University of Sistan and Baluchestan

Journal of Epigenetics

Online ISSN: 2676 - 4350

Homepage: https://jep.usb.ac.ir

Iranian Genetics Society

Correlation between Promoter Hypermethylation and Expression Profiles of P15^{INK4b} and P16^{INK4a} Genes on Development of Pterygium Disease

Tahereh Dianat[1,*], Mohammad Naiem Aminifard[2], Mohammad Arish[3], Mohammad Hussein Sangtrash[4], Robab Poyandeh[5],

[1] *Corresponding author*, Department of Biology, University of Sistan and Baluchestan, Zahedan, Iran. E-mail: dianat.tahere@gmail.com.
[2] Department of Ophthalmology, Al-Zahra Eye Hospital, Zahedan University of Medical Sciences, Zahedan, Iran. E-mail: aminifard@zums.ac.ir.
[3] Department of Ophthalmology, Al-Zahra Eye Hospital, Zahedan University of Medical Sciences, Zahedan, Iran. E-mail: arish.mohammed@gmail.com
[4] Department of Biology, University of Sistan and Baluchestan, Zahedan, Iran. E-mail: sangtarash@science.usb.ac.ir.
[5] Department of Biology, University of Sistan and Baluchestan, Zahedan, Iran. E-mail: rp.ba2om@gmail.com.

ARTICLE INFO

Article type:
Research Article

Article history:
Received: 04 September 2019
Revised: 01 November 2020
Accepted: 10 November 2020

Keywords:
Pterygium,
Hypermethylation,
Expression,
P15^{INK4b} Gene,
P16^{INK4a} Gene.

A B S T R A C T

It is not thoroughly clear what the exact etiology of pterygium. Presently it has been illustrated that pterygium is a benign and destructive condition. The aim of this study was to examine the correlation between promoter hypermethylation of *P15^{INK4b}* and *P16^{INK4a}* genes on progress of pterygium. We extracted DNA from 81 primary pterugium and 75 normal conjunctiva tissues. Methylation specific polymerase chain reaction (MSP) technique was used to analyze of promoter hypermethylation of *P15^{INK4b}* and *P16^{INK4}*genes. The expression levels of these genes were also assessed in mRNA from 23 pterygium and 18 normal conjunctiva tissue samples using real-time quantitative reverse transcriptase PCR. The frequency of methylation for *P15^{INK4b}* was 97.5% among cases and 72% among controls. *P16^{INK4}* gene methylation at promoter site was 69.1% and 33.3% for pterygium and normal conjunctiva tissue, respectively. A statistically significant relationship was found for methylation of *P15^{INK4b}* and *P16^{INK4}* genes between cases and controls (P<0.0001). The relative expression of *P16^{INK4}* gene also in Pterygium tissues was significantly different in comparison to conjunctiva tissues of healthy controls (P<0.0001). But, it was not significant for *P15^{INK4b}*. These data suggest that reduced expression of *P15^{INK4b}* and especially *P16^{INK4}* genes through promoter hypermethylation of these two critical genes in cell cycle between cases and controls is probably an early and common epigenetic alteration in Pterygium. More investigations are required to be performed to analyze of epigenetic variations effect on the progression of Pterygium in different stages.

Introduction

A pterygium is an abnormal condition, along with a raised wedge-shaped growth on the surface of the eye; it is more common in people who are living in sunny areas. Therefore, It is thought to be related to increased exposure to ultra-violet (UV) light (1, 2). Ultimately, the pterygium might interfere with the vision, either by distorting the cornea or by extending over the pupil. Most studies have shown a geographical variation in incidence in countries that are closer to the equator which gives rise to higher rates of occur-rence(3). Although, there is no consensus on its pathogenesis, recent evidence suggests a diverse failure in cells proliferation, rather than

DOI: 10.22111/jep.2020.31529.1015
© Tahereh Dianat
Publisher: University of Sistan and Baluchestan

How to Cite: Dianat, T., Naiem Aminifard, M., Arish, M., Hussein Sangtrash, M., Poyandeh, R. (2021). Correlation between Promoter Hypermethylation and Expression Profiles of P15INK4b and P16INK4a Genes on Development of Pterygium Disease. Journal of Epigenetics, 2(1), 25-30. DOI: 10.22111/jep.2020.31529.1015

degenerative condition that strongly correlated with exposure to ultraviolet radiation (UVR) of solar light (3-6). So, analyzing of genes and proteins that are involved in cell cycle regulation, possibly more expose the ambiguous points in pterygium molecular mechanisms. So, various genetic and epigenetic factors likely made an individual susceptible to pterygium. Epigenetic represents the heritable changes on DNA such as DNA methylation and which affect the efficiency of genome(7-8). Mostly, methylation of CpG islands in regulatory regions of DNA can lead to transcriptional silencing in various carcinogenesis processes (7, 9-16). For that reason, the shape and behavior of pterygium is similar to cancerous process, first for more knowledge we have focused on some genes invloved in cell cycle regulation. *P15*^{INK4b} and *P16*^{INK4a} genes are located on chromosome 9p21 which are frequently be mutated and deleted in a wide variety of tumors(17). The *P15*^{INK4b} and *P16*^{INK4a} gene show great homology together and compete with cyclin D for binding to CDK4, 6 to regulate of cell cycle with inhibition of G1 phase progression (18-22). In this case control study, we investigated promoter methylation of *p15*^{INK4b} and *p16*^{INK4a} genes and correlation of their expression profile with development of the pterygium.

2. Materials and methods

2.1. Study population and DNA amplification

Eighty one biopsy from individuals with primary pterygium and 75 normal conjunctiva tissues have been collected from alzahra eye hospital for this case -control study. Phenol chloroform protocol has been used for extraction of genomic DNA, then 1–2 mg of isolated genomic DNA was diluted in 50 µl of water and used for DNA modification as previously published (23). After that, purification of the bisulfite-treated DNA has been done by the Wizard® DNA Clean-Up System (USA, Promega) according to the manufacturer's instructions. Eventually, the modified DNA has diluted in 20 µl of water and kept at -20 ° C for further experiments. As previously given (23) designed methylated and unmethylated primers for the first promoter of the genes was used to carry out the Methylation-specific PCR (MSP) analysis. Each 25 µL PCR reaction included 1 µL of bisulfite-modified DNA , 1 µL of dNTP mix (10 mmol/L), 0.4 µL of HotStarTaq® (Parstus: 2.5 U/mL), 0.5 µL each of primer (10 mmol/L), 3 µL of RNase free double distilled water, 2.5 µL of 10× buffer, and 2 µL of Mg2+ (25 mmol/L). The amplification program was set as follows: 95 °C for 5 min, followed by 40 cycles (95 °C for 40 s, the annealing temperature for *P15*^{INK4b} : M=57, U=60 and *P16*^{INK4a} : M=57, U=59 for 40 s and 72 °C for 40 s). Final incubation was completed at 72 °C for 10 min.

2.2. Analysis of mRNA expression

We extracted total RNA from 23 Pterygiums and 18 conjunctiva tissues using the RNX™- Plus solution (Cat No: MR7713C). A Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Cat. no. K1621) was used to reverse-transcribe 1 mg of RNA in a final volume of 20 µL. An AB15700 sequence detection system (Applied Biosystems) was used to estimate the involved cDNA using real-time quantitative PCR .The normalization data has been done by *RNA18s* as an internal standard. The sequence of primers has been published (24) .

2.3. Statistical analysis

All statistical analyses have been done with SPSS version 19.0 (SPSS, Chicago, IL). Pearson's $\chi 2$ has been used to analysis the categorical data and the binary logistic regression test detected the estimating odds ratios (OR) and 95% confidence intervals (95% CI) for the methylation status of *p15*^{INK4b} and *p16*^{INK4a} genes and the risk of pterygium. Expression data have been assessed using the Mann–Whitney test between groups (healthy subjects and patients). The significance level was set at $p \leq 0.05$.

Table 1. Promoter methylation frequency of *P15*^{INK4b} and *P16*^{INK4a} genes in patients with Pterygium and estimation the risk of disease.

Genes	Methylation status	Controls (N=75)	Cases (N=81)	[a]OR	p-Value
P15^{INK4b}	M U	54(72%) 21(28%)	79(97.5%) 2(2.5%)	15.36	<0.0001
P16^{INK4a}	M U	25(33.3%) 50(66.7%)	56(69.1%) 25(30.9%)	4.48	<0.0001

Abbreviations: M, methylation; U, unmethylation used as reference

[a] OR: Odds Ratio; It was calculated using OpenEpi (http://www.openepi.com/v37/TwobyTwo.htm)

Journal of Epigenetics

Table 2. Adjusting the gene promoter methylation status between cases and controls [a].

Genes		Unadjusted			Adjusted		
		OR	95%CI	P value	OR	95%CI	P value
$P15^{INK4b}$	U(ref) M	0.054	0.010_0.283	0.001	0.057	0.011_0.298	0.001
$P16^{INK4a}$	U(ref) M	0.238	0.101_0.556	0.001	0.253	0.106_0.606	0.002

OR = odds ratio; 95% CI = 95% confidence interval, ref = reference.
[a] Binary logistic regression analysis.

3. Results

As shown in Table 1, promoter methylation frequency of $p15^{INK4b}$ (97.5% versus 72.0%) and $p16^{INK4a}$ (69.1% versus 33.3%) genes was significantly different between pterygium tissues and normal conjunctiva (p= <0.0001), The greatly decreased risk of pterygium in comparison to the reference unmethylated pattern has been determined for the promoter hypermethylation of $p15^{INK4b}$ and $p16^{INK4a}$ [OR=0.57, 95% CI=0.011-0.3, p=0.001; OR=0.25, 95% CI= 0.1-0. 6, p=0.002] Table 2. The outcomes of expression analysis exposed a statistically significant variation between cases and healthy controls concerning the relative expression of $p16^{INK4a}$ (P=0.007) and no significant for $p15^{INK4b}$.Table3.

Table 3. Comparison of relative gene expression for $P15^{INK4b}$ and $P16^{INK4a}$ genes between patients with schizophrenia and healthy controls.

Genes		No.	Mean±SD	p-Value[a]
$P15^{INK4b}$	Cases	23	0.8 ± 1.02	0.2
	Controls	18	1.4 ± 1. 3	
$P16^{INK4a}$	Cases	23	1.2 ± 0.9	0. 7
	Controls	18	1.4 ± 0.6	

[a] Mann-Whitney-Test

	group	N	Mean	Std. Deviation	Std. Error Mean
p15.h	case	23	.885752	1.0228807	.2132854
	control	18	1.422928	1.3980462	.3295227
p16.h	case	23	1.155770	.8923266	.1860630
	control	18	1.367617	.6586789	.1552521

4. Discussion

The exact etiology of ptergium is yet unidentified, it is said that ultraviolet (UV) light from the sun is the main risk factor for development of pterygium (2). Some characterizations of pterygium such as: Angiogenesis, tissue structure and frequent recurrence ,draw consideration to compare its molecular mechanisms with cancerous cell (1, 25) . The majority of recent studies have focused on the study of epigenetic changes resulting in many types of neoplasia (26, 27). DNA methylation was the first epigenetic alteration to be observed in cancer cells (28). Recently, Some studies have been highlighted that hypermethylation of the p16, E-cadherinor and disturbance in the P53 function may lead to pterygium configuration (5, 29). The vast review literatures were confirming the hypermethylation of $p15^{INK4b}$ and $p16^{INK4a}$ genes in development of various cancer such as ; head and neck, cervical, ovarian, thyroid, vulvar and skin cancer (30) . Osman *et al.*, (2013) have reported that 9p21 and 14q23 are susceptible loci for primary open angle glaucoma in Japanese population. Huang Q *et al.*, (1999) have found that alteration in $p15^{INK4b}$ and $p16^{INK4a}$ genes may be involved in development of retinoblastoma in chains population (31-32).

Finally, although the relevant data are sparse regarding the pathogenesis of ptrygium specifically in case of genetic and epigenetic variations, our outcomes showed a difference in promoter methylation of theses critical genes in cell cycle between cases and controls. These genes may be used as a significant and reliable biomarker in ptrygium in the Iranian population. For validation of this data, more studies with large sample size in various populations and in different grades of ptrygium are suggested.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards.

Consent

Informed consent was obtained from all individual participants included in the study.

Journal of Epigenetics

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments:

The authors would like to express their gratitude to the Department of Ophthalmology, Al-Zahra Eye Hospital, Zahedan University of Medical Sciences, and the Department of Biology, University of Sistan and Baluchestan, for supporting this project financially.

References

Tradjutrisno N. Pterygium: degeneration, exuberant wound healing or benign neoplasm?. Univ Med. 2009;28:179-87.

Detorakis ET, Spandidos DA.. Pathogenetic mechanisms and treatment options for ophthalmic pterygium: Trends and perspectives (Review). Int J Mol Med. 2009;23:439-447.

Chui J, Coroneo MT, Tat LT, Crouch R, Wakefield D, Di Girolamo N. Ophthalmic Pterygium A Stem Cell Disorder with Premalignant Features. Am J Pathol. 2011;178:817-827.

Donald T H Tan, Wen Ying Tang, Yan Ping Liu, Hak-Su Goh, Duncan R Smith. Apoptosis and apoptosis related gene expression in normal conjunctiva and pterygium. Br J Ophthalmol. 2000;84:212-216.

5. Young CH, Chiu YT, Shih TS, Lin WR, Chiang CC, Chou YE, et al. E-cadherin promoter hypermethylation may contribute to protein inactivation in pterygia. Mol Vis. 2010;16:1047-1053.

Balci M, Sahin S, Mutlu FM, Yağci R, Karanci P, Yildiz M. Investigation of oxidative stress in pterygium tissue. Mol Vis. 2011;17:443-447.

Andrew G. Muntean , Jay L. Hess. Epigenetic Dysregulation in Cancer. Am J Pathol. 2009;175:1353-1361.

Jones PA, Baylin SB.. The fundamendal role of epigenetic events in cancer. Nat Rev Genet. 2002;3:415-428.

Kim JK, Samaranayake M, Pradhan S. Epigenetic mechanisms in mammals. Cell Mol Life Sci. 2008;66:596 – 612.

Manel E. Epigenetics in Cancer. N Engl J Med. 2008;358:1148-1159.

Ellis L, Atadja PW, Johnstone RW. Epigenetics in cancer: Targeting chromatin modifications. Mol Cancer Ther. 2009;8:1409-20.

Sharma S , Kelly TK , Jones PA. Epigenetics in cancer. Carcinogenesis. 2010;31:27–36.

Ho E, Beaver LM, Williams DE, Dashwood RH. Dietary Factors and Epigenetic Regulation for Prostate Cancer Prevention. Adv Nutr. 2011;2:497-510.

Agarwal A, Polineni R, Hussein Z, Vigoda I, Bhagat TD, Bhattacharyya S et al. Role of epigenetic alterations in the pathogenesis of Barrett's esophagus and esophageal adenocarcinoma. Int J Clin Exp Pathol. 2012;5:382-396.

Tabish AM, Poels K,Hoet P, Godderis L. Epigenetic Factors in Cancer Risk: Effect of Chemical Carcinogens on Global DNA Methylation Pattern in Human TK6 Cells. PLoS ONE. 2012;7.

Mascolo M , Siano M, Ilardi G, Russo D, Merolla F, Rosa GD et al. Epigenetic Disregulation in Oral Cancer. Int J Mol Sci. 2012;13:2331-2353.

Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene. 2002;21:5427-5440.

Holdt LM, Teupser D. Recent Studies of the Human Chromosome 9p21 Locus, Which Is Associated With Atherosclerosis in Human Populations. Arterioscler Thromb Vasc Biol. 2012;32:196-206.

kumar jha A, Nikbakht M, Jain V, Capalash N, Kaur J. p16INK4a and p15INK4b gene promoter methylation in cervical cancer patients. Oncol Lett. 2012;3:1331-1335.

Zhuang J, Peng W, Li H, Wang W, Wei Y, Li W et al. Methylation of p15INK4b and Expression of ANRIL on Chromosome 9p21 Are Associated with Coronary Artery Disease. PLoS ONE. 2012;7.

Henley SA , Dick FA. The retinoblastoma family of proteins and their regulatory functions in the mammalian cell division cycle. Cell div. 2012;7.

Vermeulen K, Van Bockstaele DR, Berneman ZN.. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell prolif. 2003;36:131–149.

Kordi-Tamandani DM, Moazeni-Roodi AK, Rigi-Ladiz MA, Hashemi M, Birjandian E, Torkamanzehi A.. Promoter hypermethylation and expression profile of MGMT and CDH1 genes in oral cavity cancer. Arch Oral Biol. 2010;55:809-814.

Kordi-Tamandani DM, Ladies MA, Hashemi M, Moazeni-Roodi AK, Krishna S, Torkamanzehi A. Analysis of p15INK4b and p16INK4a gene methylation in patients with oral squamous cell carcinoma. Biochem Genet. 2012;50:448-453.

Džunić B, Jovanović P, Veselinović D, Petrović A, Stefanović I, Kovačević I. Analysis of pathohistological characteristics of pterygium. Bosn J Basic Med Sci. 2010;10:307-3013.

Iraji, R., Arish, M., Aminifard, M., Dianat, T. Expression and promoter-hyper methylation analysis of MGMT gene in patients with pterygium. Journal of Epigenetics, 2019; (): 1-6. doi: 10.22111/jep.2019.28421.1009.

Jha AK, Nikbakht M, Parashar G, Shrivastava A, Capalash N, Kaur J. Reversal of hypermethylation and reactivation of the RARβ2 gene by natural compounds in cervical cancer lines. Folia Biol (Praha) 2010;56:195–200.

Jha AK, Kumar S, Nikbakht M, Sharma V, Kaur J. Epigenetics and its role in ageing and cancer. J Med Med Sci. 2011;2:696–713.

Chen PL, Cheng YW, Chiang CC, Tseng SH, Chau PS, Tsai YY. Hypermethylation of the p16 gene promoter in pterygia and its association with the expression of DNA methyltransferase 3b. Mol vis. 2006;12:1411-1416.

Wong TS, Man MW, Lam AKY, Wei WI, Kwong YL, Yuen APW. The study of p16 and p15 gene methylation in head and neck squamous cell carcinoma and their quantitative evaluation in plasma by real-time PCR. Eur J Cancer. 2003;39:1881–1887.

Osman W, Low SK, Takahashi A, Kubo M, Nakamura Y. A genome-wide association study in the Japanese population confirms 9p21 and 14q23 as susceptibility

Journal of Epigenetics

loci for primary open angle glaucoma. Hum Mol Genet. 2012;21:2836-2842.

Huang Q, Tao Y, Yandell DW. Mutations of several tumor suppressor genes in primary retinoblastoma. Zhonghua Zhong Liu Za Zhi. 1999 ;21:10-12.

Journal of Epigenetics