**A novel role for CRIM1 in the corneal response to UV and pterygium development**

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**ABSTRACT**

Pterygium is a pathological proliferative condition of the ocular surface, characterized by formation of a highly vascularized, fibrous tissue arising from the limbus that invades the central cornea leading to visual disturbance and, if untreated, blindness. Whilst chronic ultraviolet (UV) light exposure plays a major role in its pathogenesis, higher susceptibility to pterygium is observed in some families, suggesting a genetic component.

In this study, a Northern Irish family affected by pterygium but reporting little direct exposure to UV was identified carrying a missense variant in *CRIM1* NM\_016441.2: c.1235 A>C (H412P) through whole-exome sequencing and subsequent analysis. *CRIM1* is expressed in the developing eye, adult cornea and conjunctiva, having a role in cell differentiation and migration but also in angiogenesis, all processes involved in pterygium formation. We demonstrate elevated *CRIM1* expression in pterygium tissue from additional individual Northern Irish patients compared to unaffected conjunctival controls.

UV irradiation of HCE-S cells resulted in an increase in ERK phosphorylation and *CRIM1* expression, the latter further elevated by the addition of the MEK1/2 inhibitor, U0126. Conversely, siRNA knockdown of *CRIM1* led to decreased UV-induced ERK phosphorylation and increased *BCL2* expression.

Transient expression of the mutant H412P *CRIM1* in corneal epithelial HCE-S cells showed that, unlike wild-type *CRIM1*, it was unable to reduce the cell proliferation, increased ERK phosphorylation and apoptosis induced through a decrease of *BCL2* expression levels.

We propose here a series of intracellular events where *CRIM1* regulation of the ERK pathway prevents UV-induced cell proliferation and may play an important role in the in the pathogenesis of pterygium.

**Keywords:** CRIM1; pterygium; UV; proliferation; ERK; apoptosis; variant

**Abbreviations: BCL2**, B-cell lymphoma 2; **BMP,** Bone Morphogenetic Proteins; **CRIM1**, Cysteine Rich Motoneuron protein1; **EMT,** Epithelial mesenchymal transition; **ERK (I)**, [Extracellular signal–regulated kinases](https://en.wikipedia.org/wiki/Extracellular_signal%E2%80%93regulated_kinases) (Inhibitor); **HCE-S**, Human Corneal Epithelial cells; **IC,** Impression Cytology; **IGV,** Integrative Genomic Viewer; **MAF,** Minor allele frequency; **MAPK**, Mitogen-activated protein kinases; **MTT,** 3-(4,5-[di](https://en.wikipedia.org/wiki/Di-)[methyl](https://en.wikipedia.org/wiki/Methyl)[thiazol](https://en.wikipedia.org/wiki/Thiazole)-2-yl)-2,5-di[phenyl](https://en.wikipedia.org/wiki/Phenyl)tetrazolium bromide; **NGS,** Next Generation sequencing; **NI,** Northern Irish; **PolyPhen,** Polymorphism Phenotyping; **SIFT**, Sorting Intolerant From Tolerant**; TiGER,** Tissue-specific Gene Expression and Regulation; **TGF-**ransforming Growth Factor- (Induced); **TUNEL,** [Terminal deoxynucleotidyl transferase](https://en.wikipedia.org/wiki/Terminal_deoxynucleotidyl_transferase) dUTP nick end labelling; **VEGFA,** Vascular Endothelial Growth FactorA; **VW(F)-C,** Von Willebrand (Factor) C; **WES,** Whole Exome Sequencing.

# INTRODUCTION

Pterygium (OMIM 178000) is a triangular shaped proliferative fibrovascular growth arising from the corneal-scleral limbus that invades the cornea centripetally[1](#_ENREF_1),[2](#_ENREF_2). The corneal invasion, through an active process of cell proliferation, matrix remodelling, angiogenesis and inflammation[1](#_ENREF_1),[3](#_ENREF_3), results in astigmatism, irritation, tearing, and, if the visual axis is impinged upon, blindness in the most severe cases[4](#_ENREF_4). The only effective treatment for pterygium is surgical excision which, together with various adjuvant therapies, still presents a 12% recurrence rate[5](#_ENREF_5). A small conjunctival lesion, pinguecula, is considered to be related to pterygium with a similar aetiology[6](#_ENREF_6), limbal localisation[7](#_ENREF_7) and histology[8](#_ENREF_8).

The main trigger for pterygium has long been attributed to UV radiation[9-11](#_ENREF_9), with epidemiologic studies showing an average prevalence of 22% (as high as 40% in some Chinese populations[12](#_ENREF_12) or 45% in provincial Indonesia[13](#_ENREF_13)) in the “pterygium belt”, an equatorial zone between latitudes 40°N and 40°S, compared to only 2% outside this area[4](#_ENREF_4). The human cornea represents a shield to protect the anterior eye from UV light and its anisotropic properties ensure that UV transmittance is reduced with a lower wavelength: while UVB (290-320nm) is completely absorbed by corneal epithelium, UVA (320-400nm) is absorbed only by 20% by the epithelial layer with the rest reaching the underlying stroma[14](#_ENREF_14). Focussing of incident light upon the nasal limbus has been proposed as the mechanism for the more frequent occurrence of pterygia at this location[15](#_ENREF_15).

In pterygium, while UVB mediates oxidative DNA damage[16](#_ENREF_16),[17](#_ENREF_17) and induction of cytokines and growth factors[18](#_ENREF_18), both UVB[19](#_ENREF_19) and UVA[20](#_ENREF_20) are responsible for activation of ERK intracellular pathway.

Irritation and chronic inflammation caused by sand, dust and wind[21](#_ENREF_21), as well as viral infections[22](#_ENREF_22),[23](#_ENREF_23), have been suggested as additional environmental triggers for pterygium.

A genetic contribution to the incidence of pterygium was suggested in an Australian study[24](#_ENREF_24), which revealed that 38% of patients admitted to hospital for excision of pterygia reported relatives with the disease, and in studies where different ethnic groups living at the same geographical location display differing prevalence of pterygium[12](#_ENREF_12),[25](#_ENREF_25).

High incidence of pterygium has been reported in a number of multigenerational families[26](#_ENREF_26),[27](#_ENREF_27) and in monozygotic twins[28](#_ENREF_28),[29](#_ENREF_29), where the most commonly reported mode of inheritance is autosomal dominant with reduced penetrance[4](#_ENREF_4),[30](#_ENREF_30),[31](#_ENREF_31).

The lack of large families presenting with multiple affected individuals, compounded by the late-onset of the disease, together with incomplete penetrance, has hampered identification of causative genes for pterygium.

Candidate gene association studies, which derive from hypotheses in which the cause of pterygium is already assumed, have identified an increased predisposition to pterygium in individuals carrying germline mutations in genes related to: oxidative stress[16](#_ENREF_16),[32](#_ENREF_32), carcinogenesis[33](#_ENREF_33) or angiogenesis[34](#_ENREF_34); even though these analyses have been found subject to false-positive associations[35](#_ENREF_35). Next-generation sequencing techniques, such as Whole Exome Sequencing (WES) and Whole Genome Sequencing (WGS), provide a largely hypothesis-free approach to identifying causative genes in smaller families with fewer affected members[36](#_ENREF_36).

In this study, using a WES approach, combined with bioinformatic and functional analysis, we identified a missense variant, p.His412Pro, in the *CRIM1* (cysteine rich motor neuron protein 1) gene, found in pterygium affected members of large multigenerational Northern Irish family with a documented small exposure to sunlight. *CRIM1* response to UV exposure revealed, for the first time, a central role within an intracellular mechanism involving ERK phosphorylation and ultimately leading to cellular proliferation or apoptosis. Introduction of the H412P variant in *CRIM1* resulted in an impairment of the whole pathway, demonstrating its possible involvement in the NI family’s pterygium pathogenesis.

# MATERIALS AND METHODS

## 2.1 Patient clinical examination

Clinical examinations were performed at Cathedral Eye Clinic, Belfast, UK (pterygium family members, pterygium and control individual samples from Northern Ireland).

A total of 24 patients from three consecutive generations of a Caucasian Northern Irish family affected by pterygium were investigated: three with pterygium, two with pinguecula and one unaffected family member participated to the WES study. Additional Northern Irish and Bolivian individuals (pterygium affected and unaffected controls) were recruited for sequence analysis of *CRIM1* and *CRIM1* expression analysis.

Following informed consent, collection of blood, tissues and a completed questionnaire was obtained from each participating individual under ethical approval from ORECNI Northern Ireland, UK (09/NIR01/14) and Comité de Bioética de la Facultad de Medicina, Santa Cruz, Bolivia.

## 2.2 Whole Exome Sequencing and Ingenuity Variant Analysis

WES was performed at the Wellcome Trust Centre for Human Genetics, University of Oxford. Briefly, genomic DNA was extracted from blood leukocytes using the QIAamp DNA Blood mini kit (QIAGEN, Manchester, UK), quantified by the high sensitivity Qubit system (Thermo Fisher Scientific, Loughborough, UK) and integrity of the DNA confirmed by electrophoresis on a 1% agarose gel.

The SureSelect Human All Exon v2 kit was used for Whole Exome capture according to the manufacturer’s instructions (Agilent Technologies UK, Wokingham, Berkshire, UK). The SureSelect n.2100 Bioanalyser (Agilent Technologies) allowed an assessment of the quality of the exome enriched library obtained.

Parallel sequencing was then performed by Illumina GAIIx using 150bp-paired-end reads. Generated reads were aligned to the Human 37 reference genome with a short read mapper (Stampy) generating data in BAM format[37](#_ENREF_37).

Coverage of the target region was verified to be in excess of 70% (greater than 10 reads). Platypus, an in-house variant caller able to detect Single Nucleotide Variants (SNVs) and short (<50bp) insertion/deletions (INDEL), was used to detect variant sites and alleles. Once the false positive rate was reduced, the resulting variants were stored as Variant Call Format (VCF) files. Ingenuity Variant analysis (Qiagen) was used to filter and select a smaller number of candidate genes. Aligned WES reads were viewed with the IGV platform. Clustal X2.1 was used to align CRIM1 sequences and results were visualized with EsPript 3.0 (http://espript.ibcp.fr)[38](#_ENREF_38).

## 2.3 Sanger Sequencing

Sanger sequencing was performed on genomic DNA extracted from the blood leukocytes using QIAamp DNA Blood mini kit.

Presence of H412P in *CRIM1* was verified using the following primers: CRIM1\_F: CTTCTTTTGCATGCACCCCC and CRIM1\_R: TCACATGTGCAACCTTTCCTC while *CRIM1* VWFs were sequenced using genomic primers designed using Primer3[39](#_ENREF_39) (Supplementary Table2). The PCR products were verified on a 1% agarose gel and Sanger sequenced at the Department of Zoology, University of Oxford.

## 2.4 Tissues and Impression cytology samples

Tissues samples (pterygium and normal cornea) were collected during surgeries while impression cytology samples were obtained using 4 x 4 mm strips of sterile LCR biopore membrane ﬁlter (pore size, 0.45 um; Millipore, Watford, UK) post pterygium surgery as previously described[40](#_ENREF_40) and stored in RNAlater (Qiagen). Prior to IHC staining tissues were fixed in 95% ethanol for 20 minutes at room temperature. The RNA yield obtained from the impression cytology samples was never lower than 8ng/ul and never higher than 15ng/ul. The ratio of absorbance at 260 and 280 nm (A260/280) of the RNA samples was between 1.8 and 2.2. Samples for which the RNA yield was too low or the quality fell outside the limits were excluded.

## 2.5 Quantitative real-time PCR

RNA extracted using the RNeasy Plus Mini Kit (Qiagen, Manchester, UK) was quantified with the Nanodrop 1000 (Thermo Fisher Scientific) and 1 g of total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Paisley, UK). cDNA was used for qRT-PCR assays, performed using a Lightcycler 480 II (Roche, West Sussex UK).

Real Time Ready Assays for *CRIM1* (assay id. 112278), *VEGFA* (assay id. 140396), *SRCAP* (assay id. 126413), *TGF-βI* (assay id. 104720), Glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)* (assay id. 141139) and hypoxanthine phosphoribosyltransferase *(HPRT*) (assay id. 102079) were purchased from Roche (Burgess Hill, West Sussex, UK).

SYBR green (Fermentas, Cambridge, UK) technology qRT-PCR was performed using *BCL2* primers (For AGCATGGGAGCCACGACCCT, Rev GGCCAAGGCCACACAGCCAA) and HPRT primers (For AGCTTGCGACCTTGACCAT, Rev GACCACTCAACAGGGGACAT), a kind gift from H. Nesbitt[41](#_ENREF_41).

Data were normalised using *HPRT* and *GAPDH* as housekeeping controls for ΔCt and ΔΔCt calculations[42](#_ENREF_42). *HPRT* and *GAPDH* were chosen as they have been shown to be the most stable corneal housekeeping genes[43](#_ENREF_43). For each condition all complementary cDNA samples were run in duplicate in two independent experiments.

## 2.6 HCE-S culture

Human Corneal Epithelial cells (HCE-S), a spontaneously generated corneal cell line[44](#_ENREF_44) (a kind gift from Prof. Julie Daniels), were cultured (37°C, 5% CO2) in Dulbecco’s modified Eagle’s medium (DMEM) containing 4 g/L glucose (Thermo Fisher Scientific, UK), and supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, UK).

## 2.7 TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was performed on HCE-S cells which had been reverse transfected with Lipofectamine 2000 using a mock transfection with pGL4.17 [luc2/Neo] plasmid (Promega Madison, WI USA), *CRIM1* wild type or *CRIM1* H412P plasmids. After 72 hours cells were fixed with 4% paraformaldehyde (PFA) and stained using the In Situ Cell Death Detection kit (Fluorescein; Roche, Burgess Hill, Surrey, UK) following the manufacturer’s instructions. Images were obtained using a ﬂuorescent AxioScope A1 microscope equipped with an AxioCam MRc camera (Carl Zeiss, Germany); 10x objective. Twelve images for each condition (n=3) were quantified using ImageJ software (US National Institutes of Health) and then normalised to total DAPI cells in each field.

## 2.8 Immunohistochemistry (IHC)

Pterygium and conjunctival tissues were formalin fixed and paraffin embedded. Sections of 7μm were permeabilised with 0.5% Triton X-100 before staining, treated with Proteinase K solution (Fisher Bioreagents, BP1700-50, 10ug/ml in PBS) and blocked with 5% goat serum (Sigma). A rabbit polyclonal CRIM1 antibody (Abcam- ab189203) was incubated at 1:100, with a rabbit IgG (Abcam) used as an isotype control. Secondary antibody fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Santa Cruz, USA) was used at a 1:100 dilution. Each section was mounted with fluorescent mounting medium (DAKO) and imaged using a 20× N Archoplan lens on an [AxioScope.A1](http://www.zeiss.com/microscopy/en_de/products/light-microscopes/axio-scope-a1-for-biology.html) microscope equipped with an AxioCam MRc camera (Carl Zeiss, Germany).

## 2.9 Site Directed Mutagenesis

*Human* *CRIM1* cloned into a pcDNA3.1 plasmid was a kind gift from Dr. L Wilkinson, Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia[45](#_ENREF_45). Site directed mutagenesis was performed to obtain the H412P *CRIM1* clone, using the Quick Change II kit (Agilent Technologies), following the manufacturer’s instructions. The entire *CRIM1* sequence was checked for integrity by Sanger Sequencing (Department of Zoology, University of Oxford), primers used are listed in Supplementary Table1.

## 2.10 MTT assay

Reverse transfection was performed in 12 well plates using HCE-S cells with either pCas9D10A\_GFP (Addgene/Zhang lab), *CRIM1* wild-type or H412P plasmids with Lipofectamine 2000, according to the manufacturer’s instructions (Falcon #353043, BD Corning Life Sciences, MA, USA). The following day cells were transferred in 96 well plates and 3-(4,5-[di](https://en.wikipedia.org/wiki/Di-)[methyl](https://en.wikipedia.org/wiki/Methyl)[thiazol](https://en.wikipedia.org/wiki/Thiazole)-2-yl)-2,5-di[phenyl](https://en.wikipedia.org/wiki/Phenyl)tetrazolium bromide (MTT) was added to the medium. Following 2 hours of incubation, the cells were resuspended in DMSO (Dimethyl sulfoxide, Sigma), absorbance was measured at 570 nm using the FLUOstar Omega (BMG Labtech, Aylesbury, UK) and quantified as relative percentages compared to control conditions. Absorbance measurements for the MTT were taken at 24, 48, 72 and 96 hours post transfection.

## 2.11 Western Blotting

HCE-S cells were cultured as described above. Low cell seeding densities were used to avoid cells becoming too confluent as this causes a decrease in ERK phosphorylation independent of the effect of CRIM1[46](#_ENREF_46),[47](#_ENREF_47).

Proteins were extracted using Complete Lysis-M (Roche Diagnostics) and proteinase inhibitor (Sigma-Aldrich P8340), quantification of total protein was performed using the Bradford assay (BioRad), 25μg of the extracted proteins was then resolved on a 4-12% NuPAGE® Bis-Tris Precast Gels (Thermo Fisher Scientific UK) using NuPAGE® MOPS SDS Running Buffer and transferred onto Amersham TM Hybond ECL membrane ([GE Healthcare Life Sciences](http://www.gelifesciences.com/webapp/wcs/stores/servlet/productById/en/GELifeSciences-us/25006201)).

Phospho ERK (#9101) and ERK (#9102) primary antibodies (Cell Signalling) were used at dilutions of 1:100 and 1:500 respectively with an overnight incubation at 4ºC. A secondary swine anti-rabbit antibody (DakoCytomation, Ely, UK) was used at a 1:2000 dilution for 1 hour at room temperature. Protein binding was detected by standard chemiluminescence: SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific UK) and imaged using the G:BOX transilluminator (Syngene). Quantification was performed using GeneTools image analysis software: average peak values of phosphoERK were normalised against the average ERK values. All the results obtained were then normalised to the transfection control.

## 2.12 UV treatment and MEK1/2 inhibitor

HCE-S cells were seeded in a 24-well plate at 1×105 cells per well in growth medium and left to adhere overnight at 37oC and 5% CO2. The following day they were treated using a UVA cross-linker (IROC Innocross AG, Ramsen, Switzerland) delivering a dose of 5.4 J/cm2 as previously described [48](#_ENREF_48) or UVB at a final dose of 0.5 J/cm2 through Arcadia D3 6% lamp (Arcadia, UK) with an aluminium reflector at a distance of 15 cm from the cells for 34 minutes.

The same doses of UVA and UVB irradiation were used in experiments in which the inhibitor of ERK phosphorylation (MEK1/2 inhibitor, U0126) was added to culture media an hour prior to the UV treatment as previously described[20](#_ENREF_20), at a concentration of 10M.

After irradiation, HCE-S cells were incubated in culture medium at 37°C with 5% CO2 and harvested at 1, 6, 12, 24 and 48 hours.

## 2.13 siRNA transfection

Four different siRNAs targeting the *CRIM1* sequence (Set of 4 Upgrade: ON-TARGETplus CRIM1 siRNA, LU-008492-00-0002, 2nmol, Dharmacon) were reverse transfected in HCE-S cells using Lipofectamine RNAiMAX (Fisher Thermo Scientific), following the manufacturer’s instructions. The four siRNAs were transfected singularly or as a pool at a final concentration of 10nM and normalised to the results from a non-specific siRNA control (NSC4) [49](#_ENREF_49). A range of different concentrations (0.2-0.5-1-10nM) of the siRNA pool reverse transfected in HCE-S cells was subsequently tested.

## 2.14 Statistical Analysis

All error bars represent the standard error of the mean (SEM) calculated between sample replicates of the same biological group. qRT-PCR, MTT and TUNEL assays significance was estimated using a Student's t-test calculation or the Mann-Whitney U test in GraphPad Prism 5 software with data illustrated using Box plots (qRT-PCR in Fig.3). p value ≤ 0.05 was deemed to be significant (\*p value ≤ 0.05, \*\*p value ≤ 0.01 and \*\*\*p value ≤ 0.001).

# RESULTS

## 3.1 WES analysis in a Northern Irish family affected by pterygium and pinguecula

A multigenerational Northern Irish family presenting with pterygium and pinguecula, but not other eye abnormalities, was examined (Fig.1A). Pterygium affected both males and females and was diagnosed at an average age of 48 years. No history of unusual sun exposure was recorded for any family member, suggesting a familial predisposition for development of pterygium/pinguecula (Table 1).

Six members of this family underwent WES: three were affected by pterygium (II.2, II.4 and II.14, 72, 70 and 65 years old respectively), two by pinguecula (III.5 and III.6, 48 and 46 years old respectively), shown in Fig.2B, and one was unaffected (II.9, 58 years old). The other two younger unaffected members of the family (III.2 and III.3, age 49 and 34) did not participate in the WES but were subsequently analysed by Sanger Sequencing.

WES resulted in the identification of 451,153 variants in 18,858 different genes which were filtered in a stepwise manner by Variant analysis software (Ingenuity**®**) (Fig.1C). Selecting only those variants with a call quality ≥50 in any case and ≥20 in the control and a read depth ≥10; 30,000 variants were obtained. When variants with a minor allele frequency (MAF) greater than 0.5% (pterygium prevalence in Europe is 2%) in 1000 Genomes Project, ESP EA exomes and Complete Genomics genome were excluded, the number of variants was reduced to 25,000. Analysis using either of the two algorithms Polyphen and SIFT, predicted as deleterious 11,000 variants, while 40 variants were finally selected as displaying an autosomal dominant segregation pattern within the family (Table 2).

Genes carrying mutations previously associated with pterygium such as *Ku70*, *GSTM1*, *ACE* *hOGG1*[*16*](#_ENREF_16)*,*[*17*](#_ENREF_17)*,*[*33*](#_ENREF_33)*,[34](#_ENREF_34" \o "Demurtas, 2014 #76)* were not observed in the selected list of variants.

Each single variant obtained by this analysis was then manually reviewed for determination of protein structure/function in Polyphen and SIFT, conservation of the amino acid among species running BLAST or eye expression interrogating Tiger Expression database. Literature was reviewed for any possible known correlation between gene mutations and pterygium or other diseases, either of the eye or otherwise. Moreover, adequate sequencing coverage and co-segregation of the variant within the family were verified through Integrative Genomic Viewer (IGV) (Table 2).

Based on the gene expression profile and on the known role in the eye, we selected *CRIM1* as our most plausible candidate gene. Recent studies have elucidated the importance of CRIM1 expression in corneal and conjunctival development[50-52](#_ENREF_50) and the same variant, H412P, was identified from another WES study on patients affected by Coloboma eye developmental disorder[53](#_ENREF_53). Moreover, CRIM1 revealed a role in cell proliferation[54-56](#_ENREF_54), adhesion and migration[57-59](#_ENREF_57), angiogenesis through VEGFA[60-62](#_ENREF_60) and UV-related diseases[63](#_ENREF_63),[64](#_ENREF_64), the main processes involved in pterygium development[4](#_ENREF_4).

## 3.2 CRIM1 structure and sequence analysis in individuals from Northern Ireland and Bolivia.

Presence of the variant in each affected family member, and absence from the unaffected sibling (II.9), was confirmed by direct Sanger sequencing (Fig.2A, example) of PCR products spanning the variant in VWFC-2.

Sequence alignment of *CRIM1* orthologues revealed residue H412 being conserved throughout human (*Homo sapiens*), cattle (*Bos Taurus*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), frog (*Xenopous laevis*), zebrafish (*Danio rerio*) (Fig.2B).

CRIM1 is a 1002 amino acid transmembrane protein (once the 34 amino acid signal peptide has been cleaved) and consists of a large N-terminal extracellular portion composed of 11 domains (six Von Willebrand factor C, four antistasin-like and one Insulin-like Growth Factor-binding Protein (IGFBP)), a 21 amino acids transmembrane domain and a small 76 amino acid C-terminal cytoplasmic domain[65](#_ENREF_65) (Fig.2C).

Although H412, located in the second VWFC domain, is not conserved in the other five VWFC of *CRIM1* (Fig.2D), it is conserved in the VWFC-2 domain of human chordin and in the VWFC-2 and 3 domains of human neuralin[66](#_ENREF_66" \o "O'Leary, 2004 #251), two related bone morphogenic protein (BMP) antagonists, like CRIM1, all of which have a proven role during embryogenesis[45](#_ENREF_45) (Fig.2E).

Sequence analysis of VWFC-2 in two additional younger family members (III.2 and III.3, age 49 and 34) revealed them to possess the same H412P variant; despite these individuals not having developed any signs of the disease. This is not incompatible with the mutation predisposing to the pathology, since a pattern of reduced penetrance has been widely described in familial pterygium[1](#_ENREF_1),[4](#_ENREF_4),[26](#_ENREF_26).

All the CRIM1 VWFC domains are important for interaction with BMPs 4 and 7[45](#_ENREF_45),[67](#_ENREF_67) as well as with VEGFA, TGF- and PDGF: if any of those domains are deleted, the interaction with the TGF-superfamily members is disrupted[62](#_ENREF_62). VEGFA, TGF-s and PDGF have an important role in pterygium formation[3](#_ENREF_3),[68](#_ENREF_68): an altered interaction of those factors with CRIM1 VWFC domains could therefore have a role in pterygium development. Sanger sequencing analysis was subsequently performed for all the six VWFC domains of *CRIM1* (primers listed in Supplementary Table2) in genomic DNA obtained from two ethnically different populations: 12 affected individuals from Northern Ireland and 9 from Bolivia (Table3).

While the H412P variant was found in none of the pterygium affected individuals, one patient from Bolivia, B1 (Table3), presented with a cytosine to thymine transition in the first position of codon 745, predicted to result in an Arginine to Cysteine (R745C) amino acid change (dbSNP: rs145721446) between VWFC-4 and VWFC-5 (exon 13). Although the MAF of this variant is 0.000008237 (Supplementary Fig.1A) and the R745C variant is predicted to be possibly damaging by PolyPhen, this arginine residue is not conserved in other species and is substituted by histidine in cattle, mouse, and rat, and by serine in both frog and zebrafish (Supplementary Fig.1B). The significance of this sequence variant is unclear and not explored here.

## 3.3 CRIM1 expression analysis

Global expression databases indicated *CRIM1* expression in the whole eye, without differentiating between specific tissues. The location of CRIM1expression in pterygium tissue and normal conjunctiva from sporadic Northern Irish individuals was therefore assessed by immunohistochemistry (IHC) and qRT-PCR (Fig.3).

CRIM1was detected, by IHC, lengthwise across the whole pterygium tissue: from the anterior head (Fig.3A) to the posterior tail (Fig.3C). Moreover, CRIM1 was observed both in the external hypertrophic conjunctival epithelium and in the internal fibroblasts, in particular in the vascular endothelial cells surrounding the blood vessels (Fig.3C arrows).

Within the pterygium stroma, CRIM1 was found around unusual structures such as hair follicles and inside sebaceous glands (Fig.3B). Finally, CRIM1 was detected in normal conjunctiva (Fig.3E) and in post-surgical impression cytology samples of unaffected individuals (Fig.3F).

*CRIM1* expression level was further investigated using qRT-PCR on RNA obtained from post-surgical impression cytology samples (Fig.3F) of unrelated Northern Irish (4 pterygium and 4 controls) individuals, together with a sample from a Northern Irish affected family member (II.2), found with the *CRIM1* H412P variant (Fig.3G).

*CRIM1* expression is significantly higher in pterygium tissues when compared to unaffected conjunctival controls (2-Ct mean values are 4.22 ± 0.76 and 1.28 ± 0.122, while median values are 3.4 and 1.3 respectively, p = 0.028). Intriguingly, *CRIM1* expression in the Northern Irish family member II.2 pterygium is lower than that of the all unaffected controls from NI (2-Ct value of 0.32).

Thus, we have shown that CRIM1 expression, both at the RNA and protein level, is higher in pterygium than in unaffected control tissue.

## 3.4 Effects of UV on CRIM1 expression in corneal epithelial (HCE-S) cells

UV radiation is considered the main epidemiologic factor responsible for pterygium and pinguecula development[9-11](#_ENREF_9). Although some papers state that pterygium originates from conjunctiva and Tenon’s fibroblasts[69](#_ENREF_69), the common consensus is that the first trigger for pterygium development occurs at the limbal area[1](#_ENREF_1),[2](#_ENREF_2),[70-72](#_ENREF_70) and pterygium growth has often been described as a localised limbal stem cell deficiency[70](#_ENREF_70). Since the aim of this paper is studying the early events triggering pterygium development, we considered that the best model would be a cell line coming from the zone where the initial events characterizing the disease have been described: the limbus. For this reason, for *in vitro* experiments we chose the HCE-S cell line, which spontaneously originated from corneal cells of the limbal area[44](#_ENREF_44).

The effects of UV light exposure upon HCE-S cells *in vitro* were therefore investigated. The average daily dose of UV light has been estimated to be 60-70 J/cm2 in central Europe[74](#_ENREF_74). Considering one hour exposure daily in general population, we irradiated an HCE-S monolayer to a dose of 5.4 J/cm2 UVA[20](#_ENREF_20),[75](#_ENREF_75), and a dose of 0.5 J/cm2 UVB.

Following UVA exposure, *CRIM1* expression started increasing from 3 hours, becoming significantly different from untreated control by 6 hours and continuing up to 24 hours (2-Ct ± SEM values at 3, 6 and 24 hours are respectively: 0.90 ± 0.15, 8.59 ± 0.22 p ≤ 0.05 and 10.39 ± 0.40 p ≤ 0.01) (Fig.4A). UVB resulted in a significant increase in *CRIM1* expression at 24 hours after the treatment when compared to the untreated control (2-Ct ± SEM values at 3, 6 and 24 hours are respectively: 1.31 ± 0.03, 0.90 ± 0.01 and 1.95 ± 0.008, the last with a p ≤ 0.05). Since irradiation with UVA has similar, but greater effects on *CRIM1* expression than UVB, subsequent experiments were conducted using UVA alone.

ERK phosphorylation, previously shown to be increased in pterygium cells following UVA exposure [19](#_ENREF_19),[20](#_ENREF_20), was assessed in UVA-treated HCE-S cells as described above. This resulted in a 5.6-fold increase in ERK phosphorylation compared to the untreated control at 6 hours after UVA treatment, rising to 32.1-fold higher at 24 hours (Fig.4B). Treatment of HCE-S cells with the MEK1/2 inhibitor, U0126, prior to UVA irradiation resulted in a complete inhibition of UVA-induced ERK phosphorylation.

Since UVA significantly elevated both *CRIM1* expression and ERK phosphorylation at 24 hours after treatment, the effects of inhibition of ERK phosphorylation on *CRIM1* expression following UV irradiation were studied. Surprisingly, inhibition of ERK phosphorylation in HCE-S cells significantly (p<0.001) potentiated the UV-induced increase in *CRIM1* expression at 24 hours (UVA + inhibitor: 3.75 ± 0.12, UVA only: 1.82 ± 0.15, inhibitor only 0.77 ± 0.02, untreated control: 0.63 ± 0.07; values expressed as 2-Ct) (Fig.4C).

Several studies have shown that increased ERK activity is associated with a decreased expression of the anti-apoptotic transcription factor, B*CL2* and an increased apoptosis[76](#_ENREF_76). Increased expression of *BCL2* and decreased expression of apoptosis markers has been observed in pterygia[1](#_ENREF_1),[77](#_ENREF_77). With this in mind, the expression level of *BCL2* was investigated following UVA treatment activating the ERK pathway in HCE-S cells.

Either UVA treatment or inhibition of ERK phosphorylation in HCE-S cells alone significantly decreased *BCL2* expression (2-Ct ± SEM values for ERK-I: 0.53 ± 0.05 and UVA 0.48 ± 0.06; both p ≤ 0.05) (Fig.4D). In comparison to those, when HCE-S cells were treated with MEK1/2 inhibitor, U0126, and UVA irradiation, *BCL2* expression was significantly increased (2-Ct ± SEM of 1.45 ± 0.13; p ≤ 0.001) and restored levels seen in untreated cells (p = 0.06).

UV irradiation has therefore a fundamental role in triggering a series of events, including the increase of *CRIM1* expression and the phosphorylation of ERK, corresponding to a decrease in *BCL2* levels in HCE-S cells.

## 3.5 CRIM1 intracellular pathway triggered by UVA

CRIM1, ERK and BCL2, as shown above, are interrelated in playing a pivotal role in the intracellular pathway triggered by UV exposure in HCE-S.

To further investigate the relationship between those factors, we sought to assess the effects of knocking down expression of *CRIM1* following siRNA transfection. Four siRNAs targeting *CRIM1*, both separately and in a pool, efficiently knocked down endogenous *CRIM1* expression in HCE-S cells to less than 30% of untreated level, 48 hours after transfection (p < 0.001) (Supplementary Fig.2).

Endogenous levels of *CRIM1* expression in HCE-S, normalised to cells transfected with NSC siRNA, increased after UVA treatment by 1.7 ± 0.05 fold, p ≤ 0.05. Because levels of *CRIM1* expression appear critical and finely regulated following UV irradiation, the concentration of siCRIM1 was titrated (0.2nM, 0.5nM, 1nm and 10nM) to establish the concentration at which *CRIM1* expression was the same as non-UVA treated, NSC4-tranfected HCE-S cells (Supplementary Fig.3). The siCRIM1 concentration at which this was achieved was 0.5nM (Fig.5A).

Since pterygium is considered a proliferative rather than degenerative condition[4](#_ENREF_4),[70](#_ENREF_70) and UV light alters cell proliferation at the limbal area as the first event promoting pterygium formation[78](#_ENREF_78), an MTT proliferation assay was then performed on HCE-S cells after UVA treatment (Fig.5B). UVA significantly increased HCE-S proliferation at 72 hours (absorbance at72-24 hours, control 0.53 ± 0.02 OD vs UVA 0.6 ± 0.03 OD; p ≤ 0.05). When cells were transfected with 0.5nM siCRIM1 prior to UVA treatment, HCE-S proliferation was further increased relative to UVA treatment alone (absorbance at 72-24 hours: 0.7 ± 0.02 OD, with p ≤ 0.05 compared to UVA and p ≤ 0.01 compared to the mock NSC4 control).

As previously shown (Fig.4B), ERK phosphorylation was significantly increased 24 hours after UV exposure. Transfection of cells with 0.5nM siCRIM1 prior to UV exposure almost completely abolished the increase in ERK phosphorylation (Fig.5C).

Finally, ERK and *CRIM1* regulation were related to *BCL2* expression levels (Fig.5D), which decreased significantly 24 hours after UVA treatment of HCE-S cells when compared with the untreated control (2-Ct values for UVA treated HCE-S: 0.61 ± 0.06, p ≤ 0.01). However, prior transfection with 0.5nM siCRIM1 prevented the decrease in *BCL2* expression, and was not significantly different from mock NSC4 transfected cells (2-Ct values of siCRIM1+UVA: 0.96 ± 0.08).

The latest results obtained using siCRIM1 were able not only to confirm the previous data showing a UVA-mediated increase in *CRIM1* expression, ERK phosphorylation and a decrease in *BCL2* expression, but also to demonstrate that by modulating only *CRIM1* expression, the whole signalling pathway leading to cell proliferation in response to UVA exposure can be regulated.

## 3.6 Effects of wild type and H412P mutant CRIM1 over-expression in HCE-S cells

Based on the outlined pathway involving CRIM1 regulation upon UV exposed HCE-S cells, the consequences of the H412P *CRIM1* variant in a pterygium pathogenic context were investigated.

The H412P variant was introduced into the human *CRIM1* sequence by site directed mutagenesis and the complete *CRIM1* sequence checked by Sanger sequencing (Supplementary Table1), confirming the presence of the c.1235 A>C substitution and absence of any other variation in the sequence.

HCE-S cells were transfected with an empty plasmid, wild type or the *CRIM1*:p.H412P expression plasmid. A significant *CRIM1* overexpression with respect to endogenous *CRIM1* was revealed by qRT-PCR both at 48 hours (*CRIM1* wt 6 ± 0.82, p<0.01 and *CRIM1* H412P 5.8 ± 0.97, p<0.05) and at 72 hours (*CRIM1* wt 6.4 ± 1.075, p<0.01 and *CRIM1* H412P 6.3 ± 1.4 p<0.01) after transfection (Fig.6A).

Since a decrease in proliferation was reported in vascular endothelial cells after transient CRIM1 overexpression[54](#_ENREF_54), an *in vitro* MTT proliferation assay was then performed in HCE-S cells transfected with *CRIM1* wild type or H412P constructs (Fig.6B). Compared to mock transfected HCE-S cells, wild type *CRIM1* overexpression had a relevant anti-proliferative effect, which was most significant at 72 hours post-transfection (absorbance 72-24 hours 0.60 ± 0.02 OD; p<0.01). This effect was not observed in the *CRIM1* H412P transfected cells (absorbance 72-24 hours 0.52 ± 0.02 OD), which did not differ significantly from the mock transfected control (absorbance 72-24 hours 0.57 ± 0.01 OD).

ERK phosphorylation is increased, simultaneously with CRIM1 expression, in vascular endothelial cells after VEGFA treatment[79](#_ENREF_79). Here, ERK phosphorylation was strongly increased in the wild type *CRIM1* transfected HCE-S compared to mock transfected control and to H412P *CRIM1* transfected cells at 72 hours post-transfection (Fig.6C).

Other gene expression levels, possibly affected by *CRIM1* H412P variant, were investigated by qRT-PCR: *VEGFA* for its role in pterygium angiogenesis[3](#_ENREF_3),[72](#_ENREF_72) and its direct interaction with *CRIM1*[73](#_ENREF_73), Transforming Growth Factor beta I (*TGF-βI*) for its involvement in several other eye diseases[80](#_ENREF_80) and *BCL2* for its importance in apoptosis, previously documented in pterygium[77](#_ENREF_77),[81](#_ENREF_81).

HCE-S cells were transfected with wild type and H412P *CRIM1* plasmids and gene expression was assessed at 48 and 72 hours after transfection (Fig.6D). Levels of *VEGFA* and *TGF-βI* expression were not significantly different between the wild type and the H412P *CRIM1* transfected cells (*VEGFA*: wild type 48 hours 0.8351 ± 0.0740, H412P 48 hours 0.8966 ± 0.0630, wild type 72 hours 1.1447 ± 0.0940, H412P 72 h 1.0443 ± 0.1100, *TGF-βI*: wild type 48h 0.7410 ± 0.0730, H412P 48h 0.8630 ± 0.0480, wild type 72h 0.9428 ± 0.0650, H412P 72h 1.1810 ± 0.1080, all values are expressed in 2-Ct). In contrast, a significant decrease in *BCL2* expression level was observed in the wild type *CRIM1* transfected cells with respect to H412P mutant and mock transfected cells (p value <0.05) both at 48 and 72 hours (wild type 48 hours: 0.5453 ± 0.0720, H412P 48 hours: 1.1647 ± 0.1800 and wild type 72 hours: 0.5977 ± 0.0240, H412P 72 hours: 1.2376 ± 0.1350, all values are expressed in 2-Ct), suggesting that overexpression of *CRIM1* may play a role in up-regulation of apoptosis, which is normally induced in cornea and conjunctiva by UV radiation[82](#_ENREF_82),[83](#_ENREF_83).

The effect of increased *CRIM1* expression upon apoptosis in HCE-S cells was investigated in *CRIM1* transfected cells by TUNEL assay (Fig.6E). At 72 hours after transfection the wild type *CRIM1* transfected HCE-S cells showed a significantly higher rate of apoptosis compared to either the H412P *CRIM1* or mock transfected HCE-S. The percentage of apoptotic (TUNEL positive), wild type *CRIM1* transfected cells (25.6 ± 1.8) was significantly higher than either H412P *CRIM1* plasmid or mock transfected HCE-S cells (3.9 ± 0.4 and 2.7 ± 0.5 respectively; p<0.001) (Fig.6E).

We have shown here that *CRIM1* plasmid overexpression exerts the same effects of UV induced *CRIM1* increased intracellular levels, in terms of decreased cell proliferation, increase in ERK phosphorylation and reduction in *BCL2* expression, followed by a higher rate of apoptosis.

The presence of H412P variant in *CRIM1* however abolished any of those effects, supporting a role for this variant in the increased susceptibility of members of the Northern Irish family to pterygium and pinguecula.

# DISCUSSION

UV light presents a chronic stimulus to the eye surface, altering the normal processes of growth control in cornea and conjunctiva, and is associated with several pathologies affecting the anterior eye[84](#_ENREF_84) such as photokeratitis[85](#_ENREF_85" \o "Cullen, 2002 #211), climatic droplet keratopathy[10](#_ENREF_10), cortical cataract[86](#_ENREF_86), squamous cell carcinoma[87](#_ENREF_87) and pterygium[9](#_ENREF_9),[21](#_ENREF_21). Pterygium, resulting in 1% of all the ocular surgeries in developed countries[88](#_ENREF_88) and presenting a 12% surgery recurrence rate[5](#_ENREF_5), represents a substantial cost to National Health Services and the community, estimated in Australia as US $100M per year[89](#_ENREF_89). In the identified Northern Irish family (Fig.1), affected by pterygium or pinguecula despite not being exposed to high levels of sunlight, genetic predisposition may play a fundamental role in the etiologic process (Table 1). A WES approach to candidate gene identification with *in silico* and literature analysis led to selection of H412P variant in *CRIM1* gene for several reasons. Firstly, the substitution of a highly conserved, positively charged histidine (H) residue with an apolar proline (P) is predicted to interfere with CRIM1 structure and function.

Moreover, the interaction between F1 domains of fibronectin, structurally similar to VWF domains of CRIM1 where H412P is located, and VEGF is enhanced at acidic pH (5.5-7)[66](#_ENREF_66). As Histidine works at this pH range (pKa3=6), a mutation in this residue may interfere with the electrostatic binding of other ligands.

CRIM1’s VWF domains (Fig.2) were also shown essential for interaction with VEGFA[62](#_ENREF_62), the angiogenic factor overexpressed in the vascularised pterygium tissue following UV exposure[3](#_ENREF_3),[72](#_ENREF_72). CRIM1 is thus proposed as a VEGFA antagonist[60](#_ENREF_60),[73](#_ENREF_73), possibly resisting pterygium formation by preventing angiogenesis as it has been shown to prevent proliferation[54](#_ENREF_54),[55](#_ENREF_55). Proliferation in cardiomyocytes is reduced by CRIM1 upregulation following miR199a silencing[55](#_ENREF_55),[56](#_ENREF_56), the same miRNA which was found overexpressed in pterygium[90](#_ENREF_90" \o "Raghunath, 2015 #31). In addition to proliferation and angiogenesis, tissue remodelling through cell adhesion and migration also occurs during pterygium formation[4](#_ENREF_4). This process has been shown to be regulated by CRIM1 in neurons[57](#_ENREF_57), lung cancer[59](#_ENREF_59) and lens epithelial cells[58](#_ENREF_58), the latter showing a premature fibre differentiation when *CRIM1* is lost[91](#_ENREF_91).

As previously described, pterygium etiopathogenesis is generally ascribed to UV eye exposure and *CRIM1* was found to be involved in UV related diseases such as bovine eye cancer[63](#_ENREF_63) and melanoma[64](#_ENREF_64). Finally, recent studies have elucidated the importance of *CRIM1* expression during the anterior eye development[50](#_ENREF_50),[51](#_ENREF_51),[58](#_ENREF_58).

*CRIM1* was thus selected as our best candidate responsible for pterygium pathogenesis within the NI family. However, a reduced penetrance, as previously described in pterygium[1](#_ENREF_1),[26](#_ENREF_26),[31](#_ENREF_31), makes interpretation of genetic data challenging[36](#_ENREF_36). While two unaffected members of the NI family (III.2 and III.3) harbour the H412P variant in *CRIM*1, no affected member was found that did not have the H412P variant. Moreover, given the younger age of the unaffected members (34 and 48 years old), penetrance in this case could be age-related, as described in MEN1 syndrome, in which tumour occurrence increases with the age, becoming fully penetrant only after sixty years[92](#_ENREF_92). It is therefore possible that III.2 and III.3 family members may never develop pterygium or will do so later in their lifetime; in any case, they should be monitored closely for development of pterygium in the future.

Neither the *CRIM1* H412P variant nor any other alteration within the six VWFC domains of *CRIM1* were found in other affected unrelated individuals from Northern Ireland.

Only one other patient from Bolivia was identified with a missense variant located between VWFC-4 and VWFC-5: R745C (Supplementary Fig.1). While this arginine residue is not conserved between the species, the introduction of a cysteine residue may facilitate disulphide bridge formation with highly conserved cysteine residues in the previous or subsequent VWFC domain, vital to the VWF domain’s structure[45](#_ENREF_45),[93](#_ENREF_93).

While no variants in *CRIM1* were found in the NI patients, CRIM1 expression was elevated throughout the pterygium tissue compared to unaffected conjunctiva (Fig.3). In particular, *CRIM1* expression was observed around pterygium blood vessels which, together with its previously described expression in endothelial cells during capillary formation[60](#_ENREF_60),[61](#_ENREF_61) and its interaction with VEGFA[73](#_ENREF_73), suggests a role for CRIM1 during the critical angiogenic processes of pterygium formation[1](#_ENREF_1),[21](#_ENREF_21). CRIM1 was also detected in unusual structures such as hair follicles and sebaceous glands, previously identified as characteristic features of some pterygia[70](#_ENREF_70" \o "Chui, 2011 #51). These structures are representative of an uncontrolled transdifferentiation program[94](#_ENREF_94) occurring in pterygium in addition to an epithelial-mesenchymal transition (EMT) or fibrosis process[95](#_ENREF_95), and likely reflects similarities between pterygia, limbal dermoids and hair epithelia[96](#_ENREF_96).

Comparing *CRIM1* expression levels between pterygium-affected and -unaffected individuals, those were significantly increased in the affected samples (Fig.3G), while the lowest *CRIM1* expression was recorded for the affected family member (II.2). A high *CRIM1* expression in pterygium-affected individuals could therefore represent a defensive cellular response mechanism against UV damaging effects, which is impaired by the H412P variant within the NI family.

Involvement of CRIM1 in UV-related diseases either in the eye[63](#_ENREF_63) or the skin[64](#_ENREF_64) is not surprising considering that UV irradiation affects the balance between proliferation and apoptosis in several cell types, including skin keratinocyes[97](#_ENREF_97),[98](#_ENREF_98), corneal epithelium[82](#_ENREF_82) and pterygium basal epithelial cells[77](#_ENREF_77). However, while the eye is exposed to both UVA and UVB radiation, the intensity of UVA radiation reaching the Earth’s surface has 10-100 times the intensity of UVB[99](#_ENREF_99) and it is known to induce oxidative stress in exposed cells and tissues, including pterygium[100](#_ENREF_100" \o "Tsai, 2005 #73). Accordingly, UVA induced a more rapid and larger response than UVB in HCE-S cells (Fig.4A).

Exposure of HCE-S cells to UVA radiation leads to an increase in *CRIM1* (Fig.4A) expression. The elevated ERK phosphorylation upon UVA irradiation we observed (Fig.4B) was previously described in UVA or UVB irradiated pterygium and conjunctival cells[19](#_ENREF_19),[20](#_ENREF_20). While inhibition of ERK phosphorylation alone had no effect on the expression of *CRIM1*, it potentiated the *CRIM1* up-regulation when HCE-S cells were treated with UV (Fig.4C). This underscores the importance of UV irradiation as the primary trigger for the intracellular pathway but underlines also the capacity of ERK phosphorylation to regulate *CRIM1* expression levels. Moreover, inhibition of ERK phosphorylation in HCE-S cells treated with UV abolished the UV-induced decrease in *BCL2* expression (Fig.4D): UV decreases *BCL2* levels through ERK phosphorylation, which results therefore in upstream *BCL2* regulation within this pathway.

The role of CRIM1 in the cellular response to UV exposure was confirmed in HCE-S cells in which *CRIM1* over-expression induced by UV treatment was inhibited by siRNA transfection (Fig.5A). An increased cell proliferation in siCRIM1-treated cells upon UV exposure (Fig.5B), suggests that CRIM1 has a role in protecting against pterygium formation by minimising cell proliferation in response to UV radiation. When siCRIM1 was added to UV treatment, HCE-S cells decreased ERK phosphorylation to that seen in untreated cells (Fig.5C) and restored *BCL2* basal expression (Fig.5D), revealing that also *CRIM1* is able to control ERK phosphorylation as well as *BCL2* expression and locates it upstream of those two factors within the pathway. Those experiments showed how CRIM1 exerts a central role within the UV triggered intracellular pathway and silencing its expression is enough to counter all those alterations we examined.

Previous studies have shown how *CRIM1* alterations influences cellular function:multiple exon deletions of *CRIM1* lead to syndromic disease[52](#_ENREF_52) while a minor alternately spliced *Crim1* isoform determines perinatal lethality with multiple organ dysfunction[51](#_ENREF_51); however, the effects of *CRIM1* missense mutations still remain unexplored. Therefore, based on the intracellular mechanism described, the effects of the H412P variant found within the Northern Irish kindred were studied.

*CRIM1* over-expression in HCE-S cells leads to reduced proliferation (Fig.6B) and a parallel increase in apoptosis (Fig.6D), as expected following the previous results where *CRIM1* was shown having a protective role in pterygium formation, counteracting the UV-induced proliferation (Fig.4B). This is in accordance with the downregulation of vascular endothelial cell proliferation after transient *CRIM1* overexpression[54](#_ENREF_54) and with the increase in cardiomyocyte proliferation following *CRIM1* silencing through miR-199a[55](#_ENREF_55),[56](#_ENREF_56). Since the same miR-199a was found upregulated in pterygium[90](#_ENREF_90" \o "Raghunath, 2015 #31), this could analogously increase cell proliferation in pterygium formation through *CRIM1* silencing, counteracting the protective effects of *CRIM1* overexpression.

*CRIM1* overexpression was also able to increase ERK phosphorylation (Fig.6B) following UVA irradiation (Fig.4B). This result confirms previous data (Fig.5C) showing that the effects of *CRIM1* overexpression lie upstream of ERK phosphorylation and demonstrates that it does not need UV to exert its function.

All the effects of *CRIM1* overexpression in HCE-S cells were almost completely abolished by the H412P variant in *CRIM1* (Fig.6).

The results obtained delineated a finely regulated pathway where either UV (Figure 4B) or elevated CRIM1 levels (Figure 6C) increase ERK phosphorylation, demonstrating that CRIM1 lies upstream of, and is responsible for ERK phosphorylation.

CRIM1 expression was increased upon UV exposure and this increase was potentiated when the U0126 inhibitor was added to the UV-irradiated cells. This suggests a feedback mechanism in which ERK phosphorylation, increased by elevated *CRIM1* levels, serves to limit the rise in *CRIM1* expression triggered by UV (Fig.7). UV is therefore necessary for the activation of the whole pathway because the U0126 inhibitor alone, without UV treatment, does not increase CRIM1 expression (Figure 4C). For the first time an increase in *CRIM1* expression has been directly correlated with a consequent activation of the ERK pathway in corneal cells.

Based on the *in vitro* experimental evidence and on the feedback mechanism outlined, H412P can be considered either a loss-of-function or a dominant-negative mutation. Similarly, a study on a shortened mouse *Crim1* isoform was unable to conclude whether the mechanism was hypomorphic (reduced activity) or dominant negative[51](#_ENREF_51), although the latter have been described in growth factor receptors which bind the same CRIM1 interactors such as PDGF[101](#_ENREF_101) or VEGF[102](#_ENREF_102) and in VWF domains similar to those found in CRIM1[103](#_ENREF_103),[104](#_ENREF_104).

1. **CONCLUSIONS**

In summary, we have shown that *CRIM1* plays an important role in the response of corneal cells to UV irradiation by activation of a finely regulated intracellular pathway where increased *CRIM1* expression counteracts cell proliferation and increases apoptosis. We demonstrate that *CRIM1* H412P variant impairs this response, suggesting its involvement in the increased susceptibility to pterygium observed in a Northern Irish family. Further investigation of the role of CRIM1 in UV triggered pathways and pterygium holds promise as a target for the prevention and treatment of development or recurrence of pterygium or other UV related diseases.

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1. **FIGURE LEGENDS**

**Figure 1.** Northern Irish family affected by pterygium or pinguecula and WES analysis. **(A).** Pedigree of a Northern Irish family affected with pterygium. Open symbols denote unaffected individuals; filled black symbols denote pterygium affected individuals and filled black symbols with open circles inside denote pinguecula affected individuals. Squares represent male and circles represent female individuals. Slashed symbols denote deceased family members and question marks are for individuals who have not participated in the study. Although all family members were invited to participate in this study, only those who gave informed consent were enrolled. **(B).** Pinguecula visible at the limbal area, between the cornea and conjunctiva (arrowhead). The image was obtained from family member III:5. **(C).** WES variants screening. Ingenuity variant analysis screened the 451,153 variants obtained with WES using four subsequent filters: Confidence to ensure quality, Common Variants (MAF < 0.05), Predicted deleterious using Polyphen and SIFT and Genetic, assuming an autosomal dominant inheritance pattern. A final *In silico* analysis based on the residue conservation through BLAST and Clustal Omega, expression analysis using TiGER database and literature research allowed selection of a single candidate variant in *CRIM1* gene.

**Figure 2.** CRIM1 (Cysteine RIch transMembrane BMP regulator 1) sequence in the NI family, conservation and structure analysis. **(A).** Electropherogram of Sanger sequencing performed in the family members confirmed the c.1235 A>C heterozygous transversion mutation in affected individuals and which was homozygous for wild-type A allele in the unaffected control. **(B).** Clustal X2.1 multiple sequence alignment of orthologous CRIM1 protein sequences from Human (NP\_057525.1), Cattle (NP\_001192227.1), Mouse (NP\_056615.1), Rat (NP\_001162574.1), Frog (NP\_001163917.1), Zebrafish (NP\_997986.1) indicates that the H412P missense mutation occurs in a highly conserved residue. EsPript 3.0. Legend: Red box, white character: strict identity; Red character: similarity in a group; Blue frame: Similarity **(C).** Schematic ideogram of Human CRIM1, characterised by multiple domains: one transmembrane (blue), six von Willebrand factor (VWF) (yellow), four antistasin-like (green) and one IGFBP (red). The position of the H412P and the R745C mutation are indicated. **(D).** Clustal X2.1 alignment of the six VWFC domains in *CRIM1* shows that, other than in VWFC-2, H412 is not conserved in the other five VWFC domains*.* **(E).** Clustal X2.1 alignment of CRIM1 with other BMP antagonists indicates that the residue H412 is conserved in the VWFC-2 domain of human chordin and in the VWFC-2 and 3 domains of human neuralin.

across groups (if more than 70% of the residues within the frame share similar physico-chemical properties).

**Figure 3.** CRIM1 expression analysis in pterygium and conjunctiva from Northern Ireland. **A-F** images represent immunohistochemical (IHC) staining of CRIM1 expression (green) and nuclei (DAPI, blue) in pterygium and conjunctival tissues obtained from sporadic Northern Irish patients. Scale bar, 100m **(A).** CRIM1 expression in a pterygium head, visible in both the external epithelial layer (E) as well as the internal stroma (S). **(B)**. CRIM1 expression shown in peculiar structures identified in pterygium stroma: a hair follicle (empty arrowhead) and a sebaceous gland (plain arrowhead). **(C).** CRIM1 expression in pterygium tail, arrowheads indicate CRIM1 expression in cells surrounding the blood vessels. **(D).** Negative IgG control in pterygium tail tissue. **(E).** CRIM1 expression in conjunctival epithelial tissue of an unaffected individual. **(F).** IHC of a post-surgical impression cytology sample obtained from unaffected superficial epithelial conjunctival cells: CRIM1 is expressed in all the cells captured on the membrane filter. **(G)** *CRIM1* expression is increased in pterygium patients from Northern Ireland. qRT-PCR analysis of *CRIM1* carried out in cDNA from post-surgical impression cytology samples: NI conjunctival individual controls, NI pterygium individuals and one affected NI family member (II.2 in Fig. 1). All values are expressed in 2-Ct ± SEM. n=2 with two technical replicates each sample.

**Figure 4.** UV treatment of HCE-S cells increases CRIM1 expression, ERK phosphorylation and decreases BCL2 expression. **(A).** qRT-PCR revealed significantly increased CRIM1 expression levels in HCE-S cells at 6 (p ≤ 0.05) and 24 hours (p ≤ 0.01) after UVA treatment and at 24 hours (p ≤ 0.05) after UVB treatment compared to the untreated control. **(B).** Western blot analysis of HCE-S cell lysates revealed an increase in ERK phosphorylation at 6 and 24 hours after UVA irradiation with respect to ERK phosphorylation levels of untreated control. Pretreatment of cells with U0126 inhibitor abolished ERK phosphorylation in UV treated cells. **(C).** qRT-PCR was used to evaluate *CRIM1* expression following UV treatment in the presence of MEK1/2 inhibitor, U0126. Inhibition of ERK pathway activation enhances the increase in *CRIM1* expression seen in UV irradiated HCE-S cells whilst inhibition of ERK phosphorylation alone has no significant effect. **(D).** *BCL2* expression measured by qRT-PCR. HCE-S cells were UVA irradiated with and without U0126 and harvested 24 hours after treatment. A significant decrease in *BCL2* expression was observed when the cells were treated with either U0126 or UVA alone but not in combination when compared to the HCE-S untreated cells. qRT-PCR data represent fold change of the 2-Ct mean ± SEM compared to untreated HCE-S. n=3 with three technical replicates each.

**Figure 5.** siCRIM1 0.5nM is able to restore normal HCE-S conditions after UVA treatment. **(A).** qRT-PCR shows the amount of siCRIM1 (0.5nM) able to restore endogenous CRIM1 levels in HCE-S after UVA exposure. For all the experiments HCE-S cells were treated with: NSC4, UVA + NSC4 and UVA+ siCRIM1 (0.5nM) and harvested 24 hours later. Data represent 2-Ct ± SEM. n=3 with three technical replicates for each sample. **(B).** An MTT assay demonstrates an increased proliferation upon UVA exposure, which is further increased if *CRIM1* expression is restored to pre-treatment endogenous levels (siRNA 0.5nM), confirming the antiproliferative effect of CRIM1. n=6 with eight technical replicates for each condition. **(C).** Western Blot analysis shows ERK phosphorylation (pERK) at 24 hours after UVA treatment in HCE-S cells. The increased ERK phosphorylation due to UVA exposure was brought back to normal levels following transfection with 0.5nM CRIM1 siRNA. **(D).** *BCL2* expression, measured by qRT-PCR, decreases upon UVA irradiation eliciting apoptosis but is restored in HCE-S cells treated with siCRIM1. Data represent 2-Ct ± SEM with respect to untransfected HCE-S. n=3 with three technical replicates each sample.

**Figure 6.** *CRIM1* wild type overexpression in HCE-S cells results in a decrease in cell proliferation and an increase in ERK phosphorylation and apoptosis. **(A).** qRT-PCR showing *CRIM1* overexpression in mRNA obtained from HCE-S cells transfected with Human *CRIM1* wild type and mutant (H412P) in pcDNA3.1 expression plasmid. Both *CRIM1* wild type (wt) and H412P mutant were significantly overexpressed at 48 and 72 hours after transfection with respect to the mock control. Data represent fold change of the 2-Ct mean ± SEM. n=3 with 3 technical replicates for each condition. **(B).** An MTT assay of HCE-S cell proliferation was performed at 72 hours after transfection with empty plasmid (mock), *CRIM1* wt and H412P mutant constructs. *CRIM1* wt, when overexpressed, has an anti-proliferative effect when compared to the mock transfected control (p<0.001). Overexpression of the mutated H412P *CRIM1* does not have the same anti-proliferative effect. n=6 with 8 technical replicates for each condition. **(C).** ERK phosphorylation (pERK) was detected by Western Blot analysis at 72 hours post transfection with mock, *CRIM1* wt and H412P plasmids in HCE-S cells. *CRIM1* wt overexpression resulted in a high level of ERK phosphorylation in comparison to *CRIM1* H412P and mock control. **(D).** qRT-PCR analysis of *VEGFA*, *TGF-I* and *BCL2* expression in HCE-S cells transfected with mock, CRIM1 wt and H412P plasmids and harvested after 48 and 72 hours. No significant variation of *VEGFA* and *TGF-I* expression was observed between mock, *CRIM1* wt and *CRIM1* H412P while *BCL2* expression significantly decreased in H412 wt CRIM1 transfected cells compared with mock transfected HCE-S both at 48 and 72 hours post transfection. Data represent fold change of the 2-Ct mean ± SEM respect to mock transfected HCE-S. n=3 with three technical replicates each. **(E).** TUNEL assay in HCE-S cells transfected with wt and H412P mutant *CRIM1* plasmids. TUNEL-positive cells are stained green and nuclei are stained blue with DAPI. Overexpression of CRIM1 wt results in increased apoptosis compared to either the *CRIM1* H412P or mock transfected cells. Scale bar, 100 m. TUNEL assay quantification was performed in 12 fields per condition using ImageJ. n=3

**Figure 7.** CRIM1 regulates pERK in a looped pathway. Here, based on our experimental evidence, we propose, within a schematic, an intracellular pathway triggered by UV that may act as a protective mechanism against pterygium development. When cells are exposed to UV light, CRIM1 expression is increased, which in turn increases ERK phosphorylation. This, in turn, blocks further CRIM1 expression in the feedback loop shown. Inhibition of ERK phosphorylation prevents this negative feedback, resulting in further increases of CRIM1 expression.

ERK phosphorylation induces decreased expression of the anti-apoptotic BCL2, initiating cell apoptosis. This proposed pathway was shown to be impaired in the case of the H412P mutation in CRIM1, found in the Northern Irish family affected by pterygium.

**Supplementary Figure 1.** R745C variant found in a pterygium affected patient from Bolivia. **(A).** Electropherogram showing the novel c.2299C>T transition variant from the Bolivian patient (B1), which corresponds to the R745C variant**.** **(B).** Multiple *CRIM1* sequence alignment shows that the R745 residue is not conserved across species; it is conserved however the absence of cysteine between VWFC-4 (residues 677-735) and VWFC-5 (residues 751-809).

**Supplementary Figure 2.** siRNAs targeting *CRIM1* efficiently knock down its expression in HCE-S cells.*CRIM1* expression obtained using qRT-PCR of HCE-S cell cDNA 48 hours post transfection. All four siRNAs tested were able to knock down *CRIM1* expression, including the pool of four siRNAs used at the same final concentration of 10 nM. qRT-PCR data represent fold change of the 2-Ct mean ± SEM with respect to untreated HCE-S. n=2 with three technical replicates each.

**Supplementary Figure 3.** A dose response curve shows that 0.5nM siRNA pool restores HCE-S endogenous *CRIM1* levels. *CRIM1* expression obtained using qRT-PCR of HCE-S cell cDNA 48 hours post transfection with UVA and the siRNA pool at different concentrations. qRT-PCR data represent fold change of the 2-Ct mean ± SEM with respect to untreated HCE-S. n=2 with three technical replicates each.

**TABLES**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Family member** | **Year of birth** | **Sex** | **Eye condition** | **Age of diagnosis** | **Lived abroad** | **Time in sunny climates** | **Wear sunglasses** |
| II.2 | 1944 | F | pterygium | 40 years | No | 1/year, 3 weeks | Sometimes |
| II.4 | 1946 | F | pterygium | 59 years | Canada 13 years | 1/year, 2 weeks | No |
| II.9 | 1958 | M | unaffected | - | No | 1/year, 3 weeks | Yes |
| II.14 | 1951 | M | pterygium | 62 years | No | 1/year, 1 week | Yes |
| III.2 | 1967 | M | unaffected | - | No | 1/year, 1 week | Yes |
| III.3 | 1982 | F | unaffected | - | No | 1/year, 1 week | Sometimes |
| III.5 | 1968 | F | pinguecula | 38 years | Canada 13 years | 1/year, 2 weeks | Sometimes |
| III.6 | 1970 | F | pinguecula | 40 years | Canada 13 years | 1/year, 2 weeks | Sometimes |

**Table 1.** Questionnaire results. The Northern Irish family members participating in the study are listed with age of pterygium diagnosis, averaged 48 years old. The family were shown not to have had excessive exposure to the sun, spending time in sunny climates from 1 to 3 weeks per years with most stating that they wear sunglasses.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Gene Symbol** | **Chr** | **Position** | **Gene Region** | **Protein Variant** | **Conservation to:** | **Gene expressed in:** | **Associated Diseases**  |
| CTRC | 1 | 15764869 | Promoter |  | Human | pancreas, liver, spleen | chronic pancreatitis |
| KIF21B | 1 | 200978030 | Exonic | p.T105M | Elephant | eye (cornea), brain, blood | multiple sclerosis, inflammatory bowel disease  |
| GALNT14 | 2 | 31348076 | Intronic; Exonic; 5'UTR | p.A58V | Human | kidney, PNS, blood, eye | breast cancer |
| CRIM1 | 2 | 36706700 | Exonic | p.H412P | Lamprey | placenta, kidney, PNS, eye | syndactyly, neuronitis, bovine ocular carcinoma |
| SPTBN1 | 2 | 54886366 | Exonic | p.T2107A; p.T2094A | Dog | tongue, soft tissue, eye |  fissured tongue, and buphthalmos |
| VRK2 | 2 | 58313555 | Exonic; ncRNA | p.G113E; p.G90E | Elephant | lymph node, testis, colon |  vaccinia, and hypoxia |
| PLEK | 2 | 68615546 | Exonic | p.N229H | X\_tropicalis | bone marrow, lymph node, eye |  aarskog-scott syndrome, centronuclear myopathy |
| HNMT | 2 | 138772049 | 3'UTR |  | Dog | bladder, kidney, liver, eye |  asthma, eosinophilia-myalgia syndrome |
| ITGB6 | 2 | 160994200 | Exonic | p.H469N | Mouse | pancreas, tongue, stomach, eye |  bullous keratopathy,  mouth disease |
| WDR12 | 2 | 203760891 | Exonic | p.L169S | Zebrafish | blood, intestine, thymus, eye | gigantism, and neuronal ceroid lipofuscinosis |
| CLDN16 | 3 | 190106072 | Exonic | p.A56fs\*16 | Rhesus | kidney, ovary, uterus | nephrocalcinosis, and hypomagnesemia primary |
| CLDN16 | 3 | 190106074 | Exonic | p.A56P | Rhesus | kidney, ovary, uterus | nephrocalcinosis, and hypomagnesemia primary |
| PRIM2 | 6 | 57398226 | Exonic | p.G310V | Human | thymus, ovary, mammary, eye |  |
| STK31 | 7 | 23811795 | Exonic; ncRNA | p.N598\_S600delinsKKI | Elephant | testis |  |
| TYW1 | 7 | 66660242 | Exonic | p.H632R | Human | stomach, heart, colon, eye | fanconi's anemia, and cytochrome p450 |
| GLDC | 9 | 6556206 | Exonic | p.I717V | Zebrafish | small intestine, kidney, liver, eye | glycine encephalopathy |
| IFNA5 | 9 | 21304891 | Exonic | p.C122S | Elephant | \_\_\_\_\_\_\_\_\_\_ | hemorrhagic fever |
| DMRTA1 | 9 | 22451120 | Exonic | p.K242R | Rhesus | \_\_\_\_\_\_\_\_\_\_ |  metabolic acidosis, prostatitis |
| MIR4289 | 9 | 91360776 | microRNA |  | Dog | \_\_\_\_\_\_\_\_\_\_ |  |
| HBG2 | 11 | 5276282 | Promoter |  | Rhesus | spleen, thymus, liver |  sulfhemoglobinemia, cavernous hemangioma |
| OR4C16 | 11 | 55339462 | Promoter |  | Human | \_\_\_\_\_\_\_\_\_\_ |  neuronitis |
| OR10AG1 | 11 | 55735808 | Exonic | p.H43D | Rhesus | \_\_\_\_\_\_\_\_\_\_ |  neuronitis |
| C12orf56 | 12 | 64712620 | Exonic; Intronic | p.T210I | Human | \_\_\_\_\_\_\_\_\_\_ |  |
| ATP8B4 | 15 | 50223420 | Exonic; ncRNA | p.R513Q | Lamprey | blood, bone marrow | intrahepatic cholestasis |
| DMXL2 | 15 | 51773329 | Exonic | p.D1356N; p.D1992N | Chicken | blood, testis, heart, eye |  gynecomastia, and myocardial infarction |
| TPSD1 | 16 | 1306802 | Exonic | p.I87V | Human | \_\_\_\_\_\_\_\_\_\_ |  asthma, allergic and inflammatory disorders |
| TPSD1 | 16 | 1306817 | Exonic | p.A92T | Human | \_\_\_\_\_\_\_\_\_\_ | asthma, allergic and inflammatory disorders |
| NOMO1  | 16 | 18544469 | Exonic | p.R418H | Chicken | intestine, cervix, colon, eye |  pseudoxanthoma elasticum (PXE) |
| SRCAP | 16 | 30731568 | Exonic | p.R968H | Chicken | Larynx, thymus, spleen, eye (cornea) |  floating-harbor syndrome, Eosinophilic angiocentric fibrosis |
| ZNF319 | 16 | 58031913 | Exonic | p.A86V | Elephant | bladder, blood, tongue |  |
| PDPR | 16 | 70154480 | Exonic | p.T29A | Human | heart, bone, Eye |  sarcosinemia |
| ZFHX3 | 16 | 72832474 | Exonic | p.454\_455insH; p.1368\_1369insH | X\_tropicalis | \_\_\_\_\_\_\_\_\_\_ |  prostate cancer, acute myocardial infarction |
| WWOX | 16 | 78466409 | Exonic | p.L272F | Rhesus | mammary, tongue, PNS, eye |  toxic pneumonitis, andaspiration pneumonitis |
| GSDMA | 17 | 38122680 | Exonic | p.V128L | Human | \_\_\_\_\_\_\_\_\_\_ |  atopy, gastric cancer |
| DNAI2 | 17 | 72306188 | Intronic; Exonic | p.V460V | Elephant | testis, uterus, lung, brain | ciliary dyskinesia |
| MAN2B1 | 19 | 12774537 | Exonic | p.P248L | Dog | bone, PNS, intestine, eye | alpha-mannosidosis, alpha-mannosidosis, adult form |
| CYP4F2 | 19 | 15989730 | Exonic | p.T472A | Mouse | intestine, blood, liver, muscle |  warfarin sensitivity, cytochrome p450 |
| PRKD2 | 19 | 47204207 | Exonic | p.V167M; p.V324M | Lamprey | Spleen, tongue, ovary, eye |  polycystic kidney disease, gastric cancer  |
| NLRP7 | 19 | 55451405 | Exonic | p.P261Q | X\_tropicalis | \_\_\_\_\_\_\_\_\_\_ |  gestational trophoblastic neoplasm, hydatidiform mole |
| TTC3 | 21 | 38569884 | Exonic | p.G1865S | X\_tropicalis | Tongue, uterus, heart, eye | down syndrome critical region, down syndrome |

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Gene Symbol** | **Translation Impact** | **SIFT FunctionPrediction** | **SIFT Score** | **PolyPhen-2 FunctionPrediction** | **dbSNP ID** | **NHLBI ESP European Frequency (%)** | **Co-segregation within the family (IGV)** |
| **II.2** | **II.4** | **III.5** | **III.6** | **II.14** | **II.9** |
| CTRC |  |  |  |  | 144717165 |  | Het | Het | Hom WT | Het | Het | Hom WT |
| KIF21B | missense | Damaging | 0,01 | Benign | 150551633 | 0,01 | Het | Het | Het | Het | Het | Hom WT |
| GALNT14 | missense | Tolerated | 0,26 | Benign | 41280621 |  | Het | Het | Het | Het | Het | Hom WT |
| CRIM1 | missense | Damaging | 0,01 | Probably Damaging | 113372122 | 0,29 | Het | Het | Het | Het | Het | Hom WT |
| SPTBN1 | missense | Tolerated | 0,72 | Benign | 147989241 | 0,16 | Het | Het | Het | Hom WT | Het | Hom WT |
| VRK2 | missense | Damaging | 0,02 | Benign | 147530902 | 0,36 | Het | Het | Het | Hom WT | Het | Hom WT |
| PLEK | missense | Tolerated | 0,52 | Benign | 34338164 | 1,14 | Het | Het | Het | Hom WT | Het | Hom WT |
| HNMT |  |  |  |  |  |  | Het | Het | Het | Hom WT | Het | Hom WT |
| ITGB6 | missense | Tolerated | 0,65 | Benign | 142197545 | 0,29 | Het | Het | Hom WT | Het | Het | Hom WT |
| WDR12 | missense | Damaging | 0 | Possibly Damaging |  | 0 | Het | Het | Hom WT | Het | Het | Hom WT |
| CLDN16 | frameshift |  |  |  | 56086318 | 0 | Het | Het | Het | Het | Het | Hom WT |
| CLDN16 | missense | Damaging | 0,04 | Benign | 3214506 | 0 | Het | Het | Het | Het | Het | Hom WT |
| PRIM2 | missense |  |  |  | 77436138 |  | Het | Het | Het | Het | Het | Hom WT |
| STK31 | in-frame |  |  |  |  | 0 | Het | Het | Het | Het | Het | Hom WT |
| TYW1 | in-frame | Tolerated | 0,5 | Benign |  | 0 | Het | Het | Het | Het | Het | Hom WT |
| GLDC | missense | Tolerated | 1 | Benign | 117460214 | 0,03 | Het | Het | Hom WT | Het | Het | Hom WT |
| IFNA5 | missense | Damaging | 0 | Probably Damaging | 140371188 | 0,59 | Het | Het | Hom WT | Hom WT | Het | Hom WT |
| DMRTA1 | missense | Tolerated | 0,54 | Benign | 145718826 | 0,38 | Het | Het | Hom WT | Hom WT | Het | Hom WT |
| MIR4289 |  |  |  |  |  |  | Het | Het | Het | Hom WT | Het | Hom WT |
| HBG2 |  |  |  |  | 113622787 |  | Het | Het | Het | Hom WT | Het | Hom WT |
| OR4C16 |  |  |  |  |  |  | Het | Het | Hom m | Het | Het | Hom WT |
| OR10AG1 | in-frame | Activating | 1 | Benign |  | 0 | Het | Het | Hom m | Het | Het | Hom WT  |
| C12orf56 | missense | Tolerated | 0,13 |  | 367932023 | 0,03 | Het | Het | Hom WT | Het | Het | Hom WT |
| ATP8B4 | missense | Damaging | 0 | Probably Damaging |  | 0 | Het | Het | Hom WT | Het | Het | Hom WT |
| DMXL2 | missense | Tolerated | 0,17 | Benign | 144241909 | 0,02 | Het | Het | Hom WT | Het | Het | Hom WT |
| TPSD1 | missense | Tolerated | 0,31 | Benign | 2401930 | 0 | Het  | Het | Het  | Het  | Het  | 0 |
| TPSD1 | missense | Tolerated | 0,75 | Benign | 3993983 | 0 | Het  | Het | Het | Het | Het  | 0 |
| NOMO1  | missense | Tolerated | 0,23 | Benign | 140359200 | 0,12 | Het | Het | Hom WT | Hom WT | Het | Hom WT |
| SRCAP | missense | Damaging | 0 | Possibly Damaging | 368876335 | 0,01 | Het | Het | Hom WT | Het | Het | Hom WT |
| ZNF319 | missense | Tolerated | 0,08 | Benign |  | 0 | Het | Het  | Hom WT | Het | Het | 0 |
| PDPR | missense | Tolerated | 1 | Benign | 200469748 | 0 | Het | Het | Het | Het | Het | Het |
| ZFHX3 | in-frame |  |  |  |  | 0 | Het | Het | Hom WT | Het | Het | Hom WT |
| WWOX | missense | Tolerated | 0,7 | Benign | 186745328 | 0,46 | Het | Het | Hom WT | Het | Het | Hom WT |
| GSDMA | missense | Activating | 1 | Benign |  | 0 | Het | Het | Het | Het | Het  | Hom WT |
| DNAI2 | synonymous |  |  |  | 148947094 | 0,05 | Het | Het | Hom WT | Het | Het | Hom WT |
| MAN2B1 | missense | Damaging | 0,01 | Possibly Damaging | 117843968 | 0,42 | Het | Het  | Het  | Het | Het  | Hom WT |
| CYP4F2 | missense | Tolerated | 0,39 | Benign | 4020346 | 0 | Het | Het | Hom WT | Hom WT | Het | Hom WT |
| PRKD2 | missense | Tolerated | 0,15 | Benign | 45455991 | 0,98 | Het | Het | Hom WT | Het  | Het  | Hom WT |
| NLRP7 | missense | Damaging | 0 | Probably Damaging |  | 0 | Het | Het | Het | Hom WT | Het | Hom WT |
| TTC3 | missense | Tolerated | 0,55 | Possibly Damaging |  | 0 | Het | Het | Het | Hom WT | Het | Hom WT |

**Table 2.** Candidate genes analysis.The table shows the 40 genes obtained from Ingenuity and the analysis done for each of the associated variants.The selection was mainly based on the expression of the gene in the eye (TIGER database), the SIFT and Polyphen predictions and known diseases association (literature review). Subsequently the aminoacid conservation through vertebrate species (BLAST analysis) and co-segregation of the mutation in pterygium (II.2, II.4, II.14) and pinguecula (III.5, III.6) affected members and not in the II.9 unaffected family member (IGV analysis) was considered. This screening led to the selection of *CRIM1* as the best candidate considering those parameters.

A.

|  |  |  |  |
| --- | --- | --- | --- |
| **Patient** **NI** | **Year of birth** | **Sex** | **Eye condition** |
| 1 | 1942 | M | pterygium |
| 2 | 1950 | M | minor pterygium |
| 3 | 1931 | M | pterygium |
| 4 | 1954 | M | pterygium |
| 5 | 1946 | M | pterygium |
| 6 | 1975 | M | pterygium |
| 7 | 1941 | M | pterygium |
| 8 | 1958 | F | pterygium |
| 9 | 1984 | F | pterygium |
| 10 | 1934 | M | pterygium |
| 11 | 1974 | F | pterygium |
| 12\* | 1967 | F | pterygium family |

B.

|  |  |  |  |
| --- | --- | --- | --- |
| **Patient****Bolivia**  | **Year of birth**  | **Sex** | **Eye condition** |
| B1 | 1965 | F | pterygium |
| B2 |  | M | pterygium |
| B3 | 1949 | M | pterygium |
| B4 | 1957 | M | pterygium |
| B5 | 1999 | M | pterygium |
| B6 | 1952 | M | pterygium |
| B7 | 1947 | F | pterygium |
| B8 | 1941 | M | pterygium |
| B9 | 1990 | M | pterygium |

**Table 3.** Individual pterygium participants. **(A).** A list of pterygium affected individuals collected in the UK and the relevant information obtained through the questionnaire. \*Patient 12 denotes the pterygium affected II.1 family member previously studied[27](#_ENREF_27). **(B).** List of pterygium affected individuals collected in Bolivia and the year of birth and sex of participants.

|  |  |
| --- | --- |
| **CRIM1 sequence** | **Primer sequence (5’ to 3’)** |
| T7\_F | TAATACGACTCACTATAGGG  |
| Seq1\_R  | GCAGAATGTGCAGTCGTCTT  |
| Seq1\_F  | TGATCGAGGGTTATGCTCCT  |
| Seq2\_F  | TACTACGTGCCCGAAGGAGA |
| Seq2\_R  | GGCACTTTCACAGGGTTTGT |
| Seq3\_F  | TGCCGGGAATGCTACTGT  |
| Seq3\_R  | ACAGAAGGGCAGGACTCAGA |
| Seq4\_F  | CTGAGTCCTGGAAGCCTGAC |
| Seq4\_R | CCTGGAGGTGACCCATATCT |
| Seq5\_F  |  AACCATCGAGGAGAGGTTGA |
| Seq5\_R  | TCGTCTTCCGTCTTTTGAAAC |

**Supplementary Table 1.** Primers used to validate that the whole of the CRIM1 sequence was inserted into the pcDNA3.1 plasmid and to check the presence of the H412P mutation introduced by site directed mutagenesis.

|  |  |
| --- | --- |
| **CRIM1 VWFs** | **Primer sequence (5’ to 3’)** |
| Exon6\_F (VWF1) | TTGAAAAACATCAAAGGACACAA |
| Exon6\_R (VWF1) | CCATGTATGCTCCTGTTAATCTG  |
| Exon7\_F (VWF2) | GATGACTAGAACCCAGGGAAAA |
| Exon7\_R (VWF2) | AGCAGACATTATGCCCAAGG  |
| Exon11\_F (VWF3) | GCCTGTTTCTCCTGTGCAGT  |
| Exon11\_R (VWF3)  | TGCAAGGCAGAAGTCATTTG |
| Exon12\_F (VWF4) | CCAGGCTTTCAAGAGTTGGA |
| Exon12\_R (VWF4) | GGGTCCCACAGAATGACAAC |
| Exon13\_F (VWF5) | CTGGCCAACAGCATCTTCTT |
| Exon13\_R (VWF5) | GACATGTCAAGCAGGGAAAAA  |
| Exon14\_F (VWF6) | AAGATCGTGTGCGTTGTCAC |
| Exon14\_R (VWF6) | GTCGAGCTCTGCTTCGATTT |

**Supplementary Table 2.** Primers used to verify the presence of other mutations in all the VWF domains of CRIM1.

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