MICRORNA EXPRESSION PROFILING IN TEARS OF CHILDREN WITH VERNAL KERATOCONJUNCTIVITIS

by

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LIST OF ABBREVIATIONS

AC	Allergic conjunctivitis
AGO	Argonaute
AKC	Atopic keratoconjunctivitis
ARHGEF5	Rho guanine nucleotide exchange factor 5
AUC	Area under curve
B3GAT1	Beta-1,3-Glucuronyltransferase 1
CCL22	C-C motif chemokine 22
CD276	Cluster of differentiation 276
CIP	Calf intestine phosphatase
DMSO	Dimethyl sulfoxide
FC	Fold change
FE	Feature extraction
FOXP3	Forkhead box protein P3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gene ontology
GPC	Giant papillae conjunctivitis
HRH1	Histamine Receptor H1
ICOSLG	Inducible T Cell Costimulator Ligand
IL-13	Interleukin - 13
JEPeM	Jawatankuasa Etika Penyelidikan Manusia
KEGG	Kyoto encyclopedia of genes and genome
LGALS9	Galectin-9
MIF	Macrophage migration inhibitory factor

miRISC	Microribonucleoprotein complex
MiRNA	Micro ribonucleic acid
MMP25	Matrix metalloproteinase-25
mRNA	Messenger ribonucleic acid
NF-kB	Nuclear factor - kappa B
NGF	Nerve growth factor
NGFR	Nerve growth factor receptor
NGS	Next generation sequencing
PGF	Placental growth factor
PPI	Protein-protein interaction
PRELP	Prolargin precursor
Pre-miRNA	Premature micro ribonucleic acid
Pri-miRNA	Primary micro ribonucleic acid
PTGDS	Prostaglandin-H2 D-isomerase
PTGER	Prostaglandin EP1 receptor
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RT-qPCR	Reverse transcriptase - q Polymerase chain reaction
SEA	Single experiment analysis
SiRNAs	Small interfering ribonucleic acid
SOCS3	Suppressor of cytokine signaling 3
sRNA	Small ribonucleic acid
STRING	Search tool for the retrieval of interacting genes/proteins

TGM2	Transglutaminase 2
TRBP	Transactivation response element RNA-binding protein
VKC	Vernal keratoconjunctivitis

PROFIL EXPRESI MIKRORNA DALAM AIR MATA KANAK-KANAK MENGALAMI VERNAL KERATOKONJUNKTIVITIS

ABSTRAK

Vernal keratokonjunktivitis (VKC) adalah keadaan keradangan konjunktiva kronik yang mempengaruhi kanak-kanak. Biasanya terdapat manifestasi klinikal yang teruk dan boleh menyebabkan parut kornea dan buta. Air mata mengandung kepekatan mikroRNA (miRNA) yang pelbagai. miRNA merupakan molekul RNA bukan pengekodan kecil yang ekspresinya dilaporkan mengatur berbagai proses sel dalam berbagai penyakit mata. Kajian ini bertujuan untuk menghasilkan profil ekspresi miRNA yang diperolehi dari air mata kanak-kanak yang mengalami VKC berbanding dengan kumpulan kawalan dan juga untuk menilai miRNA dalam air mata sebagai potensi biomarker diagnostik VKC (Fasa II). Namun begitu, melalui kajian perintis, kuantiti miRNA yang optimum dalam air mata antara pelbagai kumpulan penyediaan spesimen air mata iaitu, keseluruhan air mata, eksosom keseluruhan air mata, dan air mata tanpa-eksosom (Fasa I) diselidik. Pengasingan RNA dilakukan menggunakan Kit MiRNeasy dan pengukuran kuantiti RNA melalui kit nano Bioanalyzer RNA 6000 dan Small RNA kit. VKC dan sampel kawalan disaring untuk ekspresi miRNA menggunakan teknik mikroarray Agilent. Ini adalah kajian kawalan kes eksperimen yang dilakukan di Klinik Oftalmologi, Hospital Universiti Sains Malaysia, dari Januari 2020 hingga Disember 2020. Fasa I kajian melibatkan subjek normal manakala Fasa II melibatkan kumpulan kanak-kanak dengan diagnosis klinikal VKC dan kumpulan kawalan. Pengasingan RNA dilakukan menggunakan miRNeasy Micro kit dan pengukuran melalui Bioanalyzer RNA 6000 nano kit dan Small RNA kit. Sampel

kumpulan VKC dan kumpulan kawalan disaring untuk ekspresi miRNA menggunakan teknik mikroarray Agilent. Penyediaan spesimen air mata dari sampel air mata keseluruhan menghasilkan kepekatan miRNA yang lebih baik dan optimum. Melalui microarray, sejumlah 51 miRNA dalam air mata kanak-kanak dengan VKC dikenal pasti dengan signifikan apabila dibandingkan dengan kumpulan kawalan. Dari keseluruhan miRNA yang dikenalpasti, didapati 48 miRNA dengan signifikannya dikawal selia secara menaik dan tiga miRNA dengan signifikannya dikawal selia secara menurun. Hsa-miR-1229-5p, hsa-miR-6821-5p, dan hsa-miR-6800-5p adalah tiga miRNA teratas yang dikawal selia menaik. Manakala hsa-miR-7975, hsa-miR-7977, dan hsa-miR-1260a adalah tiga miRNA yang dikawal selia menurun. Kesemua 48 miRNA yang dikawal selia dapat digunakan sebagai biomarker miRNA diagnostik yang berpotensi untuk VKC kerana nilai AUC diskriminasi yang lebih tinggi. Analisis sasaran miRNA telah menentukan beberapa sasaran gen di mana 16 gen sasaran bertindih (ARHGEF5, CCL22, CD276, LGALS9, MIF, PGF, PTGDS, PTGER, B3GAT1, SOCS3, ICOSLG, TGM2, MMP25, NGFR, FOXP3, dan HRH1) diketahui berperanan dalam menyebabkan keradangan konjunktiva. Fosforilasi oksidatif, glikolisis/glukoneogenesis, dan gula amino dan metabolisme gula nukleotida adalah tiga jalur KEGG teratas yang terlibat. Kesimpulannya, miRNA dari spesimen keseluruhan air mata menunjukkan ekspresi secara berbeza di kalangan kanak-kanak VKC dan kumpulan kawalan. Setelah validasi dilakukan, miRNA ini berpotensi sebagai biomarker diagnostik untuk VKC dan memberi pandangan yang lebih jelas dalam patogenesis VKC.

MICRORNA EXPRESSION PROFILING IN TEARS OF CHILDREN WITH VERNAL KERATOCONJUNCTIVITIS

ABSTRACT

Vernal keratoconjunctivitis (VKC) is a chronic conjunctival inflammatory condition usually affecting children. It is often present with severe manifestations leading to corneal scar and blindness. Tears contain diverse concentrations of microRNAs (miRNAs), which are small non-coding RNA molecules regulating various cellular processes in various eye diseases. In this study we aim to generate miRNA expression profile in tears of children with VKC in comparison to controls and evaluate these miRNAs as the potential diagnostic biomarkers of VKC (phase II). However, through a pilot study, we initially investigated the optimal miRNA quantity in tears among three sampling groups, namely, unfractionated whole tears, whole-tear-derived exosomes, and exosome-depleted (phase I). This was an experimental case-control study conducted at Ophthalmology Clinic, Hospital Universiti Sains Malaysia, from January 2020 till December 2020. Phase I of the study involved normal subjects while phase II involved children with clinical diagnosis of VKC and control. RNA isolation was performed using the miRNeasy Micro Kit and quantification through Bioanalyzer RNA 6000 nano kit and Small RNA kit. VKC and control samples were screened for miRNAs expression using Agilent microarray technique. The unfractionated whole tears sampling group yielded better and optimal miRNA concentrations. Microarray results revealed a total of 51 miRNAs that were differentially expressed among children with VKC and controls. Out of these miRNAs, 48 were up-regulated and three were down-regulated. The hsa-miR-1229-5p, hsa-miR-6821-5p, and hsa-miR-6800-5p were

the three top up-regulated miRNAs, while the miRNAs, hsa-miR-7975, hsa-miR-7977, and hsa-miR-1260a were the three down-regulated miRNAs. All the 48 up-regulated miRNAs can be used as the potential diagnostic miRNA biomarkers for VKC due to their higher discriminatory area under the curve (AUC) values. The miRNA target prediction analysis has determined multiple gene targets out of which 16 overlapping target genes (ARHGEF5, CCL22, CD276, LGALS9, MIF, PGF, PTGDS, PTGER, B3GAT1, SOCS3, ICOSLG, TGM2, MMP25, NGFR, FOXP3, and HRH1) were known to play role in causing conjunctival inflammation. The oxidative phosphorylation, glycolysis/gluconeogenesis, and amino sugar and nucleotide sugar metabolism were the top three KEGG pathways involved. In conclusion, miRNAs from unfractionated whole tears were differentially expressed among children with VKC and controls. Once validated, these miRNAs could serve as potential diagnostic biomarkers for VKC and provides insights into the pathogenesis of VKC.

CHAPTER 1

INTRODUCTION

1.1 Study Background

Amongst the allergic eye diseases, vernal keratoconjunctivitis (VKC) is a bilateral, chronic inflammation of the ocular surface primarily involving tarsal and bulbar conjunctiva (Addis & Jeng, 2018; Singhal et al., 2019). Common symptoms of VKC include eye redness, mucus discharge, severe itching, tearing, irritation and photophobia while, presence of Horner Trantas-Dots, papillae (tarsal or limbal), chemosis and conjunctival hyperaemia are the classical signs of VKC (Bonini et al., 2004; Mathys and Lee, 2013; Choleva et al., 2014). Size of papillae positively correlate with the worsening or persistence of symptoms over long-term follow-up (Bonini et al., 2000). Although the name vernal (spring) suggests it as a seasonally recurring disease, often the symptoms can be seen all year long (Addis & Jeng, 2018). If left untreated, there is pain and sometimes may cause complete loss of sight in severe forms where cornea is involved (Vichyanond et al., 2014).

VKC usually affects children with age of onset generally between 4-7 years of age. Males were predominantly affected than females before the age of 20. However the difference becomes lesser as the age progresses after puberty (Kansakar, 2011; Leonardi et al., 2006). In tropical countries like Nigeria and Ethiopia its prevalence is reported as 3%, 5% and 18.1% and 37.27 % (De Smedt, 2016; Jp and Hughes D, 2016). It is suggested that

neurogenic, endocrine, environmental and socio-economic factors among different ethnic populations and the geographical location play an important role in the prevalence of VKC (Hayilu et al., 2016). Initially VKC was considered to be an immunoglobulin (Ig) E-mediated disease or a type 1 hypersensitive reaction. However, recent studies have suggested important roles of many other immunological factors (Addis & Jeng, 2018; Ahmed et al., 2019). The histamine receptors (H1, H2, and H4), eotaxin-2, eosinophil cationic protein (ECP), neurotrophin-4 (NT4), and matrix metallopeptidase 1 (MMP1) are the several reported protein biomarkers in VKC (Micera et al., 2016; Shoji, 2020; Tamhane et al., 2019).

The pathogenesis of VKC is complex and multifactorial (Ahmed et al., 2019). Patients with VKC are found to have a higher rate of linked atopic conditions such as eczema, rhinitis, and asthma among them (Bonini et al., 2000). Also, we know that an allergic inflammation is followed by the coordinated expression of a numerous genes and proteins that cause, sustain, and generate immune responses and tissue remodeling (Lu & Rothenberg, 2013). Although often neglected, the genetics of an ocular allergy such as VKC is an important area that should be studied for better understanding of the disease.

MicroRNAs (miRNAs) are small, single-stranded, 19–23 nucleotide-long RNA molecules which can affect the stability of messenger RNA (mRNA) and protein synthesis through partial sequence complementation with their interacting mRNA targets (Weber et al., 2010). It is predicted that about 30% to 80% of human genes could be regulated by

miRNAs (Lu & Clark, 2012). Exosomes are small extracellular vesicles secreted by various cells containing proteins and nucleic acids including miRNAs (Gallo et al., 2012). MiRNAs in exosomes and extracellular fluid are linked and play significant roles in the pathogenesis of disease by modulating several major pathways (Xu & Hazlett, 2019). Identification of miRNAs by microarray profiling has been performed on a handful of small RNA preparations from cell lines and body fluids such as plasma, urine, saliva, and serum, including tears (Weber et al., 2010). Tears are one of the extracellular sources that contain miRNAs and these miRNAs can be used as potential informative biomarkers to assess ocular surface's pathophysiological condition (Huang, 2017; Weber et al., 2010). A recent miRNA study has used whole tears fluid to extract miRNA, while other studies have used tears pellet to extract miRNA (Kenny et al., 2019; Lande et al., 2020; Tamkovich et al., 2019).

Recent miRNA profiling studies in multiple allergic diseases like asthma, eosinophilic esophagitis, allergic rhinitis, and atopic dermatitis have identified specific miRNAs to have critical roles in regulating pathogenic mechanisms. For example miR-21 and miR-146 have role in activation of T-cells, miR-21 and miR-223 regulate eosinophil development and miR-375 modulates interleukin (IL)-13-driven responses (Lu & Rothenberg, 2013). Investigating tear composition has revealed novel biomarkers in ocular diseases such as dry eye disease and VKC (Aluru et al., 2012; Leonardi et al., 2014). It is shown that changes in the spectrum of cellular miRNA correlates with inflammatory condition. However, we could not find any study showing miRNA expression and their regulation in VKC patients.

Recent improvements in the detection sensitivities of profiling methods have allowed quantification of tear samples, thereby increasing the frequency of tear fluid investigations. Understanding roles of miRNAs and their targets is aided by miRNA expression profiling studies. Microarray profiling is a robust technique in the examination of miRNA expression (Love & Dave, 2013). The aim of the study was to optimize the miRNA concentration in tears sample as an initial phase. Then, to demonstrate the differential miRNA expression pattern in tears and identify the potential miRNA biomarkers in children with VKC in comparison to healthy controls followed by to evaluate the potential miRNA target genes for VKC.

1.2 Problem Statement

VKC is a form of chronic allergic ocular inflammatory disease which requires aggressive management to avoid visual disability secondary to corneal complications. It is reported that about 36.4% of school-going children with VKC had missed school more than or equal to one day/month and about 4.6% of patients suffered with visual impairment and total visual loss in the absence of proper management (Alemayehu et al., 2019).

Recurrent and prolonged clinical presentation of VKC makes management and treatment of VKC quite challenging (Addis & Jeng, 2018). Multiple factors such as immune responses, epigenetic, environmental, and genetic factors are involved in the pathogenesis of VKC (Hayilu et al., 2016; Mathys & Lee, 2013). Though miRNAs are known as the key regulators of epigenetic mechanisms, gene expressions, and allergic inflammatory responses, their role in causing VKC is unknown (Lu & Rothenberg, 2013; Yao et al., 2019). Since the pathophysiology of VKC is not fully understood, therefore there is a need for better understanding of the mechanism or pathogenesis of VKC to design a potential diagnostic and therapeutic option.

Collection of blood for evaluation of inflammatory biomarkers to determine the role in the pathophysiological of VKC is not only invasive but also painful to children. Monitoring the inflammatory cells in the conjunctiva of patients with VKC is another option but the procedure is complex. Tear sample is the alternative. Although tear

collection is non-invasive, but their analysis is challenging due to their low sample volume (less than 5 µl) (von Thun und Hohenstein-Blaul et al., 2013).

1.3 Justification of the Study

Though the VKC disease etiology greatly differs among different ethnic groups and geographical locations, there is a minimal data from Asian countries. Despite VKC generally being referred to as a seasonal disease, patients in our region have the disease throughout the year, indicating a different pattern of presentation. Moreover, the trigger factor may also be different.

Tears have a large and diverse concentration of miRNAs and are a source for ocular health screening. Unlike other invasive sampling techniques like blood, serum and plasma, non-invasive tear collection has better patient compliance as it is less painful, simple and cost effective (Weber et al., 2010; Quah et al., 2014). To date, no published study has quantified the concentration of miRNA between extracellular miRNA and exosomal miRNA in human tears. Thus, establishing an optimizing protocol for miRNA concentration from tears using Schirmer strips will assist researchers conducting miRNA profiling studies that focus on ocular diseases.

Previous profiling studies using microarray have found specific miRNAs (e.g. miR-21, miR-146, miR-223 and miR-375) play a critical role in monitoring key allergic inflammation mechanisms (Lu & Rothenberg, 2013; Yu et al., 2011). Identifying the different expression pattern of miRNAs and determining potential biomarkers in tear samples of children with VKC and controls will provide better understanding of VKC pathogenesis for future pharmaco-genetic treatment.

1.4 Objectives of the Study

1.4.1 General objective

To demonstrate miRNA expression profiling in tears of children with VKC and controls.

1.4.2 Specific objectives

1. To identify different miRNA expression pattern using miRNA microarray in tears of children with VKC and controls.
2. To determine the potential miRNA biomarkers among children with VKC and controls.
3. To identify the potential miRNA target genes using in silico target prediction tools.

1.5 Research Questions

1. How is the different miRNA expression pattern in tears of children with VKC and controls?
2. What are the potential miRNA biomarkers among children with VKC?
3. What are the potential target genes of miRNAs expressed among children with VKC?

1.6 Research Hypothesis

1.6.1 Null hypothesis

1. There were no significantly expressed miRNAs in tears of children with VKC and controls.
2. There were no identified potential miRNA biomarkers among children with VKC.
3. There were no identified potential miRNA target genes among children with VKC.

CHAPTER 2

LITERATURE REVIEW

2.1 Ocular Allergies

The term ocular allergy or allergic conjunctivitis refers to the collection of eye surface disorders which affects the eyelids and conjunctiva (Leonardi et al., 2017). Ocular allergic diseases are classified into different types such as allergic conjunctivitis (AC), atopic keratoconjunctivitis (AKC), giant papillae conjunctivitis (GPC) and VKC (Takamura et al., 2017). Ocular allergic diseases affect about 15-20 % of the whole population (Bielory et al., 2016).

Ocular allergy is primarily characterized by inflammation of conjunctival mucus membrane (Bielory et al., 2016). The inflammation reactions are coordinated in response to allergens. The allergic stimuli for example pollen in case of seasonal allergic conjunctivitis, the stimulus will interact first with the conjunctival and corneal epithelial cells. Then, the stimulus passes through epithelial cells into the stroma and activates different types of immune cells including eosinophils, mast cells, T-cells, dendritic cells, and macrophages respond. Their responses trigger the symptomatic responses of tear production, lid swelling and ocular chemosis. Some responses can even damage the cornea causing pain, progressive scarring and blurred vision (Dartt & Masli, 2014).

2.2 Introduction of Vernal Keratoconjunctivitis

2.2.1 Epidemiology and demographics

VKC has a great variation in racial and geographical location. It is reported to be more prevalent in hot and dry tropical and subtropical regions of Mediterranean belt, West Africa, Indian Subcontinent, South American and Asia (Addis & Jeng, 2018; Bonini et al., 2000; De Smedt, Wildner, & Kestelyn, 2013; Leonardi et al., 2015). The prevalence of VKC in African countries such as Ethiopia ranges from 5.2% to 7.3% and 3.3% in Egypt among school children (Alemayehu et al., 2019; Hayilu et al., 2016; Marey et al., 2017).

An epidemiology study in Western Europe has reported a prevalence range of 1.16% to 10.55% (Bremond-Gignac et al., 2008). Warmer regions like Italy has higher prevalence of 27.8/10000 and colder regions like Norway has prevalence of 1.9/10000 (Mathys & Lee, 2013). It is reported as less common in Northern Europe and North America (Ahmed et al., 2019).

Recent studies in Asian countries such as Indian and Japan, the prevalence of VKC was found to be 5.8% and 1.2% respectively (Hayilu et al., 2016; Miyazaki et al., 2019). The prevalence of VKC in a Southeast Asian country such as Thailand varies from 4.5 to 10.6% (Miyazaki et al., 2020). A study in Malaysia reported that 87.5% of the patients

were male and the remaining 12.5% were females with the age range of 6 to 24 years (Yogesvaran & Min, 2017). Mathys and Lee (2013) reported the mean age of diagnosis as in between 6.8±5years and 11±5years. Young boys seem to be more affected than girls in large Asian case series, but this gender distribution reduces with age.

VKC is a seasonal disease (vernal meaning spring), indicating the increase in signs and symptoms seasonally. However, the progressive forms can have signs and symptoms throughout the year (Kraus, 2016). VKC has also been linked with higher socioeconomic status (Addis & Jeng, 2018).

2.2.2 Symptoms and signs of vernal keratoconjunctivitis

VKC is an inflammatory condition of conjunctiva with signs of hyperaemia, photophobia, chemosis and mucus discharge (Bonini et al., 2004). Clinically it can be present as tarsal form or limbal form or sometimes a mixed form of both types (Zicari et al., 2019).

The characteristic sign of tarsal form is the presence of papillae ranging from 1 mm to classical giant cobble-stone papillae (Figure 2.1) (De Smedt et al., 2013). While in the limbal form the papillae look thick and opaque with white chalk like depositions of eosinophils and epithelial cells called Horner-Trantas dots (Figure 2.2). The tarsal form of VKC (44-83%) is more common than limbal form (8-11%) and mixed type of VKC is found in 9-46% of patients. Another characteristic feature of VKC is hyper-secretion of

filamentous thick mucus called ‘ropy discharge’ (Vichyanond et al., 2014). Cornea can be present in both mild forms (epithelial erosions) and severe forms (macroerosions and ulcers). Severe forms prevent re-epithelialization and causes shield ulcers on cornea (Figure 2.3) (Kraus, 2016).

The predominant symptoms of VKC include severe eye itching generally followed by photophobia, eye redness, and foreign body sensation. Symptoms usually are seasonal recurring but patients in hot and dry regions exhibit symptoms throughout the year. The longer the patients suffer from VKC, the more likely they become to develop the persistent form of disease.

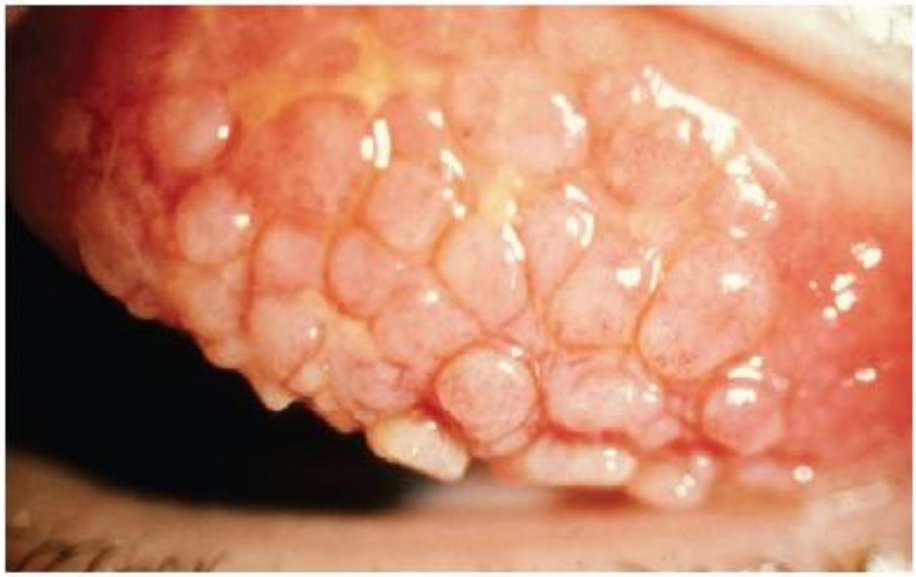

Figure 2.1: Giant cobblestone like papillary of the upper tarsal conjunctiva. Adapted from Taddio et al., (2011).

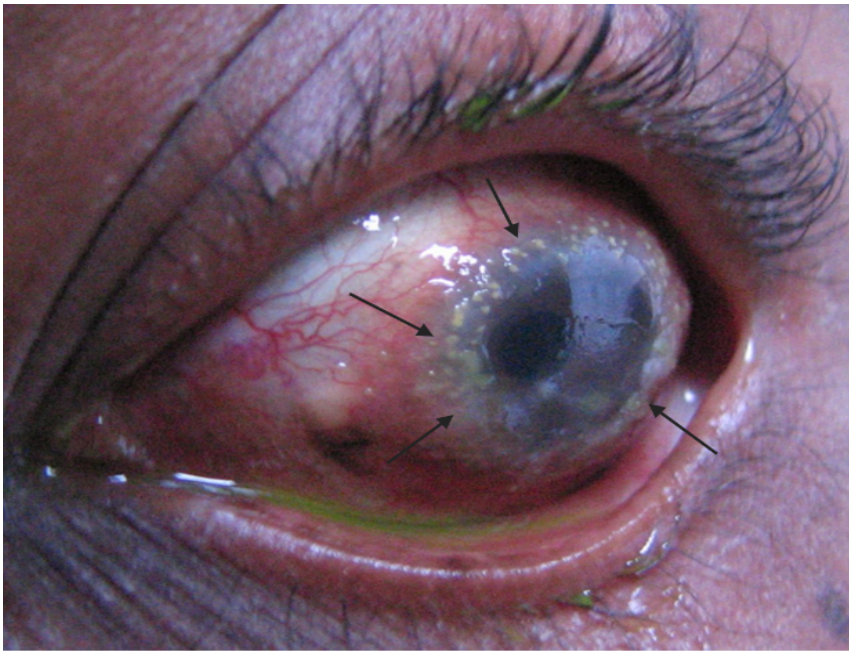

Figure 2.2: A case of limbal form of VKC where the limbus area looks thick and opaque with top deposits called Horner-Trantas dots (arrow). Adapted from Smedt et al., (2013).

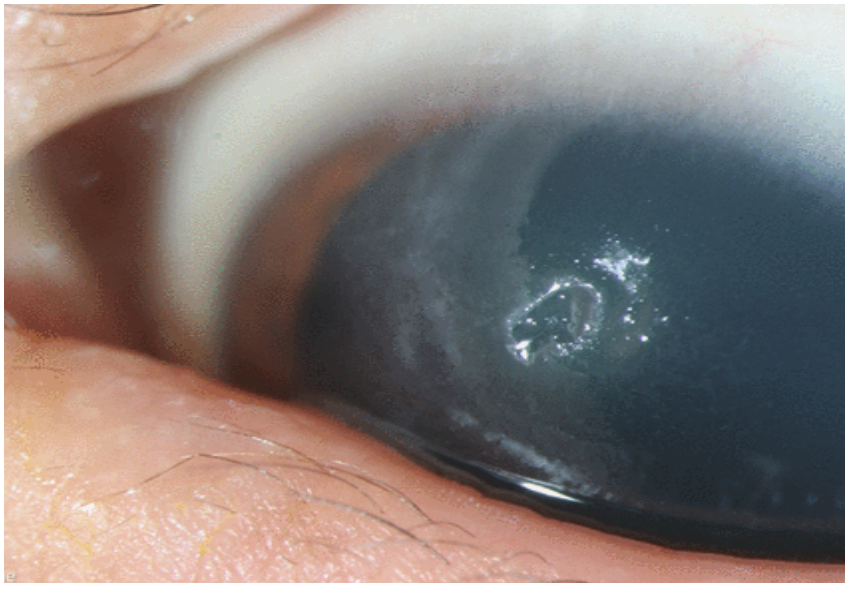

Figure 2.3: A demonstration of corneal shield ulcer. Adapted from Smedt et al., (2013).

2.2.3 Pathophysiology of vernal keratoconjunctivitis

VKC is commonly considered as the IgE-mediated type 1 hypersensitivity allergic reaction. The IgE mediated reaction is an initial or early phase reaction which activates IgE coated mast cells in releasing their preformed and newly formed mediators (cytokines/chemokines). The late phase reaction starts after six to 24 hours of the early phase reaction and is linked with the influx of leucocytes (Amin, 2012).

However, other studies have reported a non-IgE or T-cell mediated reaction involving antigen presenting cells (APCs) resulting in production of T-helper (Th)2 cells and cytokines. It is understood that the APCs also increase the influx of eosinophils by chemokines, which can attract T-cells and activate mast cells independent of Ig-E (El-Asrar et al., 2002; Pucci et al., 2003). A large presence of Th2 derived cytokines (IL-4 and IL-5) and their receptors confirms their role in onset of an immune response and the progression of inflammation seen in VKC tissues. A brief sequence of reactions in towards an antigen is illustrated in Figure 2.4 (Pattnaik & Acharya, 2015; Zicari et al., 2019b).

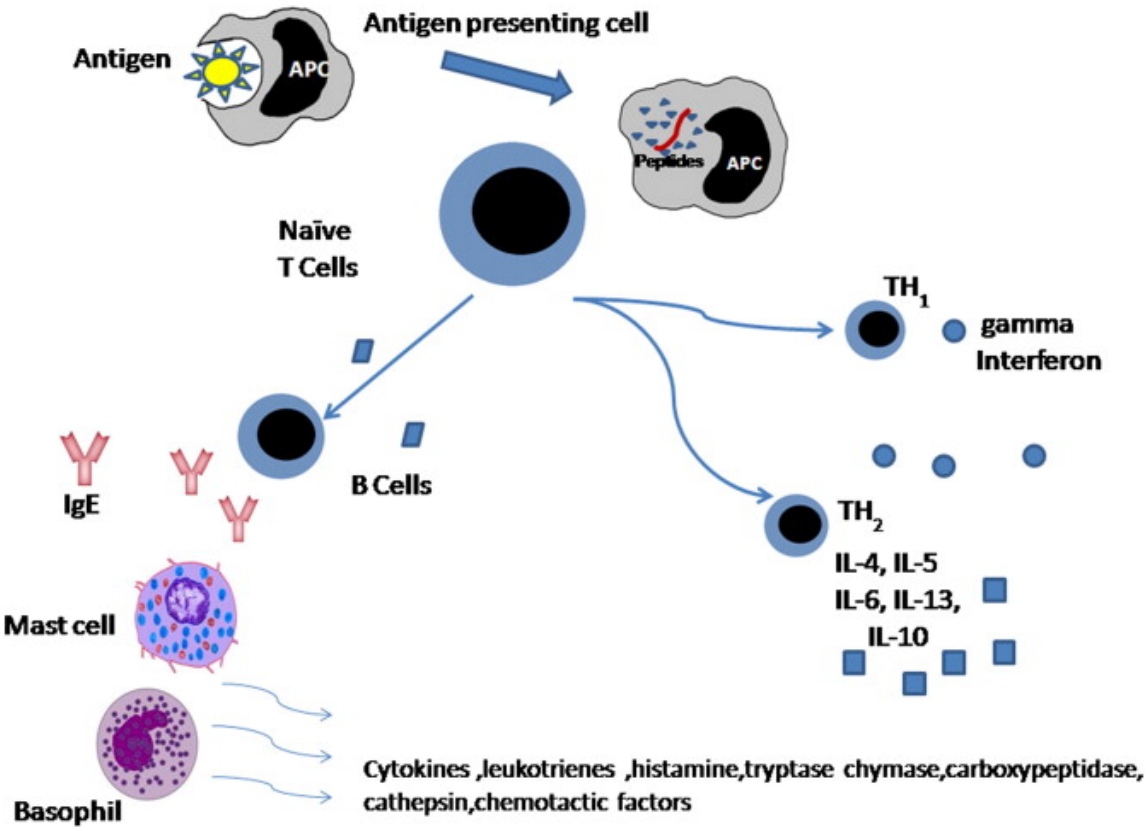

Figure 2.4: Sequence of reactions towards an antigen in VKC patients. Adapted from Pattnaik & Acharya, (2015).

The histopathological findings of the conjunctival tissue form patients reveal the presence of eosinophils, mast cells, and overgrown epithelial cells in comparison to normal tissue. The disease incidence is linked with allergic manifestations and elevated levels of specific IgE, mast cells, eosinophils, and histamine concentrations (Mathys & Lee, 2013). Also, the consistent presence of eosinophils in conjunctival scrapings and tears of patients with ocular allergies suggests their important role in pathogenesis of VKC. Though the presence of eosinophils confirms an ocular allergy or VKC, their absence cannot rule out the condition. The number of elements during an inflammatory immune response

correlates with the disease condition along with the severity of the symptom (Bruschi et al., 2020).

Moreover, the histopathological studies using conjunctival tissues have demonstrated epithelial outgrowths with proliferation of blood vessels, epithelial ingrowths, and extra cellular matrix deposition (De Smedt et al, 2013). Hence, multiple factors have been used to describe the pathogenesis making it complex and multifactorial. Although not many well-defined, non-specific responses are described in the pathophysiology of VKC, it can involve variety of factors like genetic predispositions, climate changes and environmental factors (Vichyanond et al., 2014).

2.3 Introduction of MicroRNAs

2.3.1 Nomenclature of microRNAs

To avoid the chances of labelling small interfering RNAs (siRNAs) and other fragments of small RNAs (sRNAs) as miRNAs, a characteristic criterion is set for the identification of miRNA based on their expression and biogenesis. Identification of distinct ~ 22-nt sequence RNA molecules that are complementary to the genome sequence of the species it is isolated from and the presence of a phylogenetically conserved hairpin with regulatory roles are those established criteria. A miRNA would be recognized based upon the evidence that a distinct ~22-nt product assembled in vivo which is further processed from a hairpin precursor by Dicer (Ambros et al., 2003).

Mature miRNAs are typically named using a prefix “miR” and a unique identification number (e.g., miR-1, miR-2…miR-100, etc.). However, their gene loci and pre cursors are named with a prefix “mir” followed by an identification number (e.g., mir-1, mir-2…. mir-100, etc.). The addition suffixes in terms of an identification number are assigned based on its sequence similarity with other miRNAs. Additionally, each miRNA name is preceded by three letters specific to the species they are isolated from. For human species, those three letters are “hsa” (e.g., hsa-miR-1, hsa-miR-2…hsa-miR-100, etc.). Multiple miRNAs that are evolutionarily related will be given an addition suffix letter after the identification number indicating that they are the multiple members from the same family (e.g., hsa-miR-1a and hsa-miR-1b). Furthermore, if two different loci produce same miRNA product, additional number is given after the complete name (e.g. hsa-miR-45a-1 and hsa-miR-45a-2) (Ambros et al., 2003; Griffiths-Jones et al., 2006).

2.3.2 Biogenesis of microRNA

The miRNA synthesis is a complex process involving multiple steps starting from cytoplasm and ending in nucleus. A detailed step-to-step canonical miRNA biogenesis pathway in humans is illustrated in Figure 2.5. Firstly, either RNA polymerase II or RNA polymerase III transcribes miRNA genes into primary miRNA transcripts (pri-miRNAs) (O’Brien et al., 2018). The pri-miRNA generally embedded with mature miRNA sequence, is typically long (over 1 kb) with one or more hairpin structures capped at 5’ and poly A tail (Ha & Kim, 2014).

A nuclear RNase III enzyme called Drosha along with DiGeorge syndrome critical region protein 8 (DGCR8) forms a complex called Microprocessor and initiates the maturation process by cleaving the pri-miRNAs generating smaller precursors of miRNAs (pre-miRNAs) of about 65 nucleotides length (Achkar et al., 2016). These pre-miRNAs are exported from nucleus to cytoplasm for complete maturation through a transport complex formed by Exportin-5 along with Ran-GTP protein (Kawahara, 2014).

The pre-miRNAs are further cleaved into an about 22 nucleotide miRNA duplex by Dicer in along with TAR RNA-binding protein (TRBP) (Qiu et al., 2015). Now, the functional mature strand in miRNA duplex is loaded onto Argonaute proteins (Ago) to form miRNA-induced silencing complex (miRISC). While the other passenger strand is degraded. The miRISC controls the inhibition of target gene expression through translation inhibition or mRNA degradation (Lao & Le, 2020).

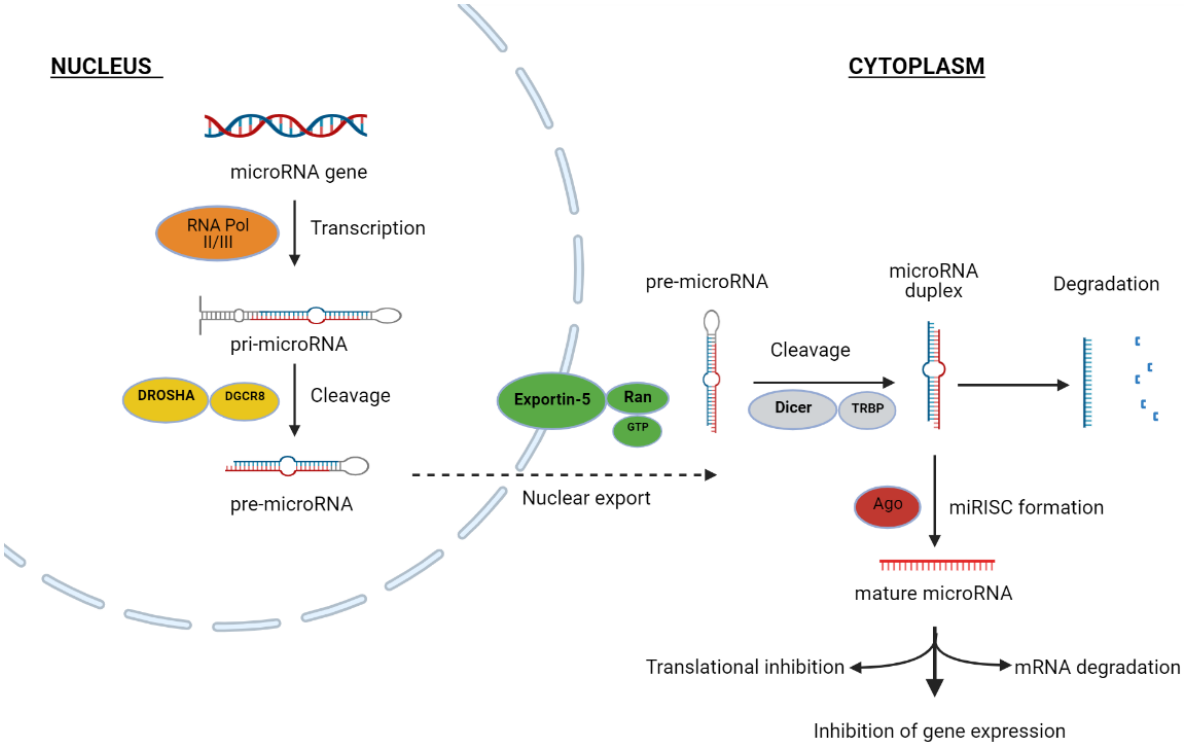

Figure 2.5: Illustration of multi-step canonical miRNA biogenesis pathway in humans. Created using Biorender.

2.3.3 Identification and quantification methods of microRNAs

The miRNAs can be obtained from various tissues and are found in different body fluids such as plasma, serum, saliva, tears, blood, and urine (Weber et al., 2010). They are generally isolated from other molecules through various commercially available total RNA extraction techniques. Different isolation methods yield distinct concentrations of total RNA, therefore it is advised to optimize the extraction method based on the samples source and volume (Brown et al., 2018).

The Qiagen miRNeasy Micro Kit is designed to isolate the total RNA including miRNAs and small RNAs even from small amounts of cells (Qiagen, 2012). Bioanalyzer is an instrument used to analyze RNA using chip based micro-capillary electrophoresis. Most of the current studies employ quantitative Real-time Polymerase Chain Reaction (RT-qPCR), microarray analysis and next generation sequencing (NGS) as the common methods to quantify active miRNAs from different body sources. The comparison of these three methods is illustrated in Table 2.1 (Moody et al., 2017). The miRNAs are generally found to be enriched in exosomes and many studies aim to extract exosomal miRNAs through ultracentrifugation for better yield, especially from body fluids such as plasma and saliva (Kamal et al., 2020).

Table 2.1: Comparison of various miRNA quantification methods (Moody et al., 2017)

Method	**Advantages**	**Limitations**
qPCR	• Gold standard for sensitivity and specificity	• No genome-wide coverage
Microarray	• commercially available reagents • Genome-wide coverage	• Specific probes and lack of reproducibility • Difficult normalization
NGS	• Many samples can run parallelly • Can detect polymorphisms • Promotes novel miRNA discovery • Genome-wide coverage	• Complicated • Non-standardized data analysis

Abbreviation: miRNA: microRNA, qPCR: quantitative polymerase chain reaction, NGS: Next generation sequencing.

MiRNA profiling can lead to identification of disease specific or tissue specific biomarkers and provide better understanding of miRNA regulation and functions (Gao & Jiang, 2016). It is estimated that about 60% of genes are targeted by one or more miRNAs (Rooda et al., 2020). Major advantage of microarray assay is that it can quickly and simultaneously detect the small amounts of miRNA in an experiment and at a lesser cost (Dell'Aversana et al., 2017).

The comparison of different microarray platforms is shown in Table 2.2. If implemented in clinical research, the genome-wide miRNA expression profiling will help in discovering the potential biomarkers and therapeutic targets. It is also reported that miRNA profiles can potentially be used in pharmacogenomics studies (Liu et al., 2008).

Table 2.2: Schematic comparison of different microarray platforms (Liu et al., 2008)

	Exiqon	**Agilent**	**Illumina**	**LC Biosciences**
Platform	miRCURY LNA microRNA arrays	Agilent 60-mer SurePrint technology	Sentrix ® Array Matrix and BeadChips	µParaFlo™ Biochip Array
Channels	Dual	Single	Single	Dual
Labelling	Hy3	Cy3	Cy3	Cy3/Cy5
Input amount of RNA	1000 ng	100 ng	200 ng	1000-3000 ng
miRBase Version	19	21	12	20

Abbreviation: Cy3: cytosine 3 dye, Cy5: cytosine 5 dye, Hy3: Fluorescent dye.

The major experimental steps in miRNA microarray profiling are shown in Figure 2.6. Short length of miRNA and precursor miRNA presence makes it challenging. Agilent technologies with its direct labelling and novel probe design strategy can simultaneously measure hundreds of mature mRNAs. Cy3-labeled pCp molecule is ligated to the 3′ end of the RNA molecule during labeling. The probes include hairpin sequence and are shortened at 5’ end, which helps in selecting mature miRNAs (D’Andrade & Fulmer-