JOURNAL OF EPIGENETICS, VOL. 02, ISS. 01 (WINTER 2020), 27-31

JOURNAL OF EPIGENETICS

Journal homepage: http://jep.usb.ac.ir/

Document Type: Original Article

Correlation between Promoter Hypermethylation and Expression Profiles of *P15^{INK4b}* and P*16^{INK4a}* Genes on Development of Pterygium Disease

Tahereh Dianat[a,*], Mohammad Naiem Aminifard[b], Mohammad Arish[b], Mohammad Hussein Sangtrash[a], Robab Poyandeh[a]

[a] *Department of Biology, University of Sistan and Baluchestan, Zahedan, Iran; Email: dianat.tahere@gmail.com, sangtarash@science.usb.ac.ir, rp.ba2om@gmail.com*

[b] *Department of Ophthalmology, Al-Zahra Eye Hospital, Zahedan University of Medical Sciences, Zahedan, Iran; Email: aminifard@zums.ac.ir, arish.mohammed@gmail.com*

***** *Corresponding author: Email Address: dianat.tahere@gmail.com*

ARTICLE INFO

Article history:

Received 4 September 2019

Accepted 10 November 2020

Available online 10 November 2020

DOI: 10.22111/jep.2020.31529.1015

KEYWORDS:

Pterygium, Hypermethylation, Expression, *P15^{INK4b}* Gene, *P16^{INK4a}* Gene

A B S T R A C T

Background: It is not thoroughly clear that what is the exact etiology of pterygium. Recently, 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. **Materials and methods**: We extracted DNA from 81 primary pterygium and 75 normal conjunctiva tissues. Methylation specific polymerase chain reaction (MSP) technique was used to analyze of promoter hypermethylation of *P15^{INK4b}* and *P16^{INK4a}* 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. **Results:** The frequency of methylation for *P15^{INK4b}* was 97.5% and 72% among cases and controls respectively. *P16^{INK4}* gene methylation at promoter 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^{INK4a}* genes between cases and controls (P<0.0001). The relative expression of *P16^{INK4a}* 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}*. **Conclusion**: These data suggest that reduced expression of *P15^{INK4b}* and especially *P16^{INK4a}* 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

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 (Detorakis and Spandidos 2009, Tradjutrisno September-December, 2009). 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

* Corresponding author: Tel: 0098-5412452335
E-mail address: dianat.tahere@gmail.com

countries that are closer to the equator which gives rise to higher rates of occurrence (Chui, Coroneo et al. 2011). Although, there is no consensus on its pathogenesis, recent evidence suggests a diverse failure in cells proliferation, rather than degenerative condition that strongly correlated with exposure to ultraviolet radiation (UVR) of solar light (Tan, Tang et al. 2000, Young, Chiu et al. 2010, Balci, Şahin et al. 2011, Chui, Coroneo et al. 2011). Therefore, analyzing of genes and proteins that are involved in cell cycle regulation, possibly more expose the ambiguous points in pterygium molecular mechanisms. Various genetic and epigenetic factors then likely made an individual susceptible to pterygium. Epigenetic represents the heritable changes on DNA (such as DNA methylation) which affect the efficiency of genome (Jones and Baylin 2002, Muntean and Hess 2009). Mostly, methylation of CpG islands in regulatory regions of DNA can lead to transcriptional silencing in various carcinogenesis processes (E. 2008, Ellis, Atadja et al. 2009, Kim, Samaranayake et al. 2009, Muntean and Hess 2009, Sharma, Kelly et al. 2010, Ho, Beaver et al. 2011, Agarwal, Polineni et al. 2012, Mascolo, Siano et al. 2012, Tabish, Poels et al. 2012). Due to this reason, the shape and behavior of pterygium is similar to cancerous process; therefore, 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 mutated and deleted in a wide variety of tumors (Esteller 2002). The $P15^{INK4b}$ and $P16^{INK4a}$ genes 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 (Vermeulen, Van Bockstaele et al. 2003, Henley and Dick 2012, Holdt and Teupser 2012, Jha, Nikbakht et al. 2012, Zhuang, Peng et al. 2012). 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.

Materials and Methods

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 (Kordi-Tamandani, Moazeni-Roodi et al. 2010). 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 (Kordi-Tamandani, Moazeni-Roodi et al. 2010) 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 Mg^{2+} (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.

Analysis of mRNA Expression

We extracted total RNA from 23 Pterygiums and 18 conjunctiva tissues using the RNX™- Plus solution (Cat No: MR7713C). A RevertAid 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 (Kordi-Tamandani, Ladies et al. 2012).

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≤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	54(72%)	79(97.5%)	15.36	<0.0001
	U	21(28%)	2(2.5%)		
P16^{INK4a}	M	25(33.3%)	56(69.1%)	4.48	<0.0001
	U	50(66.7%)	25(30.9%)		

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

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

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.

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 Pterygium 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

Discussion

The exact etiology of pterygium is not yet identified, as it is said that ultraviolet (UV) light from the sun is the main risk factor for development of pterygium (Detorakis and Spandidos 2009). Some characterizations of pterygium such as: angiogenesis, tissue structure and frequent recurrence draw consideration to compare its molecular mechanisms with cancerous cell (Džunić, Jovanović et al. 2010, Tradjutrisno 2016). The majority of recent studies have focused on the study of

DOI: 10.22111/jep.2020.31529.1015

epigenetic changes resulting in many types of neoplasia (Jha, Nikbakht et al. 2010, Iraji, Arish et al. 2018). DNA methylation was the first epigenetic alteration to be observed in cancer cells (Jha, Kumar et al. 2011). Recently, Some studies have highlighted that hypermethylation of the p16, E-cadherinor and disturbance in the P53 function may lead to pterygium configuration (Chen, Cheng et al. 2006, Young, Chiu et al. 2010). The vast review literatures confirmed 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 (Yuen and Wong 2004). 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 (Huang, Tao et al. 1999, Osman, Low et al. 2012). Finally**,** although the relevant data are sparse regarding the pathogenesis of pterygium specifically in case of genetic and epigenetic variations; Our outcomes showed a difference in promoter methylation of theses critical genes in cell cycle between studied cases and controls. These genes may be used as a significant and reliable biomarker in pterygium in the Iranian population. For validation of this data, more studies with large sample size in various populations and in different grades of pterygium 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.

Acknowledgements

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

Agarwal, A., et al. (2012). Role of epigenetic alterations in the pathogenesis of Barrett's esophagus and esophageal adenocarcinoma. *International Journal of Clinical and Experimental Pathology*, 5(5): 382.

Balci, M., et al. (2011). Investigation of oxidative stress in pterygium tissue. *Molecular Vision*, 17: 443.

Chen, P. L., et al. (2006). Hypermethylation of the p16 gene promoter in pterygia and its association with the expression of DNA methyltransferase 3b. *Mol Vis*, 12(141): l-1416.

Chui, J., et al. (2011). Ophthalmic pterygium: a stem cell disorder with premalignant features. *The American Journal of Pathology*, 178(2): 817-827.

Detorakis, E. T., & Spandidos, D. A. (2009). Pathogenetic mechanisms and treatment options for ophthalmic pterygium: trends and perspectives. *International Journal of Molecular Medicine*, 23(4): 439-447.

Džunić, B., et al. (2010). Analysis of pathohistological characteristics of pterygium. *Bosnian journal of Basic Medical Sciences*, 10(4): 307.

E., M. (2008). Epigenetics in Cancer. *N Engl J Med. 2008 Mar 13*, doi: 10.1056/NEJMra072067, 358(11):1148-59.

Ellis, L., et al. (2009). Epigenetics in cancer: targeting chromatin modifications. *Molecular Cancer Therapeutics*, 8(6): 1409-1420.

Esteller, M. (2002). CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene*, 21(35): 5427-5440.

Henley, S. A. & Dick, F. A. (2012). The retinoblastoma family of proteins and their regulatory functions in the mammalian cell division cycle. *Cell Division*, 7(1): 10.

Ho, E., et al. (2011). Dietary factors and epigenetic regulation for prostate cancer prevention. *Advances in Nutrition*, 2(6): 497-510.

Holdt, L. M., & Teupser, D. (2012). Recent studies of the human chromosome 9p21 locus, which is associated with atherosclerosis in human populations. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 32(2): 196-206.

Huang, Q., et al. (1999). Mutations of several tumor suppressor genes in primary retinoblastoma. Zhonghua zhong liu za zhi [Chinese Journal of Oncology], 21(1): 10-12.

Iraji, R., et al. (2018). Expression and promoter-hyper methylation analysis of MGMT gene in patients with pterygium.

Jha, A., et al. (2010). Reversal of hypermethylation and reactivation of the RARbeta2 gene by natural compounds in cervical cancer cell lines. *Folia Biol (Praha)*, 56(5): 195-200.

Jha, A. K., et al. (2011). Epigenetics and its role in ageing and cancer. *J Med Med Sci*, 2(3): 696-713.

Jha, A. K., et al. (2012). p16INK4a and p15INK4b gene promoter methylation in cervical cancer patients. *Oncology letters*, 3(6): 1331-1335.

Jones, P. A., & Baylin, S. B. (2002). The fundamental role of epigenetic events in cancer. *Nature Reviews Genetics*, 3(6): 415-428.

Kim, J., et al. (2009). Epigenetic mechanisms in mammals. *Cellular and Molecular Life Sciences*, 66(4): 596.

Kordi-Tamandani, D. M., et al. (2012). Analysis of p15 ink4b and p16 ink4a gene methylation in patients with oral squamous cell carcinoma. *Biochemical Genetics*, 50(5-6): 448-453.

Kordi-Tamandani, D. M., et al. (2010). Promoter hypermethylation and expression profile of MGMT and CDH1 genes in oral cavity cancer. *Archives of Oral Biology*, 55(10): 809-814.

Mascolo, M., et al. (2012). Epigenetic disregulation in oral cancer. *International Journal of Molecular Sciences*, 13(2): 2331-2353.

Muntean, A. G. and Hess, J. L. (2009). Epigenetic dysregulation in cancer. *The American Journal of Pathology*, 175(4): 1353-1361.

Osman, W., et al. (2012). A genome-wide association study in the Japanese population confirms 9p21 and 14q23 as susceptibility loci for primary open angle glaucoma. *Human Molecular Genetics*, 21(12): 2836-2842.

Sharma, S., et al. (2010). Epigenetics in cancer. *Carcinogenesis*, 31(1): 27-36.

Tabish, A. M., et al. (2012). Epigenetic factors in cancer risk: effect of chemical carcinogens on global DNA methylation pattern in human TK6 cells. *PloS One*, 7(4).

Tan, D. T., et al. (2000). Apoptosis and apoptosis related gene expression in normal conjunctiva and pterygium. *British Journal of Ophthalmology*, 84(2): 212-216.

Tradjutrisno, N. (2016). Pterygium: degeneration, exuberant wound healing or benign neoplasm?. *Universa Medicina*, 28(3): 179-187.

Tradjutrisno, N. (September-December, 2009). Pterygium: degeneration, exuberant wound healing or benign neoplasm?. *Universa Medicina*, 28(3): 179-187.

Vermeulen, K., et al. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell proliferation*, 36(3): 131-149.

Young, C. H., et al. (2010). E-cadherin promoter hypermethylation may contribute to protein inactivation in pterygia. *Molecular Vision*, 16: 1047.

Yuen, P. W. and Wong, S. (2004). 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, AACR.

Zhuang, J., et al. (2012). Methylation of p15INK4b and expression of ANRIL on chromosome 9p21 are associated with coronary artery disease. *PloS One*, 7(10).

DOI: 10.22111/jep.2020.31529.1015