Exploring the Molecular Landscape of Cutaneous Melanoma

Mitra, Shamik

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SHAMIK MITRA
FACULTY OF MEDICINE | LUND UNIVERSITY

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Shamik Mitra is a graduate bioinformatician originally from Kolkata, India and currently living in Lund, Sweden. His research interest lies in application of the data science techniques in clinical research, especially cancer.

In his spare time, he likes to travel, cook, listen to music, read both fiction and non-fiction works. He also has a keen interest in science-related discussions both within and outside his domain of research.

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Shamik Mitra

LUND
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Title and subtitle Exploring the Molecular Landscape of Cutaneous Melanoma

Abstract

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In study I, we have identified immune cell-type associated DNA methylation patterns that have offered important molecular and prognostic information for the metastatic melanoma (MM) tumors. Additionally, these immune-methylation patterns highlighted the existing microenvironmental resemblance among tumor types with diverse tissue-of-origin. We further explored the immune-microenvironment of MM tumors using single-cell RNA-sequencing derived marker genes and devised transcriptomic scores for the underlying major immune cell-types in study II. These immune cell-type scores were found to have prognostic implications and were predictive of treatment benefit from immunotherapy. In study III, we investigated predictive biomarkers for the treatment benefit to adoptive T cell (ACT) therapy. Our analyses revealed that tumor mutational and putative neoantigen burden together with immune enrichment, could work as a composite biomarker to predict treatment benefit and patient survival upon treated with ACT.

Plasticity of the melanoma cell phenotypes has garnered significant attention in recent times, especially as a possible mechanism of secondary resistance to targeted treatments. Epigenetic mechanisms such as DNA methylation is well-known to play a major role in the transcriptional process and their involvement have been highlighted in context of cancers as well. In study IV, we analyzed the possible contribution of the DNA methylation to modulate expression of the important melanoma-associated genes such as *MITF* and *SOX10*. Our results indicated that both these genes are likely to be transcriptionally modulated through DNA hyper-methylation of their promoter regions and subsequently help the underlying cells to exhibit a more proliferative, invasive and treatment-resistant phenotype.

Studies in this thesis have helped to unravel the existing molecular diversity in the CMM tumors and could potentially motivate the exploration of new therapeutic strategies.

Key words
Melanoma, Immune system, Tumor microenvironment, Epigenetics, MITF, SOX10

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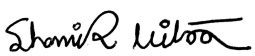
Signature  Date 2020-04-16

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To all my dear family members I have lost to Cancers

"We never are definitely right, we can only be sure we are wrong."
~ Richard P. Feynman

Table of Contents

List of papers included in thesis

The following studies were covered by this thesis,

I. **Mitra, S**., Lauss, M., Cabrita, R., Choi, J., Zhang, T., Isaksson, K., Olsson, H., Ingvar, C., Carneiro, A., Staaf, J., Ringnér, M., Nielsen, K., Brown, K.M., and Jönsson, G. (2020), Analysis of DNA methylation patterns in the tumor immune microenvironment of metastatic melanoma. *Mol Oncol.*

II. **Mitra S.**, Cabrita R., Harbst K., Lauss M., and Jönsson G. Distinct transcriptional signatures derived from single-cell RNA sequencing data predict patient prognosis and response to immune checkpoint blockade in melanoma. *Manuscript*

III. Lauss M., Donia M., Harbst K., Andersen R., **Mitra S.**, Rosengren F, Salim M., Vallon-Christersson J., Törngren T., Kvist A., Ringnér M., Svane I.M., and Jönsson G. Mutational and putative neoantigen load predict clinical benefit of adoptive T cell therapy in melanoma. *Nat Commun 8, 1738 (2017)*

IV. Sanna A., **Mitra S.,** Phung B., Lauss M., Choi, J., Zhang, T., Njauw C.N., Cordero E., Harbst K., Rosengren F., Cabrita R., Johansson I., Isaksson K., Olsson H., Ingvar C., Carneiro A., Andersson M., Tsao H., Brown K.M., Pietras K., and Jönsson G. Effects of promoter hypermethylation of melanocyte lineage genes on melanoma phenotype. *Manuscript*

List of papers not included in the thesis

Studies that are not included in this thesis,

- Betancourt L.H., Pawłowski K., Eriksson J., Szasz A.M., **Mitra S.,** Pla I., Welinder C., Ekedahl H., Broberg P., Appelqvist R., Yakovleva M., Sugihara Y., Miharada K., Ingvar C., Lundgren L., Baldetorp B., Olsson H., Rezeli M., Wieslander E., Horvatovich P., Malm J., Jönsson G., and Marko-Varga G. Improved survival prognostication of node-positive malignant melanoma patients utilizing shotgun proteomics guided by histopathological characterization and genomic data. *Sci Rep. 2019 Mar 26;9(1):5154.*
- Sanna A., Harbst K., Johansson I., Christensen G., Lauss M., **Mitra S.,** Rosengren F., Häkkinen J., Vallon-Christersson J., Olsson H., Ingvar Å., Isaksson K., Ingvar C., Nielsen K., and Jönsson G. Tumor genetic heterogeneity analysis of chronic sun-damaged melanoma. *Pigment Cell Melanoma Res. 2019; 00:1-10.*
- Cabrita R., Lauss M., Sanna A., Donia M., Larsen M.S., **Mitra S.,** Johansson I., Phung B., Harbst K., Vallon-Christersson J., Schoiack A.V., Lövgren K., Warren S., Jirström K., Olsson H., Pietras K., Ingvar C., Isaksson K., Schadendorf D., Schmidt H., Bastholt L, Carneiro A., Wargo J.A., Svane I.M., and Jönsson G. Tertiary lymphoid structures improve immunotherapy and survival in melanoma. *Nature 577, 561–565 (2020).*
- Cabrita R., **Mitra S.,** Sanna A., Ekedahl H., Lövgren K., Olsson H., Ingvar C., Isaksson K., Lauss M., Carneiro A., and Jönsson G. The Role of PTEN Loss in Immune Escape, Melanoma Prognosis and Therapy Response. *Cancers 2020, 12, 742.*

Abbreviations

AJCC	American Joint Committee on Cancer
ACT	Adoptive T-cell therapy
ALM	Acral lentiginous melanoma
BCE	Before common era
CD	Cluster of Differentiation
CMM	Cutaneous malignant melanoma
CNS	Central Nervous System
CpG	Cytosine phosphate Guanine
DC	Dendritic cell
DEG	Differential expression of genes
DMFS	Distant metastasis free survival
DSS	Disease specific survival
FDA	Food and Drug Administration (United States)
FFPE	Formalin fixed paraffin embedded
GDSC	Genomics of Drug Sensitivity
H&E	Hematoxylin and eosin
HLA	Human leukocyte antigen
ICB	Immune-Checkpoint blockade
IHC	Immunohistochemistry
LMM	Lentigo maligna melanoma
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MITF	Microphthalmia-associated transcription factor
MM	Metastatic Melanoma

mRNA	Messenger RNA
NGS	Next generation sequencing
NM	Nodular melanoma
NK cell	Natural Killer cell
OS	Overall survival
PI3K	Phosphoinositide 3-kinase
RLN	Regional lymph node
rRNA	ribosomal RNA
RTK	Receptor tyrosine kinase
SCNA	Somatic copy number alteration
SLN	Sentinel Lymph Node
scRNA-seq	Single-cell RNA-sequencing
SSM	Superficially spreading melanoma
SOX10	sex-determining region Y-box10
TCGA	The Cancer Genome Atlas
TIL	Tumor Infiltrating Lymphocyte
TIME	Tumor immune microenvironment
TLS	Tertiary Lymphoid Structures
TMA	Tissue microarray
TME	Tumor microenvironment
tRNA	Transfer RNA
TSG	Tumor suppressor gene
tSNE	t-Distributed Stochastic Neighbor Embedding
UMAP	Uniform Manifold Approximation and Projection
UVR	Ultraviolet radiation
WES	Whole exome sequencing
WGS	Whole genome sequencing
WT	Wild type
VAF	Variant Allele Frequency

Abstract

Cutaneous malignant melanoma (CMM) is one of the most aggressive skin malignancies with poor prognosis for the patients with metastatic disease. Earlier studies have highlighted the existing molecular diversity amongst CMM tumors. Such diversity does not result from the malignant cells alone but a product of multitude of complex interactions among the melanoma and non-melanoma cells in the tumor microenvironment. The studies included in this thesis aims to shed light on some aspects of this observed diversity, chiefly the roles of the tumor-enriching immune cells and melanoma cell phenotypes.

In study I, we have identified immune cell-type associated DNA methylation patterns that have offered important molecular and prognostic information for the metastatic melanoma (MM) tumors. Additionally, these immune-methylation patterns highlighted the existing microenvironmental resemblance among tumor types with diverse tissue-of-origin. We further explored the immune-microenvironment of MM tumors using single-cell RNA-sequencing derived marker genes and devised transcriptomic scores for the underlying major immune cell-types in study II. These immune cell-type scores were found to have prognostic implications and were predictive of treatment benefit from immunotherapy. In study III, we investigated predictive biomarkers for the treatment benefit to adoptive T cell (ACT) therapy. Our analyses revealed that tumor mutational and putative neoantigen burden together with immune enrichment, could work as a composite biomarker to predict treatment benefit and patient survival upon treated with ACT.

Plasticity of the melanoma cell phenotypes has garnered significant attention in recent times, especially as a possible mechanism of secondary resistance to targeted treatments. Epigenetic mechanisms such as DNA methylation is well-known to play major role in the transcriptional process and their involvement have been highlighted in context of cancers as well. In study IV, we analyzed the possible contribution of the DNA methylation to modulate expression of the important melanoma-associated genes such as *MITF* and *SOX10*. Our results indicated that both these genes are likely to be transcriptionally modulated through DNA hyper-methylation of their promoter regions and subsequently help the underlying cells to exhibit a more proliferative, invasive and treatment-resistant phenotype.

Studies in this thesis have helped to unravel the existing molecular diversity in the CMM tumors and could potentially motivate the exploration of new therapeutic strategies.

Aims of the thesis

Overall aim of this thesis was to explore the molecular diversity characteristic to the CMM tumors using computational biology-based approaches. Chiefly, we sought to explore the immune-microenvironment of melanoma tumors along with the malignant cells to understand their roles in tumor development and progression and corresponding impacts on the patient prognosis. Aims of the individual studies are listed below,

- Exploration of the MM tumor immune-microenvironment from the perspective of DNA methylation (Study I)
- Identification of the intra-tumoral immune cells using single-cell RNA-sequencing (scRNA-seq) based transcriptomic data. (Study II)
- Further applications of the scRNA-seq derived immune cell-type markers to explore corresponding enrichment landscape in both treatment-naïve and immunotherapy-treated melanoma tumor cohorts. (Study II)
- Investigation of suitable biomarkers to predict treatment benefit for ACT. (Study III)
- Exploration of the transcriptional modulation mechanism of the melanoma-associated genes such as *MITF* and *SOX10* and the overall impact of such modulation on the corresponding cellular phenotype. (Study IV)

Background

Cancer: A brief historical perspective

Cancer has been known to humanity since pre-historic times, although it likely predates humans as evidence suggests that some dinosaurs also had suffered from tumors, including metastatic ones [1]. The earliest mention of breast cancer is believed to have come from "The Edwin Smith Surgical Papyrus" discovered during the 19th century and estimated to be written around 1500-1600 BCE [2]. So far the oldest specimen of cancer was recovered from around a 2700 years old mummy of a Scythian king from southern Siberia [3]. Similar observation was made for another Egyptian Ptolemaic mummy dated around 285–30 BCE [4]. However, the credit of terming cancer for the first time most likely goes to Hippocrates as he mentioned diseases that produced masses (*onkos*) and used the word "*karkinos*" to describe ulcerating, non-healing lumps, which included both benign and malignant tumors. Later, one of his most prominent successors, Galen categorized tumorous growths into three categories, from the most benign to the most malignant [2].

During the middle ages, more information regarding cancers started to emerge as well as descriptions of the tumor resection procedures. However, it is during post-renaissance period where major progresses were made in studying cancers. Gabriele Fallopius described major clinical differences between benign and malignant tumors [2] while Henri François Le Dran postulated that the development of cancers takes place locally and then the spreading occurs through the lymphatic vessels [5]. This description of Le Dran can be called as one of the earliest attempts to explain cancer metastasis, although the term was coined later by Joseph Recamier in 1829 [2]. Around that same time, Jean-Louis Petit recommended a total mastectomy of the breast to avoid recurrences, which still survives in the current clinical practices [6, 7].

Despite these achievements, the origin of cancer was still shrouded in mystery so far. The first person to shed light on cancer's inception was Bernard Peyrilhe, who performed one of the earliest known investigation into the nature and growth pattern of cancer and postulated presence of a cancer promoting factor in the degraded or putrefied lymph [8]. Alfred Armand Louis Marie Velpeau became one of first few people to suggest that there might be unknown mechanisms at play of which the cancer cells are a mere manifestation [9]. Such an observation can be attributed as one of the earliest suggestions of genetic involvement in cancers. Velpeau's

observations were advanced further by Theodor Boveri who proposed the hypothetical involvement of somatic mutations in the cancer development [10, 11]. In another landmark study, Peyton Rous first discovered a viral link of cancer in chickens in the year 1910 [12]. Though, he did not mention the nature of the transmitting agent, this later came to be known as "Rous sarcoma virus".

With the turn of 20th century, cancer treatment got a major boost by the discovery of radioactive elements by Henry Becquerel, Marie Sklodowska-Curie and Pierre Curie [13]. Earlier, Victor Despeignes [14] and H. Gocht [15] used newly discovered X-rays by Wilhelm Conrad Röntgen [16] to treat cancer patients. Marie Sklodowska-Curie along with her husband Pierre pioneered the use of radium in treating growths including cancers and Marie later went on to found the Radium Institute for this purpose [2]. Courtesy to their remarkable discoveries, a new avenue for cancer treatment opened. [1]

Discovery of the x-rays not only established new treatment methods but also helped to create early tumor models. Pierre Marie successfully induced skin cancer in rats using x-rays [17]. Another major breakthrough came in the form of tissue culture of cancer as Alexis Carrel and Montrose T. Burrows were able to grow tumor tissue *in vitro* for the first-time, using the extract from Rous chicken sarcoma [18]. These discoveries paved the way for the future cancer research using tumor models.

The latter half of the 20th century has led to a significant progression in cancer research in an all-encompassing manner from the discovery of cancer-causing agents to the development of new treatment regimens. During this time, major progress has been made to understand molecular biology of cancer, more specifically oncogenesis.

Discovery of the Epstein-Barr virus [19] in Burkitt's lymphoma [20], established the first viral association in human cancers. However, this would not be the only link as researchers showed that hepatitis B virus significantly increases the risk of developing hepatocellular carcinoma [21]. Harald zur Hausen, a German physician discovered that human papillomavirus (HPV) plays an important role in the development of condyloma acuminata and uterine cervical carcinoma [22] and identified responsible HPV types 16 and 18 in the squamous cell carcinoma of the cervix [23]. This discovery ultimately has led to the development of an HPV vaccine which provides protection against multiple cancer types including cervical, oropharyngeal and anal cancer [24-26].

Interesting to point out in this regard, that the concept of "*oncogene*" came out of studies concerning cancer-causing RNA tumor viruses [27]. Howard M. Temin and David Baltimore's landmark discovery of the enzyme reverse-transcriptase also

[1] Historical names, events and their respective chronological order until here was adopted from "*A brief history of cancer: age-old milestones underlying our current knowledge database*" by Faguet GB; Int J Cancer. 2015;136(9):2022-36

highlighted how the information is passed on from RNA to DNA in context of proteins related to the oncogenesis [28, 29]. Nevertheless, it would be safe to say that research on oncogenesis would have still remained at its infancy if major breakthroughs had not been made in understanding the structure of DNA [30-32]. The paradigm shift in the molecular biological research in the 1950's, prompted also landmark discoveries in the field of oncology. In 1960, a reciprocal translocation between chromosome 9 and 22 was observed in chronic granulocytic leukemia patients [33], which was later named as "Philadelphia chromosome" after the city where the discovery was made [34]. Later on, many more such oncogenic alterations were discovered in the genes such as c-MYC (*MYC*) [35] and HER-2 (*ERBB2*) [36]. Also, during this period multiple growth factors and their relationship with the cancer development were uncovered [37-39]. Discovery of these growth factors have also brought the spotlight on the receptor tyrosine kinases (RTKs), some of the key players in the oncogenesis [40].

Studies on oncogenesis also brought the spotlight on the counterparts of oncogenes known as "*tumor suppressor genes*" (TSGs), whose inactivation has disruptive effects on the regulation of cellular division and subsequent promotion of the oncogenesis [41, 42]. Discovery of the important TSGs such as *RB1* [43] and *TP53* [44] has profoundly enriched our understanding of how tumors form.

Altogether, these discoveries have culminated in determining the hallmarks of cancer, which are six essential characteristics such as evasion of apoptosis, tissue invasion and metastasis, etc., that the cells need to acquire to become malignant [45]. Later, two more hallmarks deregulation of cellular metabolism and immune-evasion were added [46]. These hallmarks so far have set the directions of cancer research in the 21st century.[2]

Here, only a few of the major events were highlighted from the long history of understanding cancer. The aim of this historical overview of cancer research is only to provide the readers with an idea about how we got here, rather than a thorough chronicle of events.

[2] Historical names, events and their respective chronological order until here was adopted from "*A note from history: Landmarks in history of cancer*" series parts 5, 6 and 7 by Steven I. Hajdu, Farbod Darvishian, Manjunath Vadmal and Ping Tang. Cancer. 2013-2015.

Cutaneous malignant melanoma

Origin and Epidemiology

The first historical reference of melanoma dates back to 5th century BCE in the description of Hippocrates. However, evidence of melanocytic metastases has been found in pre-Columbian mummies estimated to be around 2400 years old from Peru [47, 48]. In the 18th century, Scottish surgeon John Hunter has been credited with the first surgical removal of a melanoma tumor. However, during the 18th and 19th century most of the information regarding melanoma came in form of description of the individual cases, whereas understanding of the etiology or mechanistic interpretation of the disease was lacking [48]. During the 1960s, Wallace H. Clark and colleagues formulated one of the first histopathological scales for categorizing malignant melanoma [49] and in 1970 Alexander Breslow observed the association between tumor thickness and prognosis in melanoma [50]. The works of Clark and Breslow not only formed the basis of the diagnosis of melanoma but are still being used in the clinical practice. Tumor thickness for the localized melanomas is the most important prognostic factor in the AJCC staging system for the melanomas [48, 51].

Melanomas have been shown to arise from the melanocytes. The primary function of the cutaneous melanocytes is to synthesize melanin pigments (brown/black eumelanin and orange/yellow pheomelanin). The melanocytic lineage is derived from the neural crest cells through multiple stages of differentiation [52]. Melanocytes although primarily found in the skin and eye, can also be observed at a lesser extent in a broad range of tissues such as head and neck, female genital tract and gastrointestinal tract [53, 54].

Cutaneous malignant melanomas (CMMs) form the majority of the observed melanomas [53] with worldwide 287,723 new cases being reported every year (Source: GLOBOCAN 2018) [55]. Multiple factors have been found to influence the melanoma incidence, from geography to ethnicity. In 1956, Henry Lancaster first reported the association between melanoma incidence and “latitude” or intensity of sunlight [56]. It is noteworthy that Australia reports the highest country-wise incidence of melanoma, which is likely influenced by both sun-exposure and ethnicity [57]. Regarding ethnicity, cutaneous melanoma in general is observed at a much higher rate among the Caucasian populace, however certain subtypes are observed more among the non-Caucasian ethnicities [58].

So far, several risk factors have been identified that associate with the CMM. Among these factors, exposure to the natural and artificial sunlight and more specifically ultraviolet radiation (UVR) is considered pivotal (Figure 1). Although major risk has been associated with the exposure to UVB, also UVA exposure could potentially increase the chances of developing CMM as well [57, 59]. Despite UVB

(280 nm-315 nm) forms only a small fraction of the solar UVR (~ 5%) it is known to cause the formation of DNA damage photoproducts between pyrimidine bases. Such DNA damage photoproducts are repaired through nucleotide excision repair (NER) but when left unrepaired leads to accumulation of mutations [60]. Dysregulation of the NER predisposes individuals to carcinogenesis at a higher risk [61]. On the other hand, UVA comprises almost 95% of the solar UVR, but it is much less potent to induce sunburn and inflammation. Nonetheless, the skin penetration capacity of UVA is much higher than UVB and UVA can also reach the dermis. UVA has been hypothesized to cause melanoma, but its exact role is somewhat controversial [59]. Studies have shown that UVA is able to cause DNA damage by producing reactive oxygen species (ROS) [62], which can cause single-strand breaks [63], mutations and other chromosomal alterations that in turn can induce cytotoxicity and carcinogenesis [59, 64]. Findings from these studies warrant further research into the specific roles of UVA radiation in melanoma development.

Artificial sources of UVR (both UVA and UVB) such as tanning beds are a major concern in terms of the melanoma risk. World Health Organization International Agency for Research on Cancer (IARC) has classified UVR emitted from the tanning beds as carcinogenic [65]. A recent literature review of the indoor tanning associated melanoma risk in the United States also reaffirmed this relationship [66].

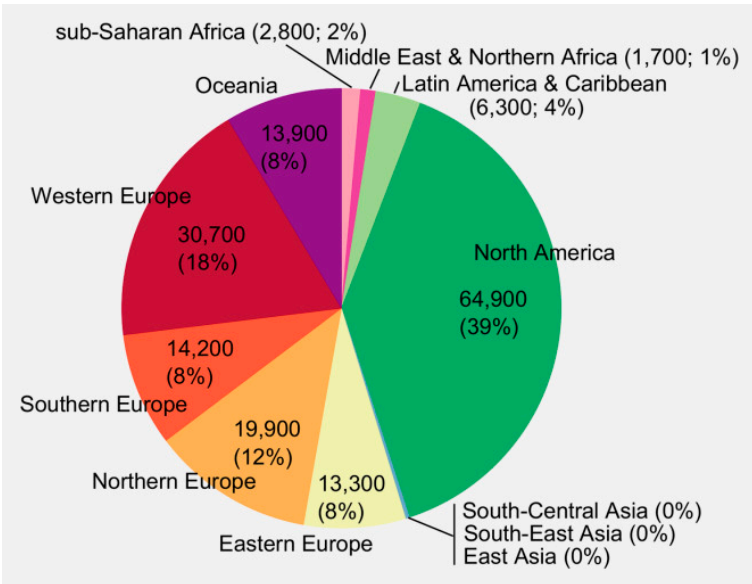

Figure 1: Melanoma cases attributable to UV radiation in 2012 by world region. *Taken from the publication by Arnold et al. [67] with kind permission from the publisher John Wiley and Sons, without any adaptation.*

Ethnicity of the population also shows major association when it comes to UV radiation. Evidences suggest that individuals with darker complexion usually have melanocytes that produce darker pigment (eumelanin) and they have bigger melanosomes compared to the individuals with lighter complexion. Eumelanin serves as a natural protective barrier by scattering the UV radiation and reducing penetration through epidermis [68, 69]. Interestingly, it is important to note that the populations of darker complexion report a higher percentage of cases when it comes to non-UV associated melanomas and melanomas at special sites [70].

Genetic predisposition of the individuals can play an important role in the development of CMM. Melanocortin 1 receptor (*MC1R*) is one of the key genes in pigment production and is found to harbor many polymorphisms [71, 72]. These polymorphisms in MC1R give rise to diverse skin pigmentation phenotypes, among which the red hair and lighter complexion group is known to express low pigmentation and also shows higher susceptibility to CMM [57, 73].

In addition to *MC1R*, *CDKN2A* has been identified as a major melanoma predisposition gene through linkage studies and positional cloning in the melanoma prone families [74, 75]. *CDKN2A* through its two proteins $p16^{INK4A}$ (p16) and $p14^{ARF}$ (p14) functions as a regulator of the cell cycle [76]. p14 also acts as a tumor suppressor and act in association with p53 [77]. Additionally, genetic alterations in *CDK4*, *BAP1* and *TERT* have also been associated with melanoma risk [70, 78].

Apart from the environmental factors like UVR and genetic predispositions, the risk of developing melanoma has been found to associate with the gender and age of the individuals. Studies have reported an increase in the melanoma incidence with advanced age [79, 80]. It has also been suggested that UVR may not be the only major factor in the melanoma development among younger individuals compared to their older counterparts [80]. Additionally, gender of the individual has also been found to play an important role. Women reported higher incidence in the younger age groups than males. However, this trend reverses after 40, when men become more prone to melanoma than females [81, 82].

Figure 2 summarizes the different risk factors for melanoma development.

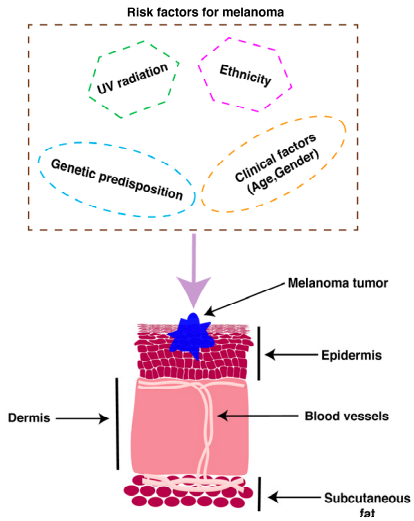

Figure 2: Schematic representation of the major melanoma associated risk factors. *Drawn using template from the Library of Science and Medical Illustrations by Idoya and Luk (https://gumroad.com/l/IxECD) with kind permission.*

Clinical characteristics

Histological subtypes of CMM

Until recently CMM tumors have been broadly categorized into four major histopathologically based subtypes, superficially spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanoma (LMM) and acral lentiginous melanoma (ALM). Among these four subtypes, SSM is mostly prevalent in the Caucasian populace and observed in the intermittent sun-exposed skin such as trunk and leg. NM is usually found in the head and neck region and lower limbs whereas LMM is predominantly observed in the chronic sun-exposed regions such as faces of the older patients. In contrast to these three subtypes, ALM is more frequently observed in the non-Caucasian populations and in more sun-shielded areas such as palms and soles of feet [70].

SSM is the most prevalent CMM subtype overall and represents around 75% of all melanomas. Histopathological characteristics of SSM includes [83],

1. Lateral spreading of the malignant melanocytes within epidermis in a haphazard manner.
2. Presence of the melanocytes above the basal layer (pagetoid spread).
3. Non-cohesive nature of the melanocytic nests.

Figure 3 upper-left panel shows an example of a SSM biopsy stained with Hematoxylin and eosin (H&E).

NM tumors share some characteristics with the SSM subtype, however unlike SSM the growth pattern is generally observed vertically. Upon invading dermis, they display rapid vertical growth with higher propensity towards metastasizing, likely attributable to their greater thickness [83]. Figure 3 upper-right panel shows an example of a NM biopsy stained with H&E.

LMM is associated with chronically sun-damaged skin and is therefore usually observed among the elderly individuals. The *in situ* phase of this subtype is known as lentigo maligna, to distinguish them from the invasive LMM. One of the most common features of LMM is the presence of hyperchromatic small melanocytes along the dermal-epidermal junction [83]. Figure 3 lower-left panel shows an example of a LMM biopsy stained with H&E.

ALM is a much rarer subtype of CMM and probably develops without major influence from the sun or UVR exposure. Typical sites of ALM includes nail beds and soles of feet and often remains un-observed or mistaken for a benign diagnosis until late. In this subtype, the spread of single and nests of melanocytes is observed along the dermal-epidermal junction. Also, upward pagetoid migration is noticed extensively across the breadth of the lesions [83]. Figure 3 lower-right panel shows an example of an ALM biopsy stained with H&E.

Histopathological subtypes of CMM can also display diversity in terms of their molecular characteristics. ALM, which is supposed to be a non-UVR induced subtype, also shows much lower mutational burden than its UVR-induced counterparts and more enriched in copy number alterations (CNAs) [84]. ALM tumors also report *BRAF* mutations less frequently and harbor mutations or CNAs in *KIT* [70].

Unfortunately, the molecular characterization of the less frequent CMM subtypes is often lacking, largely owing to the unavailability of suitable number of samples to conduct such studies. However, recent studies have highlighted the distinction between chronically sun damaged (CSD) and non-CSD melanomas based on their genomic markup and development pattern [85, 86]. Further exploration in this direction would likely enable us to probe deeper into the origins and progression of individual melanoma subtypes and might provide guidance for the suitable treatment approaches.

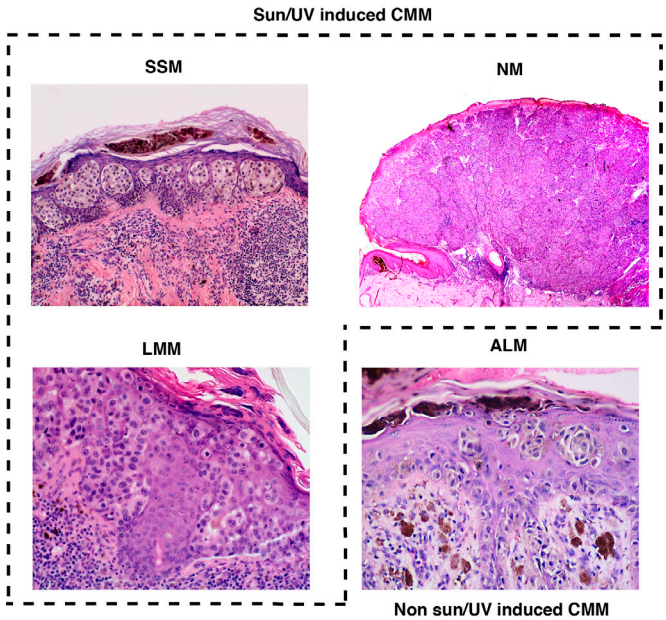

Figure 3: Images of H&E stained biopsies of histopathological subtypes of CMM. *Upper-left: Superficially spreading melanoma (SSM); Upper-right: Nodular melanoma (NM); Lower-left: Lentigo maligna melanoma (LMM); Lower-right: Acral lentiginous melanoma (ALM). Dotted box indicates subtypes associated with Sun/UVR exposure. Images adapted from Wikipedia, shared by Leszek Woźniak and Krzysztof W. Zieliński.*

Tumor staging and prognosis

Seminal works of Wallace Clark and Alexander Breslow [49, 50] played a pivotal role in the establishment of melanoma staging. In 1998, American joint committee on Cancer (AJCC) established a melanoma staging database for the first time. Over the years, by analyzing more data AJCC has updated the tumor staging manual for melanoma and revised the Tumor-Node-Metastasis (TNM) system. Currently (2020) it is in its 8th Edition [51, 87, 88].

Tumor staging can be performed clinically, i.e., with all available information before performing the surgery and *pathologically*, i.e., by adding further information to the clinical observation by microscopic examination of the tumor biopsy post-resection. Tumor stages represent the spread of the tumor expressed as I-IV, incrementally. Usually, stages I and II represent more localized tumors whereas stages III and IV indicate that the tumor has spread to the regional lymph nodes (RLNs) and distant organs respectively. TNM system serves as a guide for determining the stage [89].

In the pathological TNM system, primary melanoma tumors are classified into following pathological tumor (T) categories [88, 90, 91] (Table 1),

Table 1: Pathological primary Tumor categorization

Pathological primary tumor category	Tumor thickness[1] (mm)	Ulceration status[2]
Tx	Assessment not possible	Not Applicable
T0	Unknown or regressed primary	Not Applicable
Tis	Melanoma *in situ*	Not Applicable
T1a	< 0.8	No
T1b	< 0.8 0.8 – 1.0	Yes No
T2a	>1.0 – 2.0	No
T2b	>1.0 – 2.0	Yes
T3a	> 2.0 – 4.0	No
T3b	> 2.0 – 4.0	Yes
T4a	> 4.0	No
T4b	> 4.0	Yes

[1] Tumor thickness a.k.a. Breslow thickness measured as the distance between the top epidermal layer to the deepest part of tumor.

[2] Ulceration represents full thickness loss of the epidermis overlaying melanoma tissue [92].

Figure 4A shows a schematic representation of the pathological primary Tumor (T) stages.

Similarly, pathological regional lymph node (N) stages are categorized as [88, 90, 91] (Table 2),

Table 2: Pathological regional lymph node categories

Pathological N category	No. of tumor-involved nodes[1]	Clinically Occult/Detected[2]	In-transit / satellite / microsatellite metastasises[3]
Nx	Assessment not possible	Not Applicable	No
N0	No regional metastasis	Not Applicable	No
N1a	1	Occult	No
N1b	1	Detected	No
N1c	None	Not Applicable	Yes
N2a	2-3	Occult	No
N2b	2-3	Detected (at least 1)	No
N2c	1	Any	Yes
N3a	≥4	occult	No
N3b	≥4	Detected (at least 1)	No
N3c	≥2	Any	Yes

[1] Number of the tumor-involved regional lymph nodes (RLNs)

[2] Occult: only microscopically detectable from the sentinel lymph node (SLN) biopsy; Detected: enlarged/abnormal RLNs detectable by radiological, clinical or ultrasound examination; Any: either clinically occult or detected RLN [90].

[3] In-transit metastases: subcutaneous or cutaneous lymphatic deposits of the melanoma cells at a distance of more than 2 cm from the primary melanoma but not beyond the RLNs [93]; Satellite metastases: subcutaneous or cutaneous lymphatic deposits of the melanoma cells within 2 cm from the primary melanoma; Microsatellite metastases: microscopically detectable metastases either cutaneous or subcutaneous and adjacent or deep to the primary melanoma tumor [91].

Distant metastases pathologically (M) are staged as [88, 90, 91],

1. M0 – No evident distant metastasis

2. M1 – Evidence of distant metastasis; further classified as,
    a. Cutaneous, soft tissue including muscle and nonregional lymph nodes.
    b. Lung metastasis with or without M1a sites.
    c. Non central nervous system (CNS) visceral metastasis with or without M1a or M1b sites.
    d. CNS metastasis with or without M1a, M1b or M1c sites.

Figure 4B provides a schematic representation of the common anatomical sites for the melanoma metastases.

This *pathological* TNM categorization is further used to determine the AJCC *pathological staging* (I-IV) as given here [88, 90, 91] (Table 3), and should not be mixed up with the slightly different clinical TNM staging, which is based on the histopathologically confirmed primary tumor and clinical or radiological assessment of the regional lymph nodes and distant metastases.

***Table 2**: AJCC pathological staging with the corresponding pathological TNM categories*

Pathological Stage	Pathological T	Pathological N	Pathological M
0	Tis	N0	M0
IA	T1a-b	N0	M0
IB	T2a	N0	M0
IIA	T2b - T3a	N0	M0
IIB	T3b - T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1a - T2a	N1a or N2a	M0
IIIB	T0 T1a - T2a T2b - T3a	N1b - N1c N1b - N2b N1a - N2b	M0 M0 M0
IIIC	T0 T1a - T3a T3b - T4a T4b	N2b - N3c N2c - N3c Any N≥N1 N1a - N2c	M0 M0 M0 M0
IIID	T4b	N3a - N3c	M0
IV	Any T	Any N	M1

Prognosis for the melanoma patients varies based upon various factors such as tumor stage, age, gender, clinical subtypes, etc. [94]. However, disease stage such as localized, regional and distant metastatic diseases have reported vastly different survival. Most patients with localized disease enjoy relatively high survival times, especially those with thin melanomas as shown in this Swedish study [95]. Although, prognosis for the patients with distant metastatic disease is usually worse [96]. Nevertheless, it is important to mention that most melanoma patients are diagnosed at an early stage, thus making favorable prognosis possible.

Figure 4C depicts relative 5-years survival statistics for the different disease stages for melanoma patients.

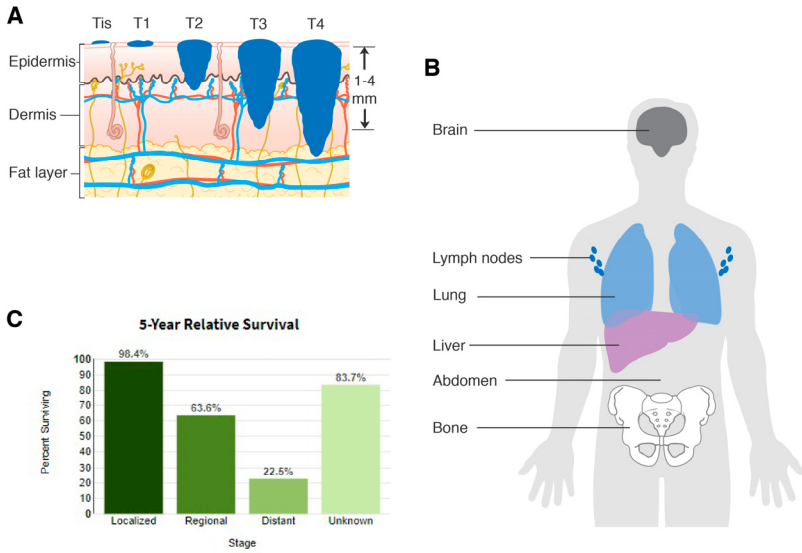

Figure 4: Staging and prognosis for melanoma patients. *A. Shows schematic representation of the pathological Tumor (T) stages according to AJCC staging guidelines 8th edition. B. Shows schematic representation of the common anatomical sites for the melanoma metastases. C. Representation of the 5-years relative survival for the melanoma patients with different disease stages in the United States in 2014 (Source: National Cancer Institute, US website). Images have been taken from Wikipedia, with B) and C) taken as original image without any adaptation. Credits: A) and B) Cancer Research UK; C) Cancer.gov.*

Molecular characteristics

Genomic and transcriptomic characterization

Diversity among CMM tumors observed clinically and histopathologically is often considered to have roots in the molecular markup of the tumors. Melanoma is considered as a largely mutation-driven disease with frequent alterations observed in the MAPK and PI3K-AKT pathways. Nevertheless, more and more studies are adding to our understanding of the underlying molecular diversity that exists in the melanoma tumors. These new-found knowledge about melanoma tumors might take an important part in shaping future clinical recommendations.

Melanocytes originate from the melanoblasts, a precursor which migrate from the neural crest to its final destination of epidermal layer of the skin and hair follicle. At the time of embryogenesis, survival and migration of the melanocytes depend on several signaling pathways such as *Wingless signaling* Wnt/β-catenin, KIT, NOTCH etc., and important transcription factors like *paired box gene 3* (PAX3),

sex-determining region Y-box10 (SOX10) and MITF [97]. The primary function of the cutaneous melanocytes is to synthesize melanin pigments (brown/black eumelanin and Orange/Yellow pheomelanin). Differentiation, proliferation and survival of the melanocytes is dependent on *MITF* and its various downstream target genes. *MITF* gene is transcribed through various isoforms, of which MITF-M isoform is exclusive to melanocytes and melanoma cells [98].

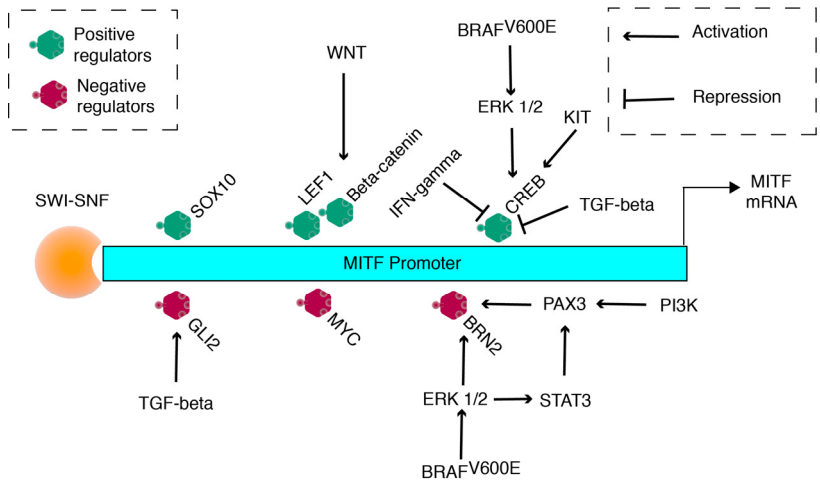

Figure 5: Schematic representation of the various transcriptional activators and repressors of the MITF gene, a simplified representation inspired from Hartman et al. [99]. *Diverse group of transcription factors belonging to various pathways involved in melanoma either promote the expression of MITF (in green) or repress (in red). Arrows indicate activation whereas dashed-head lines represent repression of the targets. Drawn using template from the Library of Science and Medical Illustrations by Idoya and Luk (https://gumroad.com/l/IxECD) with kind permission.*

MITF as a transcription factor is involved in modulation of the expression of many downstream genes and also acts as a master regulator of the melanin production by modulating pigmentation associated genes like *TYR* and *DCT*. However, the role of *MITF* in melanoma development and progression is complex. Although, *MITF* is expressed in the majority of human melanomas, nevertheless its expression is less than that of normal melanocytes [100]. Also, it has been shown that higher expression of *MITF* counteract *BRAF* mediated melanoma cell proliferation [101]. On the other hand, amplification of the *MITF* locus is observed in a fraction of human metastatic melanomas and *MITF* also promotes the expression of oncogenes such as CyclinD1 (*CCND1*), *BCL2* and c-MET along with tumor suppressors like $p16^{Ink4a}$ [100]. *MITF* also interacts with various melanoma associated oncogenic pathways (Figure 5) like Wnt/β-catenin, MAPK through BRN2 and CREB in opposing manner, TGF-β through *GLI2* and CREB, c-MYC (MYC) and PI3K. Altogether, *MITF* sits at a centerpiece of the melanoma development and progression puzzle.

Any discussion of the melanoma development and progression is incomplete without reviewing the role of mitogen-activated protein kinase (MAPK) pathway. It has been shown that a large fraction of cutaneous melanomas harbor mutations in either *BRAF*, *NRAS* or *NF1* gene in a mostly mutually exclusive manner [102]. Tumors without mutations in any of these three genes represent a heterogenous group marked by structural variation and copy number alterations [102, 103].

BRAF represent the largest fraction of melanoma driver mutations with almost 50% of the cutaneous melanomas harbor hotspot (V600E, K601E) mutations in *BRAF*. *BRAF* and *NRAS* mutations are mostly observed in a mutually exclusive manner. *NF1* mutant tumors are characterized as a distinct group associated with higher chronic sun-damage, high tumor mutational burden and older patients [104].

Interestingly, *BRAF* activating mutations are also observed in the benign melanocytic nevi at a high fraction. *BRAF* mutations has been shown to involve in the induction of senescence in the corresponding cells. $p16^{Ink4a}$ is considered to be a mediator of such senescence by blocking *CCND1-CDK4/6* associated cellular proliferation [105]. Melanoma cells can often escape this type of senescence through genomic alteration of *PTEN* and thus activating PI3K-AKT pathway. Evidences have suggested that upregulation of the PI3K-AKT pathway to be a late stage event in the melanoma tumors and thus most likely play a major role in tumor progression rather than initiation [97]. Studies on nevi have further indicated that *BRAF* or *NRAS* mutations alone are likely not sufficient in inducing oncogenesis as additional deregulation of the TSGs such as *CDKN2A*, *TP53* is required [97]. Additionally, oncogenic events of the MAPK pathway also aid in the "Epithelial-to-Mesenchymal" (EMT) transition of the melanoma cells. This results in an increased invasion capacity of the malignant cells and subsequent poor prognosis for the patients [106]. Figure 6 depicts an overview of the MAPK and PI3K pathway with their interactions.

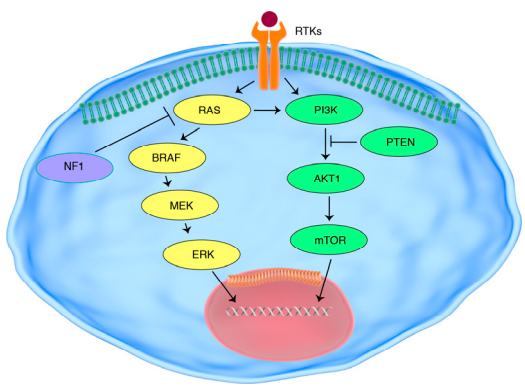

Figure 6: Schematic simplified representation of the MAPK and PI3K-AKT pathways with their interdependencies. *Yellow ellipses represent members of the MAPK pathway and green ellipses represent the same for the PI3K-AKT pathway, while NF1 is indicated in purple. Drawn using template from the Library of Science and Medical Illustrations by Idoya and Luk (https://gumroad.com/l/IxECD) with kind permission.*

Unlike somatic mutations, structural and copy number variations are somewhat less explored in melanoma. Nevertheless, studies have found rare gene fusion events in the melanoma tumors involving important TSGs such as *RB1* [102, 107]. Somatic copy number alterations (SCNAs) play a driving role in the oncogenesis across many cancers. Studies on SCNAs in melanomas revealed a rather complex landscape, where certain tumor groups were more affected than others. The Triple-WT group revealed by TCGA study on cutaneous melanoma [102] reported markedly higher amount of SCNA events than the other subtypes. Amplification events included *KIT*, *PDGFRA*, *CCND1*, *CDK4*, *MDM2* and *TERT* gene loci. Amplification of *MITF* locus and deletion of *PTEN* gene were more frequently observed in the *BRAF* mutant subtype. The locus containing tumor suppressor *CDKN2A* was found to be evenly deleted across all subtypes in the TCGA study.

Alongside genomic studies, multiple attempts have been made to identify transcriptomic signatures in the melanoma tumors. Winnepenninckx et al. in 2006 revealed a 254 gene set signature to predict metastatic dissemination and survival for the patients with primary melanoma [108]. Jönsson et al. in 2010 made one of the earliest transcriptomic classification of the metastatic melanoma tumors and identified four tumor groups characterized by the respective expression of the immune, stromal, pigmentation and cell-cycle genes [109]. Later, similar transcriptomic analyses also found CMM tumor groups based on immune, pigmentation and cell-cycle signatures [102]. Based on the gene expression phenotypes identified by Jönsson et al. [109], additional transcriptomic signatures have been identified to predict patient survival in the early stage primary melanomas [110]. More recently, a melanoblast-specific gene signature has been proposed to explore the metastatic progression [111].

Overall, the genomic and transcriptomic characterizations of CMM in the past decades, have greatly improved our understanding on how the tumors develop and helped us to predict patient prognosis. Nevertheless, a lot is still left to be understood in order to overcome the treatment resistance and enabling therapeutic benefits for every patient.

Epigenetic characterization

C.H. Waddington first coined the term “Epigenetics” to describe different internal and external interactions between the environment and the genome that leads to the development of phenotypes [112, 113]. Over the years and especially in later decades of the 20th century, epigenetic research gained traction largely due to the pivotal roles played by different epigenetic modifications in the transcriptional mechanism of the genome. Among many epigenetic modifications, methylation of the cytosine at 5’ position of its pyrimidine ring is one of the widely observed methylation events of DNA of the mammals including humans and is observed in the context of cytosine phosphate guanine (CpG). Methylated cytosines often undergo deamination to convert into thymine [114]. Such deamination leads to a GT

mismatch whose correction is often error prone and leaves a permanent change in form of A or T [115]. DNA methylation can also be observed in a non-CpG context, however its role in the mammals including humans is much less frequent and poorly understood.

In the mammalian DNA, CpGs often accumulate in regions with high GC density known as CpG islands. Gene promoters residing in these CpG islands are mostly found unmethylated whereas methylation is more common in context of the gene bodies and other regulatory regions. Figure 7 shows a schematic landscape of DNA methylation in the mammals.

In the eukaryotic cells, DNA is wrapped around histones to form structures called nucleosomes. A loosely packed structure of these nucleosomes with constituent DNA is known as euchromatin and alternatively a densely packed structure of DNA and nucleosomes is termed heterochromatin. Euchromatin structure due to its loose packing, makes underlying DNA accessible to the transcriptional machinery and thus take active participation in the transcription process. Chromatin often switch between the heterochromatin and euchromatin states to aid in the transcription of the underlying genes, a phenomenon known as chromatin remodeling.

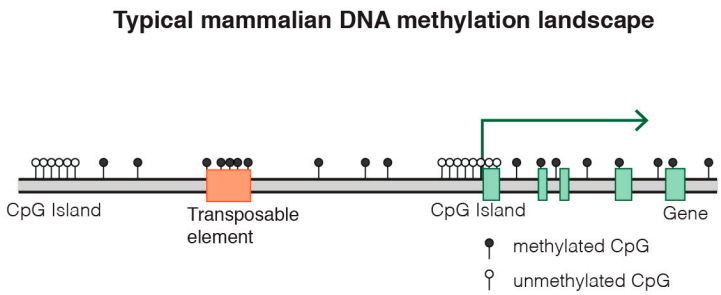

Figure 7: Schematic representation of DNA methylation in the mammals. *Image taken from wikipedia as original, shared by Mariuswalter.*

Apart from its intrinsic role in the embryonic development, DNA methylation has been shown to play a major part in the development and progression of various diseases, including cancer. Methylation of CpG island promoters have been observed across cancers along with the methylation loss of the GC-poor regions. Also, tumor suppressors like p16 and DNA damage repair genes are affected by CpG island hyper-methylation [116].

Cutaneous melanoma like other cancers, have been shown to be affected by promoter hyper- and hypo- methylation events. Studies on DNA methylation in the melanoma tumors have identified many genes associated with disease progression to be epigenetically modified [117, 118]. Furthermore, DNA methylation has also

been linked to the metastatic dissemination of melanoma [119], along with the observations of promoter hyper-methylation in key TSGs such as *PTEN* [120, 121].

Epigenetic modifications have been implicated in tumor plasticity before. Remodeling of the chromatin is often assisted by the genomic alterations in the chromatin modifier complexes and this allows malignant cells to alternatively switch to different transcriptional states and/or developmental pathways receptive to the oncogenesis [116].

In the context of the cutaneous melanoma, Lauss et al. [122] observed higher methylation of "poised promoters" that are targeted by the chromatin modifier complexes such as polycomb repressor complex 2 (PRC2), in line with the observations in other cancer types. For repetitive and heterochromatic regions, loss of methylation was reported in melanomas compared to melanocytes. Authors also identified three methylation clusters for melanoma with varying methylation patterns. Among these clusters, a promoter hyper-methylated cluster was reported with higher proliferation and up-regulation of the DNA methylation associated genes such as *DNMT3A* and *TET1* along with chromatin remodeling complex SWI/SNF members.

The *MITF* gene alongside its intrinsic role in the melanoma development and progression has also been associated with plasticity of the melanoma cells, especially upon treated with BRAF-inhibitor agents [123-126]. Alteration of *MITF* expression in such contexts could depend on epigenetic silencing through promoter hyper-methylation [127].

Altogether, epigenetic characterization of the CMM has made strides recently in unraveling diversity in the tumor cell phenotypes, however further studies are warranted to fully capture the scope of the epigenetic changes in modulating CMM development and progression.

Tumor microenvironment

Tumor stroma in cancers including CMM

Over the past few decades, interest among researchers have piqued on understanding the nature of the complex compendium of cells that enrich tumors. Malignant cells only constitute a fraction of the total cells that are present in the tumors. Non-malignant cells that form the tumor stroma, come from diverse origins and also offer various functionalities that either benefit or obstruct the tumor's growth [128]. Broader classification of the non-malignant cells in tumor microenvironment (TME) mostly reveal two major classes of cells, immune and non-immune. In this part, a general overview of the tumor stroma in cancers including CMM is presented, along with the functionalities of the underlying non-immune cells. Roles of the immune cells in the TME will be discussed in the following part.

Tumor vasculature:

Tumor vasculature counts among the most important structures within a tumor. These vessels not only supply necessary nutrients for a tumor to survive and grow but at the same time also aid in the immune surveillance by trafficking anti-tumorigenic immune cells [129]. Two major classes of vasculatures are observed within a tumor, blood and lymphatic. Blood vessels within tumors are complex structures. Their formation is abated by the release of multiple pro-angiogenic growth factors such as VEGFs from the malignant cells and stromal cells like fibroblasts [130]. Tumor blood vessels differ significantly from their normal counterparts in terms of the structure, organization and functionality. Unlike blood vessels in the normal tissues, tumor associated blood vessels lack proper structure and often have blunt ends, disorganized network of immature vessels that are leaky and unstable in nature and usually result in an inconsistent and limited blood flow within the tumor. These characteristics of tumor blood vessels make the microenvironment hypoxic and acidic due to higher anerobic glycolysis and thus facilitate selection of the malignant cells of more aggressive nature [129].

In contrast to the blood vessels, lymphatic vessels are hierarchical structures of vessels in a unidirectional manner that drain fluid and cells from the nearby tissue into the lymph nodes. They function in consort with the blood vessels and perform important functions like lipid absorption and maintaining the balance of tissue fluid [131].

In context of tumors, lymphatic vessels take important part in the metastatic dissemination. Proliferation and sprouting of the lymphatic vessels in the tumor are collectively known as lymphangiogenesis and this is instrumental in connecting the primary tumor to the lymph nodes. Tumor lymphangiogenesis has been reported in lung metastases of the CMM and higher density and area of the lymphatic vessels has been associated with poor prognosis for the patients [132].

Among the major cell-types associated with the tumor vasculature, endothelial cells (ECs) are one of the most well-studied. ECs form the inner lining of the vessels and play important roles in the trafficking of the cells, tissue fluid and other factors. ECs have been implicated in melanoma as up-regulation of the Notch signaling is observed in the melanoma cell-lines in co-culture with ECs. Overexpression of Notch3 as observed in the co-culture, associated with higher metastatic and invasive capabilities of the melanoma cells [133]. Such communication between malignant melanoma cells and ECs are likely bi-directional, as overexpression of the intercellular adhesion molecule 1 (ICAM-1) and E-selectin was reported in the EC cell-line in co-culture with metastatic melanoma cell-line. Crosstalk between the melanoma cells and ECs likely result in a better cellular adhesion during metastasis, as suggested [134]. Apart from their contribution to the metastatic dissemination, ECs have been shown to produce pro-angiogenic factors like VEGF-C to render

effector $CD8^+$ T cells ineffective in a murine melanoma model, thus aiding in the tumor immune-exclusion [135].

Although, angiogenesis inhibiting agents such as anti-VEGF drug have been tried as a therapeutic intervention, nevertheless a clearer picture of the broad scope of tumor vasculature in anti-tumor immune response, is yet to emerge.

Extracellular matrix:

Extracellular matrix (ECM) is a major structural component of the tumor and comprises two major classes of macromolecules, proteoglycans and fibrous proteins. Main fibrous proteins of ECM constitute collagen, fibronectin, laminin and elastin [136, 137]. ECM has heterogenous behavior across tissues attributable to the interaction between various cellular components involved in its formation, such as fibroblasts and epithelial cells. Tumor ECM is significantly different from its normal counterpart as the tumor ECM tends to be more rigid due to the deposition and remodeling by the underlying fibroblasts. Secretion of Matrix metalloproteinases (MMPs) by malignant cells and fibroblast further aid in the remodeling of the ECM [137].

ECM proteins play important roles in modulating the TME in cancers including melanoma. Osteopontin (OPN), a glycoprotein has been shown to be secreted by tumor cells and take part in the metastatic progression in melanoma [138]. OPN has also been found to be associated with several oncogenic driver pathways in melanoma such as PI3K [139], NIK/ERK and MEKK1/JNK1 [140]. Tenacin C (TNC) another glycoprotein, involved in the metastasis and angiogenesis [141], interact with different molecular pathways like transforming growth factor β (TGF-β) and contribute to remodeling of the stroma and tumor growth.

Among major cell-types that modulate the formation and remodeling of the ECM, cancer-associated fibroblasts (CAFs) are one of the most important. Fibroblasts get activated during the wound healing process and produce several growth factors and signaling molecules such as cytokines and chemokines [142]. In tumors, the situation resembles a non-healing wound and the activated fibroblasts or myofibroblasts remain in this state and becomes prominent contributors to the tumor growth [143].

CAFs in the tumor derive from different tissues including non-fibroblast progenitors. Their transcriptomic profiles differ significantly from their normal counterparts with the expression of factors like α-smooth muscle actin (ACTA2), Fibroblast activation protein (FAP) and platelet-derived growth factor receptor α and β (PDGFRα/β) [143]. In addition to this, CAFs produce an array of growth factors and pro-inflammatory cytokines such as TGF-β, VEGF, interleukin 6 (IL-6)

and CXC-chemokine ligand (CXCL12) to promote the tumor angiogenesis, metastasis and immune evasion [144, 145].

Keratinocytes:

Keratinocytes are among major cellular components of the skin and they are found in the epidermal layer, along with melanocytes. Keratinocytes are protected from UVR by the melanocytes and their organelles melanosomes containing the pigment melanin. The melanosomes are transported and transferred from the dendritic tips of the melanocytes to the surrounding keratinocytes [146, 147]. Keratinocytes can regulate proliferation and transformation of the melanocytes through E-cadherin mediated cell-to-cell contacts, which is lost during melanogenesis [148]. As undifferentiated keratinocytes control the melanocytic differentiation [149], contrarily differentiated distal keratinocytes expressing Notch ligands aid in the invasion of melanomas through impairment of the *MITF* functioning [150]. The dual roles performed by keratinocytes in the malignant transformation of melanocytes is intriguing and further studies in this domain is required for a better understanding.

Immune cells in CMM

In recent years, roles of the immune cells in melanoma have attracted significant attention thanks to the progress made in the immunotherapeutic treatments. Cutaneous melanoma is considered to be one of the most immunogenic tumor types and presence of the effector immune cells generally associate with a better prognosis for the patients [151]. Several attempts have been made so far by the researchers to unravel the nature of the diverse immune cell-types occurring in the melanoma TME and the crosstalk between immune, malignant cells and other stromal cells such as CAFs. The interaction between malignant cells and the immune system can be broadly categorized into two main distinct phases, *elimination* and *evasion*. The elimination phase is an umbrella term to distinguish the cascade of events that lead to the apoptotic destruction of the malignant cells through cytotoxic immune cell-mediated anti-tumor response. On the contrary, the evasion phase encompasses a likely even broader chain of events negotiated through diverse mechanisms, that result in continued survival of the malignant cells. Both phases employ multiple players and complex mechanisms, which shall be discussed in the following parts.

Elimination of the malignant cells through anti-tumor immune response is carried out by the immune cells associated with both innate and adaptive immune system. Innate immune response is considered as the first line of defense for the host against foreign pathogens. In cancer context, cells belonging to the innate immunity perform both elimination of some of the cancer cells (non-MHC expressing and apoptotic) as well as aid the cells of the adaptive immune system in the malignant

cell destruction [152]. Some of the major cell-types associated with innate immune system are discussed below.

Natural killer cells:

Natural killer (NK) cells are among the primary cytotoxic effector cell-types that recognize tumor cells lacking MHC-I antigens and facilitate their apoptosis by releasing proteins like granzymes and perforin. NK cells form a distinct class of lymphocytes that are characteristically different from the T and B cells. Nevertheless, they share many surface markers with the T cells due to their common lineage. NK cells undergo maturation in the bone-marrow assisted by a number of cytokines such as IL-7 and IL-15. Human NK cells express CD56 on their surface along with the CD16A for the majority of the group. Along with their direct anti-tumor response, these cells also produce a diverse range of cytokines including interferon-γ (IFN-γ) which plays a pivotal role in stimulating antigen presentation for the adaptive immune system [153].

Dendritic cells:

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) that also act as the main connection between innate and the adaptive immune system. DCs are a heterogenous class of cells including subsets such as conventional (cDCs), myeloid (mDCs), plasmacytoid (pDCs) and Langerhans cells (LCs) [154]. DCs in cancer context, perform a range of different functions from the tumor-antigen recognition to the removal of apoptotic cells. When encountered with the apoptotic malignant cells, DCs phagocytose them with the help of integrins and CD36 receptors. Activated LCs secrete IL-15 and help $CD4^+$ and $CD8^+$ T cells with the antigen priming through cellular immunity. Additionally, dermal DCs stimulate B cells to invoke humoral immune system [152].

The role of DCs in melanoma however has been more complicated as pDCs have been shown to associate with pro-tumorigenic activities and likely to be a facilitator of the immune-evasion [155]. It has been suggested that future successful immunotherapeutic treatments for melanoma might require enhancement of the antigen-presentation functioning of the DCs [154].

Macrophages:

Macrophages are myeloid cells that derive from the monocytes in the peripheral blood and undergo transformation upon reaching tissues, often in response to foreign pathogens. They perform many critical functions in context of the tumors. Being an intrinsic part of the innate immune response, they perform phagocytosis

of the apoptotic tumor cells with the help of scavenger receptors such as CD36, CD68, CLA-1 etc. Macrophages also take important part in the antigen-presentation mechanism through expression of the tumor-antigen epitopes on the surface using MHC-II molecules and presenting them to the T cells [152, 156]. However, tumor associated macrophages (TAMs) can also take a pro-tumorigenic role and can actively aid in the immune-exclusion process. Such alternative functionalities of the TAMs are mostly dependent on M1/M2 polarization where M1 subtype associate with the T helper cells 1 (Th1) in anti-tumor response; M2 contrarily activated by the Th2 cells release anti-inflammatory cytokines like IL-10 and TGF-β, to aid tumor survival [157].

Interaction between the melanoma cells and TAMs occur through multiple proteins with diverse functionalities. Recruitment of TAMs in the melanoma tumors is mediated through chemotactic cytokines such as monocyte chemotactic protein (MCP-1). In association to this, melanoma cells can also express high levels of macrophage migration inhibitory factor (MIF) to inhibit the random migration of macrophages and promote angiogenesis and tumor growth. On the other hand, macrophages can help in the enhancement of invasive potential of the melanoma cells through melanoma inhibitory activity (MIA) protein [152]. Overall, crosstalk between the macrophages and melanoma tumor play an important role in shaping the anti-tumor immunity.

Adaptive immune response is one of the most crucial determinants of the anti-tumor immunity. It is a more sophisticated system to combat the foreign pathogens and malignant cells in the host that aims to provide antigen-specific, long-term immunity. Lymphocytes are the key factors in mounting the adaptive immune response, which can be broadly categorized into cell-mediated immunity led by the T cells and humoral immunity (immunity against extracellular and freely circulating pathogens) aided by the B cells.

T cells originate from the progenitors in the bone marrow and undergo maturation in the thymus. Initial T cells lack the expression of both CD4 and CD8. However, through the rearrangement of the T cell receptor (TCR) and subsequent selections, the differentiate into the $CD4^+$ and $CD8^+$ naïve T cells. Naïve peripheral T cells are activated when presented with antigens by the DCs in the lymph nodes and differentiate into the effector phenotype. Post-response, the majority of the effector T cells undergo apoptosis and a fraction of the antigen-experienced T cells transform into the memory T cell phenotype [158, 159]. Here, two major classes of the T cells, $CD8^+$ and $CD4^+$ T cells are reviewed.

$CD8^+$ T cells:

$CD8^+$ T cells are the main effector cells in the adaptive immune response. They are activated by the MHC-I mediated antigen presentation and undergo an antigen-

specific clonal expansion to transform into the cytotoxic $CD8^+$ T cell phenotype. These cytotoxic T cells (CTLs) secret pro-inflammatory cytokines like IL-2 and IFN-γ that in turn promote cytotoxic functionalities of these cells through tumor necrosis factor α (TNF-α), perforin and granzymes [159].

However, in the tumors cytotoxic functionalities of the $CD8^+$ T cells can be rendered ineffective by a range of factors expressed by the malignant cells and other cells in the TME. Malignant cells often express checkpoint molecules like programmed-death ligand 1 (PD-L1) [160] and enzymes such as Indoleamine-2,3-Dioxygenase 1 (IDO1) [161]. Similar blocking of cytotoxic functionalities can be achieved by the expression of anti-inflammatory cytokines like IL-10 and TGF-β [162].

It is interesting to note that this microenvironment-mediated exhaustion of the cytotoxic T cells is of gradient nature. Single-cell transcriptomic analyses of the $CD8^+$ T cells in melanoma revealed multiple stages of dysfunctionality through the expression of markers such as granzyme K (GZMK), PD1, CTLA4 and TIM3 [163, 164]. Similar observations have been made in other cancers as well [165].

Apart from the cytotoxic subset, memory $CD8^+$ T cells have also garnered significant attention recently, largely due to their association with the long-term prognosis and treatment benefit from immunotherapies, as shown in melanoma [163, 166].

$CD4^+$ T cells:

The $CD4^+$ subset of the T cells is a diverse group of immune cells with both pro and anti-tumorigenic properties. Among many $CD4^+$ T cells, two major classes stand out in context of the tumor immune response, the T helper (Th) cells and regulatory T cells.

The T helper (Th) subset primarily constitutes Th1, Th2, Th17 and T follicular helper (Tfh) cells. Among these cell-types, Th1 and Th2 are more well established in terms of the studies on their roles in the TME. These cells are characterized by their secretion of the signature cytokines such as IFN-γ and TNF-α for the Th1 and interleukins 4, 5 and 13 for the Th2. In terms of the anti-tumor immunity, Th1 cells are perceived to be superior due to their ability to recruit $CD8^+$ T cells, NK cells and M1 macrophages to the tumor [167]. Additionally, Th cells can inhibit tumor angiogenesis in an IFN-γ dependent manner [168]. The roles of Th2 cells in the immune response is rather controversial. However, Th2 mediated adoptive T cell transfer therapy for the metastatic melanoma has shown their ability to utilize innate immune cells to mount successful anti-tumor response [167, 169].

The role of the Th17 subset of the $CD4^+$ T cells in anti-tumor immunity is also quite unclear and likely to be more context dependent like the Th2. It has also been

observed that Th17 cells are less differentiated and more stem-like. However, their stemness also enables them to assume an effector role when necessary [170].

Tfh cells are distinguished by their capacity to migrate to the secondary lymphoid organs (spleen, lymph nodes etc.) and interact with the B cells to provide anti-tumor immune response [167]. Recent studies on the role of tertiary lymphoid structures (TLSs) that form within the tumor, highlight the role of $CD4^+$ T cells in the formation of these structures and overall association with immune response and prognosis in melanoma [171, 172].

Regulatory T cells (Tregs) are a distinct subset of the $CD4^+$ T cells. They are characterized by their expression of FoxP3 and CD25, are one of the major immunosuppressive cell-types. Intra-tumoral enrichment of the Tregs might result as an immunomodulatory behavior of the TME, as reported in melanoma [173].

B cells and the tertiary lymphoid structures:

B lymphocytes are the main contributor to the humoral immune response and derive from the bone marrow of the host. B cells upon activation of the B cell receptor (BCR) differentiate into the effector and regulatory phenotypes. Inactive B cells do not possess much antigen presentation potential. However, when activated through multiple mediators such as CD40 ligand, their antigen presentation activity is greatly enhanced through the expression of the cytokines and chemokines such as CCL2, CXCR4, CXCL5, etc., and in turn prime both the $CD8^+$ and $CD4^+$ T cells [174].

The role of B cells in cancer has been controversial for a long time. For some cancers including melanoma, they have been shown to promote tumor angiogenesis by secreting factors like lymphotoxin and by triggering pro-tumorigenic myeloid cells [174, 175]. It has been hypothesized that the circulating immune complexes containing the B cells recognize tumor antigens lacking MHC-I antigen presentation. However, since this antigen-antibody conjugate cannot elicit $CD8^+$ T cell response, they alternatively activate Fcγ receptors and associate with the myeloid derived suppressor cells to promote tumor sustenance [176].

Nevertheless, studies on the $CD20^+$ B cells have demonstrated their capabilities of forming TLSs together with the T cells. TLSs are lymphoid aggregates that form intratumorally to assist in the immune response in actions resembling the activities in the secondary lymphoid organs like the lymph nodes [177]. Studies on TLSs in the melanoma and soft-tissue sarcoma have revealed their prognostic and therapeutic potential [171, 172, 178], which is likely to motivate further inspections on the role of B cells in cancers including melanoma.

Immune-evasion in melanoma:

Immune-evasion mediated by the TME is considered as a hallmark of cancer [46]. So far roles of the different immune cells associated with anti-tumor immunity have been discussed alongside their contribution to the immune-evasion process. Here, a brief review of the different immune-exclusion mechanisms employed by the TME to avoid the immune system-mediated elimination is given.

Immune-evasion of the tumors can primarily be described in two ways, innate and adaptive [179]. Innate immune exclusion of the tumors is characterized by the lack of the effector immune cells in their microenvironment. Tumors are hypothesized to achieve such an absence of a T cell mediated anti-tumor immunity through alterations in the immunomodulatory genes, changes in composition of the commensal microbiota and tumor cell-associated genomic alterations [179-181]. Contrarily, adaptive immune-exclusion could take place in the tumors infiltrated with effector immune cells earlier but have been rendered largely ineffective due to the selection of immune-resistant cells within TME. This type of immune-evasion is often observed in patients treated with immunotherapeutic agents as secondary resistance.

Involvement of several oncogenic pathways have been suggested in the immune-exclusion [179]. Many of these pathways are also involved in the melanoma development and progression, such as c-Myc, β-catenin dependent Wnt signaling, MAPK, PI3K through loss of *PTEN* functioning and TGF-β [182-186]. Immune evasion of melanomas is a major concern for the treatment resistance against immunotherapeutic agents and studies on how to turn immune-evasive tumors into immune-responsive is necessary to expand the scope of treatment benefit.

Treatment of melanoma

Surgery

Surgical resection is the most common form of treatment for the early-stage melanomas. For the primary, diagnostic excision (diagnostic excisional biopsy) a clinical margin of 1-3 mm to the primary tumor is recommended. After confirmed diagnosis a wide local excision (WLE) of 1-2 cm to the primary scar is recommended as first-line treatment. Additionally, for further staging, a sentinel lymph node biopsy (SLNB) can be conducted for patients with melanomas with intermediate to high Breslow thickness (>1 mm), to assess the possible microscopic metastatic spread in the closest (sentinel) regional lymph node (RLN).

The excision margin (WLE) for the tumor is dependent on the Breslow thickness of the primary tumor. A higher Breslow thickness warrants wider margins whereas for most *in situ* melanomas it is enough with a wide local excision margin of 5 mm.

Patients who have been cytologically verified for RLN metastases are also examined for any additional metastasis at distant organs. If the disease is only regional, then the lymph node removals are carried out following clinical guidelines [187]. If a patient is diagnosed with a distant metastatic disease, then lymph node resection is not carried out and the patient is referred for the systemic treatment instead.

Chemotherapy and radiotherapy

Chemotherapy was the earliest treatment option for the melanoma patients with advanced stage of disease. Dacarbazine was approved as a chemotherapy agent for melanoma by FDA in 1974 [188]. It remained as a major treatment option before the inception of targeted and immunotherapies, in spite of being largely ineffective in terms of the treatment response [189]. Temozolomide (TMZ) had also been used to treat advanced melanomas and had showed improved progression-free survival compared to dacarbazine. However, such an observation was not made for the overall survival [188, 190].

Radiotherapy unlike chemotherapy is rarely used as a first-line treatment. Nevertheless, radiotherapy is sometimes used in an adjuvant setting and as palliative treatment option for the advanced non-operable cases [191].

Immunotherapy

Harnessing patient's immune system to treat cancers has been one of the major game changers in the long history of oncological treatments. Almost a century after Paul Erlich's seminal hypothesis that positive mechanisms in the body might provide protection against the aberrant cells that rise during development [192], immunotherapeutic treatments have become standard curative therapy for some of the advanced stage cancers like metastatic melanoma.

One of the earliest immunotherapy agents approved for treating the advanced melanoma patients was high dose IL-2 in 1998 by the FDA. However, the risk of multi-organ complications and complexity of the procedure outweighed limited treatment benefits. IFN-α based treatments showed similar characteristics to that of IL-2. Issues observed in these treatment protocols bolstered further research to overcome the obstacles in the path of devising newer immunotherapies [193].

Immune-checkpoint inhibitors:

James P Allison and colleagues first demonstrated the effectiveness of blocking immune-checkpoint molecules like CTLA-4 to boost anti-tumor immunity. Lack of the CD28 mediated co-stimulation signal from the malignant cells hinders the activation of the T cells. CTLA-4 through its binding with the B7 family of costimulatory molecules acts as a negative regulator of the T cell activation. Thus,

blocking of the CTLA-4 inhibitory function allows for the enhancement of T cell activation and subsequent tumor inhibition [194].

Around the same time, Honjo and colleagues identified a novel protein programmed cell-death 1 (PD1) expressed by activated the T and B cells and whose absence is associated with several autoimmune diseases. Subsequent ligands of the PD1 (L1 and L2) that are expressed by cells of diverse tissue origin and malignant cells were also discovered. Additionally, Honjo and colleagues highlighted the functions of PD-1/ PD-L1 in mediating immune-escape in the tumors through blocking of the cytolytic activity of T cells [195].

Discovery of the immune checkpoint-blockade (ICB) molecules have led to the development of a plethora of immunotherapeutic drugs to enhance the cytotoxic T cell mediated anti-tumor immunity. Amongst these ICB agents for treating the metastatic melanoma patients, Ipilimumab (anti-CTLA4) received FDA approval in 2011 followed by Nivolumab and Pembrolizumab (anti-PD1) in 2014. Instead of stand-alone monotherapies with either Ipilimumab or Nivolumab, combination treatments showed better objective response rate (ORR) and progression-free survival and also performed better for the patients with PD-L1 negative tumors [196, 197]. Similar combination of ipilimumab with pembrolizumab has been resulted in positive treatment response among the melanoma patients [198].

Currently in many countries including Sweden, ICB treatments are used as first-line therapy for the patients with non-operable distant metastases and in adjuvant setting for the high-risk advanced stage patients post-tumor resection [187].

Adoptive T cell therapy:

Adoptive T cell therapy (ACT) was introduced by Steven Rosenberg and colleagues for the metastatic melanoma patients using autologous TILs in 1988 [199]. Currently, chimeric-antigen-receptor (CAR) T cell therapy is an approved treatment by FDA for a subset of B cell lymphomas [200]. For melanoma and other solid tumors, however a more classical approach is used using autologous TILs, cultured in-vitro together with IL-2 [201].

ACT has not been approved as a treatment for melanoma however, several clinical trials have highlighted its efficacy in treating the advanced stage melanoma patients who have failed to respond to ICB and targeted treatments [202, 203].

Targeted therapies

Importance of the oncogenic driver mutations, especially in the MAPK pathway in melanoma have prompted the development of corresponding inhibitor drugs. Among melanoma driver mutations, *BRAF* hotspot mutations are prevalent in a large fraction of tumors. Vemurafenib and Dabrafenib, two selective inhibitors of mutant BRAF have been associated with improved survival for the melanoma patients upon treatment [204, 205] and had received FDA approval in 2011 and

2013, respectively. Similarly, Trametinib a selective inhibitor of MEK which is downstream of BRAF, has demonstrated improved survival for the patients with *BRAF* hotspot mutations compared to chemotherapy [206].

KIT mutations are more rare in CMM however, they are observed at a higher frequency in the acral and mucosal melanomas [207]. Inhibitor of oncogenic *KIT* mutations and amplifications through drugs such as imatinib has been shown to offer treatment benefit in the single arm clinical trials [208, 209].

Despite the success of kinase inhibitors in the treatment of melanomas, resistance developed to these drugs is a major area of concern and long-term treatment benefits have been restricted so far [210]. Several mechanisms behind the resistance to the targeted therapies have been suggested with ideas to overcome such resistance [211]. Nevertheless, studies have suggested that combining targeted therapies with the ICB treatments could yield better treatment response than stand-alone therapies [212]. In Sweden, currently treatment with the kinase inhibitors is recommend in an adjuvant setting [187].

Tumor material

Tumor cohorts

In-house Lund cohort for the studies I, II and IV, is a historical collection of 214 patient tumors from the biobank. Majority of the tumors were surgically operated in Lund, at the Department of Surgery, Skåne University hospital during the period of 2000-2012. This cohort can be considered treatment-naïve in terms of the targeted and immunotherapeutic treatments. All the tumor samples were snap frozen after the surgery and were kept at a low temperature. Most of the tumor samples were from metastases, with the majority being lymph node metastases. Additionally, few primary tumors were also a part of this cohort. Apart from the tumors, also matching blood samples from the patients were available for most.

Another in-house cohort that was used in the study III, came from a clinical trial (NCT00937625) of stage IV melanoma patients enrolled in an adoptive T cell transfer (ACT) treatment. The cohort comprised 27 patients and all of them had received prior systemic therapy.

Along with the in-house Lund cohort, mouse tumors were also used in the study IV. This cohort constituted Male C57BL/6 mice (The Jackson Laboratory) aging between 7-9 weeks and bred at the Lund animal facility.

Studies on in-house Lund patient tumors were approved by the Regional Ethics Committee at Lund University (Dnr. 191/2007 and 101/2013). All experiments concerning animals were approved by the Malmö/Lund Ethical Committee for Experimental Animals (Permit number M47-15) and were performed according to the appropriate international guidelines for the use of research animals. Also, all sensitive patient information was stored and disseminated in accordance with the recent GDPR regulations.

Overview of the main methods

Studying the Genome, Epigenome and transcriptome

A brief history of nucleic acid sequencing

Since Sanger and colleagues' success in determining the amino acid sequences of Insulin [213-215], we have walked a long path to reach the current milestones of understanding molecular biology. Amino acid sequencing was developed in the early 1950's. However, successful sequencing of the DNA molecules was not done until 1970s. This can be largely attributed to the complexity of sequencing DNA molecules constituting longer sequence of repetitive bases [216, 217].

In this regard, RNA preceded DNA as in 1965 Holley and colleagues sequenced the alanine transfer RNA (tRNA) from *Saccharomyces cerevisiae* [218]. Also, around the same time Sanger and colleagues had developed a similar approach for sequencing the ribosomal RNA (rRNA) and tRNA [219]. Nevertheless, sequencing whole genomes consisting of longer sequences of nucleotides was still far from reality and would not have been possible without fundamentally altering the chemistry of the sequencing. This came in form of replacing the two-dimensional fractionation of the earlier methods with single separation by polynucleotide length via electrophoresis through polyacrylamide gels [217], used in two different protocols from Coulson and Sanger [220] and Maxam and Gilbert [221]. Although, the Maxam and Gilbert protocol had led to the birth of "*First generation of DNA sequencing*" [217], however Sanger and Colleagues' "Chain-termination" based method [222] became a watershed moment in the history of DNA sequencing.

Since then, the field of DNA sequencing has experienced a rapid progressive development and culminated in the discovery of "pyrosequencing" based methods [223] [224]. The success of the pyrosequencing technique led it to being licensed and developed into first commercially successful "Next-generation sequencing" (NGS) technology by former 454 Life Sciences (later acquired by Roche) [217].

However, the golden age of DNA sequencing had already began with the start of the human genome project, aiming to sequence the entire genome of *Homo sapiens* [225]. This monumental project spanning 13 years was undertaken in an age before the massive parallel sequencing was the norm. Needless to say, the completion of

whole human genome ushered in a new dawn for the genomic studies, which was then taken further by the advent of the new generation massive parallel sequencers.

Next generation sequencing – Genome, exome and transcriptome

Application of the NGS technology in DNA sequencing has made rapid progression in cancer research largely due to its scope of observing molecular alterations at a single base resolution. Whole genome sequencing (WGS) has significant advantage in observing global genomic alterations. However, in the tumor studies unavailability of the suitable amount of material, large number of samples, generated data volumes and computational requirements makes it challenging in most occasions. Whole exome sequencing (WES) mitigates some of the disadvantages of WGS by focusing only on the coding part of the genome. However, WES for a large number of samples with high base coverage is still expensive and researchers often look to the more economical targeted sequencing approaches that cover much smaller, but biologically important, regions.

NGS methods usually follow a standard protocol with minor variations. In the Illumina sequencing protocol, genomic sequences are fragmented into small pieces followed by the adaptor ligation for sequencing. Next, the adaptor-ligated sequence fragments are hybridized to the complementary oligonucleotides at the floor of the flow cell followed by amplification to form clusters. Sequencing is performed by first labelling the nucleotides with fluorescent dyes and then recording them with highly light-sensitive cameras [226]. Current Illumina sequencing reads are usually 150 base pair long and these sequencing reads can be organized *de novo* or can be mapped to specific genomic assemblies such as hg19 and hg38 for the humans [227]. Figure 8 shows a schematic representation of the NGS method in comparison with Sanger sequencing.

Sequencing of the mRNAs are performed by enriching RNAs with poly(A) tails to select the mRNAs and by removing other RNA types such as rRNA. Then mRNA is converted into complementary DNA (cDNA) sequence using reverse-transcriptase and undergo sequencing. Additional refinements for the single-cell based RNA sequencing protocols are made by using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and template-switching oligonucleotides such as switch mechanism at the 5' end of RNA templates (SMART) for the plate-based sequencing methods [228].

WES and targeted sequencing for identifying somatic mutations have been used in study III (WES) and in I and IV (targeted sequencing), respectively. For WES, extraction of the Tumor DNA and RNA was performed using AllPrep DNA/RNA Mini Kit (Qiagen) from the frozen tumor samples. Normal DNA was obtained from the PBMC or TILs using QIAamp DNA Mini Kit (Qiagen). SureSelect Target

Enrichment System for the Illumina Paired-End Sequencing Library Protocol along with Clinical Research Exome (CRE) capture oligo panel from the Agilent Technologies were used for library preparation followed by sequencing on Illumina HiSeq 2500 instrument in paired-end mode.

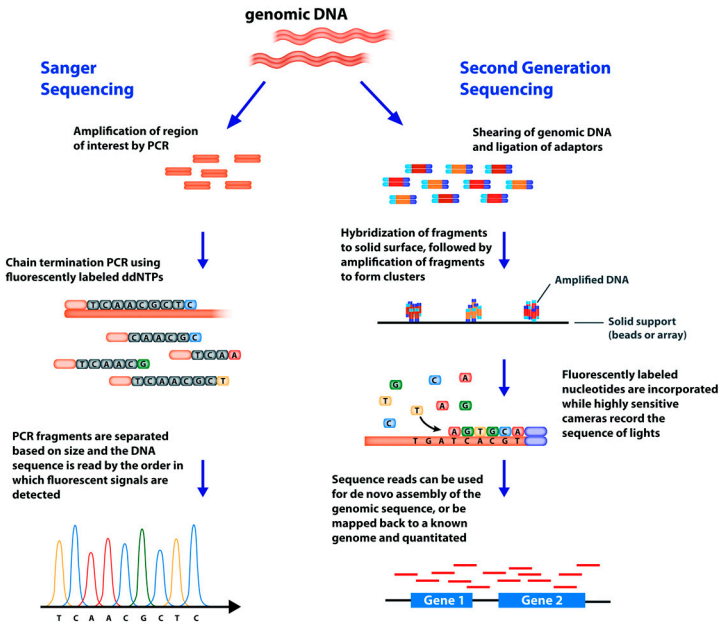

Figure 8: Comparison between Sanger sequencing and next (second) generation sequencing (NGS) technologies. Taken as original from Bunnik and Le Roch [227] with kind permission from the publisher Mary Ann Liebert, Inc.

Targeted sequencing for detecting somatic mutations was performed on 162 patient tumor samples for a previous study [229], using a 1697 cancer associated genes panel as described earlier [230].

In addition to mutational profiling, HLA typing was performed in study III using normal DNA from matching patients using Illumina TruSight HLA Sequencing Panel and the MiSeq sequencer (Illumina).

Microarray-based epigenome and transcriptome profiling

Before the advent of sequencing-based approaches to profile the genome, transcriptome and epigenome, microarrays provided a cheap and scalable alternative to investigate the molecular alterations. In cancer studies, epigenomic microarrays are still in use to a large extent due to the fact that the sequencing

alternatives in such studies are much more expensive both in terms of the material and cost.

Identification of DNA methylation is based on the concept that upon treatment with sodium bisulphite, methylated cytosines remain unchanged while unmethylated ones undergo deamination to convert into uracil and ultimately to thymine. By measuring the amount/signal of cytosine and thymine, it is possible to determine the level of methylation at a particular site.

In study I, DNA from 214 patient tumors were profiled for CpG methylation using the Illumina Infinium MethylationEPIC BeadChip technology. EPIC arrays are an extension of the earlier Illumina HumanMethylation450 BeadChip both in terms of the CpG coverage as well as in covering regulatory regions of the genome. The Infinium methylation platform uses beads containing sequence probes with multiplex technology to measure the DNA methylation for each individual CpG loci. Probe sequences are designed to be complementary to a specific genomic DNA region after bisulphite conversion with the target CpG at the 3' end. Post-hybridization, single base extension of the probe incorporates fluorescently labelled ddNTP to the target CpG site to identify C/T conversion that resulted from the bisulphite conversion earlier [231]. Finally, the level of methylation is measured from the signal intensity as β values,

$$\beta = \frac{I\ meth}{I\ meth\ + I\ unmeth + 100}$$

Where, I_{meth} is the intensity from the methylated probe and I_{unmeth} is the same from the unmethylated probe.

Transcriptomic profiling data of 214 patient tumors was obtained from a previously published study [229]. Briefly, for transcriptomic profiling, total RNA was converted to subsequent cDNA and hybridized to 50 base pair probes on HumanHT-12 v4.0 Expression BeadChips (Illumina) according to manufacturer's instruction. After performing washing and staining steps, the BeadChips were scanned using an iScan array scanner (Illumina).

Computational analyses

Pre-processing of the raw data

DNA methylation

For any computational study, pre-processing and clean-up of the raw data takes most of the time and effort. In study I, DNA methylation profiles from 214 patient tumors were pre-processed from the raw microarray image files (idat) using a custom pipeline comprising of the R packages ChAMP [232] and minfi [233]. Multiple quality control measures were used to select both probes and samples to keep and when small fraction of probes with intensity below the detection p-value cut-off were identified, they were imputed using K-nearest neighbor-based approach [234]. Missing values are often a major issue in the biological data, as a result of various technical and non-technical reasons. Thus, when imputing missing values, one must excise sufficient caution that there is no strong bias in the data regarding missing values. Also, the fraction of missing values being imputed should be noted, as large numbers of missing values being imputed could potentially bias the data in an unwarranted manner.

In addition to the missing value imputation, further caution was excised to avoid including probes that contain a single nucleotide polymorphism overlapping to the target CpG. For such CpGs, it is difficult to separate methylation from underlying genetic polymorphism. Similarly, cross-reactive probes are also avoided due to their association with spurious autosomal sex-associated methylation differences [235].

Another major issue concerning Illumina DNA methylation arrays is that they comprise of two different probe sets with different chemistry. Methylation β values coming from these probe sets show different dynamic ranges, a potential problem in further downstream analyses. Hence, it is imperative to adjust the dynamic ranges of both probe types. For this, we used BMIQ [236] that uses a three state beta mixture model to adjust the dynamic ranges of the probe sets to make them comparable.

Bulk RNA-sequencing, Microarray and NanoString based transcriptomics

In all four studies, bulk RNA-sequencing based transcriptomic data were used either as in-house cohorts and/or external datasets. RNA-sequencing based transcriptomic data from the TCGA cohorts (melanoma and 15 other solid tumor types) were downloaded from the pan-cancer atlas studies (https://gdc.cancer.gov/about-data/publications/pancanatlas). Same melanoma dataset from TCGA was used in study II, along with the additional bulk RNA-seq datasets from four ICB-treated melanoma tumor cohorts [166, 237-239]. For study III and IV, RNA-seq datasets for patient tumors and melanoma cell-lines respectively were processed using

TopHat2 [240] and Cufflinks [241] as previously described [242]. Gene-level expression was obtained by summing up FPKM values of the corresponding isoforms. For all bulk-RNA-seq datasets, transcript counts were quantile normalized followed by log-transformation.

In-house microarray based transcriptomic data was downloaded from a previous study [229]. In brief, the data was preprocessed using Illumina GenomeStudio software with the removal of outliers. Next, data normalization was performed using cubic-spline based quantile-normalization and subsequent log-transformation.

Both RNA-seq and microarray datasets were reduced to protein-coding genes only following normalization.

NanoString nCounter PanCancer Pathway Panel assay was used to profile mouse melanoma tumors in study IV. Panel contained 770 cancer and immune-system associated genes. Transcript counts were normalized using NanoString nSolver software and ERCC control probes. Post-normalization data was log-transformed and control probes were removed subsequently.

Single-cell RNA-sequencing based transcriptomics

Transcriptomics using Single-cell based RNA-sequencing (scRNA-seq) has made it possible to study molecular changes at the resolution of a single cell. However, this also came at a cost of additional technical challenges in terms of pre-processing of the raw data [243]. Technical issues for scRNA-seq range from low capture of the transcripts to cellular fusions comprising different cell-types. Noise in the data from such technical sources makes useful information retrieval difficult.

In study II, while analyzing publicly available scRNA-seq based transcriptomic datasets for the melanoma patient tumor cohorts, we experienced these challenges. Datasets were obtained as both TPM and raw transcript counts and were log-transformed following variance-stabilizing transformation using R package Seurat [244]. Noise reduction in the data was mitigated through filtering of the low-quality cells and transcripts along with adjustment for batch and other latent variables using regression-based models along with the variance-stabilizing transformation (VST). VST preserves the expression dynamics of the individual genes while normalizing them together which is important especially in context of scRNA-seq data due to the large number of drop-outs in the transcripts.

Furthermore, non-linear dimensionality reduction methods that have been demonstrated to be favorable for scRNA-seq data such as UMAP [245] were used.

Profiling of the somatic mutations

Processing, alignment and variant calling of the WES data has been done as described previously [246]. Read mapping was performed using Novoalign (Novocraft Technologies) and duplicates were marked using Picard tools (Broad

Institute). Local realignment and base quality checks were performed using GATK, and VarScan [247] and MuTect [248] were used to call mutations. DNA copy number generation and segmentation was done using CONTRA [249] and GLAD [250] respectively, for both WES and targeted sequencing data.

One of the major issues regarding pre-processing of the somatic mutations arise from the alterations with low variant allele frequency (VAF). Targeted gene panels alleviate such problems to a much larger extent by using ultra-high sequencing depth (often 30,000X), however in case of whole-exome sequencing caution must be excised to select sufficient coverage from the beginning. Additionally, the number of false positives tend to differ among mutation callers [251]. One way to mitigate the latter is to use more than one caller and then only select mutations with consensus among all callers, an approach we used in study III. In addition, in the WES studies we did not consider mutations with a VAF < 10% to avoid false positive calls.

Derivation of the HLA types were done using the bwakit or Omixon Target HLA (Omixon) and putative neoantigen prediction was performed using a custom pipeline with modified version of the pVAC-Seq method [252].

Exploratory *in silico* analyses

Not long ago, our resolution of studying an organism was limited to few genes or proteins at one go. Based on the recent developments in the field of molecular biology, we are now able to analyze thousands of genes, proteins and other molecules simultaneously. Such development has contributed to a great extent in vertical studies of the genome and has enabled us to understand functions of the molecules from a multi-omics perspective.

DNA methylation

In the study I, we had adopted an omics approach to explore immune cell-associated DNA methylation patterns in the metastatic CMM tumors. Immune cell-type associated methylation patterns were uncovered by comparing the methylation profiles of the reference immune cells, stromal cells and melanoma cell-lines to the tumors. Only CpGs with significant diversity in terms of their respective β values amongst the reference immune cells were selected and underwent further filtering against the stromal and malignant cells.

CMM tumors were then clustered across the identified immune cell-type associated CpGs using consensus clustering [253] from the R package ConsensusClusterPlus [254]. In addition to in-house data, publicly available melanoma DNA methylation data from TCGA [102] and Orozco et al. [255] were used in this study. Both datasets were processed in a similar manner as the in-house cohort and TCGA tumors were classified to the immune-methylation clusters identified in the in-house

data using methylation centroids. Methylation centroids were obtained as the median β value of each underlying CpG across clusters and only CpGs with significant methylation difference across clusters were chosen for this. TCGA tumors were then classified based on their correlation with methylation centroids in line of the common CpGs using Kendall-$\tau \geq 0.3$. Class assignment was based on the highest correlation and in case a sample does not meet the correlation cut-off, it was left unclassified.

Pan-cancer methylation datasets were processed identically to the melanoma datasets, except for that they were compared against only their respective malignant cell-lines obtained from the Genomics of Drug Sensitivity (GDSC) database (https://www.cancerrxgene.org/). Furthermore, methylation scores were created for all cohorts using median methylation of CpGs belonging to each immune cell-type.

Transcriptomics

In the study I, gene-expression scores corresponding to the methylation scores were created using matched underlying genes. Immune cell-type scores using scRNA-seq derived marker genes were calculated for both treatment-naïve and ICB-treated cohorts. MHC-antigen presentation scores were created using the associated genes for the study III.

For studies I-III, expression of the individual genes was median centered prior score calculation. Differential expression of genes (DEG) analyses were performed using significance of microarray (SAM) analysis [256] and gene-set enrichment analysis (GSEA) [257], followed by gene ontology (GO) term enrichment analysis using DAVID [258].

For the study II, immune cells were clustered based on their normalized expression using shared-nearest neighbor-based clustering on the UMAP projection. The rationale for doing this was the noisy nature of scRNA-seq data, where a bottom-up approach with reduced dimensional data proves to be advantageous in unraveling inner structures. DEG analyses for the immune marker selection were performed using the receiver operating characteristic (ROC) as a test method with the area under curve (AUC) cutoff of 0.7, in a one versus one manner.

Somatic mutations

Tumor mutational burden from the somatic mutation data was calculated as the total number of non-silent mutations per tumor and further normalized to per megabase (mb) of sequence for study I, II and IV. For study III, mutational burden was calculated as the overall somatic mutation burden per tumor. Individual driver mutations were visualized using Oncoprint function from the R package ComplexHeatmap [259].

Survival studies

Survival analyses for the patient tumor cohorts were performed using Kaplan-Meier plots and Cox regression models in both uni- and multi-variate settings. Distant-metastasis-free and progression-free survivals were used to asses association of molecular feature with disease progression, whereas disease-specific and overall survivals were used to understand similar association with the patient prognosis.

Immuno-histochemistry analyses

Immunohistochemistry (IHC) is a method for identifying the specific antigens through the antibody-antigen interaction in FFPE tissues. IHC techniques are popular among the pathologists, especially in the field of dermatological research. These methods along with the target antigens, also helps to visualize the surrounding tissue and its morphology under the light-microscope. Visualization is achieved through color signals arising from the antigen-antibody construct and the counter-staining of the surrounding tissue. The following steps are performed in the IHC protocols,

1. *Tissue processing and the retrieval of antigen* – FFPE tissues are cut into thin slices and mounted on glass slides before retrieving the antigens through either enzymatic digestion, heating or washing using buffered solutions.
2. *Antigen-antibody interaction* – This can be achieved in multiple ways, from the direct interaction through monospecific antibodies to the indirect detection involving multiple antibodies in a multi-step process.
3. *Visualization of the antigen-antibody conjugate* – Antibody molecules cannot be observed under the microscope unless they are labelled or tagged. The labelling and tagging can be done in multiple ways, either by active enzymes or more newer methods like secondary antibodies and enzymes linked to the polymer backbones.

Along with these steps, counter-staining is also used often to enhance the distinction between the target and the background using compounds like hematoxylin (blue) and eosin (red) [260, 261].

Tissue-microarray (TMA) is a high-throughput method that facilitates IHC analyses for a large number of samples. Since its introduction [262], it has become an important tool for the biomarker detection and diagnostic tests in the oncological settings.

For the construction of a TMA, first a map of the location of respective tumor cores within TMA is designed. Then using two hollow needles, tumor cores are placed on the recipient block of TMA according to the design. Post-construction, IHC stainings can be performed following sectioning of the TMA. Visualization of the stainings can be done under a light-microscope, however usually digital scanners are used for this purpose [263].

In studies I, II and IV we used TMA based IHC stainings for the immune cell markers CD3 and CD8 for the T cells, CD20 for B cells, CD68 and CD163 for the Macrophages, along with melanoma cell markers MITF and SOX10. Primary antibodies were used from Agilent/Dako except CD163 which was from Novocastra. Further information on the IHC procedure can be found in the online supplementary section of the study I.

Functional analyses

Functional studies are often performed to validate findings from the exploratory *in silico* analyses. In study IV, various functional studies were performed to validate proposed epigenetic regulation of *MITF* and *SOX10*. Additionally, *in vitro*, *in vivo* and *ex vivo* analyses were carried out to understand the nature of melanoma cells undergoing epigenetic regulation of these genes. A brief overview of some of these functional analyses are given here.

Identification and quantification of the cellular proteins

Western blot (WB) is performed to check the presence of specific proteins from tissue extracts. In study IV, it was applied to check the expression of DNA methyltransferase 1 (DNMT1) in melanoma cell-lines after treating them with demethylating agent Zebularine.

To perform WB assay, proteins from the cell-lines were extracted using a cocktail of lysis buffer, proteases and phosphatase inhibitors and were denatured prior blotting. Blotting procedure was performed using gel-electrophoresis and stained with antibodies for DNMT1 and β-actin (positive control). Visualization of the Protein bands was done by clarity western ECL substrate (Bio-Rad) in ChemiDoc MP (Bio-Rad).

Quantification of cellular proteins were performed using florescent dye-based staining using sulforhodamine B (SRB).

Global methylation assay

To assess the global DNA methylation in *MITF* promoter-hypermethylated cell-lines, dot-blot global methylation assay was used as described previously [264]. Briefly, the samples were denatured and then DNA was spot onto nitrocellulose membrane. Afterwards, the membrane was incubated subsequently with anti-5-mC monoclonal antibody and secondary antibody anti-mouse.

Cell migration and viability assay

Migration assay was performed to assess the migratory potential of melanoma cell-lines. For this, melanoma cells were starved for 15 hours and then were put in the upper compartment of transwell, whereas the lower compartment was filled with appropriate media for driving the migration. After 72 hours, stained transwell membranes were analyzed for migration under microscope.

xCELLigence real time cell analyses (RTCA) system (ACEA Biosciences) was used to monitor cell viability. Cells were seeded in microtiter plates containing microelectrodes and non-invasively monitored for viability using electrical impedance as the readout.

In vivo assay

NSG mice (10 for each SOX10 group) were injected subcutaneously with $MITF^{Met}SOX10^{Pos}$ or $MITF^{Met}SOX10^{Neg}$ cells in 1:1 ratio with matrigel (Corning). Dimensions of the primary tumors were measured at regular intervals and when the largest tumor volume reached $1cm^3$, all mice were sacrificed at once.

Ex vivo assay

$SOX10^{Pos}$ melanoma cells MM383 and $SOX10^{Neg}$ IGR-39 were cultured in appropriate media and then seeded in membrane inserts. Next, mouse brain slices were placed onto the membrane on the opposite side of the cells to observe the migration of cells towards the brain slices.

Results and Discussion

Characteristics of the immune-cell enrichment in melanoma tumors

Melanoma has long been considered as one of the most immunogenic tumor types [265]. Prognostic implications of the immune cells in melanoma tumors have been well-established [266, 267]. However, despite its immunogenicity, findings from the immunotherapy studies show that a large number of patients reported both primary and secondary immune resistance [268, 269]. Thus, it becomes necessary to explore the tumor immune microenvironment (TIME) of the melanoma tumors to explore possible factors attributing to a differential response upon immunotherapeutic treatments. Thus, in study I, we sought to explore the immune microenvironment of metastatic melanoma using DNA methylation as a tool for discovering the immune cell enrichment and its possible association with the patient survival.

DNA methylation as a major epigenetic mark has been shown to be often cellular-lineage dependent [270]. In the complex cellular assembly of the tumor-microenvironment it is often difficult to explore the enrichment of specific immune cell subsets. Therefore, we believed that harnessing the lineage specificity of the immune cell-type promoter CpG methylation, we will be enabled to characterize different immune cells present in the tumors. Indeed, we observed a gradient of immune cell-type associated methylation changes across the Lund metastatic melanoma (MM) cohort, which further revealed three clusters of varied immune-methylation levels. When we integrated the immune-methylation clusters with transcriptomic and immunohistochemical information, it further bolstered our hypothesis that these clusters reflect the diversity of the immune enrichment in these tumors. Upon validation with the TCGA MM cohort [102] and a cohort of melanoma brain metastases [255], it was noticed that combined tumor cohorts followed a characteristic separation in line of the immune-methylation cluster membership, rather than cohort or the tumor location.

Further expanding our analyses to TCGA pan-cancer solid tumor cohorts representing a diverse group of tumor types, it was observed that non-melanoma tumors often grouped together with MM tumors in line of the immune-methylation clusters. Such a finding was quite surprising considering the diverse biology of these

tumor types. Nonetheless, their spatial grouping with MM immune-methylation clusters further highlighted the possible existence of immune "*Hot*" and "*Cold*" tumors [271] across cancer spectrum with shared tumor microenvironmental characteristics.

Since the majority of the immune signatures including our immune-methylation patterns are based on the immune cells from normal tissue context, it is tempting to speculate whether phenotypic differences in the immune cells might be observed if they were extracted from the TME itself. Until recently, such exploration would have been quite difficult. However, thanks to a plethora of single-cell RNA-sequencing based studies for different tumor types including melanoma, investigating individual immune cells directly from the TME has become realizable. In our study II, we analyzed immune cell subsets from the tumor microenvironment of 33 MM tumors from a previous study by Jerby-Arnon et al. [272]. Immune cells were clustered into different lymphocyte (B, T and NK cell) subsets while the macrophages remained as a single group. When immune cell-type scores were created for these groups, reassuringly we observed clear enrichment of the scores in the target group compared to the non-target ones (including non-immune cells) both in the discovery cohort [272] and in the additional validation cohort from the previous study by Sade-Feldman et al. [163]. Next, we applied our single-cell RNA-sequencing derived immune scores to both treatment naïve and immune checkpoint-blockade (ICB) treated MM cohorts to explore their respective TIME using the bulk transcriptomic data. In the untreated datasets (Lund and TCGA MM cohorts), immune scores displayed distinct patterns of enrichment which was concordant with the previously identified melanoma transcriptomic phenotypes [102, 109]. Additionally, transcriptomic patterns were confirmed on the proteomic level using immunohistochemical information for the corresponding cell-types. Thus, we believed that our single-cell transcriptomics derived immune scores ably reflected upon the TIME of the untreated melanomas.

Further, most immune cell-type scores were found to be higher in the during-treatment patient biopsies compared to their before-treatment counterparts for responders, indicating that the ICB treatments result in an enrichment of immune cell subsets for the responding patients. In a similar fashion, we observed significant differences in the levels of immune scores between responders and non-responders for before-treatment samples. Overall, these results clearly indicated that lymphocytes share a close relationship with the response to ICB treatment.

In study III, our major aim was to establish relationship between the tumor genetic characteristics and response to the adoptive T-cell therapy (ACT). Up-regulation of the immune system associated genes along with the activation of IFN-γ signaling was observed in tumors for patients with clinical benefits. Patients without receiving clinical benefit similarly reported an upregulation of the cell cycle and proliferation associated genes in their tumors. Additionally, enrichment of some of the core

MHC-I antigen presentation genes was observed in the clinical benefit group, which was subsequently confirmed by associating MHC-I scores to the treatment response. Together, we observed that enrichment of the immune cells in TME displayed similar patterns of association for ACT as that of the other immunotherapy protocols.

It is interesting to note that we did not observe wide variation in terms of the enrichment of different immune cell subsets in the tumors. Our findings from studies I, II and III, have highlighted the fact that immune cells mostly occur in the tumors in a more "*all or none*" manner. While being quite contrary to our own expectations, this suggests that diverse immune cell subsets enrich the tumor together in the treatment naïve setting and might rely on each other for performing their respective duties. In this context, it is important to mention that we found the differences in the immune enrichment across tumors are largely driven by the cells of the lymphoid origin. Considering the active effector roles played by the lymphocytes in anti-tumor immunity [273], such observation is not very surprising. However, deeper inspection of roles of the myeloid cells is warranted, which are often considered to be harboring pro-tumorigenic properties [274].

Altogether, we found immune cell enrichment in the melanoma tumor microenvironments to be diverse, largely lymphocyte driven and in close relationship with the response to the immunotherapies.

Prognostic and predictive impacts of the immune cell enrichment in melanoma

The high immunogenicity of the melanoma tumors has for long sparked interest to study the prognostic impact of TIME in melanoma. It has been shown before, that tumor-infiltrating lymphocytes (TILs) and more specifically $CD8^+$ T cells are strongly associated with the patient survival and clinical benefit from immunotherapies [275-277]. More recently, roles of other lymphocytes such as B cells, have been established to be prognostic in the treatment naïve and predictive of the treatment benefit and survival in the immunotherapeutic treatment context for melanoma [171, 172]. In our studies I-III, we have comprehensively explored the prognostic implications of the immune enrichment in melanoma tumors. Our study I reported significant association of patient survival, both distant-metastasis free (DMFS) and disease-specific (DSS), with the immune-methylation clusters. Association of low immune-methylation (high immune-enrichment) with better prognosis for untreated patients were not limited to metastatic melanoma but also showed trends in the similar immunogenic primary tumor types such as Head and Neck and Lung carcinomas. Overall, we found immune-high melanoma tumors to

be associated with better prognosis and such a pattern was also identified in similar immunogenic tumor types across the cancer landscape.

Our study II concerning the single-cell RNA-sequencing derived immune scores not only offered a glimpse of the TIME in the bulk transcriptomic data from melanoma tumors, but also showed associations with the survival outcome in both treatment naïve and ICB-treated cohorts. Immune cell subset scores showed significant association with patient survival for most cell-types in the untreated cohorts (Lund and TCGA MM cohorts). Such patterns were identified also in the ICB treatment settings, as most cell-types displayed significant association with the overall survival for more than one cohort upon treated with the immunotherapeutic agents. Suspecting that smaller sample size might obscure true effects, we combined all four ICB-treated cohorts together to create a larger compendium of molecular and clinical data. We observed significant survival association for most immune scores in the combined cohort for both Progression-free (PFS) and Overall survival (OS), after adjusting for the cohort-related effects. Furthermore, we grouped samples based on their respective status of the individual lymphocyte scores (High vs Low) into three categories (Highly inflamed: All lymphocytes scores were high; Intermediate inflamed: Some lymphocyte scores were high; Low inflamed: None of the lymphocyte scores were high). Upon such grouping, a clear distinction in terms of both PFS and OS were observed in the combined cohort, highlighting the possible effectiveness of the immune enrichment. In summary, our immune cell-type scores derived from the TIME of the metastatic melanoma tumors, offered valuable prognostic and predictive information for the treatment naïve and ICB-treated melanoma cohorts, respectively.

Our study III reported close association between clinical benefit and high MHC-I antigen presentation in the tumors upon treated with ACT. Consequently, we derived MHC-I scores for these tumors and observed significant association with the OS but not PFS. However, considering small sample size of this cohort (n=25) such findings are needed to be interpreted with caution.

All in all, we observed that the immune enrichment in the melanoma tumor microenvironment associates closely with the patient survival and clinical benefit upon immunotherapeutic treatments.

Diversity in the immune-exclusion mechanisms in melanoma

Recently, immune-exclusion or immune-evasion of the tumors has become a major area of interest in the cancer immunology research. Multiple mechanisms have been proposed so far on how the malignant cells are able to avoid the invasion of immune

cells in the tumor microenvironment [179]. Despite high immunogenicity observed in melanomas, prevalence of immune-exclusion in a large fraction of tumors is a major area of concern for the immunotherapeutic treatments. Thus, in our study I we explored several well-known immune-evasion mechanisms in melanoma [278] with respect to our immune-methylation clusters. High immune-methylation cluster 3 was found to have the lowest immune enrichment of all three clusters. Therefore, we investigated underlying immune-exclusion mechanisms in the cluster 3 tumors. The differentiation state of the melanoma cells has been associated with treatment resistance to the targeted therapies [279, 280]. Considering this, we hypothesized that such differentiation status might play a role in the immune-evasion as well. To investigate this, we stained for MITF protein expression in these tumors as *MITF* apart from being the master regulator of melanocytic differentiation and melanoma development, have been suggested to be a major determinant of melanoma cell phenotype as well [126]. It was observed that these tumors have higher proportion of samples with low MITF protein expression compared to the low immune-methylation cluster 1. Due to the suggested differences in the transcriptional mechanisms along with the cellular states of $MITF^{+}$ and $MITF^{-}$ melanomas [126, 281], we decided to analyze them separately for possible immune-evasion. Earlier, through the pathway enrichment analysis an enrichment of tumors expressing high level of the oncogene c-MYC (*MYC*) was observed among the $MITF^{+}$ cluster 3 tumors. Subsequently, we found expression of *MYC* to be significantly different across clusters in only $MITF^{+}$ tumors. Similar characteristics were also noted for another important immune-evasion associated gene β-catenin (*CTNNB1*). *MYC* has been shown to be a major driver of the immune-exclusion across cancers [282]. Also, the role of β-catenin is well-established in the canonical WNT-signaling pathway, an important contributor of immune-evasion in cancers including melanoma [283, 284]. Together, importance of *MITF* expression on functionality of both *MYC* and *CTNNB1* [285, 286], makes it an important player in the immune-evasion of the melanomas.

Relationship of tumor mutational burden with immune enrichment and prognosis

Over the years, tumor mutational burden (TMB) has emerged to be an important factor in predicting response to immunotherapeutic treatments. However, the role of TMB as clinical benefit predictor has not been without controversies. Multiple challenges have emerged from selecting the cutoffs for high and low TMB to the mechanism of its association with the immunotherapies [287], thus contributing to the status of TMB as an enigma. In our study I, we did not observe any significant difference in levels of TMB (per mega base (MB)) across the immune-methylation clusters. Similarly, our study II did not reveal a major association between TMB

(per MB) and immune cell-type scores in terms of correlation. Also, we did not see a clear association for the high and low TMB groups with the patient survival in both treatment-naïve and ICB-treated cohorts. However, when TMB groups were joined together with the inflammation status, a difference in survival (both OS and PFS) was observed with Inflammation/TMB^{high} group reporting the best survival characteristics and vice versa, in the combined ICB-treated cohort. Such finding has led us to hypothesize that while TMB as stand-alone factor may not have a strong impact on the patient survival after immunotherapy, but still can display synergistic properties with the tumor inflammation status in predicting the survival outcome.

In the study III, we found TMB to be significantly associated with the clinical benefit in the ACT-treated cohort. When divided into tertile based groups, TMB showed significant association with the patient survival (both OS and PFS) upon ACT. However, as in the other two studies, no association was found between TMB and the immune enrichment of tumors, leading us to suggest TMB as an independent predictor of treatment response to ACT.

Unfortunately, our findings could not help to lift the veil of ambiguity from the role of TMB as an important contributor to the immunotherapeutic response. Nonetheless, these results further bolstered the hypothesis of independence between TMB and immune enrichment of the melanoma tumors.

Epigenetic regulation of the melanocytic lineage genes in melanoma cells

Melanoma cells have been hypothesized to alternate between an invasive and proliferative phenotype, where the former is considered to be intrinsically resistant to the targeted treatments such as BRAF-inhibitors [123]. *MITF*, which is considered as the master regulator of the melanocyte differentiation and melanoma development, has been shown to have decreased expression in the more invasive melanoma cell phenotype [126]. Earlier it has been shown that *MITF* expression is regulated by DNA promoter hyper-methylation [127]. In study IV, we sought to explore the impact of MITF promoter hyper-methylation on the melanoma cells and to this end we performed bisulfite Sanger sequencing of the selected CpGs as described before [127], for 65 melanoma cell-lines. Among these cell-lines 23% were found to be promoter-hypermethylated for *MITF* and consequently showed lower expression for the gene in matched RNA-sequencing data. Furthermore, to explore the underlying molecular characteristics of the MITF^{met} cell-lines, we clustered them (n=15) in line of the 1500 most varying genes using expression data. Clustering clearly delineated separation of these cell-lines into two distinct groups, chiefly in line of their SOX10 expression. Further characterization of the MITF^{met} SOX10 groups was attempted by whole-genome DNA methylation analysis using

Illumina Infinium MethylationEPIC microarrays. The $SOX10^{Neg}$ group was found to harbor higher levels of SOX10 promoter hyper-methylation along with an increased level of global DNA hyper-methylation compared to their $SOX10^{Pos}$ counterparts. Differences in the DNA methylation between SOX10 groups were further supplemented with distinct transcriptional programs underpinning these two subgroups. The $SOX10^{Pos}$ group showed up-regulation of the neuronal development associated genes whereas $SOX10^{Neg}$ group had higher enrichment for the epithelial-to-mesenchymal transformation (EMT) associated genes. In view of these findings, we hypothesize that the promoter hyper-methylation of the melanocytic lineage genes *MITF* and *SOX10*, plays an important role in determining the molecular characteristics of the underlying melanoma cells.

$MITF^{met}$ $SOX10^{Neg}$ group corresponds to an invasive treatment resistant phenotype

Our analyses of $MITF^{met}$ cell-lines indicated distinct subgrouping of these cell-lines primarily driven by SOX10 promoter hyper-methylation mediated expression changes. To characterize the phenotypes of $MITF^{met}$ SOX10 groups *in vitro*, we subjected them to proliferation, migration and colony formation assays. $SOX10^{Neg}$ cell-lines displayed significantly superior capabilities in all three assays compared to their $SOX10^{Pos}$ counterparts. Furthermore, upon treatment with the BRAF and MEK inhibitors $SOX10^{Neg}$ cell-lines reported significantly higher resistance compared to the $SOX10^{Pos}$ cell lines. We extended our analyses further *in vivo* with the injection of both $SOX10^{Pos}$ and $SOX10^{Neg}$ cells in the immunocompromised NSG mice and observed striking molecular and anatomical differences between the corresponding primary tumor groups. Additionally, $SOX10^{Neg}$ cells showed a preference towards brain as the metastatic site which was further corroborated in *ex vivo* analyses on brain slices from the mice belonging to both SOX10 groups.

Together, these results indicate the existence of a rare population of melanoma cells with promoter hyper-methylation of *MITF* and *SOX10* that are characteristically dedifferentiated with higher invasive and treatment resistance potential.

Conclusions and Future Perspectives

Molecular heterogeneity of the melanoma tumors comes from the malignant cells as well as the tumor microenvironment. Studies have shown that the cancer development and progression is a resultant of the complex interactions between the malignant and non-malignant cells that enrich tumors. In context of melanoma, immune cells in the TME play important roles in both elimination and survival of the tumor. However, true nature of many of these immune cell-types eludes us and our study I helped to shed some light on the putative presence of some of these cells along with the likely prognostic implications of such presence. Additionally, our analyses on the pan-cancer immune-methylation patterns have provided strong indications for the existence of similar immune microenvironments across tumors with diverse tissue of origin. Implications of these findings could potentially motivate future immunotherapeutic applications for a broader spectrum of immune cell-types along with targeting of immunomodulatory microenvironmental factors across multiple cancers. However, further studies on more detailed functional roles of such cell-types would be required, especially if possible, using scRNA-seq based explorations.

Identification of the immune cell-types in the TME poses significant challenge largely attributed to the cross-expression of the marker genes across cell-types from the similar cellular lineage. Complexity of the TME composition along with possible alterations of the cellular phenotype due to interactions add further problems. Nevertheless, scRNA-seq technology offers us a unique opportunity to examine each individual cell inside the TME. Our study II showed that harnessing the potential of scRNA-seq could benefit us in discovering more robust immune cell markers that are able to predict the enrichment of the corresponding cell-types in the tumor microenvironment. In future, scRNA-seq studies would likely discover additional novel cell phenotypes both in spatial and in temporal manner that could help us to answer important questions regarding tumor plasticity and treatment resistance.

The role of tumor mutational burden as a biomarker for the treatment response is increasingly coming under the scanner as studies continue to probe the underlying mechanism of tumor mutations in modulating anti-tumor immunity. Our studies have reflected on the predictive biomarker role of tumor mutation and putative neoantigen burden in immunotherapies. However, it did not reveal a clear association between TMB and immune enrichment of the tumors. Future studies on

exploring interactions between TMB, its associated antigens and immune response would be required to clear the water on this controversial subject.

Melanoma cell plasticity is an ongoing matter of debate as multiple hypotheses have emerged in recent years on how cells could alter their phenotypes especially during treatment. Our findings concerning two major melanoma associated genes, *MITF* and *SOX10* hint at the likely role of epigenetic modifications in modulating their expression pattern and concerned cell phenotype at large. Such epigenetic modulation could potentially be a part of a larger global reprogramming aimed at maintaining the invasive potential and survival capability of the melanoma cells. Further studies on likely epigenetic reprogramming of the melanoma cells needs to be conducted if possible, in a temporal manner to identify true causes and modulators of this plasticity.

Popular summary

Cancer is one of the oldest known diseases to humanity as the earliest description of cancer goes back to as late as 1500 BCE. However, over the millennia it has continued to baffle researchers with its huge diversity and complexity. In fact, it would be wise to consider cancers as a group of diseases like a vast archipelago with many interconnected islands. Malignant melanoma represents one of these islands belonging to a family of diverse skin cancers. Melanomas are primarily skin cancers arising from a group of pigment producing cells known as melanocytes although they can occur in some other organs such as eye and digestive tract as well. However, what sets melanomas apart from the rest of skin malignancies, is its superior ability to spread to other parts of the body and often resulting in fatalities.

Ultraviolet radiation (UVR) exposure is one of the major risk factors for developing skin melanoma along with other genetic factors and personal attributes such as complexion, age, gender etc. UVR from the sunlight causes damages to the DNA of melanocytes and eventually drive them towards malignancy. Artificial tanning in presence of the UVR also shows similar effect. Thus, simple lifestyle changes such as avoiding sunburn might greatly influence the number of annual melanoma cases in the western hemisphere and especially for the Caucasian population.

A melanoma tumor is a complex structure involving many different cell-types that include both cancerous and non-cancerous cells. Among these non-cancerous cells, cells from the body's immune system plays an important role in the growth and elimination of the tumor. Over the years, researchers have taken keen interest in understanding the roles different immune cells play inside the tumor, either opposing or helping it to grow. Our studies especially study I and II focused on understanding the roles of different immune cells that enrich melanoma tumors and how they influence the prognosis of the patients. We found that lymphocytes especially T and B lymphocytes associate positively with the patient survival, both without and in the context immunotherapeutic treatments. Additionally, in study III we observed that the presence of more mutations (changes in the DNA bases) in the tumor usually led to treatment benefit and prolonged survival, when the patients underwent a special kind of immunotherapy involving their own T lymphocytes. These results we believe will help to select patients in future who might perform well with the immunotherapies.

Alongside immunotherapies, treatments targeting melanoma driver mutations such as BRAF-inhibitor has shown much promise with initial tumor reduction. But over the time, melanoma tumors turn resistant to these inhibitor drugs. One possible explanation for such a phenomenon could be that upon treatment, loss of expression of the major melanoma associated genes such as *MITF* and *SOX10* occur. Loss of expression of these important genes likely make the melanoma cells resistant. In study IV, we explored the possible roles of epigenetic changes (changes in the genome without involving DNA bases) in regulating the expression of *MITF* and *SOX10*. Our results indicated important roles for epigenetic changes not only in overseeing the expression changes of these genes but also influencing the very nature of the corresponding melanoma cells. We believe further understanding of the roles of epigenetic modifications is necessary to discover novel ways to overcome the treatment resistance to the targeted therapies in melanoma.

This thesis is a culmination of the pre-clinical exploratory researches undertaken during my PhD study. Nevertheless, following up the findings of this thesis with more detailed functional studies will benefit future clinical approaches, in my humble opinion.

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