Evaluation of the Possible Role of miRNAs in the Chemical Allergen Potency

Short title: The roles of miRNAs in the allergy

miRNA'ların Kimyasal Alerjen Potensindeki Muhtemel Rollerinin Değerlendirilmesi

Kısa Başlık: miRNA'ların Alerjideki Rolleri

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ABSTRACT

Objectives: MicroRNAs (miRNAs) are short, endogenous noncoding RNA molecules that can bind to parts of target mRNAs, thereby regulating gene expression. Few studies have shown that microRNAs can be up or down-regulated in allergic skin conditions but still, there is a need for further studies. The aim of this study was to investigate the expression of miRNAs in response to the common contact allergen Bandrowski's base (BB), the principal allergen in patients reacting to p-phenylenediamine (PPD).

Materials and Methods: The human promyelocytic cell line (THP-1) was exposed to BB at the concentration of 1 µg/ml for 24, 48 and 72 hours. The dose was selected from the results of cytotoxicity assays. RNA was purified, and miRNA expression profile and real-time polymerase chain reaction (RT-PCR) were performed to identify up or down-regulated miRNAs and after, to confirm their modulation.

Results: Among the different modulated miRNAs, the up-regulation of miRNA-155 and the down-regulation of miRNA-21 were found to be important because these are related to immune systems. This expression profile of miRNAs was also confirmed by RT-PCR.

Conclusion: These preliminary results showed that miR-155 and miR-21 may have a role in the pathogenesis of allergic contact dermatitis. But further studies are needed to clarify their roles.

Keywords: microRNA, miRNA, immune system, allergic contact dermatitis, skin

ÖZ

Amaç: MikroRNA'lar (miRNA) kısa, endojen ve kodlamayan RNA molekülleri olup hedef mRNA'lara bağlanarak gen ekspresyonunu düzenlerler. Az sayıdaki çalışmada, alerjik deri hasalıklarında miRNA'ların yüksek veya düşük ekpresyonları gösterilmiştir ancak daha ileri çalışmalara ihtiyaç vardır. Bu çalışmada, p-fenilendiamine reaksiyon gösteren hastalarda ana alerjen madde olan ve aynı zamanda yaygın bir kontakt alerjen olan Bandrowski's base (BB) maruziyeti sonrası miRNA'ların ekspresyon profillerinin incelenmesi amaçlanmaktadır.

Gereç ve Yöntemler: İnsan promiyelositik hücreleri (THP-1), 1 µg/ml dozunda BB'e 24, 48 ve 72 saat maruz bırakılmıştır. Bu doz sitotoksisite deneylerinin sonuçlarına göre seçilmiştir. RNA saflaştırılarak, miRNA ekspresyon profili ve gerçek zamanlı polimeraz zincir reaksiyonu (RT-PCR) ile yüksek veya düşük ekspresyona sahip miRNA'lar ortaya konulmuş ve sonrasında bu değişimler doğrulanmıştır.

Bulgular: Farklı ekspresyonlara sahip miRNA'lar arasında, immün sistem ile ilişkili oldukları için, miRNA-155'in yüksek ve miRNA-21'in ise düşük ekspresyona sahip olması önemli bulunmuştur. Ayrıca bu ekspresyon profili RT-PCR ile doğrulanmıştır.

Sonuç: Bu ön sonuçlar, miR-155 ve miR-21'in alerjik kontakt dermatit patogenezinde rolü olabileceğini göstermektedir. Ancak bu rollerin açıklanabilmesi için ileri çalışmalara ihtiyaç vardır.

Anahtar kelimeler: mikroRNA, miRNA, immün sistem, alerjik kontakt dermatit, deri

INTRODUCTION

Immunotoxic agents are xenobiotics which can initiate or exacerbate the adverse immune responses in genetically-susceptible persons. Drugs and various chemicals can be classified as immunotoxic compounds.[1] When low molecular weight chemicals contact with skin that can lead to contact allergy and can cause allergic contact dermatitis (ACD) if exposure exceeds the personal threshold. ACD is a more common form of immunotoxic reactions in industrialized countries.[2] There are more than 4000 chemicals are linked to contact allergy and ACD in humans. ACD can be prevented by proper hazard identification and labeling; by characterization of potency; by an investigating of human skin exposure; and by the implementation of proper risk assessment and management strategies.[3]

p-Phenylenediamine (PPD) is used commonly in the hair dyeing but it has a potential skin allergen. It is found that the mechanism of reaction to PPD is linked by its oxidation products and/or metabolites. Bandrowski's base (BB) (Figure 1), 1,4-benzoquinone, is a trimer which occurs quickly upon storage of PPD and it has been suggested as the principal allergen in patients reacting to PPD.[4]

*insert fig.1

THP-1 is a human leukemia promyelocytic cell line and it has been widely used in the immunotoxicology studies which investigate the monocyte/macrophage functions, its mechanisms, and signaling pathways. This cell line has become a proper model to estimate the modulation of monocyte and macrophage activities and very well suited to the *in vitro* studies of chemical allergens.[5-7]

miRNAs are a class of evolutionarily conserved, single-stranded, noncoding RNA molecules including 19–24 nucleotides, that play an important role in various of biological processes via regulating the gene expression through affecting the transcriptional and translation processes.[8] miRNAs have also been implicated in several inflammatory and immunological disorders as well as cancer.[9] It was demonstrated that miR-21 and miR-155 have a significant role in the development of

the immune system. miR-21 controls the apoptosis of immune cells and miR-155 is an important factor controlling lymphocyte differentiation and functions.[10,11] Analysis of microRNAs (miRNAs) has powerful potential for the identification of novel prognostic or predictive biomarkers. However several studies have evaluated the impact of miRNAs on the immunotoxic processes and allergic skin conditions, these studies are not enough to draw a conclusion.[12] Therefore the aim of the study was to evaluation of miRNA profiles that might have a role in the chemical allergen potency.

MATERIALS AND METHODS

Cell culture and treatment

THP-1 cell line was obtained from Istituto Zooprofilattico (Brescia, Italy). Cell culture media (RPMI-1640) from Euroclone Diagnostica SpA (Pero, Milano, Italy) and other substances were from Sigma-Aldrich Co. (St. Louis, Mo, USA). First of all, THP-1 cells were diluted to 10^6cells/ml in RPMI 1640 containing 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 0.1 mg/ml streptomycin, 100 IU/ml penicillin and 10% heated-inactivated fetal calf serum. Cells were cultured at 37 °C in 5% CO_2 incubator. The medium was changed every 2-3 days. Cells were treated with BB (Santa Cruz Tecnology Inc., Texas, USA; CAS N° 20048-27-5) at the concentration of 1 µg/ml in dimethyl sulfoxide (DMSO) for 24, 48 and 72 hours. The dose was selected from the results of cytotoxicity assays. Cells were treated with only DMSO was used as a control (0.2% final concentration).

Total RNA extraction and complementary DNA (cDNA) synthesis

For total RNA extraction, after the treatment, cells were centrifuged for 5 min at 1200 rpm at room temperature. Then, culture media was discarded and cell pellets lysed with 700 µl of Tri-reagent (Sigma-Aldrich, St. Louis, Mo, USA). After, ‘’Quiagen miRNeasy mini kit’’ was used to extract total RNA from the cells following the manufacturer’s procedure. Purity and quantity of the total RNA were analyzed with a Nanodrop instrument (NanoVue Plus). After determining the RNA concentrations, cDNA was synthesized by using ‘’Qiagen script miRNA PCR array kit’’ according to manufacturer’s protocol. 0.25 µg and 2.0 µg of total RNA were retrotranscribed for miRNA expression profile and real-time PCR analysis, respectively.

miRNA expression profiling

After retrotranscription, miRNA expression profiling (including 86 miRNAs) was determined by the ‘’Qiagen miScript miRNA PCR Arrays’’ following the manufacturer’s procedure with ABI Prism® 7000 Sequence Detection System. The miRNA expression profiling was done in cells treated with BB (1 µg/ml) or DMSO as the control for 24 h. The amplification conditions consisted of an initial activation at 95 °C for 15 min, then 40 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and the extension step for 30 s at 70 °C. Data were analyzed with miScript miRNA PCR Arrays (http://pcrdataanalysis.sabiosciences.com/mirna).

Real-time PCR for detection of mature miRNA or noncoding RNA

For the confirmation of miRNA expression profile, miR-155, and miR-21 were selected as immune system associated miRNAs, and their expression evaluated by Real Time-PCR using an ABI Prism® 7000 Sequence Detection System with the same amplification conditions with miRNA expression profiling using the miScript SYBR Green PCR Kit (provided by Qiagen). Small nucleolar RNA 61 (SNORD61), SNORD68, SNORD72, SNORD95, SNORD96A, and RNA, U6 small nuclear 6, pseudogene (RNU 6P) were used as endogenous miRNA controls in every reaction. All PCR reactions were performed in double in a total of 25 µl reaction volume. In Real-time PCR assay, the evaluation was done by the calculation of the fluorescent signal. The Ct (cycle threshold) was the required number of cycles for the fluorescent signal overshoot the threshold which means exceed the background level. Ct levels are conversely proportional to the quantity of target nucleic acid in the sample. When normalized gene expression in the test sample divided to normalized gene expression in the control, fold-Change ($2^{-\Delta\Delta CT}$) values are obtained which is bigger than one indicate a positive or an up-regulation and less than one indicate a negative or down-regulation.[13]

Statistical analysis

Data were analyzed by SPSS software, version 23.0 (SPSS, Chicago, IL, USA). Normal distribution and homogeneity of the variances were evaluated by Shapiro–Wilk and Levene's tests, respectively. Student's t-test was used for the comparisons. p values less than 0.05 were considered statistically significant.

RESULTS

miRNA expression profiling

miRNA expression profile was evaluated in THP-1 cells treated for 24 h with BB (1 µg/ml) or DMSO as control. As shown in Figure 2 and 3, the expression of 32 miRNAs was up-regulated and the expression of 31 miRNAs was down-regulated in the cells after the treatment with BB.

*insert Figure 2 and 3

Real-time PCR for the detection of miRNAs

Following bibliographic research, we focused on miR-21 and miR-155 because these are believed to be involved in immune responses. THP-1 cells were exposed to BB (1 µg/ml) or DMSO as a control for 24, 48 and 72 h. After the miRNA extraction, miR-21 and miR-155 levels were evaluated by Real Time-PCR. As shown in Figure 4 and 5, the expression of miR-21 was down-regulated and the expression of miR-155 was up-regulated, confirming the miRNA expression profile data.

*insert Figure 4 and 5

DISCUSSION

ACD is one of the significant diseases which occurred after the topical exposure to low molecular weight chemicals.[14, 15] It is one of the delayed-type hypersensitivity reaction which needs previous sensitization by the same chemical.[16] It is important and crucial to the identification of potential sensitizing agents because ACD is a common and serious health problem worldwide.[17]

It has been shown that miRNAs are involved in the processes of innate and adaptive immune systems. Among the whole miRNAs, miR-21, miR-146a, and miR-155 are focused by the scientists. Signal Transducer and Activator of Transcription-3 (STAT3) and nuclear factor kappa-B (NF-κB) regulated the expression of miR-21.[18] miR-155 involved in the especially pro-inflammatory processes. It has been observed that the expression of miR-21 is increased by vesicular stomatitis virus infection in macrophages.[19] Also, miR-155 can suppress the SH2 domain-containing inositol-5-phosphatase (SHIP1) that can lead to activation of Akt kinase and up-regulation of interferon response genes during the cellular response to lipopolysaccharide.[20] In an animal study, it has been demonstrated that miR-155 stimulate atherosclerosis in

mice via directly suppress B-cell lymphoma 6 protein (BCL6) which is a transcription factor that attenuates NF-κB signaling.[21] miR-155 has also involved in the development and activation of adaptive immune cells such as effector T cell subsets.[22]

In this study, we showed that there were an up-regulation and down-regulation, in the expressions of miR-155 and miR-21 in THP-1 cells after exposure to BB, respectively. The up and down-regulation of the miRNAs were validated by RT-PCR. Similarly, in a study by Sonkoly et al[23], the expression of miR-155 was found to be highest in the skin samples from patients with atopic dermatitis compared to healthy controls. Also, it has been observed that after the topical exposure of relevant allergens to non-lesional skin of atopic dermatitis patients, miR-155 expression was induced. Cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4), a negative regulator of T-cell function has been repressed by miR-155 and in animal models blocking of CTLA-4 stimulated a much severe allergic reaction and inflammation by increasing the number of eosinophils and immunoglobulin-E, while increasing in the expression of CTLA-4 ameliorated the symptoms of allergic pulmonary inflammation in humans.[24, 25] Besides, in patients with atopic dermatitis miR-155 has significantly higher expression compared to healthy subjects and the levels of expression correlated with the severity of atopic dermatitis.[26] In contrast to these studies, miR-155 levels were lower in sputum from allergic asthmatics than in the healthy subjects.[27]

miR-21 levels were evaluated in a few studies. In line with the results obtained from this study, it was shown that miR-21 was lower in monocytes from children with allergic rhinitis and in patients with metabolic syndrome[28,29]. On the contrary, in an animal study, miR-21 levels were higher in mice skin with contact dermatitis and in esophageal tissue of Eosinophilic esophagitis of mice, which can be reflected interspecies differences between mice and humans in the miRNA expressions.[30,31]

In addition to involvement in various biological processes, miRNAs have potential in disease diagnostics and therapies. Due to their stability, miRNAs could be used as biomarkers. Currently, miRNA panel has been used by clinicians in order to determine the origins of cancer cells. The development of miRNA therapeutics has proved more challenging because of the delivery problems. Besides, relations between gene expression and miRNAs are complex. As a result, administration or

silencing of one miRNA could modify the expression of numerous genes with unknown consequences. In multifactorial diseases, successful silencing of a single gene may not be efficient in clinical practice. Therefore, there is only one miRNA drug in clinical trials (SPC3649: inhibitor/antagomir of miR-122, Santaris Pharma, Denmark) and several more await entering clinical phases to date.[32] From this view, our study has some limitations. We evaluated only gene expression profile but protein products made from genes and other immune systems biomarkers such as interleukins and interferons should be analyzed. These are our aims in future projects.

CONCLUSIONS

In conclusion, our results showed that the miR-155 expression was up-regulated and miR-21 expression was down-regulated in THP-1 cells treated with the BB. It has been demonstrated that miRNAs are involved in many biologic processes and they are important in the pathogenesis of allergic inflammation. But relations between gene expression and miRNAs are complex so further investigation of these preliminary results is needed to explain the precise functional roles of these two miRNAs in the regulation of chemical allergen potency and also it's potential to serve as novel therapeutic targets.

Conflict of interest: The authors declare that they have no conflicts of interest.

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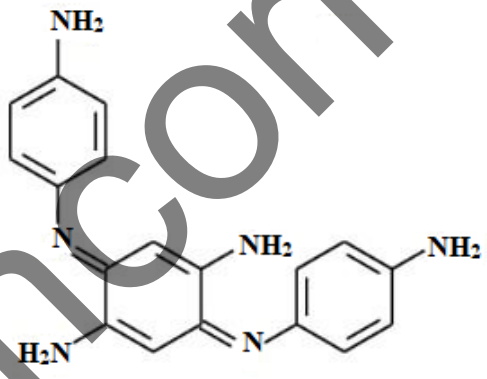

Figure 1. Chemical structure of Bandrowski's base

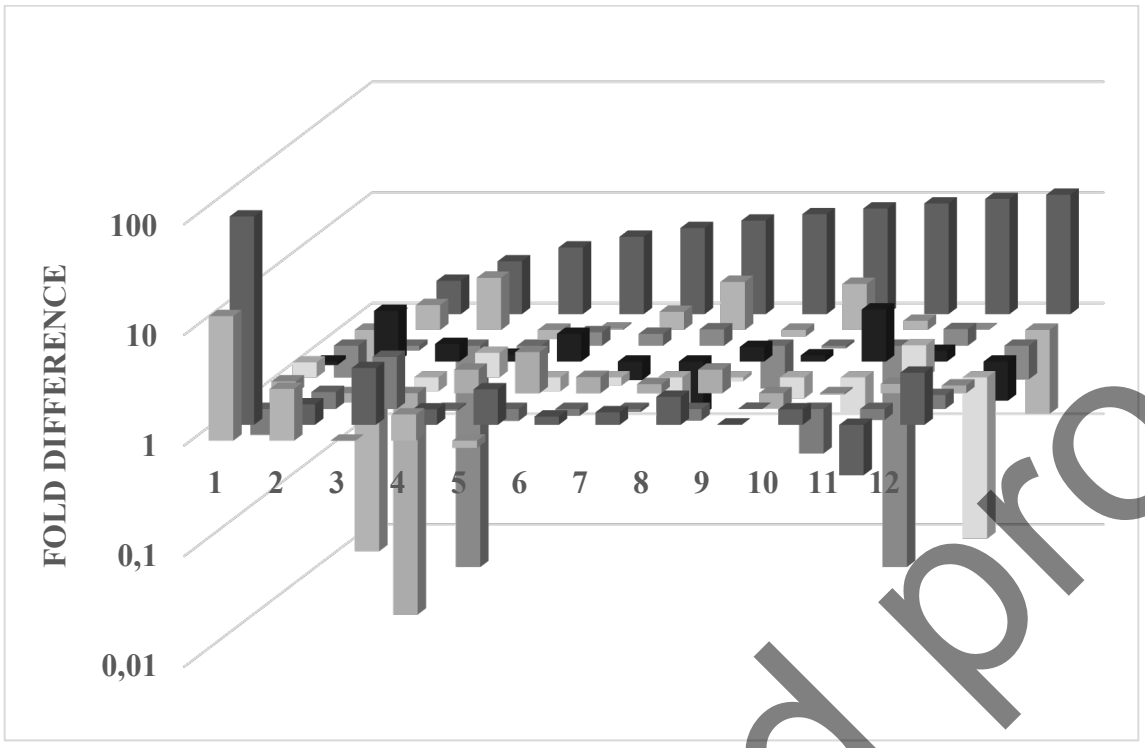

Figure 2. 3D-Profile of miRNA expressions

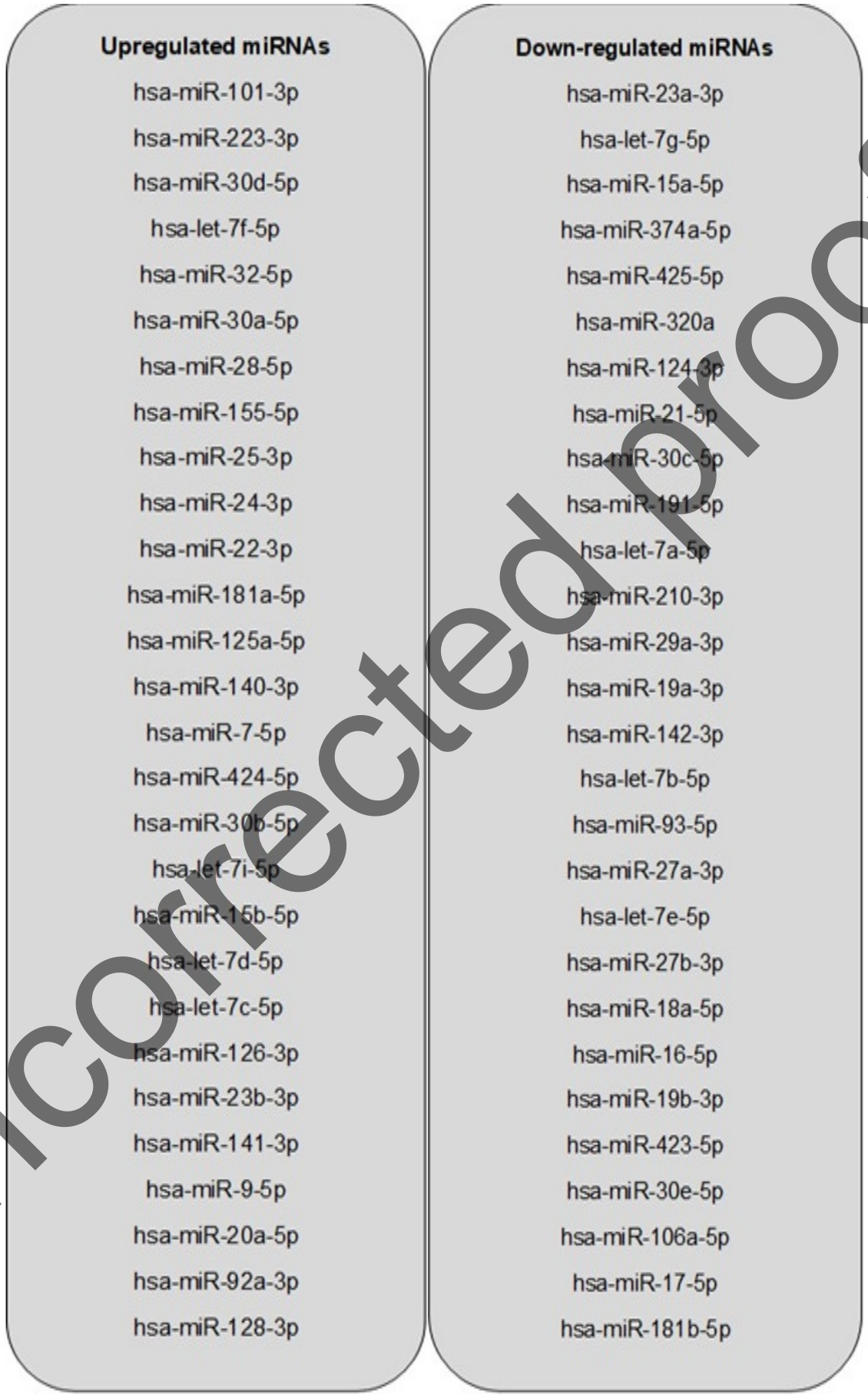

Figure 3. Upregulated and downregulated miRNAs

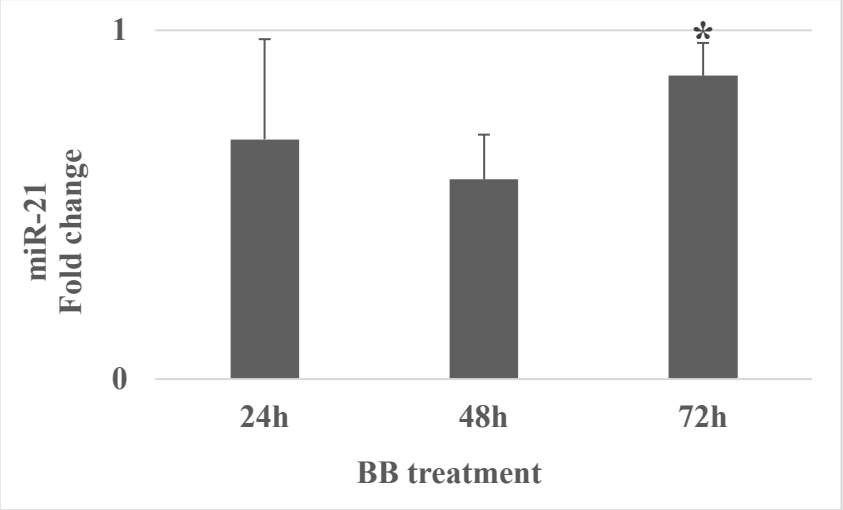

Figure 4. Expression of miR-21 in THP-1 cells following exposure to BB (1 µg/ml) or DMSO for 24, 48 and 72 h. Fold changes in miR-21 were calculated for each sample for each time point and expressed as mean±standard deviation, *p<0.05 vs control at the different time points of three independent experiments.

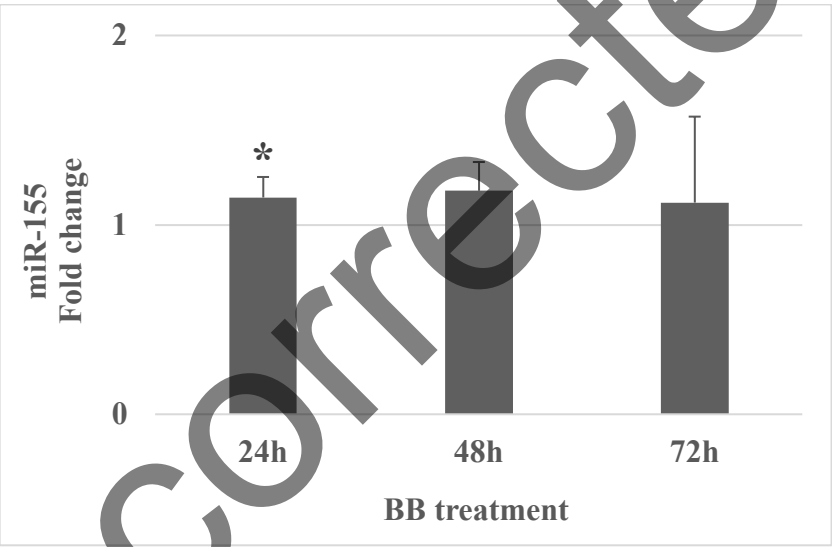

Figure 5. Expression of miR-155 in THP-1 cells following exposure to BB (1 µg/ml) or DMSO for 24, 48 and 72 h. Fold changes in miR-21 were calculated for each sample for each time point and expressed as mean±standard deviation, *p<0.05 vs control at the different time points of three independent experiments.