Chapter 3
Molecular Diagnostics and Tumor Mutational Analysis

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Abstract Genetic and genomic analysis of melanoma tumor samples has identified a number of somatic mutations integral to melanoma pathogenesis, with the most prevalent mutation being the *BRAF* V600 mutation. Targeted inhibitors directed against this mutation have produced improved overall survival compared to chemotherapy. Multiple additional somatic mutations have been identified, and some also have prompted the development of therapy targeted against them. In this chapter, we review common techniques used to identify gene mutations and genomic aberrations, and briefly describe mutations important in melanoma pathogenesis. We also describe massively parallel sequencing and discuss advances that have been made in the identification of novel driver mutations in melanoma tumors. Finally, the application of these techniques with respect to clinical testing is addressed, specifically as they pertain to the development and advancement of personalized medicine.

Keywords BRAF · Melanoma · Molecular diagnostics · Molecular testing · Mutational analysis · Massively parallel sequencing · Somatic mutations · Tumor analysis

3.1 Introduction

Genetic mutations and genomic aberrations have been identified in all tumor types and implicated in multiple aspects of pathogenesis, including examples such as in *EGFR* in lung cancer, *KRAS* in colon cancer, and *BRAF* in melanoma [1–5].

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Identification of these genetic events has been integral in guiding the development of targeted therapies, which have improved progression free or overall survival of patients in some cases, as compared to prior standard of care therapies. Cutaneous melanoma is the most aggressive form of skin cancer and its incidence continues to increase, in contrast to some other cancers such as breast, colon, and lung [6]. Genetic and molecular studies have detected a number of somatic mutations in melanoma cell lines and tumor samples integral to the pathogenesis of melanoma (reviewed in [7–10]). The discovery of mutant *BRAF* as a driver mutation in melanoma led to the development of targeted inhibitors, which have demonstrated an increased overall survival in patients with advanced melanomas containing mutant *BRAF* [1, 3, 4, 11, 12]. In addition, mixed responses have been observed in *KIT* mutant melanomas, treated with the tyrosine kinase inhibitors imatinib or dasatinib [13–19]. Moreover, new driver mutations are continually being identified in melanoma tumor samples [20, 21] which may serve as future targets for therapeutic intervention.

Recently, genetic and genomic profiling of tumors has moved from research to clinical laboratories as targeted therapies have become the new standard of care for the treatment of a sub-set of malignancies, including melanoma, and as techniques have been optimized for high-throughput analysis of somatic mutations. Moreover, tumor mutational analysis is advancing beyond single gene mutation testing; new sequencing methods allow for the simultaneous analysis of multiple genetic mutations within an individual tumor sample. These advances in sequencing techniques continue to have a large impact on the clinical testing of patient tumor samples, with the results having implications for therapeutic interventions such as inclusion or exclusion from clinical trials and initial therapy choices. This chapter will focus on techniques used in molecular diagnostics and mutational analysis of melanoma tumor samples, review sequencing methods, and discuss current and future technologies integral to the field of genetic sequencing. In addition, we will highlight relevant somatic mutations in melanoma tumors, which may be important in the development of future targeted therapies.

3.2 Somatic Mutations in Melanoma

3.2.1 UV Damage-Induced Mutations

Somatic driver mutations identified in patient tumors, both in melanoma and other cancers, tend to be recurrent single nucleotide changes in oncogenes, with mutations leading to stop codons and frameshift mutations, and insertion and deletions as observed in tumor suppressor genes. Likewise, critical genomic aberrations also exist and include loss of heterozygosity or amplification at specific loci, splice variants, and epigenetic dysregulation. As such, the molecular diagnostic testing of melanoma tumor samples needs to reliably detect these diverse somatic mutations and genomic aberrations, since identification of these mutations in patient tumors is critical for the determination of appropriate therapy.

Notably, samples from melanoma cell lines and tumor samples have demonstrated mutations consistent with UV exposure [22], which is a known risk factor for the development of melanoma [23–25]. Pyrimidine dimers are characteristic of UV-induced DNA damage and mutations are predominantly C to T/G to A transitions, along with CC to TT transitions [26, 27]. These mutations are frequently observed in adjacent pyrimidine sequences and at higher frequencies of CpG dinucleotides. Indeed, massively parallel sequencing of a melanoma cell line derived from a metastatic tumor sample demonstrated increased C to T transitions in bases at pyrimidine dinucleotides (92%, as compared to predicted 53% due to chance) and at CpG dinucleotides (10%, as compared to predicted 4.4% due to chance) [22]. Whole exome sequencing of larger numbers of melanoma tumor samples, 121 melanoma tumor/normal pairs and 147 melanoma tumor samples, confirmed these observations of UV-induced damage [20, 21] along with identification of different mutation patterns in tumors from sun-exposed and sun-shielded sites [21]. Cells have a number of mechanisms for repairing DNA damage. In UV-induced DNA damage, nucleotide excision repair (NER) is the predominant mechanism for DNA damage repair [22, 28, 29], with preferential repair of actively transcribed strands [22, 27, 30, 31]. Results from massively parallel sequencing of melanoma tumor samples demonstrate that fewer somatic mutations are identified on transcribed DNA strands within genes than non-transcribed strands, consistent with transcription coupled repair [21, 22]. UV-induced DNA damage is highly prevalent in melanoma tumor samples from sun-exposed areas. However, somatic mutations due to UV-induced DNA damage are under-represented as causative driver mutations in melanoma, as cells have developed mechanisms to repair DNA damage through NER. The converse is observed in tumors derived from xeroderma pigmentosum (XP), a hereditary syndrome characterized by deficient nucleotide excision repair. In these tumor samples, patterns of somatic mutations in key tumor suppressor genes, including *TP53*, are a result of deficient NER, with significant UV-induced DNA damage, although preferential transcription-coupled repair is preserved [26, 27].

3.2.2 BRAF *Mutations*

BRAF is the most common driver mutation identified in melanoma tumor samples and is mutated in approximately 50% of melanomas [32–34]. Within *BRAF*, the most prevalent mutation is a glutamic acid substitution for valine at codon 600 (*BRAF* V600E) which occurs in the kinase domain and results in a constitutively active protein [32–34]. Additional *BRAF* V600 and proximate mutations are observed in melanoma cell lines and tumor samples, as well as in the loop domain (exon 11) [34–36]. The *BRAF* V600E mutation is associated with younger age of diagnosis and truncal site of primary lesion [36, 37]. The *BRAF* V600K mutation is a result of a two base change within codon 600; it has been observed in 9–19% of melanomas and is associated with increased age and higher cumulative sun damage [37, 38]. Improved clinical response to targeted BRAF inhibition compared to chemotherapy

has been observed in patients whose melanomas carry *BRAF* V600E and V600K mutations [3, 11, 12, 36]. However, lower response rates to targeted BRAF inhibition are observed in patients with *BRAF* V600K mutant melanoma [39, 40]. The BRAF V600 inhibitors were developed to target the mutated protein. Thus, it is not entirely clear whether patients with melanomas harboring the non-V600 *BRAF* mutations will respond similarly to *BRAF* inhibition. Dahlman et al. [41] demonstrate both preclinical and clinical data supporting the use of targeted inhibition of the MAPK pathway in *BRAF* L597 mutated melanoma. A patient with *BRAF* L597S mutated metastatic melanoma responded to treatment with the MEK inhibitor, TAK-733 [41]. In addition, preclinical data suggest that *BRAF* K601 mutant melanomas may respond to treatment with MEK inhibitors; as expression of *BRAF* K601E induced signaling through the MAPK pathway was abrogated with MEK inhibition [41]. Further studies are needed to determine the role of BRAF and/or MEK inhibition in non-*BRAF* V600 mutant melanoma.

3.2.3 NRAS *Mutations*

NRAS mutations are the second most prevalent mutations, and are found in 15–20% of melanomas [42–44]. The predominant mutations in *NRAS* occur in exon 2 at codon 61 with substitution of glutamine with several different amino acids (Q61) [45, 46], resulting in activation leading to uncontrolled cell proliferation. In addition, somatic mutations have been identified in exon 1 at codons G12 and G13 [47]. *NRAS* Q61 mutations are associated with the nodular subtype of melanoma, increased tumor thickness, and worsened clinical outcome, demonstrating shorter melanoma specific survival time [38, 42, 48, 49]. It has been challenging to target RAS mutations in tumors generally, however, current clinical trials are underway investigating the use of MEK inhibitors (*MAP2K1* and *MAP2K2*, mitogen-activated protein kinase 1 and 2) either as single agents or in combination with parallel intracellular signaling pathway inhibitors, such as PI3K/mammalian target of rapamycin (mTOR) inhibitors to treat *NRAS*-mutant melanomas [50–53] (www.clinicaltrials.gov).

3.2.4 KIT *Mutations*

KIT is a receptor tyrosine kinase and is mutated in a small percentage of cutaneous melanomas. However, mucosal and acral lentiginous melanomas, along with melanomas arising in chronic sun-damaged skin, have an increased prevalence of *KIT* mutations; mutations and increased copy number have been identified in approximately 30% of these specific melanoma subtypes [54, 55]. *S*omatic mutations in *KIT* have been observed in a number of different exons including 9, 11, 13, and 17. As there is no single predominant mutation in *KIT*, molecular testing must evaluate multiple exons within the gene. Variable responses to treatment

with imatinib, a KIT and PDGFR tyrosine kinase inhibitor, have been observed in patients with melanomas with *KIT* mutations [13–18]. Several studies have found that the maximal response to imatinib is seen in patients whose melanomas have *KIT* mutations in exons 11 and 13 [13, 14]. Responses have also been observed upon treatment with dasatinib, a tyrosine kinase inhibitor similar to imatinib, in melanoma [19].

Approximately 30% of melanoma tumors do not contain mutations in *BRAF*, *NRAS*, or *KIT* genes, and therefore do not currently have mutations that can be therapeutically targeted. However, additional driver mutations in melanomas have been identified, which may lead to the eventual development of appropriate targeted therapies. In particular, massively parallel sequencing has delineated mutations in *ARID2*, *NF1*, *PPP6C, RAC1*, *SNX31*, *STK19*, and *TACC1* [20, 21]. PPP6C is a component of the PP6 protein phosphatase complex and a proposed tumor suppressor; it functions to regulate cyclin D1 during cell cycle progression [56, 57]. *STK19* is thought to encode a kinase of unknown function and mutations within this gene are identified within hotspot regions in melanoma tumor samples [20]. RAC1 is a member of the Rho family of GTPases and functions in melanocyte proliferation and cell migration through its role in cell adhesion, migration, and invasion [20, 21, 58]. With the data from several published studies using whole exome and genome massively parallel sequencing in melanoma [20–22,59–62], as well as the on-going Cancer Genome Atlas effort, the spectrum of genetic mutations and genomic aberrations in untreated cutaneous melanoma is likely to be well described in the near future. With the routine use of targeted therapies in the treatment of *BRAF* mutated melanoma, clinicians have observed resistance to therapy. Discovery of additional or acquired mutations in these tumor samples is important for identification of resistance mechanisms, which may fall outside the spectrum of mutations observed in untreated melanomas, with the eventual goal of preventing and overcoming these mechanisms of resistance.

3.3 Somatic Mutation Testing—Technology

Understanding of the genetic underpinnings of melanoma has led to current treatment advances for advanced stage melanoma and will continue to aid in the development of future therapies. Therefore, it is important to identify known mutations in melanoma tumors in order to stratify patients for therapeutic options, as well as identify mechanisms and mutations involved in treatment resistance. A number of techniques have been used to identify somatic mutations and genomic aberrations providing clinicians with tools to genotype melanoma tumor samples from patients, at all stages of disease.

For many years, molecular diagnostic techniques have evaluated single gene mutations individually or a small number of genes through reaction multiplexing. Massively parallel sequencing allows for the simultaneous testing and identification of multiple mutations and genomic aberrations within tumor samples concurrently.

Although knowledge of all mutations and genomic aberrations within tumor samples would appear on the surface to be most helpful, currently there are limited gene mutations that are clinically actionable. Thus, assaying individual genes and/or mutations is still appropriate in many circumstances. It is important to note that although full profiling of tumors may shed light on future research and clinical trial endeavors, it is very possible that mutations will be identified for which no therapeutic intervention is currently available.

Tumor samples are heterogeneous, which may result in only a fraction of tumor cells harboring a specific mutation, and also may contain surrounding normal tissue resulting in decreased amount of mutated DNA in tumor samples (admixture). Thus, assay sensitivity is important so that mutations can be detected even when they represent a small portion of the DNA extracted from the tumor sample. Advances in several technologies have allowed for the detection of mutations in samples with as little as 5 % mutant DNA in the total DNA sample. Different sources of tumor samples are available for testing including fresh frozen tumor samples and formalin fixed paraffin-embedded (FFPE) tumor samples. FFPE tumor samples, which are commonly used for clinical mutation detection, can have DNA which is degraded and fragmented [63]. A specimen of large enough size for DNA extraction also needs to be available, which can be particularly an issue for primary melanomas. However, in the vast majority of cases, FFPE specimens from metastatic melanomas can be used for mutation identification, even for alleles at relatively low frequency.

When evaluating tumor samples with gene specific mutation testing, consideration must be given to the type of mutation being sought. Some somatic point mutations occur at specific sites in a given gene, known as hotspot mutations (which can be seen in oncogenes), whereas other mutations can occur anywhere within a gene (which can be seen in tumor suppressor genes). Mutational patterns will dictate the type of analysis optimal for mutational detection, as does the number of samples being analyzed, as some methods are better suited for processing of multiple samples, and others more appropriate for limited numbers of samples. We will review techniques used in clinical laboratories focusing on individual gene testing, along with newer sequencing technologies used to identify mutations within melanoma tumor samples.

3.3.1 Direct Sequencing

DNA isolated from tumor samples can undergo direct sequencing to identify point mutations in a specific stretch of DNA. Sanger sequencing, or chain terminating method, can be performed on DNA from tumor samples using a variety of dye-terminators, but is relatively insensitive with a mutation detection rate of ~25 % allele frequency [64, 65]. Pyrosequencing™ (Qiagen, Inc., Alameda, CA) is another direct sequencing technique [66] and can be used to sequence specific short regions of DNA up to 50 bases. Somatic mutations can be identified when clustered within a small region of interest providing for the identification of mutations within a given

DNA locus. Pyrosequencing™ is used by many molecular pathology laboratories to evaluate somatic mutations located within mutation hotspots, and has the advantage of detecting mutant DNA alleles at frequencies as low as 5–15% of the total, depending on the gene being investigated [63, 66]. This method is useful for sequencing *BRAF* mutations in tumor samples, as mutations have been identified in several different nucleotides within and around *BRAF* V600 [32–36].

Allele-specific primers are used to detect single nucleotide changes in tumor samples. For single mutations, Taqman® mutation detection assays (Life Technologies, Carlsbad, CA) is a popular choice. Single nucleotide extension assays also can be used to identify specific point mutations in a given gene, as the technique evaluates changes at an individual nucleotide. Two commonly used platforms for multiplexed single nucleotide extension assays include iPlex™ (Sequenom, Inc, San Diego, CA) [67, 68] and SNaPshot™ (Applied Biosystems, Inc, Foster City, CA) [69]. These techniques make use of primer sets to amplify the DNA and detect the mutated base, along with specific tags, which results in amplification and multiplexing [70]; the tags vary depending upon the platform that is employed. For the iPlex™ platform, nucleotides are detected by matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF) analysis [71]. For the SNaPshot™ platform, nucleotides are fluorescently labeled and nucleotide incorporation into extension products are detected [70].

The iPlex™ [72] and SNaPshot™ [70] technologies can detect mutant DNA with a sensitivity of 5–10% of DNA, thus demonstrating a higher sensitivity for mutation detection than direct sequencing. Additionally, these platforms are effective in genotyping DNA from FFPE tumor samples, allowing for mutation detection in lower quality DNA. Given the use of primer tags, multiple single nucleotide extension assays can be multiplexed, allowing for the interrogation of a number of different mutations within a given reaction. Multiple mutations or single nucleotide polymorphisms within a given region can be assessed in specific tumor samples, albeit not within the same multiplex. These platforms are also commonly used by molecular pathology laboratories and are well suited to assess genes which demonstrate mutational hotspots, such as *NRAS*, *BRAF*, and *GNA11/GNAQ*.

In 2011, the United States Food and Drug Administration (FDA) approved the targeted mutant BRAF inhibitor, vemurafenib, for the treatment of advanced melanoma in patients with the *BRAF* V600E mutation [3]. As this therapy is targeted to a specific somatic mutation identified in patient tumor samples, tumor samples must undergo molecular testing to detect this mutation prior to initiation of therapy. Simultaneously, with the approval of vemurafenib, the FDA also approved a commercially available test for the *BRAF* V600E mutation, the cobas® 4800 BRAF V600 Mutation Test, in order to determine the presence of the *BRAF* mutation and to receive treatment with vemurafenib. The cobas® test probes are specific for and bind to wild-type and mutant V600E *BRAF* sequences and are detected when the probes bind to their correct sequence. However, the cobas® test is limited in its ability to detect *BRAF* non-V600E mutations, with a 66% cross-sensitivity for *BRAF* V600K, and V600E mutations that are a result of a two base pair mutation (package insert). Evidence

suggests that patients with non-V600E *BRAF* mutations, such as V600K, also respond to therapy with targeted BRAF inhibitors, such as vemurafenib and dabrafenib [3, 11, 12]. As such, it is important that somatic mutations in patient tumor samples are accurately detected, thus using the cobas® test alone may not be adequate.

3.3.2 Genomic Aberrations

DNA copy number alterations have been shown to be involved in the pathogenesis of a number of cancers [73] and may have predictive value relating to disease progression or clinical outcome in different tumor types [74–78]. Copy gains or losses are structural variants of segments of DNA, and thought to exert their effects through dysregulation of gene expression. Techniques to determine cancer copy number profiles have improved over the years. Initially copy number determination was performed with probe sequences derived from bacterial artificial chromosomes. Single nucleotide polymorphism (SNP)-based arrays and array-based Comparative Genomic Hybridization (aCGH) are used currently, with massively parallel sequencing the next step for copy number determination, with the development of precise analytical techniques needed. SNP arrays also provide increased identification of loss of heterozygosity and allele copy number, however, these arrays are less suitable for analysis of FFPE tumor samples given the concerns of DNA quality. DNA degradation is common in FFPE tumor samples. As such, shorter DNA fragments are present and limit accurate detection of copy gains and losses in tumor samples. These array techniques rely on the presence of longer DNA fragment sizes to map regions of copy gain and loss, thus, these shorter fragments can result in increased background signal in assay data and could contribute to imprecise DNA copy gains and losses in tumor samples.

Copy number alterations have been analyzed in melanoma cell lines and tumor samples in order to detect genomic aberrations and distinct genomic changes involved in melanoma pathogenesis [79]. A number of genetic regions have been found to be altered in melanoma tumor samples, including gain of chromosomes 5 and 7 and loss of chromosomes 4, 10, 11, 12, 17, and 22 [35,80]. Amplifications of *BRAF*, *NRAS*, *MITF*, *CCND1*, *MDM2*, and *NOTCH2*, and homozygous deletions of *CDKN2A* and *PTEN* have been identified as driver aberrations [35, 81–83]. Moreover, specific patterns of chromosomal gains and losses have been associated with *BRAF* and *NRAS* mutation status [35, 80], suggesting that additional genetic alterations or aberrations cooperate in the pathogenesis of these melanomas.

Other techniques evaluating genomic aberrations have been used to provide supplemental information which, in different tumor types, can be used in risk stratification and prognostic implications in clinical settings. Larger genomic alterations, deletions and rearrangements (over 100,000 base pairs) can be detected using Fluorescence in Situ Hybridization (FISH). FISH is routinely used in hematologic malignancies to delineate cytogenetic characteristics with direct impact on disease stratification and treatment decisions. FISH is gaining popularity to evaluate solid malignancies as well [84, 85]. FISH is being used to

detect *ALK* rearrangement in lung cancers, the presence of which provides the rationale for treatment with the ALK inhibitor crizotinib [86]. FISH based assays are emerging as tools to assist in the diagnosis of histologically indeterminate melanoma. Using three specific probes for *RREB1*, *MYB*, and *CCND1* genes and a centromere specific control probe, Senetta et al. [87]. assessed their use in distinguishing between benign nevi and melanoma. Although specific probe patterns were established in benign nevi vs. melanoma in the validation samples, results were ambiguous in the indeterminate samples in their sample set. Hossain et al. [88] evaluated the use chromosome specific probes to categorize benign lesions vs. melanoma. Results from these studies established chromosomal abnormalities in 94 % melanoma samples, 6 % compound nevi, and 0 % normal skin. Moreover, the most frequent abnormality was gain of chromosomal 11, along with observed gains in chromosomes 6, 7, and 20 [88]. Clinicians can use results from FISH analysis, due to these characteristic genetic events, to guide clinical decisions in the setting of indeterminate histology.

Multiplex probe ligation amplification (MLPA, MRC-Holland, Amsterdam, Netherlands) is used to perform targeted analysis in tumor samples in order to evaluate specific, localized amplifications and deletions [89, 90]. Probes are annealed adjacent to the genomic region of interest, ligated together, and amplified. Quantification and determination of copy number is determined by normalization to controls. MLPA provides copy number profiles for specific genes of interest, requiring less tumor DNA as starting material relative to aCGH. In addition, MLPA can be multiplexed to evaluate a number of genes within the same reaction. MLPA had been successfully used to identify genetic rearrangements in genes associated with inherited syndromes, contiguous gene deletion syndromes, and somatic copy number alterations [91–94]. Moreover, evaluation of specific chromosomal loci, including chr 9p21 (*CDKN2A*), for genetic changes by MLPA has been used to evaluate genetic heterogeneity of uveal melanomas [95] and to distinguish between Sptiz nevi and atypical spitzoid melanocytic tumors [96], as it can be difficult to distinguish these two lesion based on histology alone.

Identification of genetic alterations in melanoma tumor samples and cell lines provides investigators with pertinent information regarding genetic alterations which may contribute to melanoma pathogenesis, which also has the potential to lead to development of novel targeted therapeutics. Additionally, detection of genetic events known to be associated with melanoma can help to guide clinical decisions and treatment plans in the setting of indeterminate lesions.

3.3.3 Massively Parallel Sequencing

The development of massively parallel sequencing (MPS), also referred to as next-generation sequencing, has revolutionized the way in which DNA from tumor samples is analyzed. MPS allows for the analysis of whole genomes, exomes, or targeted regions (i.e. select genes) in individual tumor samples providing simultaneous information regarding mutational analysis of a wide range of genes

and identified mutations, genetic alterations (including deletion and insertions), and copy number gains and losses. A number of different platforms are available to perform massively parallel sequencing such as the HiSeq™2000, HiSeq™2500 and miSeq (Illumina, San Diego, CA) and IonTorrent™ and IonProton™ (Life Technologies, Grand Island, NY), which are reviewed in detail by Ross and Cronin [97]. To perform MPS, DNA libraries are prepared from individual samples. In brief, genomic DNA is sheared to 150–200 bp fragments, blunt ended, and ligated with tagged adaptors and indexes (bar codes), which allow for sample identification. Optimal fragment sizes of DNA within these libraries depend on the length of the sequence reads. DNA libraries are combined with capture baits for targeted sequencing and whole exome sequencing (WES) or remain uncaptured for whole genome sequencing (WGS). The DNA then is sequenced, undergoing amplification and repetitive cycles of sequencing and detection. With improving technology, an increasing number of samples can be multiplexed while retaining mutation detection capability. The ideal read depth, which is the number of sequence reads of a particular nucleotide, varies depending upon whether whole genome, whole exome, or targeted MPS is being done [98].

Somatic mutations in tumor DNA can be challenging to identify given possible admixture of surrounding normal cells and tumor heterogeneity. Identification of low frequency mutations is crucial in the characterization of all tumor samples, including melanoma tumor samples, as it has implications for treatment options, including targeted therapies. Initial platforms for MPS were higher in cost per sample compared to traditional sequencing techniques, which was prohibitive for running large number of samples. However, over time, as technology has advanced, the cost per sample has decreased making it more attractive to use these methodologies to analyze multiple tumor DNA samples. Initially, the source DNA was restricted to fresh frozen tumor samples; however, several studies have demonstrated that adequate results can be achieved using FFPE tumor samples [98–101]. Despite these advances, DNA quality remains a crucial determinant of MPS success.

In addition to whole genome and whole exome analysis, massively parallel sequencing with targeted capture is also used to evaluate tumor samples focusing on specific genes of interest. A number of targeted capture platforms are commercially available to test for common cancer somatic mutations, including such examples as TruSeq Amplicon Cancer Panel (Illumina, San Diego, CA) and Somatic Mutation Analysis (SOMA) panel (Ambry Genetics, Aliso Viejo, CA). These targeted captures provide the advantage of deep sequencing of select, known genes. Of note, whole exome captures generally only select for ~85 % of the complete exome [102–105], so if there is poor coverage over your gene of choice, it will provide limited information.

An immense amount of data is generated from massively parallel sequencing and analysis remains a challenge. The softwares available for data analysis are constantly evolving. Moreover, methodologies used for analysis also depend upon whether germline or somatic genomes are being sequenced. Mutations are first identified and then annotated in order to best assess their potential function. Briefly, sequence data is aligned to the human genome most commonly with the Burrows-Wheeler Aligner (BWA) [106, 107]. Variants are found using programs which detect single

nucleotide variants (SNVs), as compared to the reference sequence, as well as insertions and deletions (indels), though programs such as the Genome Analysis Toolkit (GATK) [108] and Pindel [109]. The analysis of genomic rearrangements and copy number alterations for targeted massively parallel sequencing lags behind that of SNVs and small indels, but are evaluated using programs specific to these types of genetic aberrations, such as VarScan2 [110, 111]. Annotation with programs, such as ANNOVAR, provides information regarding the potential function of identified genetic variants [112]. ANNOVAR calls variants as frameshift indel, non-frameshift indel, stopgain, stoploss, synonymous, non-synonymous and splicing (intronic and exonic). ANNOVAR automatically identifies variants previously reported in pubic databases, including EVS6500, 1000 Genome (1000G), dbSNP (Flagged/Nonflagged) and COSMIC [113]. ANNOVAR also annotates SNVs using SIFT, Polyphen2, MutationTaster and PhyloP to make predictions about function [113–118]. Mutation information obtained using ANNOVAR can be used to filter variants based on specific score cutoffs for the different software programs. The pipeline for mutation identification and annotation will differ depending on input DNA, that is, germline DNA versus tumor DNA. Software has been developed specifically for the analysis of somatic genomes and mutations including BreakPointer, Indelocator, and MuTect (www.broadinstitute.org/cancer/cga/). MuTect is a sequence analysis program that uses the sequence of both normal and tumor to identify somatic point mutations [119]. Despite technology and software advances, the pathogenicity of a number of the detected genetic variants, both germline and somatic, will have unknown significance. These variants of unknown significance pose challenges for clinicians as these variants are not clinically actionable and it is not clear whether these variants are involved in tumor pathogenesis.

3.3.4 Results of Whole Exome Sequencing/Whole Genome Sequencing in Melanoma

Melanoma tumor samples have been evaluated using whole genome and whole exome sequencing. An initial whole genome sequencing study identified 33, 345 somatic mutations, 680 deletions, 303 insertions, and 51 rearrangements in a single melanoma cell line derived from metastatic melanoma when compared to matched germline DNA [22]. Whole genome sequencing detected known somatic mutations involved in melanoma pathogenesis including *BRAF* V600E, *PTEN* deletion, and a two base pair deletion within *CDKN2A*. Potential driver mutations were also identified in transcription factors, including *SPDEF*; genes thought to be involved in metastasis, including *MMP28*; and proposed tumor suppressor genes, including *UVRAG* [22]. Wei et al. [62] described the identification of recurrent mutations within *TRRAP* in 4% (6/167) metastatic tumor samples with functional studies of TRRAP suggesting it functions as an oncogene. Additional somatic mutations were identified in *GRIN2A*, which was mutated in 33% (17/52) of melanoma samples [62]. Somatic mutations in *GRIN2A* also were identified by whole genome sequencing of a melanoma tumor sample/normal DNA pair [41], but other

somatic mutations suggested by the study have not been validated in subsequent massively parallel sequencing analyses. In addition to these novel genes, whole exome sequencing detected known somatic mutations including *BRAF* mutations in 50 % of samples, consistent with previously published observations. However, no *NRAS* mutations were identified in these melanoma samples, in contrast to the multiple publications showing a frequency of mutations in 15–20 % of melanomas [42–44]. Additional whole exome studies also identified gain of function mutations in genes found in pathways known to be involved in melanoma pathogenesis, such as *MAP2K1* and *MAP2K2* [61]. Evaluation of an expanded panel of melanoma samples identified mutations within these two genes in 8 % (10/127) samples. Additional previously unidentified somatic mutations were observed in *FAT4*, *DSC1*, and *LRP1B*, but their role in melanoma pathogenesis in unknown [61]. However, it is important to note that subsequent studies have not validated the *FAT4*, *DSC1*, and *LRP1B* mutations in independent analysis of multiple melanoma tumor samples.

Two recent studies using whole exome sequencing of a large number of samples generated a more comprehensive understanding of the genetic landscape of somatic mutations in melanoma [20, 21]. Hodis et al. [20] reported on the results from whole exome sequencing analysis of 121 melanoma/normal DNA pairs. In this study, the authors used a statistical approach comparing the frequency of mutations in intron sequences adjacent to exon sequences to identify novel driver mutations in melanoma. Six genes demonstrated recurrent somatic mutations novel in melanoma. Activating mutations were described in *PPP6C*, catalytic subunit of PP6 protein phosphatase and potential tumor suppressor [20, 57]; *RAC1*, member of Rho family of GTPases [58, 120]; *SNX31*, protein sorting nexin 31, a possible Ras effector protein [121]; *TACC1*, transforming acidic coiled-coil protein 1 which potentially stimulates Ras and PI3K pathways [122]; and *STK19*, a predicted kinase, generally clustered around hotspot regions. Loss of function mutations were observed in *ARID2*, component of the SWI/SNF chromatin remodeling complex [123]. In addition to these novel somatic mutations, mutations were identified in known genes, such as *BRAF*, *NRAS*, *PTEN*, *TP53*, *CDKN2A*, and *MAP2K1* [20]. All mutations were identified in over 4 % of melanoma samples. Whole exome sequencing also was done by Halaban and colleagues at Yale University, in 147 melanoma samples, either primary melanomas or metastases. They also identified novel somatic mutations at higher rates in *NF1*, *PPP6C*, *RAC1*, and *ARID2*. In addition, Krauthammer et al. [21] also identified additional novel somatic mutations in melanoma samples in *PTPRK*, protein tyrosine phosphatase, receptor type K; *PTPRD*, protein tyrosine phosphatase receptor type D; and *DYNC1I1*, dynein, cytoplasmic 1, intermediate chain 1, which may be involved in chromosomal segregation [124]. Some of these newly identified driver mutations are associated with *BRAF*/*NRAS* mutations, but others have been specifically identified in melanomas lacking these mutations.

In addition to the individual examination of melanoma tumor samples by investigators, mutational data on a large number of melanoma tumor samples, from tumor metastases, are publically available through the Cancer Genome Atlas (TCGA) (http.//www.cbioportal.org/public-portal/; http.//gdac.broadinstitute.org/; and https.//tcga-data.nci.nih.gov/tcga/), and are continuing to be collected.

Currently, the skin cutaneous melanoma (SKCM) TCGA dataset reports the results of available mutational analysis of 337 biospecimens from metastatic melanoma tumor samples including somatic mutations, copy number, methylation clustering, protein activities, and gene expression analyses. Within the skin cutaneous melanoma (metastatic) dataset, specific copy number changes can be identified as well as somatic mutations and the types are available, highlighting those that are UV-induced. Co-mutation plots provide information regarding simultaneous mutations in different samples, allowing for grouping of melanoma tumor samples. Moreover, the available dataset can be queried to investigate specific genes of interest, either singly or for pathway analysis, and results provide information regarding copy number alterations and somatic mutations. Detailed information regarding specific types of somatic mutations are available, and provide insight into types of mutations commonly identified within a particular gene. mRNA and protein expression data is provided along with methylation profiling. The TCGA endeavor undertaken by a number of collaborators provides a large dataset of metastatic melanoma tumor samples and subsequent analysis in one central repository, making it available to all investigators to use this information for research and clinical purposes.

3.4 Conclusion

Treatment options for advanced stage cancers, especially metastatic melanoma, have advanced with the advent of effective targeted therapy, necessitating the use of molecular diagnostics for clinical decision making. Standard mutation detection techniques may still remain the optimal choice in somatic testing, in particular when evaluating an individual mutation or gene (e.g. *BRAF* or *KIT*), as these tests have been validated and are cost effective with relative quick turnaround time. The information garnered with next generation sequencing provides clinicians with a large amount of information, extending past the presence or absence of a particular mutation (e.g. V600E in *BRAF*). However, many of the mutations identified beyond those available with targeted mutation screening may not be clinically actionable. As MPS strategies are used to profile tumors, they have become available in Clinical Laboratory Improvement Amendments (CLIA) certified laboratories, so the data generated will be usable in the clinical setting. As concurrent mutations are evaluated and detected, it is possible to ascertain the presence of mutations which would predict for resistance to a specific targeted therapy (e.g. additional pathway mutations, such as in *MAP2K1*). Thus, MPS provides valuable information with potential implications regarding treatment options for patients at the time of initial evaluation. More importantly, the use of MPS at the time of disease relapse, progression, or development of resistance to therapy to classify the genetic landscape within an individual's tumor will provide information regarding potential therapeutic options. From a research perspective, the identification of somatic mutations and mechanisms of resistance will further guide research endeavors and clinical trial development as clinicians seek out improved therapeutic options.

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