), which is consistent with the view that more structural variation events continuously accumulate as the tumor progresses. Among all seven patients, melanoma had a mean CLI value of 5.4%; and six out of seven nevi samples showed a mean CLI near 0. The nevi sample captured from P3 (P3\_N1) exhibited a significantly different CLI (0.9%) and a slightly noisier CNA profile compared with nevi samples from other patients. This sample also showed several small CNAs such as gains in chromosomes 8 and 22 and losses in chromosome 1 (see Supplementary Figure S1). These signatures could indicate premelanoma, which has been challenging to identify in clinical practice through morphological assessment, and illustrate the potential power of accurate diagnosis of early melanoma with an integrated morphology-genomics approach.

## L2 genomic variation: CNA heterogeneity among patients

We compared the CNA profiles of tumor samples (Figure 2b) with those from The Cancer Genome Atlas (TCGA) Skin Cutaneous Melanoma project (TCGA Network, 2015). Some hotspot CNAs could be observed, including gains of chromosome arm 1q and chromosomes 7, 8, and 22 and losses of chromosome arms 6q, 9p, and 11q (see Supplementary Materials and Methods). We further analyzed 17 genes that



**Figure 1.** Microdissections of primary melanoma and list of patient samples list. (a) Clinical images of primary tumors. The site marked by the red circle was cut and sliced into serial sections for staining. Scale bar = 5 mm. (b) Immunohistochemical staining with Melan-A (Abcam, Cambridge, UK). (c) The section adjacent to that pictured in **b** was stained with hematoxylin and eosin to visualize pathology. (d) Image after laser microdissection of **c**. Scale bar = 100  $\mu$ m in **b**–**d**. (e) Diagrams illustrating the four levels of CNA heterogeneity. (f) Information of patients and samples. Staging of the disease was determined according to the American Joint Committee on Cancer staging system. CNA, copy number alteration; F, female; L, level; M, male.

were associated with CNAs in melanoma (Figure 2c) and found that most of them exhibited great diversity. For example, the copy number gain of 11q13, where CCND1 (cyclin D1) is located, was previously reported in AM (Bastian, 2014) but was not significantly reflected in the TCGA data set, possibly because of low representation of acral types in the data set. In our results, four of the seven patients investigated (P1, P3, P5, and P6) had significant copy number gains in 11q13 (CN>3), and especially for P1, the copy number exceeded 10. Two patients (P4 and P7) showed reduced copy number in 9p21.3, where CDKN2A is located, which is a signature for metastatic events (Bastian, 2014). Patient P7 had copy number loss in chromosome 10, which encompasses the PTEN gene. Such heterogeneity of CNA also clearly indicates the diversity of melanoma and the diversity in treatment response and prognosis. From these results, we further speculate that CNA analysis in primary tumors can be used to aid precise diagnosis. For example, patients P2, P3, and P7 were all classified as having in situ stage melanoma by conventional pathology, but P7 actually exhibited more severe CNA events (CLI = 6.1%), including the 9g gain, that could indicate a more dangerous tumor subtype.

# L3 genomic variation: intratumoral CNA heterogeneity among phenotypically different lesions

Patient P1 had three types of tumor sites with distinct gross appearance and pathological features: dark patch, regression,

and nodule (Figure 3a). The dark patch lesion had characteristic radial growth pattern of melanoma cells; the regression type exhibited obvious infiltration of inflammatory cells and melanophages around melanoma cells; and the nodule showed vertical growth pattern of melanoma cells. CNA analyses showed both similarities and diversities of these phenotypically different lesions. All samples had copy number losses at chromosomes/chromosome arms 6q and 16q, gains at 7 and 22q, and disorders at 11 and 17, indicating their common predecessors. The technical noise is characterized by a 95% confidence interval (see Supplementary Materials and Methods and Supplementary Figure S4 online). Thus, the true biological variation can be confidently retrieved in pairwise between the samples with different phenotypes (Figure 3b). The results depict that gains in chromosomes 1 and 2 are shared in dark and regression and that nodule has a unique gain in chromosome 8. There also exist subtle but valid copy number variances between dark and regression phenotypes. These observations are helpful in generating the evolution course among samples.

# Super-CNAs are potential indicators of phylogenetic relationship

We observed that whole-genome sequencing data of melanoma LCM samples, with high cellular purity, exhibited several dramatic CNA events, some with copy numbers as high as 10 at a size of several to tens of mega base pairs (Mb).



**Figure 2.** Overview of CNAs from all seven patients. The patients are ordered by progressed disease stages. (a) The copy number density distribution of nevus and melanoma samples for each patient. The CLI (percentage of bins with significant CNAs) (see Methods section) are listed to the right of each plot for both the nevus (blue) and melanoma (red). (b) Heatmap showing copy numbers for all melanoma samples. The gain-and-loss ratios for each bin in TCGA Skin Cutaneous Melanoma project samples were plotted for comparison. (c) The copy numbers of representative melanoma-associated genes. Genes are ordered by the median copy number across all samples. The corresponding copy numbers in TCGA Skin Cutaneous Melanoma samples are represented in background boxplot. CLI, copy number alteration level index; CN, copy number; CNA, copy number alteration; P, patient; TCGA, The Cancer Genome Atlas Network.

These unusually high copy numbers, which may be underestimated in other studies because of their lower sample purity, were also highly heterogeneous among samples. We defined these high copy number variations, *super-CNA*, as those CNA events having at least three 1-Mb bins in a 20-Mb window with copy number larger than 6. Patient P3 had two types of melanoma with differences in their appearance: dark (P3\_1, P3\_2) and regression (P3\_3, P3\_4). These two tumor sites had common super-CNAs at chromosomes 11 and 22, whereas the dark sample exhibited a unique super-CNA at chromosome 12 and the regression sample a unique super-CNA at chromosome 5 (Figure 4a and b). The super-CNA

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**Figure 3.** The intratumoral heterogeneity of CNA in patient P1. (a) Three types of tumor sites were analyzed, each with two biological replicates: dark patch (D1/2), regression (R1/2), and nodule (N1/2). (Left) Hematoxylin and eosin staining and immunohistochemical staining images of each type. (Right) Scatterplots showing CNAs for normal skin, nevi, and six melanoma samples. Scale bar = 1 cm for tumor images. Scale bar =  $200 \,\mu$ m for staining images. (b) The pairwise comparison of copy number among the three types with technical noise level displayed in background (grey dots). The valid copy number differences exceeding the technical noise range are labeled and colored by originating chromosome. chr, chromosome; CN, copy number; CNA, copy number alteration.

in chromosome 1 further differentiated the two regression samples. We found that, by using super-CNAs alone, a phylogenetic relationship of these cell clusters could be robustly constructed (Figure 4c).

# L4 genomic variation: intratumoral heterogeneity among adjacent tumor cell clusters

We hypothesized that, in the highly heterogeneous tumors such as melanoma, even tumor cell clusters in very close proximity to each other would harbor significantly different genomic variations. From a single tumor, we carefully isolated cell clusters in close proximity to each other and with identical gross and pathological appearance. Adjacent cell clusters with a distance of 100 to 200  $\mu$ m apart in the same tumor superficially showed similar CNA patterns. However, subtle differences were found upon in-depth inspection (Figure 5c). For patient P2, one tumor nest showed copy

number gain in chromosome 6, whereas the other had copy number loss in the same region. In patients P6 and P7, we also noticed the difference of copy number in chromosome 1 from two adjacent cell clusters. These small CNAs, from a few Mb to hundreds of Mb in size, are easily obscured by the larger regions of apparent genomic similarity among microscopically adjacent melanoma cell clusters, and difficulty in identifying these subtle differences could be one reason why molecular typing and diagnostics of melanomas is so challenging.

#### Hierarchical heterogeneity of CNA in melanoma

Although each level shows distinct CNA diversity among samples, the degree of diversity is not the same in these levels. The quantification of degree of heterogeneity (DOH) involves two aspects, the extent and the intensity. The copy number profiles between two samples are compared, and the ratio of genomic



**Figure 4. Super-CNAs and their utility in reconstructing a timeline of tumorigenesis and evolution. (a)** A scatterplot of the copy numbers for dark patch (P3\_1&2) and regression (P3\_3&4) phenotypes of the tumor from patient P3, with the super-CNAs labeled by arrows. **(b)** The super-CNAs and CNA events table of the four samples. **(c)** The inferred phylogenetic tree of tumor samples from the super-CNA events. chr, chromosome; CNA, copy number alteration; P, patient.

regions or bins showing deviations greater than 1 between them are used as the index for the extent of heterogeneity (Figure 5a). In our small cohort, there was at least one pair for L1 and L4 samples for each patient, whereas only patients P1 and P3 had two or more apparently different tumor phenotypes to provide quantification of L3 DOH. L2 DOH quantification was done by crossmatching samples between different patients. The technical replicate pairs were also included to estimate the level of technical noise. The highest DOH was observed in L2, and the lowest was in L4. Moreover, in L4, DOH was significantly higher than the technical noise, validating the existence of distinct heterogeneity among adjacent tumor cell clusters (P = 0.0027, Student t test). In addition, L1 and L3 DOHs are comparable. We also quantified the heterogeneity intensity by using the top 10 percentile of copy number differences in pairwise comparison, and a similar distribution result was identified (Figure 5b).

### Single nucleotide variations

Identification of SNVs is challenging for the minute amount of DNA samples because of the various types of errors and artifacts introduced during library preparation. Whole-exome sequencing is performed for each tumor sample (see the Methods section). We first filtered out sequencing and alignment errors and then further reduced the amplification errors and DNA damage by using the consensus SNVs from two or more biological or technical replicates (Figure 6a). Ultimately, we identified 515 SNVs from all seven patients. We calculated the percentage of C-T/G-A mutations among all SNVs for each patient. Although C-T/G-A is overall more frequent compared with other mutations, contrary to TCGA data sets that describe Western populations, it is not always the most dominant mutation type in each patient (Figure 6b). This is expected, because AM is not caused by sunlight/UV exposure-induced DNA damage.

### Intratumor heterogeneity and branched evolution

In total, we identified 173 SNVs in patient P1, and among them, dark patch, regression, and nodule had 23, 56, and 71 private SNVs, respectively. Specifically, SNVs in the loci of representative melanoma-associated genes showed a subset of 21 SNVs shared by all three tumor sites, whereas two SNVs were shared by only the dark and regression sites, in addition to several disjoint sets of SNVs that were found in only one of the three sites (Figure 6c and 6d). When we checked the shared SNVs between nodules and dark patches, or between nodules and regression samples, they were completely overlapped. With this mutational information, we reconstructed a simple phylogenetic tree of this primary melanoma (Figure 6e), postulating that all three tumor sites originated from the same predecessor, which had accumulated key mutations, leading to tumor initiation. Subsequently, the nodule type diverged by acquiring some new SNVs. Shortly after this, the rest of the tumor cells diverged as a result of other mutations produced. This branched evolution process reconstructed through SNVs is also in agreement with the result of the CNA analysis.

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**Figure 5. Summarizing the four levels of CNA heterogeneity. (a)** Comparison of heterogeneity extent among levels. The extent is defined as the ratio of genomic bins showing copy number deviances greater than 1 between the two samples. **(b)** Comparison of heterogeneity intensity among levels. The intensity represents the upper 10th percentile of all bins' copy number absolute deviation between two samples. **(c)** Three examples of L4-type heterogeneity. The spatial relationship and copy number patterns are both shown for adjacent tumor nests. The error bars represents standard deviation of index values within each level of heterogeneity. chr, chromosome; CNA, copy number alteration; L, level; M, mega base pairs; P, patient.

We also analyzed the SNVs of patient P3, which contain two types of phenotypes (dark patch and regression), each with two biological replicates (see Supplementary Figure S5 online). In total, we detected 81 SNVs, 23 of which were shared between two phenotypes. We have identified 7 and 51 private SNVs in the dark patch and regression samples, respectively. Based on these SNVs, we built a phylogenetic tree, which was in accordance with the result from super-CNA analysis. The list of SNVs from all patients has been shown in Supplementary Table S3 online .

#### DISCUSSION

In this work, we applied LCM to ensure cellular purity of the dissected tumor sample, sequencing tissue samples with sizes of approximately 0.01 mm<sup>2</sup> and containing as few as 20–50 cells. We used MALBAC, previously shown to be efficient and to have low bias for single-cell genomic DNA inputs, to amplify genomic DNA from each small tissue sample. Our results of CNA distribution reflect the four hierarchical heterogeneity levels of melanoma: comparing CNAs between lesions and nevi (L1), among individuals (L2), among phenotypically different tumor sites (L3), and among adjacent tumor cell clusters with identical phenotypes (L4).

Our data also indicate that the prominence and signature of CNAs could potentially distinguish early stage melanoma from benign nevi. CLI is an indicator of the severity of the lesion and shows better correlation to the pathological staging. Purity of the tumor samples is essential for highconfidence CNA identification and quantification. Low-purity samples blur the heterogeneity in an artificial ensemble measurement. Thus, LCM is necessary to avoid underestimating the heterogeneity between two adjacent tumor cell clusters. We also identified distinct super-CNA signatures in two morphologically identical, closely attached cell clusters within a regression type of melanoma. This type of heterogeneity (L4) is likely the origin of all other levels of heterogeneities we have observed and could be a major cause of tumor drug resistance and poor prognosis.

We also conducted the quantification of DOH through CNA analyses. Besides the well-known diversities among patients, we found in patients P1 and P3 that heterogeneity between different morphological tumor sites (L3 heterogeneity) is comparable to that between nevi and tumor (L1 heterogeneity). This observation indicates that the lesion appearance is highly associated with genomic variations and that each melanoma patient can harbor high degrees of heterogeneity in each of their tumors.

For identification of SNVs from low-input DNA material, stringent filtering criteria are necessary to identify SNVs with high confidence. Although the size of filtered data is limited, we were able to perform phylogenetic analysis based on the intratumor heterogeneous SNVs among samples. By

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**Figure 6. SNV** analysis of WES data. (a) Diagram of SNV processing pipeline to remove artifacts and generate reliable results. (b) The ratio of different types of somatic mutations in all seven patient tumors. (c) Heatmap showing SNVs in three types of tumor sites – dark patch, regression, and nodule – from P1. Representative tumor-associated genes that were found to be mutated are shown. The dropout positions failed to call SNVs are labeled as missed (green bars). (d) A Venn diagram illustrating shared and unique SNVs found in the three types of tumor sites (see Supplementary Table 3 for the SNV list). (e) A phylogenetic tree illustrating possible tumor evolutionary structure and relationship for the three types of tumors. D, dark patch; HE, hematoxylin and eosin; MALBAC, multiple annealing and looping-based amplification cycles; N, nodule; P, patient; R, regression; SNV, single nucleotide variation.

combining the results of both CNAs and SNVs, it was possible to retrospectively construct the branched evolution process of early stage primary AM.

In conclusion, we show the utility of our workflow for samples with limited starting material—early stage primary AM—and applied both laser microdissection and wholegenome amplification to quantitatively assess the genomic variations at multiple hierarchical levels: between tumor and nevi, among patients, among different phenotypes within the same tumor, and among adjacent tumor cell clusters with identical phenotypic appearance. We confirmed that intratumor heterogeneity, as characterized by both CNA and SNV landscapes, exists among cell clusters in close proximity even in very early stage melanoma. We were able to derive the branched evolution process of a melanoma and build its phylogenetic tree using genomic data of phenotypically addressable cell clusters. We envision that the diagnosis and personalized treatment of melanoma will be facilitated by our efforts to explore the diversity and similarity between and within tumors.

#### MATERIALS AND METHODS

#### Patients, samples, and experimental process

Patients (n = 7) with early stage primary plantar AM were enrolled, and 22 individual tumor cell cluster samples were obtained for sequencing analysis (Figure 1f, see Supplementary Table S1 online for patient information). We received written, informed patient consent from all seven patients. The nevi and normal skin tissues for each patient were also collected for comparison. The study was approved by the Peking University First Hospital Ethics Committee, and all patients consented. The details of hematoxylin and eosin and immunohistochemical staining, microdissection, whole-genome amplification, and whole-exome capture and sequencing are described in the Supplementary Materials and Methods.

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#### Sequencing data analysis

Quality control of the raw data, as well as trimming of the MALBAC primers, library adaptors, and sequencing primers, was performed by using a custom Python script (https://github.com/beiseq/ AcralMelanomaSNV). The steps of calling CNAs include alignment, bin-counting normalization, and segmentation. For SNV analysis of whole-exome sequencing data, we used a GATK3 toolset (Van der Auwera et al., 2013) and MuTect (Cibulskis et al., 2013)based pipeline. The functional impact and pathogenicity of the mutations were estimated using CADD (combined annotation dependent depletion) (DePristo et al., 2011). The details are available in the Supplementary Materials and Methods.

#### Data availability

Raw sequencing data and mapped data have been deposited to the Sequence Read Archive under the study or BioProject of SRP133650.

#### **CONFLICT OF INTEREST**

The authors state no conflict of interest.

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#### AUTHOR CONTRIBUTIONS

YH and HL conceived and designed the study. XZ performed bioinformatics analyses. YP, CL, ZY, and YP conducted the experiments. XZ, YP, ARW, YH, and HL wrote the paper with help from all authors. All authors read and approved the final manuscript.

### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www. jidonline.org, and at https://doi.org/10.1016/j.jid.2019.01.019.

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