miR-15a: A Potential Diagnostic Biomarker and a Candidate for Non-operative Therapeutic Modality for Age-related Cataract

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Abstract

The aim of this study was to estimate and investigate the expression levels of hsa-miR-15a with the target anti-apoptotic genes, BCL-2 and MCL-1, in lens epithelial apoptotic cells amongst age-related cataract patients. This in turn will help us better understand the role of hsa-miR-15a in the pathogenesis of this disease.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was applied to quantify the expression levels of hsa-miR-15a and the target genes BCL-2 and MCL-1 in lens epithelial cells of 120 age-related cataract patients compared to 40 normal patients; including 40 patients with cortical cataracts, 40 patients with nuclear cataracts and 40 patients with posterior subcapsular cataracts. Moreover, 60 specimens (15 normal and 45 cataracts) were stained immunohistochemically with BCL-2 and MCL-1 markers.

The expression level of hsa-miR-15a was significantly increased ($p < 0.001$) in lens epithelial cells of cataract patients compared to the control group. Regarding BCL-2 & MCL-1genes, their expression levels were significantly decreased in cataract patients. ($p < 0.001$). A significant increase in hsa-miR-15a expression in the cortical subtype compared to the posterior subcapsular subtype & a significant decrease in BCL-2 & MCL-1expressions in the cortical subtype compared to the nuclear & the posterior subcapsular subtype was detected.

The results of this study suggest that the increased expression of hsa-miR-15a in lens epithelial cells of cataract patients may repress the expression of the anti-apoptotic genes BCL-2 and MCL-1. Thus, the authors propose that the expression of hsa-miR-15a and the subsequent apoptosis of lens epithelial cells is part of the pathogenesis of age-related cataracts.

Keywords: Age-related Cataract; hsa-miR-15a; BCL-2, MCL-1; Apoptosis

Introduction

Cataract, one of the prevalent age-related eye diseases, continues to exist as the prominent cause of blindness worldwide [1]. The group of age-related cataract includes nuclear, cortical & posterior subcapsular cataract [2]. Nuclear cataract is the most common subtype of cataracts, and accounts for approximately 60% of age-related cataract cases, cortical cataract accounts for about 30% and the remaining 10% of age-related cataract cases are of the posterior subcapsular type [3]. It has already been established that varying levels of apoptosis are present in the epithelium of human cataract lenses [4].

MicroRNAs are small RNAs (20 to 25 nucleotides) which are non-coding but play an important role in the regulation of gene expression through a post transcriptional modification [5, 6]. miRNAs regulate degradation or translation of mRNA in binding to a complementary sequence in the 3′-untranslated regions (UTR) of mRNAs of the target gene [7]. Many previously published studies have revealed that abnormal miRNAs expression is strongly associated with the development of many age-related pathological conditions, including cataract [8, 9].

The miRNA hsa-miR-15a has been involved in the apoptosis of several cell types. It can negatively control the expression of anti-apoptotic genes like BCL-2 & MCL-1 which inhibits cell growth and arrests the cell cycle causing apoptosis [10]. This study aimed to compare the expression of hsa-miR-15a and its target anti-apoptotic genes BCL-2 and MCL-1 as apoptotic markers in age-related cataract patients compared to a group of control patients with no age-related cataract disease. This in turn will help us better understand the role of this microRNA in the pathogenesis of age-related cataract disease.

Patients and Methods

This research study was conducted at Benha University, Faculty of Medicine, Egypt as a collaborative intradepartmental project between the departments of Ophthalmology, Medical Biochemistry & Molecular Biology and Pathology between May 2016 and July 2017. This study was approved by Benha University Research Ethics Committee and the 1964 Helsinki Declaration including later amendments to the study design.

This study included 120 eyes of 120 patients with Age-related Cataracts; 40 patients with cortical cataracts, 40 patients with nuclear cataracts, and 40 patients with posterior subcapsular cataract. Forty-five specimens (15 from each patient group) were fixed in 10% formalin for immunostaining. Patients were excluded with previous ophthalmic injuries or surgeries, glaucoma, diabetes, intraocular tumors, intraocular inflammation, either infectious or autoimmune, or long-term eye exposure to radiation.

A control group of 40 age matched normal lens patients indicated for refractive lens exchange were used to compare with the age-related cataract disease group *(see Table 1).* Fifteen specimens were fixed in 10% formalin for immunostaining. All the patients in the study signed a detailed consent form in Arabic.

Pre-operative examination was done for all patients in the form of a visual acuity test, intra-ocular pressure, fundus examination and slit lamp to examine the anterior eye segment and determine the cataract subtype. The anterior capsule including anterior sub-capsular epithelial cells were obtained from all patients during cataract extraction by phacoemulsification. The control patients' anterior capsules were harvested during refractive lens exchange to treat high myopia.

Cataractous epithelial tags were taken out within minutes after cataract surgery, they were extracted and graded by K.M.M. Contaminating fiber cells were gently removed from both

normal & cataractous lens epithelia and the resulting tissues were washed to remove potential contaminates as described previously [11, 12]. All samples were immediately stored at -80°C after dissection for further molecular assays.

Total RNA extraction

Total RNA including miRNA was extracted from human lens epithelial cells using the RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol.

Molecular assays of the expression of hsa-miR-15a, BCL-2 and MCL-1 by real-time PCR

Two-step RT-PCR for hsa-miR-15a, BCL-2, and MCL-1 was performed using Qiagen miScript preAMP RT-PCR kit (Qiagen GmbH, Hilden, Germany) for conversion of microRNA to cDNA in a G-storm thermocycler (Gene technologies Ltd., Essex, UK). Then, amplification and quantification of RNAs was done by real time PCR in ABI7900 (Applied Biosystem, CA, USA) using SuperReal Premix Plus QuantiTect. Kit, SYBR Green (Tiagen, Shanghai) according to manufacturer's instructions and using the specific primers for each. Real time cycler conditions were 95°C for 15 min for initial denaturation, followed by 40 cycles of 95°C within 30 Secs for denaturation, 55°C for one min for annealing and 72°C for one min for extension step. The target sequences were hsa-miR-15a, BCL-2, and MCL-1 and the calibrator sample is normal lens. The reference gene (housekeeping gene) was RUN6B. PCR primer for miR-15a: **forward** 5′-GCGGCTAGCAGCACATAATGG-3′, **reverse** 5′-GTGCAGGGTCCGAGGT-3′ [13]. Primers for apoptotic genes: BCL-2 **forward**: 5′-GGAGGATTGTGGCCTTCTTT-3′; BCL-2 **reverse**: 5′-GGCCGTACAGTTCCACAAAT-3′ [14]. MCL-1 **forward**: 5′-TGGTGCCTTTGTGGCTAAA-3′; MCL-1 **reverse**: 5′-CCACCTTCTAGGTCCTCTACAT-3′ [15]. Primer sequence of RUN6B: **forward** 5'- CTC GCT TCG GCAGCACA -3', **reverse** 5'-AACGCTTCACGAATTTGCGT -3' [16].

The Threshold Cycle (CT) serves as a tool for the calculation of the starting template amount in each sample and gene fold expression changes are calculated using the equation $2^{-\Delta\Delta ct}$. Due to the relative nature of quantification using the $2^{-\Delta\Delta CT}$ method an adjustment is required for each sample. Briefly, cDNA was diluted 10-, 10^2-, 10^3-, 10^4-, 10^5-, and 10^6-fold prior to amplification by real-time PCR and a standard curve was derived in order to obtain optimal amplification conditions [17].

Histology and Immunostaining

Capsulotomy specimens (n=60, 45 cataractous specimen and 15 non cataractous) were processed for conventional light microscopy and immunohistochemistry. Specimens were processed and embedded in Paraffin blocks. Three slides of each block of 4 µmm thick sections were cut. The sections were dewaxed at 56˚C for 2 hours and then one slide processed for staining with haematoxylin- eosin. The other 2 slides were processed to detect BCL-2 and MCL-1 by immunohistochemical method. Envision tm FLEX/HRP (Envision Flex Target Retrieval solution High PH, Dako) method was used according to manufactures' instruction for immunostaining. The two sections from paraffin block were microwave treated to unmask epitopes. The monoclonal antibody BCL-2 (Thermo Scientific, USA) conc. in dilution 1:100 and polyclonal MCL-1 antibody (Thermo Scientific, USA) concentration in dilution 1:50 were used.

The number of immune-positive cells was counted in 4 representative fields, the total number of cells in 1 microscopic field was counted and the percentage of positively stained cells was detected (positive cells/total cells ×100%). The average of the 4 fields was calculated for both BCL-2 and MCL-1 and then analyzed for scoring. The scoring system was based on a scale of 0 to 4+ as follows: 4+: very high (75% to 100% positive cells); 3+: high (50% to 75% positive cells); 2+: moderate (25% to 50% positive cells); 1+: low (<25% positive cells) and 0: negative (0 positive cells) [18].

Statistical Analysis

These data were tabulated, coded then analyzed using the computer program SPSS (Statistical package for social science) version 24; the results were expressed as median and interquartile range (IQR), numbers and percent. Normality was verified by the Kolmogorov-Smirnov test. In the statistical comparison of quantitative data between the different groups, the significance of difference was tested using Student's t-test to compare between mean of two groups of numerical (parametric) data, For non- parametric data, Mann-Whitney U- test was used, Kruskal-Wallis was Used to compare between more than two groups of non-parametric data. While in the statistical comparison of qualitative data between the different groups, chi square and fisher-exact test were used. Significance was accepted at $p < 0.05$.

Results

Expression Pattern of hsa-miR-15a:

Patients with cataracts showed a significant increase in hsa-miR-15a expression compared to the non-cataractous control group *(p<0.001) (see Table 1)*. The comparison between cataract subtypes showed a significant increase in hsa-miR-15a expression in the cortical subtype compared to the posterior subcapsular subtype *(p<0.05) (see Table 2)*.

Expression Pattern of BCL-2

Patients with cataracts showed a significant decrease in BCL-2 expression compared to the control group *(p<0.001) (see Table 1)*. The comparison between cataract subtypes showed a significant decrease in BCL-2 expression in the cortical subtype compared to the nuclear & the posterior subcapsular subtype *(p<0.05) (see Table 2)*.

Expression Pattern of MCL-1

Patients with cataracts showed a significant decrease in MCL-1 expression compared to the control group *(p<0.001) (see Table 1)*. The comparison between cataract subtypes showed a

significant decrease in MCL-1 expression in the cortical subtype compared to the nuclear & the posterior subcapsular subtype *(p<0.05) (see Table 2).*

Correlation between miRNA-15a and both BCL-2 and Mcl-1

There is an inverse correlation between hsa-miR-15a expression and the expression level of both BCL-2 genes (r=-0.82, p<0.001) and MCL-1 genes (r=-0.87, p<0.001). These results show irrespective of subtype of cataract the expression levels of genes BCL-2 and MCL-1 were significantly reduced.

Immunohistochemical results

Cytoplasmic staining in the lining epithelium of the lens capsule showed BCL-2 expression in 7/15 (46.6%) of cortical cataract patients, 9/15 (60 %) of nuclear cataract patients and 9/15 (60%) of posterior subcapsular cataract patients compared to 12/15 (80%) in the control group *(see Figure 1 and Table 3).*

Cytoplasmic staining in the lining epithelium of the lens capsule showed MCL-1 expression in 7/15 (46.6%) of cortical cataract patients, 8/15 (53.3%) of nuclear cataract patients and 7/15 (46.6%) of posterior subcapsular cataract patients compared to 13/15 (86.6%) of the control group *(see Figure 2 and Table 3).*

Discussion

Studying the molecular base of lens epithelial cell apoptosis may shed new light in understanding the mechanism of cataract development and progression. This in turn could provide new insights into a potential non-operative therapeutic modality for cataracts.

Abnormal miRNAs expression has been shown to be strongly associated with the development of many age-related pathological conditions [19–22] including cataract formation [23, 24]. Informations gained about miRNA has provided new opportunities in studying lens epithelial cell apoptosis, which appears to be a common cellular basis for non-congenital cataract development [25]. B cell lymphoma 2 (BCL-2) is a central player in the

gene program of eukaryotic cells favoring survival by inhibiting cell death [26] and known to have protective effects against varied age-related diseases [27, 28]. MCL-1 is a pro-survival member of the BCL-2 family that also plays a role in antagonizing apoptosis [15].

The dysregulation of BCL-2 protein family expression & function has been virtually implicated in all malignancies and a number of other pathologies [29]. Cimmino et al. showed that miR-15a & miR-16-1 expression were inversely correlated with BCL-2 expression in Chronic Lymphocytic Leukaemia and they both negatively regulated BCL-2 at a post-transcriptional repression level. They also demonstrated that BCL-2 repression by these miRNAs promoted apoptosis in a leukemic cell line model. Therefore, miR-15 & miR-16 have been considered as natural antisense BCL-2 interactors that could have a valuable therapeutic significance in BCL-2 overexpressing tumors [30].

MCL-1 is a member of the BCL-2 protein family and the MCL-1 transcript is also a target of miR-15a and miR-16-1. Calin et al. showed that MCL-1 expression is inhibited by these miRNAs [31]. BCL2 and MCL1 regulation by miR-15a has been widely investigated on malignancies and a number of pathological conditions other than age-related cataract. So, in this study, we aimed to demonstrate the expression levels of hsa-miR-15a in lens epithelial cells and investigate the associations of this miRNA with the expression of its target anti-apoptotic genes BCL-2 and MCL-1 and subsequently with lens epithelial cells apoptosis among Egyptian age-related cataract patients.

We found that the expression of hsa-miR-15a was significantly increased in lens epithelial cells of cataract patients compared to normal lens epithelial cells *($p<0.01$)*. These results are consistent with a trial by Yuanbin et al. who revealed that hsa-miR-15a-5p, hsa-miR-15a-3p, & hsa-miR-16-1-5p were expressed at lower levels in normal lens epithelial cells compared to patients with age-related cataract of all subtypes (cortical cataracts, nuclear cataracts and posterior subcapsular cataracts) [15]. As regards BCL-2 & MCL-1, their mRNA expression

levels were significantly decreased in lens epithelial cells in age-related cataract patients compared to control patients (*p* value <0.001). These findings were further supported by the results of immunohistochemistry (IHC) staining, which revealed that BCL2 and MCL1 proteins were highly expressed in lens epithelial cells of the non-cataractous control group compared to patients with all subtypes of cataract. This corroborates the study of LIU et al who demonstrated reduced protein levels of BCL2 and MCL1 in the miR-15a-3p mimic transfected HLE-B3 human lens epithelial cell line [32]. Regarding the comparison between cataract subtypes, significant differences in the expression of hsa-miR-15a, BCL2 and MCL1 were observed as there was a significant increase in hsa-miR-15a expression in the cortical subtype compared to the posterior subcapsular subtype and a significant decrease in BCL-2 & MCL-1 expressions in the cortical subtype compared to the nuclear & the posterior subcapsular subtype. We think these differences between cataract subtypes could be related to differences in the disease severity as reported by Chien et.al who demonstrated positive correlation between high miR-34a levels and high lense opacity severity in nuclear, cortical or posterior subcapsular cataracts [33]. Further studies are needed to evaluate the correlation between the severity of lens opacity and hsa-miR-15a, BCL-2 & MCL-1 expression levels in the lens epithelium of age-related cataracts.

This study demonstrated an inverse correlation between hsa-miR-15a expression and the expression levels of BCL2 and MCL1 genes as their expression levels were significantly decreased in cataract patients, irrespective of subtype *(p<0.001)*. This agrees with findings previously shown by Cimmino et al. [30], Li et al. [15] and Willimott & Wagner [34] who reported that decreased expression of miR-15a and miR-16-1 were associated with increased BCL-2 expression and resistance to apoptosis.

In conclusion, this study identifies the impact of hsa-miR-15a and its target genes, BCL2 and MCL1, on age-related cataract patients. These findings may give a share in understanding the

molecular mechanisms implicated in the pathogenesis of cataract development and progression that could provide new insights into a potential non-operative therapeutic modality for cataract. miRNA is a good candidate for therapeutic applications through antisense inhibition or replacement that could significantly affect disease progression.

What is known about this subject:

• Apoptosis is associated with human cataractogenesis.

• Both BCL-2 & MCL-1 play a role in the genetic program of eukaryotic cells antagonizing apoptosis

• hsa-miR-15a has been implicated in the apoptosis of several cell types & may target BCL-2 & MCL-1 at the posttranscriptional level.

What this paper adds:

• This study identifies the impact of hsa-miR-15a and its target genes, BCL-2 and MCL-1, on age-related cataract patients.

• BCL-2 and MCL-1 expressions were significantly decreased in lens epithelial cells in age-related cataract patients compared to control patients

• hsa-miR-15a expression was significantly increased in lens epithelial cells of patients with all subtypes of cataract.

This work represents an advance in biomedical science because it participates in understanding the molecular mechanisms involved in the pathogenesis of cataract development and progression.

Compliance with Ethical Standards

Declaration of interest: All authors state that they have no involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript.

This study was approved by the Benha University Research Ethics Committee and with the 1964 Helsinki Declaration and its later amendments. All patients in the study subjects signed a detailed written consent form in Arabic.

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Table 1 Comparison between cases and control groups regarding age and sex, fold change and median (IQR) levels of the studied parameters expressed in FRU.

		All cataract patients (n=120)	**Control group (n=40)**	***p-value***
Age (years) Mean ± SD		58.8 ± 2.9	57.9 ± 1.2	0.06
Sex	**Male** No. (%)	63 (52.5%)	21 (52.5%)	0.9
	Female No. (%)	57 (47.5%)	19 (47.5%)	
miRNA-15a (FRU)	**Fold**	2.56	1.00	0.003
	Median (IQR)	4.6 (1.2-8.1)	2.0 **(1.2-2.8)**	
Bcl-2 mRNA (FRU)	**Fold**	0.13	1.00	<0.001
	Median (IQR)	559.5 (341.6-623.8)	7549.5 **(2643.3-12455.7)**	
Mcl-1 mRNA (FRU)	**Fold**	0.07	1.00	<0.001
	Median (IQR)	1.8 (0.8-3.6)	21.7 **(15.5-27.9)**	

Table 2 Comparison between cataract subtypes regarding age, sex and median (IQR) levels of the studied parameters expressed in FRU.

		Cortical cataract (n=40)	**Nuclear cataract (n=40)**	**Posterior subcapsular cataract (n=40)**	***p-value***
Age (years) Mean ± SD		58.3 ± 2.5	58.6 ± 2.8	59.5 ± 3.3	0.2
Sex	**Male** No. (%)	20 (50%)	21 (52.5%)	22 (55%)	0.9
	Female No. (%)	20 (50%)	19 (47.5%)	18 (45%)	
miRNA-15a (FRU)	**Median (IQR)**	5.7**(1.1-7)** †	4.6 **(1.2-8.4)**	5 **(2.1-12.6)**	0.003
Bcl-2 mRNA (FRU)	**Median (IQR)**	442.2**(277.4-561.3)** *†	605**(341.6-868.4)**	607.0 **(330.9-883.1)**	0.002
Mcl-1 mRNA (FRU)	**Median (IQR)**	1.8**(0.2-3)** *†	2.9**(1.1-3.1)**	3.6 **(0.8-5.7)**	0.001

*** →Significant in comparison with nuclear cataract**
† →Significant in comparison with posterior subcapsular cataract

Table 3: Immunohistochemical staining for BCl-2 and MCL-1 in lens capsule of control and cataract groups

Positive expression	Score	Control No=15	Cataract patients Cortical No=15	Nuclear No=15	Posterior subcapsular No=15	P-value
BCL-2 N (%)	**0**	3 (20%)	8 (53.3%)	6 (40%)	6 (40%)	<0.001
	+1	0 (0%)	6 (40%)	7 (46.7%)	7 (46.7%)	
	+2	2 (13.3%)	1 (6.7%)	0 (0%)	1 (6.7%)	
	+3	8 (53.3%)	0 (0%)	2 (13.3%)	1 (6.7%)	
	+4	2 (13.3%)	0 (0%)	0 (0%)	0 (0%)	
MCL-1 N (%)	**0**	2 (13.3%)	8 (53.3%)	7 (46.7%)	8 (53.3%)	<0.001
	+1	1 (6.7%)	4 (26.7%)	5 (33.3%)	3 (20%)	
	+2	1 (6.7%)	3 (20%)	3 (20%)	4 (26.7%)	
	+3	6 (40%)	0 (0%)	0 (0%)	0 (0%)	
	+4	5 (33.3%)	0 (0%)	0 (0%)	0 (0%)	

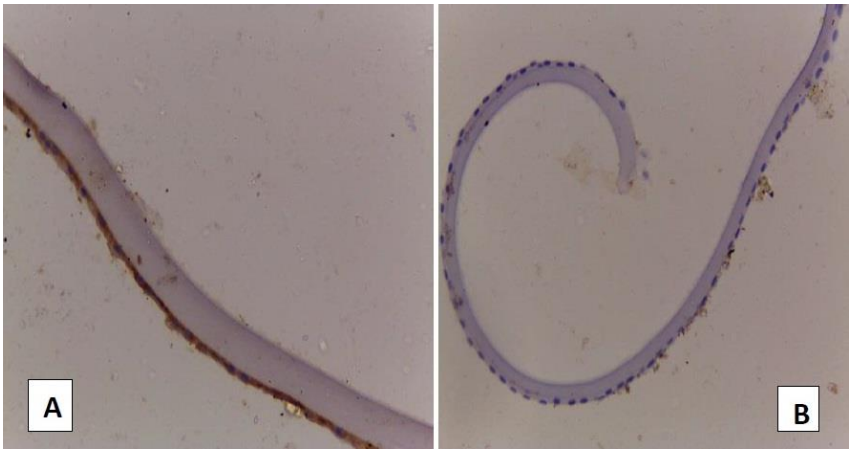

Figure 1: A: lens capsule of control group showing positive cytoplasmic immunostaining of BCL-2, B: lens capsule of cortical cataract patients showing negative immunostaining of BCL-2 (IHCX400).

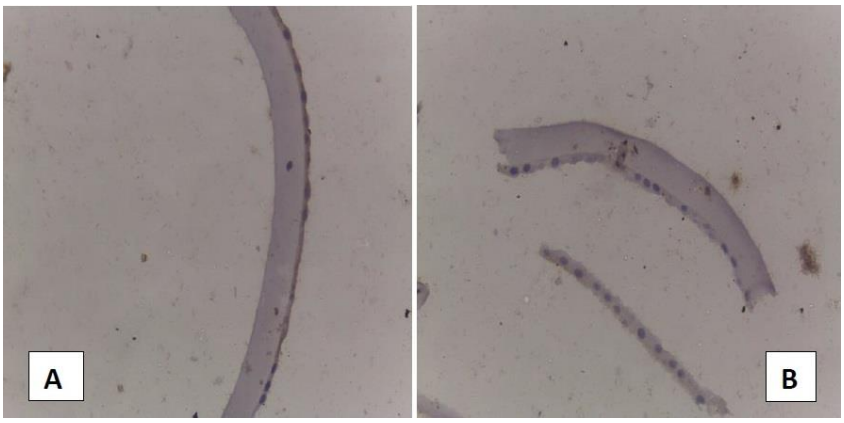

Figure 2: A: lens capsule of control group showing positive cytoplasmic immunostaining of MCL-1, **B:** lens capsule of cortical cataract patients showing negative immunostaining of MCL-1 (IHCX400).