Multiregion Whole-Exome Sequencing Uncovers the Genetic Evolution and Mutational Heterogeneity of Early-Stage Metastatic Melanoma

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Abstract

Cancer genome sequencing has shed light on the underlying genetic aberrations that drive tumorigenesis. However, current sequencing-based strategies, which focus on a single tumor biopsy, fail to take into account intratumoral heterogeneity. To address this challenge and elucidate the evolutionary history of melanoma, we performed whole-exome and transcriptome sequencing of 41 multiple melanoma biopsies from eight individual tumors. This approach revealed heterogeneous somatic mutations in the range of 3%–38% in individual tumors. Known mutations in melanoma drivers BRAF and NRAS were always ubiquitous, whereas mutations of other drivers showed a high degree of mutational heterogeneity were associated with more aggressive disease progression. In conclusion, our multiregion tumor-sequencing approach highlights the genetic evolution and non-UVB mutational signatures associated with melanoma development and progression, and may provide a more comprehensive perspective of patient outcome.

Introduction

Intratumor heterogeneity (ITH) has recently been thoroughly examined in cancer using whole-exome sequencing (WES) of multiple regions of a sample (1–8). ITH could have major implications for clinical strategies concerning biopsy representation of the tumor, clonality of alterations in targetable genes, and drug resistance. In renal cell carcinoma, substantial ITH was demonstrated with up to 70% of mutations occurring in a heterogeneous pattern across spatially separated regions in individual tumors. Several known cancer gene mutations were confined to distinct regions (2). Lung adenocarcinomas have also been analyzed using a similar multiregion approach (3, 4). Although lung adenocarcinomas are also characterized by ITH, 76% of all mutations were identified in all tumor regions. Furthermore, somatic mutations acquired during the progression of the tumors tended to be less smoking induced and instead had evidence of being acquired through upregulation of APOBEC cytidine deaminases (9). Other studies using multiregion sequencing of tumors revealed different extent of heterogeneity depending on the tumor type, for example, close to 100% heterogeneous genetic alterations in multifocal prostate cancer (6, 7), 53% in esophageal adenocarcinoma (8), and 49% in ovarian cancer (5). Overall, these data point to the fact that single-biopsy strategies may be inadequate to inform on mutation status and may lead to significant molecular information being missed.

We recently found evidence of intrapatient tumor heterogeneity, using targeted gene sequencing and gene expression microarray analysis, in treatment-naïve metastatic melanoma patients (10). Furthermore, individual metastases have been shown to be founded by multiple cell populations in the primary melanoma (11) highlighting the complexity of metastatic progression. Although few treatments have been available in metastatic melanoma, the recent development of inhibitors targeting BRAF and MEK changed the clinical outcome of patients with metastatic disease (12). However, in most cases,
the success is limited by the development of resistance in the majority of patients within 6–9 months from treatment initiation. Several groups have studied whether genetic heterogeneity can explain therapy resistance (4, 13, 14) and further highlighting the complex heterogeneity of therapy resistance, it has been shown that different mechanisms of resistance occur simultaneously in a single melanoma patient (15). Whether these genetic lesions exist in small subclones prior to treatment initiation still remains unclear.

To address these challenges, we applied multiregion DNA and RNA sequencing to melanoma tumors from eight patients. Our findings shed light on the extent of ITH in treatment-naive melanoma, help to separate early from late genetic events, and decipher mutational spectra occurring during the course of early metastatic disease.

**Patients and Methods**

**Patient selection**

Patients (n = 8; Table 1) were selected for the study by a surgeon or oncologist based on the size of the tumor to allow for sampling of each quadrant of the tumor (see Supplementary Information). The biopsies were taken at surgery and immediately stored at −80°C (see Supplementary Fig. S1 for study workflow). We collected frozen tissue from multiple spatially separated tumor regions (Supplementary Table S1). Six patients had metastatic disease at the time of surgery, and for two patients (Mm1765, Mm1837) the primary melanoma was biopsied. In one case (Mm1742), the inguinal metastasis was removed during temozolomide treatment due to local progress and should be considered separately in all subsequent analyses. All other cases were untreated at the time of surgical removal of tumor.

For patient Mm1702, only three different core-needle biopsies were analyzed. Blood samples were available from all patients except for Mm1837, for whom normal skin was used as matched normal control. All patients consented and the study was approved by the Regional Ethical Committee (Dnr. 101/2013).

**Nucleic acid extraction**

DNA and RNA were extracted from tumor and normal skin using AllPrep DNA/RNA Mini Kit (Qiagen). DNA was extracted from blood using QIAamp DNA Blood Mini Kit (Qiagen).

**Whole-exome sequencing**

Tumor and matched normal DNA samples were subjected to library preparation. All sample libraries except Mm1641 were prepared according to SureSelect Target Enrichment System for Illumina Paired-End Sequencing Library Protocol (Agilent Technologies), using Clinical Research Exome (CBE) capture oligo panel. For Mm1641, libraries were prepared using the following three platforms: SureSelect All Exon V5+UTR (Agilent Technologies); TruSeqProtocol with TruSeq Exome Enrichment (Illumina); and Nextra Rapid Capture Protocol with Expanded Exome (Illumina). All biopsies were sequenced with a physical coverage of at least 120× with the exception of Mm1641 (coverage 220×). Libraries were sequenced on a HiSeq 2500 using normal or rapid run mode.

**WES data analysis**

Reads were mapped to the human reference genome (hg19) with decoy using Novoalign (Novocraft Technologies). Postalignment processing is described in the Supplementary Information. For variant calling, MuTect (16) and VarScan (17) were used, and consensus calls between the two in the coding parts of the genes by Annovar annotation (18) were retained (see Supplementary Information). Copy number log ratios of sequenced exons were generated from bam files of tumor–normal pairs using CONTRA 2.03, with default parameters (19). Exons with insufficient coverage in the normal sample were removed. Copy number data were segmented using GLAD (20).

**Phylogenetic and subclonality analysis**

Phylogenetic trees of single-nucleotide variation (SNV) data were generated using the maximum parsimony method. ABSCO-LITE (21) was used for the identification of cancer cell fractions of SNVs with support from segmented copy number data and FACS ploidy analysis (for details see Supplementary Information).

**Gene expression analysis**

Gene expression levels of multiregion samples were assayed using the Illumina HT12 microarray platform according to the manufacturer's instructions at the Swegene Center for Integrative Biology at Lund University. Data analysis included integration of the multiregion samples with a reference set of 214 melanomas (22) and subtype classification (Supplementary Information).

**Allele-specific expression at mutation sites**

RNA-seq was performed using the Illumina NextSeq for the multiregion samples largely as described by Saal and colleagues (23) but using Illumina TruSeq Stranded mRNA Library Prep Kit and NextSeq500. Mm1641:R1 and Mm1749:R6 were omitted due to lack of input mRNA. For details on RNA-seq...
and allele-specific expression analysis see Supplementary Information.

Results

Analysis of intratumor heterogeneity in melanoma tumors

WES was performed on 41 tumor samples obtained from eight patients with an average target depth of 120× per sample (220× for Mm1641). Overall, three samples were excluded due to high normal tissue admixture (see Supplementary Information). We called a mutation being present in a tumor if a nucleotide substitution was present in at least 10% of reads in at least one tumor region. We then determined the regional distribution of nonsynonymous mutations based on the WES data. We found on average 499 (range 45–814) nonsynonymous mutations per tumor whereof 13% (range 2.7%–37.8%) of the nonsynonymous somatic mutations were heterogeneous, that is, not detected across all sampled regions in the individual tumors (Fig. 1). Notably, mutational ITH was not obvious at the morphologic level, with uniform appearance of the tumor throughout its regions (Supplementary Fig. S2). In melanoma, three genes (BRAF, NRAS, and NF1), all key components of the MAPK pathway, are frequently mutated and overall demonstrate a mutually exclusive pattern (24). We found BRAF V600E mutation in two patients, three cases harbored NRAS Q61K mutation, one case had both NRAS Q61K and NF1 mutation, and one case had both KIT and NF1 mutation. KIT is rarely mutated in cutaneous melanoma; however, it is a known oncogenic driver in acral and mucosal melanoma (25). These mutations were present across the tumor regions at similar allele frequencies (Supplementary Table S1). The Mm1767 tumor, which did not receive treatment regimens prior to surgical removal, had the lowest degree of mutational heterogeneity with 546 nonsynonymous somatic mutations, of which only 2.7% were not detected in all spatially separated regions. Three patients (Mm1837, Mm1641, Mm1702) died of their disease within six months of the analyzed biopsy (Table 1). These had higher degree of mutational heterogeneity as compared with the other patients (P = 0.03, t test). Seven spatially separated regions from a lymph node metastasis from patient Mm1641 exhibited 14.2% mutational heterogeneity. At the time of surgery, the patient had an aggressive disease and developed lung metastases. The patient subsequently received BRAF inhibitor therapy; however, after six months on therapy, the patient had a progressive disease course. From Mm1702, three core-needle biopsies in a lymph node metastasis were analyzed and revealed 20.9% mutational heterogeneity. In addition, FACS ploidy analysis revealed one hypo- and one hyper-tetraploid subclone in all tumor regions (Supplementary Fig. S3; Supplementary Table S2). At the time of diagnosis the patient presented with distant metastases to the lungs and shortly thereafter the patient developed meningi carcinoma. The third patient (Mm1837) had 45 somatic mutations, which exhibited 14.2% mutational heterogeneity. At the time of surgery, the patient had a large primary melanoma (Breslow thickness 18 mm) of a nondefined pathologic subtype that was MLANA negative, and developed an aggressive metastatic disease six months after initial diagnosis. Two patients are currently alive, of which, one received both ipilimumab and pembrolizumab. These two patients had the lowest degree of heterogeneous mutations (2.7% and 5.4%, respectively; Table 1). Although intriguing, larger series are required to determine the usage of ITH as a biomarker in melanoma.

Analysis of regional subclones and mapping melanoma tumor evolution by phylogenetic tree analysis

Next, we mapped driver genes from Catalogue of Somatic Mutations in Cancer (COSMIC) Cancer Gene Census list (26) to determine whether mutations in previously identified cancer driver genes were predominantly trunk or branch mutations within the phylogenetic tree. We found 129 mutations in cancer driver genes with 113 mutations (88%) being trunk mutations while only 16 (12%) were branch mutations (Fig. 2). Mutations in the melanoma driver genes, BRAF, NRAS, and NF1, were always present on the trunk consistent with the critical role of constitutive activation of the MAPK pathway in the majority of melanomas. Although the majority of driver mutations detected in these melanomas appear to be present in all spatially separated regions (Figs. 1, 2A, and B), we also observed parallel evolution of subclones within the melanoma. For example, Mm1641 harbored three distinct and spatially separated activating mutations in PIK3CA (encoding p.R388H, p.V344G, and p.H1047R). Furthermore, a PIK3R1 (encoding p.Q384X) truncating mutation was detected as part of the dominant clone in a distinct region (R5) wild-type for the three different PIK3CA mutations. In addition, a mutation in the phosphatase domain of Pten (p.K125E) was present at high allele frequencies in all regions except R1. This mutation alters a highly conserved amino acid residue and was predicted “probably damaging” by PolyPhen-2 with a score of 0.999. Importantly, R1 harbored the H1047R PIK3CA kinase domain mutation frequently found to be mutually exclusive to Pten mutations (27). The R7 region demonstrated the highest degree of subclonality that presumably includes the clones from R5 and R1 due to subclonal presence of mutations in PIK3CA (p.H1047R), Pten, and PIK3R1 (Fig. 2C and Supplementary Figs. S4–S8). Overall, parallel evolution converging on distinct genes in the same pathway indicates a strong selection for aberrations in these genes in the progression of melanoma. Although the presence of private mutations was observed in all analyzed tumors, there was no evidence of subclonality of cancer driver genes in the other patients.

It is well established that most somatic mutations acquired in melanomas are C>T transitions preceded by a pyrimidinerepresenting UVB-induced mutations. We found that approximately 80% of all trunk mutations harbored the UVB signature, while there was a span of 20%–70% of branch mutations having the UVB signature (Fig. 3; P < 0.001). On the other hand, T>G transversions characteristic of UVA mutation signature (28) were significantly increased among the branch mutations as compared with trunk mutations (P < 0.001, Fisher exact test). These findings indicate that different mutational processes are operative during the progression of melanoma (Supplementary Fig. S9). Notably, the only case biopsied during treatment (Mm1742) did not show a decrease of UVB- or increase of UVA-induced mutations (Fig. 3).

Finally, we constructed phylogenetic trees for these tumors by maximum parsimony analysis (Fig. 4). We observed a branched evolutionary pattern in tumor Mm1641 where seven distinct regions were subjected to WES. In contrast, Mm1749 showed limited degree of branching, even though these were different in-transit metastases removed simultaneously. Inclusion of three additional asynchronous in-transit metastases...
supported the limited branched evolution in Mm1749. In the phylogenetic tree it is clearly observed that M1-3 is closely intermingled with R1-6. In conclusion, the branched evolutionary pattern indicates that a linear progression model does not sufficiently explain melanoma evolution.

Transcriptome analysis of intratumor heterogeneity
We then performed microarray-based gene expression profiling of all samples to determine the transcriptional heterogeneity within individual tumors. Importantly, we pooled the data from the multiregion samples with a reference set of 214 metastatic
melanoma tumors previously profiled using the same platform, allowing to relate the gene expression levels of the ITH regions in relation to the full melanoma expression space (22). We determined the intratumoral expression of a subtype signature shown to classify melanomas into four molecular subgroups: high-immune (associated with a good prognosis), microphthalmia-associated transcription factor (MITF)-low proliferative (associated with a poor prognosis), MITF-high pigmentation (associated with a poor prognosis), and normal-like. In 4 of 8 patients, we could observe gene expression–based ITH when taking into account genes that have been used to develop the subtype classification. Inconsistent with the phylogenetic tree analysis, we observed that two regions evolutionary similar by mutation analysis could be assigned to different gene expression subtypes (Fig. 5). For example, based on phylogenetic tree analysis in Mm1641, R1, and R7 displayed genetic divergence. However, based on gene expression subtype analysis, R3 and R5 demonstrated different gene expression classification. Such differences were also observed patients Mm1702, Mm1749, and Mm1742.

Next, we investigated mRNA levels of 10 genes involved in melanocyte differentiation (MITF, MLANA, TYR), immune checkpoint (CTLA4, PD1), T-cell functions (CD3, CD247, CD8A), and cell proliferation (CCNE1, AURKA; Fig. 5). MITF and its target genes (MLANA and TYR) displayed intratumor gene expression differences and showed agreement with the subtype classification. Furthermore, mRNA levels of immune checkpoint genes (CTLA4 and PD1) showed some consistency with the phylogenetic tree analysis. For example, R1 and R2 from Mm1765 had decreased levels of CTLA4 and these two regions formed a distinct branch that seemed to have evolved later than R3 and R4, suggesting that some clones may evolve to escape the immune response (Figs. 3 and 5). Gene copy number values extracted from the WES data were used to determine an additional layer of ITH and unveiled additional information using GISTIC regions from the melanoma Cancer Genome Atlas study (24). The amount of copy number changes per tumor varied between patients. For example, Mm1765 harbored copy number changes exclusively on chromosomes 1q, 6p24.3, and 9p23 with a heterogeneous pattern at chromosomes 9p23 (Fig. 5). In Mm1749, several copy number changes were identified with three in-transit metastases lacking a CDKN2A homozygous deletion detected in all the other six metastases. Mm1837 harbored the highest degree of mutational ITH; however, we found no gene copy number differences. Overall, this suggests that ITH may independently be dictated by mutations, DNA copy number changes, or gene expression.

RNA-seq reveals allele-specific expression

Finally, we performed RNA sequencing to determine spatially restricted mutation allele–specific expression. The majority of mutated genes had low or absent expression, as only about 40% of mutated genes were expressed and <20% had >20 reads (Fig. 6A). To determine allele-specific expression, we used the >20 read cutoff to obtain robust frequencies of the mutated allele, and correlated the variant allele frequency (VAF) of the RNA to the VAF of the DNA. In line with Castle and colleagues (29), we observed a good correlation between RNA and DNA VAF for the majority of
mutations, that is, the mutated allele was expressed according to its frequency in the DNA (Fig. 6B). As an exception from this rule and in accordance with the concept of nonsense-mediated mRNA decay, we found that alleles harboring stop mutations had significantly lower expression as compared with alleles harboring synonymous or missense mutations (Fig. 6C). As expected, a significant fraction of genes with mutations at chromosomal loci undergoing loss of heterozygosity (LOH) demonstrated preferential expression of the mutated allele. Subsequently, we used a binomial test to identify genes with preferential expression of the mutated allele. Subsequently, we used a binomial test to identify genes with preferential expression of the mutated allele. Additionally, we performed a Fisher exact test to identify genes with preferential expression of the mutated allele. Subsequently, we used a Fisher exact test to identify genes with preferential expression of the mutated allele.

Discussion

We have identified ITH of somatic mutations, DNA copy number, and transcriptomic changes in multiple regions from eight melanoma tumors and demonstrated that 3%–38% of somatic mutations were not identified in all regions of the tumors. In contrast to renal cell cancer, where approximately 75% of cancer driver mutations are affected by ITH (2), we found only 12% of cancer driver mutations to be affected by ITH, indicating that heterogeneous mutations were predominantly passenger mutations. Moreover, ITH seemed to be independent of the number of biopsies analyzed, as the tumor with only three regions analyzed showed the highest degree of ITH, suggesting that the degree of ITH could vary in melanomas.

In line with other melanoma sequencing studies (9, 30), we detected UVB-induced C>T transitions. Our analysis confirms that this signature is enriched early in melanoma tumor evolution occurring in all tumor regions. We found a significant reduction of the UVB-induced mutational signature in branch (nontrunk) mutations, suggesting that mutations arising later in the progression of melanoma may occur as a result of other mutational processes. This is in line with recent studies in lung cancer, where heterogeneous mutations are passenger mutations. Furthermore, we found that the exception of one patient in our current study none received any systemic treatment prior to surgical removal of tumor; therefore, the switch in mutational processes is likely due to evolutionary changes occurring during melanoma progression and not due to extrinsic factors. This further highlights the benefits of multiregion sequencing as different mutational processes operate during tumor evolution.

We confirmed previous findings consistent with activating somatic mutations in the MAPK pathway being driver events in the majority of melanomas (24). We showed that constitutive activation of BRAF, KIT, and NRAS through somatic mutations was ubiquitous in seven patients. The last patient was wild-type for BRAF, NRAS, and KIT; however, the tumor harbored a nonkinase domain PDGFRα mutation. In contrast, we found evidence of genetic mutations in the PI3K pathway occurring as nontrunk events. In particular, we found parallel evolution of three distinct activating PIK3CA mutations (p.V344G, p.R38H, and p.H1047R) and a stop mutation in PIK3R1 in a large lymph node metastasis (Mm1641). Mutational convergence affecting
different PI3K members in single melanoma tumors implicate that activation of this pathway is involved in later stages of metastatic melanoma progression. In addition, activation of the PI3K pathway via somatic mutations may be a reason for acquired resistance to BRAF inhibition as previously described (31). Indeed, patient Mm1641 received BRAF inhibition after surgery but subsequently developed resistance to the therapy.

Although it is difficult to discern the full extent of ITH at all levels using multiregion sequencing only, we observed that patients having melanoma tumors with high degree of ITH as revealed by our approach experienced a more aggressive disease course. These findings are further supported by data from similar studies in lung cancer, esophageal adenocarcinoma, and pediatric tumors (4, 8, 32). However, given that this study included a...
limited number of patients all treated differently, additional larger studies are required to further confirm these data given the significance of prognostic markers in melanoma.

Addressing an additional layer of genomic heterogeneity we investigated the expression of mutated alleles using RNA sequencing. In line with the results of Castle and colleagues (29), we found a good correlation (corr. = 0.57) between DNA and RNA VAFs. Our molecular exploration of ITH in melanoma further showed that there is considerable variation in prognostic gene expression signatures within individual melanoma tumors in line with results from studies in renal cell cancer (2). However, the analyzed gene signatures are partly dependent on the tumor microenvironment, highlighting another layer of genomic complexity within melanoma tumors.

Taken together, we have used a multiregion sequencing approach in early-stage treatment-naïve metastatic melanomas

Figure 5.
Gene expression and DNA copy number aberrations analysis for the eight melanoma patients. For each tumor site/patient (A), the following information is shown (panels from top): B, molecular subtype [high-immune (orange), pigmentation (green), proliferative (red), normal-like (blue), and unclassified (gray; ref. 22). C, expression of pigmentation, immune response, and proliferation genes (green, downregulation; red, upregulation). D, copy number levels of GISTIC regions defined by the The Cancer Genome Atlas melanoma study (24). For copy number aberrations, blue denotes loss of genetic material and red denotes gain thereof.
and showed that ITH was directed by either mutational or gene expression heterogeneity, suggesting a dual layer of genomic complexity in the heterogeneity of melanoma tumors. This study represents the first comprehensive genomic investigation of intratumor heterogeneity in metastatic melanoma and complements the findings by Shain and colleagues on genetic evolution of melanoma from precursor lesions (33). Although the majority of mutations in cancer driver genes such as \( \text{BRAF}^{V600E} \) occurred ubiquitously throughout the tumor, mutations in the PI3K pathway demonstrated a heterogeneous pattern, suggesting that genetic events occurring in this pathway represent later acquired mutations in metastatic melanoma evolution. Furthermore, Shain and colleagues (33) found that UVB is the main factor in both the initiation of precursor lesions and their progression to primary melanoma; however, our results implicate that there are other mutational processes operating during the evolution of metastatic melanoma. Thus, early melanoma progression may be dependent on UVB-induced mutagenesis, while later metastatic evolution is associated with additional non-UVB mutational processes. Importantly, our results further indicate that mutation based ITH may be a prognostic biomarker, as patients with high degree of mutational heterogeneity presented with an aggressive disease course. Extension of research to epigenetic and clinical assessment in much larger cohorts, preferably with repeated biopsies at relapse after treatment, is needed to fully understand the clinical and biologic impact of ITH in melanoma.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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