Medical bioinformatics in melanoma

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Purpose of review
Bioinformatic insights from next-generation sequencing has been integral in understanding melanoma biology, resistance to treatment and provided new avenues for melanoma treatment. Whole-genome sequencing, whole-exome sequencing and RNA sequencing has redefined the molecular classification of melanoma, revealed distinct genetic aberrations that define clinical subtypes of melanoma and uncovered the diverse heterogeneity that resides in an individual tumor.

Recent findings
In this review, we will summarize the recent whole-genome study that catalogs the genomic landscape across many melanoma subtypes, the single-cell RNA sequencing studies that interrogates tumor heterogeneity and the personalized vaccine approaches to melanoma treatment.

Summary
Whole-genome sequencing of diverse subtypes of melanoma revealed acral and mucosal subtypes to have a different genomic landscape compared with cutaneous melanoma. Acral and mucosal melanomas are characterized by low mutation burden and high structural variants. Single-cell RNA sequencing revealed high intratumoral heterogeneity and the existence of rare intrinsic drug-resistant populations. Lastly, vaccination against tumor neoantigens could be a potential personalized medicine therapy for melanoma patients. In summary, bioinformatics research is deeply ingrained in all aspects of melanoma research and will continue to blossom together for many years to come.

Keywords
bioinformatics, genomics, melanoma

INTRODUCTION

A period of 15 years ago, the first genomic study discovered the mutual exclusivity v-Raf murine sarcoma viral oncogene homolog B (BRAF) and neuroblastoma Rat sarcoma viral oncogene homolog (NRAS) mutations in 65% of melanoma samples [1]. That discovery has paved the way to our understanding of the genetics of melanoma and the importance of aberrant mitogen-activated protein kinase (MAPK) pathway signaling. This has lead to the development of several pharmacological compounds to target mutant BRAF V600E and mitogen-activated protein kinase 1 with great success in the treatment of melanoma [2–4]. Checkpoint blockade inhibitors anti-cytotoxic T-lymphocyte associated protein 4 and anti-programmed cell death 1 (PD1) are also quite successful in the treatment of melanoma [5–7]. Bioinformatics has played a part in establishing a link of mutation burden to positive response to these checkpoint blockade inhibitors [8,9].

MELANOMA GENOMICS

Next-generation sequencing has become integral in evaluating the genomic landscape of melanoma and uncovering biological insights about the disease. Two of the largest whole-exome sequencing (WES) studies looking at 121 melanoma tumors and 147 melanoma tumors confirmed an ultraviolet radiation (UVR) signature, defined by C>T substitutions in a dipyrimidine context, for sun-exposed melanomas. For nonsun-exposed melanomas, a different mutation signature was seen, and these melanomas had a lower mutation rate. This finding supported the notion that UVR is an environmental risk factor for melanoma. The mutation signature differences highlight the genomic diversity in melanoma subtypes, such as sun-exposed melanomas like lentigo maligna and nonsun-exposed melanomas like acral and mucosal melanomas [10,11]. These two studies together detected hotspot mutations in known melanoma oncogenes such as BRAF and NRAS and in

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KEY POINTS

- Subtypes of melanoma arise from different molecular events.
- Intrinsic drug-resistant populations occur spontaneous in melanoma cell cultures.
- Neoantigen vaccination is an effective strategy for melanoma treatment.

addition uncovered new hotspot mutations in Rac family small GTPase 1 (RAC1), serine/threonine-protein phosphatase 6 catalytic subunit (PPP6C) and serine/threonine kinase 19 and new tumor suppressors like AT-rich interaction domain 2 (ARID2), DCC netrin 1 receptor, transforming acidic coiled-coil containing protein 1, sorting nexin 31, neurofibromin 1 (NF1), Zinc finger protein 560, family with sequence similarity 58 member A and malic enzyme 1. Copy number analysis revealed losses in known tumor suppressors like phosphatase and tensin homolog (PTEN) and cyclin dependent kinase inhibitor 2A (CDKN2A) and gains in known melanoma oncogenes like microphthalmia-associated transcription factor (MITF), cyclin D1, cyclin dependent kinase 4 and telomerase reverse transcriptase (TERT). These two studies provided a great insight into the genomic landscape of melanoma and also highlighted the driver mutations for BRAF and NRAS do not occur from UVR, suggesting the initial transformation is UV independent. The next largest WES study came from The Cancer Genome Atlas melanoma working group [12▪]. In this study, 333 cutaneous melanoma samples comprising 67 primary tumors and 266 metastatic tumors were analyzed by WES. Two new molecular subtypes were defined in this study. Tumors that were both BRAF and NRAS wild-type often had a mutation in NF1 (45%). As no high-frequency hotspot mutations occurred in NF1, it was assumed that NF1 was a tumor suppressor. NF1 is known as a negative regulator of Rat sarcoma; thus, loss of function mutations would lead to activation of the MAPK pathway. Tumors that were triple wild-type for BRAF, NRAS and NF1 were typically less likely to have a UVR signature and were more likely to have copy-number alterations and complex structural rearrangements. Significantly, there were no significant mutations associated with metastasis suggesting progression to metastasis maybe patient specific.

One of the first whole genome sequencing (WGS) studies investigated 25 metastatic melanoma samples revealed frequent phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 2 (PREX2) mutations, translocations at the PREX2 locus and TERT promoter mutations [13,14]. WGS also confirmed the UVR signature and as well revealed many structural rearrangements in the genome that alter known melanoma oncogenes like ETS variant 1. The one acral sample in this study contained the second highest number of structural variants but one of the lowest mutation rates.

To build on the existing genomic landscape of melanoma, Hayward *et al.* [15▪▪] performed WGS on 183 melanoma samples which is the largest whole-genome study in melanoma to date. This study interrogated 75 primary melanomas, 93 metastatic melanomas and 15 cell lines cultured from metastatic melanomas. A total of 35 were acral melanoma, eight were mucosal and the rest were cutaneous. The acral and mucosal subtypes had a significant higher frequency of structural variants than the cutaneous subtype. Structural variants included deletions, duplications, tandem duplications, foldback inversions and more complex rearrangements like breakage-fusion-bridge and chromothripsis. For noncoding mutations, TERT was the most common, with 115 of 167 overall. These TERT promoter mutations generate a new transcription factor binding motif [14,16] that increases the transcriptional activity of TERT. The significant coding mutations seen from WGS included BRAF, CDKN2A, NRAS, TP53, ARID2, cell wall biogenesis 43 C-terminal homolog, NF1, PTEN and retinoblastoma 1 (RB1) which largely confirms with the results from the previous exome studies. Significantly, acral and mucosal melanomas did not have mutations in known melanoma oncogenes TP53, PTEN, DEAD-box helicase 3, X-linked, RAS p21 protein activator 2, PPP6C, RAC1 or RB1, suggesting that molecular cause of these subtypes are distinct from cutaneous melanoma. Another advantage of WGS is the detection in structural variants in the NF1 locus which would be missed from exome sequencing. From the 32 samples with NF1 alterations, nine had a structural variant affected the NF1 locus. Thus, exome sequencing would underestimate the NF1 subgroup. WGS would be informative in a clinical setting, 177 out of the 183 melanoma samples had a minimum of one genetic aberration that could be treated with a therapeutic treatment approved by the Food and Drug Administration or in a clinical trial setting. The WGS study from Hayward *et al.* highlights the diverse genetic events that define the subtypes of melanoma and emphasizes that now many of these genetic aberrations could be targetable by currently available therapeutics.

MELANOMA HETEROGENEITY

Melanoma heterogeneity has been a well studied topic for many years. It is clear now that gene

expression patterns in melanoma define physical characteristics like proliferation and invasion, can imbue resistance to current targeted therapies and most importantly switch from one to the other [17–19]. These phenomena were termed 'Phenotype switching' and first described from microarray data. Microarray analysis of 218 melanoma cell cultures across six different studies revealed two gene expression patterns, a 'proliferative' signature and an 'invasive' signature [20]. The 'proliferative' signature was defined by high expression of typical melanocytic genes like MITF, tyrosinase, lymphoid enhancer binding factor 1 and SRY-Box 10 (SOX10) and the 'invasive' signature was defined by high expression of Wnt family member 5A (WNT5A), SRY-box 9, transforming growth factor beta and transcription factor 4. This signature was found to be independent of primary or metastatic lesions and of mutated BRAF V600E. These two signatures had phenotypic consequences for the melanoma cell. 'Proliferative' signature melanoma cells had a fast doubling time and limited invasive capacity, whereas 'invasive' signature melanoma cells had a slow doubling time and high invasive capacity, and the melanoma can switch back and forth between the two phenotypes [21▪]. Bulk RNA sequencing (RNAseq) could only reveal the overall gene expression signature of the tumor and a question in the field was if all cells from the tumor had the proliferative or invasive gene expression signature or if the tumor was a mix of the two gene expression signatures. Single-cell RNA sequencing could address this question and the single-cell RNA-seq study from Tirosh *et al*. [22▪▪] interrogated 4645 cells from 19 melanoma tumors. One of the main findings from the study was the individual tumor cells from a single tumor could have MITF high signature or an AXL receptor tyrosine kinase (AXL) high signature which corresponds to the proliferative and invasive signatures described before. At the tumor level, the gene expression signature was either MITF high or AXL high, but at the single-cell level, an anticorrelative spectrum of MITF and AXL cells could be detected. This is the first direct evidence that a melanoma tumor could have cells with both phenotypes. Another interesting finding was the observation in postrelapse samples from targeted therapy (vemurafenib or combination dabrafenib and trametinib) shift toward an AXL program signature compared with the pretreatment sample, even if the pretreatment sample was already correlated with the AXL signature. These data demonstrate that drug-resistant tumor cell populations already exist in the tumor before treatment and targeted therapy selectively increases these drug-resistant populations. Along with the tumor cells, stromal cells and immune cells were also sequenced. Cancer-associated fibroblasts (CAFs) had a significant association with the AXL high signature and an anticorrelative association with the MITF high signature. CAFs and melanoma cells could both express an AXL signature suggesting tumor–stromal interactions in which melanoma cells could adopt a fibroblast-like fate. The T-cell compartment could be distinguished to their main identities, CD8+, CD4+ and T-regulatory cells. Naïve, cytotoxic and exhausted T cells could also be elucidated. Exhausted T cells were linked to T-cell expansion, whereas nonexhausted T cells were not expanded. Overall, this study has provided great insight into the transcriptomic heterogeneity of melanoma and interaction of melanoma cells with the environment of stromal and lymphocytic cells.

Another study addressed the tumor heterogeneity in the context of drug resistance with single-cell RNA fluorescence *in situ* hybridization (FISH) [23▪▪]. Shaffer *et al*. isolated a single cell from a melanoma cell line and expanded the single cell for seven to eight divisions. This population of cells was treated with vemurafenib and resistant colonies would remain. They had two hypothesis for this experiment, first, if drug resistance was a genetic heritable factor and during the expansion from a single cell if the drug-resistant mutation occurred early then many resistant colonies would form. Second, if drug resistance was transient, then all cells in the culture have an equal likelihood of becoming resistant and few resistant colonies would form. From the experiment, only few resistant colonies formed supporting the second hypothesis of transient drug-resistant subpopulations. Upon RNA sequencing of the resistant colonies, known markers for drug resistance were found such as WNT5A, AXL, epidermal growth factor receptor, platelet derived growth factor receptor beta and Jun proto-oncogene, AP-1 transcription factor subunit. These genes are part of the AXL and invasive signatures described previously. To detect these drug-resistant populations, they performed RNA FISH for 19 genes on the four melanoma cell lines, primary melanocytes and four other cancer cell types. Each of the cell lines had subpopulations that expressed multiple resistant markers, suggesting that these drug-resistant populations exist across many cancer cell types. Next, they hypothesized if drug resistance occurs in a step-wise fashion. They used a combination of RNA sequencing and assay for transposase-accessible chromatin (ATAC) using sequencing at baseline, at 1-week vemurafenib treatment and at 4-week vemurafenib treatment. The resistant cells gradually increased in population from baseline to 1 week and again increased in frequency from 1 to 4 weeks of vemurafenib

treatment. ATAC-seq showed large-scale changes in transcription factor occupancy with a loss of accessible sites from baseline to week 1 and a gain in new sites from week 1 to 4. The sites that were lost between baseline and week 1 were mainly SOX10 binding sites. SOX10 is an important factor melanocyte differentiation [24,25]. The sites that were gained were mostly TEA domain transcription factor (TEAD) sites. TEADs have been found to regulate invasion in melanoma [26]. Thus, as the melanoma cell becomes drug resistant, it switches from a proliferative state to an invasive state supporting the phenotype switching model.

The two single-cell studies from Tirosh and Shaffer highlight the existence of intrinsic drug-resistant subpopulations occurring in melanoma tumors and elegantly show that treatment with targeted therapy enriches for these subpopulations, thus finding new drugs that could target these subpopulations would lead to better survival for melanoma patients.

TUMOR NEOANTIGEN VACCINES

Neoantigens are peptides formed from somatic mutations in genes expressed by the tumor. These neoepitopes are only expressed by the tumor not normal healthy tissue. This makes them an attractive target for personalized cancer therapy as only the tumor would be targeted sparing the normal tissue. One of the first studies in melanoma to analyze antigens detected by T cells leads to the identification of several neoantigens [27]. In this study, the authors found the majority of reactive T cells were targeting the neoantigens, thus highlighting the importance of the T-cell response to tumor-specific antigens. As mutations between patients are usually not shared, neoantigens would be patient specific. Thus, each patient's tumor would have be sequence to identify the potential neoantigens. Sequencing each patient's tumor wasn't feasible till recently with the decreased costs in next-generation sequencing. With WES, all coding mutations in the tumor could be identified, peptide binding to major histocompatibility complex (MHC) specific for the patient's human leukocyte antigen (HLA) type could be predicted and the resulting neoantigens could be queried for T-cell reactivity. Recently, Ott *et al.* [28▪▪] and Sahin *et al.* [29▪▪] presented their phase 1 vaccination studies against melanoma neoantigens. This is the first time this treatment method has been performed in humans. Both studies used WES and RNA sequencing to identify expressed mutated genes. Ott used an algorithm to predict neoantigen binding to MHC class 1 and formulated a vaccine that contained up to 20 clinical grade peptides (15–30 amino acids long) per patient. Six patients who previously undergone surgery to remove the melanoma tumor received the vaccination, and 2 years later, four patients are still tumor free. Sahin used an algorithm to predict neoantigen binding to MHC class 2 and MHC class 1 and selected 10 mutations for production of an RNA vaccine consisting of two synthetic RNAs each encoding five linker-connected 27-mer peptides with the mutation at position 14. A total of 13 patients received the vaccine and eight patients remain tumor free a year later. Both vaccine strategies produced $CD4^+$ and $CD8^+$ T-cell responses, but in both cases, the $CD4^+$ T-cell response was stronger. Multiple validation methods showed the reactive $CD4^+$ and $CD8^+$ T cells against the neoantigens were not detected before vaccination and were enriched after vaccination and functional. Two patients from Ott's study progressed, but tumor regressed upon subsequent anti-PD1 antibody treatment. Out of the five progressive patients from Sahin's study, one patient regressed on anti-PD1 treatment. Significantly, in one of the patients that progressed, the tumor cells did not have expression of HLA class 1 which was caused by a deletion-inversion event at the Beta-2-microglobulin (*B2M*) gene. Tumor cells transfected B2M reestablish HLA class 1 expression, and T-cell recognition and killing. This finding proposes a resistant mechanism against vaccination and the importance of HLA class 1 for T-cell recognition and killing. Overall, the two studies from Ott and Sahin show the promise of bioinformatic analysis in the production of personalized vaccines against melanoma.

CONCLUSION

Bioinformatics is a growing field and is becoming highly integrated in cancer research. As more next-generation sequencing data become available, the next analysis efforts would focus on individual private mutation or structural variants when the main driver mutations or structural variants are absent in the melanoma sample. Single-cell RNAseq could also be applied to longitudinal melanoma samples under therapy. Analysis of these samples would reveal simultaneously the phenotypic expression changes in the microenvironment, immune compartment and tumor from the therapy. These data would be useful for discovering cell autonomous resistance mechanisms and noncell autonomous resistance mechanisms from the same sample. Lastly, bioinformatics analysis can be informative for treatment in a personalized medicine setting. As sequencing costs decrease, and algorithms become better at predicting epitope binding and T-cell

receptor recognition, it could be possible that many melanoma patients could receive a personalized vaccine for their tumor. In conclusion, interdisciplinary teams of cancer biologists, immunologists, bioinformaticians and clinicians will be needed to drive melanoma research and treatment forward.

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Conflicts of interest

There are no conflicts of interest.

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