HHS Public Access

Author manuscript

Semin Cancer Biol. Author manuscript; available in PMC 2021 April 01.

Published in final edited form as:

Semin Cancer Biol. 2020 April ; 61: 139–148. doi:10.1016/j.semcancer.2019.09.013.

The Mutational Landscape of Mucosal Melanoma

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Abstract

Mucosal melanoma is a rare and aggressive subtype of melanoma that has a less favorable prognosis due to the lack of understanding and identification of oncogenic drivers. Recently, whole genome and whole exome sequencing have unveiled the molecular landscape and potential oncogenic drivers of mucosal melanoma, which remains distinct from cutaneous melanoma. In this review, we provide an overview of the genomic landscape of mucosal melanoma, with a focus on molecular studies identifying potential oncogenic drivers allowing for a better mechanistic understanding of the biology of mucosal melanoma. We summarized the published genomics and clinical data supporting the observations that mucosal melanoma harbors distinct genetic alterations and oncogenic drivers from cutaneous melanoma, and thus should be treated accordingly. The common drivers (*BRAF* and *NRAS*) found in cutaneous melanoma have lower mutation rate in mucosal melanoma. In contrast, *SF3B1* and *KIT* have higher mutation rate in mucosal melanoma as compared to cutaneous melanoma. From the meta-analysis, we also observed that the mutational profiles are slightly different between the “upper” and “lower” regions of mucosal melanoma, providing new insights and therapeutic options for the mucosal melanoma patients. Mutations identified in mucosal melanoma should be incorporated into routine clinical testing, as there are targeted therapies already developed for treating patients with these mutations in the precision medicine era.

Keywords

Mucosal Melanoma; KIT; SF3B1; Mutational Landscape; Druggable Targets

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Conflict of Interest: None

1. Introduction

Mucosal melanoma is an aggressive rare sub-type of melanoma, arising from melanocytes in mucosal tissues lining the respiratory, gastrointestinal and urogenital tracts[1]. Mucosal melanoma is markedly different from cutaneous melanoma at both the molecular and clinical level. Unlike cutaneous melanoma, mucosal melanoma has a significantly lower somatic mutational burden, lower frequency of the common targetable *BRAF*-V600E mutation and poorer responses to immunotherapy. Clinical presentation of mucosal melanoma is aggressive, the 5-year survival of mucosal melanoma, considering all stages at time of diagnosis, is 14% as compared to 80% for cutaneous melanoma[2,3]. While major advancements have been made in the understanding and treatment of UV-exposed cutaneous melanoma, there remains a lack of understanding and identification of oncogenic drivers in mucosal melanoma, likely due to the rarity of samples and lack of available preclinical models. Recently, whole genome and whole exome sequencing have unveiled the molecular landscape and potential oncogenic drivers of mucosal melanoma, which remains distinct from cutaneous melanoma. Such studies have identified that somatic mutation rates are considerably lower in mucosal melanoma, and do not display the UV mutational signatures, as compared to UV-exposed cutaneous melanoma[4]. Interestingly, the somatic mutation rates in mucosal melanoma are comparable to the rates seen in cancers that are not associated with exposure to known mutagens[4]. It has also been demonstrated that mucosal melanoma display increased genomic instability which is characterized by structural variants, amplifications and deletions[4–7]. In this review, we provide an overview of the genomic landscape of mucosal melanoma, with a focus on molecular studies identifying potential oncogenic drivers allowing for a better mechanistic understanding of the biology of mucosal melanoma. We systematically reviewed published literatures and identified 65 key studies that define the mutational landscape of mucosal melanoma (Table 1). We classify the somatic mutations as “druggable” based on the published clinical trial results and discuss the recent advances in systemic treatment of this disease. We summarized the published genomics and clinical data supporting the observations that mucosal melanoma harbors distinct genetic alterations and oncogenic drivers from cutaneous melanoma, and thus should be treated accordingly.

2. Melanocyte biology and clinical characteristics of mucosal melanoma

Melanoma comprises all skin cancers that arise from melanocytes, which are specialized cells whose primary role is the production of melanin that serves as a shield to protect DNA from UV-radiation. The presence of melanocytes has been demonstrated in mucosal membranes, however, the definitive role and function of mucosal melanocytes in non-UV exposed mucosal tissues remains unclear. It is hypothesized that melanocytes localized to mucosal tissues potentially due to errors in migration from the neural crest during development[3]. Interestingly, melanin is involved in antimicrobial defense, supporting a role for melanin in innate immunity[8]. It is hypothesized that mucosal melanocytes have an immunogenic role, especially given their location in immunologically critical mucosal surfaces[3]. In order to understand the biology of mucosal melanoma tumorigenesis, examining the different functions of melanocytes situated in various mucosal tissues may be of importance.

It is estimated that mucosal melanoma accounts for 0.8–1.8% of all melanomas in the US[9], while the incidence in Chinese populations has been reported to reach 23%, likely due to the lower prevalence of cutaneous melanoma in Asian populations[10]. While mucosal melanomas can arise from any mucosal epithelium, the most common areas are vulvovaginal (18–40% of cases), anorectal (17–24% of cases) and the head and neck region (31–55% of cases)[11,12]. In rare occasions, primary mucosal melanoma has been observed in the urinary tract, esophagus, stomach, small and large intestine and cervix[13]. So far, the definitive risk factors for the development of mucosal melanoma remain unknown.

The five year overall survival rate for all subtypes of mucosal melanoma is only 25%, however mucosal melanoma of the head and neck region has a significantly better five year overall survival rate of 31.7%, as compared to anorectal (19.8) and vulvovaginal (11.4%)[14]. The median age of diagnosis for mucosal melanoma is 70[11,15]. Overall, the incidence of mucosal melanoma is stable, except for anorectal melanomas which have an increasing incidence, although one cannot rule out this increase as being attributed to awareness of clinicians and better diagnostic resources for mucosal melanoma[16].

Mucosal melanomas often present and are diagnosed at later stages, likely due to the fact that they occur in more concealed areas of the body. Locoregional nodal metastasis at diagnosis is highly common in mucosal melanoma, specifically 21% of head and neck, 23% of vulvovaginal and 61% of anorectal mucosal melanomas present with involved lymph nodes[14]. Even considering the stage at diagnosis, mucosal melanoma is associated with significantly worse survival outcomes compared to cutaneous and acral melanoma[17]. Currently, the best approved treatment modality for mucosal melanoma is complete surgical excision of the primary tumor. However, the anatomical surgical constraints and multifocal growth pattern significantly limit the ability for wide negative margins, and must be heavily weighed on the patient's quality of life. Unfortunately, 50–90% of patients exhibit postoperative local recurrence, even in the context of achieving negative margins[14]. Adjuvant radiotherapy improves local control of the disease but does not change the overall survival[18,19]. Therefore, there is an urgent need to better molecularly characterize mucosal melanoma and to identify "druggable" targets to improve clinical outcomes in this rare cancer.

3. Mutated driver genes and molecular landscape of Mucosal Melanoma

Recently, several studies have been conducted using targeted sequencing, whole-exome sequencing (WES) or whole-genome sequencing (WGS) approaches to characterize and identify somatic mutations in mucosal melanoma. However, due to the limited samples of this rare cancer, most of these global genome studies (WES/WGS) have small sample sizes (range from 2–67, average of 27)[4–7,20–24]. To systematically define the mutational landscape of mucosal melanoma, we performed a meta-analysis of 4009 patients reported in 65 published studies utilizing either targeted sequencing or WES/WGS technologies (Table 1). We found that mucosal melanoma has a different mutational landscape as compared to cutaneous melanoma (Figure 1A & 1B). The mutation burden is much lower in mucosal melanoma, as compared to cutaneous melanoma[4,6,7,21]. Moreover, we also observed that the mutational landscape is different between mucosal melanoma in "upper" and "lower"

regions (Figure 1C & 1D). The results from the meta-analysis provide mechanistic insights to potential oncogenic drivers and some clinical and therapeutic implications for mucosal melanoma.

3.1 MAPK pathway

The mitogen-associated protein kinase (MAPK) pathway is an important intracellular signaling pathway and is commonly activated in melanoma, promoting tumorigenesis. The MAPK pathway responds to extracellular binding of growth factors to receptor tyrosine kinases (RTKs), that activates downstream signaling starting with activation of a GTPase (Ras) followed by tyrosine kinases that are activated by phosphorylation. The signal transduction typically includes activation of the following proteins: Ras/Raf/MEK/ERK. In cutaneous melanoma, the MAPK pathway is commonly activated by mutations in the key signaling components, BRAF, NRAS and NF1. Recently, the Cancer Genome Atlas Network (TCGA) proposed a genomic classification for cutaneous melanoma defined by four subgroups, each harboring mutations in *BRAF, NRAS, NF1*, or "triple wildtype", corresponding to tumors lacking these mutations[25]. Figure 1B outlines the cutaneous melanoma TCGA cohort as defined by the four subgroups, however for the purposes of this study, we define the triple wildtype group as "unknown". A vast majority of cutaneous melanomas (94%) contain MAPK pathway activating mutations (*BRAF, NRAS, NF1*), whereas only a 28% of mucosal melanomas harbor these mutations (Figure 1 A–B). Although found at a lower rate, MAPK activating pathway mutations can be therapeutically targeted, thus it remains important to understand the role that mutations in the MAPK pathway may be playing in mucosal melanoma tumorigenesis.

BRAF is a serine/threonine kinase involved in signal transduction in the MAPK pathway promoting cellular proliferation and survival. The *BRAF* oncogene is found to be highly mutated at codon V600 in multiple cancers and known to occur in approximately 35–50% of cutaneous melanomas (Figure 1B). BRAF-V600 mutations result in constitutive activation of the BRAF protein, and hyperactive MAPK pathway activity promoting tumorigenesis. The MAPK pathway can be therapeutically targeted with FDA approved small molecule inhibitors directly targeting BRAF-V600 and MEK. Clinically, combined inhibition of BRAF and MEK has been approved for *BRAF*-mutated cutaneous melanoma[26–28]. For the current meta-analysis of mucosal melanoma, we focused on *BRAF*-V600 mutations, since those are known to strongly activate MAPK pathway and is a relevant clinical target. We observed that only approximately 6% of mucosal melanomas harbor *BRAF*-V600 mutations (Figure 1A), which was seen at similar mutational rates in both the upper and lower mucosal melanoma regions (Figure 1C–D). Interestingly, in mucosal melanoma, there is an increased number of non-canonical *BRAF* mutations (L505H, G469A, L597R, and T599I), which are known to lead to weaker MAPK activation as compared to *BRAF*-V600[29]. However, it remains unclear if these non-canonical *BRAF* mutations will be clinically responsive to MAPK pathway inhibition, indicating the importance of understanding the effects of non-canonical *BRAF* mutations.

NRAS is an oncogene that is part of the Ras family of oncogenes that encode small GTP-binding proteins that respond to RTK activation and facilitate downstream activation of Raf.

Activating point mutations in *NRAS* are commonly found at the G12, G13 and Q61 sites, which are the somatic mutations that we report for *NRAS* in our meta-analysis. Mucosal melanomas harbor *NRAS* mutations at a rate of 8%, which is lower than the rate seen in cutaneous melanoma (28%) (Figure 1A–B). Previous studies have reported conflicting observations regarding the enrichment of *NRAS* mutations in mucosal melanomas arising from upper or lower regions. In a pan-mucosal melanoma study, 10% (7/71) of tumors were *NRAS* mutated, at the G12, G13 or Q61 sites. Interestingly, they noticed that vaginal melanomas have a significantly higher proportion of *NRAS* mutations (43%) as compared to other mucosal melanoma subtypes, and were associated with a significantly worse overall survival[30]. However, a study of 16 esophageal melanomas identified *NRAS* (Q61, G12/13) mutations in 37.5% of cases (6/16)[31], which the authors conclude that this data suggests that esophageal mucosal melanomas may display an enrichment of *NRAS* mutations. In the present study, we observed that there is not a significant difference in *NRAS* mutations in upper (13%) or lower (9%) region of mucosal melanomas (Figure 1C–D), suggesting that *NRAS* mutations may not be specific to a particular mucosal melanoma sub type.

NF1, Neurofibromin 1, is a negative regulator of Ras, and is commonly lost or harbors loss of function mutations in cancers, and thus is considered to be a tumor suppressor. Loss of *NF1* is associated with increased MAPK pathway activity, and has been shown to be significantly enriched in cutaneous melanoma tumors lacking either *BRAF* or *NRAS* mutations[22]. In our current meta-analysis, we observed that *NF1* is mutated at a rate of 14% in mucosal melanoma, which is also found at the same rate observed in the TCGA cohort of cutaneous melanoma (14%) (Figure 1 A–B). Of interest, one study found that *NF1* was significantly co-mutated with *KIT* in 32% of mucosal melanomas, which is a significantly higher rate than in cutaneous melanoma (4%)[21].

SPRED1 (sprout-related, EVH1 domain containing protein 1), a negative regulator of the MAPK pathway, recruits NF1 to the plasma membrane to convert active Ras-GTP into the inactive form bound to GDP. It has recently been reported that *SPRED1* may function as a tumor suppressor in mucosal melanoma. *SPRED1* loss was found in 26% (11/43) of mucosal melanomas, which included bi-allelic inactivation through either deep deletion or by truncating mutation combined with loss of the wild type *SPRED1* allele[32]. Consistent with this, more recently Newell et. al. identified *SPRED1* aberrations in 5 of 67 mucosal melanomas through whole genome sequencing[7]. Ablain et. al. observed a trend towards a pattern of mutual exclusivity with *SPRED1* loss and *NF1* loss of function mutations, suggesting that *SPRED1* loss and *NF1* loss may play similar roles in tumor progression in mucosal melanoma[32]. *SPRED1* loss co-occurred significantly with *KIT* alterations (30%, 7/23 cases). *In vitro* and *in vivo* models demonstrated that in the context of *KIT* mutations, *SPRED1* loss resulted in increased MAPK pathway activity and conferred resistance to the KIT tyrosine kinase inhibitor dasatinib. These results lay the groundwork to establish *SPRED1* as a tumor suppressor gene that cooperates with activating *KIT* mutations to sustain MAPK signaling and may confer resistance to KIT inhibition. However, the clinical impact of *SPRED1* loss remains to be defined in mucosal melanoma.

3.2 Receptor Tyrosine Kinase: KIT

KIT is a transmembrane receptor tyrosine kinase (RTK) that is commonly expressed in a variety of normal cell types, and its activation plays an important role in normal melanocyte development regulating growth, differentiation and migration[33]. Once it is activated through dimerization, it regulates the activation of several oncogenic downstream signaling pathways such as MAPK and AKT pathways[33]. The common *KIT* alterations observed in melanomas are amplifications and missense mutations, which occur throughout the coding region at a high frequency in the juxta-membrane autoinhibitory domain (encoded by exon 11) and the tyrosine kinase domains (encoded by exons 12–21)[34]. In the current meta-analysis, we reported all non-synonymous *KIT* mutations in mucosal melanoma. We found *KIT* mutations at a rate of 13% in all mucosal melanomas, and we observed a similar frequency in both upper (15%) and lower (13%) regions (Figure 1C–D). While *KIT* mutations are found at a lower rate in cutaneous melanoma, previous reports identify that cutaneous melanomas lacking recurrent mutations in *BRAF* or *NRAS* have a significant enrichment of alterations in *KIT*[22]. Given that *KIT* mutations are enriched in mucosal melanomas, it is important to understand the clinical implications of *KIT* mutations as a prognostic factor, as there are conflicting reports on the prognostic impact.

In a cohort of 86 French patients, of various mucosal melanomas, 11.6% (5/96) harbored *KIT* mutations, however there was no prognostic impact of *KIT* mutant patients compared to *KIT* wild type patients[35]. Further, in a pan mucosal melanoma study, *KIT* mutations were most frequently found in 35% (8/23) mucosal melanomas of the vulva as compared to all other sites[30]. Additionally, when KIT protein levels were analyzed by immunohistochemistry, there was a significant increase in KIT protein expression in *KIT* mutant tumors as compared to *KIT* wildtype tumors. There was no significant association with *KIT* mutational status or KIT protein expression on overall survival. In a 28 patient cohort, mixed with nasal and oral mucosal melanomas, 25% (7/28) of patients harbored *KIT* mutations. Again, there was no prognostic impact of *KIT* mutations as compared to *KIT* wildtype patients in this cohort[36]. In contrast to the previously mentioned studies, *KIT* mutational analysis from a large cohort of 755 mucosal melanoma Asian patients found that KIT mutant positive patients (8.7%, 66/755) had worse overall survival in mucosal melanomas, as compared to *NRAS* and *BRAF* mutations, which did not have any effect on prognosis[37].

However, it is important to note that none of these previously mentioned studies of the prognostic factor of *KIT* mutations was done in the context of KIT targeted therapy, which is discussed in section 4.2. Further, these studies did not address the differential effect on prognosis based off of the location of the *KIT* mutation, which is suggested to play a role in the sensitivity of response to KIT inhibition.

3.3 mRNA splicing factor: SF3B1

The spliceosomal protein SF3B1 is a core component of the U2 snRNP which recognizes the branch point sequence at the 3’ splice site at intron-exon junctions. One of the major roles of SF3B1 is RNA splicing, which involves the removal of nucleotide sequences from precursor RNAs into mature RNA transcripts. Mutations in *SF3B1* are considered to be

neomorphic resulting in alternative splicing that promotes global transcriptomic dysregulation[38,39]. The fate of alternatively spliced transcripts can either be (1) translation into aberrant proteins, or (2) undergo nonsense mediated decay (NMD) resulting in downregulation at the mRNA and protein levels (Figure 2A)[40].

SF3B1 mutations have been identified in myelodysplastic syndrome (MDS)[41], chronic lymphocytic leukemia (CLL)[42], prostate cancer[43], breast cancer[44], and uveal melanomas[4,45] (Figure 2B). The C-terminal domain of SF3B1 contains 22 HEAT domains (Huntington, elongation factor 3, protein phosphatase 2A, and the yeast PI3-Kinase TOR1), where hotspot mutations are typically localized in domains 4–6 (Figure 2B)[46]. Across cancer types, *SF3B1* mutations are heterozygous and enriched for the R625, K666 and K700 residues, here defined as “canonical hotspot mutations”. Interestingly, *SF3B1* mutations in mucosal and uveal melanoma are almost exclusively enriched for the R625 residue, which is found at a lower frequency in hematologic malignancies and breast cancer, where the K666 and K700 residues predominate (Figure 2B). While *SF3B1*-R625 is found at a high frequency in mucosal melanoma and we consider it to be a canonical hotspot mutation, it remains unclear how additional non-canonical HEAT domain mutations may affect SF3B1 function. Thus, for our current meta-analysis we reported all non-synonymous *SF3B1* mutations in mucosal melanoma. Interestingly, we observed that *SF3B1* mutations may be enriched in lower regions of mucosal melanomas (27%) as compared to the mucosal melanomas in the upper regions (7%) (Figure 1 C–D). In support of our observation, recent studies using WES or WGS on oral mucosal melanomas (n=84) failed to identify any mutations in *SF3B1*[5,20].

Hintzsche et. al. observed that *SF3B1* mutations were present in 7/19 (35%) of mucosal melanomas, the most common sites being anorectal (3/5,60%) and vulvovaginal (4/9, 44.4%), as compared to nasopharyngeal (1/5, 20%). It is important to note that only the lower regions of mucosal melanomas (anorectal and vulvovaginal) harbored *SF3B1*-R635 hotspot mutations, whereas the upper regions (nasal) harbored a non-canonical *SF3B1*-E1105G mutation located in the HEAT domain of SF3B1[21]. In agreement, Quek et. al. observed that *SF3B1* mutations were the most common mutation (6/27, 22%), where *SF3B1*-R625 hotspot mutations were enriched (5/6, 83%) and were exclusively found in vulvovaginal (5/19, 26%) and anorectal (3/5, 60%) sites, as compared to oral and nasal locations. Furthermore, *SF3B1* mutations were associated with shorter overall and progression free survival (34.9 and 16.9 months, respectively) compared to *SF3B1* wild type mucosal melanomas (79.9 and 35.7 months, respectively)[47]. We performed meta-analysis of *SF3B1* mutations on overall survival from three mucosal melanoma studies (total patients, n=53) and found that *SF3B1* mutations trend towards an increase in overall survival (Figure 2C). The differential effect of clinical outcomes associated with *SF3B1* mutations indicates that the prognostic value of *SF3B1* mutations still needs to be explored in a larger patient cohort.

3.4 Structural variants and fusion genes in mucosal melanoma

Whole genome sequencing provides the platform for the analysis of structural variants at the genome-scale[4,6]. WGS has been performed in an oral mucosal melanoma cohort, and the authors identified specific structural variants were associated with worse prognosis[5].

Specifically, patients with clustered inter-chromosomal translocations between chromosome 5 and chromosome 12 have significantly worse overall survival (9.0 vs 28.0 months, respectively)[5]. Additionally, break fusion bridges, characterized by the loss of telomeric regions and a high number of inversions, were tightly associated with a worse prognosis (median overall survival 9.0 vs 34.0 months)[5].

BRAF fusion genes represent an alternate mechanism of MAPK pathway activation and are commonly identified in identified in a small percentage of "Triple-Wild Type" tumors, lacking *BRAF, NRAS* or *NF1* mutations, in cutaneous melanoma[48]. The frequency of *BRAF* fusions in mucosal melanoma is comparable to the frequency of *BRAF* fusions found in triple wild type cutaneous melanomas[49]. Kim et. al. discovered and biologically characterized a novel *BRAF* fusion (*ZNF767-BRAF*) in a vemurafenib resistant respiratory tract mucosal melanoma patient. This *BRAF* fusion was the result of two successive microhomolgy mediated end joining of exon 1 of *ZNF767* with exon 11 of *BRAF*, retaining the kinase domain of BRAF. *ZNF767* is a pseudogene, and its biological role remains unclear. Melanoma cells harboring the *ZNF767-BRAF* fusion displayed resistance to the BRAF inhibitor vemurafenib *in vitro*, which recapitulated the clinical response seen in the mucosal melanoma patient harboring the *BRAF* fusion, but demonstrated sensitivity to the MEK inhibitor trametinib *in vitro*[49]. Mechanistically, the *ZNF767-BRAF* fusion activates the MAPK pathway through the formation of RAF homo- and hetero-dimers. Lastly, the *ZNF767-BRAF* fusion cells were sensitive to MEK inhibition with either the combination of PI3K or CDK4/6 inhibitors in vitro and in vivo[49].

Anaplastic lymphoma kinase (*ALK*) fusions are oncogenic and occur in 3–7% of NSCLC, and clinically are sensitive to small molecule inhibitors targeting ALK. *ALK* fusions have been identified in ~11% of cutaneous melanomas, however the clinical impact of *ALK* fusions in melanoma has been understudied. Recently, Couts et. al. identified a mucosal melanoma that contained several novel *EML4-ALK* fusion variants, and was sensitive to ALK inhibition *in vitro* and *in vivo*. Interestingly, a novel ALK isoform, *ALK-ATI*, was also identified. *ALK-ATI* results from an alternative transcript initiation site located in intro 19 that includes a portion of intron 19 and exons 20–29[50]. In other cancers it was previously shown that *ALK-ATI* induces tumorigenesis and sensitized cells to ALK inhibitors. Couts et. al. also identified a subset of mucosal melanomas that expressed *ALK-ATI*, however, these cells were not sensitive to ALK inhibition *in vitro* and *in vivo*.

3.5 Copy number alterations in mucosal melanoma

One of the benefits of WGS is the ability to assess genome-wide copy number variations (CNV) in mucosal melanoma. Whole genome sequencing of 65 oral mucosal melanomas identified significant amplifications in *KIT, TERT, CDK4, CCND1* and *NOTCH2*; along with significant losses in *CDKN2A/B* and *TP53*[5]. Amplifications in the 12q13–15, containing *CDK4* and *TERT*, in >50% of oral mucosal melanomas (33/65 samples), representing the most commonly altered genomic region. In addition, chromosome 5p15, containing *TERT*, was also significantly co-associated with *CDK4* amplifications, suggesting a potential functional relevance of *TERT* and *CDK4* as co-amplified genes. A small whole exome sequencing study of 19 oral mucosal melanomas identified that 11/19

(57.9%) harbored amplifications of chromosome 12q14, which contains *CDK4*[20]. However, whole exome and whole genome studies of other subtypes of mucosal melanoma, such as head and heck, vulvovaginal and anorectal, did not describe the presence of chromosome 12q14 or *CDK4* amplifications[4,21,24,32,47,51]. This warrants further interrogation of *CDK4* amplification status in other subtypes of mucosal melanoma.

4. Precision medicine: Druggable targets and immunotherapy in mucosal melanoma

One of the immediate clinical implications from the mutational analysis is the identification of actionable driver mutations for mucosal melanoma. Leveraging the success from the development of targeted therapies, many of the identified mutations in mucosal melanoma have drugs or clinical investigated compounds available to treat these patients (Figure 3).

4.1 BRAF and MEK kinase inhibitors

BRAF mutations result in hyperactivation of MAPK pathway signaling and represent a promising therapeutic target in mucosal melanoma. The ATP-competitive small molecule inhibitors (vemurafenib, dabrafenib and encorafinib) specifically targeting mutant BRAF have resulted in remarkable responses in cutaneous melanoma patients harboring BRAF-V600 mutations, increasing progression free survival and overall survival when compared to chemotherapy[52,53]. More strikingly the combination of BRAF and MEK inhibitors have demonstrated superior clinical benefits over BRAF inhibitor monotherapy. There are currently three FDA approved non-ATP competitive allosteric inhibitors of MEK which target MEK1 (cobimetinib) or both MEK1 and MEK2 (trametinib and binimetinib). Dual inhibition of BRAF and MEK with the combination of the following BRAF/MEK inhibitors vemurafenib/cobimetinib, dabrafenib/trametinib and encorafinib/bimimetinib are FDA approved for the treatment of *BRAF* mutant metastatic melanoma[26–28]. Based on promising results of the combination of BRAF and MEK inhibition in cutaneous melanoma, these BRAF/MEK inhibitors represent an attractive treatment option for *BRAF*V600 mutant mucosal melanoma patients.

Although mucosal melanomas do not harbor a high rate of *BRAF* mutations, they do have a high rate of *NF1* alterations. Preclinical studies have demonstrated that tumors with alterations in *NF1*, either loss of function mutations or deletions, are more resistant to BRAF inhibitors[54]. Thus, it remains important to understand how *NF1* mutations, *RAS* mutations and other mutations that activated the MAPK pathway may be targeted by MEK inhibition. Recent reports in cutaneous melanoma suggest that BRAF fusions may function as a novel resistance mechanism to vemurafenib through promoting reactivation of the MAPK pathway[55]. Further preclinical and clinical research needs to be conducted to identify the best targeted therapy for such BRAF fusions, and mucosal melanomas should be screened for such fusions as they may represent sensitivity to MEK inhibition.

4.2 Tyrosine kinase inhibitors

Clinical trials in *KIT* mutant tumors, such as gastrointestinal stromal tumors (GISTs) and cutaneous melanoma, have observed that patients with *KIT* exon 11 or exon 13 mutations

are shown to have a better response to KIT targeted therapy, suggesting that certain *KIT* alterations may be more sensitive to KIT inhibition[30,56]. There are a number of small molecule tyrosine kinase KIT inhibitors, such as imatinib, nilotinib and dasatinib, that have shown variable clinical activity in the treatment of *KIT* mutated mucosal melanoma.

Imatinib: Imatinib is a tyrosine kinase inhibitor (TKI) that targets KIT, BCR-ABL and platelet-derived growth factor receptor alpha (PDGFRA)[57]. A seminal study demonstrated that certain *KIT* alterations may render the tumor more or less sensitive to KIT targeted therapy with imatinib in metastatic melanoma. This open arm phase II trial with imatinib in 25 patients with *KIT* mutant metastatic melanoma, consisting of mucosal (n=13), acral (n=10) and chronically sun-damaged (CSD) (n=5) subtypes, displaying a range of mutations in exons 9, 11, 13, 17 and 18 of KIT (21/25). *KIT* amplifications were present in 15/25 patients and co-occurred with mutations in 11/25 patients. In this study, the overall durable response rate of 16%, with a median time to progression of 12 weeks, and a median overall survival of 10.7 months. There was no significant association with clinical melanoma subtype and response to imatinib. Two patients achieved durable complete responses, and harbored a *KIT*-L576P mutation in exon 11 and amplification. Patients harboring recurrent *KIT* mutations previously identified in GIST and melanoma (V559C, L576P, V642E and N822J), had a higher proportion of response (46%) compared to other *KIT* mutations. Furthermore, all patients with a partial, durable or complete response (n=6) harbored either L576P or K642E *KIT* mutations. In addition, tumors with a mutant *KIT* allele in greater abundance that the wild type *KIT* allele demonstrated a better response rate, time to progression and overall survival as compared to other cases. This study suggests that some *KIT* mutations, such as L576P and K642E, may possess a greater oncogenic driver capacity, and thus increased sensitivity to *KIT* inhibition[34].

Consistent with this clinical observation, several phase II clinical trials were conducted to evaluate the clinical benefit of imatinib in *KIT* mutated melanoma patients, including mucosal melanoma[58–60]. Taken together, these clinical studies of treating *KIT*-mutated mucosal patients observed response rates that ranged from 20%−30%. Overall, *KIT*-mutated patients, as compared to patients with *KIT* amplifications, have better clinical responses to imatinib and the mutations in exons 11 and 13 were more predictive of imatinib response as compared to other *KIT* mutations. These clinical studies warrant further investigation of treating imatinib for *KIT*-mutated mucosal melanoma.

Nilotinib: Nilotinib is a second-generation TKI structurally derived from imatinib, and has a similar target profile to imatinib but exhibits greater potency does not require an active transport mechanism to enter cells. Nilotinib binds to and inhibits KIT, DDR, ABL/BCR-ABL, PDGF and several EPH RTKs, and maintains activity against a variety of *KIT* mutations in exons 9, 11 and 13[61]. One of the first clinical trials with nilotinib in *KIT* altered metastatic melanoma, including mucosal melanoma, tested the clinical efficacy in two cohorts, one that was refractory to previous KIT targeted therapy (cohort A), with the other testing nilotinib in *KIT*-mutant patients with CNS metastasis (cohort B)[62]. The primary endpoint of this study was to determine the proportion of patients who were alive and without progression of disease 4 months post nilotinib treatment. The cohort included 19

patients, with 90% (17/19) patients harbored *KIT* mutations, consisted of acral, CSD and mucosal melanoma, with mucosal consisting of 63% (12/19) patients, 5 of which harbored CNS brain metastases. Of note, 17/19 patients previously received prior treatment with imatinib. Patients in cohort A, previously treated with imatinib, displayed a time to progression (TTP) of 3.4 months and an OS of 14.2 months. In cohort A, 3/11 patients achieved disease control at 4 months, with a range of progression free survival times of 5.5, 11.5 and 37.5+ months. Of interest, two patients achieved durable partial responses to nilotinib, both of which had previously demonstrated either a partial or complete response to imatinib, 12.4 and 20 months, respectively, demonstrating that nilotinib has a clinical effect in overcoming acquired resistance to imatinib. Both of the responding patients had mucosal melanoma harboring either L576P or K642E *KIT* mutations. Patients in cohort B, with CNS involvement, had a TTP of 2.6 months, and a short OS of 4.3 months. One partial response was observed in an anorectal mucosal melanoma with CNS involvement, harboring a V560D *KIT* mutation, however this patient did not receive prior imatinib therapy.

Additional phase II clinical trials with nilotinib in *KIT*-mutated melanoma patients (including mucosal melanoma) exhibited similar responses as seen with imatinib, and observed an average overall response rate of 20.9% (range 16.7%−26.2%)[63–65]. For example, the French Skin Cancer network conducted a phase II study using nilotinib in 25 patients with metastatic melanoma having *KIT* mutations or amplifications, where mucosal melanoma accounted for 40% of the patients (n=10)[65]. At 6 months, there was a 16% overall response rate, which included three patients with a partial response and one patient with a complete response to nilotinib. In this cohort, *KIT* mutations in exon 11 and 13 were the most common and found in 44% and 32% of patients, respectively. Furthermore, all patients with partial or complete response had either exon 11 or 13 mutations. This study collected serial tumor biopsies at baseline and post nilotinib treatment from 8 patients and monitored oncogenic signaling pathways downstream of KIT, such as the MAPK, PI3K/AKT and JAK/STAT pathways. At baseline, all tumors were positive for phosphorylation of STAT3 at the Serine-727 site, which is implicated in tumorigenesis and survival in melanoma. Following nilotinib treatment, phospho-STAT3 levels significantly decreased in good responders, and remained unchanged in poor responders. This data suggests a phospho-STAT3 levels as potential biomarker of response to nilotinib and warrants future investigation into the mechanism of KIT inhibition, potentially through downregulation of STAT3, in melanoma.

Dasatinib: Dasatinib is another multi-kinase “second-generation” small molecule inhibitor that targets KIT, BCR-ABL, PDGFR-β and the SRC family kinases. Previous preclinical studies demonstrated that dasatinib has superior activity compared to other KIT inhibitors such as imatinib. However, this did not prove to be true in a two stage phase II clinical trial for 73 patients with locally advanced or stage IV melanoma, where 52% (n=38) of patients had mucosal or vulvovaginal melanoma treated with dasatinib[66]. Stage one consisted of 51 total patients, where 3 patients harbored *KIT* mutations, and 6 patients were not tested (n=51 total patients). However, the patients that achieved a partial response (n=3) did not harbor *KIT* mutations. In stage two of the study, only *KIT* mutant positive melanomas were tested (n=22), and 7/22 patients had a partial response, all containing either exon 11 or exon 13 mutations, but did not include L576P mutations. Patients harboring *KIT* mutations in exon

11 or 13 demonstrated a median progression free survival of 4.7 months and a median overall survival of 12.3 months. In this study, *KIT* mutational status had no significant effect in progression free and overall survival with dasatinib. There were no complete responses observed in either stage one or stage two. It is important to note that *KIT* amplifications were not tested in this cohort.

Other Kinase inhibitors: From the published studies, a small subset of mucosal melanoma patients may harbor gene fusions and could be exploited as therapeutic targets. In a recent study, a cell line derived from mucosal melanoma was detected to harbor *EML4-ALK* fusion and responded to ALK inhibitors such as crizotinib and ceritinib *in vitro* and *in vivo*[50]. This preclinical data indicates that targeting *ALK* fusions may represent a therapeutic option. This highlights the importance of screening patients for *ALK* rearrangements and alternative isoforms in mucosal melanoma as patients may benefit for the targeted treatment for these gene fusions.

4.3 Spliceosomal inhibitors

From the recent WES/WGS studies, *SF3B1* was found to be commonly mutated in ~15% in mucosal melanoma. *In vitro* analysis of a subset of alternatively spliced genes identified in *SF3B1* mutant breast cancer and uveal melanoma was validated in a cohort of *SF3B1*-R625 mutant mucosal melanomas[21]. This demonstrates that *SF3B1*-R625 mutations are functionally involved in alternative splicing in mucosal melanoma. Currently, no drugs have been approved by FDA for targeting *SF3B1*-mutant patients, however, several compounds have been recently developed targeting the spliceosome, that has shown preferential lethality for *SF3B1*-mutant cells in preclinical studies[67]. The leading clinical investigated compound is H3B-8800, which is an orally available spliceosomal inhibitor entering phase I clinical trials patients with advanced myeloid malignancies, including patients with *SF3B1*-mutations[68]. In the future, this compound could be investigated in mucosal melanoma patients harboring mutations in *SF3B1*.

4.4 Cell cycle inhibitors

Cell cycle progression from G1 (pre-DNA synthesis) to S phase (DNA synthesis) commonly results from activation of CDK4/6 and forms a complex with Cyclin D1 (*CCND1*) and hyper-phosphorylates retinoblastoma (RB), leading to dissociation of RB from and activation of transcription factor E2F, and cell cycle progression. Inhibition of CDK4/6 with small molecule tyrosine kinase inhibitors result in ablation of CDK4/6 kinase activity and result in RB remaining dephosphorylated and bound to E2F, preventing cell cycle progression. Currently there are three FDA approved drugs, palbociclib, ribociclib and abemaciclib, that target CDK4/6, which are used for the treatment of hormone receptor positive, HER2 negative advanced breast cancer[69]. The anti-tumor effect of palbociclib, was used in an *in vivo* study with oral mucosal melanoma patient derived xenograft (PDX) harboring *CDK4* amplification and resulted in sustained tumor suppression for 8 weeks, which was not observed in a *CDK4* wildtype PDX model[5]. Suggesting that oral mucosal melanomas harboring *CDK4* amplifications may be more likely to benefit from palbociclib treatment. Given that there is a subset of mucosal melanomas that harbor *CDK4*

amplifications, and *CDKN2A* gene deletions, these patients may be candidates for CDK4/6 inhibition.

4.5 Immunotherapy in Mucosal Melanoma.

Immunomodulatory antibodies directly effecting and enhancing the function of T cells have shown promising results in many cancers, especially cutaneous melanoma. Such agents are referred to as “check point inhibitors”, and function to block negative regulators of T cell immunity such as cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1). Three immune check point monoclonal antibody inhibitors are currently approved for patients with metastatic cutaneous melanoma that either target CTLA-4 (ipilimumab) or PD-1 (nivolumab and pembrolizumab). One of the biggest hurdles in understanding the mechanism of response to immunotherapies is the lack of large studies analyzing the efficacy of anti-PD-1 and anti-CTLA-4 in mucosal melanoma.

Recently, pooled retrospective analysis of immunotherapy responses from clinical trials have been published and have shown that single agent anti-PD-1 may be more effective that anti-CTLA-4 in mucosal melanoma. One single center cohort analysis of 44 mucosal melanoma patients analyzed the response to either anti-PD-1 (n=20) or anti-CTLA-4 (n=24), and found that the overall response rate (ORR) for both therapies was 20%[70]. However, when stratifying by treatment, patients treated with anti-PD-1 demonstrated an increased ORR compared to anti-CTLA-4, 35% vs 8%, respectively. In line with this, anti-PD-1 therapy demonstrated a statistically significant improvement in progression free survival (PFS), but not in overall survival, compared to anti-CTLA-4, which was independent of primary tumor site.

Mignard et. al. analyzed the response to immunotherapy (n=151) compared to chemotherapy (n=78) in mucosal melanoma, and observed that the median overall survival of patients treated with immunotherapy (OS: 15.97 months) was significantly longer than treatment with chemotherapy (OS: 8.82 months). Consistent with previous studies, this group observed higher response rates in patients receiving anti-PD1 (20%) as compared to patients receiving anti-CTLA-4 (3.9%), suggesting that anti-PD1 as a single agent may be more effective than anti-CTLA-4[71].

A retrospective multicenter analysis evaluated the efficacy of anti-PD-1, either nivolumab or pembrolizumab, in 35 patients with MM, and found that the objective response rate was 23%, consisting of all partial responses[72]. The median progression free survival was 3.9 months, and the responses were not dependent on the primary site of disease. Of the 80% of patients received prior systemic therapy, 93% received anti-CTLA4 (ipilimumab), and a majority of patients (92%) did not respond, again highlighting the limited efficacy of anti-CTLA-4. Mutational analysis identified that 64% of patients lacked driver mutations in *BRAF, NRAS* and *KIT*. Interestingly, this study found that the responses to anti-PD-1 therapy were not associated with primary tumor location, mutational burden or primary therapy, and may overcome therapeutic resistance to anti-CTLA-4, supporting the routine use of PD-1 blockade for patients with unresectable mucosal melanoma.

A pooled analysis from phase III clinical trials comparing immunotherapy responses in mucosal and cutaneous melanoma found that the combination of anti-PD1 and anti-CTLA-4 demonstrated a superior objective response rate of 37.1% as compared to single agent treatment with anti-PD-1 (23.3%) or anti-CTLA-4 (8.3%) in mucosal melanoma[73]. While mucosal melanomas showed a favorable response to combination immunotherapy, the rates were still lower when compared to cutaneous melanoma (60.4%), which is consistent with previous clinical observations in cutaneous melanoma. This study analyzed the role of PD-L1 expression (measured by immunohistochemistry) as a clinical biomarker of response to immunotherapy, however it remains unclear in both mucosal and cutaneous melanoma.

More recently, the FDA has approved several PD-L1 inhibitors (atezalizumab, avelumab, durvalumab) as another class of immunotherapy in cancer. These PD-L1 inhibitors should be evaluated in the mucosal melanoma, either alone or in combination with other drugs as another therapy option to this rare disease. More pre-clinical and clinical studies are needed to better understand predictive biomarkers and identify mucosal melanoma patients responsive to immunotherapy.

5. Concluding Remarks

In summary, the mutational landscape of mucosal melanoma is significantly different from cutaneous melanoma. The common drivers (*BRAF* and *NRAS*) found in cutaneous melanoma have lower mutation rate in mucosal melanoma. However, *SF3B1* and *KIT* have higher mutation rate in mucosal melanoma as compared to cutaneous melanoma. From the meta-analysis, we also observed that the mutational profiles are slightly different between the “upper” and “lower” regions of mucosal melanoma, providing new insights and therapeutic options for the mucosal melanoma patients. This review highlights the need to perform meta-analysis to further define the mutational landscape of mucosal melanoma, and further established and developed new preclinical models to study rare cancers. Mutations identified in mucosal melanoma should be incorporated into routine clinical testing, as there are targeted therapies already developed for treating patients with these mutations in the precision medicine era.

Acknowledgements:

This study was supported by the National Institutes of Health P50CA058187, P30CA046934, T32CA190216 (KWN), the RNA Bioscience Initiative RNA Scholars Program (KWN) at the University of Colorado Anschutz Medical Campus, and the David F. and Margaret T. Grohne Family Foundation.

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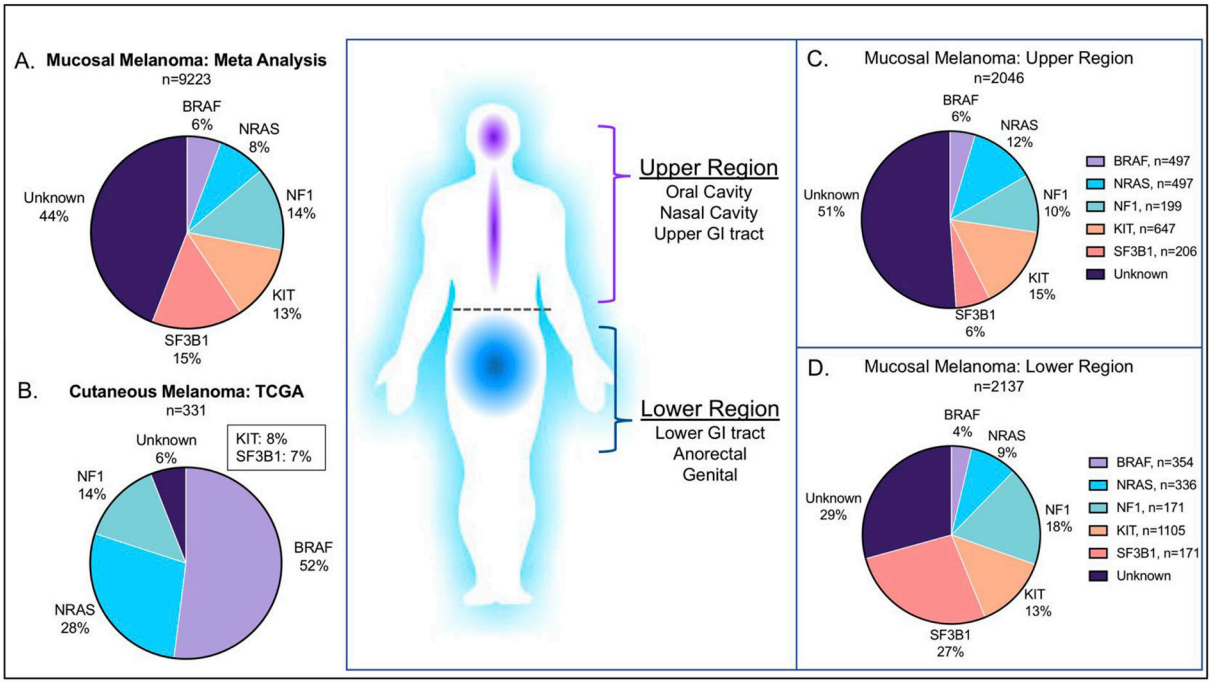

Figure 1:

Mutational landscape of Mucosal Melanoma. Molecular classification of melanoma with BRAF (V600), NRAS (G12, G13, Q61), NF1, KIT and SF3B1 mutations in (A) Mucosal melanoma meta-analysis from 64 studies (B) cutaneous melanoma from TCGA. (C-D) The difference in molecular classifications between mucosal melanomas arising in upper and lower anatomical sites (C) Upper sites include: Head and neck and upper GI. (D) Lower sites include: Lower GI, anorectal, and genital.

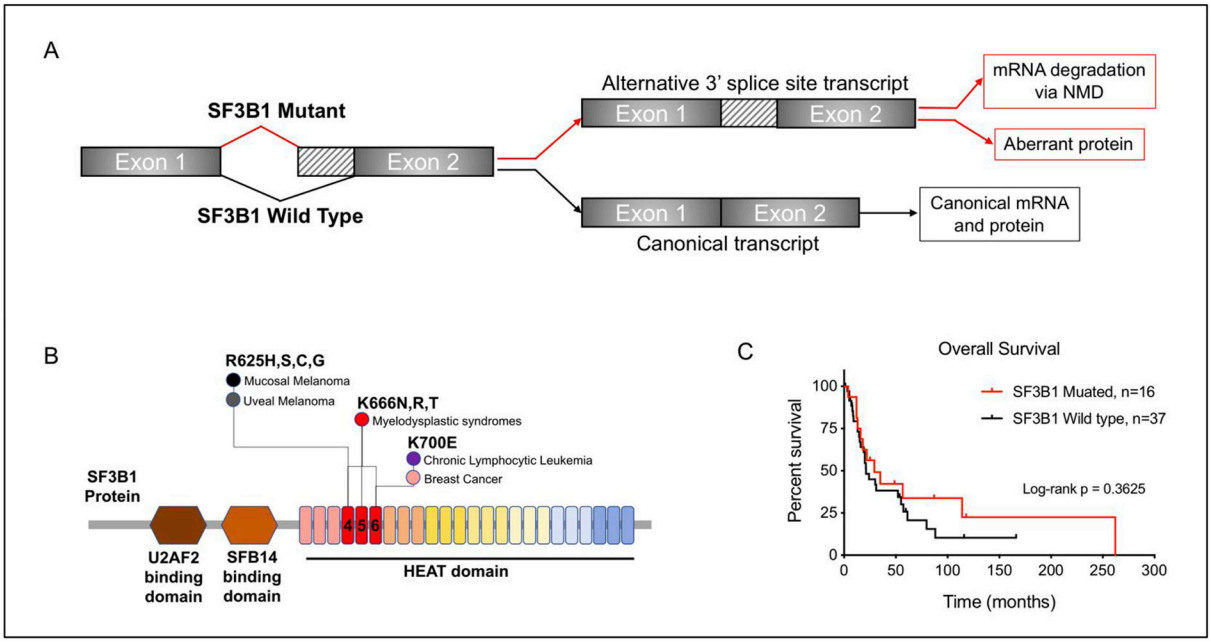

Figure 2:

SF3B1 mutations in cancer. (A) Mutations in SF3B1 are associated with alternative branch point usage and result in increases in alternative 3' splice sites. (B) Hotspot SF3B1 mutations represented in SF3B1 protein, highlighting the locations that predominate in specific cancer types. (C) Kaplan-Meier curve of overall survival comparing SF3B1 Mutant (n=16) and SF3B1-WT (n=37) from 3 studies (Hintzsche et. al. 2017, Yang et. al. 2017, Quek et. al. 2019), p-value is calculated by log-rank test.

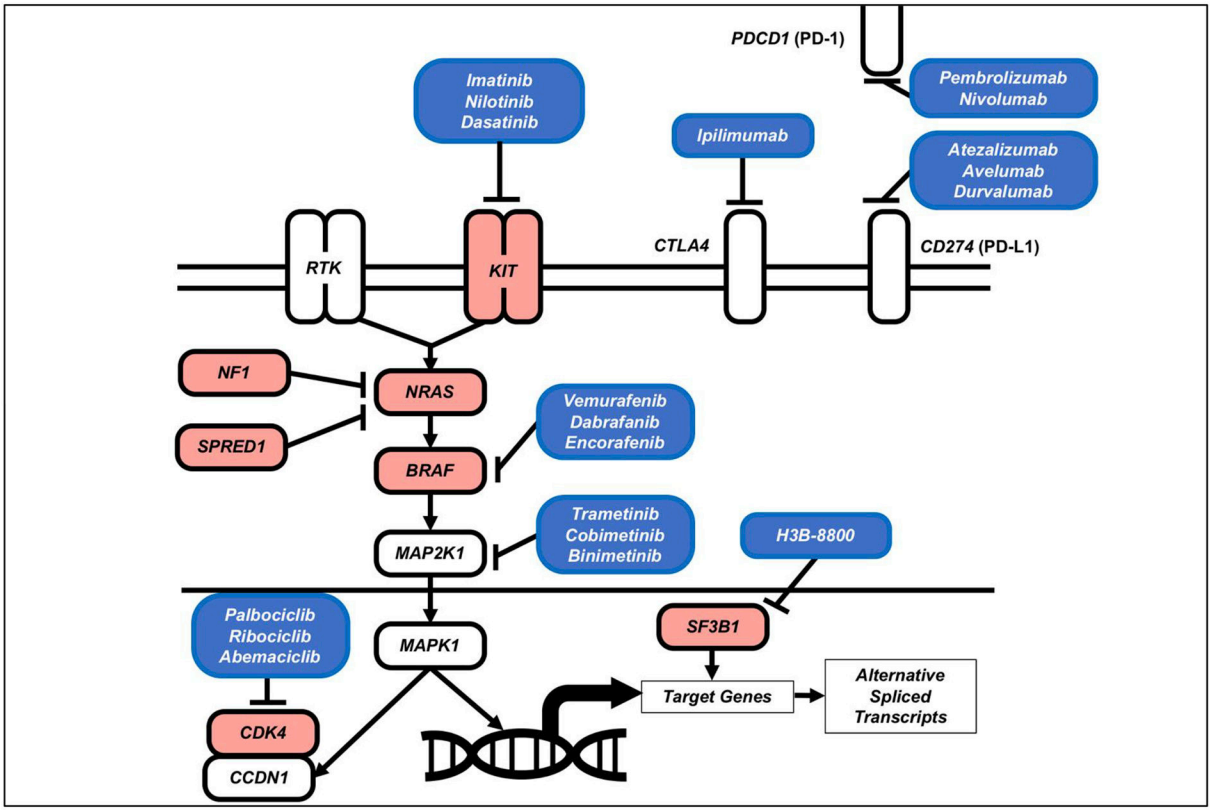

Figure 3:
Oncogenic signaling and therapeutic targets in Mucosal Melanoma.

Table 1:

Mucosal melanoma mutational studies covered in this review.

		Number of Mutations					Region			
Mucosal Melanoma Site	**Number of Patients**	**BRAF**	**NRAS**	**NF1**	**KIT**	**SF3B1**	**Upper**	**Lower**	**Detection Method**	**Reference**
Whole Genome Sequencing/Whole Exome Sequencing Studies										
Oral	65	2	1	5	15	---	X		WGS	5
Oral	19	0	1	0	4	0	X		WES	20
Various	19	0	1	7	9	8	X	X	WES	21
Various	8	0	2	0	2	3	X	X	WGS	6
Various	67	3	11	11	10	8	X	X	WGS	7
Various	10	0	0	1	2	1			WGS and WES	4
Various	2	1	0	0	0	0			WES/WGS	22
Various	8	0	1	1	0	0			WES	23
Various	46	0	8	---	3	9			WES	24
Targeted Sequencing Studies										
Anorectal	15	1	1	3	5	3		X	Targeted NGS	74
Vulvovaginal	51	6 (5 NA)	1 (26 NA)	---	10 (5 NA)	---		X	Sanger / NGS	75
Vulvovaginal	20	---	---	---	3 (7 NA)	---		X	Targeted NGS	76
Various	27				3	6	X	X	Targeted NGS	47
Various	71	5	6	13	5	7	X	X	Targeted NGS	77
Various	43	3	4	6	6	5	X	X	Targeted NGS	32
Various	45	3	7	8	10	12	X	X	Targeted NGS	78
Various	41	0	---	9	4	---			Targeted NGS	79
Various	46	2	6	5	3	---			Targeted NGS	49
Various	28	2	4	---	2	---			Targeted NGS	80
Oral	57	0	0	---	4	---	X		Sanger	81
Oral	139	---	---	---	22	---	X		Sanger	60
Oral	18	---	---	---	4 (3 NA)	---	X		Sanger	82
Sinonasal	72	4	8	---	16	5	X		Sanger	83
Sinonasal	32	0	5	---	4	---	X		Sanger	84
Sinonasal	17	0	3	---	1	---	X		Sanger	85

		Number of Mutations					Region			
Mucosal Melanoma Site	**Number of Patients**	**BRAF**	**NRAS**	**NF1**	**KIT**	**SF3B1**	**Upper**	**Lower**	**Detection Method**	**Reference**
Sinonasal	56	2	8	---	2	---	X		Sanger	86
Head and neck	42	2	2		4		X		Sanger	87
Head and Neck	28	---	---	---	7	---	X		Sanger	36
Esophageal	16	1	6	---	1	---	X		Sanger	31
Anorectal	20	0 (1NA)	1 (1NA	---	3	---		X	Sanger	88
Anorectal	31	---	---	---	11	---		X	Sanger	89
Vulvar and Vaginal	65	0 (11 NA)	(8 NA)	---	7 (11 NA)	---		X	Sanger	90
Vulvovaginal	24	0	4	---	1	---		X	Sanger	91
Vulvovaginal	24	0	4	---	4	---		X	Sanger	92
Vulvovaginal	16	1	0	---	0	---		X	Sanger	93
Various	86	0 (44 NA)	---	---	5 (43 NA)	---			Sanger	35
Various	755	66 (35 NA)	20 (200 NA)	---	66	---			Sanger	37
Various	71	4	7	---	7	---			Sanger	30
Various	38	1 (2NA)	2 (4 NA)	---	8	---			Sanger	94
Various	55	1	1		3	---			Sanger	95
Various	120	13	9	---	---	---			Sanger	96
Various	167	---	---	---	16	---			Sanger	97
Various	39	3 (12 NA)	---	---	6 (2 NA)	---			Sanger	98
Various	52	---	---	---	9	---			Sanger	99
Various	25	---	---	---	7	---			Sanger	100
Various	25	0 (8 NA)	1 (8NA)	---	4 (8 NA)	---			Sanger	101
Various	35	---	---	---	3	---			Sanger	102
Various	36	1	5	---	---	---			Sanger	103
Various	13	0	---	---	---	---			Sanger	104
Various	25	1	---	---	---	---			Sanger	105
Various	31	---	---	---	4	---			Sanger	106
Various	22	0	2	---	4	---			Sanger	107
Various	21	---	1	---	---	---			Sanger	108
Oral	14	3	4	---	---	---	X		Pyrosequencing	109
Various	62	2 (5 NA)			7				Pyrosequencing/Sanger	110

Semin Cancer Biol. Author manuscript; available in PMC 2021 April 01.

Mucosal Melanoma Site	Number of Patients	Number of Mutations					Region		Detection Method	Reference
		BRAF	NRAS	NF1	KIT	SF3B1	Upper	Lower		
Various	23	0	1	---	0 (7 NA)	---			Pyrosequencing/Sanger	111
Various	26	0	---	---	---	---			Pyrosequencing	112
Various	93	8 (1 NA)	11 (1 NA)	---	17	---			PCR assay	34
Various	30	1	1	---	5	---			PCR assay	113
Various	37	---	---	---	16	---			PCR assay	66
Various	5	---	---	---	0	---			PCR assay	114
Various	16	---	---	---	6	---			HRM/sequencing	115
Various	48	---	---	---	8	---			HRM/sequencing	116
Various	45	0	9	---	7	---			DHPLC / Sequencing	117
Various	706				70			X	ND: from patient database	2

Note: NA = not available, --- = not reported, ND = not determined.