Single nucleotide polymorphism rs3746444 in miR-499a affects susceptibility to non-small cell lung carcinoma by regulating the expression of CD200

NAN GE1*, CHUNXIA MAO2*, QINGBO YANG1, BIN HAN1, YONGJIE WANG1, LINHAO XU1, XIUZHI YANG3, WENJIE JIAO1 and CHUAN LI1

Departments of 1Thoracic Surgery, 2Hematology and 3Operation Room, The Affiliated Hospital of Qingdao University, Qingdao, Shandong 266003, P.R. China

Received February 6, 2018; Accepted March 4, 2019

DOI: 10.3892/ijmm.2019.4124

Correspondence to: Professor Wenjie Jiao or Professor Chuan Li, Department of Thoracic Surgery, The Affiliated Hospital of Qingdao University, 16 Jiangsu Road, Qingdao, Shandong 266003, P.R. China
E-mail: drlung@yeah.net
E-mail: lcxlh1218@sina.com

*Contributed equally

Key words: microRNA-499a, cluster of differentiation 200, non-small cell lung carcinoma, rs3746444

Abstract. The present study evaluated the association between single nucleotide polymorphism (SNP) rs3746444 and the risk of non-small cell lung carcinoma (NSCLC) in a Chinese population. Computational analyses and luciferase assays were performed to investigate the regulatory relationship between miR-499a and CD200. In addition, reverse transcription-quantitative polymerase chain reaction and western blot assays were performed to examine the effect of rs3746444 on the expression of miR-499a and CD200. The results demonstrated a significant difference in the smoking history of patients carrying malignant pulmonary nodules and those carrying benign pulmonary nodules. Furthermore, CD200 was demonstrated to be a direct target of miR-499a, and a miR-499a binding site was located in the 3'UTR of CD200. Notably, the levels of miR-499a in malignant pulmonary nodules were higher compared with benign pulmonary nodules, while the levels of CD200 were higher in benign pulmonary nodules compared with malignant pulmonary nodules. In addition, the subjects carrying the AA genotype of SNP rs3746444 exhibited upregulated miR-499a expression and reduced CD200 expression, compared with the subjects carrying AG and GG genotypes. These findings indicate that the SNP rs3746444 in miR-499a could affect the prognosis of NSCLC patients by regulating the expression of CD200.

Introduction

In total, >80% of lung carcinomas are non-small cell lung carcinoma (NSCLC), which includes large cell carcinoma, squamous cell carcinoma, adenocarcinoma, and unclassifiable NSCLC. The prognosis of patients with lung cancer is significantly affected by the cell type (for example, small cell vs. NSCLC), clinical staging, and the overall condition of the patients. Unfortunately, NSCLC is frequently diagnosed at a late stage, thus limiting the number of available treatment options. Since pulmonary nodules may be observed in specific NSCLC cases, the difference between malignant and benign pulmonary nodules has been used as a diagnostic criterion of NSCLC (1).

As a family member of immunoglobulin (Ig) proteins, cluster of differentiation 200 (CD200; also known as OX-2) carries two extracellular immunoglobulin domains and one small 19 amino acid intracellular domain with no known signaling motifs (2). CD200 is expressed in a wide range of human cancer types, including malignant B carcinoma, myeloid leukemia, ovarian cancer and human melanoma (3-6). A previous study has demonstrated that intravenous injection of CD200+ B16 cells into CD200R-deficient mice induced significant tumor growth in multiple organs, including kidney, lung, liver and the peritoneal cavity (7).

As small non-coding RNAs containing 19-25 nucleotides, microRNAs (miRNAs) are able to mediate the expression of numerous protein-coding genes in animals and plants. In addition, miRNAs have important roles in a number of cellular processes, including stem cell renewal, cell metabolism, proliferation, apoptosis and differentiation (8). Due to the important functions of miRNAs, their abnormal expression has been implicated in many human diseases, including obesity, cardiovascular diseases, cancer, psoriasis, schizophrenia, chronic hepatitis and diabetes (9-14). For example, a previous study (15) demonstrated that miR-499a-5p serves as a tumor suppressor to regulate the expression of vav guanine nucleotide exchange factor 3 (VAV3), and that low expression levels of miR-499a-5p are correlated with a poor prognosis in NSCLC. Experimental data has additionally demonstrated that overexpression of miR-499a-5p inhibits the proliferation and
metastasis of NSCLC cells by inducing their apoptosis (15). As a type of genetic variation discovered in precursor miRNAs (pre-miRNAs), single nucleotide polymorphisms (SNPs) may influence the expression of their host miRNAs. As a result, SNPs have been implicated in altered biological functions and gene expression. For example, the miR-499a>A>G (rs3746444) SNP is closely associated with the pathogenesis of diabetes, cardiovascular disease, hypertension and cancer (16).

It has been reported that the presence of SNP rs3746444 in miR-499a affects miR-499a expression (17). In the present study, by searching an online miRNA database, cd200 was identified as a possible target of miR-499a. Notably, CD200 has been reported to function as a tumor suppressor in the development of lung cancer (18,19). Therefore, the association between SNP rs3746444 and the risk of lung cancer was evaluated in a Chinese population. In addition, the effect of SNP rs3746444 on the expression of miR-499a and CD200 was investigated.

**Patients and methods**

**Sample collection.** All participants underwent imaging examinations to confirm the presence of pulmonary nodules. Peripheral blood and tissue samples were collected from the participants at the Affiliated Hospital of Qingdao University (Qingdao, China) between January 2017 and August 2017 to determine the malignance of their pulmonary nodules. In total, 476 patients with benign pulmonary nodules and 425 patients with malignant pulmonary nodules were enrolled into the study. The tissue samples were additionally examined by genotyping analyses, and 30 samples were determined as benign while 32 were determined as malignant.

The Human Research Ethics Committee of The Affiliated Hospital of Qingdao University approved the present study. Written informed consent was obtained from all patients or their first-degree relatives prior to the start of the research project. The research protocols additionally complied with the latest version of the Declaration of Helsinki.

**TaqMan assay.** A QuantStudio 12K Flex instrument (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to perform TaqMan assays and to identify the genotypes of SNP rs3746444 in all samples.

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** TRIZol® reagent (Thermo Fisher Scientific, Inc.) was used to extract total RNA from tissue samples and A549 and A427 cells. The extracted RNA samples were transcribed to cDNA using a ThermoScript RT-PCR system (Invitrogen; Thermo Fisher Scientific, Inc.) at 50˚C for 15 min. Subsequently, the expression of CD200 mRNA [CD200 mRNA-forward (F), 5'-cAG AGG cAT AGT GGT AAc 50˚C for 15 min. Subsequently, the expression of CD200 mRNA

**Cell culture and transfection.** Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; both Gibco; Thermo Fisher Scientific, Inc.), 100 mg/ml streptomycin and 100 U/ml penicillin was used to culture A549 and A427 cells. A427 cells, at 37˚C and 5% CO₂, A549 cells were cultured from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). A427 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The possibility of contamination was excluded using a mycoplasma stain assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. When the cell confluence reached 80%, the cells were transfected with miR-499a mimics (sequence, 5'-UUAGACUGUCUGAGUAG UUU-3'; 50 nM), miR-499a inhibitors (sequence, 5'-AAACAUC ACCUGCAAGCUUA-3'; 50 nM), CD200 short hairpin RNA 1 (shRNA1; sequence, 5'-CGTACGTTAGCGTGGTCTG GATA-3'; 50 nM) or CD200 shRNA2 (sequence, 5'-CGACGT ACCATCGTAACCA-3'; 50 nM; all Shanghai GenePharma Co., Ltd., Shanghai, China) for 24 h using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For the shRNA, an empty vector was used as the control, and for the anti-miR, a scramble sequence (5'-GCAGACATACCAAGAGCTGTG-3') was used as the control.

**Luciferase assay.** A fragment of the 3'-untranslated region (UTR) of CD200 mRNA containing the miR-499a binding site was amplified by PCR. At the same time, a site-directed mutagenesis kit was used to generate a mutation in the miR-499a binding site of CD200. The two different PCR products were respectively inserted into pGL3-Basic vectors (Promega Corporation, Madison, WI, USA) to generate luciferase reporter constructs for wild-type and mutant CD200. Subsequently, A549 and A427 cells were seeded into 48-well plates and cotransfected with wild-type or mutant CD200 luciferase reporter vectors and miR-499a mimics using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 24 h of transfection, a 96-well plate lumino meter (Berthold Detection System, Pforzheim, Germany) was used to analyze the luciferase activity (normalized to Renilla luciferase activity) of transfected cells with a Dual-Luciferase Reporter Assay System (Promega Corporation).

**Western blot analysis.** A urea buffer (containing 200 mM ammoniumbicarbonate, 6 M urea, and 2% SDS) containing a protease inhibitor cocktail (Complete mini) or a complete lysis-M reagent (both Roche Diagnostics GmbH, Mannheim, Germany) was used to extract proteins from A549 and A427 cells, according to the manufacturer's protocol. A bicinechonic acid protein assay (Pierce; Thermo Fisher Scientific, Inc.) was used to measure the protein concentration. The extracted proteins (35 µg/lane) were subsequently denatured
in hot water and resolved by 15% SDS-PAGE, followed by semi-dry transfer onto a nitrocellulose membrane (Trans-Blot; both Bio-Rad Laboratories, Inc.). TBS with Tween-20 containing 5% bovine serum albumin (Thermo Fisher Scientific, Inc.) or 5% non-fat dry milk was used to block the membrane for 60 min at room temperature. Subsequently, the membrane was incubated at 4°C for 12 h with anti-CD200 primary antibodies (cat. no. ab173498; 1:2,000; Abcam, Cambridge, UK), followed by another 2 h of incubation with horseradish peroxidase-conjugated anti-mouse secondary anti-
bodies (cat. no. 7076s; 1:10,000; Cell Signaling Technology, Inc., Danvers, MA, USA). The membrane was subsequently developed with an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and exposed to X-rays. ImageJ software (v1.8.0; National Institutes of Health, Bethesda, MD, USA) was used to perform protein densitometric analyses.

Statistical analysis. All data are presented as the mean ± standard deviation. The bioinformatics tool TargetScan (21) was used for the sequence analysis between CD200 mRNA and miR-499a. Student's t-test was used to evaluate the difference between two different groups, while Student-Newman-Keuls multiple comparison test and one-way analysis of variance with Bonferroni's test was used to examine the difference among multiple groups. Count data were compared using χ² tests. Logistic regression analysis was used to analyze the correlation between SNP rs3746444 and the risk of NSCLC, in addition to the correlation between miR-499a and CD200 expression. P<0.05 was considered to indicate a statistically significant difference. SPSS 15.0 statistical package (SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analysis. Each experiment was repeated three times.

Results

Patient characteristics. The demographic and clinicopatho-
logical characteristics of the 425 patients with malignant pulmonary nodules and 476 patients with benign pulmonary nodules are listed in Table I. No obvious difference was found between the two groups of subjects in terms of sex and age (P>0.05). However, a significant difference in smoking history was observed between the two groups. The 476 patients with benign pulmonary nodules were further divided into four groups according to the genotypes of their SNP rs3746444: AA (n=346), AG (n=118), and AG+GG (n=120). At the same time, the 425 patients with malignant pulmonary nodules were also divided into four groups: AA (n=32), AG (n=14), and AG+GG (n=12). RT-qPCR and western blot analyses were then performed to compare the expression of miR-499a and CD200 among the AA, AG and GG groups. As illustrated in Fig. 5A, the levels of miR-499a in malignant pulmonary nodule samples were significantly higher compared with benign pulmonary nodule samples. In addition, miR-499a expression in the AA group was significantly higher compared with the AG and GG groups, while miR-499a expression in the AG and GG groups was comparable (Fig. 5B). By contrast, the levels of CD200 mRNA (Fig. 6B) and protein (Fig. 7B) in the AA group were lower compared with the AG and GG groups, whereas, the expression levels of CD200 mRNA (Fig. 6B) and protein (Fig. 7B) in the AG and GG groups were comparable.

miR-499a inhibits CD200 expression. To confirm the effect of miR-499a on CD200 expression, A549 and A427 cells were transfected with miR-499a mimics, miR-499a inhibitors, CD200 shRNA1 or CD200 shRNA2. Subsequently, the mRNA and protein expression of CD200 was measured using RT-qPCR and western blot analyses. As presented in Fig. 2, the expression of CD200 mRNA (Fig. 2A) and protein (Fig. 2B and C) in A549 cells was significantly reduced upon successful transfection with miR-499a mimics (Fig. 2D), CD200 shRNA1 or CD200 shRNA2. As presented Fig. 3, the expression of CD200 mRNA (Fig. 3A) and protein (Fig. 3B and C) in A427 cells was significantly reduced upon successful transfection with miR-499a mimics (Fig. 3D), CD200 shRNA1 or CD200 shRNA2. By contrast, the expression of CD200 mRNA (Fig. 4A and D) and protein (Fig. 4B, C, E and F) was significantly increased in A549 (Fig. 4A-C) and A427 (Fig. 4D-F) cells transfected with miR-499a inhibitors.

CD200 is a direct target of miR-499a. As illustrated in Fig. 1A, CD200 mRNA was identified as a potential target of miR-499a by the online bioinformatics tool TargetScan (21). To determine if CD200 was a direct target of miR-499a, a luciferase reporter assay was conducted in A549 and A427 cells. As presented in Fig. 1, the luciferase activity in A549 (Fig. 1B) and A427 (Fig. 1C) cells was significantly reduced when cotransfected with miR-499a mimics and wild-type 3'UTR of CD200, suggesting that CD200 is a direct target of miR-499a.

miR-499a expression in NSCLC. The results demonstrated that the AG allele of SNP rs3746444 was significantly associated with the risk of NSCLC (95% CI=1.34-2.18; OR=1.71; P<0.0001).
miRNAs have been reported to serve essential roles in the pathogenesis of many diseases (22). In addition, SNPs located in miRNAs may affect their expression, thus inducing diseases, including colorectal cancer (23). Furthermore, it is hypothesized that such SNPs may additionally affect the binding between the host miRNAs and the target mRNAs, thus altering the expression of target mRNAs (24).

SNP rs3746444 (A>G) is located in the seed sequence of miR-499a-3p, a region crucial to the process of miRNA-mediated gene silencing. For example, the SNP rs3746444 has been demonstrated to increase the risk of ankylosing spondylitis, Behcet's disease, coronary artery disease and rheumatoid arthritis (25-28). The SNP rs3746444 additionally increases the risk of hepatocellular carcinoma among the Chinese population, and is used as a biomarker to predict the susceptibility to coronary artery disease (29).

In the present study, 476 samples of malignant pulmonary nodules and 425 samples of benign pulmonary nodules were collected, and it was demonstrated that the SNP rs3746444 was significantly associated with the risk of NSCLC. Furthermore, the expression of miR-499a was demonstrated to be higher, whereas the expression of CD200 was lower.
Figure 2. miR-499a negatively regulates CD200 expression in A549 cells. (A) Expression levels of CD200 mRNA in cells transfected with miR-499a mimics, CD200 shRNA1 or CD200 shRNA2. (B) Representative images and (C) quantification of CD200 protein expression levels in cells transfected with miR-499a mimics, CD200 shRNA1 or CD200 shRNA2, detected by western blot analysis. (D) Levels of miR-499a in cells transfected with miR-499a mimics, confirming that the mimics transfection was successful. All experiments were repeated at least three times. *P<0.05 vs. NC. CD200, cluster of differentiation 200; NC, negative control; sh, short hairpin.

Figure 3. miR-499a negatively regulates CD200 expression in A427 cells. (A) Expression levels of CD200 mRNA in cells transfected with miR-499a mimics, CD200 shRNA1 or CD200 shRNA2. (B) Representative images and (C) quantification of CD200 protein expression levels in cells transfected with miR-499a mimics, CD200 shRNA1 or CD200 shRNA2, detected by western blot analysis. (D) Levels of miR-499a in cells transfected with miR-499a mimics, confirming that the mimics transfection was successful. All experiments were repeated at least three times. *P<0.05 vs. NC. CD200, cluster of differentiation 200; NC, negative control; sh, short hairpin.
GE et al: rs3746444 IN miR-499a IS ASSOCIATED WITH SUSCEPTIBILITY TO NSCLC

Figure 4. Anti-miR-499a upregulates CD200 expression. (A) Expression levels of CD200 mRNA in A549 cells transfected with anti-miR-499a. (B) Expression levels of CD200 protein in A549 cells transfected with anti-miR-499a. (C) Expression levels of miR-499a were significantly downregulated in A549 cells transfected with anti-miR-499a. (D) Expression levels of CD200 mRNA in A427 cells transfected with anti-miR-499a. (E) Expression levels of CD200 protein in A427 cells transfected with anti-miR-499a. (F) Expression levels of miR-499a were significantly downregulated in A427 cells transfected with anti-miR-499a. All experiments were repeated at least three times. *P<0.05 vs. NC. CD200, cluster of differentiation 200; NC, negative control.

Figure 5. miR-499a is differentially expressed in various patient groups. (A) miR-499a levels in malignant pulmonary nodules were higher compared with benign pulmonary nodules, as detected by RT-qPCR (malignant group, n=32; benign group, n=30). *P<0.05 vs. benign PN. (B) miR-499a levels in subjects carrying the AA genotype were higher compared with the subjects carrying the AG and GG genotypes, as detected by RT-qPCR (AA group, n=32; AG group, n=14; GG group, n=6). *P<0.05 vs. AA group. PN, pulmonary nodules; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

in malignant nodules. Lung tissue samples were additionally divided based on their genotypes of SNP rs3746444 and the results identified that the expression levels of miR-499a in the AA group were much higher, whereas, the CD200 expression in the AA group was significantly reduced.

To validate the regulatory relationship between miR-499a and CD200, in silico analyses and luciferase assays were performed to confirm the role of CD200 as a direct target of miR-499a. In addition, the effect of miR-499a on CD200 expression was investigated using RT-qPCR and western blot analyses. The results demonstrated that transfection of miR-499a mimics, CD200 shRNA1 and CD200 shRNA2 into A549 and A427 cells significantly reduced the mRNA and protein expression levels of CD200. By contrast, transfection of miR-499a inhibitors into the cells increased the mRNA and protein expression of CD200.

As a family member of Ig proteins, CD200 carries two extracellular immunoglobulin domains and one small 19 amino acid intracellular domain with no known signaling motifs (30). CD200 is expressed in a wide range of normal tissues and lymphoid cells, such as activated T cells and B lymphocytes (31,32). CD200R, a receptor of CD200, is mainly expressed in mast cells, neutrophils and macrophages, and the expression levels of CD200R are similar in human and mouse (4). The activation of CD200R inhibits the in vitro activity of myeloid cells, while the binding between CD200R and CD200 suppresses the activation of myeloid cells (33). Unlike other Ig receptors,
CD200R does not contain immunoreceptor tyrosine-based inhibition motif (ITIM) domains (34). In mast cells and macrophages, the signaling cascade of CD200 suppresses the phosphorylation of P38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) (35). The expression of CD200 can also be detected in most cases of plasma cell myeloma. Nevertheless, the results regarding the levels of CD200 in polytypic plasma cells are inconsistent (36-39). In addition, the post-treatment levels of CD200 are stable in myeloma, suggesting that CD200 can be used as a diagnostic biomarker for minimal residual disease of myeloma (36). Despite this, the effort to evaluate the prognostic value of CD200 in multiple myeloma produced conflicting results (36,39). In addition, the post-treatment levels of CD200 are stable in myeloma, suggesting that CD200 can be used as a diagnostic biomarker for minimal residual disease of myeloma (36). Despite this, the effort to evaluate the prognostic value of CD200 in multiple myeloma produced conflicting results (36,39). For example, CD200 is expressed in normal hematogones, while the abnormal expression of CD200 was detected in ~95% of B-cell acute lymphocytic leukemia (B-ALL) (40). Consequently, the levels of CD200 may be used in the evaluation of minimal residual disease of B-ALL (41). In addition, significantly upregulated CD200 expression has been observed in the TEL-AML1 subtype of B-ALL, although the relationship between CD200 expression and the prognosis of B-ALL (TEL-AML1) remains elusive (41). Nevertheless, the abnormal level of CD200 may suggest a poor prognosis for ALL (5). Additionally, CD200 can increase the efficacy of gefitinib in the treatment of lung cancer by decreasing the number of drug-resistant cancer cells (21). Similarly, a microarray analysis study revealed that the levels of CD200 were significantly increased in cancer associated fibroblasts (CAFs), whereas the silencing of CD200 decreased the sensitizing potential of CAFs. An immunohistochemical analysis of samples collected from patients undergoing gefitinib treatments revealed that the patients harboring CD200+ CAFs were associated with longer progression-free survival (PFS) (42). Expression of CD200 has been observed in melanoma, neuroblastoma, ovarian carcinoma, and renal carcinoma, but not in glioblastoma, astrocytoma, breast carcinoma, lung carcinoma, and prostatic carcinoma (43). In addition, a sub-group of basal-cell carcinoma cells have been found to express CD200 during the initiation of tumor growth (44). CD200 has also been demonstrated to induce the metastasis of cutaneous squamous cell carcinoma (45). However, the expression profile of CD200 has not been studied in pulmonary small cell carcinoma.

In summary, using an online miRNA database and luciferase reporter assays, CD200 was confirmed to be a target of miR-499a. Furthermore, the SNP rs3746444 located in miR-499a was demonstrated to affect the risk of NSCLC by regulating the expression of CD200. Given that CD200 functions as a tumor suppressor in lung cancer development, the genotypes of SNP rs3746444 may be used as the biomarker to differentiate malignant and benign pulmonary nodules.
Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

WJ and CL contributed to the conception of the study, guaranteed the integrity of the entire study and reviewed the manuscript. NG defined the intellectual content, designed the study, was involved in data collection and wrote the manuscript. CM and QU collected and analyzed the data, and prepared the draft of the manuscript. LX and XY collected the literature, and prepared the draft of the manuscript. BH and YW visualized the data, and prepared the draft of the manuscript. All authors contributed to the data collection and analysis, and read and approved the final manuscript.

Ethics approval and consent to participate

The Human Research Ethics Committee of The Affiliated Hospital of Qingdao University approved the present study. Written informed consent was obtained from all patients or their first-degree relatives prior to the start of the research project.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

24. Saunders MA, Liang H and Li WH: Human polymorphism at rs3746444 IN miR-499a IS ASSOCIATEd WITH SUScEPTIBILITY TO NS cLc


