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ANALYSIS OF ACTR-3 GENE EXPRESSION IN PTERYGIUM

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Master Thesis

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DARYA SARDAR MUHAMMADRASHID

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ABSTRACT

ANALYSIS OF ACTR-3 GENE EXPRESSION IN PTERYGIUM

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Pterygium is a disorder of the ocular surface defined by uncontrolled cell proliferation, fibrovascular growth that crosses the cornea of the eye and disruption of cell shape and morphology, moreover ACTR-3 is a gene that encodes for one of the major subunits of ARP2/3 complex that help regulate actin assembling in which it gives cell its shape and motility. The purpose of this study is whether there's a link between ACTR-3 gene expression and pterygium development.The study included 18 pterygium patients and 18 volunteers with normal conjuctiva.The expression of the ACTR-3 gene in each group of individuals was evaluated using a quantitative reverse transcription PCR technique.. The result of the study was statistically significant (P=0.015) and there was an increase in ACTR-3 gene (13.62±4.91) in pterygium tissues compare to normal conjuctiva. We concluded that this increase in expression of ACTR-3 gene may have role in pterygium formation.

Key words: pterygium: PCR, PTERYGIUM, ACTR-3

ÖZET

PTERJİUMDA ACTR-3 GEN İFADESİNİN ANALİZİ

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Tokat , 2021

Pterjium, hücrelerin kontrolsüz büyümesi ile karakterize edilen bir oküler yüzey hastalığıdır, fibrovasküler büyüme gözün korneasını geçen, hücre şekli ve morfolojisinin bozulmasıdır. Ayrıca ACTR-3, hücreye şeklini ve hareketliliğini verdiği aktin oluşumunu düzenlemeye yardımcı olan ARP2/3 kompleksinin ana alt birimlerinden birini kodlayan bir gendir. Bu çalışmanın amacı, ACTR-3 geninin (ekspresyonu) ifadesi ile pterjium gelişimi arasındaki ilişkiyi araştırmaktır. Calışma 18 pterjiyumlu ve 18 normal konjuktiva dokusu üzerinde yapıldı. ACTR-3 geninin ekspresyonunu değerlendirmek için her grupta kantitatif ters transkripsiyon PCR yöntemi kullanıldı. Çalışma sonucu istatistiksel olarak anlamlı bulundu (p=0.015), Normal konjuktivaya göre pterjium dokularında ACTR-3 geninde (13.62+4.91) artış oldu. ACTR-3 geninin ekspresyonundaki bu artışın pterjiyum oluşumunda rolü olabileceği sonucuna vardık.

Anahtar Kelimeler: Pterjium, PCR, ACTR-3

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ABBREVIATIONS

UV	:	ultraviolet ray
UVA	:	ultraviolet A-rays
MMP	:	Metalloproteinase
CsA	:	Cyclosporine
ACTR3	:	actin related protein3
BC	:	before century
MMC	:	mitomycin3
5-FU	:	Fluorouracil
nm	:	Nanometer
ROS	:	radical oxygen species
8-ohdG	:	8-hydroxydeoxyguanosine
8-hOGGI	:	8-oxoguanine glycosylase
IL-6	:	interleukin-6
IL-8	:	interleukin-8
VEGF	:	vascular endothelial growth factor
HB-EGF	:	heparin binding epidermal growth
VEGF	:	vascular endothelial growth factor
CTGF	:	connective tissue growth factor
bFGF	:	basic fibroblast growth factor
TGF-β	:	transforming growth factor-beta
IGF	:	insulin-like growth factor

NGF	**:**	nerve growth factor
VEGFR	**:**	vascular endothelial growth factor receptor
PlGF	**:**	placental growth factor
5-FU	**:**	Fluorouracil
IGFBPs	**:**	insulin-like growth factor binding protein
HSPs	**:**	Heat shock proteins
CMV	**:**	Cytomegalovirus
HPV	**:**	Human papillomavirus
HSV	**:**	Herpes Simplex Virus
EBV	**:**	Epstein-Barr virus
P53	**:**	Tumor suppresser gene
ECM	**:**	Extracellular matrix protein
MMPs	**:**	matrix metalloproteinase
TIMs	**:**	Tissue inhibitors of metalloproteinase
Bcl-2	**:**	B-cell lymphoma/leukemia-2 gene)
BAX	**:**	Bcl-2 Associated X-protein
ICAM-1	**:**	intercellular adhesion molecule-1
VCAM-1	**:**	Vascular cell adhesion molecule-1
CD4	**:**	cluster of differentiation 4
NPF	**:**	nucleation promoting factor
HER2	**:**	human epidermal growth factor receptor 2

INTRODUCTION

Pterygium is an eye surface disorder with a triangular form that can induce astigmatism in the cornea, it can even lead to visual disturbance or loss (Coroneo, 1993). It is defined by the development of bulbar conjunctiva tissue across the cornea of the eye (Hirst, 2003). It is divided into head, neck and body, the head which covers the cornea of the eye and a cap in front of head the neck is in the limbus area of the eye and body near nasal side of the eye covers the sclera (Seet et al., 2012). The histology of pterygium is a fibrovascular tissue that grows with unstoppable proliferation and angiogenesis (Džunić et al., 2010).

The main factor that cause pterygium is UV light which damages DNA, protein and lipid (Balci et al., 2011; Coroneo, 1993). UVB has more effects on ocular surface of the eye and may lead to changes in the corneal of the eye and cause pterygium (Taylor, 1989). Eyes can be protected from UV light damage and continuous exposure to the sunlight by using (UV)-blocking spectacle lenses (Liou et al., 2015). Old aged male gender and countryside residence increase the probability of having pterygium because of the difference of their life style, they are more prone to the sun, (Nemesure et al., 2008). Viruses including the herpes simplex virus, the cytomegalovirus, and the human papillomavirus may potentially have a greater impact on pterygium illness. (Reid & Dushku, 2003). Disruption of programmed cell death (apoptosis) process that occur in conjunctiva may result pterygia (Tan et al., 2000), and there are some other factors related to pterygium such as extracellular matrix breakdown primarily collagen by over-expression of matrix metalloproteinases (MMPs) in epithelial cells of pterygium head cells. Angiogenesis, fibrosis and inflammatory infiltrates all considered to have relation to pterygium but it's still not fully understood (An et al., 2011)

Surgical removal of pterygium, which includes bare sclera excision, conjunctival transpositional flaps, conjunctival autograft, and amniotic membrane grafting, is the most appropriate cure (Mohammed, 2011). However, the risk of recurrence following surgery is significant, uncontrollable, and continues to be a problem. Surgery with conjunctival graft or amniotic membrane graftis done to reduce the rate of recurrence by few degree (Hovanesian et al., 2017), besides using antibiotics

and anti-metabolite have effect on reducing recurrence such as mitomycin-C and 5-fluorouracil they all have different effects on recurrence rate of pterygium (Hirst, 2003). Ethanol can cause great amount of cell apoptosis and has been reported that it can reduces the rate of recurrence after surgery with low complications (K.-H. Chen & Hsu, 2006), another two drugs that has been reported to reduce the rate of pterygium are Bevacizumab and Cyclosporine A (CsA) which both have different functions (Kim et al., 2017). There are several factors that affect recurrence after surgery including patient genetic, surgical or environmental factors may tent to recurrence again (Hovanesian et al., 2017). Some other factors may give rise to recurrence of pterygium and they are angiogenesis and lymphangiogenes by few levels formation of blood vessels and lymphatic vessels proves the relationship between reccurent ptreygium and immunological mechanism (Qi et al., 2012).

ACTR-3 gene is responsible for encoding Arp3 protein which is a part of Arp 2/3 complex that have role in regulating actin assembly and formation of new branch. Arp complex which consists of seven subunits. With two major subunits (Arp2 and Arp3) that go under conformational change when Arp 2/3 complex is activated, and five other subunits (ARPC1 – ARPC5) with each have different role in formation of new filament from parent actin. Actin is filament which is in form of a network in cell which gives cell shape and drives cell phagocytosis, cell locomotion and intracellular motility of vesicles, organelles, and certain pathogens (Rouiller et al., 2008). In which cellular metaplasia has been shown in pterygium in which the cells have changed shape. Moreover, Arp3 has been reported that is involved in formation, growth, invasion and progressing of gastric carcinomas. And pterygium has shown cancer like traits such as invading cells and uncontrolled recurrence (Chui et al., 2011; Pajic et al., 2016; Zheng et al., 2008, p. 3).

My aim in this study is to further investigate ACTR-3 gene to find the relation between pterygium disease and ACTR-3 gene, there isn't a clear factor for pterygium to occur there are many studies and investigation about this disease but there is not clear one about ACTR3.

2. GENERAL BACKGROUND

2.1 Pterygium

2.1.1 Pterygium Definition and Histology

Pterygium is abnormal cell division and growth of bulbar conjunctiva continuously then invading the cornea of the eye. Cornea is the outer layer of the eye it's clear with no blood vessel, its durable and protects the eye (Figure 2.1). Pterygium is considered as a proliferation tissue disorder (Parker et al., 2002). It's winged like shape with pinkish color, it can sometimes cause cosmetic issues, visual irregularity, irritation and dryness of the eye (Clearfield et al., 2016). Pterygium was first known by the first ophthalmic surgeon Susruta in 1000 BC. (A & Sk, 2015). Many ways used for pterygium treatment throughout the years such as bile, urine, acids, radiotherapy, thiotepa, 5-fluorouracil and mitomycin C (MMC). Horse hair used to remove pterygium in the past (Martins et al., 2016). Based on encroachment of pterygium over the cornea it's classified into four grades: grade1: the head of pterygium crosses over the limbus, grade II:the head of pterygium goes between the limbus and the pupillary margin, grade III: pterygium head leads on and goes over the pupillary margin not crossing it, grade IV: the pterygium head passes the pupillary margin (figure 2.2) (Thatte et al., 2019).

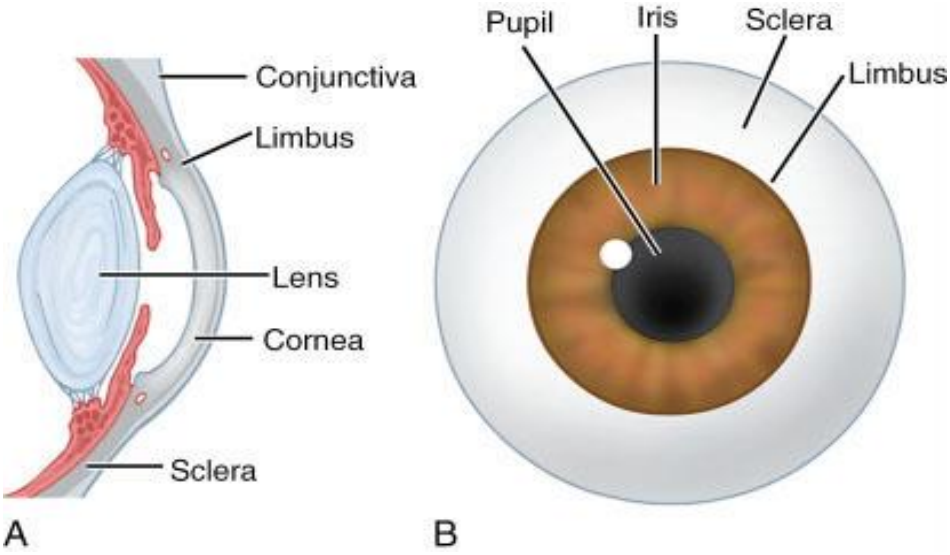


Figure 2.1: A- cornea, limbus in cross section of the eye. B- pupil, human eye anatomy. https://newsomeye.com/cornea-surgery-newsom-eye/

Surgical treatment of pterygium, such as bare sclera method, conjunctival autograft, amniotic membrane, and conjunctival transpositional flap, are the best treatment for pterygium. These techniques are done in case the pterygium was in advance stage such as astigmatism, inflammation and discomfort however after surgery the recurrent rate is high. To reduce recurrence, we can use mytomycin C which is a substance extracted from Streptomyces caespitosus, it reduces recurrent rate by 9%, also there's antimetabolite substance Fluorouracil (5-FU) can reduce the recurrent rate of pterygium after surgery (Nuzzi & Tridico, 2018). Bare sclera technique is a simple removal of pterygium tissue and was the first technique used for removal of pterygium however it has high recurrent rate due to simple excision without anything else (Kaufman et al., 2013). Conjunctival autograph is another technique in which after excision of the pterygium an autograph from another part of the eye is removed and put over the excision place of pterygium. It was first discovered by Kenyon et al in 1985, it became more successful than bare sclera excision (Figueira et al., 2007), effective reducing rate of pterygium recurrence its best to use conjunctival autograph than bare sclera excision (Dt et al., 1997). Amniotic Membrane Transplantation is another technique to remove pterygium and minimize the recurrence rate, it has many benefits, it does not cause any scar, does not cause inflammation and also it is anti- angeogenesis, the recurrence rate of this technique can be between 12-40%(A & Sk, 2015).

It was also suggested that amniotic membrane transplantation has higher rate of recurrence which means that conjunctival autograph is better than amniotic membrane (Tananuvat & Martin, 2004), Conjunctival transpositional flap is using conjunctival flap by rotating it to cover the site of pterygium excision has recurrence rate from 1% to 5% and it was suggested that its better than bare sclera by which it has lower recurrence rate, the only complication from this technique is that sometimes folds over the conjunctiva because of the tissue rotating (Figure2.3)(Alpay et al., 2009; Asghar, 2007).

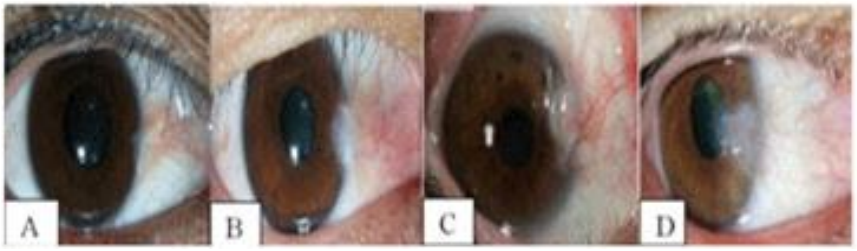


Figure 2.2. grades of pterygium according to its extension.
https://biomedpharmajournal.org/wpcontent/uploads/2019/09/Vol12No3_His_Put_fig1.jpg

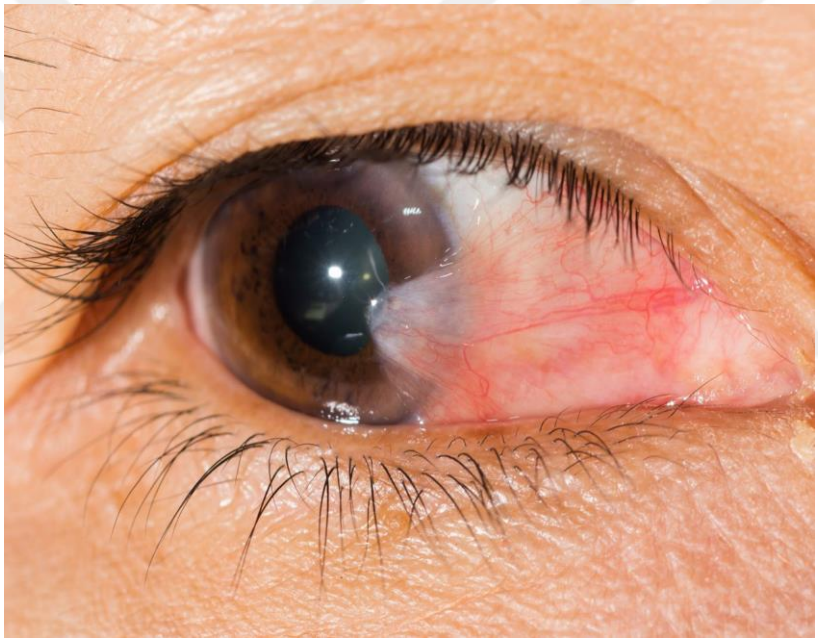


Figure 2.3. human eye showing pterygium.
https://www.shepardeye.com/pterygium.html

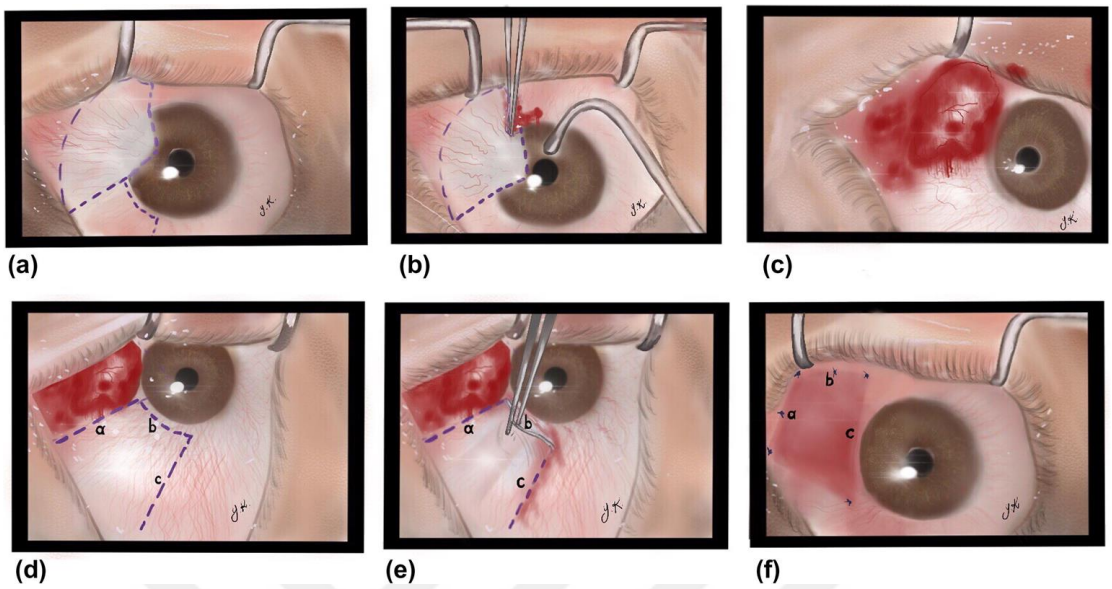


Figure 2.4: conjunctival flap technique. https://ars.els-cdn.com/content/image/1-s2.0-S1319453417301418-gr1.jp

2.2 Epidemiology and Etiology:

Dust, dryness, wind and UV considered to induce pterygium though the etiology is still not known (H. Zhong et al., 2012). Pterygium is observed to be found with a high ratio of occurrence in welders due to high rate of exposure to ultra-violate light in relation to the year of working as a welder (Karai & Horiguchi, 1984). In some study it's suggested that pterygium is more common in men compared to women and in relation to age it is more common in elderly people ≥40 years old (Cajucom-Uy et al., 2010, 2010; Li & Cui, 2013). Some other studies showed that the ratio of pterygium in female is higher compared to male also in elderly people (Chun et al., 2018; K. Wu et al., 2002). There is a strong relation between pterygium and outdoor working, indoor workers are less exposed to sunlight and dust in contrast to outdoor workers which they are more affected without any protection from sunlight (Khoo et al., 1998). There is association between long term sunlight exposure and pterygium formation because of the UV radiation absorption by the eyes for long time, UVB is the major factor for damaging the eye and cause pterygium. (S.-Q. Wu et al., 2020) this study show that the UVB radiation from sun has direct effect to cause pterygium (McCarty et al., 2000).

Viruses have also been hypothesized as possible causes of pterygium production, such as HPV, which has been found to have a role in the activation of pterygium and the growth of pterygium. Furthermore, growth factors have been linked to the development of pterygium. MMPs and TIMPs have been reported that they might contribute to inflammation, tissue remodeling and angiogenesis of pterygium. Evidence of disruption of apoptosis process in normal conjunctiva may lead to pterygium development and formation has been reported (Detorakis et al., 2010; N. Di Girolamo et al., 2000; Tan et al., 2000).

There have been published studies on the epidemiology of the pterygium. It has been shown that the rate of pterygium is high in "pterygium belt" which is a geographical area located between 37-degree north and 37-degree south of the earths equator (Hashemi et al., 2016). There are many countries based studies have been carried out, in indigenous populations of the Brazilian Amazonian rain forest data collected from 624 adult Indians, the pterygium rate was 36.6% there was difference in the rate of the pterygium in the groups of the indigenous populations due to rate of sun exposure (Paula et al., 2006).

In Singapore pterygium rate was 10.1% in ≥40 years it was associated with outdoor working (Ang et al., 2012). In rural areas as china pterygium rate was 10.53% and In people 50 year and above it was associated with age and outdoor working without protection (Jiao et al., 2014). In southern indian population, the rate of pterygium was 9.5% in age above 40 years, this was higher in rural areas than urban population due to more exposure to UV radiation (Asokan et al., 2012). In spain the rate of pterygium is suggested to be lower than pinguecula, in population 40 years and above because of alcohol consumption the rate of pinguecula considered to be 47.9% and pterygium in 5.9% (Viso et al., 2011).

2.3 Factors of pterygium

2.3.1 UV Radiation

UV (beyond violet) is an electromagnetic radiation that is between visible light and x-ray. It has power to ionize molecules and can induce chemical reaction, it can cause mutation and many dangerous cancers. It has also beneficial factors such as inducing vitamin D. a UV light are subdivided into UVA (320–400 nm), UVB (280–320nm) and UVC (200–280 nm) according to their wavelengths and the UV that reaches the earth. UVA makes up 95%, UVB makes up 10% and UVC completely blocked by ozone layer (Figure 2.5) (Maverakis et al., 2010). UV radiation exposure is thought to be the primary cause of pterygium, an eye condition characterized by growth, winged like shape and fibrovascular growth which covers the cornea of the eye (Zhou et al., 2016). From all the types of UV wavelengths eye absorbs UVB radiation, to protect the inner side of the eye while long term exposure to UVB damages corneal and conjunctiva of the eye (B.-Y. Chen et al., 2013). UV has ability to dissolve the limbus which is a zone that separates conjunctiva and cornea and lets entry of conjunctiva into cornea and may lead to pterygium development (Chui et al., 2011).

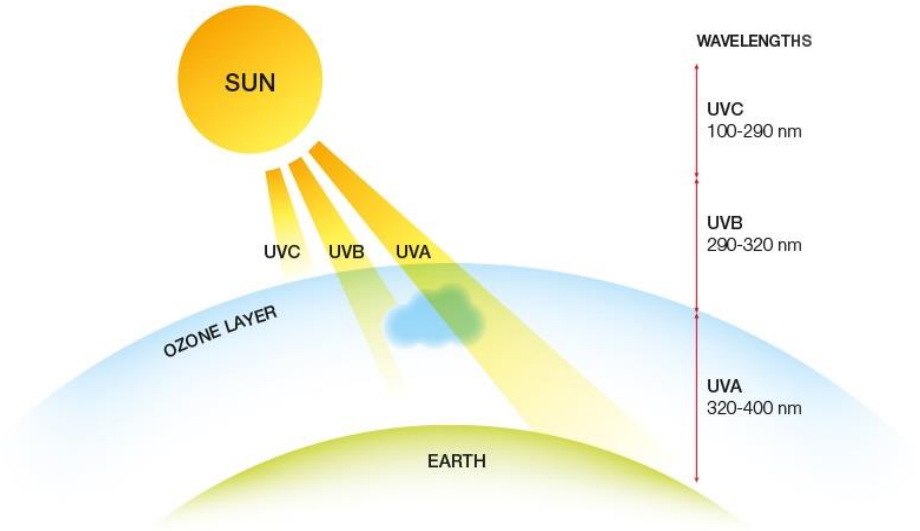


Figure 2.5: The UV rays coming from the sun.
https://www.ultraceuticals.com/blog/skin-science/do-you-know-the-difference-between-uv-rays/

UV indirectly has ability to form radical oxygen species (ROS) which can damage the cell in different ways including damaging DNA, lipid and proteins which it leads to oxidative stress in cell. In a study by Tsai et al show that the expression of 8-hydroxydeoxyguanosine (8-OhdG) has induced increasing in human 8-oxoguanine glycosylase (hOGG1) expression, and reveal that these cause oxidative stress to eye and may cause pterygium development (Tsai et al., 2005), also a study by Perra et al suggest that 8-OhdG expression is connected to p53 expression which there are evidence show that the expression of P53 has increased in pterygium and led to uncontrolled proliferation of epithelial cells and may have role in pterygium development (Perra et al., 2006). UVB has ability to mutate DNA and RNA, and induce expression of both growth factors and cytokines such as IL-6, IL-8, and VEGF which are considered as a risk factors in pterygium formation (Nick Di Girolamo et al., 2006). It was shown that UVB stimulated the expression of MMP-1 in the epithelial cells of eye and they suggested that UVB has role in development of pterygium (Nick Di Girolamo et al., 2003), Nolan et al. discovered heparin-binding epidermal growth factor-like growth factor (HB-EGF). it was elevated and expressed more due to exposure to UVB and they suggested that UV has major role in developing of pterygium disorder (Nolan et al., 2003).

2.3.2 Growth Factor

Growth factors are molecules that promote cell growth and proliferation. There are several growth factors have been reported to have role in pterygium pathogenesis. For example: vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF),basic fibroblast growth factor (bFGF),transforming growth factor-beta (TGF-β), insulin-like growth factor (IGF) and nerve growth factor (NGF).(Nick Di Girolamo et al., 2004).

VEGF is a growth factor with various roles, including angiogenesis, proliferation, and vasopermeability. It is a key growth factor in eye illness, VEGF family consist: VEGF-A (referred to as VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and PlGF (placental growth factor) and have a receptor called vascular endothelial growth factor receptor (VEGFR)-1-2-3 (Penn et al., 2008), VEGF and

VEGFR-2 have been reported that both were in higher level of expression than normal conjunctiva (Gumus et al., 2014).It has been presented that VEGF-C/VEGFR-3 pathaway have role in formation of lymphatic vessels more than VEGF mainly in recurrent pterygium (Ling et al., 2012). VEGF exposure 5-fluorouracil (5-FU) was suggested to reduce its expression but it has been confirmed it doesn't have any influence on its expression (Hoyama et al., 2015).It has been reported that (anti-VEGF) Pterygium can be treated with anti-vascular endothelial growth factor, although its safety has yet to be proved(Mak et al., 2017).

CTGF is a member of CCN family, a multi-functional protein which its name come from the first three members of this family in which they are six members, the first three members are cysteine-rich 61 (CYR61; CCN1), (CTGF; CCN2), nephroblastoma overexpressed (NOV; CCN3) and the other three members are CCN4 (WISP1), CCN5 (WISP2), and CCN6 (WISP3) which they all have role in cell adhesion, apoptosis and migration activities (C.-C. Chen & Lau, 2009). Setten et al discovered connective tissue growth factors in pterygium epithelium cells that were not seen in normal conjunctiva epithelium(Setten et al., 2003).

bFGF is another growth factor one of 18 members from FGF family that they are involved in angiogenesis, tumor progression, wound healing, endocrine signaling and development of some organs during embryogenesis (Beenken & Mohammadi, 2009) bFGF known as fibroblast growth factor 2 (FGF2), it was reported that its level is high in epithelium, mast cell and blood vessels of pterygium by(Y. Zhong et al., 2001), also another study shows that pterygium had a higher level of FGF2 mRNA than normal conjunctiva(Detorakis et al., 2010).bFGF was found that it induces formation of Cyclooxygease-2 (COX-2) which is the key enzyme for inflammatory cytokine-induced angiogenesis and it may be one of the factors infecting pterygium (Gao et al., 2007; Park et al., 2011).

(TGF-β) are large family of dimeric proteins that have many signaling rolls and several functions, they induce angiogenesis, induction of cellular proliferation, apoptosis, migration, adhesion, extracellular matrix (ECM) protein laysis , production,

vasculogenesis, and lymphangiogenesis (Goumans & ten Dijke, 2018). There are studies shows that the expression of TGF-β1 and TGF-β2 were up-regulated and their receptors were down regulated and they may which play a role in pterygium pathogenesis (M. Zhong et al., n.d.).

IGF is growth factor that can induce cell proliferation and prevent apoptosis also Insulin-like growth factor binding protein (IGFBPs) have same function as anti-apoptosis beside its main role in IGF transport (Firth & Baxter, 2002). It has been suggested that IGFBP-2 and IGFBP-3 have role in pterygium pathogenesis. Solomon et al reported that IGFBP-2 was over-expressed and was correlated with over-expression of MMP-1 in pterygium fibroblast (Solomon et al., 2003).

However, it was shown by Wong et al that the expression rate of IGFBP-3 was down regulated in pterygium (Wong et al., 2006), and there are evidence sates that the down regulation of IGFBP-3 related to cancer (Bruning et al., 1995). Lastly high level of growth factor NGF and its receptor may have connection to pterygium formation. It was reported that the endothelial cell of pterygium were immunoreactivity to NGF and its receptor TrkA, and that this immunoreactivity is related to microvascular density (Ribatti et al., 2009).

2.3.3 Interleukins

Interleukins are a group of cytokines which are unstable and cause non-lasting synthesis. They are secreted by many types of cells which are involved in immunity, cell proliferation, differentiation, growth, migration, survival and adhesion. Interleukins have role in immune, inflammatory response and functions in both autocrine and paracrine signaling. They are 40 (IL-1-IL-40) each type of interleukins have different functions and many of them work together to do the same function (Justiz Vaillant & Qurie, 2020). IL-6 and IL-8 have been confirmed that they are strongly connected to pterygium, they are expressed abundantly in pterygium's epithelial cells by UV and they are involved in inflammation, proliferation and producing blood vessel, so they may play a great role in pterygium pathogenesis (Nick Di Girolamo et al., 2002). According to Tiong et al research, the two pro-inflammatory interleukins IL17 and IL21 expressed much higher in pterygium than normal conjuctiva cells, may play role in pterygium development by inducing inflammatory process (Tiong et al., 2017). by Zidi et al it was suggested that inflammatory and antiapoptotic process are involved in pterygium pathgogenesis such as the roles of IL-17A, IL-6 and NO in pterygium pathogenesis (Zidi et al., 2017).

2.3.4 Heat Shock Protein (HSPs)

Heat shock proteins are proteins secreted when a cell is exposed to stress (J. Wu et al., 2017). According to their molecular weight they have been classified into members. There are six members, the smallest Hsps is 5-30KDa and the others are HSPs40, HSPs60, HSPs70, HSPs90, HSPs110. HSPs acts as chaperon helping protein folding, assembly of protein, protein degradation. By defect of HSPs many serious diseases happen such as cancer and inflammation (Mellati, 2006). in a report by Tiong et- al the rate of Hsp90 is up regulated in pterygium compare normal conjunctiva (Tiong et al., 2017). In addition, according to pagoulatos et al the expression of Hsp90, Hsp70, and Hsp27 were reported in higher level of expression in pterygia than normal conjunctiva (Pagoulatos et al., 2014)

2.3.5 Virus

Viruses also have been confirmed to have role in conjunctival neoplastic lesion Conjunctival papilloma, conjunctival intraepithelial neoplasia (CIN), ocular surface squamous neoplasia (OSSN) have role in pterygium pathogenesis. Viruses that have been involved in pterygium pathogenesis include: HPV (Human papillomavirus), CMV (cytomegalo virus), (HSV) Herpes Simplex Virus and EBV (Epstein-Barr virus) which they are oncogenic viruses. Most common viruses which have been reported to have relation to pterygium development is HPV (CHALKIA et al., 2013). It was shown by spandidos et al that HSV and CMV were found in pterygium tissue and they suggested that they may have a role in pterygium pathogenesis (Spandidos et al., 1994), chen et al unconfirmed that HSV has role in pterygium development or pathogenesis (Y.-F. Chen et al., 2008). according to Gallagher et al theisis HPV virus have a role in development of pterygium and may lead to pterygium recurrence after surgery (Gallagher et al., 2001), also in Tsai et al research HPV16 and HPV18 was present in pterygium tissue and was not found in normal conjuctiva, and suggested that it has relation with pterygium pathogenesis (Tsai et al., 2009)

2.3.6 Tumer supresser gene (P53)

Tumor suppressor gene is a gene that controls cell cycle. when DNA is damaged P53 repairs it by not allowing cell division to occur and stopping cell cycle in G1 before DNA synthesis occur in S phase. It also induces apoptosis and inhibition of angeogenesis and metastasis (Benjamin et al., 2008). Mutation in p53 gene is mediated by chronic exposure to UVB caused skin cancer in mouse (Berg et al., 1996). Onur et al found very low level of P53 gene expression in pterygium, 3 out of 38 samples showed P53 expression in them and they concluded that altered P53 is not associated with pterygium formation (Onur et al., 1998).A study shows that p53 mutation is not induced by UV rather than it is uncontrolled cell proliferation (Pelit et al., 2009). In another study it has been suggested that p53 was not mutated and which they were in high level in pterygium cells, however they have been found that they are inactive which does not prevent apoptosis or prevent cell cycle(Schneider et al., 2006).P53 was

found very high in pterygium epithelial cells compared to normal conjunctiva and was suggested pterygium is a growth disorder not a degenerative disorder because of uncontrolled growth of cells (Weinstein et al., 2002), in a study by Liang et al also showed that there are no apoptosis and cellular proliferation activity in epithelial cells of pterygium (Liang et al., 2011),

2.3.7 Extracellular Matrix Protein

ECM are networks of macromolecules in which can hold the cells and provides the structure of the tissues (Järveläinen et al., 2009). ECM consists of collagens, elastin, proteoglycans, microfibrillar proteins, laminin and fibronectin, they provide chemical and physical property of the cell. Cells can feel and interact with outside environment and acts as a physical strength to the cell (Figure 2.6) (Yue, 2014).

Collagen type I, type III and type IV present in both normal conjunctiva and pterygium epithelia while type II has been shown only presented in pterygium epithelium and may have role in its development (Dake et al., 1989). Evidence the use of collagen matrix implant to reduce the rate the recurrence of pterygium has been tested and it was shown that it reduces the risk rate of pterygium recurrence (Arish et al., 2013). In a study by Zhang et al also they showed that keratins such as K8, K10, K14, K16 and AE3 are found in pterygium cells but not in normal conjunctiva and concluded that they may role on causing high proliferation of pterygium and lead to its development (M. Zhang et al., 2000),

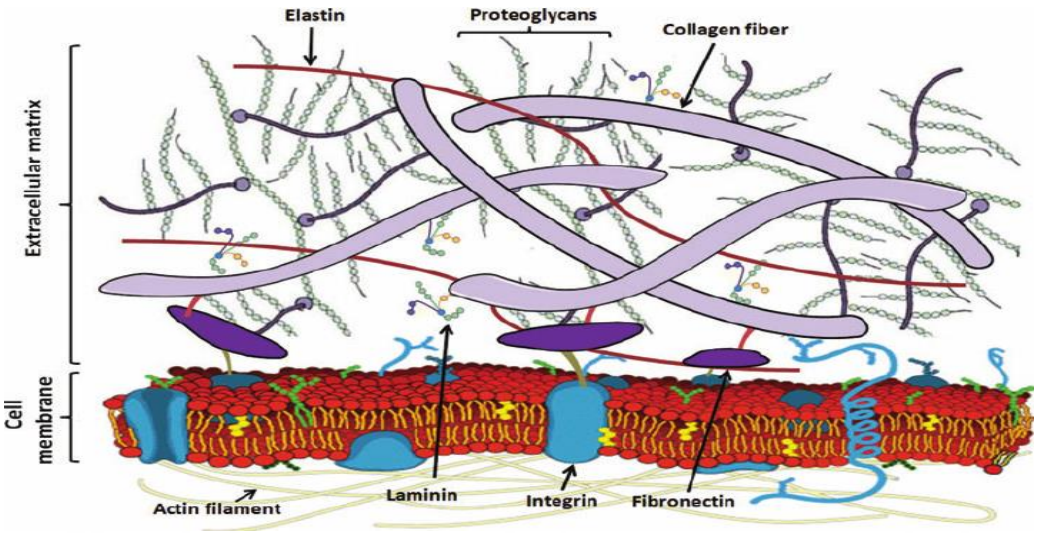


Figure 2.6: the components of ECM. https://www.researchgate.net/figure/Major-extracellular-matrix-components-and-their-interactions-with-each-other-and-cell_fig2_269838392

2.3.8 Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases

Matrix metaloproteinase(MMPs) are proteiteinase which degredate extracellular matrix(ECM). They are divided into 6 groups based on the substrate they work on. MMP-1, MMP-8, MMP-13, and MMP-18 are in collagenase group which are able to degredade collagen I II III.elatinase include gelatinase A (MMP-2) and gelatinase B (MMP-9), they can digest denatured collagens and gelatins. Stromelysins include Stromelysin 1 (MMP-3) and stromelysin 2 (MMP-10) both work on the same substance.Matrilysins are characterized by(MMP-7)and (MMP-26),Membrane-Type MMPs includes six types which are MMP-14, MMP-15, MMP-16, MMP-24,MMP-17 and MMP-25, they lysis collagene and have role in angeogenesis and digest some extracellular matrix and others such as MMP-12,MMP-19 ,MMP-20 digest amelogenin, MMP-22 (Visse Robert & Nagase Hideaki, 2003).

Tissue inhibitors of metalloproteinases(TIMs) are proteins which bind to MMPs to alter its function, they are TIM1, TIM2, TIM3, TIM4 (Brew et al., 2000). In a study which shows that both MMP and TIMP were present in pterygium, MMP-9 and MMP-10 were found in cytoplasm of epithelial cells of pterygium and also TIMP was found

in epithelium and fibroblasts of pterygium and corneal epithelial cells which they concluded they may have role in pterygium formation (Tsai et al., 2010), In another study MMP-2 and MMP-9 were both found over-expressed in pterygium tissue and fibroblast in advance stages of pterygium and shown that they may have role in pterygium pathogenesis and development (Yang et al., 2009), also MMP-1 and MMP3 both has been found with different levels in primary and secondary pterygium (An et al., 2011). Moreover, characteristics such as Angeogenesis and inflammation in pterygium maybe be due to presence of MMP and TIMP sugessted by (N. Di Girolamo et al., 2000)

2.3.9 Apoptosis Realated Proteins

Apoptosis is a programmed mechanism of cell death that work for maintaining and developing of cells and tissues it prevents producing malignant cells and prevents metastasis (Su et al., 2015). Apoptosis related protein are proteins which take part in apoptosis include (survivin, Bcl-2, Bax and Bcl-w) (Feng et al., 2017), it was suggested that pterygium development is related to disruption of normal process of apoptosis (Tan et al., 2000).

Survivin is a protein which can block caspase activation to inhibit apoptosis, and reports show that it is mostly found in human cancers. Moreover, over-expression of survivin has relation to inhibition of apoptosis (X. Chen et al., 2016). Over-expression of survivin and p53 has been reported in cells of primary and recurrent pterygium and suggested that they have role in development and progression of pterygium (L.-W. Zhang et al., n.d.).

Bcl-2 (B-cell lymphoma/leukemia-2 gene) are a group of proteins nearly 15 members. They have role in preventing or inducing apoptosis, and they are encoded by a gene Bcl-2 on chromosome location of 18q21.3 (Papaliagkas et al., 2007),zhang et al concluded that the expression of Bcl-2 was higher in pterygium than normal comjuctiva and suggested that it may have role in its progression and formation (D.-N. Zhang et al., 2012).

2.3.10 Cell adhesion proteins

Cell adhesion proteins are proteins found on cell surface which functions as adhering the cells together (Figure 2.7) (Gumbiner, 1996). ICAM-1 is a molecule which expressed by leukocytes and endothelial cells and other cells, it is a reason for the process of inflamation and T-cell mediated host defense system(van de Stolpe & van der Saag, 1996).According to beden et al, intercellular adhesion molecule-1 is present in pterygium but not in normal conjunctiva and concluded that its one of the factors that induce pterygium formation (Beden et al., 2003), also Tekelioglu et al found that there are high rates of intercellular adhesion molecule-1 (ICAM-1). Vascular cell adhesion molecule-1 (VCAM-1) in pterygium tissue and low rates in normal conjunctiva tissue which led to suggestion that they may have relation to the pathogenesis of pterygium disorder by increasing CD4 and CD8 lymphocytes (Tekelioglu et al., 2006), and Kase et al showed that E-cadherin may have role in formation pterygium by inducing cell proliferation and adhesion (Kase et al., 2007).

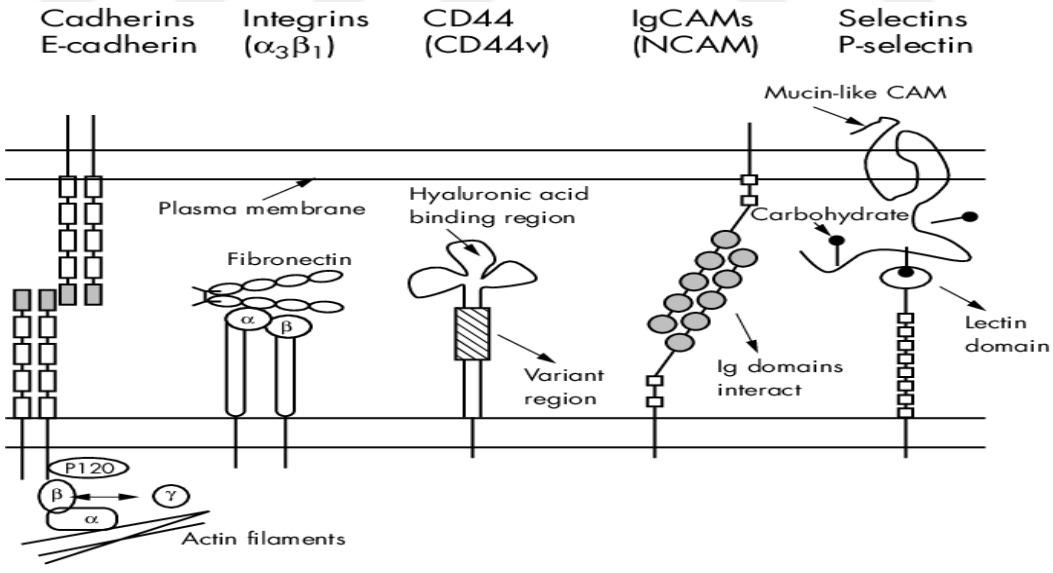


Figure 2.7: showing cell adhesion Molecules.
https://www.researchgate.net/figure/Diagrammatic-representation-of-five-families-of-cell-adhesion-molecules-CAMS_fig2_7944627

2.3.11 Actin related protein 3 (ACTR3)

This gene is located on chromosome 2q14.1 (Figure2.8) it has 13 exons and 5499 base pairs. Scientists could not yet find the specific function of this gene but this gene encodes for protein (ARP3) that is important component of ARP2/3 complex which is located on the surface of the cells in which it gives each cell its shape and motility (ACTR3 Actin Related Protein 3 [Homo Sapiens (Human)] - Gene - NCBI, n.d.)

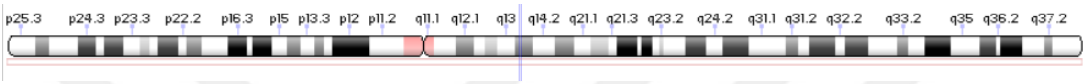


Figure 2.8: location of ACTR3 gene on chromosome.
https://www.ncbi.nlm.nih.gov/genome/gdv/browser/gene/?id=101060091

The ARP2/3 complex induces formation and assembly of new branch (daughter filament) of actin filaments (nucleation of actin) from mother filament which results in formation of branched actin network it controls phagocytosis, cell locomotion and intracellular motility of vesicles, organelles, and certain pathogens. ARP2/3 is composed of seven complexes, there are two major components they are Arp2 and Arp3 which they form the first two subunits of daughter filament and they resemble in structure to actin, and five other subunits which are ARPC1–5. This seven protein complex bind to the mother actin filament and it starts forming new actin filaments in presence of ATP and nucleation-promoting factors (Figure2.9) (Rouiller et al., 2008). Arp2/3 complex by itself is inactive it needs nucleation promoting factor (NPF) such as WASP, WAVE and SCAR. Which they have VCA, (verprolin homology) sequences (also known as WASP homology 2 or WH2 domains), C (central or connecting) an A (acidic) (Figurer.13) which they each have role as following, actin monomers binds to V and C is important for nucleation and A which activates Arp complex (Kelly et al., 2006). Actin gives cell shape and support to the cell. Moreover, change in cell morphology and shape in pterygium has been reported (Zoroquiain et al., 2016). Arp3 has been reported that has role in formation, growth, invading and development of some

type of tumor such as gastric carcinomas. And pterygium has been reported that have characteristics of tumor development including invading and uncontrolled rate of recurrence (Chui et al., 2011; Zheng et al., 2008)

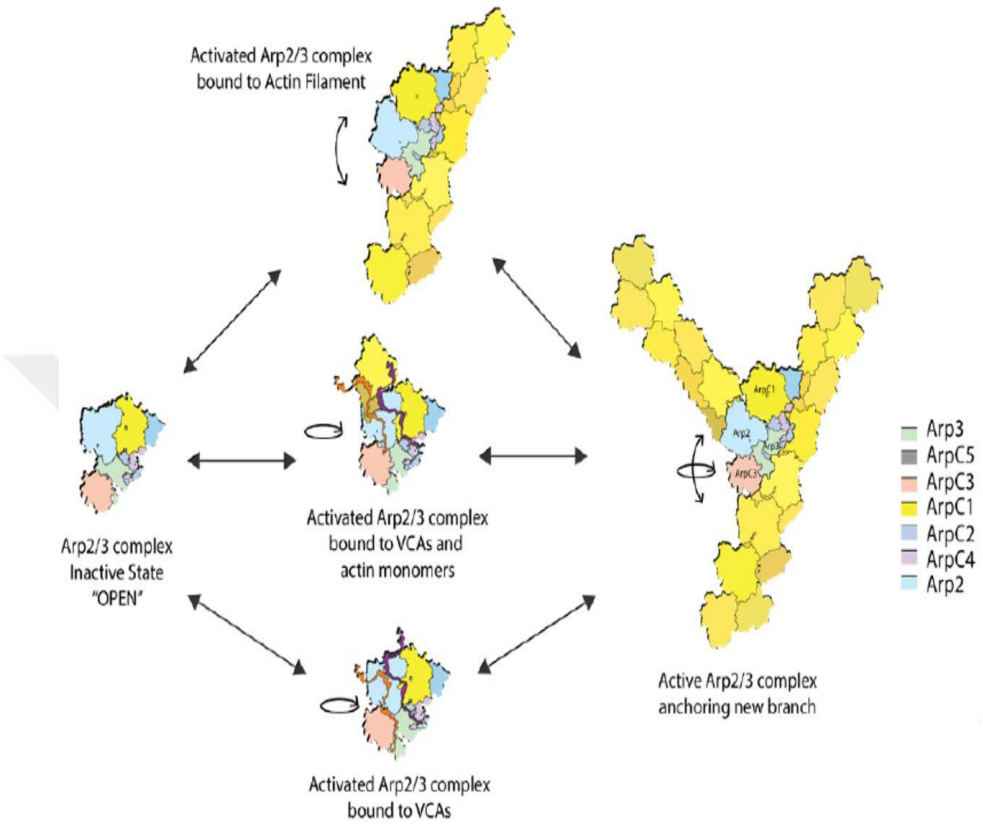


Figure 2.9: Arp2/3 complex, formation of new actin filament.
https://www.biorxiv.org/content/10.1101/164319v1.full

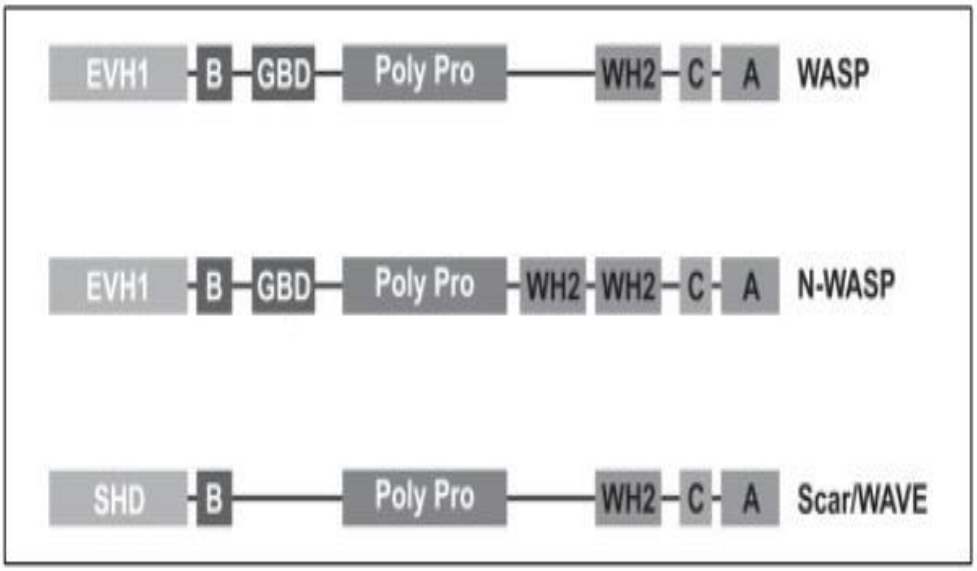


Figure 2.10: WASP, WAVE and SCAR VCA domain.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3077991/

3. MATERIAL AND METHOD

3.1 Material

3.1.1. Working Group

36 samples (18 pterygium; 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 control group of the patient group and the normal conjunctival tissues belonging to the same eye of the same patients. 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

Some of the tissue samples taken during surgery were sent for histopathological examination and a diagnosis of pterygium was made. at -80° C, Gene expression analysis by obtaining cDNA from the tissue pieces are used to detect by real-time PCR.

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, Thermo brand RNA mini kit (Cat No: 12183018A) RNA. An isolation kit was used; the stages of RNA isolation are carried out as listed below.

- An environment free of RNase was prepared for Caligma, tissue samples removed from -80 ° C and were cut into small pieces with a scalpel on ice and 20-25 mg of each sample was used.

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

Tissue samples

Using a homogenizer, homogenize 100 mg of tissue samples in 1 ml RiboExTM.

The sample volume should not be more than 10% (w/v) of the RiboExTM homogenization volume.

2. . Allow the homogenate to sit at room temperature for 5 minutes.

This phase permits nucleoprotein complexes to dissolve entirely.

Homogenized samples can be kept for at least one month at -70°C.

3. Centrifuge at 12,000 x g for 10 minutes at 4°C and transfer the supernatant to a fresh tube. This optional step is only required for homogenate with high contents of proteins, fats, polysaccharides or extracellular materials such as muscles, fat, tissue, and tuberous parts of plants. The resulting pellet contains extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernatant contains RNA. Fat tissue samples will form a layer on top of the aqueous phase, therefore, remove and discard this layer.

4. To 1 mL RiboExTM, add 0.2 mL chloroform.

Shake vigorously for 15 seconds and store for 2 minutes at room temperature, instead you can use 0.1 ml of BCP (1-bromo-3-chloropropane) instead of chloroform.

5. Centrifuge at 12,000 x g for 15 minutes at 4°C and transfer the aqueous phase to a fresh tube. The mixture will be separated into three phases; a lower layer, an interphase, and a colorless upper aqueous layer. The upper aqueous layer is about 50% of the volume of RiboExTM used for homogenization. Centrifugation at temperatures >8°C may 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.

7. Transfer up to 700 ul of the mixture to a mini spin column.

8. Centrifuge at at room temperature for 30 seconds at 10,000 x g, Replace the tiny spin column in the same tube after discarding the pass-through.

9. Repeat step 7 ~ 8 using the remainder of the sample

10. To the mini spin column, add 500 ul of buffer SW1..

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

12. to the mini spin column Add 500 ul of buffer RNW.

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

14. Centrifuge at ≥ 10,000 x g for an additional 1 minute at room temperature to remove residual wash buffer. Transfer the mini spin column to a new 1.5 ml tube (provided). Residual ethanol may interfere with downstream reactions. Care must be taken at this step for eliminating the carryover of buffer RNW.

15. Fill the mini spin column with 50 to 100 ul of RNase-free water in the center of the membrane, 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 rapid analysis or at -70°C for long-term preservation.

3.2.2. Measurement of mRNA concentration

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 Standard1 and Standard2 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

For cDNA (copied DNA) synthesis I used A.B.T. cDNA Synthesis Kit with RNase Inhibitor, which it contains all the components to obtain cDNA except the RNA sample in table 1.3 and RNase Inhibitor presented in the kit adequately protects RNA templates against degradation.

TABLE 3.1. Reverse transcriptase reaction volume (20 µl)

For 20 µl Reverse Transcriptase Reaction	**Volume**
10X Reaction Buffer	2 µl
dNTP mix (2.5 mM each)	1 µl
Random hexamer (50 µM)	2 µl
Reverse Transcriptase (200 U/µl)	1 µl
RNase Inhibitor	0.5 µl
RNase free Water	3.5 µl
RNA Template	10 µl

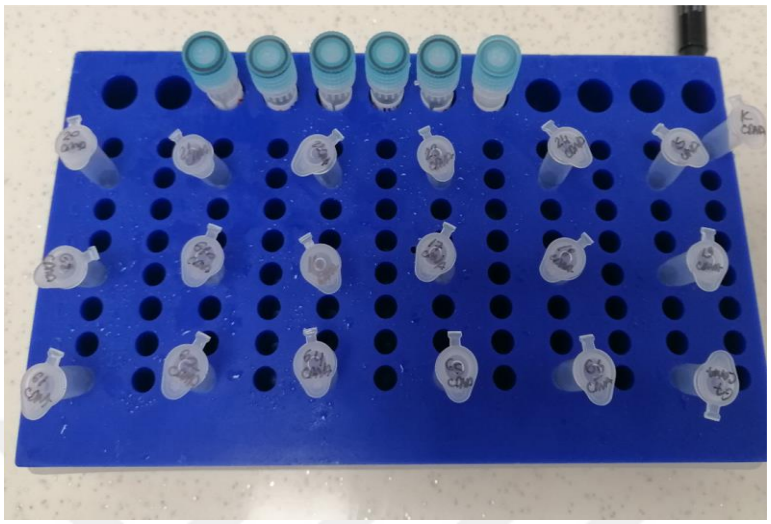
CDNA synthesis was done in two steps.

1. The amount information of the kit used for cDNA synthesis is given in the table above.

We mix all the components together and prepare 220 µl of all the components without the RNA sample, we obtained this by multiplying each components volume to 22 samples although we had 19 samples but we did this for spare volume.

we transfer 10µl of the mixture we prepared into 19 new tubes and after that we also transfer the RNA samples to each new tube with the same labeled names (Figure 3.1)

- A-



B-

Figure 3.1: A- purified RNA, B- the kit components in each tube without RNA

2. After the cDNA mastermix was prepared on ice, the following conditions were followed.

Table 3. 2 is the PCR program applied for cDNA synthesis

Then we put the tubes that contain mixture of RNA samples and reverse transcriptase reaction component into the thermocycler (Figure 3.2) with these conditions Table-3.

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 synthesized 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: The 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, Standard2: 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-ACTR-3 mRNA Expression

ACTR-3 gene expressions in pterygium and normal conjunctiva tissues were performed in Applied Biosystem StepOnePlus device (Figure 3.4**)** using SYBR-Green based Real-time PCR method.

3.2.6 Real-time PCR

After obtaining cDNA from the isolated RNAs, the real time PCR Applied Biosystem StepOnePlus device was started for expression analysis. At this stage, the control and study groups for the target ACTR-3 gene and beta-actin(ACTB) as the internal control gene were used. A.B.T.TM 2X qPCR SYBR-Green MasterMix (with ROX) (Cat no: Q03-02-01) was used as the reaction mixture.

The reaction mixture was prepared as follows:

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

For 20 µl PCR Reaction	**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	5 µl

It was carried out in 20ul volume using the components given in in the expression analysis of the ACTR-3 gene, Beta actin (B-actin) was used as an internal control, and a PCR mixture without cDNA sample was used as a negative control.

Added the mixture to the PCR plate (Figure 3.3) for with two gene target gene (ACTR-3) and a control gene (ACTB), and then cDNA of each gene added to their place on the plate and mix with their forward and reverse primers of their own and the plate placed in the real-time PCR (Figure 3.4).

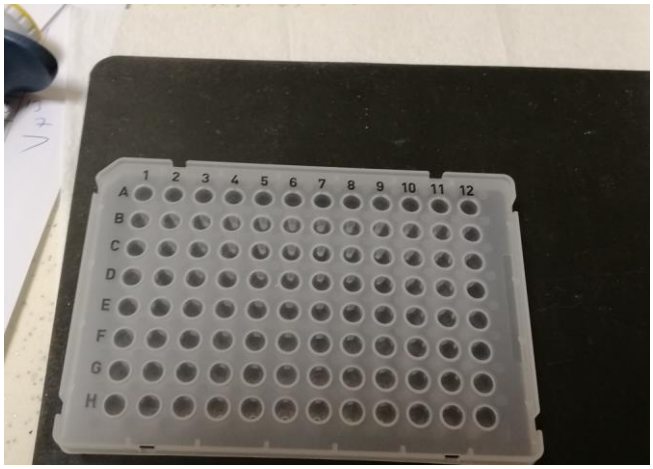


Figure 3.3: The PCR plate.

Figure 3.4: Applied Biosystem StepOnePlus device real-time PCR

The real-time PCR machine system was prepared for the cycling reaction (Table 3.6) 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

4.1. Result to the Working Groups

In Gaziosmanpasa university hospital 18 invidividual were diagnosed with pterygium. Two groups were created, first group was tissues with pterygium and the second tissue was healthy conjunctiva tissue of the same person.

The age of the patients was between 27-93 and the mean was found to be 56 ± 13.84. 15 12 of the Patients were male meanwhile 6 of the patients were female and 15 was taken from the right eye and 3 was taken from the left eye

Demographic data of patient group with pterygium are shown in Table 3.7

TABLE 4.1. The Demographic data of patient group with pterygium

	Pterygium (n=18)
Age	56±13, 29
Gender	12M/6F
Right/left eye	15Right/3Left

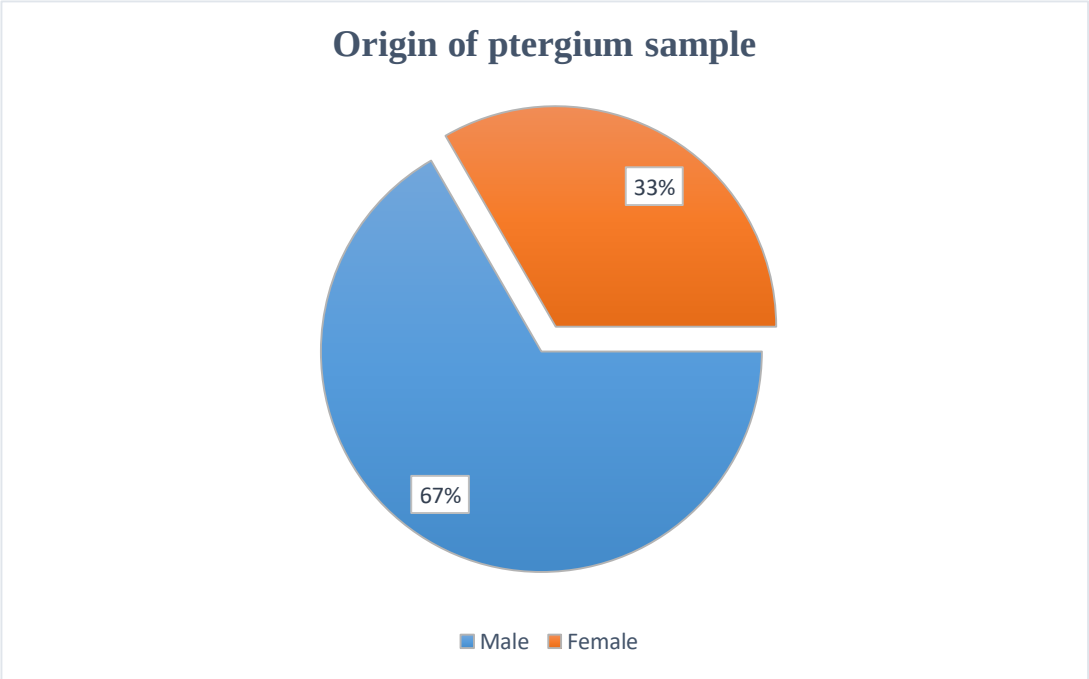


Figure 4.1: Showing gender distribution.

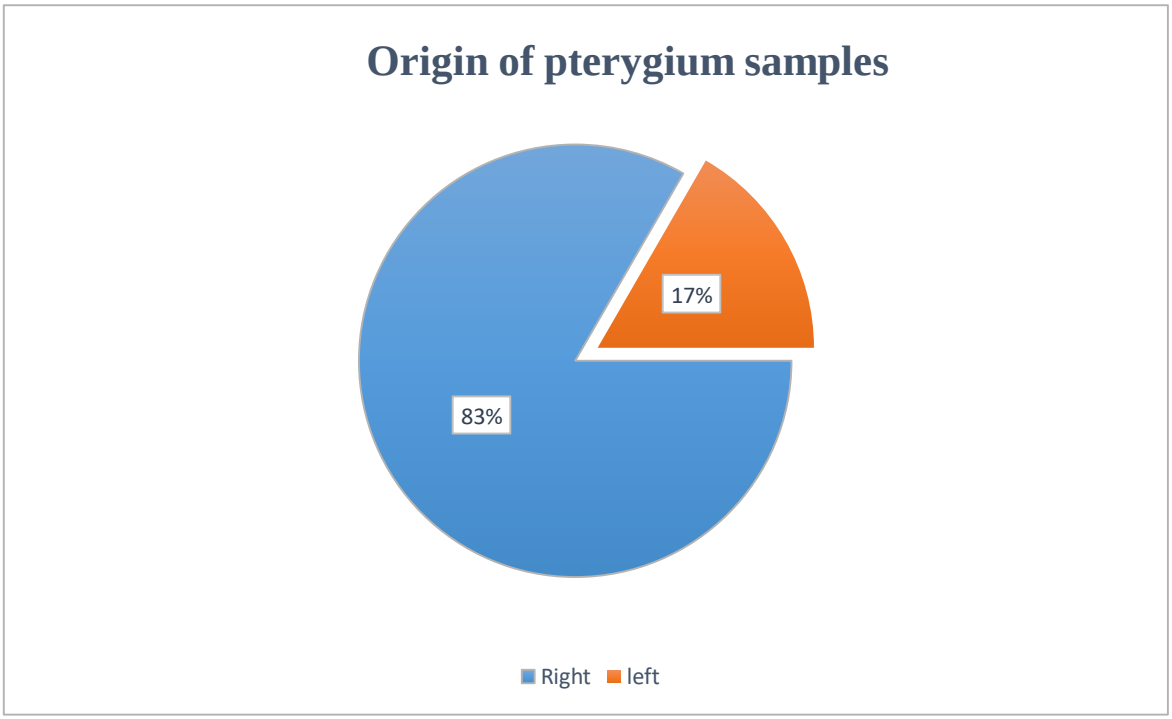


Figure 4.2: the difference between numbers of right and left eyes.

4.2 Analysis of Quantitative Real-Time PCR (qRT-PcR) results

4.2.1. qRT-PCR Images and Evaluation of the ACTR-3 Gene

The expression level of ACTR-3 mRNA was determined using a quantitative Real-Time PCR technique based on SYBR Green. For ACTR-3 and the gene preferred as reference gene (Beta Actin : ACTB), cDNA samples were made, and dilutions at various concentrations were performed. For each sample, amplification efficiencies were calculated by performing 3 repeated readings on the Applied Biosystems StepOnePlus device. After calculating the amplification efficiencies, cDNA and standard samples of pterygium and control conjunctiva tissues were studied in 3 repetitions and the mean CT (cycle threshold) value was obtained. In the study, as the reference (control) gene; ACTB gene: as negative control; Real-Time mix without cDNA template and finally for positive control; an example with a good result was used before. In the figures below (Figure 4.3, 4.4, 4.5, 4.6) respectively shows the amplification curve and melting peak of ACTR-3 and ACTB gene that was observed as a given result of qRT-PCR.

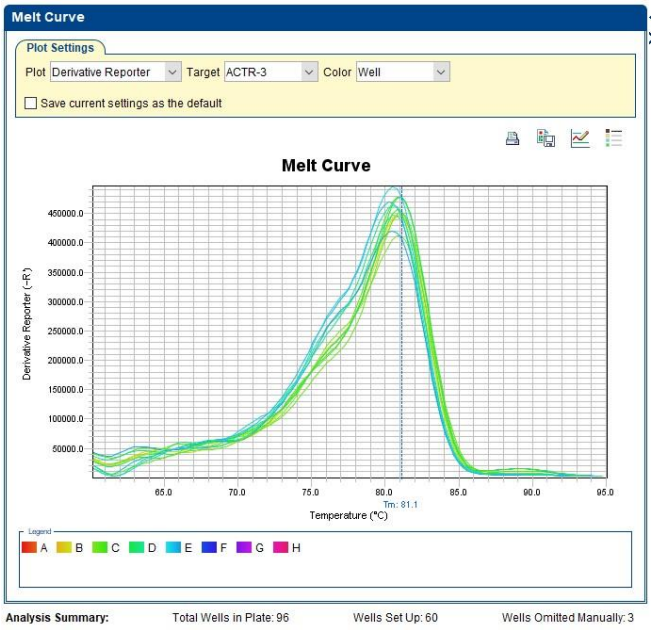


Figure 4.3: Melting curve of ACTR-3

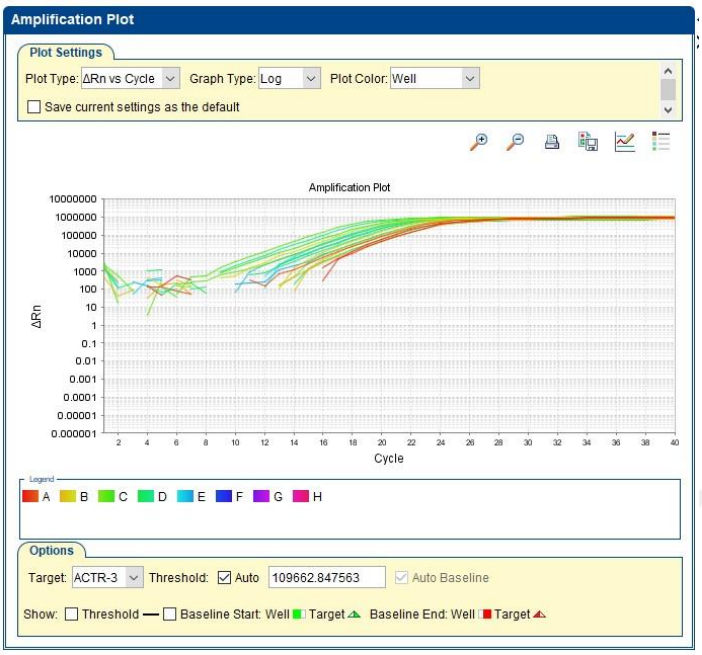


Figure 4.4: Amplification plot of ACTR-3

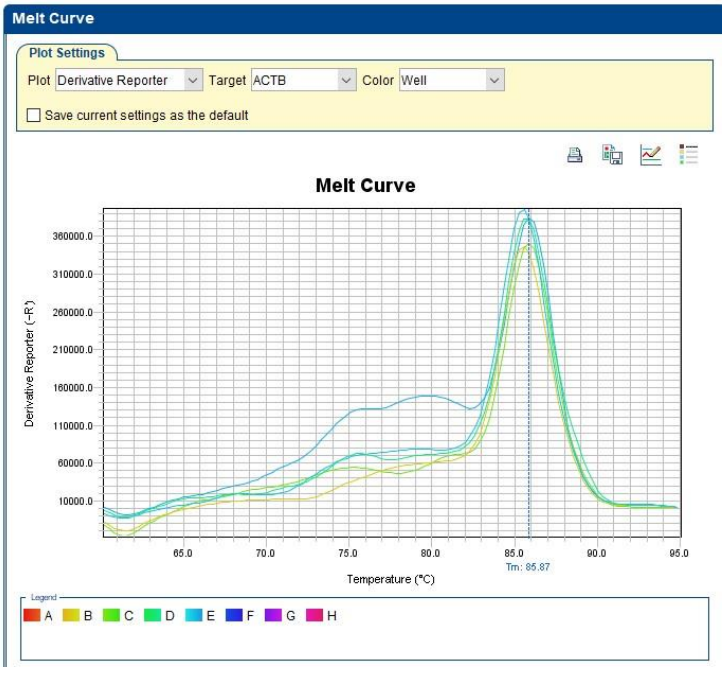


Figure 4.5: Melting curve of ACTB

Figure 4.6: Amplification plot of ACTB

4.3. Statistical analysis of qRT-PCR findings of the ACTR-3mRNA gene

QRT-PCR was used to evaluate the expression of the ACTR-3 gene at the mRNA level in pterygium and control conjunctiva tissues. The ACTR-3 and ACTB genes' QRT-PCR results were quantified using the step one plus software v2.3 program.The ACTR-3 gene data was standardized against the ACTB gene, by using the 2-ΔΔCt formula fold change was calculated (Table4.2). The information acquired throughout this method was statistically analyzed. All data was presented as mean standard error of the mean (SEM). As a guideline, the results were analyzed using a range of 0.9 to 1.1. When compared to normal conjunctival tissue, the expression level of the relevant gene in the pterygium tissue was reduced, with values less than 0.9, when compared to normal conjunctival tissue in the range of 0.9-1.1, remained unchanged, and when compared to normal conjunctival tissue, the gene's expression was higher in the pterygium tissue than in normal conjunctival tissue, with a value greater than 1.1. Statistical analysis of expression data was performed using SPSS 16.0 software. The significance levels of the data were analyzed with Student's test. The statistical significance level was accepted as 0.05, if p value was less or equal to 0.05 ($p \leq 0.05$) then it is significant, and if p value is greater than 0.05 ($p > 0.05$), it is not significant and suggested that there was no difference (Schmittgen & Livak, 2008).

TABLE 4.2. the complete 2-ΔΔCt formula.

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: ARPC-2 CT - ACTB CT = Δ CT1 For pterygium: ARPC-2 CT - ACTB CT = Δ CT2 Δ CT1- Δ CT2 = ΔΔ CT2

TABLE 4.3. Statistical analysis of 2 -$\Delta\Delta C_T$ values of ACTR-3 in conjunctiva tissues.

gene	Tissue	
ACTR-3 mean ± SEM	Pterygium N=18	Normal conjunctiva N=18
	13.62±4.91	
P	0.015	

As shown in Table 3.8 that presents the results of the qRT-PCR which was obtained from C_T of both target gene and control gene of pterygium and normal conjunctiva tissue. According to this statistical values (13.62±4.91-fold change, P = 0.015) shows that the rate of ACTR-3 gene expression has extremely increased in pterygium when it is compared to normal conjunctiva tissues and the difference between the two samples is statistically significant according to ($p \leq 0.05$) which is taken as a standard value.

5. DISCUSSION

Pterygium is winged like shape pinkish color with fibrovascular growth which grows over cornea of the eye, and some other studies consider it as a degenerative disease. It is composed of three parts (head, neck and body). There are some recognizable distinguishing features of this disease which they are: induction of cell proliferation, inflammation, fibrosis, angiogenesis, squamous metaplasia, goblet cell hyperplasia, and extracellular matrix breakdown. Pterygium causes eye irritation and cosmetic disorder, the main factor for causing pterygium considered to be UV, mostly people who live in rural areas exposed to prolonged exposure of the sun have the most probability to be diagnosed with pterygium. The main treatment for pterygium is surgery which there are several types each with different recurrence rate which is the main problem for treating pterygium (Engelsvold et al., 2013).

To this date there is no clear understanding. There have been several studies, each describing a distinct cause for the occurrence of this eye disorder. Other factors such as viruses which there are variety of oncogenic viruses have been reported that controversially have role in development of pterygia and some other eye disorder such as: conjunctival neoplastic lesions, Conjunctival papilloma, conjunctival intraepithelial neoplasia, ocular surface squamous neoplasia and (Chalkia et al., 2019) apoptosis is a natural process that cells die disruption of this process thought to induce pterygia. Survivin, Bcl-2 and p53 have been reported in high rate of expression and may have role in pterygium by inducing high cellular proliferating (D.-N. Zhang et al., 2012; L.-W. Zhang et al., 2011).

Growth factors which stimulate cell growth and proliferation have been also reported that they induce angiogenesis which is one the characteristics of pterygium growth factors that has been reported to have role in pterygia include: VEGF, CTGF, bFGF, TGF-β, IGF and NGF (Di Girolamo et al., 2004). Interleukins have role in immunity, inflammatory, cellular proliferation, differentiation and growth. IL-6, IL-8, IL17 and IL21 have been reported that they are over-expressed in pterygium cells and involved in its development and induce angiogenesis (Di Girolamo et al., 2002; Ki et

al., 2017). Intercellular adhesion molecule-1, E-cadherin and Hsp90 both has been shown with high rate of expression in pterygial cells which may lead to cellular proliferation, and protein degradation and have role in cancer development which this leads to suggestion that heat shock protein and cell adhesion proteins are both may involve in development of pterygia (Beden et al., 2003; Kase et al., 2007; Sebastiá et al., 2013).

Matrix metalloproteinases and Tissue Inhibitors of Metalloproteinases also both has been suggested as a factor to induce angiogenesis and inflammation due to high expression rate in pterygium cells (Tsai et al., 2010). ECM which support the cell physically hand help in cellular integrity. Evidence of these macromolecules in pterygium development has been established, few types of collagen and keratin both has been reported that are present in pterygia cells but not in normal conjunctiva cells led to suggestion that they might induce its development (Dake et al., 1989; M. Zhang et al., 2000).

ACTR-3 is a gene that encodes for an important protein (Arp3) which one of the seven subunits of ARP 2/3 complex that have role in assembling and regulating new actin branch formation. This gene is located on chromosome 2q14.1, it has 13 exons and 5499 base pairs structurally Arp3 resembles actin with Arp2 they form the first two subunits in nucleating actin after the complex is activated by nucleating promoting factor, with all other subunits (ARPC 1- 5) they complete the assembling and form a new branch of actin, in which Actin filament has several important role which include controlling phagocytosis, cell locomotion and intracellular motility of vesicles, organelles, certain pathogens, and gives cell shape and support while cell shape and morphology changes in pterygium which may have role in its development (Zoroquiain et al., 2016).

Over expression of ARP 2/3 has been linked to cancer development and used as a marker for diagnosing cancerous diseases. Over the years, deregulation of the Arp2/3 regulatory system has been reported in many types of cancer. Immunohistochemistry has revealed that one or more Arp2/3 subunits are overexpressed in a number of

malignancies, including lung, breast, gliomas, gastric, and colorectal cancers. Also Arp2/3 has been shown to have role in formation of lamellopodia and appendages such as invadopodia which they help in invasion and metastatic characteristic of cancers. Overexpression of the Arp2/3 complex in patient samples was more evident in strong invasive colorectal carcinomas and was associated with liver cancer that came from another part of body. Invasive carcinoma cells were discovered to overexpress genes encoding Arp2/3 subunits in breast cancer cell lines and mammary tumors produced in a mouse model. The two genes encoding the two paralogous ARPC1 subunits, ARPC1a and ARPC1b, are overexpressed in pancreatic cancer. In this cancer, these two genes are commonly amplified. It was confirmed in pancreatic cancer cell lines that RNAi-mediated reduction of the Arp2/3 complex reduces migration and invasion, much as it has in other cell models. There are been studies ensure that upregulation ARP2/3 complex has been shown that it has direct proportional connection to upregulation of HER2 in breast cancer. It's also has been confirmed that ARPC2 and ACTR2 both have role in cancer development and progression such as gliomas and breast cancer respectively, and in cancers such as lung cancer Arp2/3 complex has role in lowering rate of mortality of the patent.

The N-WASP, WAVE, WASH and WHAMM families of NPFs all also have been studied to find their relation to cancer development and inducing their invasive trait. The N-WASP family has been shown to have been over-expressed in lung cancer, pancreatic ductal adenocarcinomas, and hepatocellular carcinomas but have shown downregulation in breast carcinomas. N-WASP also have role in formation protrusions and lamellipodia in transformed cells which they help in invasion and metastasis of cancer such lung metastasis from breast cancer.

WAVE family have important function such as control of cellular migration. This family also has been reported to have role in cancer formation and helping in its cellular invasion by lamellipodia. In cancers such as breast, colon, liver, lung, ovary, and prostate this family's proteins were shown to be overexpressed.

The nucleating factors which had role more in intracellular trafficking rather than having role in development, progression and contributing in their invasive and metastatic traits were WASH, WHAMM, and JMY (Swaney et al., 2016; Molinie et al., 2018; Rothschild et al., 2006)

In addition, evidence of ACTR-3 or its protein Arp3 have role in promoting some types of cancer, such as their involvement has been reported in growth, invading, proliferating and inducing invasive trait of metastatic cancer such as gastric carcinomas and colorectal cancer. And it's thought to have role in pterygium development because pterygia also reported that has cancer like traits including invading other cells and high rate of recurrence after surgery (Chui et al., 2011; Zheng et al., 2008)

The statistical analysis of ACTR-3 which was done according to results of qRT-PCR in which the results (13.62±4.91-fold change, P = 0.015) showed that expression in of ACTR-3 gene expression has significantly ($p \leq 0.05$) increased in pterygium compared to normal conjunctiva tissues.

In this thesis a high over-expression in the rate of ACTR-3 expression in pterygium samples has been shown compare to normal conjunctiva samples and there was significance difference between the two groups. Which leads to conclusion that ACTR-3 may have role in pterygium formation and development. With Further studies a strong connection between ACTR-3 and pterygium pathogenesis may be found.

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