JOURNAL OF EPIGENETICS, VOL. 01, ISS. 02 (SPRING 2019), 01-08

JOURNAL OF EPIGENETICS

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

Document Type: Original Article

Expression and Promoter-Hyper Methylation Analysis of MGMT Gene in Patients with Pterygium

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ARTICLE INFO

Article history

Received 14 January 2019

Accepted 22 April 2019

Available online 22 April 2019

DOI: 10.22111/jep.2019.28421.1009

KEYWORDS:

Pterygium, MGMT gene, promoter methylation, gene expression, epigenetic mechanisms

A B S T R A C T

1) Background: Pterygium is a benign legion and is observed as aggressive growth of conjunctiva fibro-vascular tissue on the cornea. The alkylating agents are observed as considerable threats for human health because alkylated lesions lead to cytotoxic, teratogenic and cancerizing effects. MGMT is one of the repair proteins of DNA which repairs the alkylated lesions. Expression and activity of MGMT is controlled by epigenetic mechanisms such as DNA methylation in the promoter regions like transcription factors which are connected to MGMT promoter and lead to positive or negative induction of that activity, protein-protein interactions, and negative regulation. 2) Materials and methods: In order to study methylation, DNA samples of 43 patients and 40 healthy individuals were extracted, bisulfited and then were studied. Also in order to study the expression, RNA was extracted from 15 other patients and 15 other healthy individuals; and then, the technique of Real-time PCR was used. 3) Results: analysis of promoter methylation of MGTM gene showed that there is no significant relationship in the situation of promoter methylation between the patients and control individuals (P value = 0.43; 95%Cl = 0.66-2.40; OR = 1.52). However, analysis of MGMT gene expression showed significant difference between the patients and control individuals (Mean ± SD: 1.25 ± 0.10 and 1.52 ± 2.91, respectively; P value = 0.009). 4) Conclusion: since there are no significant changes of promoter methylation of MGMT gene, there seems to be other unknown procedures that regulate this gene's expression levels. In this respect, expression of MGMT gene in the pterygium increases through unknown procedures. In order to approve this data, further studies are suggested in more populations with bigger sample sizes by the use of advanced molecular techniques.

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1. Introduction

Among the pathological eye conditions, human pterygium is one of the most controversial eye problems. Pterygium is overgrowth of abnormal conjunctiva in cornea which happens because of exposure to ultraviolet radiation. This lesion has a shape like a wing and in more developed cases, it can change the visual performance and lead to inflammation, redness and irritation in the area(Xu, Tao et al. 2013, Ozturk, Yıldırım et al. 2017). Despite the fact that it is categorized as a benign lesion by pathologists, the epithelial and surface fibro-vascular growth of the eye has a microscopic appearance that is propagative, progressive and vascular. In addition to that, recurrence is one of the unexplainable features of human pterygium. Also, pterygium has the potential of growing into some eye cancers, such as squamous neoplasia of the eye surface. The pterygium tissue has many common features with tumor and neoplasia such as proliferation, aggression, and recurrence. Recently, pterygium has been identified as hyperactivity in conjunctiva epithelium(Liu, Liu et al. 2013, Xu, Tao et al. 2013).

Pterygium is a very common legion in the populations located near the equator; that zone is called “pterygium zone”. Almost 22% of the population in some regions of that zone suffers from pterygium. These epidemiologic observ - ations show strong effect of the environment on development of pterygium. The effects of environmental factors on incidence of pterygium has been reported to be lower (below 2%) in the countries located out of the pterygium zone(Liu, Liu et al. 2013). Its prevalence in public population is estimated to be around 7-13% (Tradjutrisno 2016).

Although pathogenesis of this benign fibro-vascular proliferation on cornea has not been fully understood yet, there are many theories about the reasons for occurrence of pterygium; exposure to UV is a main risk factor for pterygium which leads to overgrowth of abnormal conjunctiva in the cornea; other factors include viruses, oxidative stress, DNA methylation, oncogene proteins and apoptosis, loss of heterozygosity, microsatellite instability, inflammatory mediators, extracellular matrix modifiers, lymphangiogenesis, epithelial mesenchymal transition, and alterations in cholesterol metabolism(Liu, Xu et al. 2017). Treatment is limited to surgical removal and the recurrence level is very high. This has made the researchers look for potential precautionary and predicting factors so that they can establish new alternative methods and purposeful treatment instead of surgery(Ozturk, Yıldırım et al. 2017).

DNA repair is one of the important subjects in cancer biology. Further attention is paid to mismatch repair, recombination and nucleotide excision repair. While these pathways have identified importance for evolution and develop - pment of cancer in human beings, other DNA repair mechanisms have not been studied in details. MGMT gene is one of the rarely studied pathways which is effective on repair capacity and is involved in the biology of cancer shape and behavior(Ozturk, Yıldırım et al. 2017).

Human genome, like other genomes, encodes information in order to preserve its unity. DNA repairing enzymes continuously supervise the chromosomes so that they can correct the damaged nucleotide remnants produced through exposure to carcinogens materials and cytotoxic combinations. If DNA repair did not exist, genome instability resulting from different types of DNA damaging factors would be a funda - mental problem for cells and organisms(Esteller and Herman 2004).

Alkylating agents are a group of reacting chemicals that transfer carbon-alkyl groups on a vast range of biological molecules, as a result, they change their structures and potentially confuse their performance. Because of their abundant presence in the environment and within the living cells, they cannot by fully avoided(Fu, Calvo et al. 2012).

The main sources of external alkylating agents are: components of air, water and food, biological ingredients (e.g. abiotic herbal elements) and pollutants (e.g. cigarette smoke and fuel smoke). Internal alkylating agents can be products that create oxidative damage, or could be originated from methyl donor cells like S-

adenosylmethionine which is a common co-factor in the biochemical reactions(Fu, Calvo et al. 2012).

Different pathways of cellular repair altogether regulate the alkylation sensitivity. The main repair mechanisms of alkylation damages include direct DNA repair which is done by homologous members of the α-ketoglutrate-dependent dioxy - genase enzymes and repair protein 06-methyl guanine DNA methyl transferase (MGMT), mult-istage base excision repair (BER) and nucleotide excision repair (NER).The results of direct repair in reverting an alkylate base into anatural base are without any mediating procedures or breaking DNA bonds(Fu, Calvo et al. 2012).

MGMT is a highly conserved enzyme and is fully expressed in DNA repair. This enzyme is located in position 10 q 26[9]. MGMT separates methyl group from O6-meG through covalent transfer and in this way, it brings back guanine to its normal situation. This process irreversibly deacti-vates MGMT and it remains apt to destruction by mediation of ubiquitin(Wick, Weller et al. 2014).

Expression and activity of MGMT is regulated through epigenetic mechanisms. One of its classic examples is methylation promoter (by H3K9 or MeCP2) or gene body. Other regulating mecha-nisms include: transcription factors which are connected to promoter MGMT and lead to induction (+) or reduction (-) of activity, protein-protein interactions (in the case of NDRG1) and negative regulating through microRNAs. How - ever expression of MGMT is also regulated through multiple molecular mechanisms, one of these molecular mechanisms is Wnt / B-catenin signaling cascade which causes induction of MGMT expression, and controlling of Wnt signaling causes the increase of the effects of alkylating drugs and causes sensitivity to chemotherapy in different cancers. Considering the importance of Wnt signal during the embryonic development and its importance in accurate DNA replication in the stem cells, it is fair that Wnt signaling might regulate the activity of DNA repair enzymes (Wick, Weller et al. 2014, Wickström, Dyberg et al. 2015).

2. Materials and Methods

2.1. The Study Samples: In this survey which is a case-control study, the study samples were collected from eye tissues of several patients suffering from pterygium, and the control samples were selected from several healthy individuals who were not suffering from pterygium (individuals suffering from cataract or strabismus) during one year (2016-2017) from Alzahra Eye Hospital in the city of Zahedan, Sistan and Baluchistan Province. The studied group included 58 sample patients and 55 healthy samples. Out of them, DNA of 43 sample patients and 40 healthy samples were selected, bisulfited and then were studied. Also because of smallness of pterygium tissue and the healthy tissue, in order to analyze the expression, RNA was extracted from 15 other patients and 15 other healthy individuals, and then, Real-time PCR technique was used.

The patients group included 30 male and 13 female patients and the control group included 21 male and 19 female samples.

DOI: 10.22111/jep.2019.28421.1009

Table 1- Profile of the Age and Sex of the Studied Samples

		Case(N=43)	Control(N=40)	P value
Mean age ± SD		4.40±2.02	3.45±2.58	0.00001
Median age		45	27	
Age range		20-90	7-87	
Gender	Male	30(69%)	21(53%)	0.00001
	Female	13(31%)	19(47%)	

2.2. DNA Extraction and Bisulfite Changes

DNA extraction from biopsy samples was performed through phenol chloroform method. Then, its quality was estimated by the use of spectrophotometer. Then, DNA of healthy samples and patients was bisulfited by the use of Wizard DNA Clean-Up System (Promega) kit.

2.3. MSP Conducting Method for MGMT Gene

In order to study methylation of MGMT gene by the use of MSP technique, DNA samples from the patient and healthy samples, which had been bisulfited previously, were treated by methylated and non-methylated specific primers for MGMT gene and their sequences are shown in table 2.

The used materials for conducting the MS-PCR reaction of promoter MGMT gene along with the related quantities and concentrations in the volume unit of 20 microliter in every tube are as follows in Table3:

Table 2- Primer Sequence Used to Study the Status of Methylation and Expression of MGMT Gene

Gene	Primer sequences (5′ → 3′)	Annealing temperature(°C)	Product size(bp)
MGMT M	F:TTTCGAACGTTCGTAGGTTTTCGC	59	93
	R: GCACTCTTCCGAAAACGAACG		
MGMT U	F:TTTGTGTTTTGATGTTTGTAGGTTTTTGT	59	81
	R:AACTCCACACTCTTCCAAAAACAAAACA		
MGMT(	F: TGAATGCCTATTTCCACC	46	102
Real-time PCR)	R: CTTCCATAACACCTGTCT		

Table3- The Materials used for the MS-PCR Reaction of Promoter MGMT Gene

Buffer PCR (10X)	2/5 µl	2X
Mgcl2(25mM)	3 µl	3 Mm
dntps(10Mm)	1 µl	400 nM
Each Primer(10mM)	1 µl	400 nM
Hot Start Taq(5u/µl)	4,0 µl	2U
Template DNA	1 µl	-
Depc Water	11,1 µl	-

The conditions of conducting PCR reaction for methylated and non-methylated primers were as follows: after heating the reaction tubes in 95 °C for 5 minutes, a cycle (including 40 seconds in 95 °C, 30 seconds in 59 °C and then 30 seconds in 72 °C) was repeated 40 times. After that, they were placed in 72 °C for 10 minutes; and finally, they were preserved in 4 °C.

Then, PCR products were electrophoresed on 2% agarose gel and were studied under UV radiation after staining with ethidium bromide, as shown in Fig. 1.

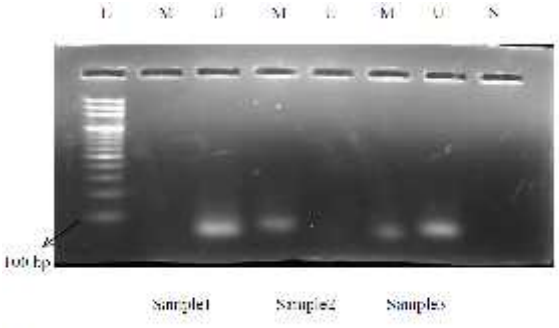

Fig.1- Results of MGMT Methylation Analysis

2.4. Extraction of rRNA

In this study, in order to extract rRNA, the Total RNA Extraction kit of Pars Tous Bio-technology Co. was used.

2.5. Convert RNA to cDNA

In order to convert RNA to cDNA, Easy cDNA kit of Pars Tous Bio-technology Co. was used.

2.6. Real-Time PCR

In this study, in order to study gene expression, Applied Biosystm Step One Real-Time PCR and specific SYBER green pigments were used, and B-Actin gene was used as the housekeeping gene. The SYBR® Green Master Mix used in this thesis includes tag polymerase enzymes, Mgcl2, dNTPs, buffer PCR and SYBR® Green pigments.

2.7. Statistical Analysis

Data analysis was performed using statistical software program (IBM SPSS Statistics version 22.0).An online software (Epi Info™) was used for estimation odds ratios [OR]. And 95% confidence intervals95% [CI] of methylation status between cases and controls. Pearson's ANOVA test was applied to analyze the methylation data, while the T-Test was used to analyze the expression level (2−ΔΔCT) between cases and controls.

3. Results

The results of statistical studies showed that there are not significant differences between the samples of the two patient and control groups in the methylation situation of MGMT gene(OR = 1.52; 95% CI = 0.66–2.40; P value = 0.43) (Table4).

Table4- The Results of Methylation Analysis of MGMT Gene

Gene	Methylation Status	Controls (N=40)	Cases (N=43)	P value	OR	CI 95%
MGMT	Present Absent	32(80%) 8(20%)	38(88.37%) 5(11.63%)	0.43	1.52	0.66-2.40

The frequency of methylation statuses is shown in Figure 2.

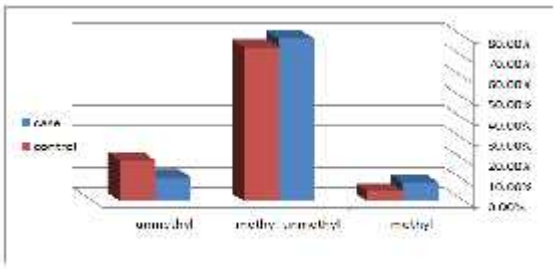

Fig.2- The Frequency of Methylation Statuses

The results of statistical analysis showed that there is a significant difference in the expression of MGMT gene between the patient group and control group (P-value = 0.009) which has been shown in table5. (Mean ± SD: 1.25 ±0.10 and 1.52 ± 2.91, respectively; P value = 0.009).

DOI: 10.22111/jep.2019.28421.1009

Table 5- Analysis of MGMT Gene Expression

Gene		N	Mean ± SD	P value
MGMT	Cases Controls	15 15	1.25± 0.10 1.52±2.91	0.009

Figure 3 shows that pterygium is observed approximately two times more in males compared to females; it shows the role of environmental factors in formation and development of pterygium.

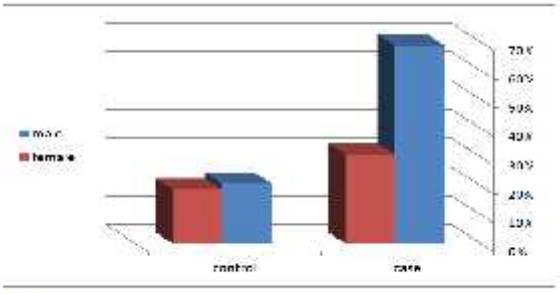

Fig. 3- Demographic Chart of Comparing Gender Frequency for MGMT Gene between the Two Patient and Control Groups

4. Discussion

Pterygium is a triangular part which grows from the conjunctival epithelium to the cornea. It is categorized according to its size, the amount of development, largeness and eyesight defect. There are several theories about etiology of pterygium. Both external and internal factors have been suggested. The prevalence is different in the world. The studies show that UV light is a main participant in appearance of pterygium. The effects of UV-A and UV-B (280-400 nm) are specifically harmful. The biological mechanisms that guarantee pterygium have not been fully understood. If pterygium affects eyesight, the only used treatment is removal through surgery; however, recurrence is common(Koranyi 2010)(Liu J.et al. 2017).

MGMT gene is one of the rarely studied pathways which is effective in the capacity of DNA repair and involved in the biology of the shape and behavior of cancer. Expression and activity of MGMT is regulated through epigenetic mechanisms; its classic example is methylation of promoter(Esteller and Herman 2004).

In this study, the relationship between epigenetic changes of MGMT gene and pathogens of pterygium has been studied. Our results showed that frequency of methylation of MGMT gene in the pterygium samples, compared to healthy control samples, does not have significant differences (P-value = 0.43). However, expression of MGMT gene showed significant difference between the patients and the control samples so much that it has been reported to be higher in the patients than in the healthy individuals (P-value = 0.009).

In addition to epigenetic mechanisms, expression and activity of MGMT is also regulated by molecular mechanisms. One of these mechanisms is WNT / B-catenin signaling cascade which leads to induction of MGMT expression(Wick, Weller et al. 2014).

5. Conclusion

In this study, since the methylation promoter changes of MGMT gene did not show significant changes, there are probably other unknown procedures in setting expression levels of this gene. In this regard, consequently, the expression of MGMT gene in the pterygium tissue increases through unknown procedures.

High expression of MGMT gene in pterygium shows that base reversal repair mechanisms are activated in pterygium. Repair mechanisms in pterygium are formed as a result of damages to DNA under the effect of environmental factors and intracellular factors. Therefore, interaction of genetic and environmental factors is approved in the pathogenic of pterygium which shows that

exposure to environmental conditions and also genetic sensitivity of the host lead to formation and development of the disease.

Also, the results showed that there is a significant difference in age and gender between samples of two patient and control groups (P-value = 0.00001) which showed that pterygium is observed approximately two times more in males than in females; it might be because men, compared to women, spend more time in the outside environment. It was also observed that generally pterygium appears in the ages over 20 which shows the effective roles of environmental factors in formation and development of pterygium.

Acknowledgements

This study was carried out with the assistance of Dr. Dor Muhammad Kordi Tamandani and Dr. Muhammad Arish as well as with the cooperation of Alzahra Eye Hospital. Hereby we appreciate their valuable contribution to this survey.

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DOI: 10.22111/jep.2019.28421.1009

JOURNAL OF EPIGENETICS, VOL. 01, ISS. 02 (SPRING 2019), 01-08 DOI: 10.22111/jep.2019.28421.1009

JOURNAL OF EPIGENETICS

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

آنالیز بیان و متیلاسیون پروموتر ژن MGMT در بیماران مبتلا به Pterygium

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چکیده

ناخنک چشم یک ضایعه خوش خیم است و به عنوان رشد بافت ملتحمه بر روی قرنیه دیده می شود. عوامل آلکیله کننده به عنوان تهدیدات قابل توجهی برای سلامت انسان دیده می شوند؛ زیرا ضایعات آلکیل شده منجر به اثرات سیتوتوکسیک، تراتوژنیک و سرطان می شود. MGMT یکی از پروتئین های تعمیر DNA است که ضایعات آلکیل شده را تعمیر می کند. بیان و فعالیت MGMT بوسیله مکانیسم های اپی ژنتیک مانند متیلاسیون DNA در ناحیه پروموتر کنترل می‌شود. به منظور مطالعه متیلاسیون، نمونه های DNA از ۴۳ بیمار و ۴۰ فرد سالم استخراج گردیدند و بی سولفیت شدند و سپس مورد بررسی قرار گرفتند. همچنین به منظور مطالعه بیان، RNA از ۱۵ بیمار دیگر و ۱۵ فرد سالم دیگر استخراج شد. سپس تکنیک Real-Time PCR استفاده شد. تجزیه و تحلیل متیلاسیون پروموتر ژن MGTM نشان داد که در وضعیت متیلاسیون پروموتر بین بیماران و افراد کنترل رابطه معنی داری وجود ندارد (P value = 0.43؛ ٪۹۵ Cl = 0.66-2.40؛ OR = 1.52). با این حال، آنالیز بیان ژن MGMT تفاوت معنی داری بین بیماران و افراد کنترل نشان داد (SD: 1.25 ± 0.10 و 1.52 ± 2.91؛ P value = 0.009. از آنجاییکه تغییرات قابل توجهی در متیلاسیون پروموتر ژن MGMT وجود ندارد، به نظر می رسد روش های ناشناخته دیگری وجود دارد که سطح بیان این ژن را تنظیم می کند. در این راستا، بیان ژن MGMT در ناخنک چشم از طریق روش های ناشناخته افزایش می یابد. به منظور تایید این داده ها، مطالعات بیشتر در جمعیت های بیشتر با اندازه نمونه های بزرگتر با استفاده از تکنیک های مولکولی پیشرفته پیشنهاد می شود.

واژگان کلیدی: ناخنک چشم، ژن MGMT، متیلاسیون پروموتر، بیان ژن، مکانیسم های اپی ژنتیک

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