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Applications of Genomics in Melanoma Oncogene Discovery

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Abstract

SYNOPSIS (for table of contents)—The identification of recurrent alterations in the melanoma genome has provided key insights into the biology of melanoma genesis and progression. These discoveries have come about as a result of the systematic deployment and integration of diverse genomic technologies, including DNA sequencing, chromosomal copy number analysis, and gene expression profiling. Here, we chronicle the discoveries of several key melanoma oncogenes affecting critical cell pathways and examine the role played by evolving genomics technologies in melanoma oncogene discovery. These advances are being exploited to improve prognosis and treatment of melanoma patients through the development of genome-based diagnostic and targeted therapeutic avenues.

Keywords

melanoma; oncogene; DNA sequencing; microarrays; gene amplification; targeted therapy

The notion of cancer as a genetic disease is, by now, well-established [1]. The accumulation of genomic mutations and epigenetic changes that dysregulate genes controlling key hallmarks of malignancy [2] (proliferation, survival, invasion, etc.) is considered to be the main cause of cancer. The last decade has witnessed the discovery of mutations in many genes driving the progression of a host of different cancers. These include activating mutations in growth-promoting oncogenes, inactivating mutations in growth-inhibiting tumor-suppressor genes, and alterations to stability genes. Collectively, somatic base pair mutations have been identified in almost 10% of genes in human cancers, although only a subset of these are believed to be driver events [3]. Ongoing efforts to elucidate these genes and their roles in promoting the growth of different tumor types constitute a major component of molecular cancer research.

Melanoma, a malignant tumor of melanocytes, is responsible for 80% of all deaths from skin cancer. An estimated 62,000 new melanoma cases were diagnosed in the United States in 2008 [4]. The poor prognosis of advanced melanoma and the relative ineffectiveness of conventional therapies for patients with metastatic disease underscore a dire need for improved therapeutic agents. Recent discoveries of recurrent mutations in the melanoma genome have provided an

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improved understanding of the biology of melanoma genesis and progression as well as an opportunity for the development of new classes of targeted drugs. In this review, we chronicle the discoveries of several critical melanoma oncogenes and highlight the role played by genomic technologies. Our goal is not to provide a comprehensive list of all genetic alterations observed in melanoma. Rather, we hope to illustrate through the lens of melanoma oncogene discovery how genomic analysis can lead to a better understanding of tumor biology and novel therapeutic avenues. We conclude with a discussion of emerging “omic” technologies and how these might lead to the discovery of additional oncogenes that articulate a path to improved prognosis and treatment of patients suffering from this deadly malignancy.

MELANOMA ONCOGENES

Genetic mutations involving numerous genes have been linked to melanoma genesis and progression. These genes encompass many signaling pathways, including the receptor tyrosine kinase (RTK), phosphatidylinositol-3-kinase (PI(3)K), retinoblastoma (RB), p53, Wnt, and NF- κ B pathways. The supporting evidence implicating these oncogenes in melanoma ranges from positional cloning studies in familial melanoma to elevated frequencies of mutation or amplification in patient cohorts to functional studies *in vitro* and *in vivo*. Below, we consider five well-studied melanoma oncogenes and their contributions to overall morbidity (Table 1). Each of these genes was identified by a different approach, highlighting alternate strategies for melanoma oncogene discovery and emphasizing the expanding importance of technology and integrative genomic analysis.

NRAS

One of the first genes shown to be specifically mutated in melanoma was *NRAS*, a gene encoding a member of the RAS family of small GTP-binding proteins. The RAS proteins lie at the “top” of the RAS/RAF/MEK/ERK MAP kinase pathway, which activates a large number of growth-promoting genes in response to signals transmitted from growth factors and cytokines. Regulation of RAS signaling is achieved by alternating between active (GTP-bound) and inactive (GDP-bound) forms of the RAS protein. The prototypic members of the RAS family, *HRAS* and *KRAS*, were originally discovered as the transforming oncoproteins in Harvey and Kirsten murine sarcoma viruses [5]. Human cellular versions of the *HRAS* and *KRAS* genes were subsequently identified, and single nucleotide mutations were shown to confer transforming capabilities on these genes upon transfection into the mouse embryonic fibroblast NIH 3T3 cell line [6-8]. *NRAS* was later discovered as the transforming gene of a neuroblastoma cell line, and sequence analysis revealed considerable homology with *HRAS* and *KRAS* [9,10]. Mutations converting all three genes to active oncogenes nearly always occur in residues 12, 13, or 61 of the protein [11]. These mutations impair the GTPase catalytic activity of RAS, resulting in a constitutively GTP-bound and activated state. Altogether, approximately 20-30% of human tumors carry a mutation in one of the *RAS* genes.

NRAS was first shown to be mutated in melanoma in the mid-1980s when two studies demonstrated that *NRAS* DNA isolated from four human melanoma cell lines was sufficient to transform NIH 3T3 cells [12,13]. Subsequent sequencing and genotyping efforts revealed that *NRAS* is mutated in 15-30% of melanomas, >10 times more frequently than *HRAS* or *KRAS* [14-18]. Notably, the mutations occur most often at residue 61. The spatial distribution of *NRAS*-mutated tumors on the skin and their proclivity for dipyrimidine mutations suggest a possible correlation with UV exposure [14,17]. Transgenic mice with oncogenic *NRAS* targeted to the melanocytic compartment show hyperpigmented skin and develop cutaneous metastasizing melanoma [19]. In addition to its role in tumor formation, mutant *RAS* is also critical for tumor maintenance [20]. Knockdown of mutant *NRAS* (but not wild-type *NRAS*) by RNA interference reduces the viability of melanoma cell lines, suggesting a possible therapeutic avenue in patients whose tumors harbor this mutation [21]. However, the

development of small molecules that target RAS proteins directly has proved exceedingly difficult.

As stated above, RAS operates in the MAP kinase pathway. This pathway is hyperactivated in up to 90% of human melanomas [22]. This underscores the contribution of *NRAS* mutations to the development of melanoma but also indicates the involvement of other genes in the pathway, discussed below. At the same time, RAS-activating mutations can influence the phosphoinositide-3-OH kinase (PI(3)K) pathway. The specificity of mutations in melanoma for *NRAS* compared to *HRAS* and *KRAS* is notable considering that all three isoforms are expressed in primary melanomas and melanoma-derived short-term cultures [23,24], and that *HRAS* and *KRAS* are mutated in a variety of other human cancers. For example, *KRAS* mutations are found in 15-20% of lung cancers, ~30% of colorectal cancers, and up to 90% of pancreatic adenocarcinomas [11]. *HRAS* mutations have been detected in Spitz nevi, yet they rarely occur in malignant melanoma [25]. Comparisons of the transformation efficiencies of different RAS isoforms suggest that the activity of different isoforms may depend on the cellular context [26].

In summary, the recurrence and high transforming potential of oncogenic *NRAS* mutations in human melanomas demonstrate the critical role of this gene and its downstream effector mechanisms in melanoma genesis and maintenance. As with many other “classic” oncogenes described in cancer, the original discovery and validation of *NRAS* as a melanoma oncogene required a laborious series of low-throughput experiments spanning many years and multiple research groups. The following examples illustrate how recent melanoma oncogene discoveries have emerged from powerful systematic approaches enabled by the massive technology revolution that accompanied the genomic era.

BRAF

BRAF, a serine-threonine kinase, lies downstream of RAS in the MAP kinase signaling pathway (Fig. 1) (BRAF induces MEK to phosphorylate ERK, which enhances cell growth and proliferation. In 2002, as part of a large-scale DNA sequencing effort to identify mutant oncogenes in the MAP kinase pathway, Davies *et al.* discovered highly recurrent oncogenic BRAF mutations in melanoma through systematic re-sequencing across 545 cancer cell lines [27]. The *BRAF* gene was observed to be mutated in 20 of 34 (59%) melanoma cell lines and an additional 18 of 24 (75%) patient-derived primary melanomas and short-term cultures, compared to an overall mutation frequency of 8% in all cancers. (After melanoma, *BRAF* was mutated in 15% of colon cancers, 11% of gliomas, and 10% of ovarian cancers, considering cell lines and primary tumors.) Notably, >90% of the *BRAF* mutations in melanoma involved a single substitution of valine to glutamic acid in the kinase domain (V600E) [27]. Subsequent studies have confirmed the specificity for V600E mutations and established the overall frequency of *BRAF* mutations at 50-70% in cutaneous melanomas [28-32]. Unlike the mutation patterns observed in *NRAS*, the T>A transversion associated with the V600E mutation is not suggestive of UV-induced DNA damage, although there is an apparent association between this mutation and intermittent exposure to sun [31]. Surprisingly, the V600E mutation was also observed in 39 of 44 (89%) benign melanocytic nevi, suggesting that mutation of *BRAF* represents an early event in melanocytic neoplasia but one that is insufficient by itself for tumorigenesis [29]. However, mutant *BRAF* does appear to induce transformation of NIH 3T3 cells and murine melanocytes and allow them to grow as tumors in nude mice, clearly implicating it as an oncogene [27,33-35].

As noted above, the oncogenic potential of BRAF lies in its ability to phosphorylate MEK, leading to ERK activation and cell proliferation (Fig. 1). The kinase activity of BRAF is normally regulated by the phosphorylation of two amino acids, T599 and S602, which disrupts the interaction between the activation segment and P loop and causes a conformational

change to the active state [36]. The V600E mutant mimics these phosphorylation events by inserting a negatively charged residue in the activation segment, resulting in constitutively active BRAF [27,34]. The absence of comparable mutations from *ARAF* and *CRAF* (paralogs of *BRAF* that also function in MAP kinase signaling) is likely due to the further requirement of phosphorylation of an additional regulatory region (N region) in the kinase domain of these proteins. Unlike *ARAF* and *CRAF*, the N region of *BRAF* is constitutively phosphorylated such that *BRAF* is primed for activation [33]. Recent studies have identified several genes functioning downstream of *BRAF* in melanoma, including critical cell cycle regulators, tumor maintenance enzymes, and microphthalmia-associated transcription factor (*MITF*, discussed below) [37]. These findings underscore the key role of *BRAF* in melanoma genesis and progression.

Depletion of oncogenic *BRAF* (but not *ARAF* or *CRAF*) by RNA interference in cultured human melanoma cells and mouse xenograft models has been shown to inhibit proliferation and induce apoptosis [38,39]. This suggests an essential role for the MAP kinase pathway in tumor maintenance. Consequently, inhibitors of *BRAF* and *MEK* are of considerable interest as potential targeted therapies for melanomas harboring *BRAF* mutations. To this end, several *BRAF* inhibitors (such as BAY 43-9006 and CHIR-265) and *MEK* inhibitors (such as PD0325901 and AZD6244) are currently in various stages of development and/or clinical trials [37]. One well-studied *MEK* inhibitor, CI-1040, clearly inhibits proliferation and soft-agar colony formation *in vitro* and causes rapid regression of *BRAF* mutant xenografts in mouse [40]. Given the additional role of *RAS* in MAP kinase signaling, it would stand to reason that *MEK* inhibition would produce a similar effect in *NRAS* mutant cells. However, human melanoma cell lines with *BRAF* mutations proved to be demonstrably more sensitive to CI-1040 and the related (but more potent) *MEK* inhibitor PD0325901 than did many *NRAS* mutant melanoma cell lines [41]. This suggests that activation of additional pathways by *RAS*, such as the PI(3)K/*PTEN* pathway, decreases the dependence of melanoma cells on *MEK*. Consistent with this, *NRAS* and *BRAF* mutations, as well as *NRAS* and *PTEN* mutations, are mutually exclusive in melanoma due to their shared pathway membership, whereas *BRAF* and *PTEN* mutations are coincident in up to 20% of melanomas [42]. *BRAF* and *PTEN* operate in different pathways downstream of *RAS*, such that separate mutations are required to activate both pathways. It thus remains uncertain as to why *BRAF* would be mutated so much more frequently than *NRAS* in melanoma.

MITF

In addition to nucleotide substitutions, DNA copy number alterations are frequently observed in cancer. More than just passive reflections of the genomic instability of cancer, such amplifications and deletions often function as driver events in tumorigenesis by altering the expression levels of important genes regulating cell growth, survival, etc. Changes in DNA copy number are readily profiled at high resolution by hybridizing tumor genomic DNA to microarrays comprised of oligonucleotides spanning the entire human genome [43]. As an illustration of the use of recurrent chromosomal aberrations to identify a novel melanoma oncogene, Garraway *et al.* profiled genomic copy gains and losses in the NCI-60 cancer cell line collection using high-density single nucleotide polymorphism (SNP) microarrays and observed a focal amplification at 3p14-3p13 shared among 6 out of 8 melanoma cell lines [44]. By integrating these copy number data with gene expression signatures for this cell line collection, the authors identified only one highly expressed gene located within the amplified region. This product of this gene, microphthalmia-associated transcription factor (*MITF*), was already known to be a master regulator of the melanocyte lineage and a sensitive marker for melanoma diagnosis [45,46]. However, this integrative genomics approach provided the first evidence that *MITF* is an oncogene in human melanoma and the target of a specific somatic alteration in this malignancy.

Detailed characterization this locus by fluorescence *in situ* hybridization (FISH) established the frequency of *MITF* amplification in melanomas at 10-20%, with a higher incidence among advanced tumors and an associated decrease of 5 years in survival [44]. Interestingly, *MITF* amplifications are coincident with mutations in *BRAF*. Growth of NCI-60 cell lines harboring *MITF* amplification was inhibited following RNAi-mediated knockdown of MITF or introduction of dominant-negative MITF [44]. In an apparent contradiction, very high levels of MITF also seem to reduce tumorigenicity and promote melanocyte differentiation, perhaps by transcriptionally activating p16INK4A and p21, two cyclin-dependent kinase inhibitors [37]. Tight regulation of MITF levels may therefore be necessary for maximal proliferation.

As a master regulator of melanocyte development and differentiation, MITF represents an emerging class of “lineage-survival” oncogenes [47]. Unlike oncogenic *NRAS* and *BRAF*, which acquire new and tumor-specific cellular functions through nucleotide mutations, *MITF* becomes oncogenic via deregulation, affecting survival mechanisms that are also present in the normal melanocyte lineage. It is well established that wild-type MITF is essential for lineage survival and that deficiency of MITF results in the absence (or loss) of melanocytes during development [48]. The same survival mechanisms that govern melanocyte proliferation and development may subsequently persist or become deregulated during tumor progression. Additional lineage-survival oncogenes have been implicated in other cancers for which normal lineage programs become aberrantly regulated in cancer. For example, the androgen receptor (AR) and thyroid transcription factor 1 (*TTF1*) are targeted by genetic alterations in carcinomas of the prostate and lung, respectively [49,50]. Like *MITF*, these genes encode transcription factors that are essential master regulators during development for their particular lineage, but whose functions provide key tumor survival roles in prostate and lung adenocarcinoma. These observations emphasize the importance of lineage in providing a context for additional oncogenic alterations and help to explain why certain mutations occur at different frequencies discrete tumor types.

NEDD9

The identification of *MITF* within a locus of recurrent amplification in cancer was enabled by the integration of copy number and gene expression data. Subsequently, Kim *et al.* used a related integrative genomics approach that incorporated evolutionary conservation to implicate *NEDD9* as a melanoma metastasis gene from within a broad gain of chromosome 6p [51]. Amplification of 6p had been previously associated with 36% of human metastatic, but not primary, melanomas [52]. However, the shared amplified region spans >35 megabases, making recognition of the underlying driver gene(s) nearly impossible by analysis of copy number data alone. Using high-resolution microarray comparative genome hybridization, Kim *et al.* identified a recurrent focal amplification associated with metastatic potential in a genetically-engineered inducible mouse model of melanoma. By performing a cross-species comparison between the mouse and human genomes, the authors dramatically narrowed the minimal region of overlap to an 850-kilobase region on human chromosome 6p24-25. Further integration with expression data revealed *NEDD9* (neural precursor cell expressed, developmentally downregulated 9) as the only resident gene that was overexpressed when compared to nontransformed primary melanocyte cultures. Accordingly, *NEDD9* is amplified in 36% of human metastatic melanomas and overexpressed at both mRNA and protein levels in approximately 50%. *NEDD9* protein expression was shown to be up-regulated in a manner correlated with tumor progression [51].

Functional studies provided further support of *NEDD9* as a melanoma gene important for invasion and metastasis [51]. Knockdown of *NEDD9* by RNAi reduced the number of distal metastases *in vivo* and limited cellular proliferation and invasion *in vitro*. Additionally, gain-of-function studies demonstrated that overexpression of *NEDD9* enhances metastatic potential

of primary melanocytes and nonmetastatic melanoma cells. This increased invasiveness is conferred by a functional and physical interaction between NEDD9 and focal adhesion kinase (FAK) at the cell periphery. These results demonstrate the utility of genome-wide cross-species comparisons for oncogene discovery; in particular, the integration of genomic data from both a mouse model and human tumors led to the identification of *NEDD9*. Genetically-engineered mouse models also enabled the design of appropriate functional assays and suggested the genetic context in which these assays would be most informative.

KIT

As discussed above, *NRAS* and *BRAF* mutations represent common mechanisms of upregulating the MAP kinase pathway in melanoma. In particular, *BRAF* mutations are prevalent in cutaneous melanomas without chronic sun-induced damage, while they almost never occur in melanomas of the palms and soles (acral melanomas), mucosal membranes (mucosal melanomas), and occur less frequently on skin with chronic sun-induced damage (CSD melanomas). In an attempt to discover oncogenic mutations in melanomas with wild-type *NRAS* and *BRAF*, Curtin *et al.* identified a shared region of amplification at 4q12 using comparative genomic hybridization (CGH) to microarrays [53]. This amplicon was specific to the acral and mucosal melanoma subtypes where *BRAF* mutations are rare, and it contained an attractive candidate oncogene, *KIT*. *KIT* encodes a receptor tyrosine kinase (RTK) for the stem cell factor (SCF) ligand and functions as an upstream activator of the MAP kinase signaling pathway (Fig. 1). Like *MITF*, *KIT* is an essential gene for melanocyte survival and development [54], although its expression is usually lost during cutaneous melanoma progression. Re-sequencing of *KIT* in melanomas with amplification of 4q12 revealed mutations in 3 of 7 cases. More extensive tumor sequencing confirmed that while they may occur in only 2–5% of all melanomas [55,56], *KIT* mutations are much more prevalent in acral (12–23%), mucosal (16–25%), and CSD (8–20%) melanomas [53,57]. Just as many additional cases exhibit amplifications without observed sequence mutations [53].

Of note, the *KIT* mutations that have been observed in melanoma nearly always occur in exon 11. This is similar to the spectrum of activating *KIT* mutations in patients with gastrointestinal stromal tumors (GISTs) [58]. GISTs have been successfully treated by the KIT inhibitor, imatinib mesylate [59]. (Imatinib, or Gleevec, also targets the BCR-ABL tyrosine kinase produced by the Philadelphia chromosome in chronic myelogenous leukemia (CML) and leads to response in more than 95% of cases [60].) GIST patients whose tumors express an exon 11 mutant KIT protein exhibit an 84% partial response rate to imatinib [58]. These results have prompted multiple phase II clinical trials of imatinib in patients with metastatic melanoma. Results are inconclusive to date, but at least two melanoma patients with activating *KIT* mutations in exon 11 have demonstrated a marked response to imatinib treatment [61,62]. These results speak to the emerging need for systematic cancer mutation profiling prior to enrolling patients in clinical trials for targeted agents, and suggest an immediate benefit of profiling melanoma patients for *KIT* mutations. Further, they anticipate a scenario that may ultimately typify personalized cancer medicine, in which a drug used to treat one type of cancer can be applied in a new cancer based on a shared genetic mechanism.

Additional Oncogenes

The genes described above account for only a fraction of the altered loci observed in melanoma. Oncogenes and tumor suppressor genes in many critical pathways have been implicated from a variety of experimental systems. The MAP kinase signaling pathway is commonly altered as evidenced by mutations in *NRAS*, *BRAF*, and *KIT*, but *NRAS* and receptor tyrosine kinases also contribute to the PI(3) kinase (PI(3)K) pathway. Oncogenic signaling through PI(3)K leads to the activation of AKT, a prominent downstream oncogenic effector in many tumor types. Interestingly, the AKT3 isoform is highly expressed in neural crest-derived cells such as

melanocytes, and *AKT3* has been found to undergo chromosomal copy gains and/or overexpression in up to 60% of melanomas [63]. Recently, activating point mutations in *AKT3* have also been described in melanoma [64]. Hyperactivation of the PI(3)K/AKT pathway arises from a variety of additional perturbations as well, including activating PI(3)K mutations in 3% of metastatic melanomas [65], and deletions or loss-of-function mutations in *PTEN* in 40% of melanoma cell lines [66,67]. The p16/RB pathway is another common target of melanoma genomic alterations; lesions affecting this pathway enable cells to avoid senescence and acquire extended proliferative potential. Germline mutations in the cyclin-dependent kinase inhibitor p16 (encoded by *CDKN2A*), as well as in the cyclin-dependent kinase *CDK4*, preventing binding by p16, are common in familial melanoma [68-71]. *CDKN2A* also encodes p14/ARF in an alternate reading frame, which serves to inhibit the p53 antagonist MDM2. Likewise, p53 is lost or mutated in 10–20% of melanomas [72,73]. Finally, the Wnt pathway can provide additional proliferative and survival signals in melanoma through loss of adenomatous polyposis coli (*APC*) [74] and/or or mutation of beta-catenin (*CTNNB1*) [75,76]. The complex interplay amongst each of these pathways warrants further study in order to determine their total contribution to melanoma genesis and progression.

MELANOMA ONCOGENES AND PERSONALIZED MEDICINE

The completion of the human genome project marked the beginning of the genomic era in biology. However, for this knowledge to truly transform medicine, accessible and affordable technologies are needed that can probe the genomes of many individuals in order to understand the genetic basis of disease and, ultimately, to stratify cancer patients in the clinical arena for optimal therapy. For cancer in particular, the ability to profile the genetic make-up of a patient's tumor can illuminate which pathways are activated, thus enabling the prediction of disease outcome and the most appropriate course of treatment. The discovery of critical oncogenes, even if mutated in only a small percentage of tumors, can provide new putative targets for drugs tailored for specific patient subpopulations. DNA sequencing, copy number measurements, and gene expression profiling have already led to the identification of many key genes underlying melanoma progression. As described below, these technologies are likely to turn up many new candidate genes in the coming years and shed light on the dependencies operant among these genes and pathways in cancer subtypes defined by genomic or other molecular criteria (Table 2). Further, the emergence of “next-generation” DNA sequencing technologies is expected to dramatically accelerate the search for genes and the movement towards personalized medicine.

Discovery of Additional Oncogenes

Despite the success of previous oncogene discovery efforts, a large part of the genetic basis of melanoma remains uncharacterized. As discussed above, the MAP kinase pathway is hyperactivated in 90% of melanomas [22]. This is frequently the result of *BRAF* mutations, but *NRAS* and *KIT* are often mutated as well. However, many melanomas harbor wild-type sequences for all three genes, suggesting that additional mutations may perturb this pathway. (The recent discovery of *FGFR1* mutations in two melanoma lines with wild-type *BRAF* and *NRAS* may indicate a possible alternate mechanism for MAP kinase activation [23].) Further, perturbations to this pathway alone are not sufficient to drive melanoma progression. While *BRAF* is mutated in 50–70% of malignant melanomas, it is also mutated in as many as 90% of benign melanocytic nevi [29]. *MITF* overexpression and *BRAF* mutations together are sufficient to transform primary human melanocytes (in the context of deficient p53 and p16/RB pathways), but only ~15% of *BRAF* mutated melanomas also exhibit *MITF* amplification [44]. Undoubtedly, additional melanoma oncogenes in many other pathways await discovery.

Large-scale, systematic DNA sequencing and copy number profiling efforts will undoubtedly aid the search for new oncogenes. Several groups have utilized large-scale Sanger sequencing

to identify novel mutations in other tumor types. Greenman *et al.* sequenced the coding exons of 518 protein kinase genes in 210 diverse human cancers and found evidence for high-frequency “driver” mutations in more than 100 genes [77]. A separate, multi-institutional study identified 26 genes significantly mutated in a single tumor type upon sequencing 623 genes in 188 lung adenocarcinoma samples [78]. Vogelstein, Velculescu, Kinzler, and colleagues have impressively sequenced the coding exons of approximately 20,000 genes in breast, colorectal, and pancreatic cancers, as well as glioblastoma multiforme, albeit in relatively small discovery cohorts, which may have constrained the statistical power [79-82]. Genome-wide copy number profiling across many samples can also reveal regions of shared copy gain or loss, leading to the discovery of novel lineage-specific oncogenes and tumor suppressors [50]. To provide an organizational and scientific framework for systematically mapping genetic abnormalities that lead to cancer, the U.S. National Cancer Institute and National Human Genome Research Institute established The Cancer Genome Atlas (TCGA) initiative [83]. By integrating genomic data generated by multiple scientific teams using different platforms, including DNA sequence, gene expression, copy number alterations, and DNA methylation, TCGA will lead to the identification of genes and pathways significantly altered in different cancers [84]. Though the pilot stage of TCGA only includes glioblastoma, lung cancer, and ovarian cancer, it will serve as a model for similar large-scale efforts to comprehensively catalog and characterize the genomic changes involved in melanoma.

Most of the DNA mutations discussed to this point involve somatic changes that accumulate over the lifetime of an individual tumor. However, allelic variants of certain genes can be transmitted through the germline and result in an inherited predisposition to developing melanoma. Much remains to be understood about this heritable component of melanoma. Genetic linkage studies have demonstrated that rare mutations in *CRCCKN2A* on 9p21 segregate with melanoma susceptibility in large melanoma-prone families [68,69]. More common physical characteristics, such as fair complexion and red hair, are also often accompanied by an increased risk of melanoma. To identify the common genetic variants underlying susceptibility to melanoma in large populations, genome-wide association studies will be particularly useful. Genome-wide association studies involve simultaneously genotyping hundreds of thousands of genetic polymorphisms in large sets of cases and controls. One recent study identified a susceptibility locus for melanoma on chromosome 20q11 by pooling 2,019 cases and 2,105 controls [85]. Additional genetic loci known to affect hair, eye, and skin MD pigmentation in Europeans show clear associations with cutaneous melanoma and basal cell carcinoma [86]. Further studies utilizing additional populations and larger cohorts will likely narrow these loci and reveal novel associations with melanoma susceptibility genes.

Further Characterization of Genetic Lesions

Even as more oncogenic mutations in melanoma become known, the precise mechanisms by which existing mutations confer tumorigenicity remain poorly understood. *In vitro* transformation assays monitoring proliferation, pathway activation, and anchorage-independent growth have been informative, yet these assays must be performed in the appropriate genetic background. Further, for certain types of mutations, the underlying oncogene may not always be apparent. Amplifications and deletions typically encompass many genes. To narrow down regions of copy gain or loss, one can incorporate additional genomic information. The examples of MITF and NEDD9 discussed above demonstrate how gene expression and evolutionary conservation can be successfully used to pinpoint the driver oncogene within a broader locus. However, many additional significant regions of copy number alteration remain uncharacterized. For example, a recent study identified 27 significant copy gains and losses across 101 melanoma cell lines and short-term cultures using high-density SNP arrays [23].

An additional strategy for the functional characterization of commonly amplified regions involves systematic gene knockdown by RNA interference. Though many regions harbor attractive candidate oncogenes, all constituent genes can be examined through systematic RNAi screens. For instance, one would expect RNAi knockdown of an oncogene to result in decreased proliferation of all cell lines harboring an amplification of that locus. Similarly, restoring the expression of a tumor suppressor gene that has been deleted should produce a similar anti-proliferative effect.

The high prevalence of particular mutations in melanoma underscores the critical roles played by the associated pathways. However, no single mutation is sufficient to lead to malignant melanoma. The interdependence among various mutations affecting different pathways needs to be further examined. From the distribution of mutations in *NRAS*, *BRAF*, and *PTEN*, it is clear that the MAP kinase pathway and the PI(3)K/PTEN pathway are often simultaneously targeted. The context-dependence of oncogenic mutations can be explored by RNAi screens in multiple cell lines with different genetic backgrounds [87]. Additional genes whose knockdown reduces proliferation in some, but not all, cell lines can be linked to common alterations through supervised analysis. The temporal order in which mutations occur can also be deduced from their distribution. *BRAF* is mutated in ~90% of benign melanocytic nevi, whereas *MITF* amplification is restricted to malignant melanoma [29,44]. This suggests that *BRAF* mutations occur early during melanoma progression, perhaps predisposing cells to hyperproliferation following additional genetic alterations.

Next-Generation Technologies for Oncogene Discovery

Looking forward, the discovery of new oncogenes in melanoma and other tumors will largely come about from genome-scale DNA sequencing efforts. Until recently, large-scale DNA sequencing in cancer has been conducted entirely by traditional capillary-based Sanger sequencing methods [77-82,84]. New technologies have now emerged that promise to dramatically reduce the cost and increase the throughput of DNA sequencing [88]. These massively parallel “next-generation” sequencing platforms involve the clonal amplification of millions of individual template molecules without the need for multi-well plates and large quantities of costly reagents. The short sequence reads (ranging from 20-50 to a few hundred bases) that are generated can be mapped onto the reference human genome, and the nucleotide identity at each position is determined by counting discrete reads. This results in increased sensitivity for detecting rare sequence variants, which is especially important in cancer where confounding factors such as stromal contamination and genetic heterogeneity are common [89]. The first whole-genome sequence of a cancer patient’s tumor was recently completed using massively parallel sequencing [90]. In this study, 33-fold coverage was obtained for an acute myeloid leukemia genome, and 14-fold coverage was obtained for a normal skin sample from the sample patient, leading to the discovery and validation of several novel somatic mutations. For the purposes of oncogene discovery, it is currently more economical to sequence only protein-coding regions rather than sequence across the entire genome. Several groups have developed exon capture methods for targeted resequencing to overcome the cost limitations of parallel PCR reactions [91,92]. Consequently, it will soon be feasible to sequence all human exons (or a subset thereof) in hundreds of tumors at reasonable cost.

The value of next-generation DNA sequencing technologies in cancer is not limited to genome sequencing applications. Microarray-based assays can be adapted to use DNA sequencing as a read-out, providing more sensitivity and a greater dynamic range. For instance, gene expression levels can be monitored by counting the number of sequence reads arising from different cDNAs. In doing so, novel transcription events and alternatively-spliced isoforms can be readily identified [93,94]. To demonstrate its utility for cancer, Sugarbaker *et al.* used next-generation sequencing of cDNA to characterize RNA mutations and gene expression levels in

malignant mesotheliomas [95]. In addition to expression, DNA methylation patterns can be evaluated in different samples using reduced representation bisulfite sequencing [96]. Chromatin modifications and transcription factor binding can be analyzed by DNA sequencing following chromatin immunoprecipitation (ChIP-Seq) [97,98]. Further, copy number alterations can be identified in tumors at high-resolution from low-coverage shotgun sequencing of genomic DNA [99]. It is likely that integrative genomic characterization efforts such as The Cancer Genome Atlas will soon deal predominantly (if not exclusively) in various forms of DNA sequence data.

Targeted Therapies and Personalized Medicine

Ultimately, the key cancer-associated mutations uncovered through these discovery efforts can serve as important clinical biomarkers for the diagnosis and prognosis of melanoma, the prediction of a patient's response to a particular treatment, and the development of novel rational therapeutics targeting specific subsets of patients. With regard to mutations as prognostic biomarkers, Curtin *et al.* used genome-wide copy number measurements and *BRAF* and *NRAS* mutational status to classify 126 melanomas into four clinically distinct groups with 70% percent accuracy [100]. Additionally, as discussed above, *MITF* amplification in melanoma correlates with a 5-year decrease in survival [44]. Global gene expression patterns have also revealed discernible subclasses of cutaneous melanoma [101]. The FDA has recently approved several anti-cancer drugs that specifically target the products of mutated genes in other malignancies. These include imatinib (*BCR-ABL* translocation in CML; *KIT* mutation in GIST) [59,60], trastuzumab (*ERBB2* amplification in breast cancer) [102], and gefitinib and erlotinib (*EGFR* mutation in non-small cell lung cancer) [103-105]. Imatinib represents a potential therapy for malignant melanomas harboring *KIT* mutations, as evidenced by the marked response of at least two patients to imatinib treatment [61,62]. Given the large number of melanomas with activating mutations in the MAP kinase pathway (*NRAS* and *BRAF*), MEK inhibitors such as PD0325901 and AZD6244 are currently being developed and investigated in clinical trials [106].

The utility of these genetic biomarkers for the development and administration of targeted therapies hinges on being able to profile patients for mutations in an efficient, cost-effective manner. Although this goal has not yet been attempted categorically, recent advances suggest that there is room for optimism. Thomas *et al.* described a mass spectrometry-based strategy whereby they genotyped more than 200 high-frequency cancer-associated mutations in 1,000 human tumors [107]. Using this platform, termed OncoMap, the authors discovered that certain mutations commonly associated with particular cancers were also present at lower frequencies in many other cancers. This reinforced the notion that existing targeted treatments such as imatinib may be applied to additional cancers with shared genetic lesions. In its current embodiment, OncoMap relies on the observation that a relatively small number of recurrent base pair mutations accounts for the majority of somatic events known to drive tumor progression. However, extending this strategy to next-generation sequencing of whole exons will enable the rapid profiling of all mutations—both base pair mutations and copy number alterations—in cancer-associated genes. The ability to interrogate large panels of tumors will have a large impact on the discovery of targeted therapies, as pharmacological data from drug screens can be correlated with the mutational status of the associated tumor-derived cell lines [23,108]. It will also enable efficient patient stratification for clinical trial design. Finally, such an approach, if applied universally, can transform patient care through improved diagnosis and prognosis in the movement towards personalized medicine.

SUMMARY

The identification of melanoma oncogenes, such as *NRAS*, *BRAF*, *MITF*, *NEDD9*, and *KIT*, have led to an improved understanding of the biology of melanoma. These discoveries have

come about from a variety of genomic approaches, including systematic DNA sequencing, chromosomal copy number profiling, gene expression profiling evolutionary conservation analysis, and RNA interference. Despite these initial successes, the genetic basis of melanoma progression remains largely uncharacterized. It is clear that additional knowledge will come from the utilization of improved genomic technologies with increased throughput and sensitivity for mutation detection and discovery as well as analyses that integrate these diverse data types. These technologies (and the biological insight gained from their deployment) hold great promise for the development of improved diagnostics and targeted therapies and carry the potential to dramatically transform patient care.

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Figure 1.

Cell signaling pathways that undergo oncogenic dysregulation in melanoma. The receptor tyrosine kinase-driven MAP kinase and PI(3)K pathways are featured. Genes mutated in melanoma are marked with an asterisk. These include *KIT*, *NRAS*, *BRAF*, *MITF*, *PI(3)K*, *PTEN*, *AKT3*, and *NEDD9*.

Table 1

Summary of exemplary melanoma oncogenes

Gene	Function	Alteration	Prevalence	Discovery Method
NRAS	Growth, Proliferation	Mutation	15-30%	Transformation assay, genotyping
BRAF	Proliferation, Survival	Mutation	50-70%	Systematic DNA sequencing
MITF	Lineage survival	Amplification	10-20%	Copy number and gene expression profiling
NEDD9	Invasion, Metastasis	Amplification	36%	Copy number profiling and evolutionary conservation
KIT	Proliferation, Survival	Mutation, Amplification	2-5%	DNA sequencing

Table 2

Genomic technologies for oncogene discovery

Genomic Approach	Type of Tumor Data
SNP Microarrays, Comparative Genomic Hybridization (CGH)	Chromosomal copy number
	Loss of heterozygosity
Gene Expression Microarrays	Gene expression profiles miRNA profiles
Sanger DNA Sequencing	Point mutations
	Small insertions and deletions
Mutation Profiling	Known and/or “druggable” mutations
Next-Generation DNA Sequencing	Point mutations
	Small insertions and deletions
	Chromosomal rearrangements translocations
	Chromosomal copy number
	DNA methylation (with bisulphite treatment)
	Epigenetics (with chromatin immunoprecipitation)
RNAi Screening	Essential genes
	Suppressor genes