Conjunctival Melanoma from an Epigenetic Perspective

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Abstract

This article is an examination of literature regarding the epigenetic understanding of conjunctival melanoma (CjM), with a special emphasis on current knowledge gaps and future research directions. CjM is a rare but highly aggressive cancer that mainly affects the older population. With the expected increase in affected individuals in the Western world due to aging population, CjM has public health relevance. Local recurrences and distant metastases are common in CjM patients, but their prediction and management remain challenging. There is therefore an urgent need for new clinically useful biomarkers and more effective treatments to improve survival rates in these patients. Based on the articles collected for this review, there is an evident understanding of the pathology, genetic mechanisms, and environmental risks surrounding CjM. Like other cancers, CjM development and progression are believed to be driven by multiple genetic and epigenetic factors that contribute to tumorigenesis/spread, impaired immune response, and primary/acquired resistance to therapies. While the cancer field has recently witnessed a rapid increase in available epigenetic technologies and treatments, which have enabled the development of various epi-drugs with promising results in combinatorial therapies, the epigenetic understanding of CjM remains largely incomplete due to a small number of related studies published to date. These epigenetic studies primarily focused on DNA methylation, non-coding RNA (miRNA or circRNA) expression, or RNA modification/methylation. While they have revealed some potential biomarker or therapeutic candidates, these initial studies suffered from

some limitations and their results warrant replication in larger studies/samples. Therefore, an *in-depth understanding of CjM epigenetics*, which is currently a critical unmet need, remains essential for improving the clinical management and outcomes of this deadly disease, and overall, the public health.

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1.0 Introduction & Aims

The purpose of this essay is to provide an overview of the molecular (genetic and epigenetic) mechanisms in conjunctival melanoma (CjM), with a special emphasis on current knowledge gaps in CjM-related epigenetic mechanisms and future research directions and opportunities. The aims of this review are as follows:

- Define CjM and explain why a better understanding of its development and progression may impact the prevention and management of the disease, and overall, the public health
- Review the current knowledge on the genetics and epigenetics of CjM and identify the knowledge gaps in its epigenetic understanding
- Outline specific avenues for future research to fill the knowledge gaps in epigenetic understanding of CjM while explaining how an advanced knowledge can provide future opportunities to improve clinical outcomes.

2.0 Disease Background

Conjunctival melanoma (CjM) is a rare cancerous growth on the surface of the eye. CjM arises from melanocytes located in the basal layer of the conjunctival epithelium.[1] Conjunctiva is a clear vascularized mucous membrane that lines the inner surface of eyelids (*palpebral conjunctiva*) and folds back (*conjunctival fornix*) to cover the front surface of the eye globe (*bulbar conjunctiva*) (Figure 1). It produces mucus and tears to keep the eye surface lubricated and functions as a protective barrier against foreign bodies and pathogens .[2]

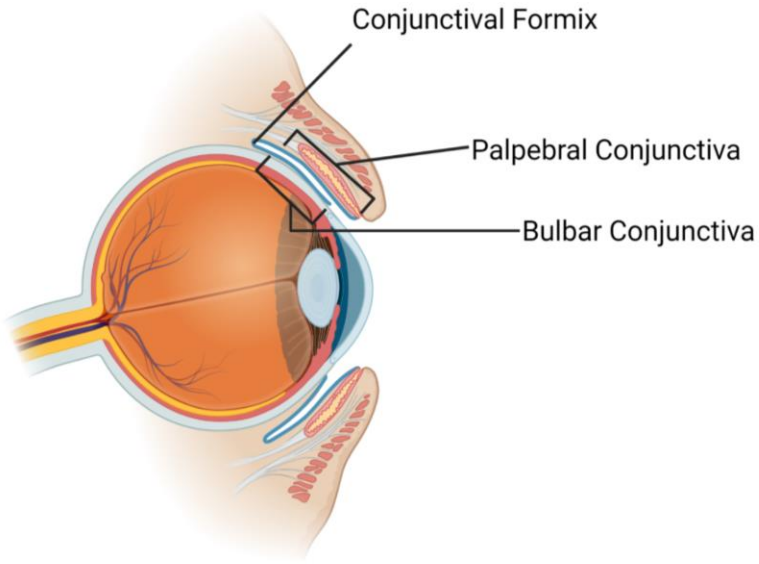

Figure 1 Conjunctiva Sections of the Eye

The conjunctiva sections are the distinct blue lines surrounding the anterior ocular surface, the point of the eyelid contacting the ocular surface, and inner eyelid surface (*Created with BioRender.com*).

CjM most commonly occurs in the bulbar conjunctiva, which is directly exposed to the sunlight. The bulbar conjunctiva covers the front part of the sclera, which is the opaque and rigid tissue that forms the white of the eye .[2] CjM cases account for about 5% of all ocular melanoma cases and about 0.25% of all melanoma cases.[1,3,4]

2.1 Epidemiology & Environmental Risk Factors

The patients predominantly affected by this disease are European-descent adults and >50 years of age.[5] While some studies reported more common occurrence in men, others did not find significant sex-related differences.[5-10] CjM is overall a rare condition, with annual incidence rate estimates ranging from 0.24 to 0.9 cases per million persons per year in Western countries.[6-8] While earlier studies (using the data before 2008) suggested a rising incidence of CjM, more recent studies do not suggest the continuation of that trend, although geographical differences may exist.[6-10]

Changes in the reported incidence trends may be related to various factors. One possibility is the effect of a change in generational shift in global population.[1,5,11] Post WWII was the baby boomer generation with a large influx of children in Western countries from 1945-1960. Those children were raised in a world of medical advances and public health improvement that has allowed them to live longer than any other generation before. The baby boomer generation is now in their 60s and 70s, when age-related conditions are more likely to develop. The aging population is increasing; therefore, it is only logical to anticipate a rise in number of individuals affected by late-onset diseases. On the other hand, with the improved health care/screening and increased awareness, more individuals can be diagnosed with high-risk precursor lesions that can be treated

before developing into melanoma. Another possible explanation is changes in ultraviolet (UV) light exposure given that a significant amount of evidence supports the role of UV light as a mutagenic risk factor for CjM, especially in sun-exposed areas similar to skin melanoma.[12,13] CjM is a biologically distinct form of melanoma that bears certain similarities to both sun-exposed skin melanomas and other (non-sun-exposed) mucosal melanomas, as supported by the genetic changes that overlap with those seen in either (or both) of these two major melanoma types.[12]

2.2 Precursors

There are two known precursor conditions, nevi and primary-acquired melanosis (PAM) with atypia, which can lead to the development of CjM. PAM with atypia is responsible for up to 75% of CjM cases.[1,11] PAM is clinically defined as an acquired, flat, noncystic pigmentation of conjunctiva.[1,5] While PAM without atypia is benign in nature, PAM with atypia is a serious melanocytic lesion that carries about 13% risk of transformation into CjM.[1,5] To quantify the severity of the PAM lesion, four categories were developed: "mild atypia, moderate atypia, severe atypia, and melanoma in situ".[1] Mild and moderate atypia are less likely to develop into CjM, but they still pose a risk, whereas severe atypia presents the highest risk of CjM development.[1,14]

The other approximately 25% of CjMs arise from conjunctival nevi or *de novo*.[11] Conjunctival nevi are common, colored growths on the eye, like a freckle. The growths are usually disregarded due to their benign state but increase the risk of developing CjM, albeit rarely.[5] Nevi that are present in childhood and show growth into adulthood present suspicion for malignant transformation.[14]

De novo cases develop in the absence of any precursors and account for about 20% of all CjM cases.[11] These cases are usually associated with less favorable outcomes.[5,14]

2.3 Diagnosis & Prognosis

CjM is usually identified when patients receive an eye examination after the growth is recognized by patients or their eye doctors.[14] While CjMs often arise as pigmented lesions, the pigmentation level can vary among the tumors and amelanotic lesions can also be occasionally observed.[14] Initial diagnosis of CjM is made by clinical examination and determined through an excisional biopsy and histopathological examination.[15] The significance of CjM is that it is very prone to local spread and distant metastasis through mainly the lymphatic but also vascular system.[1] Currently, 10 years after the CjM diagnosis, the mortality rate is around 30% and local recurrence rate is about 50%.[11,16] These mortality and recurrence rates suggest that CjM is an aggressive cancer and identifying the risk factors and better understanding its development and progression can help to increase survival rates and decrease recurrence and metastasis rates.

The American Joint committee on Cancer Classification (AJCC) regularly releases clinical and pathologic cancer-staging manuals that assess the extent of malignancy to guide the prognosis prediction in patients with cancers including CjM.[14,17,18] The clinical staging for CjM follows the TNM system where the T category is based on tumor size and location ("bulbar [T1]; nonbulbar [T2]; local invasion into globe, eyelid, orbit, sinus [T3]; and invasion into brain [T4]"), and the N and M categories identify regional lymph node metastasis (N1) and distant metastasis (M1), respectively.[14,17,18] (Appendix Table 1) The pathological stages outlined by the AJCC are based upon tumor thickness and depth of cell invasion.[14] The clinical studies have consistently linked

the advanced AJCC T-subcategories with metastasis and mortality risk in CjM.[14,18-20] The AJCC T-staging can also be used as a predictor for local tumor control/recurrence after the CjM treatment.[17,20] While the clinicopathological features of CjM can be helpful for prognostic assessment of patients, a better understanding of tumor development and progression can be achieved by molecular studies of CjM, including those on underlying genetic and epigenetic mechanisms that may inform the prevention and better management strategies for this aggressive cancer.

2.4 Current Treatment & Management Strategies

The rarity of this condition has proved a challenge to conduct the clinical trials. To receive the appropriate treatment, the appropriate diagnosis needs to be made. Clinicians can sometimes face diagnostic challenges related to technical reasons (e.g., limited biopsy material for histopathology), atypical histopathologic findings, or unusual clinical presentations (e.g., younger age or uncommon tumor location).[14]

The current standard of care is local excision, proceeded by various forms of adjuvant therapy.[15] Focal cryotherapy is one form of adjuvant therapy that is done at the time of excision to the surgical margins to reduce risk for recurrence and metastatic spread.[15] Other adjuvant therapy options include topical chemotherapy and brachytherapy.[14,15] Advanced cases may require an extensive and disfiguring surgical procedure called orbital exenteration.[14,15]

Management therapies available are crucial to CjM patients because of the high recurrence rate and risk for distal metastasis as discussed earlier. Risk factors associated with recurrence are the size of primary tumor, location of tumor on the eye, and perceived success of surgical

removal.[15] The AJCC clinical staging (Appendix Table 1) was predictive of local recurrence as twice the risk in eyes with T3 tumors compared to eyes with T1 tumors.[17] Patients who only receive local excision more frequently experience local recurrence and distant metastasis.[21] Patients who experience recurrence in turn experience higher mortality rates than non-recurrent cases.[21] Regardless of the adjuvant therapies following excisional biopsies, there still is a high rate of distant metastasis.[21]

The management of CjM is burdensome on the patient, but our molecular understanding of the disease has greatly increased in recent years and opened the door to new systemic therapies such as targeted therapies driven by specific mutations (e.g., BRAF/MEK inhibitors for tumors with *BRAF* mutations) and immunotherapies. Ocular oncologists have recently started investigating these new treatments in CjM patients.[1,5,22,23] For systemic immunotherapy, clinical researchers have been specifically focusing on immune checkpoint inhibitors (ICIs).[23] ICIs that are currently approved by the FDA include nivolumab and pembrolizumab (anti-PD-1), avelumab and atezolizumab (anti-PDL-1), and ipilimumab (anti-CTLA-4 inhibitors).[23] The expectation of further immunotherapy research for patients with CjM is to avoid recurrence after eradication, orbital exenteration, and ultimately mortality.[23]

3.0 Genetic Background

3.1 Pathogenesis & Major Pathways/Gene Groups Affected by Genetic Alterations

CjM occurs sporadically, caused by somatic genetic alterations, and it is not linked to any cancer predisposing syndrome to date.[5] The pathogenesis of CjM is closely related to two major molecular pathways: the *Mitogen-Activated Protein Kinase (MAPK) pathway (a.k.a. RAS-RAF-MEK-ERK pathway) and the phosphatidylinositol 3-kinase (P13K)-AKT-mTOR pathway*. These pathways are responsible for transmitting the growth signals to the nucleus and altering the expression of multiple genes involved in cellular differentiation, proliferation, and survival.[1,11] Similar to skin and mucosal melanomas, CjM is characterized by the overactivity of the MAPK and/or P13K-AKT-mTOR pathway(s),[1,11,24] and the mutations in genes relating to these pathways present a strong association with CjM development. Cancer-associated mutations are known to affect two major groups of genes: *proto-oncogenes* and *tumor suppressor genes*. Proto-oncogenes have the potential to cause cancer if/when subjected to the activating mutations transforming them into the oncogenes, which promote cell survival and proliferation. Tumor suppressor genes, on the other hand, act like anti-oncogenes by preventing uncontrollable cell division and growth, and thus the inactivating mutations leading to their reduced or loss of function contribute to cancer development.[1,11] The well-known MAPK and/or P13K-AKT-mTOR pathways-related proto-oncogenes involved in CjM pathogenesis are *BRAF*, *NRAS*, and *KIT*, whereas the tumor suppressor genes are *NF1* and *PTEN*. In addition to these signaling pathways-related genes, the *telomere maintenance*-related genes, *TERT* and *ATRX*, are believed to play important roles in CjM

pathogenesis.[22,25-29] Telomere maintenance mechanisms are commonly used by cancer cells to achieve immortality by preventing progressive telomere shortening in successive cell divisions.

3.2 Genetic Mechanisms

Genetic alterations observed in CjMs include somatic mutations and structural variations. The UV mutational signature is also frequently observed in these tumors, especially if/when subjected to UV light-induced DNA damage due to chronic sun exposure.

3.2.1 Somatic Mutations

3.2.1.1 The MAPK & P13K-AKT-mTOR Pathways-Related Genes

The MAPK (RAS-RAF-MEK-ERK) and P13K-AKT-mTOR pathways are complex signaling cascades responsible for the transduction of growth signals from the outer surface of the cell to the DNA within the nucleus.[30] (Figure 2) The signal begins outside the cell when a ligand molecule binds with a receptor tyrosine kinase (RTK), turning it 'on', and initiating the sequential activation of the proteins within those cascades. When a protein along these cascades becomes mutated, it can cause the signal to remain on, causing the uncontrollable cell survival and division, therefore leading to malignancies.[1,11] Due to the interconnectivity between the MAPK (RAS-RAF-MEK-ERK) and P13K-AKT-mTOR cascades/pathways, the mutations affecting the members or regulators of these pathways can over-activate either one or both cascades (Figure 2).

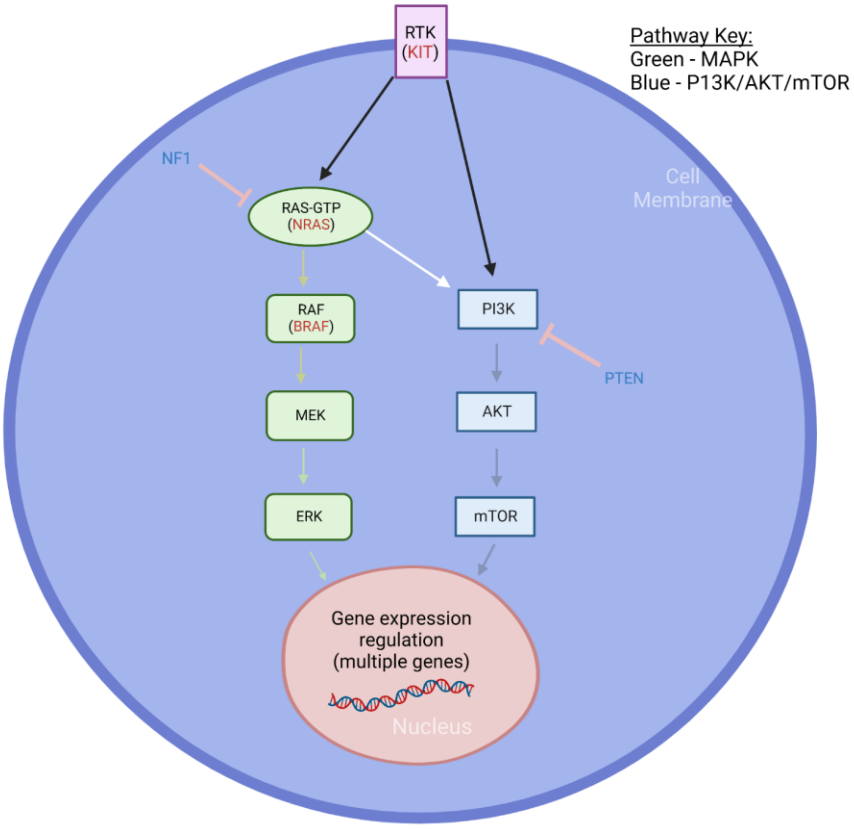

Figure 2 MAPK and PI3K-AKT-mTOR Pathways

The members (NRAS, BRAF) and regulators (KIT, NF1, PTEN) that are commonly affected in CjM are shown (proto-oncogene products are marked in red whereas tumor suppressor proteins are marked in blue) (*Created with BioRender.com using information from previous publications).* [1,5,11]

3.2.1.1.1 V-Raf Murine Sarcoma Viral Oncogene Homologue B1 (BRAF)

Located on chromosome 7, the BRAF gene encodes a RAF protein that plays a role in regulating cell growth, differentiation, proliferation, senescence, and apoptosis.[30] Activating *BRAF* mutations are detected in about a third of CjM cases,[5] resulting in overactivation of the MAPK pathway (Figure 2). Of those tumors with *BRAF* mutations, 80-90% harbor specifically the V600E

mutation, where Valine is substituted with Glutamic Acid at amino acid 600.[1,5,11] The second most common *BRAF* mutation is V600K, where Valine is substituted with Lysine at amino acid 600. The *BRAF* mutations associated with CjM are consistent with those found in skin melanoma.[1,5,11] *BRAF* mutations are more commonly detected in CjMs developing in sun-exposed conjunctiva, suggesting a potential role of UV exposure as similarly observed in skin melanomas.[12,31] Activating *BRAF* mutations are also commonly found in conjunctival nevi, thus they may represent early events during CjM development.[1,5,11] There have not been any significant associations reported between *BRAF* mutations and tumor recurrence and metastasis rates or mortality in CjM cases. However, identification of *BRAF* mutations in CjM appears to have relevance to therapeutic decision-making, given the increasing number of reports supporting the benefit of targeted therapy with BRAF/MEK inhibitors in patients with advanced CjM carrying *BRAF* mutations.[1,5,22]

3.2.1.1.2 V-Ras Neuroblastoma RAS Viral Oncogene Homolog (NRAS)

Encoded by a gene located on chromosome 1, NRAS is a GTPase protein that belongs to the RAS family of proteins, which are capable of activating both the MAPK and PI3K-AKT-mTOR pathways through the downstream activation of the RAF and PI3K proteins.[1] (Figure 2) About 20% of CjM cases can harbor activating *NRAS* mutations (most commonly affecting codon 61), which are also similarly found in skin melanomas.[1,5,11] In most cases, *NRAS* mutations occur mutually exclusively with *BRAF* mutations. Similar to *BRAF* mutations, *NRAS* mutations are also frequently found in conjunctival nevi.[32] Unlike *BRAF* mutations though, *NRAS* mutations appear to have prognostic relevance in CjM as they are reported to be associated with a less favorable prognosis.[27]

3.2.1.1.3 V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog (KIT)

Located on chromosome 4, *KIT* (a.k.a., *c-KIT*) encodes an RTK that operates in cell signal transduction,[1,11] and it can activate both the MAPK and PI3K-AKT-mTOR pathways after binding of its ligand (Figure 2). Activating *KIT* mutations occur less frequently in CjMs, primarily in those developing in non-sun-exposed areas as similarly observed in other mucosal melanomas.[12,27,33] *KIT* mutations are mutually exclusive with *BRAF* and *NRAS* mutations and are more frequently observed in geographical locations where the *BRAF* mutations are less common, such as in East Asia.[34]

3.2.1.1.4 Neurofibromin 1 (NF1)

Encoded by a gene located on chromosome 17, NF1 inhibits RAS through the hydrolysis of RAS-bound guanosine triphosphate (GTP).[1] Therefore, NF1 is a tumor suppressor protein that negatively regulates both the MAPK and PI3K-AKT-mTOR pathways, and inactivating *NF1* mutations are associated with overactivation of both pathways (Figure 2). *NF1* mutations are linked to about 30% of CjM cases, and they can co-occur with *BRAF, NRAS, or KIT* mutations.[27,35]

3.2.1.1.5 Phosphatase & Tensin Homolog (PTEN)

Located on chromosome 10, *PTEN* encodes a tumor suppressor protein that negatively regulates the PI3K-AKT-mTOR pathway through its inhibitory effect on PI3K.[11] Therefore, inactivating *PTEN* mutations are associated with overactivation of the PI3K-AKT-mTOR pathway (Figure 2). While the PTEN loss is commonly caused by copy number alterations (see section 3.2.2), recent studies have also found the *PTEN* mutations to uncommonly occur in CjMs.[12,27,29] *PTEN* mutations can co-occur with *BRAF* or *KIT* mutations.[5]

3.2.1.2 Telomere Maintenance-Related Genes

3.2.1.2.1 Telomerase Reverse Transcriptase (TERT)

Encoded by a gene located on chromosome 5, TERT is a catalytic subunit of telomerase, which prevents degradation of chromosomal ends with increasing cell division rate, therefore creating cell immortality.[11] Similar to skin melanomas, *TERT* promoter mutations that drive telomerase expression are commonly observed in CjMs (in >30% of tumors) [25,26,28,29] and are also linked to the UV signature (see section 3.2.1.3). *TERT* promoter mutations appear to have a prognostic value as they are found to be associated with shorter metastasis-free survival rates in recent studies.[25,26,28,29]

3.2.1.2.2 ATRX Chromatin Remodeler

Located on chromosome X, *ATRX* encodes a chromatin remodeler involved in alternative telomere lengthening (ALT), a mechanism commonly used by cancer cells.[36] Recent studies have shown that inactivating *ATRX* mutations are also commonly present in CjMs (in >20% of tumors).[22,27] Initial observations suggest that *ATRX* mutations more commonly occur in CjMs developing in non-sun-exposed areas, and they also appear to be associated with a more favorable prognosis.[27]

3.2.1.3 UV Signature & Tumor Mutational Burden

Skin melanoma has been confidently linked to UV exposure being a risk factor for the tumor development. As previously stated, skin melanoma and CjM share some characteristics,[11] including the role of UV exposure in tumor development on sun-exposed surfaces.[1] A UV mutational signature is characterized by the abundance of C > T and CC > TT substitutions in

tumor mutation profile, and UV light-induced DNA damage usually leads to high tumor mutational burden (TMB).[37] CjMs developing in sun-exposed areas often exhibit a UV signature, and when also associated with high TMB, they represent good targets for immunotherapy.[22,27,38-40]

3.2.2 Structural Variations

Similar to skin and mucosal melanomas, CjMs often harbor structural variations, including the chromosomal aberrations and copy number alterations (CNAs).[41] Both the genome-wide changes (e.g., polyploidy or aneuploidy) and several different gains or losses affecting whole chromosomes or their arms have been described in CjMs.[22,38,42-44] While recurring CNAs are found in all tumor groups, some are more commonly observed in specific groups, such as the 10q deletions in *BRAF*-mutated tumors that result in loss of various tumor suppressor genes (some including *PTEN*).[42,43] Several smaller scale alterations have also been detected, including those affecting some of the CM-relevant genes discussed above (in section 3.2.1), such as the amplification of *KIT* and the deletion of *NF1*.[22,38,42-44]

4.0 Epigenetic Background

4.1 Epigenetic Mechanisms

Epigenetic mechanisms include molecular processes that regulate gene expression without altering the underlying DNA sequences. Epigenetic regulation can occur at the transcriptional or post-transcriptional level, through changes in the *chromatin conformation/accessibility, post-translational histone modifications or the use of variant histones, changes in DNA methylation, the effects of long or short non-coding RNAs, or RNA modifications*.[45-49] Various synergistic or antagonistic interactions can also occur among different epigenetic mechanisms. Physiologically, these mechanisms are necessary for healthy development and maintenance of tissue-specific gene expression. In addition to the regulation of gene expression, epigenetic mechanisms can also influence DNA repair and replication.[45-49] Accumulating evidence support that epigenetic alterations play a major role in cancer development/progression, tumor-immune system interactions, and response/resistance to cancer treatments.[45-51] Because they are usually dynamic and reversible, epigenetic modifications are attractive targets for new therapeutic options.

4.1.1 Chromatin Conformation & Accessibility

Genome activity is influenced by the conformation and structure of the chromatin.[46] Chromatin accessibility (closed vs. open chromatin) is regulated by ATP-dependent remodeling complexes that can mediate nucleosome positioning or variant histone swapping within the nucleosomes, the proteins that can recognize/bind modified histones or methylated DNA, and the

enzymes that can modify histones or methylate DNA.[48,52,53] All these chromatin modifiers play a key role in epigenetic regulation at the transcriptional level (Figure 3).

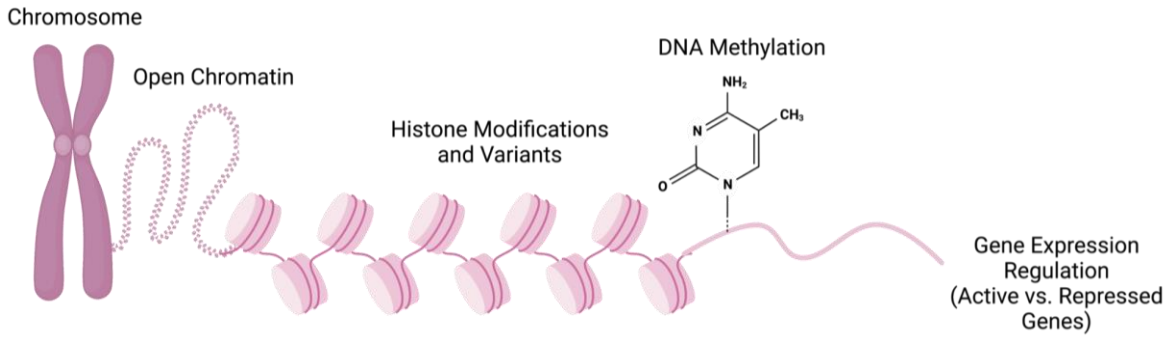

Figure 3 Epigenetic Mechanisms (at the Transcriptional Level)

(Created with BioRender.com)

4.1.2 Histone Modifications & Variants

Nucleosomes represent the basic/repeating units of the chromatin. Each nucleosome is comprised of eight histone proteins (two copies of four core histones) and ~150 base pairs of the DNA double-wrapped around them.[48,52,53] Histone modifications can influence chromatin accessibility by increasing or decreasing the contact between the nucleosomes and affecting their stability. These post-translational modifications (acetylation, methylation, as well as other alterations) predominantly occur at the N-terminal tail of the histones (at specific amino acidic residues) and can lead to either activation or repression of gene transcription.[45,46,48,52,53] Major enzyme groups responsible for dynamic regulation of these modifications include "histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs),

and histone demethylases (HDMs)".[45,46,48,52,53] Altered expression/function of these enzymes are often seen in cancers. Histone variants, which differ in protein sequences, can replace the core histones in specific regions of the genome and contribute to genome integrity.[54]

4.1.3 DNA Methylation

DNA methylation typically involves the modification of cytosine (C) of Cytosine-phosphate-Guanine (CpG) dinucleotides by addition of a methyl group to the 5-carbon position in the pyrimidine ring [5-methylcytosine (5-mC)].[47,55] DNA methylation leads to gene expression inhibition by attracting specific methyl-CpG-binding proteins for chromatin reconfiguration, and by preventing the transcription factors and other regulatory molecules from accessing/binding the DNA.[48] Thus, "DNA methyltransferases (DNMTs) and methyl-binding domain proteins (MBDs)" play a major role in transcriptional repression. CpG islands are found throughout the genome, and in about 60-70% of gene promoters, of which the methylation state determines the gene activity status.[47,55] DNA methylation represents a stable epigenetic mark causing long-term gene silencing and playing an important role in embryonic development, imprinting, X chromosome inactivation, tissue-specific gene regulation, and transposable elements silencing.[48] DNA demethylation can occur either passively (due to the failure of DNMT1 to maintain methylation through DNA replication) or actively (by removal of the methyl group from 5-mC through a chain of reactions catalyzed by TET proteins).[49,53] An intermediary molecule generated during this chain of reactions is 5-hydroxymethylcytosine (5-hmC), loss of which is implicated in cancer biology.[49,53] Alterations in DNA methylation states are frequently observed in cancers including selective promoter hypermethylation leading to tumor suppressor gene inactivation and hypomethylation leading to chromosomal instability, proto-oncogene activation, and drug resistance.[47,55]

4.1.4 Non-coding RNAs

Non-coding RNAs (ncRNAs) have mainly regulatory effects and are divided into two major groups: "(i) small ncRNAs (sncRNAs) [microRNAs (miRNAs), short interfering RNAs (siRNAs), and P-element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs)] and (ii) long ncRNAs (lncRNAs)".[53,55]

Among the sncRNAs, the miRNAs (~18–25 nucleotides in length) are the most studied. MiRNAs can downregulate gene expression post-transcriptionally via degradation of their target mRNAs or their translational repression. They can target different mRNAs by binding to their 3' untranslated region (UTR) and can function as onco-suppressors or oncomiRs when involved in cancer pathophysiology.[45] MiRNA-mediated repression of tumor suppressor transcripts or lack of miRNA-mediated degradation of oncogenic transcripts can play an active role in almost all cancers.

LncRNAs (>200 nucleotides in length) represent a large group of transcripts that can interact with various types of molecules to *cis*-/*trans*- regulate the gene expression both at the transcriptional and post-transcriptional level.[45,47] LncRNAs can contribute to miRNA biogenesis or function as miRNA sponges.[46,47] As part of their involvement in cancer, lncRNAs can act as oncogenes or tumor suppressors depending on their functions and interactions. Circular RNAs (circRNAs) are covalently closed lncRNAs (5' and 3' ends are covalently linked by back-splicing), and similar to linear lncRNAs, they are involved in multiple biological processes but are relatively more stable due to their structures.[46]

4.1.5 RNA Modifications

RNAs can undergo diverse modifications, the most common being the methylation of adenosine residue at the N-6 position [N^6-methyladenosine (m^6A)], which can occur in 5′ and 3′ UTRs and internal long exons.[48,52] This modification can therefore influence RNA processing and function, including its splicing, polyadenylation, stability, nuclear export and translation.[46,48] The m^6A is generated by N^6-methyltransferases (the METTL3-METTL14 complex) and removed by demethylases (ALKBH5 or FTO enzymes).[48,52] As an increasingly studied post-transcriptional epigenetic regulation mechanism, aberrant RNA methylation is emerging as an important player in cancer biology.[46]

4.2 What is Currently Known About Melanoma Epigenetics

Epigenetic alterations are common in melanomas and can be influenced by both genetic and non-genetic factors affecting the tumor cells or their microenvironment. These alterations can contribute to melanoma initiation, progression, and metastatic potential as well as tumor-immune system interactions and response to therapy.[45,47,55,56] Most of the current knowledge on melanoma-associated epigenetic alterations come from the studies of skin melanoma, as it corresponds to the most common melanoma type. While various epigenetic mechanisms have been increasingly studied in melanomas, DNA methylation and miRNA expression are among the most frequently investigated.

Chromatin structure/conformation can be influenced by changes in levels/function of the components of chromatin remodeling complexes, and altered chromatin remodeling can play an

important role in melanomagenesis,[47,55] as exemplified by frequently dysregulated activity of the Switch/sucrose-non-fermentable (SWI/SNF) complexes, whose components are involved in DNA repair and genomic stability maintenance.[57]

Aberrant patterns of histone modifications (e.g., hypoacetylation and hypermethylation) play active roles in melanomagenesis mainly by silencing of tumor suppressor genes, which in turn results in dysregulated signaling pathways, apoptosis, and cell cycle progression.[47,55] Altered histone modifications can also influence the expression of genes that contribute to tumor immunogenicity and/or to immune response within the tumor microenvironment.[56] Aberrant expression of *histone variants*, such as loss of macroH2A or overexpression of H2A.Z.2, have also been described in correlation with melanoma progression as related to cell cycle dysregulation and increased metastatic potential.[47,55]

DNA methylation aberrations have been frequently studied in melanomas for their pathogenetic or prognostic influences. *Focal hypermethylation* of specific tumor suppressor gene promoters is commonly seen in melanomas (such as aberrant methylation of *PTEN* and *CDKN2A*), resulting in dysregulated intracellular signaling, cell cycle progression, apoptosis, and DNA repair.[47,55] In addition to the known tumor suppressors, multiple other genes involved in cell differentiation/survival/growth or in tumor immunogenicity and/or immune response are also found to be aberrantly methylated in melanomas.[47,55,56] Some melanomas can exhibit a "CpG island methylator phenotype", which is characterized by increased *global DNA hypermethylation*.[47,55] More commonly, melanomas exhibit *global DNA hypomethylation*, which is associated with genomic instability and oncogenes activation. Melanomas can also often show partial/complete *loss of 5-hmC*, which usually results from the deficiency of enzymes/cofactors involved in active DNA demethylation.[49,58,59]

Aberrant expression of *miRNAs and lncRNAs (including circRNAs)* has been increasingly studied in recent decades as related to melanoma pathogenesis and prognosis.[45,55] Multiple miRNAs have been found to be dysregulated in melanomas, exhibiting oncogenic, anti-apoptotic, or pro-metastatic properties, or mediating tumor-specific immune response and/or resistance to therapies. Similarly, several *lncRNAs* have been found to be dysregulated in melanomas (e.g., *HOTAIR* and *MALAT1*), exhibiting oncogenic, anti-apoptotic, or pro-metastatic properties. Melanoma-associated ncRNAs are increasingly investigated as potential biomarkers or new therapeutic targets.[45]

Aberrant m^6A RNA methylation is also increasingly recognized for its involvement in in melanoma, by influencing either the immunogenicity of tumor cells or functions of tumor-infiltrating immune cells.[56]

4.3 What is Currently Known About Conjunctival Melanoma Epigenetics

Only a handful of studies have been published to date concerning the epigenetic analyses of CjMs, and they have primarily focused on *DNA methylation*,[60,61] *ncRNA expression (miRNAs or circRNAs)*,[62-65] or *RNA modification/methylation*.[66,67] As previously stated, CjM shows genetic similarities to cutaneous and mucosal melanomas, and the initial results from currently available studies suggest also some overlap in epigenetic alterations observed in these melanoma types.

A recent study by Jurmeister et al. (2022) [60] investigated *DNA methylation* landscape of 107 melanomas originating from different primary sites (25 cutaneous and 82 non-cutaneous melanomas including 9 CjMs) along with their genome-wide copy number analyses. Their results have revealed that conjunctival and other mucosal melanomas share a common *global DNA*

methylation profile with cutaneous melanomas; however, the primary tumor sites differ in *promotor methylation patterns* and copy number changes *at cancer-related genes*. While the tumor suppressor gene *APC* promoter methylation was the most common in CjMs (6/9; 67%), the *CDKN2A (p16)* promoter methylation was commonly present in both CjMs (4/9; 44%) and skin melanomas (9/25; 36%). The *CDKN2A* and *PTEN* tumor suppressor genes were found to be inactivated by *either* promoter methylation or focal deletion, as these two events appeared to occur in an exclusive manner.[60]

Given the molecular similarities between skin and conjunctival melanomas, a recent study by Stahl et al. (2021) [61] investigated whether CjMs similarly show a *global 5-hydroxymethylcytosine (5-hmC) loss* compared to the nevi as previously reported in skin melanomas.[58,59] For this purpose, the authors performed immunohistochemistry and RNA in situ hybridization (RNA ISH) in 37 CjMs and 40 conjunctival nevi to assess the expression of 5-mC, 5-hmC and TET2 (a methylcytosine dioxygenase that catalyzes the conversion of 5-mC to 5-hmC). The loss/reduction of 5-hmC has been previously linked to TET inactivation or downregulation.[59] Stahl et al. (2021) [61] were able to detect 5-hmC and TET2 in 54% and 35.1% of CjMs and in 100% and 95% of conjunctival nevi, respectively, suggesting a significant loss of 5-hmC and TET2 occurring in CjMs similar to skin melanomas.

MicroRNAs are the most studied epigenetic regulators in CjM to date, investigated in three previously published studies.[62-64] In an initial 2016 study, Larsen et al. [62] performed a "*microarray-based miRNA expression profiling"* to compare archived [formalin-fixed, paraffin-embedded (FFPE)] CjM samples (n=37) with normal conjunctiva samples (n=7). The authors also tested the association of miRNA expression patterns with TNM stage, local recurrence, and distant metastases in CjM. Their analyses identified 25 dysregulated (24 upregulated and 1

downregulated) miRNAs in CjM compared to normal conjunctiva, including many miRNAs with known roles in skin melanoma (most noteworthy being the oncomiRs miR-20b-5p, miR-146a-5p, miR-146b-5p, miR-506-3p and miR-509-3p). The authors were also able to link seven upregulated miRNAs to the T stage and increased tumor thickness, and two upregulated miRNAs (miR-3687 and miR-3916) to local recurrence risk but found no link to metastasis risk or mortality in CjM. Moreover, when they analyzed/compared fresh frozen samples of CjM (n=6) with those of head and neck mucosal melanomas, they found resemblance in miRNA expression patterns. Larsen et al. (2016) [62] concluded that CjMs show a similar pattern of differentially expressed miRNAs compared to other melanomas, and some of these dysregulated miRNAs could function as potential prognostic biomarkers or future therapeutic targets.

In a later study published by the same research group, Mikkelsen et al. (2019) [63] performed a "*microarray-based miRNA expression profiling"* of primary CjMs with and without subsequent metastases to identify prognostic miRNAs associated with metastatic spread. The study included archived FFPE samples from 25 patients with nonmetastatic CjM and 13 patients with metastatic CjM. The authors identified several hundred differentially expressed miRNAs when they compared primary CjMs (with/without subsequent metastases) with normal conjunctiva, including two miRNAs (*miR-509-3p and miR-181b-5p*) similarly found upregulated in their previous study.[62] Pathway analysis of these two miRNAs linked them to Hippo and p53 signaling pathways as relevant to tumorigenesis. The authors discovered 15 differentially expressed miRNAs when they compared primary CjMs with and without subsequent metastases, including 9 upregulated and 6 downregulated miRNAs in primary CjM with subsequent metastases. Of 15 miRNAs associated with metastasis risk, four (mir-575, mir-622, miR-1270, and miR-1290) were previously linked to other cancers. In addition, the authors compared primary CjMs with their pair-

matched metastases and detected six differentially expressed miRNAs, including two upregulated (miR-1246 and miR-302d-5p) and four downregulated (mir-6084, miR-184, mir-658, and mir-4427) in distant metastases, three of which (miR-1246, miR-184, and mir-658) were previously linked to other cancers. Using quantitative PCR (qPCR), the authors were able to confirm downregulation of *miR-184* in metastases compared to primary tumors, a miRNA involved in extracellular matrix (ECM)-interaction pathway and representing a potential therapeutic target. Pathway analysis of miRNAs dysregulated in either metastasis prone primary CjMs or distant metastases linked these miRNAs to "cancers, cell growth and death, signal transduction, metabolism, and immune system". Mikkelsen et al. (2019) [63] concluded that, while their sample size was small due to the rarity of CjM, the primary CjMs with and without subsequent metastases showed distinct global miRNA expression profiles, but larger studies would be necessary to characterize the specific set of miRNAs predictive for metastatic spread.

In a more recent study from another research group, van Ipenburg et al. (2020) [64] used "*TaqMan Low Density Array Card A to perform miRNA expression profiling"* to compare benign vs. malignant conjunctival melanocytic lesions, as well as the primary CjMs with vs. without metastases, to identify the discriminating miRNAs between these study groups. Their study included a discovery sample of 6 conjunctival nevi and 20 CjMs and a replication sample of 13 conjunctival nevi and 19 CjMs, all archived FFPE samples, and their analysis primarily focused on miRNAs upregulated in CjMs. While they found no miRNA expression differences between the primary CjMs with and without metastases, the authors were able to identify/replicate a set of five miRNAs upregulated in primary CjMs compared to the nevi, including "*miR-9-5p, miR-196b-5p, miR-450a-5p, miR-501-5p, and miR-615-3p*". Three of these miRNAs (*miR-196b-5p, miR-615-3p, and miR-9-5p*) represented the best performing miRNAs and seemed to be in involved in

a shared pathway of homeobox gene clusters. van Ipenburg et al. (2020) [64] concluded that, while further studies are needed to identify the miRNAs that could predict metastatic potential in CjM, their results suggested a potential benefit of miRNA profiling to discriminate benign from malignant lesions, especially in situations where the limited tissue availability makes such distinction difficult using routine methods.

In a single study on the role of *circRNAs* in CjM published to date, Shang et al. (2019) [65] performed RNA-seq to evaluate the expression of circRNAs in a small sample of three CjMs compared to the paired adjacent normal tissues and identified a large number of potentially CjM-relevant circRNA candidates. Upon further investigation with functional analyses, they characterized *CircMTUS1* (a circRNA derived from exons 2-3 of *MTUS1*) as an oncogenic circRNA upregulated in CjM and acting as a miRNA sponge for *miR-622* and *miR-1208* to modulate several tumor-associated pathways promoting tumorigenesis. The host gene of this circRNA, *MTUS1*, and the targeted miRNA *miR-622* are both known to function as tumor suppressors in several cancers.[68]

Finally, two recent studies [66,67] investigated the role of *RNA methylation (m^6A)* in ocular melanoma by analyzing uveal and conjunctival melanomas together. After evaluating 88 ocular melanoma and 28 normal melanocyte tissues using various analyses, Jia et al. (2019) [67] detected decreased levels of global m^6A (associated with tumor progression) and reduced methylation of the *HINT2* mRNA (hampering the translation efficiency of this tumor suppressor transcript) in ocular melanoma. The authors also mechanistically demonstrated that the methylated *HINT2* mRNA is normally recognized and promoted for translation by YTHDF1, a protein with both m^6A-containing RNA and ribosome binding activities. In a later study that examined 47 ocular melanoma and 27 normal melanocyte tissues, He at al. (2021) [66] demonstrated upregulation of the

BACE2 mRNA, which was mediated by its increased m^6A RNA methylation. The authors also found that *BACE2* upregulation was associated with intracellular calcium release leading to ocular melanoma progression.

4.4 Future Initiatives

CjM is a rare but highly aggressive cancer. Local recurrences and distant metastases are common in CjM patients, but the prediction and management of these outcomes are difficult and often unsuccessful. There is therefore an urgent need for new clinically useful biomarkers and more effective treatments to reduce the high recurrence and metastasis risk in these patients. Like other cancers, CjM development and progression are also believed to be driven by *multiple genetic and epigenetic factors* that contribute to tumorigenesis/spread, impaired immune response, and primary/acquired resistance to therapies. While our *genetic* understanding of CjM has significantly increased in recent years, our *epigenetic* understanding remains incomplete due to a limited number of studies.

The cancer field has witnessed a rapid increase in available epigenetic technologies and treatments based on recent developments and discoveries. In light of these advancements and emerging new therapies, *an in-depth understanding of CjM epigenetics* becomes increasingly essential to provide better prognosis and favorable outcomes to CjM patients. Apart from the therapeutic improvements, an advanced knowledge of CjM tumor epigenetics is also expected to guide the diagnostic/prognostic biomarker development efforts for this aggressive condition.

4.4.1 Gaps in Epigenetic Research of CjM & Areas to Focus On

The emerging epi-drugs for cancer therapy (as a single agent or combined with other drugs/therapies) include those targeting DNA methylation (e.g., *DNMT inhibitors*), histones and chromatin remodeling (e.g., *HDAC inhibitors, HMT or HDM inhibitors, and BET inhibitors*), or ncRNAs (e.g., *lncRNA or miRNA mimics or antagonists*).[48,50,51] The promising results from the latest clinical trials on combinatorial therapies (epi-drugs with other treatments) are especially encouraging, given the concerns about the commonly observed primary or acquired resistance to existing cancer therapies (immunotherapies, targeted therapies, or conventional therapies), which appears to be primarily driven by epigenetic mechanisms.[48,50,51] In order to take advantage of these emerging epi-drugs in CjM management, we first need to better understand the epigenetic mechanisms underlying the CjM development/progression, and its interactions with immune system in the tumor microenvironment, by conducting several additional studies.

Currently, the epigenetic studies on histones and chromatin remodeling are lacking in CjM. A recent study [60] on global or promoter DNA methylation described similar patterns between CjM and other mucosal and skin melanomas but included only nine CjM samples. A few recent studies that explored RNA methylation [66,67] or circRNAs [65] in CjM have revealed some interesting candidates but warrant replication in studies larger than those previously published. Three previous studies on CjM-related miRNA expression profiles (investigated by either microarray-based or TaqMan methods) have revealed some dysregulated miRNA candidates associated with CjM development (compared to normal conjunctiva), prognostic potential (associated with local recurrence or metastasis risk), or diagnostic potential (discriminatory between CjM and nevi); however, the results were not very reproducible across studies or screening platforms.[62-64] Methodological differences, the use of FFPE rather than fresh frozen tissues, and small studies

were discussed by the authors as potential factors that might be contributing to those inconsistencies. Hence there is clearly a need for additional studies using larger sample sizes, as well as using the latest technologies such as miRNA-seq, to further explore the miRNA dysregulation in CjM for therapeutic target or diagnostic/prognostic biomarker discovery. Also, investigating circulating miRNAs in biofluids (e.g., in tear or serum/plasma) from CjM patients could be an alternative and attractive strategy for biomarker discovery/development as compared to tissue-based analyses.

While researchers have already started investigating the miRNA expression differences between CjM and nevi, studies to date are lacking on comparisons between CjM and PAM, a precursor associated with greater risk for malignant transformation.[64] Because PAM with atypia is identified as an important risk factor for CjM, more objective methods targeting PAM to identify predictive factors for malignant transformation could have positive results for better follow-up and early treatment strategies.[64]

The miRNAs robustly identified/confirmed in future studies could be targeted as a form of treatment and present a possible new avenue for future research.[62] There is also a need for more T3 stage tumor-related research to identify differentially expressed miRNAs in that advanced stage group.[62] If researchers can robustly identify advanced T stage-specific miRNAs, this could lead to more accurate prognosis assessment for better follow-up and early treatment.[62]

4.4.2 How Greater Epigenetic Understanding Can Benefit the CjM Patients

It is apparent that lack of *in-depth epigenetic understanding* of CjM poses several limitations for management and treatment of this aggressive tumor. For instance, van Ipenburg et al. (2020) [64] discussed the limited treatment options for tumors that have already metastasized, and

thus the need for biomarkers that could predict tumor behaviors at earlier stages to prevent subsequent metastases. More in-depth investigation into prognostic biomarkers could help create more effective follow-up and early treatment strategies for patients with CjM.

Identifying epigenetic mechanisms contributing to CjM development and progression will allow patients to receive more effective treatments through the use of epi-drugs, alone or in combination with other therapies. Various epigenetic factors may be involved in promoting the tumor cells to metastasize. Exploring these factors will improve potential targeted and personalized therapies for future patients diagnosed with CjM. Focusing on improving treatment methods will lead to more favorable outcomes. CjM is a chronic condition, and as the population ages and advances in medicine prolong the average lifespan, it is unpredictable how many more cases could develop in the future, emphasizing the importance of making more discoveries in CjM.

5.0 Public Health Significance

5.1 Who is Affected Most & Why it is Important to Help

Those who are most affected are elderly persons of European descent, slightly more commonly male patients. CjM metastasizes early and frequently, thus it is life threatening. Early diagnosis and therapeutic interventions are crucial for the best possible patient outcome in terms of recurrence-/metastasis-free and overall survival. There are standard treatment practices in place, however, they do not produce the most successful results in terms of preventing local recurrences and distal metastases. Sometimes, if the malignancy is too advanced upon diagnosis, an extensive and disfiguring surgery is necessary that involves removing the eye and surrounding tissues from the orbital socket (orbital exenteration). Unfortunately, for those who already have metastasis at the time of initial diagnosis, the prognosis is poor and survival rate is low. The racial diversity of those affected by this disease is currently limited, however, as longevity in populations increases, one can predict that cases in other racial groups could be expected to increase as well.

5.2 Strategy Development & Applications to Improve CjM Molecular Understanding

Guidelines for clinical staging of CjM are established, but not ideal, and there is currently no standard practice when it comes to therapeutic strategies.[69] Therefore, improved molecular understanding of CjM may help to develop better prognostication and treatment strategies, and in turn lead to improved clinical outcomes. Unfortunately, the studies on molecular understanding of

CjM are limited and hampered by the rarity of this condition. Collecting larger numbers of CjM samples, through long-term and/or collaborative studies, to compare molecular development and diversity is important for greater molecular understanding of this aggressive disease. A retrospective analysis of patients who had/have CjM could be done to compare molecular development of the disease to map out patterns and consistencies.

5.3 Preventative Measures & Early Diagnosis/Treatment

5.3.1 Education About Modifiable Risk Factors

Obviously, age is a risk factor in any late-onset chronic condition that is inevitable. UV exposure is the most prominent environmental risk factor that can be actively controlled by individuals to decrease their risk of CjM development. Generally, people understand that being in direct UV exposure is detrimental to their visual capabilities, but do not consider how it could affect ocular tumor development. It is important to provide this information to the public in a comprehensive way to promote preventive strategies as well as to increase screening to diagnose CjM earlier to improve survival rates. While this is a sporadic cancer influenced by multiple factors, educating people, especially those with conjunctival precursor lesions, would be important in terms of informing them about UV exposure risk and encouraging them to have regular eye exam visits.

5.3.2 Screening

Typical screening for common melanoma includes dermatology and oncology practices. However, CjM occurs on the eye and thus requires an eye exam for initial diagnosis and then referral to an ocular oncologist. Since the population most affected is ~65 or older, eyesight typically declines with age due to various age-related eye conditions. Therefore, older individuals typically visit the eye doctor for related treatments and regular checkups, although not always at sufficient frequency. It would be beneficial to encourage patients of a certain age group to keep up with their regular eye exams to enable early diagnosis. This could improve early diagnosis rates and the odds of recurrence-/metastasis-free and overall survival.

5.3.3 Proactive/Early Diagnosis & Treatment

The screening opportunities previously discussed could improve survival rates by decreasing recurrence and metastasis rates of CjM. Diagnosing CjM at earlier stages of development will improve the effectiveness of current treatments, as well as that of future therapeutic strategies driven by increased molecular understanding, and it will help to eradicate the tumor with a lower chance of spread of the malignancy. Advanced molecular understanding of CjM is also likely to improve diagnostic and prognostic strategies, which in turn will improve early diagnosis and treatment of this rare but deadly disease.

Appendix A Tables

Table 1 The Eighth Edition of American Joint Committee on Cancer (AJCC) Staging Chart Below Categorizes Conjunctival Melanoma in Order of Tumor Severity and Location (T category)[17,19]

Tumor Category	Staging (Clinical and Pathologic)
TX	Primary tumor cannot be assessed clinically or pathologically
T0	No evidence of primary tumor clinically or pathologically
Tis	Pathological assessment of melanoma confined to conjunctive epithelium
T1	Clinical tumor of the bulbar conjunctiva
T1a	Pathological assessment of tumor of bulbar conjunctiva with invasion of substantia propria, not more than 2.0 mm in thickness; clinical assessment of tumor localized in less than 1 quadrant
T1b	Pathological assessment of tumor of bulbar conjunctiva >2.0 mm in thickness with invasion of substantia propria; clinically the tumor localized in ≥1 but less than 2 quadrants
T1c	Clinical assessment ≥2 but less than 3 quadrants
T1d	Clinical assessment ≥3 quadrants
T2	Clinical assessment of tumor of the non-bulbar (tarsal, palpebral, forniceal, caruncular)
T2a	Pathological assessment of tumor of the nonbulbar conjunctiva involved with invasion of substantia propria, not more than 2.0 mm in thickness; clinically non-caruncular, ≤1 quadrant of non-bulbar conjunctiva involved
T2b	Pathological assessment of tumor of the nonbulbar conjunctiva with invasion of substantia propria, more than 2.0 mm in thickness; clinically non-caruncular, more than 1 quadrant of non-bulbar conjunctiva involved
T2c	Clinically assessed as any caruncular, ≤1 quadrant of the non-bulbar conjunctiva involved
T2d	Clinically assessed as any caruncular, more than 1 quadrant of the non-bulbar conjunctiva involved

T3	Tumor of any size with local invasion
T3a	Clinically and pathologically assessed as tumor on the globe
T3b	Clinically and pathologically assessed as tumor on the eyelid
T3c	Clinically and pathologically assessed as tumor on the orbit
T3d	Clinical and pathological nasolacrimal duct and/or lacrimal sac and/or paranasal sinuses
T4	Clinical and pathological tumor of any size with invasion of central nervous system

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