CHEST Online Supplement

MicroRNA-199a-5p Is Associated With Hypoxia-Inducible Factor-1α Expression in Lungs From Patients With COPD

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e-Appendix 1.

Detailed methods:

Chemicals

Chemicals and materials were obtained from the following sources: the RNA later - ice kit frozen tissue transition solution was from Ambion Inc. (Austin, TX); the high capacity cDNA Reverse Transcription kit was from Applied Biosystems Inc. (Foster City, CA); the ECL system (Western Lightening and Western Lightening plus-ECL) was from PerkinElmer (Waltham, MA); High-Capacity cDNA Archive Kit and the Power SYBR Green PCR master mix was from Applied Biosystems (Foster City, CA); NE-PER Nuclear and Cytoplasmic Extraction Reagents was from Thermo Scientific (Rockford, IL); 4–12% Bis-Tris Nupage gels, and MES-SDS running buffer were from Invitrogen (Carlsbad, CA, USA); the polyvinylidene difluoride (PVDF) membranes was from Bio-Rad Laboratories (Richmond, CA); the protease inhibitor cocktail tablets was from Roche Applied Science (Indianapolis, IN); miRNeasy Mini kit, miScript Reverse Transcription Kit and miScript SYBR Green dye were from Qiagen, (Valencia, CA); Mouse anti-HIF-1α antibody, mouse anti-p53 antibody, rabbit anti-phospho AKT antibody, mouse anti-AKT antibody, and mouse anti-Lamin A/C antibody were from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA); MK2206 and Perifosine were from (Selleck Chemicals, Houston, TX); All other chemicals were purchased from Sigma (St. Louis, MO). Real-time RT-PCR analysis of mRNA and microRNA (miRNA)

Real-time RT-PCR analysis of mRNA and miRNA

Fresh frozen lung tissue samples were immersed in RNA later - ice kit according to the manufacturer's instructions. After overnight soaking, the tissue samples were homogenized by FastPrep-24 (MP biomedicals,

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Solon, OH). Isolation of total RNA and miRNA from lung tissue and HPMVEC was performed using a miRNeasy Mini kit according to the manufacture's protocol. Total RNA (1 μg) was reverse-transcribed using random primer and MultiScribe RT (High-Capacity cDNA Archive Kit) for mRNA analysis and miScript Reverse Transcription Kit for miRNA analysis. PCR was performed with the resulting reverse transcription products using specific oligonucleotide primers. The sequence of the forward primer for HIF-1α was 5'-CCCATTTTCTACTCAGGACACAG -3' and the reverse primer 5'- CTGATCGAAGGAACGTAACTGG -3'. The sequence of the forward primer for VEGF was 5'- CTTGCCTTGCTGCTCTACCT -3' and the reverse primer 5'- GTGATGATTCTGCCCTCCTC -3'. The sequence of the forward primer for AKT1 was 5'-GCACAAACGAGGGGAGTACA -3' and the reverse primer 5'- CGCTCCTTGTAGCCAATGAA -3'. The sequence of the forward primer for ß-actin was 5'- GCAAGCAGGAGTATGACGAG -3' and the reverse primer 5'- CAAATAAAGCCATGCCAATC -3'. The sequence of the primer for miR-34a was 5'-GGCAGTGTCTTAGCTGGTTGT - 3', The sequence of the primer for miR-199a-5p was 5'-CCCAGTGTTCAGACTACCTGTTC - 3', and the sequence of the primer for miR-103 was 5'-CAGCATTGTACAGGGCTATGA -3'. All PCR reactions were performed with a LightCycler480TM PCR system (Roche Diagnostics, Meylan, France) using DNA binding SYBR Green dye for mRNA analysis and miScript SYBR Green dye for miRNA analysis for the detection of PCR products. The ß-actin gene was used as the reference of mRNA, and miR-103 was used as the reference of miRNA.

Western blot analysis

Cytoplasmic and nuclear proteins from lungs and cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents based on the manufacturer's protocol, and the protein extracts were analyzed for protein content using a Bradford method. Each sample was quantified, and then 40 μg of protein (cytoplasmic protein) or 20 μg of protein (nuclear protein) was loaded into each lane of a 4–12% Bis-Tris Nupage gel with MES SDS running buffer, according to the manufacturer's protocol. The gel was transferred to a PVDF membrane by electrophoresis, and the membrane was then probed with the primary and secondary antibodies. The ECL system was used for detection of the proteins. The Lamin A/C protein was used as the reference of nuclear protein, and ß-actin protein was used as the reference of cytosolic protein, and AKT protein was used as the reference of phospho-AKT protein.

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Transfection of small interfering RNA (siRNA), precursor miRNA and miRNA inhibitor into HPMVECs

AKT1, p53 and control siRNAs were designed and synthesized by Invitrogen. Ambion Pre-miR™ miRNA Precursors of control, miR-34a, miR-199a-5p miRNA (Ambion® Anti-miR™ miRNA Precursors) were purchased from Applied Biosystems. The AKT1, p53 and control RNA target sequences were 5'-GCACCUUCAUUGGCUACAATT-3', 5'-GCGCACAGAGGAAGAGAAU-3', 5'-CCUAGAACCUAAGACCCUU-3', respectively. HPMVEC were seeded into 6-cm dishes and incubated until the time they had reached about 60% confluence. After rinsing, the cells were incubated with liposome solution comprised of Opti-MEM medium, 10 µl/ml of Lipofectamine 2000 (Invitorogen), and 100 nM of siRNA or 200 nM of precursor miRNA with 10% FBS. After 8 hours of incubation, the same amount of Opti-MEM medium containing 10% FBS was added to the dishes and the incubation was continued for 16 hours. After 24 h of transfection, the liposome solutions were replaced and cultured for 24 hours. After incubation, RT-PCR, and Western blot analysis was performed.

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