Research Article

Evaluation of \textit{LATS1} and \textit{LATS2} Promoter Methylation with the Risk of Pterygium Formation

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**Purpose.** Pterygium is a serious eye problem in countries with high exposure to UV. However, despite numerous studies, the molecular etiology of pterygium is unclear. Recent studies have indicated that \textit{LATS1} and \textit{LATS2} genes are involved in DDR signaling pathways against continuous UV exposure. Our aim was to evaluate the \textit{LATS1} and \textit{LATS2} promoter methylation with the risk of pterygium formation. Methods. We evaluated the promoter methylation status of \textit{LATS1} and \textit{LATS2} using methylation-specific PCR technique. Also, mRNA expression of \textit{LATS1} and \textit{LATS2} was assessed in 14 cases of pterygium and 14 normal specimens by real-time PCR. Results. Promoter methylation of \textit{LATS1} and \textit{LATS2} was detected significantly between pterygium tissues and normal tissues \([\textit{LATS1}}; \text{OR} = 4.9; 95\% \text{ CI: 1.54 to 15.48}, P = 0.003; \textit{LATS2}}; \text{OR} = 7.1; 95\% \text{ CI: 1.53 to 33.19}, P = 0.004\]. The gene expression analysis showed a statistically significant difference between pterygium tissues and healthy controls for both \textit{LATS1} and \textit{LATS2} \((P < 0.05)\). Conclusions. The data of this study is the first report regarding the effect of promoter methylation of the \textit{LATS1} and \textit{LATS2} in the pterygium. To confirm these data, doing further studies in various genetic populations with large sample sizes using advanced molecular techniques is proposed.

1. Introduction

Pterygium is a common wing-shaped and oriented fibrovascular lesion coating the surface of the eye. According to the population-based studies, its prevalence rate varies from 0.7\% to 33\% [1]. This abnormality arises from the conjunctiva and extends into the cornea and can result in remarkable cosmetic problems, visual impairment, recurrent inflammation, and mild irritation [2]. Surgery is needed for cases when lesion expands to the central part of the cornea [3] (Figure 1).

There are countless theories regarding the causes of pterygium including UV light exposure, viruses, oxidative stress, DNA methylation, apoptotic and oncogenic proteins, loss of heterozygosity, microsatellite instability, inflammatory mediators, extracellular matrix modulators, lymphangiogenesis, cell epithelial-mesenchymal transition, and alterations in cholesterol metabolism [4]. Most studies have implicated that pterygium is a UV light associated disease. Therefore, we focused on \textit{LATS1} and \textit{LATS2} genes which are the common tumor suppressor genes in the UV-induced DNA Damage Response (DDR) signaling pathways. \textit{Lats} (large tumor suppressor) gene, a Ser/Thr kinase, belongs to the Ndr/LATS subfamily of AGC (protein kinase A/PKG/PKC) kinases originally isolated from \textit{Drosophila melanogaster}. Two mammalian homologs of fly \textit{lats}, \textit{LATS1} and \textit{LATS2}, are located in 6q25.1 and 13q12.11 chromosomes, respectively [5].

One of the DDR signaling pathways, which facilitate apoptosis following high levels of UV-induced damage, is \textit{Chk1-Lats2-p21} axis [6]. And \textit{Chk1-Lats2-(14-3-3)} regulates the P-body formation as a unique signaling pathway in response to UV-induced DNA damage [7]. \textit{LATS1} and \textit{LATS2} are also engaged in the regulation of cell cycle through G2-M arrest and G1-S arrest, respectively [8, 9]. After DNA damage the integrity of genome is warranted through \textit{RASSFIA-LATSI/2-MDM2-P53} signaling pathway [10]. In addition, a large amount of literature has reported the function of these genes in morphogenesis, cell division, and apoptosis [11].

Epigenetic modifications such as DNA methylation of CpG islands in promoter regions are the main cause of tumor suppressor gene silencing and can result in tumor
Table 1: Methylation primer sequences and annealing temperature.

| Genes | Sequences (5'-3') | Annealing temperature (°C) | Product size |
|-------|-------------------|----------------------------|-------------|
| LATS1 M | F: GGAGTTTCGTTTTGTC  
        R: CGACGTAATAACG AACGCCTA | 53°C | 138 bp |
| LATS1 U | F: TAGGTGGGATGTGGTGGT  
        R: CCC AACATAATAACACACCT | 57°C | 121 bp |
| LATS2 M | F: ATTTGGTTATTTGTATTTTTC  
        R: AACCAACATAATAAACCCCCG | 55°C | 148 bp |
| LATS2 U | F: TTGGTTTTTTGAAAAATTTAGT  
        R: CCACACATAAAACCCCCCA | 55°C | 130 bp |

M, methylated; U, unmethylated.

Development [12]. Some tumors such as breast cancer and astrocytoma have shown downregulation of LATS1 and LATS2 mRNA expression through promoter methylation [13]. To our knowledge for the first time, this study highlights the status of LATS1 and LATS2 promoter methylation and mRNA expression profiles in pterygium.

2. Materials and Methods

2.1. Subject. This case-control study was performed from 2010 to 2013 consisting of 70 primary pterygium tissues (35 males and 35 females with a mean age of 52.44 ± 20.611) and 70 normal conjunctiva tissues of the patients who had undergone cataract surgery (35 males and 35 females with a mean age of 50.67 ± 23.318). The biopsy tissue samples were frozen in −80°C until molecular analysis. These samples were collected from Al-Zahra Eye Hospital. All procedures in this study were approved by the Ethical Board at the Zahedan University of Medical Sciences. Informed consent was taken from all participants. Arish et al. 2016 described the clinical information of the patients who have participated in this study [14].

2.2. DNA Extraction and Modification. Genomic DNA was extracted from frozen tissues by phenol chloroform isoamyl alcohol extraction protocol. Then 1-2 μg of isolated DNA was diluted in 20 μL of water and used for bisulfite treatment by Wizard DNA Clean-Up System (Promega) kit which converts unmethylated cytosine to uracil and leaves methylated cytosine unaltered. According to the manufacturer’s instructions of Promega, the treated DNA should be diluted in 20 μL of water and kept at −20°C for using in the further experiments.

2.3. Methylation-Specific PCR (MSP). To carry out the MSP analysis, promoters of the genes were recognized through online data analysis (http://www.ensembl.org) and then the preferred sequences were used to design methylated and unmethylated primers by MethPrime online software. Our selection for the site of methylated and unmethylated was consistent with the related literature. AccuPower HotStart PCR Premix from Bioneer Company (Cat. Number: K-5050) was used for each PCR reaction. Each PCR reaction contained 1 μL of modified DNA and 0.5 μL of each primer which is dissolved in the lyophilized blue pellet of AccuPower HotStart PCR Premix reached a final volume of 20 μL with water. The MSP amplification was set as follows: 95°C for 5 min, followed by 40 cycles (95°C for 40 s, the annealing temperature for LATS1: M = 53, U = 57; LATS2: M = 55.5, U = 55 for 40 s and extension at 72°C for 40 s). Final incubation was completed at 72°C for 10 min. The designed primers were listed in Table 1. PCR products were detected by electrophoresis in 2% agarose gel, 80–100 volts for an hour until being well separated (Figure 2).

2.3.1. RNA Extraction and Modification. Total RNA was extracted from pterygium and control tissues using the RNX-Plus solution (Cat. Number: MR7713C). A Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Cat. Number: K1621) was used to reverse-transcribe 1 mg of RNA to cDNA in a final volume of 20 μL.

2.3.2. mRNA Quantification by Real-Time PCR. Real-time PCR was performed using SYBR green in ABI 5700 sequence detection system (Applied Biosystems). We compared the mRNA expression in pterygium tissues related to normal tissues. 18S-rRNA was used as an internal standard. PCR efficiencies (E) were calculated for all used primers from the given slopes of standard curves, generated from serial dilutions of positive controls, according to the following equation: $E = 2^{(−\Delta\Delta CT)}$ [15]. The designed primers for expression analysis are shown in Table 2.
both genes (and controls revealed a statistically significant difference in LATS1 between groups. The significance level was set at P ≤ 0.05. The Mann-Whitney test was employed for statistical analyses. The Stata SE (version 13.1) was employed for statistical analyses. The methods including exact, Cornfield, and Woolf. The Stata in estimating OR, we calculated confidence intervals by three intervals (95% CI), using Logistic Regression. Avoiding bias detected by estimating odds ratios (OR) and 95% confidence intervals (95% CI), using Logistic Regression.

4. Discussion

Pterygium is a benign lesion that extends from conjunctiva to the cornea where it may interfere with vision. Since the current treatment of pterygium is invasive, mainly based on surgery, studies for new markers should be conducted. The current knowledge regarding the molecular basis of pterygium needs to be widened. Presently pterygium is considered as a UV light exposure-related uncontrolled cell proliferation [16]. Environmental factors such as UV radiation play an important role in tumor promotion through the epigenetic dysregulation of the cell cycle genes [17].

Table 2: Expression primer sequences and annealing temperatures.

| Gene  | Forward Primer | Reverse Primer | Annealing Temp |
|-------|----------------|----------------|----------------|
| LATS1 | F: GTTAAGGGAGGAGCCAGGTTCTT | R: TCAAGGAGATCCTCCCAAGGACTGT | 60°C 132 bp |
| LATS2 | F: ACTTTTTCTGGCCAGCATTTATTC | R: GATGGCTGTTTTAACCCTCA | 60°C 77 bp |
| 18S RNA | F: GTAAACCGTTGAACCCCATTT | R: CCATCCCAAACGGTAGTACGC | 60°C 112 bp |

LATS1 and LATS2 as a part of DDR signaling pathways are putative serine/threonine kinase proteins that localize to the mitotic apparatus and constitute a complex with cell cycle control system [9, 18]. LATS1 acts as a negative regulator of CDC2/cyclin A, which reduces H1 histone kinase activity of CDC2 and results in a G2-M cell-cycle arrest [19]. Also, LATS1 is activated by RASSF1A (Ras association domain family 1 isoform A) that stimulates response to DNA damage [20]. The activation of LATS1 promotes genomic stability via stabilizing replication forks by restricting Cdk2-mediated phosphorylation of BRCA2. This modulation not only has a fundamental role in error-free DNA repair but also maintains nucleofilamentation at stalled replication forks [21]. LATS2 localizes to centrosomes during interphase, both early and late metaphase. It interacts with the centrosomal proteins aurora-A and ajuva and also is required for the accumulation of gamma-tubulin and spindle formation at the onset of mitosis [18]. It also interacts with a negative regulator of p53 and may function in a positive feedback loop with p53 that responds to the cytoskeleton damage. The Lats2-Mdm2-p53 axis thus constitutes an innovative checkpoint pathway critical for the maintenance of proper chromosome number [22]. Both LATS1 and LATS2 as tumor suppressors are part of hippo signaling pathway which has profound effects on normal cell fate and tumorigenesis [23]. Therefore, silencing of LATS1 and LATS2 putative tumor suppressor genes through promoter methylation may cause the development of pterygium. The methylation of LATS1/LATS2 has been demonstrated in Japanese lung cancer patients [24]. Decreased expression of LATS1 in colorectal cancer was in association with the promoter methylation [25]. In addition, promoter hypermethylation mediates decreased expression of LATS1 and LATS2 in human astrocytoma [13]. Downregulation of LATS1 and LATS2 mRNA expression by promoter hypermethylation has been reported in breast cancer [5]. Consistent with the abovementioned studies, our results confirmed the significant relationship between reduced expression of the LATS1 and LATS2 through methylation and the risk of pterygium formation. Besides our data, the literature reviews showed the aberrant DNA methylation and decreased expression of P16, E Cadherin, TGM 2, MMP2, and CD24 genes in pterygium [26–28]. Exploring pterygium methylation markers and their subsequent effects on mRNA expression paves the road for better therapy such as discovering drugs with the regulation of methylation characteristic. Further studies are required to identify the exact molecular function of LATS1/LATS2 genes in pterygium in various and larger genetic populations using advanced molecular techniques.
Table 3: Risk of pterygium formation based on gene promoter methylation.

| Gene  | Methylation status | Pterygium tissues \( n = 70 \) | Normal tissues \( n = 70 \) | OR   | 95% CI      | \( P \) |
|-------|--------------------|-----------------------------|-------------------------|-------|------------|-------|
|       |                    | \( n (%) \)                | \( n (%) \)            |       |            |       |
|       |                    | Exact method               | Cornfield method        | Woolf method |
| LATS1 | U (ref)            | 4 (5.71)                   | 16 (22.86)              | 4.9   | 1.44 to 21.06 | 0.003 |
|       | M                  | 66 (94.28)                 | 54 (77.14)              |       |            |       |
| LATS2 | U (ref)            | 2 (2.85)                   | 12 (17.14)              | 7.1   | 1.47 to 67.42 | 0.004 |
|       | M                  | 69 (98.57)                 | 58 (82.86)              |       |            |       |

Table 4: Comparison of relative gene expression for LATS1 and LATS2 genes between patients with pterygium and healthy controls.

| Genes | Number | Mean ± SD | \( P \) value | \( P \) value |
|-------|--------|-----------|---------------|---------------|
| LATS1 | Cases  | 14        | 0.42 ± 0.030  | <0.001        |
|       | Controls | 14      | 0.57 ± 0.068  |               |
| LATS2 | Cases  | 14        | 0.44 ± 0.028  | <0.001        |
|       | Controls | 14      | 0.57 ± 0.061  |               |

\( ^{a} \) Binary logistic regression analysis.
\( U \): unmethyl, ref: reference, and M: methyl.
\( OR \): odds ratio; 95\% CI: 95\% confidence interval.
\( NC \): not calculated.

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.

Conflict of Interests
The authors declare that they have no conflict of interests.

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