



MicroRNA screening and functional study of obliterative bronchiolitis in a rat model simulating lung transplantation

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ABSTRACT. The aim of this study was to provide the experimental basis for effective prevention and treatment of obliterative bronchiolitis (OB) by studying the changes on the microRNA (miRNA) expression profile after an orthotopic tracheal transplantation (OTT) simulating lung transplantation (LT). The OTT was performed on inbred rats to establish an OB animal model simulating LT, which was confirmed successful through pathological examination after 4 weeks. A miRNA microarray was used to screen for the most significantly differentially expressed miRNA in the OB tissues of donor transplanted trachea and real-time quantitative PCR was then used to validate the reliability of the microarray results. The microarray detection obtained 29 OB-related miRNAs, composed of 15 and 14 significantly up- and down-regulated miRNAs, respectively, among which miR-146a, miR-155, and miR-451, whose function is involved in the immune and inflammatory reactions, were subjected to relative quantitation research. The LT-simulated OTT-induced OB showed significantly differential expressions of multiple miRNAs, among which miR-146a and miR-155

were highly expressed, while miR-451 was lowly expressed, suggesting that these miRNAs may play an important regulatory role in the OB pathological process after LT.

Key words: Lung transplantation; Tracheal transplantation; Tiny RNA; Obliterative bronchiolitis; Gene expression profile

INTRODUCTION

Lung transplantation (LT) or heart-lung transplantation (HLT) is the most effective treatment for end-stage cardiopulmonary function failure or pulmonary disease. With the gradual development of surgical technologies, the early survival rate of the treated patients is improving, but the chronic rejection reaction of late-stage lung transplantation, in the form of obliterative bronchiolitis (OB), is still a major problem in clinical medicine (Trulock et al., 2007). Clinically, OB is the most important cause of chronic functional loss of the transplanted lung and affects the long-term survival of patients, with no effective treatments against it (Belperio et al., 2009; Todd and Palmer, 2011). The microRNA (miRNA) is a class of endogenous non-coding single-stranded small-molecule RNA, with about 19-24 nucleotides and evolutionary conserved, that can regulate the expression of specific genes through mRNA degradation or protein translation inhibition. Studies have shown that miRNAs have the potential of diagnosis and treatment assistance (Lanford et al., 2010) and that therapeutic strategies based on miRNA molecular mechanisms have great significance in preventing the post-transplantation complications and improving the post-transplantation long-term survival rate (Shan et al., 2011). Therefore, in this study, we established a rat OB model simulating a post-LT situation, and studied the changes in the miRNA expression profile of the graft, aiming to provide the experimental basis toward OB pathogenesis and the possible diagnosis and prevention strategies after LT or HLT.

MATERIAL AND METHODS

Animals

The Brown Norway (BN) inbred and Lewis male rats (SPF grade, 250 ± 50 g, Certificate No. SCXK (Hu) 2003-0002) were purchased from Shanghai SIPPR-BK Laboratory Animal Co., Ltd. The animal breeding environment was an SPF-grade barrier system (breeding facility: SYXK (Hu) 2004-0005) in the Experimental Animal Center, a medical translational platform of Shanghai East Hospital affiliated with Tongji University. All the experiments in this research study followed the National Laboratory Animal Regulations and the Implementing Rules of National Laboratory Animal Regulations.

Preparation of rat OB model

The establishment of our rat orthotopic tracheal transplantation (OTT) model was based on and improved from the relevant literature (Deuse et al., 2007; Mantovani et al., 2011). In the experimental group, the BN and Lewis rats were set as donor and recipient, respectively, while in the control group, both donor and recipient rats were Lewis rats. The donor rat (BN or Lewis) was intraperitoneally injected with chloral hydrate (350 mg/kg) for the anesthesia and then fixed on

the operating table in supine position. The SM-2000L ophthalmic surgical microscope (Shanghai Eder Medical Equipment Co., Ltd.) and Aesculap microsurgical system including surgical scissors, micro-ring forceps, micro-tweezers and needle holding forceps (Aesculap AG & Co. KG, Tuttlingen, Germany) were used to perform the cervical anterior median incision and expose the trachea. The tracheae with the maximum length (13 to 15 tracheal rings) were then extracted. After isolation and disinfection, the tracheae were washed with saline at 4°C and segmented into two pieces, with five tracheal rings in each piece, for the transplantation. The recipient rat (Lewis) was subjected to the same anesthesia, fixation, and cervical anterior median incision as the donor one, in order to isolate and expose the tracheal neck. Under the OPTON ophthalmic surgical microscope (Germany), the 3rd and 8th tracheal rings were sutured once and stretched with a 7-0 suture thread, with the 5th and 6th tracheal rings being then resected. The donor trachea and recipient trachea were then connected using an end-to-end continuous anastomosis of the tracheal membranacea and an intermittent anastomosis of the proximal tracheal cartilages, followed by the anastomosis of distal tracheal cartilages with a 7-0 Prolene suture thread to complete the airway reconstruction. After positioning the transplanted trachea, the skin was sutured. During the whole transplantation process, the vital signs of the rats were monitored with the MPA-2000 biological signal acquisition and analysis system (Shanghai Alcott Biotech Co., Ltd.), and the natural breathing was maintained, without the need of mechanical ventilation.

Grouping and specimen collection

The experiment was divided into the tracheal transplantation OB group and the tracheal transplantation control group (N = 6). Four weeks after the successful modeling, the recipient rats were sacrificed and each transplanted trachea was taken out and divided into 2 equal parts. One part of the trachea was meant for the histopathological examination: the transplanted trachea was fixed in 10% neutral formalin and subjected to the conventional paraffin embedding to make a 4- μ m slice, followed by the hematoxylin and eosin staining. The remaining part of the trachea was meant for the miRNA expression profile analysis: the transplanted trachea was stored in liquid nitrogen for the miRNA microarray screening and real-time quantitative PCR (RT-qPCR) validation.

miRNA microarray detection

The liquid nitrogen grinding method was used to grind a 100 mg graft (tracheal tissues) into powder and it was mixed with 1 mL TRIzol reagent for the total RNA extraction following the manufacturer protocol. The microarray mammalian V3.0 microarray detection (Bo'ao Biological Technology Co., Beijing) was performed, with three chips tested in each experimental group and each chip repeated once. The Significance Analysis of Microarrays (SAM, version 3.0) software was used to determine the differentially expressed miRNA. The Cluster 3.0 software (de Hoon et al., 2004) was used to perform the cluster analysis on the differentially expressed miRNA to screen the up-regulated and down-regulated miRNA. The ratio of sample standard value versus the control group standard value was used. While a ratio greater than 2 meant a significant up-regulation, a ratio less than 0.5 meant a significant down-regulation, with all other ratios meaning a non-significant differential expression.

miRNA validation by RT-qPCR

The RT-qPCR instrument (MJ Research Inc., USA) and mirVana miRNA Isolation Kit (Ambion, USA) were used to extract miRNA. The stem-loop RT primer, with the target specificity

structure, was used to perform the reverse transcription RT reaction (RT kits were purchased from ABI, USA). The PCR amplification conditions were: 95°C, 15 s; 60°C, 30 s; 40 cycles, with U6 as the internal reference. The melting curve was plotted from 75° to 95°C, the TaqMan® MicroRNA Assays (ABI) was used for the PCR amplification, and the non-denatured agarose gel electrophoresis was used to detect the results of the PCR amplification. The measured data were converted for the statistical processing and the relative expression of miRNA was calculated to validate the microarray results.

Statistical analysis

SPSS statistical package (version 17.0) was used for the analysis and processing of the data. The measurement data are reported as means \pm SD and the data were analyzed by the Student *t*-test, with a $P < 0.05$ considered as statistically significant.

RESULTS

General data

All experimental rats survived until the sampling. After the transplantation, the rats exhibited a little worse mental state, despite normal eating. After activities, the rats exhibited mild to moderate wheezing, but no signs of respiratory failure, suffocation, infection, or others.

Histopathological examination of transplanted tracheal tissues

This study compared the transplanted tracheae, which had the typical signs of medium-term OB formation (4 weeks after the transplantation), with the same-period grafts of the corresponding control group. The tracheal anastomotic stomas of both groups were good, with the neck muscles covering around, the cartilages exhibited no softening, and the morphology was intact. The histopathological hematoxylin and eosin staining of the transplanted trachea is shown in Figure 1. In the control group, the transplanted trachea wall presented columnar ciliated epithelial cells, and the endothelium was slightly thickened, but exhibited no significant morphological abnormality or mild inflammatory cell infiltration. In the experimental group, the transplanted tracheal epithelium presented abnormalities and/or the disappearing of mucous glands. The partial tracheal intima had the growth of columnar ciliated epithelial cells, with the inferior part of mucous membrane exhibiting periphery inflammatory cell infiltration, matrix deposition, and proliferation of fibrous tissues. Granulation tissues may form inside the lumina, which showed various degrees of OB, with an occurrence rate of 100%.

Detection of miRNA microarray

The miRNA microarray was used to compare and analyze the miRNA expression profile of transplanted trachea between the two groups. The total number of miRNAs detected was 699, among which 62 were found to be differentially expressed. The expression ratio (as defined in the Material and Methods section) values of 33 of those 62 (53.23%) miRNAs were between 0.5 and 2.0, i.e., exhibiting no significant differences. The remaining 29 miRNAs (46.77%) were significantly differentially expressed, among which 14 were significantly down-regulated, accounting for 48.28%, and 15 were significantly up-regulated, accounting for 51.72% (Table 1).

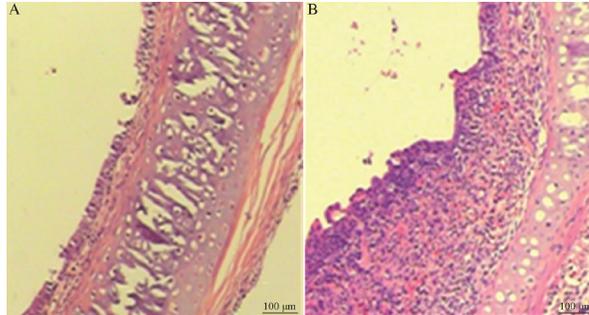


Figure 1. Pathological changes of the transplanted trachea (100X magnification). **A**, Control group; **B**, experimental group.

Table 1. Significantly differentially expressed miRNAs (chip signal intensity values).

miRNA	Control group	OB group	Ratio
miR-27a	495	1001	2.022
miR-99a	306	614	2.007
miR-146a	226	788	3.487
miR-146b	183	599	3.273
miR-151-5p	353	869	2.462
miR-155	410	1285	3.134
miR-378	648	1524	2.352
miR-690	300	637	2.124
miR-720	272	578	2.123
miR-762	1161	2476	2.133
miR-1937a	374	1008	2.695
miR-1937b	370	805	2.177
miR-2141	720	1576	2.189
miR-2142	14388	39410	2.739
miR-2146	253	788	3.113
miR-15	1923	907	0.472
miR-25	854	388	0.454
miR-451	6412	66	0.010
miR-486	2328	1136	0.488
miR-574-5p	5640	1022	0.181
miR-698	596	29	0.048
miR-705	2580	1091	0.423
miR-764-5p	391	27	0.068
miR-805	1582	706	0.446
miR-1187	5380	990	0.184
miR-1196	990	335	0.339
miR-1224	1480	318	0.215
miR-1894-3p	683	86	0.125
miR-1895	404	192	0.475

Validation of miRNA microarray by RT-qPCR

From the results of the miRNA microarray, the significantly up-regulated miR-146a and miR-155, and the significantly down-regulated miR-451 were selected and subjected to a relative quantification study, which confirmed the microarray results. The results showed that the expression levels of miR-146a and miR-155 genes in the OB group were, respectively, 3.581 ± 0.886 and 3.163 ± 0.529 times higher than the control group, suggesting that the expression levels were significantly increased ($P < 0.05$). The expression level of miR-451 gene in the OB group was 0.032 ± 0.012 times lower than the control group, suggesting that the expression level was significantly lower ($P < 0.05$).

The commonly used TargetScan database (Garcia et al., 2011) was used to predict target genes of the significant differentially expressed miRNA. The miR-146a target genes were IRAK1 and TRAF6, with the former being correlated with the IL-1-induced NF- κ B up-regulation, while the latter is involved in the inflammatory cell response. The miR-155 target gene was TSHZ3, which participates in the transcriptional regulation, while the miR-451 target gene was Tollip, which is related to the inflammation regulation.

DISCUSSION

LT or HLT is an important clinical means of treating certain end-stage heart and lung diseases. With the gradual maturation of surgical technologies and continuous introduction of new immunosuppressants, the early survival rate from these transplantations has been continuously improving. However, the long-term survival is still affected by chronic complications such as OB and others, with the 5-year survival rate at only 30 to 40% (Burton et al., 2007; Weber and Wilkes, 2013). Currently, the clinical treatment for post-LT OB is poor, thus the pathogenesis of OB and prevention strategies against it need to be addressed so that this problem can be solved in clinical medicine (Trulock et al., 2007). Post-LT OB is a complex process involving a variety of immune pathways and multiple factors. In recent years, it was found that, besides the rejection response, the inflammation and autoimmune responses play an important role in the development of OB (Mantovani et al., 2011). The first step to study post-LT OB was to choose the right ideal animal model. A previous study from Stanford University (Deuse et al., 2007) reported that the BN \rightarrow Lewis rat OTT could form pathological features similar to the human post-LT OB, thus it was considered to be an ideal experimental model to study the mechanism of post-LT OB. In this study, the BN inbred and Lewis rats, which had mature, reliable, and clear genetic background, were used for the tracheal transplantation, and in a relatively short time after the surgery (4 weeks), the post-LT OB animal model was successfully established (Lanford et al., 2010). The method was simpler than whole-lung transplantation, with low operative mortality, and this model exhibited the same pathophysiological changes as the post-LT OB (Deuse et al., 2007).

Post-LT OB is the repairing process of immune and/or non-immune factors causing chronic inflammation injury and abnormality on small bronchi, with most scholars believing that it is the primary cause of immune rejection response (Todd and Palmer, 2011). miRNA is an important class of biomolecules that play a key role in many important life processes, including immune and inflammation responses. A recent study found that, miRNA can act as an immune regulatory factor, involved in the regulation of innate immune response and inflammatory response (Luo et al., 2013). Furthermore, miRNA plays a huge action in the regulation of immune cell signaling transduction (Hashimi et al., 2009). Therefore, the detection of miRNA differential expression and its functional analysis are new angles for elucidating the molecular mechanisms of OB after the transplantation. The miRNA genome microarray is currently the most widely used method for screening abnormally expressed miRNA, but its false-positive rate is still a concern, and the results need further verification using the RT-qPCR method. This study detected changes in the miRNA expression profile of the post-LT OB animal model, and found that 29 miRNAs were significantly differential expressed, among which 15 were up-regulated and 14 were down-regulated. This indicates that miRNA may play an important regulatory role in the OB process after LT or HLT, and provides a theoretical basis for subsequent target gene determination and function analysis.

Searching, screening, and determining the target genes that miRNAs directly affect can

clarify their regulatory mechanisms, which are important for miRNA mechanism studies. miRNA is a small RNA expressed by the regulatory genes at the post-transcriptional level, and it can partner with transcription factors to perform the inflammation fine regulation of the co-acted target genes in the immune response and inflammation, thus playing a key role in the immune system (Contreras and Rao, 2012). It has been shown that miR-155 plays a very important role in the processes of cell differentiation, immune, infection, cardiovascular diseases, viral infections, and tumor occurrence (Famoni et al., 2009). miR-146a plays a role in the feedback regulation of the immune signaling transduction, and is closely related to the activities of various inflammatory cytokines and transcription factors, playing fine regulatory roles in the immune response (Taganov et al., 2007) and participating in the pathological process of chronic inflammation (Sonkoly et al., 2007). Additionally, miR-146a is highly expressed in the regulatory T cells, regulating the immune tolerance and mediating the damages of organs and tissues (Lu et al., 2010). Another study showed that, miR-155 and miR-146a could be highly expressed inside the macrophages, playing an important role in the blood vessel immune response (Urbich et al., 2008). Currently, the number of studies about miRNA-451 in the blood system has been increasing, revealing that it plays a key role in the maturation of red blood cells by regulating its target genes (GATA-1 and GATA-2), playing its consistent function together with miRNA-144 (Ashton-Chess et al., 2003). Furthermore, recent studies showed that miR-451 might change the expression levels of oncogene C-myc and positive regulatory factors of cellular cycle, thus slowing the progress of the cell cycle and reducing the rate of cell proliferation (Amendola et al., 2009). In this study, the miRNA microarray technology found that, in the post-LT OB animal model, miR-451 was significantly down-regulated, while miR-155 and miR-146a were significantly up-regulated. The target genes of miR-146a, IRAK1 and TRAF6, are correlated with the IL-1-induced NF- κ B up-regulation and involved in the inflammatory cell response, suggesting that the function of miR-146a is to negatively regulate the TLR signaling pathway in the innate immune responses, thus playing the role of controlling the inflammatory response (Guo et al., 2010). The target gene of miR-155, TSHZ3, is involved in the transcriptional regulation of the development, differentiation, and function of T cells and B cells, thus affecting the formation of germinal center and antibody class switching (Pauley et al., 2009). The target gene of miR-451, Tollip [codes for an ubiquitin-binding protein (Lo et al., 2009)], is the negative regulator of TLR signal (Hosiawa et al., 2005), and is closely related to the activation of IL-1R and IRAK-1. All these expression changes suggest that these three miRNAs may be related with the post-LT immune inflammatory reactions, and further research about the functions of these three miRNA may provide new ways for preventing and treating the occurrence of OB after LT or HLT.

Conflicts of interest

The authors declare no conflict of interest.

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