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TOKAT GAZİOSMANPAŞA UNIVERSITY

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ANALYSIS OF ACTIN RELATED PROTEIN 2/3 COMPLEX SUBUNIT 2 GENE EXPRESSION IN PTERYGIUM

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Master Thesis

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TOKAT – 2021

The defense examination of the thesis study titled “Analysis of Actin Related Protein 2/3 Complex Subunit 2 Gene Expression in Pterygium’’ is prepared by **BALEN ZAINAL** was held on 29/07/2021 it has been accepted as a Master Thesis at Tokat Gaziosmanpaşa University Graduate School Institute Department of MEDICAL BIOLOGY.

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29/7/2021

BALEN ANWAR ZAINAL

I Present This to My Parents

May God have Mercy on Them

ACKNOWLEDGMENT

First I'm thankful and grateful to Almighty God for making this research possible and allowing me to finish it without problem. All praise to God.

I would like express my gratitude and give many thanks to my supervisor Prof. Dr. Hacı Ömer ATEŞ. Who helped me and supported me in conducting my research, and offered help with his kindness in a place we didn't know anything about. I am grateful to the Institute of Health Sciences for allowing me to pursue my research at TOKAT GAZİOSMANPAŞA UNIVERSITY Department of Medical Biology. Also to Emel ENSARİ and Nihan BOZKURT for their unforgettable help and advice throughout my research in conducting experimental studies.

And I'm very grateful and thankful to Dr. Sardar Muhammadrashid Karim for his tremendous help, geniality, guidance and advice in writing my thesis. To my dear wife who was a source of encouragement and energy for me during my thesis. May God bless her.

And I would like to express my sincere love, respect and thanks to my parents and mother and father in law who supported spiritually, prayed for me and believed in me, and to all my brothers and sisters who were as source of joy for me and kept me going for being there.

The Scientific Research Projects Commission of Tokat Gaziosmanpaşa University supported the thesis.

(Project No: 2019/110) (Tokat Gaziosmanpaşa University)

Project No: 215S692 (TUBITAK)

ABSTRACT

ANALYSIS OF ACTIN RELATED PROTEIN 2/3 COMPLEX SUBUNIT 2 GENE EXPRESSION IN PTERYGIUM

Tokat Gaziosmanpaşa University, Institute of Health and Science, Department of Medical Biology

Balen Anwar Zainal, Master thesis

Tokat- 2021

The most important aspects of diagnosing a disease are determining its cause and then attempting to find a treatment. Pterygium is a disorder that isn't completely understood yet and it's described as a growth disorder by some and a degenerative disorder by others. However, several studies have been conducted in this field, and certain factors for this disease have been identified, including UV, virus, growth factors, apoptosis, ECM, MMP, and interleukins. Uncontrolled proliferation of conjunctiva cells onto the cornea, metaplasia, and certain reports of malignant traits have been documented. Furthermore, the ARPC-2 gene has been discovered to play a role in the progression and induction of a certain type of cancer, which is one of the Arp 2/3 complex's subunits that is involved in actin assembly and therefore sustains and maintains cellular integrity, the objective of this paper is to determine how ARPC-2 gene expression relates to pterygium development and formation. The quantitative reverse transcription PCR method was used to determine the rate of gene expression in 18 samples with pterygium and 18 normal conjunctiva of the same eye. The gathered data showed that the expression of ARPC-2 in pterygium has up-regulated (3.24±1,75) compared to normal conjunctiva, although there was not a significant difference between the two groups ($p = 0.209$). In conclusion the up-regulation of ARPC-2 gene is thought that it may contribute to pterygium development and formation.

Key words: pterygium, Arp2/3, ARPC-2, qRT-PCR

ÖZET

PTERYGIUMDA ACTIN ILE ILGILI PROTEIN 2/3 KOMPLEKS ALT BIRA 2 GEN EKSPRESYONUNUN ANALIZI

Tokat Gaziosmanpaşa Üniversitesi Sağlık ve Fen Bilimleri Enstitüsü Tıbbi Biyoloji Anabilim Dalı

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Tokat-2021

Bir hastalığı teşhis etmenin en önemli yönleri nedenini belirlemek ve daha sonra bir tedavi bulmaya çalışmaktır. Pterygium henüz tam olarak anlaşılamayan bir hastalıktır ve bazıları tarafından büyüme bozukluğu ve diğerleri tarafından dejeneratif bir bozukluk olarak tanımlanır. Bununla birlikte, bu alanda çeşitli çalışmalar yapılmıştır ve bu hastalık için UV, virüs, büyüme faktörleri, apoptoz, ECM, MMP ve interlokinler dahil olmak üzere bazı faktörler tanımlanmıştır. Konjonktiva hücrelerinin kornea, metaplazi ve kötü huylu özelliklerin belirli raporları üzerine kontrolsüz çoğalması belgelenmiştir. Ayrıca, ARPC-2 geninin, Arp 2/3 kompleksinin aksin montajında yer alan ve bu nedenle hücresel bütünlüğü koruyan ve koruyan alt birliğinden biri olan belirli bir kanser türünün ilerlemesinde ve indüksiyonunda rol oynadığı keşfedilmiştir, Bu makalenin amacı ARPC-2 gen ekspresyonunun pterygium gelişimi ve oluşumu ile nasıl ilişkili olduğunu belirlemektir. Pterygiumlu 18 örnekte ve aynı gözün 18 normal konjonktivasında gen ekspresyon oranını belirlemek için nicel ters transkripsiyon PCR yöntemi kullanılmıştır. Toplanan veriler, pterygium'daki ARPC-2 ekspresyonunun normal konjonktivaya kıyasla yukarı düzenlenmiş (3.24±1,75) olduğunu, ancak iki grup arasında anlamlı bir fark olmadığını göstermiştir ($p = 0.209$). Sonuç olarak ARPC-2 geninin yukarı regülasyonunun pterygium gelişimine ve oluşumuna katkıda bulunabileceği düşünülmektedir.

Anahtar Kelimeler: pterygium, Arp2/3, ARPC-2, qRT-PZR

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviation	Meaning
UV	: Ultraviolet Radiation
DNA	: Doxyribinucleic Acid
MMC	: Mitomycin-C
5-FU	: 5-Fluorouracil
RNA	: Ribonucleic Acid
Arp2/3 complex	: Actin Related Protein 2/3 Complex
Arp 2	: Actin Related Protein 2
Arp 3,	: Actin Related Protein 3
ARPC 1-5	: Actin Related Protein 2/3 Complex Subunit 1-5
ARPC-2	: Actin Related Protein 2/3 Complex Subunit 2
ECM	: Extracellular Matrix
CAG	: Conjunctival Autograft
LSC	: Limbal Stem Cells
CsA	: Cyclosporine A
ROS	: Reactive Oxygen Species
8-OhdG	: 8-hydroxydeoxyguanosine
hOGG1	: Human 8-oxoguanine glycosylase
IL-1	: Interleukin-1
IL-6	: Interleukin-6
IL-8	: Interleukin-8
TNF-α	: Tumor Necrosis Factor Alpha
ERK	: Extracellular Signal-Regulated Kinase
MAPK	: 1/2 Mitogen-Activated Protein Kinase
PSMB5	: Proteasome Subunit Beta Type-5

Nrf2-AREN pathway	: Nuclear Factor Erythroid 2-Related Factor 2 - Antioxidant Response Element Pathway
HB-EGF	: Heparin-Binding Epidermal Growth Factor-Like Growth Factor
HBV	: Hepatitis B Virus
HCV	: Hepatitis C Virus
HPV	: Human Papillomavirus
EBV	: Epstein Barr Virus
HHV8	: Human Herpes Virus 8
MCPyV	: Merkel Cell Polyomavirus
HTLV-1	: Human T-lymphotropic Virus Type 1
HSV	: Herpes Simplex Virus
CMV	: Cytomegalovirus
INK4	: Cyclin Dependent Kinase Inhibitors -4
PTEN	: Phosphate and Tensin Homolog
APC	: Adenomatous Polyposis Coli
MADR2	: Mothers Against Decapentaplegic Homolog 2
BRCA1	: Breast Cancer Genes 1
BRCA2	: Breast Cancer Genes 2
ERK	: Extracellular Signal-Regulated Kinase
VEGF	: Vascular Endothelial Growth Factor
TGF-b	: Transforming Growth Factor-Beta
bFGF	: Basic Fibroblast Growth Factor
IGF	: Insulin-like Growth Factor
NGF	: Nervous Growth Factor
CTGF	: Connective Tissue Growth Factor
VEGFR-1	: Vascular Endothelial Growth Factor-1
VEGFR-2	: Vascular Endothelial Growth Factor-2
VEGFR-3	: Vascular Endothelial Growth Factor-3
NO	: Nitric Oxide
Cyr61	: Cysteine-rich Protein 61

CTGF	: Connective Tissue Growth Factor
NOV	: Nephroblastoma Overexpressed Gene
PCNA	: Proliferating Cell Nuclear Antigen
AP-1	: Activator protein-1
Bcl-2	: B-cell lymphoma 2
mTOR	: Mammalian Target of Rapamycin
MMPs	: Matrix Metalloproteinase
ECM	: Extracellular Matrix
SPARC	: Secreted Protein Acidic and Rich in Cysteine
CsA	: Cyclosporine A
LOX	: Anti-lysyl Oxidase
FBLN5	: Fibulin-5
FBN1	: Fibrillin-1
VD	: Vitamin D
VDR	: Vitamin D receptor
NPFs	: Nucleation Promoting Factors
qRT-PCR	: Quantitative Real Time PCR
CT	: Cycle Threshold
ACTB	: Actin-B
GAPDH	: Glyceraldehyde-3-phosphate dehydrogenase
H3	: Histone H3
SEM	: Standard error mean
siRNA	: Small interfering RNA

1. INTRODUCTION

The name "pterygium" comes from the Greek word "pteron," which means "wing." which is a fibrovascular growth disorder with proliferating cells that is triangular, wing-shaped which abnormally grows from bulbar conjunctiva tissue onto the cornea, also its thought that UV alters the limbal stem cells which leads to over growth of conjunctival cells over the cornea. Historically degeneration of Bowman's layer and elastosis made ptrerygium to be regarded as degenerative lesions in pterygia (Chui et al., 2011a; Shtein, 2013).

Pterygium causes distortion in vision, dryness, irritation feeling in the eye and reduce the movement of the eye and blindness in rare cases (Clearfield et al., 2016). It also induces astigmatism which is a condition in which the eyes cannot focus light on retina rather its scattered due to refractive error of the eye surfaces (Maheshwari, 2007).Other characteristics have been reported, such as epithelial proliferation, goblet cell hyperplasia, elastosis, angiogenesis, stromal plaques, and Bowman's membrane dissolution, also The coordinated actions of cytokines, growth factors, and matrix metalloproteinases in an active fibroblastic stroma mediate inflammation, neovascularization, and matrix remodeling (Chui et al., 2011a; Džunić et al., 2010).

The cause of pterygium not yet fully known its predominance is different from place to place depending on geographical location and other factors that cause the disease the such as rate of exposure to UV light, latitude, inflammation, age, some viruses and life style of the individual people under sun exposure outdoor (e.g. Farmers, construction workers, welders...etc) are more prone than those work indoor (Džunić et al., 2010; Hossain, 2011). Most occurrence of pterygium has been recorded in old age such as people above age 49 are high especially in male gender in correlation with working outdoor (Ma et al., 2007). The predominance of pterygium in rural areas is more compare to urban because of the different of life style of the people and environmental factors (Nemet et al., 2016). UV light is considered as a major factor for development of pterygia it has been studied that induces activation of pro -inflammation protein and has effect on subconjunctival connective tissue. Chronic inflammation and growth factors also UV damage DNA causes formation of dimers which there was sign of this in pterygium and may indirectly cause it by formation of UV byproducts but still

the factors of causing pterygium are being studied and not fully understood (Goodsell, 2001; C.-W. Lu et al., 2017). Viruses may also cause pterygium most common studied is Human papillomavirus (HPV) (Gallagher et al., 2001). In addition, HPV also has been linked to cause tumor of epithelial cells of conjunctiva comprising conjunctival papilloma (Sjö et al., 2001).

Anti-inflammatory drops, ointments and artificial tear used to treat pterygium but surgery has been known as best treatment for pterygium as conjunctival autograft and amniotic membrane graft most common techniques is conjunctival autograft since the day its been introduced (Kenyon et al., 1985; Marsit et al., 2016). However, pterygium has malignant trait its recurrence is still problem to reduce the recurrence there have been reports of drugs that may lower the reccurence rate such as Mitomycin-C (MMC is anti-tumor antibiotic “anti-metabolite” that repress tumor cell rapid groth by assisting DNA depolymerization and prevent RNA dependent DNA synthesis) which affect proliferation of fibroblasts and vascular endothelial (Rubinfeld et al., 1992), 5-Fluorouracil an anti-metabolite that works as DNA inhibitor strongly active against fibroblast cells reduces the proliferation rate of fibroblast cells. Moreover, Subconjunctival membrane graft is used to reduce the recurrence of pterygium by reducing fibrosis through covering the excised region of pterygium (Altay & Balta, 2016; Shusko & Hovanesian, 2016).

Microfilaments have role in forming cytoskeleton which are polymers of Actin protein which forms a network in cell that gives cell shape, support, integrity and drives cell locomotion, phagocytosis, and intracellular motility of vesicles, organelles, and certain pathogens. Arp2/3 complex is a group of proteins that regulate actin assembly and formation of new actin filament branch which is composed of two major subunits Arp 2 and Arp 3, and five other subunits ARPC 1-5 (Lodish et al., 2000; Rouiller et al., 2008). ARPC-2 gene encodes for one of Arp2/3 complex subunits that have role in regulating actin assembly. Which has been reported that have role in progress, development and proliferating cells of human gastric cancer (J. Zhang et al., 2017). In addition, pterygium has been reported that has cancer characteristics as invading normal cells and high recurrence rate, and the cells of pterygium have been shown changed in shape which they displayed squamous metaplasia with increased goblet cell density (Chui et al., 2011a; Pajic et al., 2016).

Genetic expression is regarded as mRNA copying the genetic information on DNA strand which acts as a template, codes of a specific gene on DNA is copied by means of transcription this information then translated to protein, this protein participate in cellular function and leads to expression of phenotypic traits. High or low activity of this process will lead to up-regulation or down-regulation of specific genes which results in abnormality in expression of protein that can be used to find defect in cells and understand diseases (Engelsvold et al., 2013; Is & Cs, 2016).

Since ACTR-2 has role in cell proliferation and progression of cancers, and have role in shaping of cell through regulating actin assembly. It's believed that this gene may have role in development and formation of pterygium. I aim to find the connection between ARPC-2 and pterygium through investigating expression level. To understand pterygium pathogenesis more and what is ARPC-2 role in pterygium due poorly understanding of the subjects and few studies regarding ARPC-2 related to pterygium has been conducted.

2. GENERAL BACKGROUND

2.1. Pterygium Disorder

Pterygium is a fibrovascular growth on the ocular surface that has wing-like or triangular shapes, its considered as a degenerative disorder and there are evidences consider it as a growth disorder rather than degeneration (C.-W. Lu et al., 2017; D. T. Tan et al., 1997), it's also suggested that it has characteristics of a tumor-like disorder invading other cells and high recurrence rate (Van Acker et al., 2019),With a fleshy color in which the conjunctiva over-growth and extends over cornea by passing the limbus of the eye (Figure 2.1) (Clearfield et al., 2016), Or by degrading limbus which it leads to entering of conjunctiva onto cornea which was as a barrier between the two parts (Chui et al., 2011a). The functions of these parts of the eyes mentioned above are: The first, conjunctiva which is a mucus membrane that coats the inner side of the eyelids and protects the surface of the eye from bacteria and viruses and also provides immune tissue and a mucus layer tear film. The second, cornea which is a colorless avascular part of the eye that covers the pupil and iris and is highly reflective, allowing light to enter the inner side of the eye. The third, limbus which is a line of tissue that separates the cornea from the conjunctiva and sclera and contains corneal epithelial stem cells (Figure 2.2) (Smolin et al., 2005).

Visual impairment, cosmetic and eye movement problems can occur due to the pterygium induced astigmatism and the degree of astigmatism increases by increased grading of pterygium (Maheshwari, 2007), there are four stages of pterygium (Figure 2.3) Grade 1: pterygium starts from nasal and the fibrovascular tissue reaches the margin of limbus of the eye. Grade 2: the pterygiums fibrovascular tissue goes over the cornea. Grade 3: the fibrovascular tissue it extends more to the pupil margin. Grade 4: when the fibrovascular tissue passes the margin and reaches the pupil of the eye (Wanzeler et al., 2019). If the fibrovascular tissue of pterygium starts from nasal side of the eye and reaches the pupillary margin it's called nasal pterygium which is most common and if it starts from the temporal side of the eye its and reaches the temporal pupillary margin it's called temporal pterygium (Figure 2.4) (Maheshwari, 2007). A completely grown pterygium consists of four parts (Figure2.5) a cap which in-front of

the head of pterygium, and a neck and body each on different places on eye cornea, limbus and sclera respectively (Anguria et al., 2014).

There are many studies suggested different factors that have been shown to be involved in pterygium pathogenesis such as inflammation process, ECM remodeling, cytokines, anti-apoptotic mechanism, oxidative stress, viral infection, wound healing defect, hypoxic ischemic injury, anomaly in epithelial differentiation and well known and accepted factor for pterygium considered to be UV (Maxia et al., 2017). And the major UV factor for pterygium is considered to be UVB radiation which induce some biological changes in eyes such as causing high expression of many intracellular molecules (e.g. cytokines, pro-inflammatory, interleukins and growth factors) and they may induce pterygium through uncontrolled proliferation of conjunctiva cells (O'Brien et al., 2011).It has been suggested also sex, age, windy, dry, dusty, hot and smoky environments have role in inducing and increasing in progression of pterygium disorder (Hall, 2016; C. S. H. Tan et al., 2006). Moreover, studies regarding heredity of pterygium has been conducted and suggested that pterygia a autosomal dominant trait (Hecht & Shoptaugh, 1990; Romano et al., 2016).

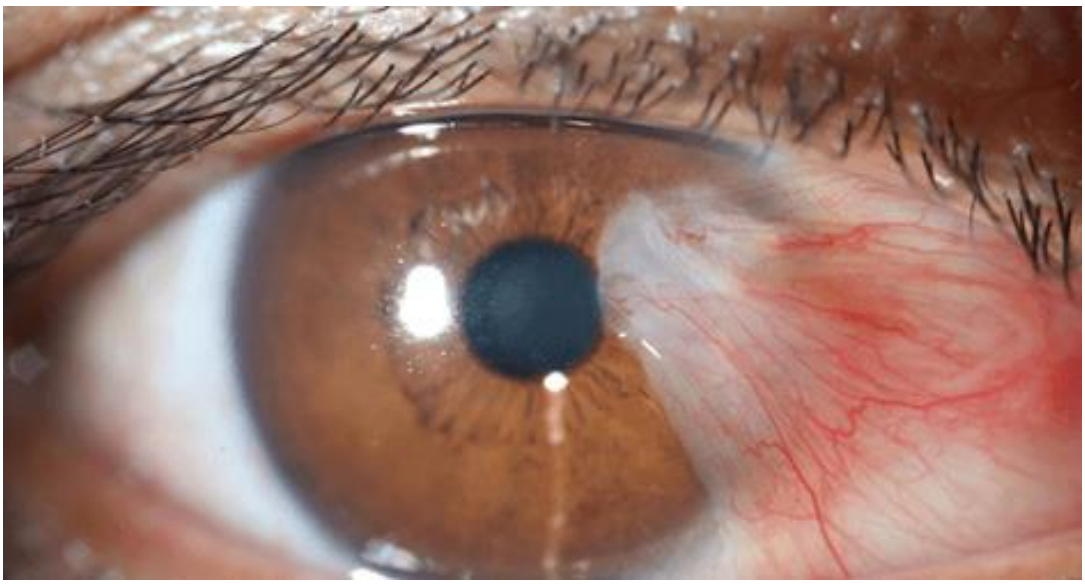

Figure 2.1: Human eye covered with Pterygium.
https://www.clarityeye.ca/pterygiums/

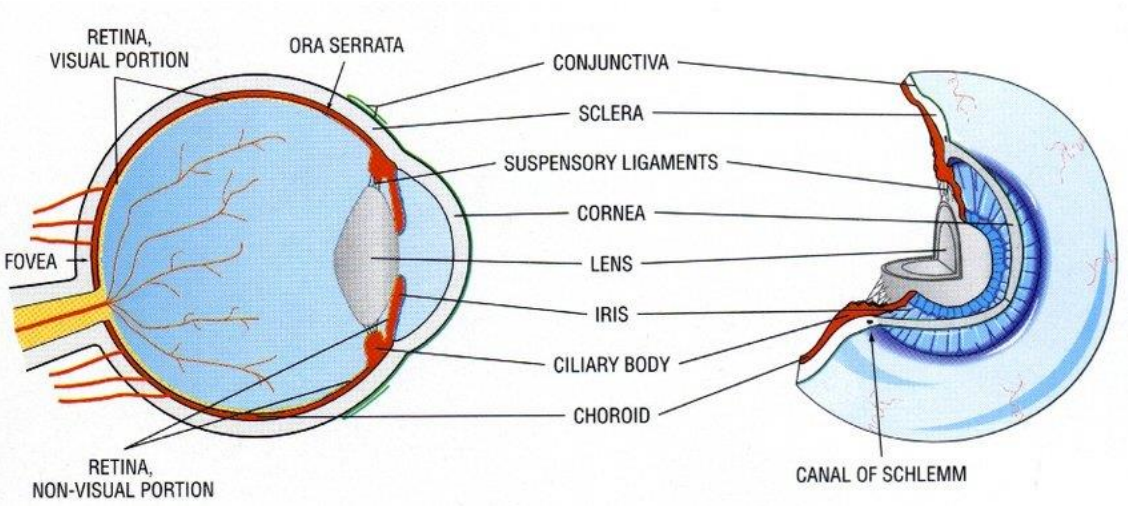

Figure 2.2: Human eye anatomy, showing cross-section and external view of eye. https://www.researchgate.net/figure/Anatomy-of-the-eye-a-External-view-The-boundary-between-the-sclera-and-the-iris-is_fig25_238690003

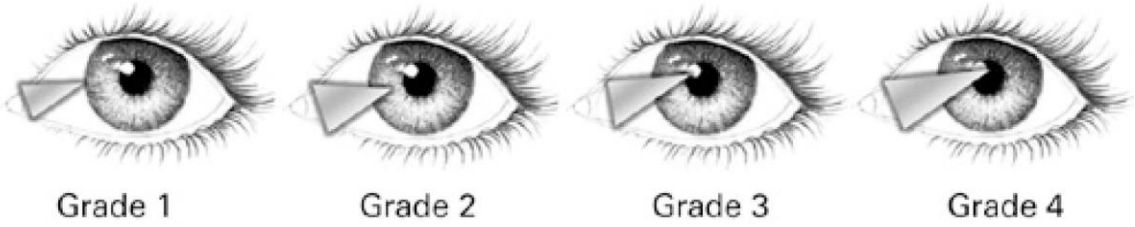

Figure 2.3: Grades of pterygium development. https://www.scielo.br/img/revistas/abo/v82n6//0004-2749-abo-20190103-gf01.jpg

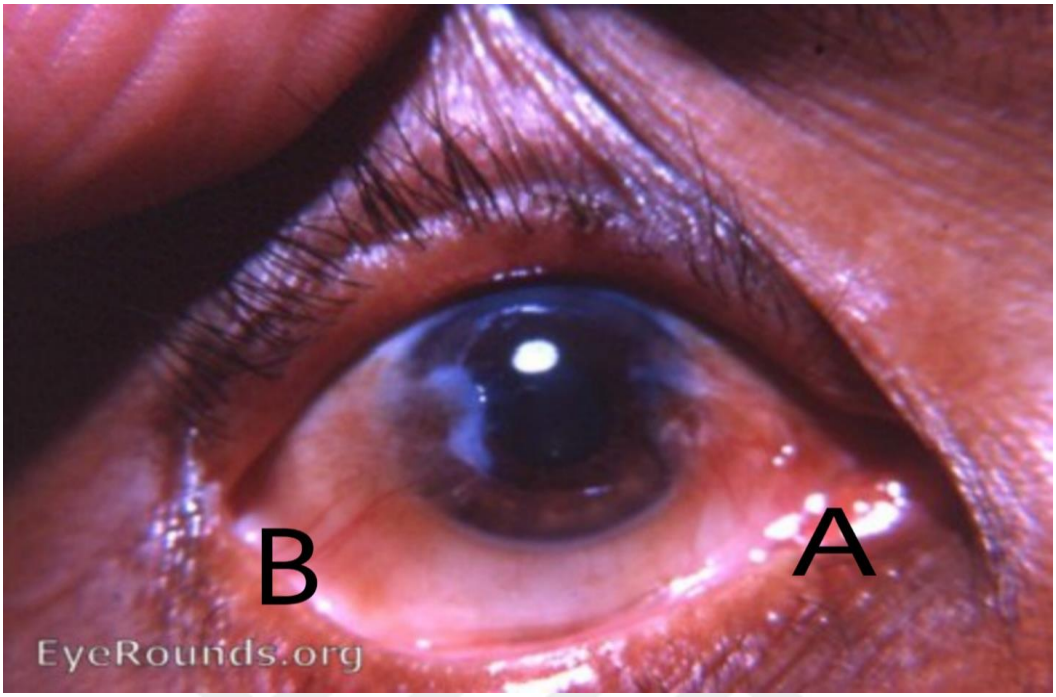

Figure 2.4: human eye showing, A: nasal pterygium, B: temporal pterygium. https://eyerounds.org/atlas/pages/pterygium.html

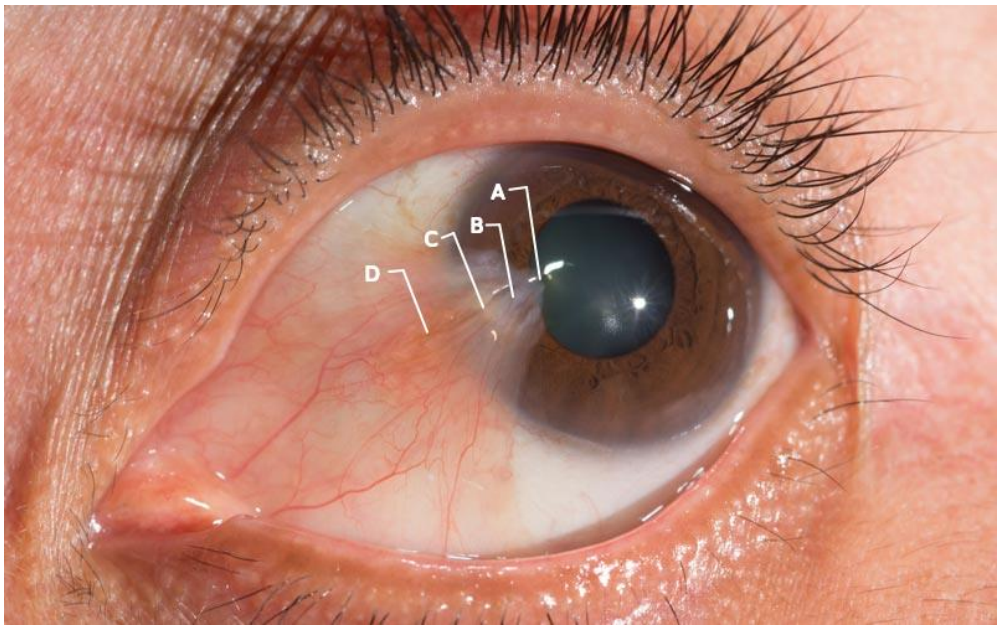

Figure 2.5: pterygium parts. A- Cap, B- head, C- Beck, D- Body https://leonau.b-cdn.net/images/site/eye-conditions/Pterygium.jpg

2.2. Treatment

2.2.1. Treatment by Excision

The best treatment for pterygium is surgical excision of the fibrovascular tissue; however, none of the treatment techniques that have been discovered to treat pterygia can totally prevent recurrence, and each technique has a variable rate of recurrence after surgery (post-surgery recurrence) (Clearfield et al., 2016). The recurrence time after surgery has been studied and 91.6% of the recurrence occurred 360 days after the pterygium excision and others suggested 50% chance of recurrence cases happened after 120 days and 97% chance happened after 12 months of the after surgery and excision of pterygium (Avisar et al., 2001; Hirst et al., 1994). Due to lack of control of this disorder's recurrence there many ways have been suggested for treatment in two ways first by surgical treatments such as bare sclera excision, excision with conjunctival autograft and amniotic membrane graft, Conjunctival transpositional flap, excision with simple conjunctival closure and second any type removal with use of antimetabolite adjuvants such as MMC and 5-Fluorouracil, and Adjuvant therapies such as radiotherapy, antimetabolite, angiogenesis inhibitor and immunosuppressive drugs (Nuzzi & Tridico, 2018).

An early pterygium doesn't require surgery unless it causes visual impairment, inflammation, motility restriction, cosmetic problems and in rare cases blindness (Maheshwari, 2007). Evidence show sometimes after surgery some problems occur to the corneal refraction and with bigger size of the pterygium the greater relation to the corneal refraction involving astigmatism, asymmetry, irregularity and spherical power of the eye (Tomidokoro et al., 2000). But with a successful and good surgery for pterygium excision the eye abnormalities get better such as eye refractive and astigmatism (Stern & Lin, 1998). Some studies have shown that conjunctival autograft is better than other techniques due its safeness and effectiveness, and regarding recurrence it has lower rates than other techniques (Mahdy & Bhatia, 2009).

The surgery treatments include first Conjunctival graft which is a technique that the pterygium fibrovascular tissue is excised and then a same size in another part of the bulbar conjunctiva is marked, cut and placed on the site of excised pterygium on conjunctiva (Figure 2.5) (Singh, 2017). In addition, using limbal stem cells with

conjunctival autograft transplantation has been shown it has very low rate of recurrence by using thin CAG and LSC may take a long time but it's very effective and has lowest rate 4.75% of reccurence of pterygium (Mahdy & Bhatia, 2009).

Second simple conjunctival closure includes surgical removing pterygium and closing the defect place of the removed tissue to sclera on each side of the wound and compared with other techniques the recurrence rate has been reported to be very high. And third removing the pterygium without doing anything else such as closing the wound or trying to cover the removed place it's called bare sclera excision (Figure 2.5)**,** which it has been known for its high rate of recurrence 38% to 88% due to this it's not recommended frequently for pterygia treatment (Noureddin & Yeung, 2016).

Fourth amniotic membrane transplantation from the innermost layer of placenta on to the removed part of the conjunctiva after the removing of the pterygium tissue with the stroma facing the bare sclera and basement membrane facing up with fibrin glue for fixation (Figure 2.6), amniotic membrane has many biological effects on the and facilitate many process such as reducing inflammation, epithelialization, vascularization, scarring considered to be better than bare sclera technique and conjunctival autograft though it's still unclear due its recurrence rate which is 3.8% and 40.9% (Nuzzi & Tridico, 2018). In addition, Conjunctival inflammation can occur after amniotic membrane transplantation with mitomycin-C if fibrin glue is not used in the surgery (Kheirkhah et al., 2008). There are also another way to use amniotic membrane by hyperdrying the fresh human amniotic membrane which its fibers are not destroyed by the hyperdrying method because it becomes fresh again when it absorbs water and ready to use and it can be used to cover large surface area of defected regions such as double-headed pterygium in this study by Pan et al showed that the rate of the group recurrence (5.06%) by this method was lower than the group by conjunctival autograft (20.97%) (Pan et al., 2018), also using amniotic membrane transplantation with limbal autografting was shown to have good result for patients with recurrent pterygium associated with symblepharon (Shimazaki et al., 1998).

Fifth the Conjunctival transpositional flap is cutting a part of conjunctival near the excised pterygium and sliding it around and covering the defected part (Figure 2.7), it has variety of recurrence rate from 3.2% to 33.33%, in this technique peribulbar anesthesia is not required and graft loss and inversion is not a problem, aslo its less time consuming (Bilge, 2018). The Conjunctival autograft for recurrent to date stays as better and preferred more technique than other ones because it has less complications and low rate of recurrence results though its hard and takes time (Alpay et al., 2009). Although another technique has been reported by Lawrence W. Hirst which is pterygium extended removal followed by extended conjunctival transplantation the P.E.R.F.E.C.T technique he showed that with this technique the recurrence rate of pterygium is really low nearly 0%, in 250 consecutive patients only had (0.4%) rate of recurrence and has few complications such as is that it takes long time (Hirst, 2008).

2.2.2. Adjuvant Treatment Therapies

Mitomycin-C (MMC) is an antibiotic used to first was used to treat pterygium recurrence in japan by Kunitomo and Mori. Its isolated from an actinobacteria (Streptomyces caespitosis) it inhibits the synthesis of DNA, RNA and protein mostly used as anticancer but in use for pterygium has shown some problems the exact dosage is unknown but using with surgery has been reported to lower rate of recurrence (Alpay et al., 2009). Use of conjunctival autograft with antimetabolite adjuvants such as mitomycin-C in low dose gives an effective result after surgery with low rate of recurrence and no complication after long time of surgery (P. P. Chen et al., 1995), also using MMC with bare sclera has shown a good result by using MMC one month before the surgery is found to be effective in a study by (Mohammed, 2013).

5 Fluorouracil (5-FU) also has been used for treatment of pterygium and preventing the recurrence of pterygium its effective and safe to use for preventing pterygium recurrence but MMC is stronger to use regarding recurrence preventing and cause fewer problems for the patients (Altay & Balta, 2016). Using 20% ethanol for pterygium recurrence after and before surgery for short time has been conducted and it showed a good result by showing low rate of recurrence, no cosmetic problems, low risks and simplicity of the procedure it was suggested as an alternative treatment for pterygium (K.-H. Chen & Hsu, 2006).

Other drugs such as Bevacizumab and Cyclosporine A (CsA) which they are an angiogenesis inhibitor and immunosuppressive drug respectively both has been shown good result in preventing the recurrence of pterygium and its management (Y.-H. Kim et al., 2017). And radiation therapy after the pterygium excision has been used and beta irradiation was confirmed that is effective and reduces the rate of pterygium recurrence and its satisfactory regarding cosmetic problem for the patient (Wilder et al., 1992).

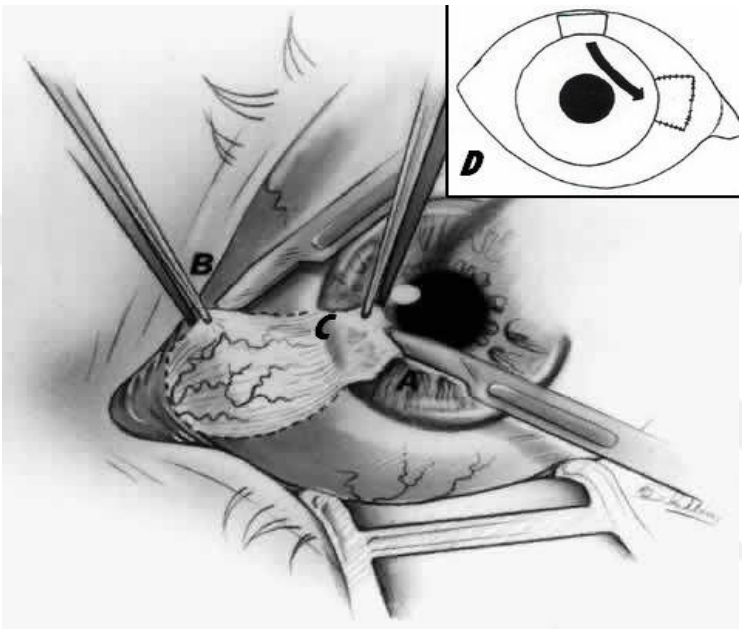

Figure 2.6: A, B, C- removing the pterygium parts using bare sclera, D- covering it by conjunctival autograf. https://epomedicine.com/wp-content/uploads/2014/06/Bare-sclera-technique-and-conjunctival-autograft-pterygium.jpg

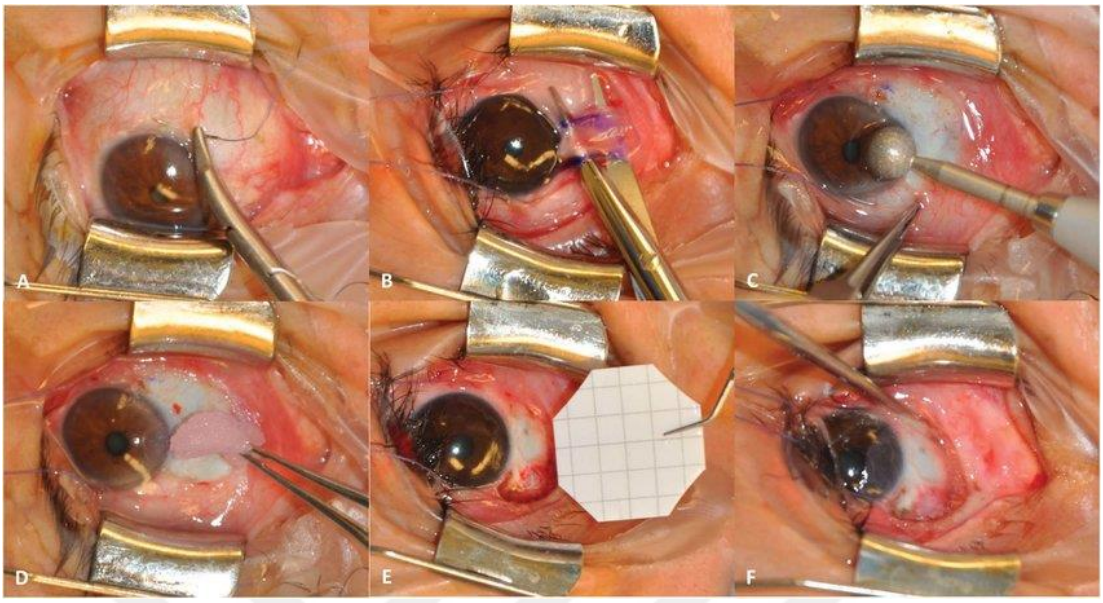

Figure 2.7: Amniotic Membrane Graft, A-Traction suture, B- pterygium removing, C-cornea polishing by diamond burr, D- applying MMC, E- Amniotic Membrane cut size, F- Amniotic Membrane covering the defected place of surgery. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5768221/figure/F1/

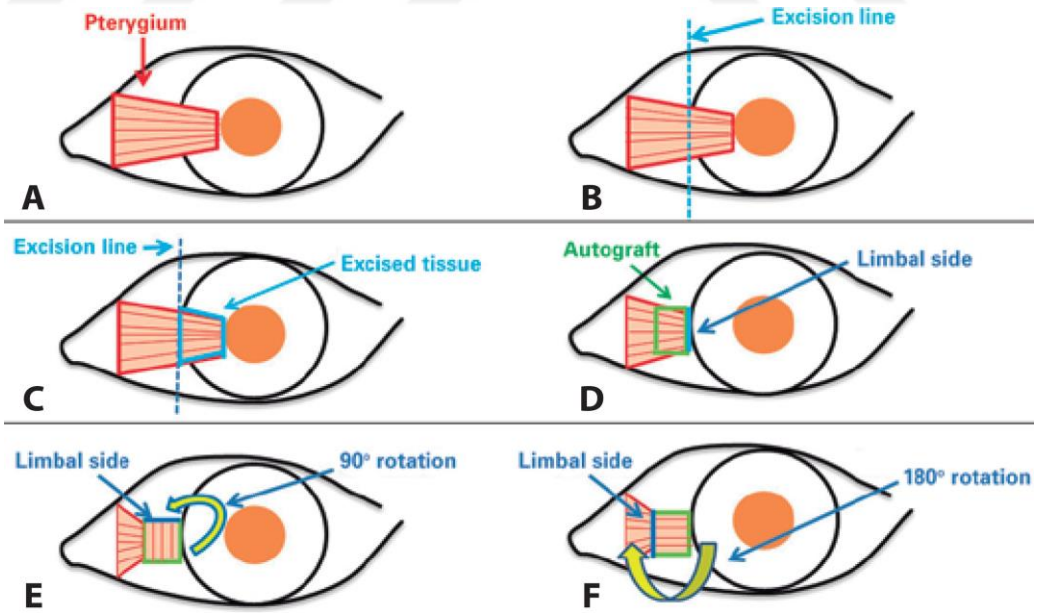

Figure 2.8: Steps of Conjunctival transpositional flap. https://www.scielo.br/img/revistas/abo/v80n6//0004-2749-abo-80-06-0373-gf01.jpg

2.3. Epidemiology and Etiology

Pterygium has been suggested that is most prevalence in “pterygium belt” which includes many Asian countries such as Japan, China, Nepal, Malaysia, Singapore, India and Indonesia which they are in between the 37 degree of south and north of the earths equatorial (Figure 2.8) (Hashemi et al., 2016). The distribution and analysis of pterygium has been studied in many countries but a large scale study about it has not yet been done. In a meta-analytic study that was conducted in 2018 included 24 countries from 68 articles the epidemiology of pterygium was 12% and the highest ratio of occurrence was in china 53% and lowest was in Saudi Arabia 0.07% (Rezvan et al., 2018). Most pterygium cases with high occurrence is in rural areas of the countries due to their lifestyle and duration of hours under the sun (T. Chen et al., 2015), According to a study conducted in indigenous tribes of the Brazilian Amazonian rain forest, the prevalence of pterygium was 36.6 %, and there were differences among the communities of populations according to social behavior and sun exposure duration (Paula et al., 2006). In a rural area population of Doumen district southern China the prevalence of pterygium was 33.01% in people 50 years or above it was higher in female than males (Wu et al., 2002). In central Myanmar there was a high ratio of pterygium suggested in correlation to outdoor working in people 40 and above (Durkin et al., 2008). In another study in Kumejima Island, Japan the rate of pterygium prevalence was very high 30.8% in 40 years or older Japanese adults the risk factors were related to outdoor working and old age and male gender (Shiroma et al., 2009). Another study suggested that the Mongolian population at high altitude in Henan County, China were prone to high risk of pterygium due to high altitude, long time sunlight exposure and low educational level, its prevalence was in 40 years and above 17.9% (J. Lu et al., 2009). Prevalence of pterygium among residents of Pulau Jaloh an island in Indonesia was high the prevalence ratio was 17.0% in different ages with 22.8 years as mean age. With increasing in age the rate of pterygium development has increased (C. S. H. Tan et al., 2006). In high altitude Tibetan population in Zeku, China a study by Lu at al it was shown that the prevalence of pterygium was high 14.49% the related factor was sunlight exposure time (P. Lu et al., 2007). In another rural area in central India the pterygium commonness was 13% in 30 years old and above, and risk factors were correlated with the disease was time spending outdoor under the sun, male

and old age were all associated with pterygium development (Nangia et al., 2013). with all these reports its shown that in urban areas pterygium prevalence is lower compare to rural areas due to difference in lifestyle (T. Chen et al., 2015). Such as in Spain, Qatar and Saudi Arabia the rate of pterygium prevalence 5.9%, 6.2% and 0.074% respectively was too low compare to other places (Alqahtani, 2013; Hosni, 1977; Viso et al., 2011). Though some studies showed that in a rural area such as Harbin pterygium prevalence was lower compare to other region in the world (Li & Cui, 2013). As in some urban areas such as in a multiethnic Population-based study in Singapore the prevalence of pterygium was 10.1% it was high in Malaysian people compare to Chinese and Indian with controlling other factors race was an important factor in this study suggested by (Ang et al., 2012).

The origin and pathogenesis of pterygium is still unclear and yet not fully known, but it's been suggested that UV has major impact on pterygium specially UVB is has been confirmed it damages cornea and induces pterygium formation. Moreover, the rate exposure of the sun light has strong connection to the pterygium pathogenesis the time spent under the sunlight it's better to be protected by any ocular protector such as sunglasses and hat (Threlfall & English, 1999). In a study regarding outdoor workers the Commercial motorcyclists in Nigeria the result showed that long time exposure to the sunlight has effect on pterygium prevalence without using proper protection against sunlight UV (Achigbu & Ezepue, 2014). The UV from excessive sunlight exposure can damage eye cells and corneal damage by absorbing the wavelength of UVB which can be absorbed by cornea of the eye to protect the inner side of the eye (B.-Y. Chen et al., 2013). Also age and male gender has been recognized as a risk factor for prevalence and increasing of pterygium (Sun et al., 2013), but in other studies have shown that pterygium prevalence was higher in females this shows that sex maybe not an important risk factor to count on due to its controversy (Wang et al., 2020). Moreover, there are evidence that sex and age has major effect on increasing prevalence of pterygium (C. S. H. Tan et al., 2006), also evidence of proliferation of pterygium cells by virus has been reported such as HPV inducing proliferation of pterygium epithelial cells (Varinli et al., 1994).

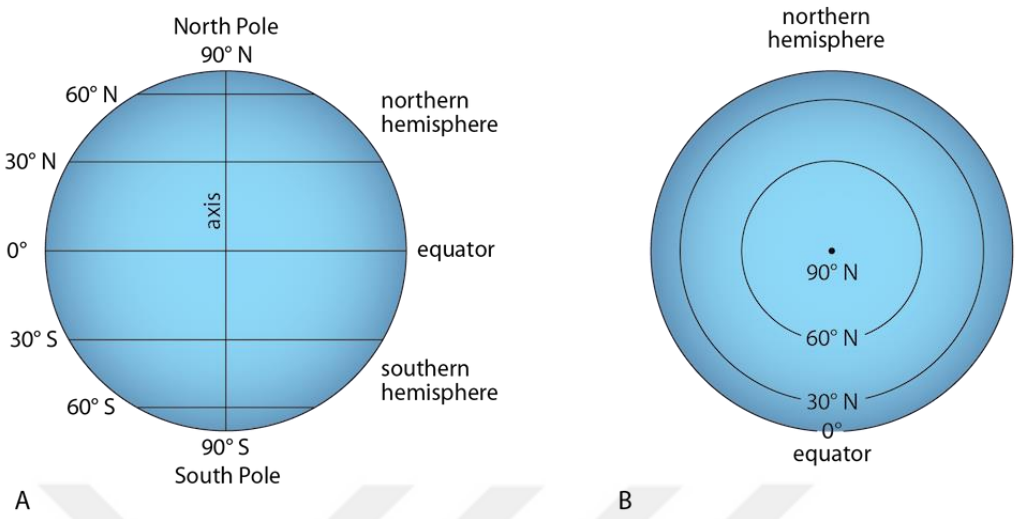

Figure 2.9: A-showing earth equator between north and south pole, B- showing the earth equatorial from either pole.
https://manoa.hawaii.edu/exploringourfluidearth/physical/world-ocean/locating-points-globe

2.4. Non Intracellular Factors

2.4.1. UV Radiation

The long term exposure to sunlight leads to extensive UV radiation absorption by human's body which can cause many complications such as hair loss, blisters, fast aging, burning of skin, immune irregularity and risk of causing cancers. There are three types of UV that comes from the sun according to their wavelength: UVA with (320nm-400nm), UVB with (290nm – 320nm) and UVC with (200nm -280nm) wavelengths, UV A and B are more to be concerned about compare to UVC because its wavelength its blocked by ozone layer (Figure 2.9) (Dale Wilson et al., 2012), In addition, relation between sunlight exposure and pterygium has been confirmed and agreed upon in which long time under sunlight substantially leads to pterygium (Threlfall & English, 1999). According to Luthra et al for Barbados eye studies concluded that wearing protective lenses or sunglasses reduces the risk of pterygium in people who work outdoor compare to those who without any protective against sunlight exposure (Luthra et al., 2001).

UVB has been shown to be the major factor for causing pterygium through inducing many changes (O'Brien et al., 2011). such as UVB has many biological effects on eyes which it leads to many changes, such as cataract, climatic droplet keratopathy, snow blindness and pterygium (Taylor, 1989). Because UVB radiation is absorbed by cornea of the eye by which it protects the inside parts of the eye but excessive exposure time to UVB causes damage to the cornea irreversibly (Black et al., 2011). Limbal stem cells (LSC) which separates conjunctiva and cornea it has been shown its degraded by long time exposure to UV and which lead to conjunctiva entering cornea and cause pterygium development (Chui et al., 2011b). UVB damages cornea by inducing the production of reactive oxygen species (ROS) and acumulation of ROS may lead oxidative stress by DNA damage, mutation of genes, and cancer (Heck et al., 2003). 8-hydroxydeoxyguanosine (8-OhdG) as oxidative stress marker which damages DNA it has been shown its expression was increased in level and with it human 8-oxoguanine glycosylase (hOGG1) has increased in cells of pterygium due to UV radiation (Tsai, Cheng, Lee, Tsai, Tseng, Lin, et al., 2005), also relation btween 8-OhdG and p53 has been reported and suggested that 8-OHdG expression is related to expression of p53 (Perra et al., 2006). Acute UV has been shown to induce IL-1, IL-6, IL-8, and TNF-α

proinflammatory cytokines (interleukins) thier expression in cornea cells of the eye has been increased and may lead to corneal inflammation (Kennedy et al., 1997). In another study by Di Girolamo et al they confirmed that UVB induces production and high expression of proinflammatory interleukins of 6 and 8 in epithelia of pterygium and suggested they may play important role in pathogenesis of pterygium by activation of cell proliferation, angiogenesis, tissue invasion and inducing inflammation (Di Girolamo et al., 2002), also in another study Di Girolamo et al concluded that UVB induced MMP-1 in limbal epithelial cells through extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase (MAPK) pathway and suggested may induce pterygium development(Di Girolamo et al., 2003). UVB also mutates the p53 gene in(G to T and GG to TT) and it leads to the skin cancer (Hn et al., 2002), and it has been demonstrated and shown high expression of p53 in conjunctiva of primary pterygium development and recurrence pterygium epithelium suggested that its a growth disorder may be cause uncontrolled proliferation (D. T. Tan et al., 1997). Proteasome subunit beta type-5 (PSMB5) which has chymotrypsin-like activity its activated by Nrf2-AREN pathway (nuclear factor erythroid 2-related factor 2-antioxidant response element) which protects the cell from oxidative damage by degrading proteins that have been oxidized and regulate expression of metalloproteinases and MMP inhibitors. It has been shown that PSMB5 down regulated in pterygium cells compare to normal conjunctiva which these may lead to pterygium development in which its suggested maybe by partially UVB exposure (Aletras et al., 2018).

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) which is a potent mitogene it has been shown that is expressed more in epithelial cells of pterygium from UVB exposure and there is evidence that maybe its major factor in inducing pterygium development, and pathogenesis of pterygium is caused by UV radiation (Nolan et al., 2003). In a research by Moran and Hollows a study were done on more than 100000 of Australian Aboriginal and non Aborigin peoples, they showed that UV radiation in climate has important role in causing pterygium and considered as major factor. In addition, there was difference between the groups due differences in lifestyles (Moran & Hollows, 1984).

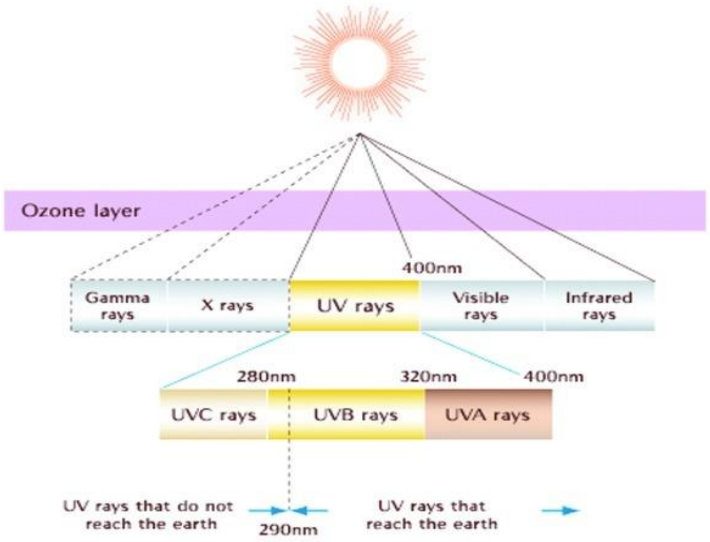

Figure 2.10: UV light spectrum from sun.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3460660/figure/F2/?report=objectonly

2.4.2. Virus Factor

Pterygium recently has been recognized as a growth and uncontrolled proliferation disorder in the epithelial cells (Ljubojević et al., 2016). There are several viruses that can cause cancers in humans which they're called oncogenic viruses that are responsible for 20% of human cancers. They insert their genomic content and combine to the host genome and chromosome and they alter the normal states and functions of the host cell, and disrupt DNA repairing and genetic abnormally which leads uncontrolled proliferation of the infected cell (Figure 2.10). Such as Human herpes virus 8 (HHV8), Epstein Barr virus (EBV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Merkel cell polyomavirus (MCPyV), Human papillomavirus (HPV) and Human T-lymphotropic virus type 1 (HTLV-1) (Luo & Ou, 2015). Moreover, Herpes simplex virus (HSV) and cytomegalovirus (CMV) also have been found to cause cancer in humans (Rafferty, 1973; Richardson et al., 2020). HPV, EBV, HSV and CMV DNA of these oncogenic viruses have been reported to be present in pterygium cells (CHALKIA et al., 2013). There Many studies showed HPV involvement in

pterygium it has different level from low to high. In a study by Piras et al found 100% presence of HPV in pterygium in Italian patents and 24% prevalence in Ecuadorian pterygium and stated that HPV difference in pterygium is due to difference in geographical location, lifestyle and environment (Piras et al., 2003). HPV with high prevalence was found and suggested its related to cellular proliferation of pterygium epithelial cells and it may play role in pterygium formation and development (Varinli et al., 1994). high prevalence of HPV has been detected in a study which states that its related to abnormal expression of p53 together they may induce formation of pterygium disorder through uncontrolled proliferation (Rodrigues et al., 2008), 27.6% prevalence of HPV in pterygium samples has been reported and concluded that it's not necessary for pterygium formation but have a synergistic role in its development (Piecyk-Sidor et al., 2009). E6 protein expression from High-risk HPV 16/18 has been showed that it binds to p53 and it inactivates it. Which in this study detected 24% HPV 16/18 E6 in pterygia tissue and was partially related inactivation of p53 but with mutation in p53, also suggested HPV 16/18 may have role in pterygium formation (Tsai et al., 2009). In addition, a very low prevalence HPV was detected in pterygium samples and was concluded that it's not necessary for pterygium formation and development (Hsiao et al., 2010; Takamura et al., 2008), and zero prevalence pf HPV was detected in some other studies and concluded that HPV it's not necessary for pterygium development (K.-H. Chen et al., 2003; Otlu et al., 2009). Other viruses that were detected such as HSV was found with HPV also may both involved in pterygium development (Detorakis et al., 2001). EBV DNA was found in pterygium and suggested to be involved in pterygium pathogenesis (Otlu et al., 2009), and DNA of both CMV and HSV were found in pterygium which aslo led to suggestion that they are involved in pterygium pathogenesis and development (Spandidos et al., 1994).

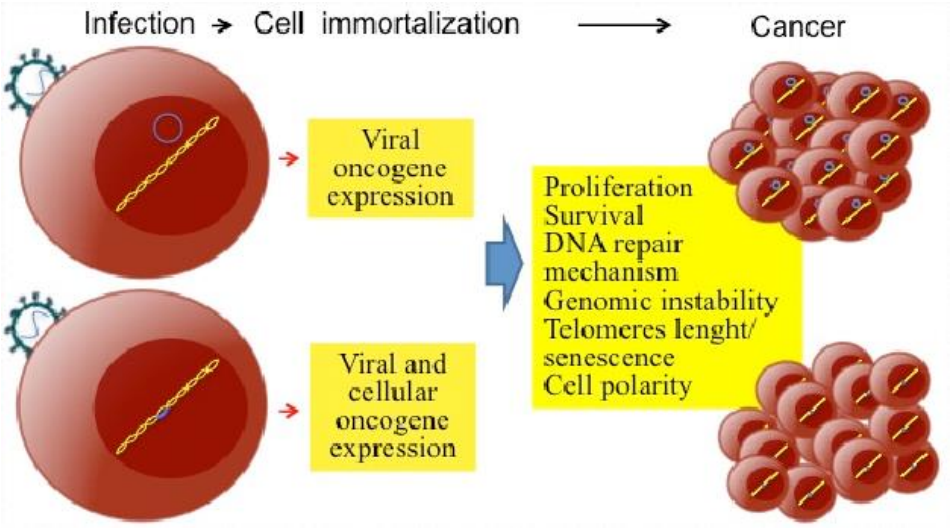

Figure 2.11: Mechanism of oncogenic viruses, infecting cell and causing cancer. https://www.researchgate.net/publication/267736070_Human_Viruses_and_Cancer

2.4.3. Dry Climate, Age and Gender

Pterygium formation has been related to sunlight. Furthermore, a high prevalence of pterygia has been reported, while living in a dry, dusty environment with long-term exposure to huge amounts of particles is considered to be a risk factor for the disorder. Outdoor workers under sun for a prolonged period of time have shown higher prevalence compare to indoor workers as a result of prolonged exposure to sun light which leads to an increase in the cumulative UV exposure time. With increasing rate of UV affect its thought that might thickening and hyperplasia in subconjunctival connective tissue. With increasing age, the frequency ratio of pterygium has been shown up-regulated. In age 40 years above and in some other studies reported above 30 and 50 of age that has shown higher tendency to pterygium development and were considered as risk factor compared to age 20 years below. Moreover, rate of pterygium has increased with increasing of age shown in a study among residents of Pulau Jaloh an island in Indonesia. Gender also has been studied that has effect on pterygia development. Male gender has been shown that are more prone to pterygium although there are studies shows that pterygium has more prevalence in female with this controversy on gender the factors such as type of the population screened, the age range of the respondents, variable exposure to environmental risk factors, occupation and

genetic differences between the populations have impact on prevalence of pterygia development in both gender. There are evidence showing that using protective factors such as sunglasses and that prevents pterygium formation and development even exposured to sunlight and living in dry and dusty environment (C. S. H. Tan et al., 2006; Threlfall & English, 1999; Wang et al., 2020)

2.5. Intracellular Factors

2.5.1. Tumor Suppressor Gene

Tumor suppressor genes inhibit uncontrolled cell proliferation and tumor development if mutation occurred in these genes it may leads to cancer formation. The first tumor suppressor gene was found was Rb gene it was discovered by studying in an eye tumor retinoblastoma which it regulates cell cycle by stopping it in G1 if there is DNA damage. Studies shown that mutation in this gene leads to development of cancer, although it was studied and shown that it was inactivated in some other cancers, also Rb protein is a target for oncogenenic viruses. The second tumor suppressor gene found was p53 which its most common target for studying of cancer because it plays role in 50% of human cancers it regulates cell cycle progress by stopping it if there is DNA damage till is repaired and if it wasn't repaired it leads to apoptosis and uncontrolled proliferation (Figure 2.11), example of other tumor suppressor genes when mutated have role in cancers INK4 and PTEN in lung cancer, prostate cancer, APC and MADR2 in colon cancers, BRCA1 and BRCA2 in breast cancer, and some others (Cooper, 2000).

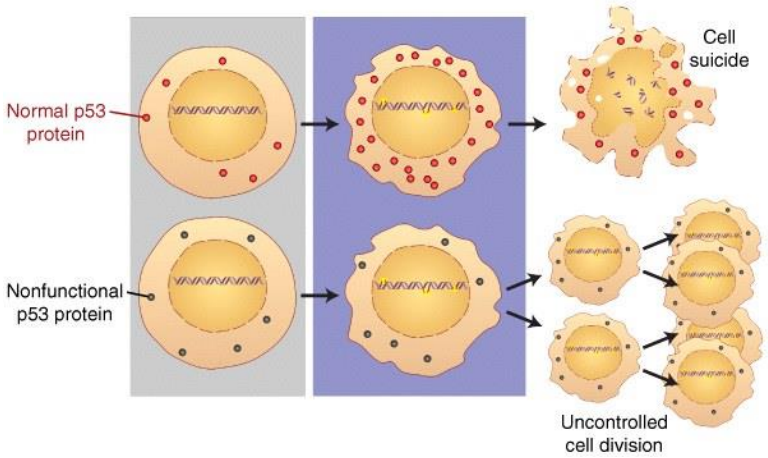

Figure 2.12: Normal p53 leads to apoptosis and inactive p53 leads to uncontrolled cell proliferation. https://inapoptosis.files.wordpress.com/2016/05/f0209.jpg

Most frequently tumor suppressor gene was studied in pterygium as a factor was p53 it was presented by Weinstein et al which was shown that there was an over expression of p53 in epithelium of pterygium they suggested that uncontrolled proliferation may cause pterygium formation due to this proliferation (Weinstein et al., 2002), aslo Tan et al found abnormal expression of p53 gene in the pterygium epithelium and suggested that pterygium is a tissue growth disorder (D. T. Tan et al., 1997). Moreover, 22.8% expression of p53 tumor suppressor gene in pterygium tissues was detected in the 129 samples that were studied by (Tsai, Chang, et al., 2005), also there are evidence that pterygium is a tissue growth disorder due to the proliferation increasing in pterygium epithelial cells with increasing in expression of p53 with prevalence of 44% in pterygium samples. In-conclusion it may have role in development of pterygium (Ljubojević et al., 2016). Mutation in p53 gene has been observed and evidence was found that this mutation may be related to pterygium pathogenesis which it was shown by Dushku & Reid that mutation in this gene has caused pterygium formation (Dushku & Reid, 1997). Dushku et al did a study regarding HPV relation to p53. It shown that HPV was not a co-factor for pterygium development rather its p53 gene mutation due to chronic UV exposure thus the normal programmed cell death disrupted and lead to mutation in other genes and lead to multi-step pterygium formation (Dushku et al., 1999). In contrast, others showed that its not mutation in p53 that leads causation of pterygium, Schneider et al in a study they suggested that the

even with increased number of p53 protein because normal function such as causing apoptosis and stopping cell proliferation has been inactivated by something else than gene mutation in pterygium (Schneider et al., 2006). the p53 gene inactivation by virus protein was reported it was shown that the E6 protein of HPV binds to tumor suppressor p53 protein and degrades it and it was hypothesize this degradation may lead to pterygium formation by HPV 16/18 E6 protein (Tsai et al., 2009), in another study by Tsai et al which they showed mutation in p53 gene in exons 4-8 in pterygium epithelial and they suggested due to mutation in some of the samples not all the pterygium cause maybe other than mutation in p53 gene(Tsai, Cheng, Lee, Tsai, Tseng, & Chang, 2005), and in a review by Tsai et al on 8 immunohistochemical studies on p53 expression in pterygium from Medline and 127 pterygial specimens and 18 normal conjunctival samples were immunohistochemically stained. the expression of p53 gene only 22.8 % of all the samples were positive to p53 gene and all other indications were the same in positive and negative to p53 gene and in the 8 other sample from Medline the rate of p53 expression was respectively 7.9%, 36.8%, 37.5%, 38.1%, 50%, 53.8%, 60%, and 100% (Tsai, Chang, et al., 2005), and in some other studies showed that p53 gene mutation is not related to pterygium formation Shimmura et al suggested that the p53 gene expression in epithelia is not associated with pterygium development (Shimmura et al., 2000), also Onur et al suggested in their investigation that on genetic bases inactivated tumor suppressor gene p53 has no relation to pterygium formation (Onur et al., 1998),

The stem-cell markers of squamous epithelia p63 and p16 genes have also been studied and shown high expression rate in the epithelial cells of recurrent and primary pterygium which this may show p63 and p16 genes have role in pterygium pathogenesis (Ramalho et al., 2006). p27 gene low expression, Cyclin D1 and Ki-67 high expression rate in pterygium has been detected. There are evidences suggest that p27 has been down-regulated probably due to phosphorylation of extracellular signal-regulated kinase (ERK) ½ and Cyclin D1 has been detected in both head and body of pterygium. Which this may led to suggestion that p53, Cyclin D1 and high expression of Ki-67 in head of pterygium may lead high rate of proliferation in pterygium samples (Kase et al., 2007).

2.5.2. Growth Factors

Growth factors are secreted molecules that have role in several important functions such as cell growth, inducing or inhibiting mitosis, cellular proliferation and wound healing (Stone et al., 2020). There are various growth factors that have been reported to have connection and role in pterygium pathogenesis which they are vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF-b), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), nervous growth factor (NGF), and connective tissue growth factor (CTGF) (Wanzeler et al., 2019). VEGF is an important target for many eye diereses which it stimulates cell proliferation, cell migration, proteolysis and angiogenesis. It consists of seven members VEGF-A (or VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and three receptors VEGFR-1, VEGFR-2 and VEGFR-3 (J. S. Penn et al., 2008). VEGF as an angiogenesis factor was proven to be over expressed in pterygium tissue and considered to have role in pathogenesis of pterygium formation (Aspiotis et al., 2007). It was shown by Liang et al that mast cells secrete VEGF and induce abnormal angiogenesis and leads to pterygium development (Liang et al., 2012), also Marcovich et al supported that over expression of VEGF may play role in pterygium formation by inducing angiogenesis in pterygium tissue (Marcovich et al., 2002). Lee et al were first to show that environmental stress cause nitric oxide (NO) and VEGF together have role in formation of pterygium by inducing conjunctival fibrovascular growth in epithelial cells of pterygium (Lee et al., 2001), and Gumus et al showed that the high level of VEGF and vascular endothelial growth factor receptor 2 (VEGFR-2) expression may play role in pterygium pathogenesis and induce pterygia recurrence (Gumus et al., 2014). High expression of VEGF-C and VEGFR-3 in lymphatic vessel endothelium was in pterygium pathogenesis by inducing pterygium formation through formation of new lymphatic vessels a process called lymphangiogenesis. In chronic wound healing the VEGFR-3 in lymphatic has been expressed abnormally which in this study by Fukuhara et al shown over-expression of its ligand VEGF-C. Through binding to VEGFR-3 the epithelial cells of pterygium my cause lymphangiogenesis and both contribute its pathogenesis (Fukuhara et al., 2013).

Secondly TGF-β has role in various significant processes such as wound healing, inflammation, angiogenesis, proliferation, synthesis and remodeling of

extracellular matrix. And it has three isoforms TGF-β1, TGF -β2, TGF -β3 with two TGF-β receptors (J. W. Penn et al., 2012). a study by Shayegan et al reported that over expression of TGF-beta in pterygium may have role in pathogenesis of pterygium in atopic patients (Shayegan et al., 2016). Bianchi et al have reported that TGF-Beta expression was higher in pterygium compare to normal conjunctiva along with VEGF and PGE_2. It was led to that they may induce pterygium through angiogenesis forming new blood vessels take role in formation and progression of pterygium and concluded that with more studies they may be a good target for pterygium treatment (Bianchi et al., 2012). The over expression of TGF-β 1 and TGF-β 2 and low expression of both of receptors TGFR-b1 and TGFR-b2 in epithelium, blood vessels and infiltrating mast cells of the pterygium may play great role in pathogenesis of pterygium and inducing its recurrence (M. Zhong et al., n.d.).

Third growth factor FGF which has role wound healing, cell proliferation, migration, differentiation and angiogenesis are 23 members and there are 18 FGFR. With four members that do not bind to receptors which include basic (bFGF aslo known as FGF2) and acidic (aFGF also known as FGF1), there are 22 members found in human being (Yun et al., 2010). bFGF (FGF2) has been reported in pterygium in a study by Powers et al suggested that bFGF may have role in pathogenesis of pterygium by having effects on epithelium and blood vessels of pterygium tissue sourced possibly from epithelial, endothelial cells and also from mast cells that contains bFGF (Powers et al., 1997). In addition, Y. Zhong et al also showed that bFGF has been increased expression in epithelium, blood vessels and mast cells of the pterygium tissue and these may be having role in pterygium formation (Y. Zhong et al., 2001), also Nakagami et al showed in their study that bFGF mRNA was highly expressed in mast cells and low expression in normal conjunctiva cells and they suggested that the bFGF expression in mast cell may have role in development of pterygium (Nakagami et al., 1998)

Fourth IGF has functions such as preventing apoptosis, proliferation and have role in growth. There are two ligands IGF-1and IGF-2, and three receptors IGF-1R, IGF-2R and insulin receptor and 6 IGF-binding proteins (IGF-BP) that have role in regulating of IGF1 (AsghariHanjani & Vafa, 2019). There are evidences of low expression of IGFBP3 to cancer and its low expression in pterygium tissue compare to normal conjunctiva this may be due to unregulated cellular proliferation and this may

be the result of the continuous growth in pterygium (Wong et al., 2006). increased IGFBP-2 mRNA and protein expression in fibroblasts of pterygium may help in understanding the growth of fibrovascular tissue in the pterygium (Solomon et al., 2003).

Fifth NGF with its receptors (trkA and p75) have role in differentiation, proliferation and survival of neurons (Aloe et al., 2016), also NGF has ability to stimulate angiogenesis, migration and proliferation and remodelling of extracellular matrix, Ribatti et al in their study suggested that the activation of autocrine loop between NGF and TrkA may play in the angiogenesis of the pterygium because endothelial cells were immunoreactive to both of them (Ribatti et al., 2009).

Sixth CTGF involved in some process including cell proliferation, migration, differentiation, angiogenesis, fibrogenesis, wound healing, and formation of bone and cartilage (Lasky, 2006). It belongs to a complex family of CCN which they are six members that the name is derived from first letters of the three members Cyr61 (cysteine-rich protein 61), CTGF (connective tissue growth factor) and NOV (nephroblastoma overexpressed gene) (Holbourn et al., 2008). Setten et al in a study suggested that fibrosis of CTGF may play a role in pathogenesis of pterygium because it was expressed in pterygium tissue and not in normal conjunctiva (Setten et al., 2003).

2.5.3. Interleukins

Interleukins are proteins secreted by different type of cells in body which bind to specific receptors. They are a large group and have different variety of functions such as Immunosuppressive effect, growth factor, development, maintenance, proliferation, differentiation for different type of cell, induction of pro-inflammatory cytokines, tumor suppression, activation and regulation of epithelial cells, have role in inflammatory response (Akdis et al., 2016). Ki et al compare to normal conjunctiva they found high expression of proinflammatory interleukin 17 and 23 (IL-17 and IL-23) in pterygium tissue (Ki et al., 2017), also interleukin 6 and 8 (IL-6 and IL-8) were found highly expressed due to UVB radiation in epithelial tissue of pterygium compared to normal conjunctiva and these pro-inflammatory interleukins may have role in pterygium pathogenesis (Di Girolamo et al., 2002). Interleukin 4 (IL-4) was reported to be high in

recurrent pterygium and induces fibroblasts to secret an extracellular protein after injury, and induction of this protein may have role in pterygium recurrence (Kuo et al., 2010). Zidi et al suggested that inflammation and anti-apoptotic process induced from IL-17A and IL-6 and high expression of Bcl2 and anti-apoptotic protein have role in pterygium development and pathogenesis through tissue damage and uncontrolled proliferation (Zidi et al., 2017). Interleukin 17 (IL-17) is a pro-inflammatory cytokine that may induce pterygium formation. Reports shown that its expressed in the epithelial and vascular endothelial cells and in the perivascular tissue of pterygium, and it was suggested that controlling IL-17 may be a way to manage primary pterygium or recurrence pterygium (Jabarin et al., 2015).

2.5.4. Proliferation Proteins

Proliferation proteins serve as a marker for indication of growth and cell proliferation in cells which are going through such the process. Ki-67 antigen (Ki-67) is a marker for cell proliferation its expressed in cell cycle during the late G1, S, G2 and M phases and absent in G0 (Bologna-Molina et al., 2013). It was shown in a study that Ki-67 was over expressed in pterygium compare to normal conjunctiva and it might be a good target for more understanding pterygium (Sebastiá et al., 2013). Another study shown that proliferation play role in pathogenesis and development of pterygium, and inducing its recurrence because of elevated expression of Ki-67 in primary pterygium and secondary pterygium epithelium compare to normal conjunctiva samples (Turan & Turan, 2020).

Proliferating Cell Nuclear Antigen (PCNA) is a protein that have role in functions such as DNA replication, DNA repairing by acting as accessory for DNA polymerase alpha, cellular proliferation, chromatin remodeling, sister-chromatid cohesion and cell cycle control (Strzalka & Ziemienowicz, 2011). Liang et al found that PCNA expression rate is higher in pterygium tissue compare to normal conjunctiva due to that they confirmed that cell proliferation with cell apoptosis plays role in pathogenesis and development of pterygium (Liang et al., 2011), also over expression of PCNA in limbal part of pterygium epithelial cells has been reported and this may induce pterygium formation by increasing cell proliferation (Das et al., 2015).

Cyclin D1 is a protein required for cell cycle progression by regulating of G1 to S phase progression. Cyclin D1 overexpression is involvement in development of some types of cancers has been presented (Alao, 2007). Increasing of cellular proliferation has been noticed with increasing expression of Cyclin D1 in limbal part of pterygium epithelial cells and this may be result in progression of pterygium (Das et al., 2015). There are evidence that Cyclin D1 is activated through activator protein-1 (AP-1) which have role in cellular proliferation and this may lead to pterygium development (Kase et al., 2007), and report of the over expression of Cyclin D1 is mediated through both nuclear/cytoplasmic β-catenin and activator protein-1 (AP-1) has been shown and this may lead to cellular proliferation of pterygium (Tung et al., 2010).

2.5.5. Apoptosis and Anti Apoptosis Factors

Apoptosis is a regulated programmed cell death process which is regulated by several proteins. Example of apoptosis regulator is first the B-cell lymphoma 2 (Bcl-2) family which are a group of proteins that have anti-apoptotic and pro-apoptotic functions work as “apoptotic switch”. There are three subgroups of Bcl-2 family depending on the BH domains one with anti-apoptotic and two with pro-apoptotic functions (Pistritto et al., 2016). In a study evidence showed that the level of Bcl-2 was elevated more in pterygium than normal conjunctiva and it was higher in recurrent pterygium compared to primary pterygium and this leads to suggestion that the anti-apoptotic process may have important role in pathogenesis and development of pterygium through uncontrolled cellular proliferation (Turan & Turan, 2020, p. 2), also Liang et al in their results showed that apoptosis process is related to pterygium pathogenesis and its formation by showing the level of apoptosis related protein Bcl-2 was higher in pterygium compare to normal conjunctiva (Liang et al., 2011).

Second Survivin is a small apoptosis related protein which protects the cell against apoptosis its expressed in development and mature proliferating cells which is encoded by BIRC5 gene (Wheatley & Altieri, 2019). Anti-apoptotic protein survivin has been shown that it has role in formation of pterygium by over-expression in recurrent and primary pterygium which regulated by p53 thus targeting survivin may be a good way to inhibit formation of pterygium (L.-W. Zhang et al., 2011), aslo Xu et al showed in their study that the level of survivin expression was higher in pterygium

compared normal conjunctiva tissue and they concluded that targeting this protein maybe used to stop pterygium because survivin may have relation to formation of pterygium and its pathogenesis (Xu et al., 2014).

The mammalian target of rapamycin (mTOR) is a protein that is activated in a variety of illnesses, including tumor formation, angiogenesis, and insulin resistance, and it becomes irregular in cancer and type 2 diabetes. It is involved in cell metabolism, growth, proliferation, and survival. mTOR forms two different protein complexes mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Laplante & Sabatini, 2009), and in a study by Liu et al has shown the mTORC1 role in pterygium formation by apoptosis inhibition and inducing proliferation by targeting autophagy which it might be a good target for treatment of pterygium and FGFR3 and there was no detection of mTORC2 in pterygium (Liu et al., 2017).

2.5.6. Matrix Metalloproteinase and Its Inhibitors

Human proteinases the matrix metalloproteinase (MMPs) are a family of enzymes that have role in normal development and diseases, they act on extracellular matrix (ECM) proteins by degrading them such as collagens, laminin, fibronectin, vitronectin, aggrecan, enactin, versican and others, also work on growth factors and cytokines, MMPs enzymes are divided in groups gelatinases (MMP-2 and MMP-9) and collagenases (MMP-1, MMP-8, MMP-13, MMP-14), membrane type-1 matrix metalloproteinase (MT1-MMP), stromelysins (MMP-3, MMP-10) and others (Cieplak & Strongin, 2017). When ECM components are produced the MMPs are responsible for the degrading of these products. The MMPs activation and prevention is under control if disrupted it may lead to many diseases such as arthritis, cardiovascular disease, cancer, pulmonary disease, nephritis and some others. MMPs are regulated by zymogen and endogenous inhibitors such as tissue inhibitors of metalloproteinases (TIMPs) there are four TIMPs (TIMPs-1, TIMPs-2, TIMPs-3, TIMPs-4) each work on different type of MMPs (Brew & Nagase, 2010). Girolamo et al in their study result showed that the MMP-1 (Collagenase-1) and MMP-13 (Collagenase-3) MMP-8 (Collagenase-2) and TIMP-1 and 3 were expressed and increased in pterygium and cultured human PECs compared with normal conjunctiva and thus they concluded that they might have role in pathogenesis and development of pterygium through inducing

the inflammation, angiogenesis and tissue remodeling. Fully understanding these proteins may make pterygium treatment easier (Girolamo et al., 2000). Also expression of MMP-9 and MMP-10 and TIMP-1 in pterygium tissue and they suggested that MMP-9 and MMP-10 expression may induce pterygium formation and TIMP may help in pterygium invasion inhibition (Tsai et al., 2010). MMP3 in collaboration with secreted protein acidic and rich in cysteine (SPARC) by both increasing in pterygium tissue compare to normal conjunctiva which they both may have role in development and pathogenesis of pterygium and may be they be good target for working on pterygium progression suggested by (Seet et al., 2012). Kim et al in their studying showed that MMP-3 and MMP-13 have role in pterygium formation and pathogenesis by induction the fibroblast migration due to high expressed MMP-3 and MMP-13 in migrated fibroblast tissues, and also tested that the migration of fibroblast is reduced by treating with bevacizumab and Cyclosporine A (CsA) together or separately and their result showed that the drugs down regulated the expression of both MMP-3 and MMP-13 and with this the migration of fibroblast is reduced (Y.-H. Kim et al., 2017). The expression of MMPs such as MMP-2 and MMP-9 was higher in pterygium tissue and pterygium fibroblast in advanced stages of pterygium compared to normal conjunctiva and it was suggested that they induce the development and progression of pterygium disease (Yang et al., 2009), and Di Girolamo et al confirmed that MMP enzymes have role in pterygium development and pathogenesis and also they suggested that MMP-7 has role in the pterygium angiogenesis (Di Girolamo et al., 2001).

2.5.7. Extracellular Matrix

Extracellular matrix is a network of macromolecules that surrounded the cells and linked together give cell mechanical support and strength, biochemical characteristics and it also has role in cell proliferation, adhesion, migration, polarity, differentiation, and apoptosis, ECMs components such as Collagen, laminin, Proteoglycans, Fibronectin and Elastin (Figure 2.12) (B. Yue, 2014).

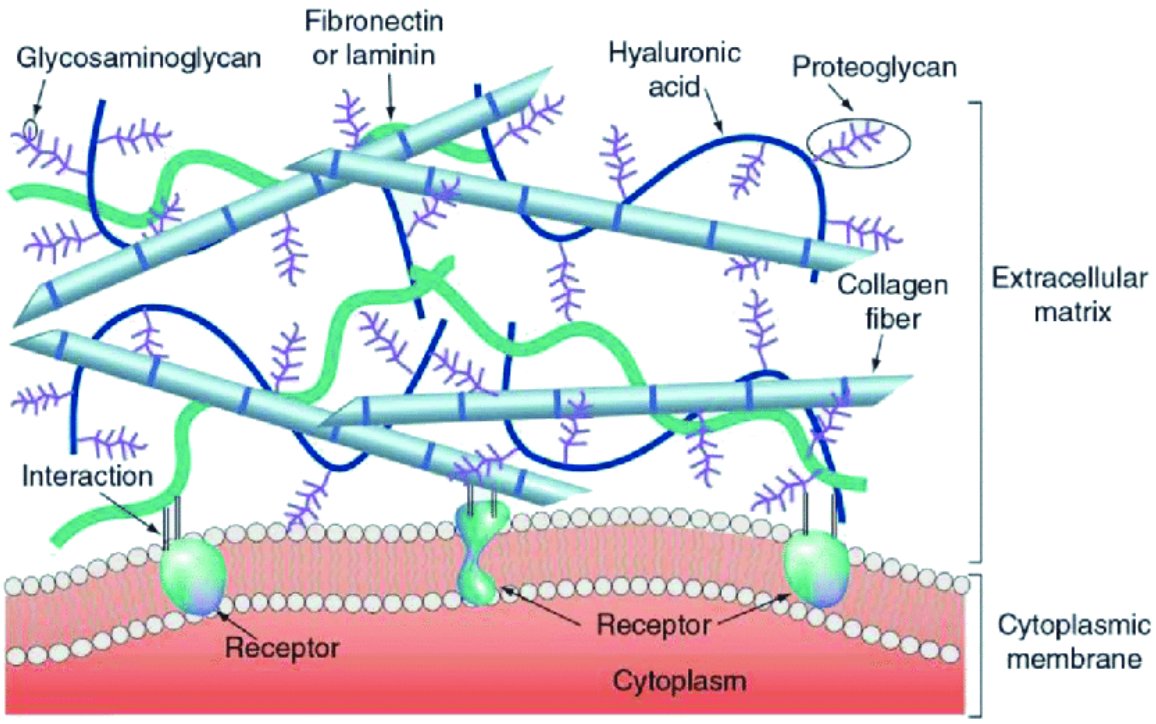

Figure 2.13: Extracellular matrix components.
https://www.researchgate.net/figure/Model-of-complex-3D-structure-of-the-natural-extracellular-matrix-and-the interactions_fig3_315363883

Tropoelastin, fibulin-2, and fibulin-3 were found to be overexpressed in subepithelial connective tissue, and elastin metabolism was shown to be irregular in pterygium disorder, as well as an increase in collagen type III in pathogenic samples, according to a study by (Pérez-Rico et al., 2011). Overexpression of LOXs, FBN1, and FBLN5, which are extracellular matrix constituents and play a role in elastin synthesis, may result in elastin dysregulation and the continuing to lead to pterygium development (Pérez-Rico et al., 2014). evidence of that the type II collagen was found in pterygium samples and collagen I, III and IV were found in both pterygium and normal conjunctiva samples which this may lead to suggestion that collagen from normal conjunctiva may be the risk factor for development of pterygium (Dake et al., 1989). Fibronectin, collagen and versican were found up regulated in pterygium in a study done in 2013 suggesting that ECM may hae role in formation of pterygia (Engelsvold et al., 2013). MMPs a family of ECM enzymes that degrade all protein of extracellular

matrix ECM found up regulated in pterygium giving conclusion to that irregularity in degrading ECM protein may lead to development of pterygium (Di Girolamo et al., 2004). SPARC (secreted protein acidic and rich in cysteine) that binds to ECM protein specially collagen which affects collagen production and assembly (Bradshaw, 2009). Has been shown there in a study that SPARC has role in cancer disease cells it affects tumor development, invasion, metastases, angiogenesis and inflammation which these are may be factors affecting pterygium pathogenesis which it has been suggested that shows cancerous traits. Moreover, some studies show that SPARC has been elevated in expression and may have primary role in pterygium development and formation (Chlenski & Cohn, 2010; Yue & Gao, 2019).

2.5.8. Hereditary and Vitamin D

Vitamin D relation to pterygium pathogenesis since VD has role stopping the proliferation of cell and activation of apoptotic pathways a study done by maxia et al regarding vitamin D concentration in pterygium to evaluate its status and vitamin D receptor (VDR) immunohistochemical expression since it controls the VD activity in 41samples from people with pterygium and 47 samples from volunteered healthy peoples the study showed that there is no significant difference between control and the patients conditioning vitamin D levels but there was a difference in vitamin D receptor Immnunohistochemical localization in pterygium patients and healthy subjects conjunctiva. Besides other studies showed that there are defects in vitamin D and VDR pathways in pterygium and this dis-regulation may lead to chronic inflammation and uncontrolled cell proliferation (Maxia et al., 2017). Some other studies have suggested and provided evidence that there is association between blood 25-hydroxyvitamin D the active form of vitamin D and sunlight exposure duration with pterygium development, the use of serum 25(OH)D level was purposed as marker for the exposure to UVB light since its considered as major risk factor for pterygium pathogenesis (Chun et al., 2018; Jee et al., 2016).

The heredity may also act as a factor in pterygium pathogenesis. Moreover, in some studies they presented the relevance of the hereditary and that the family history has role in occurrence of primary and after surgery recurrence pterygium, its described as autosomal dominant inheritance (Anguria et al., 2012; Romano et al., 2016) which is a

mode of inheritance of the Mendelian laws of inheritance. Law of Dominance which is whether one allele or both alleles are dominant it can cause the disorder and %50 of the offsprings are affected (Lewis & Simpson, 2020).

2.6. Actin Related Protein 2/3 Complex Subunit 2 (ARPC-2) Gene role in Actin nucleation

2.6.1. Actin Filament

Actin provides support in many ways to cells as an important part of cytoskeleton, there are two types monomeric globular (G actin) and when it assembles and polymerizes it forms actin filaments (F actin) by fast-growing (barbed or plus) end and a slow-growing (pointed or minus) end. Actin filaments are formed by the assembly of actin monomers. The early dimerization and trimerisation stages are inadequate in terms of energy. Nucleation factors, on the other hand, resolve slow polymerization and accelerate it such as Arp2/3 (actin-related protein 2/3) complex with help of nucleation-promoting factors (NPFs), formins and the tandem-monomer-binding nucleators. Proteins that isolate actin monomers such as profilin and thymosin β4, prevent spontaneous actin polymerisation in cells. Actin cytoskeleton arranged in cell in two common forms bundle (actin filament arranged in parallel way) and network (the actin filaments arranged in crisscross) which they have role in keeping cell shape integrity, cell motility and give cytocol gel like characteristics. Arp2/3 complex in many eukaryotes is a family of protein that has 50% homology to actin and they are responsible for actin assembly and regulation from preexisting filaments (Lodish et al., 2000).

2.6.2. Arpc2 Gene

ARPC2 gene is encodes for a protein with 300 amino acids which is one of the subunits of Arp2/3 complex which has 11 exons and 1419 base pairs its located on the chromosome 2q35 (Figure 2.13) which is also known as ARC34; PRO2446; p34-Arc; PNAS-139 (*ARPC2 Actin Related Protein 2/3 Complex Subunit 2 [Homo Sapiens (Human)] - Gene - NCBI*, n.d.). Arp2/3 complex is a group of proteins consist of seven subunits include which two are more important Arp2-Arp3 and five other additional subunits starting from ARPC1 to ARPC5 (Figure 2.14). This complex is very important

for regulating the assembly of actin protein it stimulates nucleation and formation of new branches on a pre-existing actin filament when it binds to nucleation promoting factors (NPFs), ATP and pre-existing actin filament it leads to formation of branched actin network in cell cytoplasm, which it plays a role in cell locomotion, phagocytosis, and intracellular motility of lipid vesicles, organelles, and invasive pathogens (Egile et al., 2005).

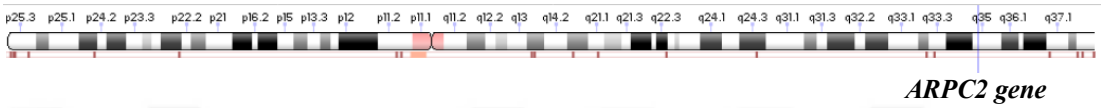

Figure 2.14: chromosome 2 showing ARPC2 gene on 2q35.
https://www.ncbi.nlm.nih.gov/genome/gdv/browser/gene/?id=10109

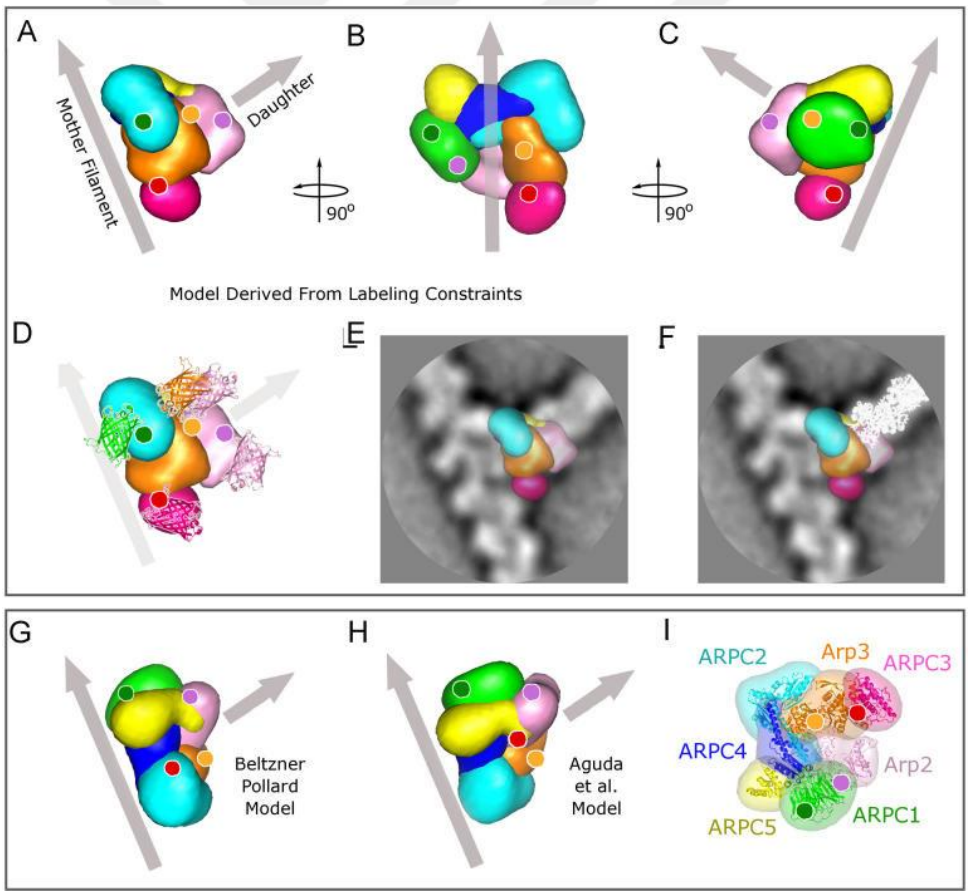

Figure 2.15: structure of ARPCs at new branched actin junction.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1278936/figure/pbio-0030383-g004/?report=objectonly

The daughter (new branch) actin is arises from mother filament (existing filament) by help of proteins such as Arp2/3 complex and in anchoring site (M1 to M6) subunits from mother filament by the connection between the Arp2/3 complex and mother filament (Figure 2.15) in which M1-M6 take part in the interaction but most part of the interaction is taken by M2 and M4. Arp2/3 complex stays inactive till its binds to NPF such as WASP, SCAR and WAVE which they have Verprolin homology (WASP Homology 2; WH2), Central and an Acidic domain (VCA or WCA domain) which they result to a conformational change to the Arp2/3 complex with using ATP, and after the Arp2/3 complex binds to the A region, two actin subunits binds to the V region of the NPFs domains. After activation Arp2 and 3 which in structure they resemble actin they both form a dimer that imitates actin dimer in which helps in nucleation of actin and formation of new filament. Together the Arp complex binds to the mother filament, NPFs is released and Arp complex binds to the existing filament. The actin subuints binds to the Arp2/3 complex and actin polymerization starts.

2.6.3 ARPC2 role in Actin nucleation

ARPC2 is important for maintaining the structural integrity of whole complex and as a part of of the complex backbone its essential for nucleation, cellular function, cell growth and endocytosis. In addition, ARPC2 with ARPC4 gives the main surface for the interaction between the mother filament they are important for the filament side binding and branch stabilization they anchor Arp3 that also plays role in binding the complex to the mother filament which the first subunit of daughter filament. ARPC3 forms a bridge, ARPC1 and ARPC5 provide some weak contact with the mother filament also during the conformational change ARPC5 helps Arp3 tie around the complex so it becomes the second subunit of the daughter filament. Moreover, loss of cellular intergrity which may lead to pterygium formation due to showing changes in cell morphology which there has been evidence of cells in pterygium has shown squamous metaplasia with increased number of goblet cells (Daugherty & Goode, 2008; Kelly et al., 2006; Rouiller et al., 2008). The high level expression of ARPC2 gene was shown that it has role in promoting tumor development and progression such as promoting both the cell proliferation and invasion of human gastric cancer cells, and pterygium has been shown that it has cancer characteristics by which it has uncontrolled

cell proliferating and unlimited growth and local tissue invasion which are some characteristics of cancer but it doesn't migrate to other organs inside body (K. W. Kim & Kim, 2018; J. Zhang et al., 2017).

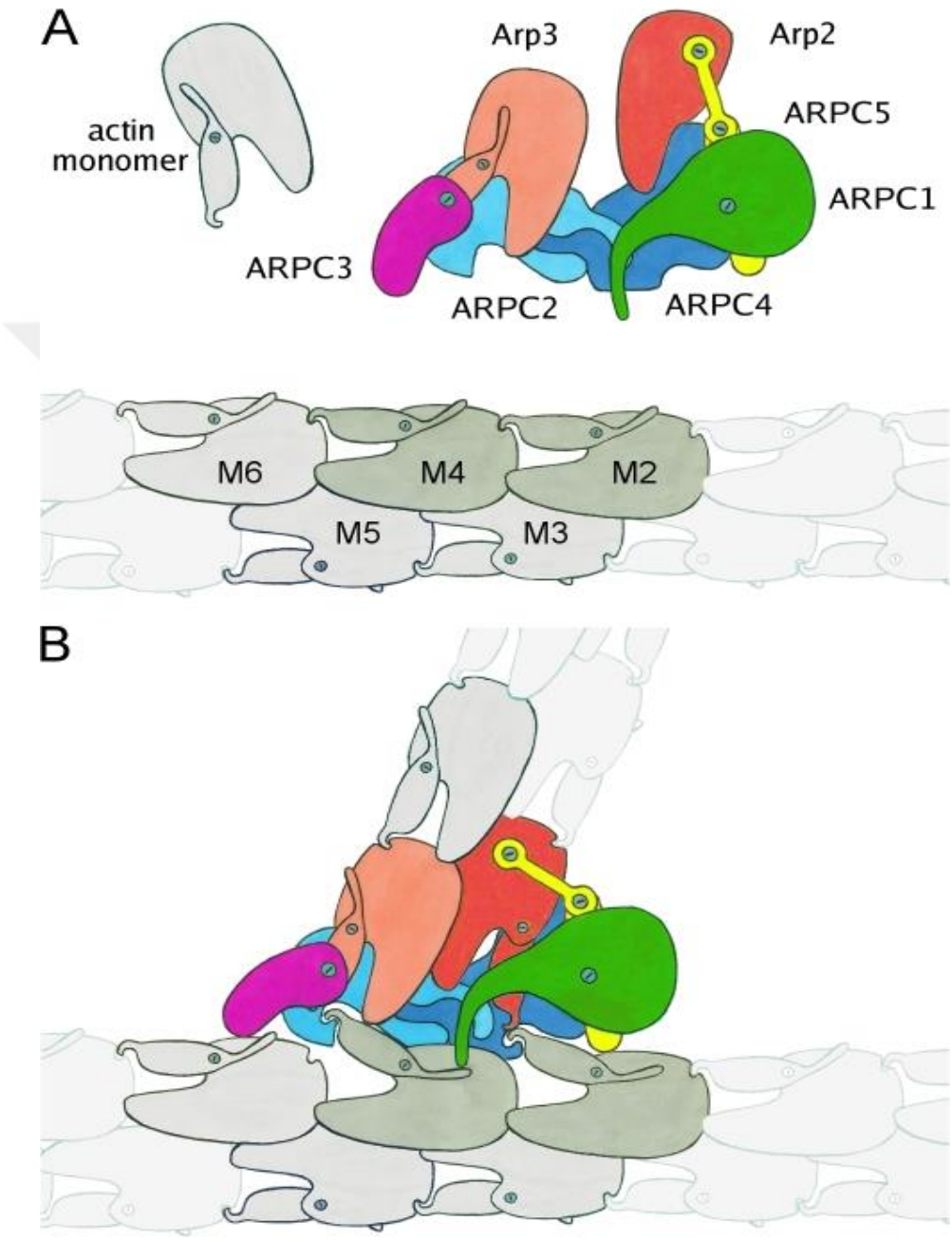

Figure 2.16: showing Arp2/3 complex, mother subunits and the junction between them. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2265399/figure/fig5

3. MATERIAL AND METHOD

3.1. Material

3.1.1. Working Group

18 pterygium and 18 control cases with pterygium who applied to the outpatient clinic of Tokat Gaziosmanpasa University Faculty of Medicine, Department of Ophthalmology were included in the study. With the permission and knowledge of the participants, pterygium tissue samples taken during surgery were used as the patient group and the normal conjunctival tissues belonging to the same eye of the same patients used as Study groups were formed by using the pterygium tissues taken during the operation as the patient group and the healthy conjunctival tissue from the same eye of each patient as the control group. For this study, the necessary permission for the study was obtained by the Clinical Research at Tokat Gaziosmanpaşa University's Faculty of Medicine by project number 2019/110. Tokat Gaziosmanpaşa University Scientific Research funded this research with project number 19.KAEK-024. The research was carried out in Medical Biology and Genetics laboratories.

3.1.2. Tissue Storage

Histopathological examinations were performed on some of the tissue samples retrieved after surgery and diagnosis of pterygium was made. The rest tissues are kept frozen at -80 degrees Celsius. By obtaining cDNA from the tissue samples collected, real-time PCR was performed to detect gene expression analysis.

3.1.3. Tools and Devices Used in the Study

1. -20 Deep Freezer (Arçelık).

2. -80 Deep Freezer (Nuaire, NU-9483E, USA).

3. Refrigerator (Arçelık, 570465 MB, Turkey).

4. shaker (ika, Germany).

5. Distilled Water Device1 (Elga-option Q7, UK).

6. Oven (Memmert, Germany).

7. Power Supply (Biorad, 1645070, USA).

8. Precision Balance (Kern, ABT, WB0750631, Germany).

9. Homogenizer (Next Advance Storme 24, BBY24M-CE, USA).

10. Magnetic Stirrer (Velp Scientifica, F20520162, Italy).

11. Microwave Oven (Argelik MD554, Turkey).

12. Micropipette Set (Gilson, Thermo Scientific, Finnpipette).

13. Microcentrifuge (Mikro120, Hettich Zentrifugen D- 78 532n).

14. Autoclave (HMC, HV25, Germany).

15. Automatic pipettes (Gilson, France).

16. pH meter (Hanna Instruments, HI2020W, USA).

17. Plate (Applied Biosystems, 4346907, UK).

18. Plate adhesive (Applied Biosystems, 2014005092, UK).

19. PCR Device (Thermal Cycle Device (Techne, UK).

20. Qubit (invitrogene).

21. Real Time (RT) PCR (Applied Biosystems, UK).

22. Centrifuge (Hettich, D78532, Germany).

23. Refrigerated Centrifuge (Hettich. Germany).

24. Vortex (Velp Scientifica; F20220176, Italy).

3.1.4. Chemical Substances and Kits Used

1. Beta mercaptoethanol (Merck, Germany).

2. cDNA Synthesis Kit (A.B.T, Hyperscript, first strand synthesis kit Catalog no: 601-005, Korea.

3. Reverse Transcriptase Enzyme.

4. 10X RTase Reaction buffer.

5. 10 mM dNTP mix.

6. RNase Blocker.

7. Random hexamer (primary).

8. Nuclease-free water.

9. Ethanol (Sigma-Aldrich Catalog No: E7023, USA).

10. Qubit ssDNA Assay Kit (Invitrogen Catalog No: Q10212, USA).

11. Qubit RNA HS-Assay Kit (Invitrogen Cat No: Q32852, USA).

12. RNA isolation kit (Thermo, Catalog No: 121830184, APD.

13. Lysis Solution.

14. Washing Solution 1.

15. Washing Solution 2.

16. RNase-free Water.

17. RNasezap (Thermo, Catalog Number: AM9780, USA).

3.1.5. Buffers and solutions

3.1.5.1. Buffers and solutions used in RNA recovery

400 uL Lys

+

4 ul B-mercaptoethanol (as 1% of Lys) = LYS Solution

3.2. Method

Tissues taken during surgery and stored at -80 ° C and tissue fragments stored is used for gene expression analysis.

3.2.1. RNA Isolation and Purification

For RNA isolation, GeneAll brand RiboEx ™ (301-001) LS kit was used. The stages of RNA isolation are carried out as listed below.

1. 100 mg tissue samples homogenized in 1 ml RiboExTM. Homogenize ~ 1 x 107 cells in 1 ml RiboExTM.

Tissue specimens using a homogenizer, homogenize 100 mg of tissue samples in 1 ml RiboExTM. The sample volume should not exceed 10% (w/v) of the RiboExTM volume used for homogenization.

2. Incubate the homogenate at room temperature for 5 minutes to allow nucleoprotein complexes to completely dissolve. Homogenized samples can be stored for at least one month at -70°C.

3. After centrifugation at 12,000 x g for 10 minutes at 4°C transfer the supernatant to a new tube.

4. To 0.75 mL RiboEx TM LS, add 0.2 mL chloroform. Shake vigorously for 15 seconds, then set aside at room temperature for 2 minutes. In substitute of chloroform, 0.1 ml BCP (1-bromo-3-chloropropane) can be used instead.

5. Transfer the aqueous phase to a new tube after centrifuging at 12,000 x g for 15 minutes at 4°C. A lower layer, an interphase, and a colorless upper aqueous layer separate from the mixture. The upper aqueous layer makes up about half of the volume of RiboExTM LS used for homogenization, centrifugation at temperatures higher than 8°C could cause some DNA to partition in the aqueous phase.

6. Add 1 volume of buffer RB1 to the sample and mix thoroughly by inverting. Do not centrifuge the mixture.

7. Up to 700 ul of the mixture should be transferred to a mini spin column.

8. Discard the pass-through and reinsert the mini spin column back into the same tube after Centrifugation at ≥ 10,000 x g for 30 seconds at room temperature.

9. Using the rest of the sample, repeat steps 7 and 8. Reinsert the mini spin column in the same tube after discarding the pass-through.

10. Fill the mini spin column with 500 ul of buffer SW1.

11. Centrifuge at room temperature for 30 seconds at ≥ 10,000 x g.

12. Fill the mini spin column with 500 ul of buffer RNW.

13. Discard the pass-through and reinsert the mini spin column back into the same tube after Centrifugation at ≥ 10,000 x g for 30 seconds at room temperature.

14. To remove any remaining wash buffer, centrifuge at ≥ 10,000 x g for 1 minute at room temperature. Transfer the mini spin column to a new 1.5 ml tube. Residual ethanol has the potential to interfere downstream reactions. This step must be done with caution to avoid buffer RNW carryover.

15. To the center of the membrane in the mini spin column, add 50 to 100 ul of RNase-free water. Allow it to sit for 1 minute.

16. Centrifuge for 1 minute at room temperature at ≥ 10,000 x g. Purified RNA can be kept at 4°C for immediate analysis and at -80°C for long-term storage.

3.2.2. Measurement of RNA Amount

RNA measurements were performed using the ABP iQuantTM RNA HS Assay kit. The necessary procedures for DNA measurement were performed. In order for the measurement to be standardized and made correctly, the standards must first be introduced to the device. All components were brought to room temperature before use. First, 1µl of iQuant Reagent and 199 µl of iQuant buffer were mixed and vortexed per sample. This solution was prepared in both standards and sample measurement. For standards, 190 µl of solution was taken into 0.5 ml tubes. 10 µl Standard 1 and Standard 2 were prepared in separate tubes and standard readings were made on the device. For RNA kit 57, Standard 1: 0 ng / µl, Standard2: 10ng / µl. The device is read as if these are samples, and control is provided. For sample measurement; According to the amount we have, samples in the range of 1µl-10µl were taken into tubes and completed to 200 µl with Working solution. The sample was mixed thoroughly with a finger tap. Vortex is not preferred. Thus, the samples whose measurements were completed were ready for the next experimental steps.

3.2.3. cDNA Synthesis from RNA

cDNA is made by the reverse transcriptase enzyme from RNA with reverse to the transcription reaction, A.B.T.^{TM} cDNA Synthesis Kit with RNase Inh was used. Which is a cDNA synthesis kit containing all the components necessary to obtain and synthesis of a cDNA (Table 3.1). In addition, the RNase Inhibitor presented in the kit adequately protects RNA templates against degradation.

In two steps cDNA is synthesized.

1-The amount of each component that is necessary for cDNA synthesis is listed below

TABLE 3.1. recommended volume for Reverse Transcriptase Reaction.

The components for the Reverse Transcriptase Reaction	**Volume**
Reaction Buffer	2 µl
dNTP mix	1 µl
Random hexamer	2 µl
Reverse Transcriptase	1 µl
RNase Inhibitor	0.5 µl
RNase free Water	3.5 µl
RNA sample	10 µl
Total V.	20 µl

- mixing all together equals to 20µl, first mixed all the components together except the RNA sample, we multiplied all the component's volume to the sample size originally we had 18 samples but we used 20 for multiplying for spare volume.

- then 10µl of the mixture added into 18 new tubes and then we transferred the RNA samples to other tube with same label as tubes of purified RNA. (Figure 3.1)

A- B-

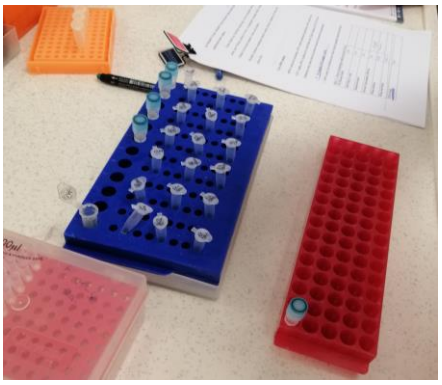

Figure 3.1: showing A- the purified RNA, B- the kit components put into the tubes.

2- Tubes that contain mixture of RNA samples with reverse transcriptase reaction component put into the Thermocycler (Figure 3.2) with these conditions Table-3.2.

TABLE 3.2. Reverse Transcriptase Reaction Conditions.

RT reaction	Temp (°C)	Time	Cycle
Step 1	25	10 min.	1
Step 2	37	120 min.	1
Step 3	85	5 min	1
Step 4	4	∞	1

Finally, the obtained cDNA can be used immediately, without purification, for end-point or real-time PCR or stored at -20°C in a freezer for future use.

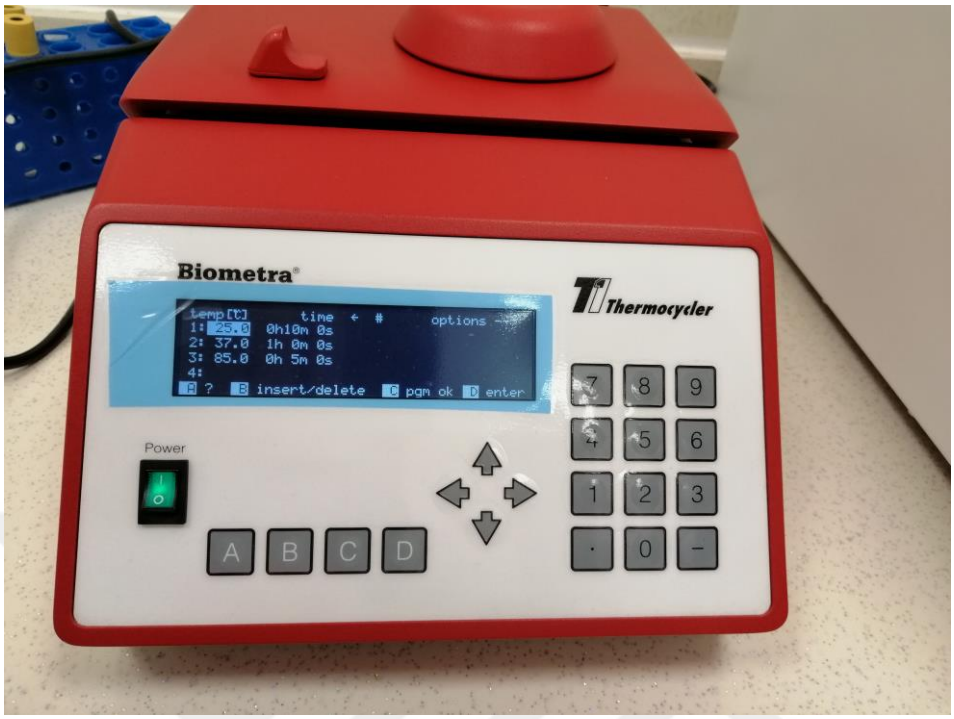

Figure 3.2: Thermocycler used for the reverse transcriptase reaction.

3.2.4. cDNA Concentration Measurements

CDNA measurements were performed using the ABP iQuantTM dbDNA HS Assay kit. The necessary procedures for DNA measurement were performed. In order for the measurement to be standardized and made correctly, the standards must first be introduced to the device. All components were brought to room temperature before use. First, 1µl of iQuant Reagent and 199 µl of iQuant buffer were mixed and vortexed per sample. This solution should be prepared in both standards and sample measurement. For standards, 190 µl of solution was taken into 0.5 ml tubes. 10 µl Standard1 and Standard2 were prepared in separate tubes and standard readings were made on the device. For the cDNA kit, Standard 1: 0 ng / µl, Standard 2: 10ng / µl. Control is provided by reading the device as if these were examples. For sample measurement; According to the amount we have, samples in the range of 1µl-10µl were taken into tubes and completed to 200 µl with Working solution. The sample was mixed thoroughly with a finger tap. Vortex is not preferred. Thus, the samples whose measurements were completed were ready for the next experimental steps.

3.2.5. ARPC-2 mRNA Expression

ARPC-2 gene expressions in pterygium and normal conjunctiva tissues were performed in Applied Biosystem StepOnePlus device using SYBR-Green Real-time PCR method (Figure 3.3).

3.2.6. Real-time PCR

The last step or endpoint of traditional PCR uses an agarose gel to measure PCR amplification (plateau). Real-time PCR, on the other hand, can detect PCR amplification during the exponential growth phase of the reaction. At each given cycle number, there is a quantifiable connection between the amount of starting target sample and the amount of PCR product. The acummulation of amplicon during the reaction is detected using real-time PCR. The data is then analyzed during the exponential phase of the PCR, making DNA and RNA quantification easier and more precise than with traditional procedures. In the laboratory, there are two methods that are frequently utilized. First, the 5′ nuclease assay, for example, uses a TaqMan® Probe oligonucleotide in the PCR reagent master mix. This probe is built using a high-energy dye called a Reporter at the 5′ end and a low-energy molecule called a Quencher at the 3′ end to anneal to a specific template sequence between the forward and reverse primers. The Reporter dye's emission is inhibited by the Quencher dye when this probe is intact and stimulated by a light source due to the dyes' close proximity. The distance between the Reporter and the Quencher rises when the probe is cleaved by the enzyme's 5′ nuclease activity, causing the energy transfer to stop. The reporter emits more fluorescent light, while the quencher emits less. The amount of amplicons created is exactly proportional to the rise in Reporter fluorescence signal. Second SYBR Green Dye, which can bind the minor groove of any double-stranded DNA molecule, is another real-time PCR method. The intensity of fluorescent emissions increases when SYBR Green dye attaches to double-stranded DNA. The SYBR Green dye signal will rise as more double-stranded amplicons are generated. The following is an example of real-time PCR done on a stepone plus real-time PCR detection system using A.B.T.TM 2X qPCR SYBR-Green MasterMix (with ROX) (Valones et al., 2009).

The A.B.T.TM 2X qPCR SYBR-Green MasterMix (with ROX) is used to discriminate between cDNA, genomic DNA, and plasmid DNA after amplifying the acquired cDNA. Then they're ready for applications for real-time PCR such as: SNP genotyping, gene expression profiling, DNA and cDNA target detection and quantification, gene knockdown verification, viral load measurement, array validation, and microbial detection.

Stepone plus real-time PCR machine was used to amplify cDNAs obtained from RNA samples by reverse transcriptase reaction from previous step and the SYBR-Green MasterMix and ROX dye were in the kit only the MasterMix used and mixed with forward and reverse primer of target gene (ARPC-2) and reference gene (ACTB) with other components (Table-4).

Figure 3.3: Applied Biosystem StepOnePlus Real time PCR device.

TABLE 3.3. The components for the real-time PCR reaction.

PCR Reaction Components	Volume
A.B.T.TM 2X qPCR SYBR-Green MasterMix (with ROX	10 µl
ROX Dye (50X) (Optional)	0.4 µl
Forward Primer	1 µl
Reverse Primer	1 µl
Template	3 µl
RNase-Free Distilled Water	4.6 µl

Reaction Table 3.3. It was carried out in 20ul volume using the components given in in the expression analysis of the ARPC-2 gene, Beta actin (B-actin) was used as an internal control gene, and a PCR mixture without cDNA sample was used as a negative control.

The mixtures were mixed without the cDNA and didn't use the ROX dye before mixing we multiplied the component volume by 20.

Added the mixture to the PCR plate (Figure 3.4) for with two gene target gene (ARPC-2) and a control gene (ACTB), and then added 3 µl of cDNA of each gene to their place on the plate and mix with their own forward and reverse primers that was prepared before in the mixture and the plate placed in the real-time PCR with required condition (Figure 3.5).

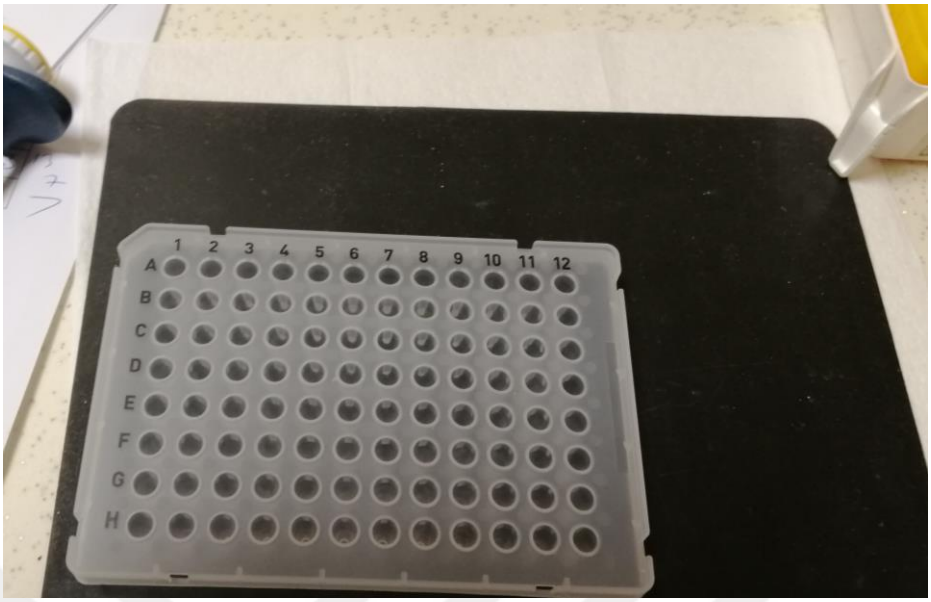

Figure 3.4: RT. PCR plate.

Figure 3.5: Real-time PCR device opened for the plate with the samples.

The real-time PCR machine system prepared for the cycling reaction (Table-3.5) and plate should have been prepared same as the plate layout for the tube places in the PCR program during preparation.

TABLE 3.4. The condition for the cycling reaction.

Temperature	time	cycles
50°C	2 min.	40x
95°C	10 min.	
95°C	10 sec.	
60°C	1 min.	

4. RESULTS

Patients in this study were diagnosed with Pterygium are those who applied to Tokat Gaziosmanpaşa University Faculty of Medicine, Ophthalmology Polyclinic. 18 of the participants have been diagnosed with pterygium. Real-Time Polymerase Chain Reaction (RT-PCR) was used to examine ARPC2 gene expression in the pterygium and normal conjunctival tissues of the same eye sample with pterygium. The patients ranged in age from 27 to 93, with a mean of 56.5 ± 7.5. Twelve of the patients were males and six were females, with 15 patients having right eye pterygium and three having left eye pterygium, as demonstrated in Figure 4.1. The distribution of demographic and clinical characteristics of the patients with pterygium included in the study is shown in Table 4.

TABLE 4.1. Demographic and clinical characteristics of the patients with pterygium.

	Pterygium (n=18)
Age/year, Mean. ± S.D.	56.5 ± 7.5
Number of Genders	12 male / 6 female
right and let eyes	15 right / 3 left

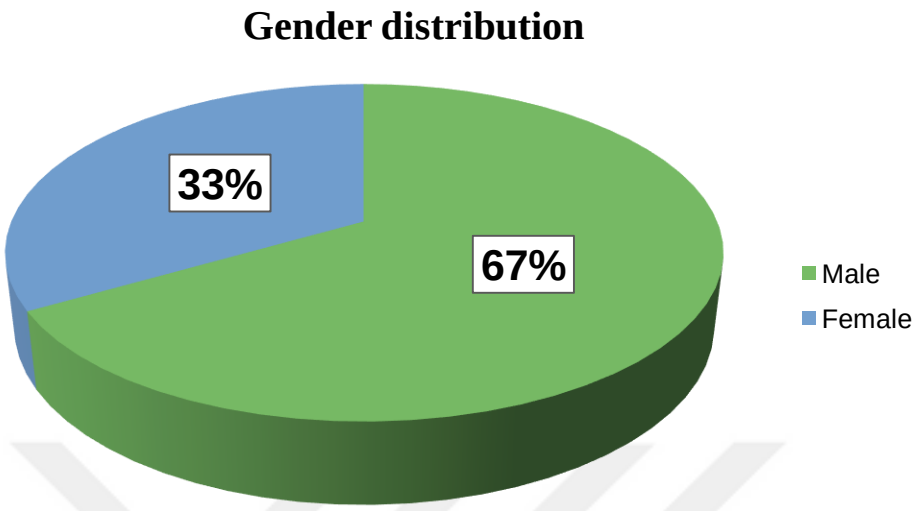

Figure 4.1: Showing Gender distribution.

4.1. The Collected Data for ARPC-2 And ACTB Genes from QRT-PCR

The data displayed during melting curve and amplification curve stages. The melting curve plot, which displays the derivative reporter view, allowing us to examine the maximum rate of change in fluorescence as temperature changes over time. While the accumulation of product over the course of the entire PCR reaction is represented by the amplification plot. The target gene is ARPC-2, while the internal control gene is ACTB. The amplification curve and melting peak of the ACTB and ARPC-2 genes were observed as a result of the qRT-PCR method, as shown in the figures below.

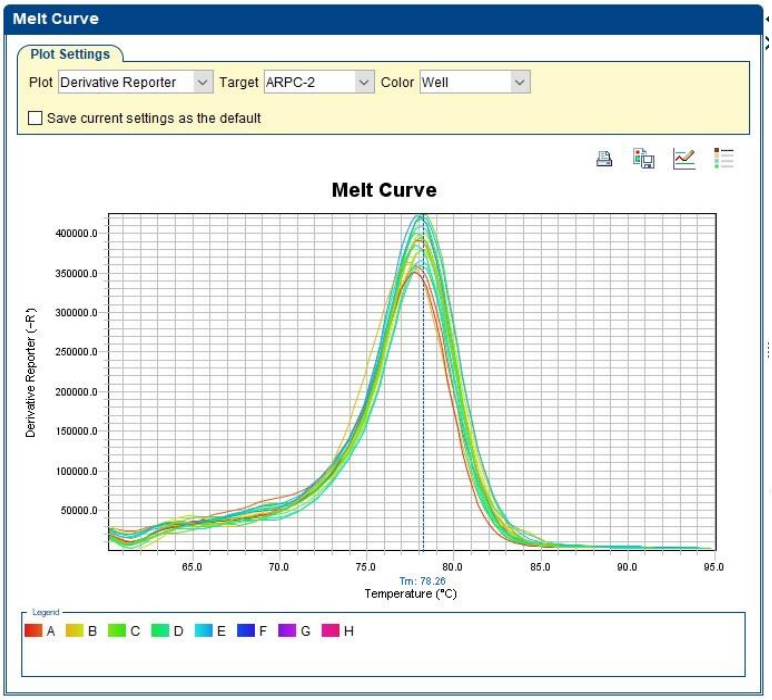

Figure 4.2: melting curve of ARPC-2 in RT-PCR.

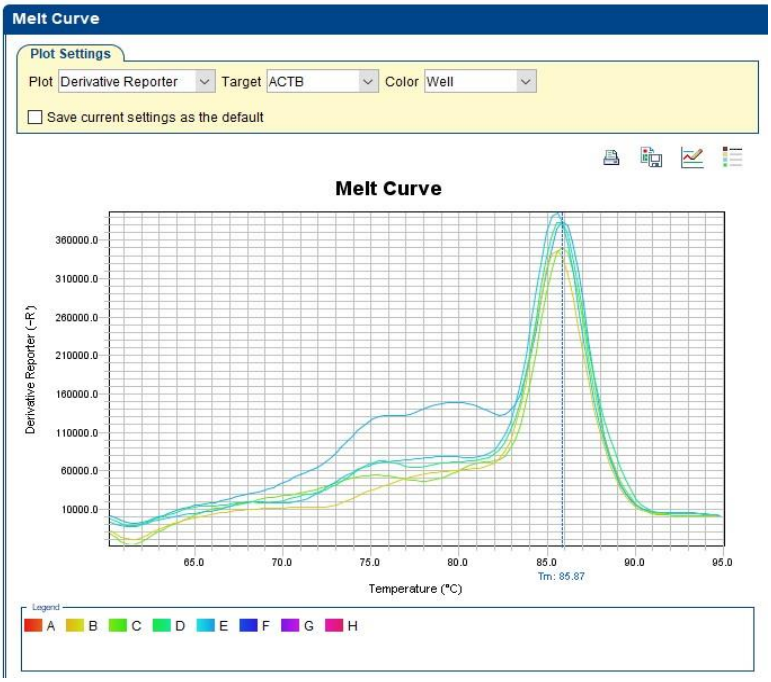

Figure 4.3: Melting curve of ACTB in RT-PCR.

Figure 4.4: Amplification plot showing ARPC-2 gene CT.

Figure 4.5: Amplification plot showing ACTB gene CT.

4.2. Analysis of ARPCc-2 mRNA gene expression results.

The level of expression of ARPC-2 mRNA was determined using a SYBR Green based quantitative Real-Time PCR technique. It was carried out utilizing the Applied Biosystems StepOnePlus device with cDNA generated from RNA isolated from pterygium and healthy conjunctival tissue samples of 18 patients with pterygium who took part in the study. Beta-actin (ACTB) was used as the reference gene (internal control gene) while ARPC-2 as a target gene, cDNA samples (1/10, 1/100, 1/1000, and 1/10000) were produced at various concentrations and serial dilution was performed. For each sample, amplification efficiencies were calculated by performing 3 repeated readings on the Applied Biosystems StepOnePlus device. cDNA and standard samples of pterygium and control conjunctiva tissues were studied in 3 repetitions and the mean value of the C_T (Cycle Threshold) values of the ARPC-2 and B-actin genes was taken then later.

TABLE 4.2. The C_T values of ARPC-2 and ACTB genes.

sample groups	CT_ACTB	CT_ ARPC-2	ΔΔCt ARPC-2 (Fold Change)
G1	1	0,606	0,6570158136
G2	1	0,793	0,5771427088
G4	1	-0,262	1,199139914
G5	1	2,864	0,1373567772
G6	1	-2,144	4,419857939
G7	1	1,433	0,3703599495
G8	1	-0,43	1,347233577
G10	1	-4,148	17,72851757
P16	1	1,081	0,4727010584
P17	1	0,725	0,6049970446
P18	1	3,036	0,1219194327
P19	1	-4,809	28,03194608
P20	1	2,76	0,1476240827
P21	1	2,785	0,1450879893
P22	1	0,474	0,7199656593
P23	1	0,017	0,9882856515
P24	1	7,306	0,006319385534
P25	1	0,545	0,6853914025

4.3.1. Statistical analysis of qRT-PCR Data of ARPC-2 mRNA gene

There are two main ways for interpreting qRT-PCR data: absolute and relative. After the expression of the ARPC-2 gene at the mRNA level in the pterygium, and control conjunctiva tissues was determined by QRT-PCR. The ARPC-2 and ACTB genes' QRT-PZR values were quantified using the step one plus software v2.3 program. When an exact number of amplification is necessary, absolute quantification, which uses a standard cure to determine the copy number of the target gene is used. In contrast, in relative quantification, the data collected from the target gene is compared to the data collected from a reference gene (internal control gene). Relative quantification was used to calculate of the data belonging to the ARPC-2 gene and normalized with the ACTB gene an internal control gene which is a commonly used reference gene to normalize and minimize errors when amplifying with target gene. There are three methods to present relative gene expression: efficiency correction method, sigmoidal curve fitting method and comparative CT method. Comparative CT was used which calculates the fold change, also known as the 2-ΔΔCt method (Table 4.3). It's also known that it makes a number of assumptions, such that PCR efficiency is near to 1, and that the PCR efficiency of both the reference gene and the target gene is identical. This approach has the advantage of presenting the collected data of the expressed gene in “fold change”. The downside is that the assumptions must hold or else the PCR will need to be optimization further. Statistical analysis was performed with the data obtained from this process. All data were presented as mean ± SEM and results were Analyzed according to the 0.9 – 1.1 range. The expression level of the related gene in the pterygium tissue compared to the normal conjunctival tissue decreased if the values were lower than 0.9. It did not change when compared with the normal conjunctival tissue if btween the range of 0.9-1.1. And if the value was higher than 1.1 then there was an increase in the expression of the gene compared to the normal conjunctival tissue (Schmittgen & Livak, 2008).

TABLE 4.3. Final form and full form of $2^{-\Delta\Delta CT}$ equation for pterygium sample and for control samples.

Fold change = $2^{-\Delta\Delta CT}$ $2^{-\Delta\Delta CT}$ = [(CT gene of interest – CT control gene) sample A – (CT gene of interest – CT control gene) sample B For pterygium sample ARPC-2 CT – ACTB CT = -Δ CT 1 for control samples ARPC-2 CT – ACTB CT = -Δ CT 2 Minus both we get ΔΔCT value for each sample Δ CT 1 – Δ CT 2 = -ΔΔCT

Statistical analysis of expression data was performed using SPSS 16.0 software. Student's t test was used to determine the significance levels of the data. The statistical significance level was accepted as 0.05, if $p \leq 0.05$ the data was significant which means there is significant level of difference. However, if $p > 0.05$, the data value was considered not significant and that there was no significant difference between the samples.

TABLE 4.4. analyzing the values of $2^{-\Delta\Delta CT}$ for ARPC-2 gene in normal and conjunctiva samples.

Gene	Samples	
	Pterygium	Normal conjunctiva
ARPC-2 mean ± SEM	3.24 ± 1,75	
P	0,209	

As shown from the statistical analysis of the result that was obtained from the QRT-PCR. The level of ARPC-2 gene expression has increased in pterygium tissues when this increase in expression is compared to normal conjunctiva tissue ARPC-2 gene expression (3.24±1,75-fold change, $p = 0.209$). This increasing of ARPC-2 gene mRNA in the result is statistically not significant because the P value is higher than 0.05 therefore there is a difference between the two groups of pterygium and normal conjunctiva sample genes. This suggests that there is an insignificant difference between the ARPC-2 gene expression in pterygium tissue and normal conjunctiva samples.

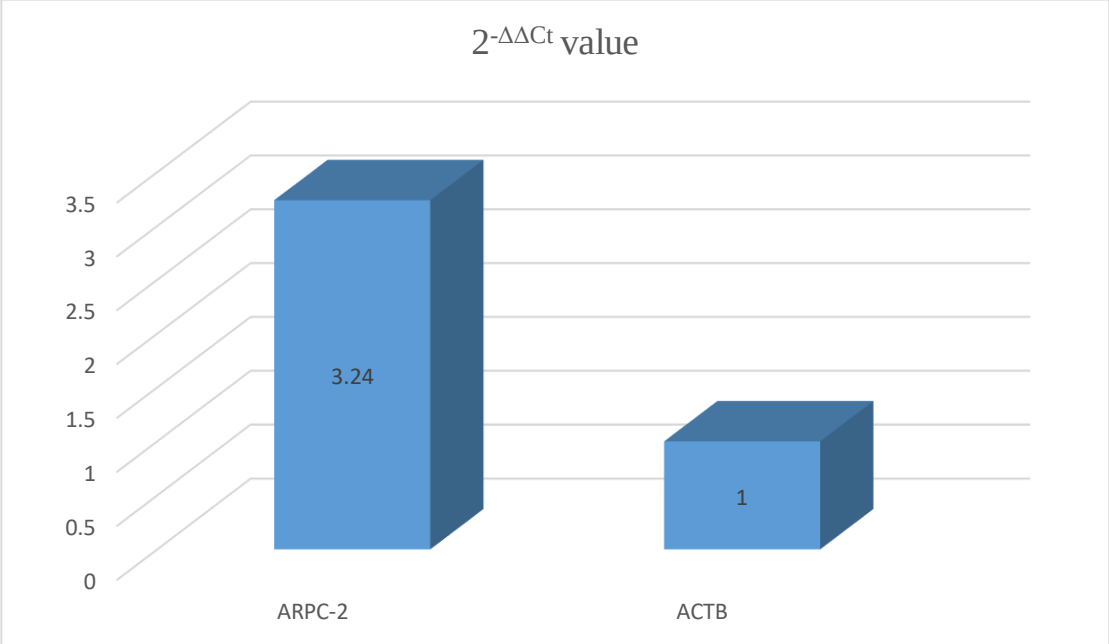

Figure 4.6: Mean values of 2-ΔΔCt values of ARPC-2 and ACTB Gens in Pterygium samples.

5. DISCUSSION

Pterygium is ocular surface disorder with uncontrolled growth of conjunctiva cells over the cornea as a vascular and fibrous tissue which is considered as a growth disorder and also considered as a degenerative disorder by which the limbus is degraded and conjunctiva overgrowths and enters the cornea and covers the eye surface (C.-W. Lu et al., 2017; D. T. Tan et al., 1997).

Conjunctiva overgrowth may cause the patient with pterygium complications such as eye irritation, discomfort and cosmetic problems and rare cases of blindness. However, it's not yet fully understood. The disease pathogenesis has been related to some factors including UV radiation which has been considered as a major factor in the development of pterygium which may induce pterygium affecting subconjunctival connective tissue and causing chronic inflammation. also dry and dusty climates with long term exposure to sunlight that may increase UV radiation impact on the eye which may cause DNA damage, viruses may induce uncontrolled proliferation which may lead to pterygium formation, and other factors such as ECM, MMP inducing angiogenesis, tissue remodeling and cellular proliferating. Apoptosis, proliferation factors and pro-inflammatory interleukins may lead to uncontrolled cellular proliferation, tumor suppressor gene also induces uncontrolled proliferation if mutated or lost its activity, and growth factors may induce cell proliferation, migration and angiogenesis. all have been reported that they may be a risk factor and have role in pterygia pathogenesis and induce its development and formation (Chui et al., 2011a; C.-W. Lu et al., 2017; Luo & Ou, 2015; C. S. H. Tan et al., 2006; Van Acker et al., 2019).

Actin as a major component of cytoskeleton gives cell support in a network like form and makes cytocol gel like material and it has important role like in driving cell locomotion, phagocytosis, and intracellular trafficking including motility of lipid vesicles, organelles and invasive pathogens. When actin is nucleated which leads to formation of new actin filament or formation of branched actin filament. Arp 2/3 complex with presence of NPFs and ATP by the help of its seven subunits which consists of two major component Arp2 and Arp3 which they resemble actin in structure

and have role in formation of a dimer that helps in formation of new branched actin filament. And five additional subunits ARPC1-ARPC5. The ARPC-2 gene encodes for one of the protein that has role in formation of branched actin. ARPC-2 it has significant role in maintaining the structure integrity of Arp complex. It serves as a main surface for interaction of the complex and mother filament with other subunits and also important for cell growth and endocytosis. (Daugherty & Goode, 2008; Egile et al., 2005; Lodish et al., 2000; Rouiller et al., 2008; *ARPC2 Actin Related Protein 2/3 Complex Subunit 2 [Homo Sapiens (Human)] - Gene - NCBI*, n.d.).

Arp 2/3 complex has been confirmed that has a great role in progression of cancer when its regulation is disrupted by over-activation. Degree of cancer lethality is higher due to its migration from its primary start point to other part of body and leading to secondary cancer development which is called metastases. Invadopodia which are means used to invade other cells by cancer cells which are actin based membrane projections that extends over by using matrix metalloproteinase enzymatic activity to degrade surrounding extracellular matrix. Arp 2/3 complex activation by contractin is critical for forming invadopodia through starting the Arp 2/3 nucleation activity to form new actin filaments which results in branched F-actin networks. Over-expression of Cortactin and its binding directly to newly formed filaments and F-actin networks which formed by Arp 2/3 complex will lead to increased invasion, motility and invadopodia activity and stabilization of the invadopodia formation. And it has been shown that the newly formed branches of actin exhibit a pushing force and this force may lead to formation of structures used for migration of the cancerous cell by lamellipodia.

Arp 2/3 complex subunits have been found to have critical roles in many types of cancer progression, development and metastases when up or down-regulated such as: lung, breast, gliomas, gastric, and colorectal cancers, since they assemble as a group deregulation of one subunits may lead to a disorder. Genes that encode for Arp 2/3 complex proteins have been found over-expressed in breast cancer and was shown to be related to human epidermal growth factor receptor 2 (HER2), also over-expressed in colorectal cancers which were shown to cause atypia in cells, and in lung cancer it led to decreased survival of the patents.

Nucleating promoting factors also has been shown to be over-expressed during cancer. WAVE family which sometimes called scar which function in control cell migration they have been fund near the tips of lamellipodia where new actin are formed and bound to the dense network of actin under the plasma membrane. WAVE family proteins were shown to be overexpressed in carcinomas of the breast, colon, liver, lung, ovary, and prostate.

Like the WAVE complex, N-WASP plays an important role at the plasma membrane. In untransformed cells, however, N-WASP appears to play only a minimal role in membrane appendages. N-WASP, on the other hand, is required for the formation of appendages in transformed cells. In addition to lamellipodia, transformed cells produce invadopodia, which break down the extracellular matrix by conveying the MT1-MMP metalloprotease to specific sites. During cancer progression, N-WASP is not consistently overexpressed. In pancreatic ductal adenocarcinomas, lung cancer, and hepatocellular carcinomas, N-WASP is overexpressed. However, in esophageal squamous cell carcinomas, N-WASP levels are often normal, and in breast carcinomas, it is even downregulated. Other NPFs involved in intracellular trafficking, such as WASH and WHAMM, have not been implicated in cancer progression until now. (Markwell et al., 2019; Molinie & Gautreau, 2018).

Pterygium has shown similar characteristics to cancer due to uncontrolled proliferation and growth of cells and local cell invasion, moreover there has also been reports regarding ARPC-2 role in cancer development and progressing its over expression has been shown to have role in development of human gastric cancer and has been reported that its over-expressed in glioma cancer (K. W. Kim & Kim, 2018; Molinie & Gautreau, 2018; J. Zhang et al., 2017). Also in recent studies, pterygium has been reported that shows dysplasia and metaplasia which maybe leads to loss of integrity and destruction of cell shape and morphology (Pajic et al., 2016).

our results shown that the that the rate of ARPC-2 mRNA gene has increased (3.24±1,75-fold change) in pterygium tissue samples compare to normal conjunctiva. But this increasing in the expression of the gene is not statistically significant ($p = 0.209$).

In this study a relationship between pterygium formation and ARPC-2 gene expression has been established. As the data was gathered form the qRT-PCR the results shown that there an increasing in expression of ARPC-2 gene in pterygium tissue samples when compared to the normal conjunctiva tissue samples. And regarding to the increasing in ARPC-2 gene mRNA in pterygia tissues is insignificant ($P>0.05$). Although with this insignificant deference in the expression between the two groups the data shows that there is a difference between the study group and control group samples. And this Leads to the conclusion that ARPC-2 may have an insignificant role in pterygium development and formation and it may contribute rather that play main role in this ocular disorder pathogenesis. More studies maybe needed to get a clear understanding of ARPC-2 connection to pterygium.

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