miR-130a regulates macrophage polarization and is associated with non-small cell lung cancer

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Received June 3, 2015; Accepted July 13, 2015

DOI: 10.3892/or.2015.4301

Abstract. Lung cancer is the most common cancer as well as the leading cause of cancer-related mortalities worldwide. Macrophages are the most abundant immune cells in primary and metastatic tumors, and contribute to tumor initiation, progression and metastasis. Macrophages have been shown to demonstrate a high level of plasticity, with the ability to undergo dynamic transition between M1 and M2 polarized phenotypes. In the present study, we investigated a pivotal role of miR-130a in macrophage polarization and whether it was associated with poor prognosis in non-small cell lung cancer (NSCLC), using RT-qPCR and western blot analyses. The in vitro experiments showed that miR-130a was expressed at a higher level in M1 compared to M2 macrophages. The enforced expression of miR-130a in macrophages resulted in a significantly increased production of proinflammatory cytokines, whereas deletion of miR-130a impaired the M2-associated gene expression and led to an M1-biased response. Mechanistically, the bioinformatics analysis revealed that proliferator-activated receptor γ (PPARγ) is a potential target of miR-130a. Additionally, the luciferase assay confirmed that PPARγ translation was suppressed by miR-130a through the interaction with the 3'UTR of PPARγ mRNA. A subsequent analysis revealed that the induction of miR-130a suppressed PPARγ protein expression. In NSCLC patients, the results showed that miR-130a downregulation exhibited clinical relevance as it was correlated with poor prognosis and increased tumor stage and metastasis. In addition, miR-130a was inversely correlated with the macrophage marker, CD163, and target gene, PPARγ. Taken together, the results established miR-130a as a molecular switch during macrophage development and as a potential target for the treatment of NSCLC.

Introduction

Lung cancer is the most common cancer as well as the leading cause of cancer-related mortalities globally (1). Histologically, non-small cell lung cancer (NSCLC) accounts for ~80% of lung cancer cases. In spite of the emergence of new cytotoxic drugs and targeted biological agents, NSCLC remains one of the most clinically challenging cancer types (2). Thus, providing better treatment strategies is crucial.

Tumors are composed of an array of cell types, including cancer and non-cancer cells. The most prominent component of these non-cancer cells are macrophages, also known as tumor-associated macrophages (TAMs) (3). There are two well-established polarized phenotypes, classically activated macrophages (M1) and alternatively activated macrophages (M2), both of which have been observed in many tumors (4-6). M1-type macrophages are known to be induced by granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon γ (IFN-γ), and/or lipopolysaccharide (LPS) and have an IL-12high, IL-23high and IL-10low phenotype (7). At the opposite extreme, M2-type macrophages are various forms of macrophages other than the classic M1 including cells exposed to IL-4, IL-13, immune complexes, IL-10, and glucocorticoids (8). Generally, M1-type macrophages are regarded as the effector cells that defend the body against pathogens and tumor cells, while M2-type macrophages suppress inflammatory responses and adaptive immunity and stimulate angiogenesis and tumor growth (9,10). The prognosis of cancer patients is dependent on the ratio of M1 and M2 macrophages (11,12). TAMs play a pivotal role in the progression of NSCLC. The cytotoxic M1 phenotype explains the extended survival of patients with NSCLC, suggesting that positive immunoresponses play a crucial role in the prevention of NSCLC progression (13-16). Therefore, the molecular mechanisms underlying TAM polarization to different phenotypes are the focus of intense investigation.

microRNAs (miRNAs) have been shown to be important mediators of the macrophage activation process. miRNAs are a small class of nucleic acids (~20-24 nt) that function in the transcriptional and post-transcriptional regulation of gene expression (17). miRNAs play a vital role in the regulation of most biological and physiological processes, including development, cell proliferation, cell cycle, apoptosis, migration, and differentiation, including those connected to cancer and...
cells were seeded in tissue culture flasks at 3 x 10^6 cells/flask. To generate macrophage-like differentiated THP-1 cells, THP-1 cells were transfected with lentiviral RNAi of miR-130a and exposed to 320 nM PMA in the culture medium for 48 h as previously described (21). Following incubation, the PMA-containing medium was removed and adherent (differentiated) cells were incubated in fresh culture medium for subsequent experiments. To generate M1-polarized THP-1 macrophages, the dTHP-1 cells were cultured with IFN-γ (100 ng/ml) for an additional 48 h. To generate M2-polarized THP-1 macrophages, the dTHP-1 cells were cultured with M-CSF (100 ng/ml) for an additional 48 h.

Tissue specimens. From December 2010 to November 2014, a total of 75 NSCLC adenocarcinoma specimens and 75 matched normal tissue from adjacent regions were collected from the patients undergoing curative resection and diagnosed histopathologically at the Department of Medical Oncology, Cancer Hospital Institute, Chinese Academy of Medical Science, Peking Union Medical College (Beijing, China). The samples were immediately frozen and stored in liquid nitrogen prior to analysis. None of the patients received chemotherapy or radiotherapy prior to the surgical excision. All the patients provided informed consent for the sample collection. The procedure was approved and supervised by the Institutional Review Board (IRB) of the Cancer Institute/Hospital of Chinese Academy of Medical Sciences and Peking Union Medical College.

Quantitative PCR. RNA was isolated using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA), RNeasy (Qiagen, Hilden, Germany), or miR-Neasy (Qiagen) as per the manufacturer's instructions. The synthesis of cDNA was performed with 0.5 µg RNA using PrimeScript® RT Master Mix (Perfect Real-Time) (Takara Bio Inc., Dalian, China). RT-qPCR analysis was performed in triplicate on LightCycler® 480 II (Roche Applied Science Indianapolis, IN, USA) using SYBR® Premix Ex Taq™ (Perfect Real-Time) (Takara Bio Inc.) and the results were normalized according to the expression levels of GAPDH RNA. Results were expressed using the 2^ΔΔCT methods. The primers for the selected genes are shown in Table I.

Western blotting. Western blot analysis was performed as previously described (22). Briefly, the cells were lysed in cell lysis buffer [1% NP-40, 20 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 3 mM EDTA, 3 mM ethylene glycol tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 20 mg/ml aprotinin and 5 mg/ml leupeptin]. Following treatment, the lysates were purified by centrifugation and denatured by boiling in loading buffer. Equal amounts of protein samples were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis ( SDS-PAGE), and electrophoretically transferred to a nitrocellulose membrane. Following blocking with 5% non-fat milk at room temperature for 1.5 h, the membrane was incubated with rabbit anti-human mono-clonal primary antibody with an appropriate dilution of antibodies (1:1,000–1:2,000) overnight at 4°C and then with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at 1:5,000 dilution for 1 h at room temperature, and detected using the Western Lightning Chemiluminescent detection reagent (Amersham, Freiburg, Germany).

Luciferase activity assay. To construct pGL3-PPARγ-3'UTR, the full length 3'UTR of the human PPARγ mRNA was amplified by PCR and cloned into the pGL3-control vector (Promega, Madison, WI, USA). For the reporter assays, 293T cells were transiently transfected with reporter plasmid and Chinese Academy of Medical Sciences and Peking Union Medical College.

Materials and methods

Cell lines. The human acute monocyte THP-1 leukemia cell line was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). To generate macrophage-like differentiated THP-1 cells, THP-1 cells were transfected with lentiviral RNAi of miR-130a and exposed to 320 nM PMA in the culture medium for 48 h as previously described (21). Following incubation, the PMA-containing medium was removed and adherent (differentiated) cells were incubated in fresh culture medium for subsequent experiments. To generate M1-polarized THP-1 macrophages, dTHP-1 cells were cultured with IFN-γ (100 ng/ml) for an additional 48 h. To generate M2-polarized THP-1 macrophages, the dTHP-1 cells were cultured with M-CSF (100 ng/ml) for an additional 48 h.

Table I. Primer sequences used for RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer</th>
<th>Antisense primer</th>
</tr>
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<tbody>
<tr>
<td>IL-1β</td>
<td>5'-TCCAGGGACAGGATAGG-3'</td>
<td>5'-TCTTTCACACGGAGACAG-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-CTGTGACCATGTGGAGC-3'</td>
<td>5'-GCTGTTTACCTCAGCTCCAC-3'</td>
</tr>
<tr>
<td>iNOS</td>
<td>5'-GCCAAGCTTGAAATGAGGA-3'</td>
<td>5'-TTCTGTGCCGCGAGCTTTAAC-3'</td>
</tr>
<tr>
<td>IL-10</td>
<td>5'-TTTAAGGGTTACCTGGTGTC-3'</td>
<td>5'-TTGATGTCTGGTGTTAGTC-3'</td>
</tr>
<tr>
<td>CCL17</td>
<td>5'-GGATGCCACGTGTTTGTAACTG-3'</td>
<td>5'-AATGCGATTTCTCCTCTTGGTTG-3'</td>
</tr>
<tr>
<td>CCL22</td>
<td>5'-TGCCGTGATTACCTGCGTTA-3'</td>
<td>5'-TCCTCTATCCCTGAGGTTAGCA-3'</td>
</tr>
<tr>
<td>CD163</td>
<td>5'-GCTGACGATGCAATGGCAGATAT-3'</td>
<td>5'-CGGGAGTAGGCGACCTGT-3'</td>
</tr>
<tr>
<td>PPARγ</td>
<td>5'-CATGTTGCGCTTCCGCTGAT-3'</td>
<td>5'-CAATGGCCATGAGGAGTTA-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GCCACCCTAAGGCTGAGA-3'</td>
<td>5'-TGGTGAAGACGAGCCAGTG-3'</td>
</tr>
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miR-130a mimic, using Lipofectamine 2000 (Invitrogen Life Technologies). Reporter assays were performed 48 h post-transfection using the dual-luciferase assay system (Promega), normalized for transfection efficiency by co-transfected Renilla luciferase.

**Flow cytometry.** The samples were incubated with PE-CD86 and FITC-CD206 (BioLegend, San Diego, CA, USA) according to the manufacturers' instructions. Fluorescent conjugated with Alexa Fluor 488 (Invitrogen Life Technologies) was used as a secondary antibody. For each sample at least 1x10⁴ cells were analyzed.

**ELISA.** Cytokine concentrations in the culture supernatants were determined by ELISA kits according to the manufacturer's instructions (eBioscience, San Diego, CA, USA).

**Bioinformatics.** Prediction of putative miR-130a targets was performed by using the online software, TargetScan (http://www.targetscan.org/) in conjunction with miRanda (http://www.microrna.org/microrna/home.do) and PicTar (http://pictar.mdc-berlin.de/). MiRanda was used for the primary screening of miRNA target sites with cut-off values for free energy (ΔG) ≤14 kcal/mole and scores >70. PicTar and TargetScan is an algorithm for the identification of miRNA targets.

**Statistical analysis.** Data are presented as mean ± SD. One-way ANOVA followed by the Bonferroni test was performed for multiple group comparisons. The Student's t-test was used for a comparison between two groups. Pearson's correlation was used to analyze the relationship between the expression of miR-130a and CD163 and PPARγ mRNA. P<0.05 was considered to indicate a statistically significant result.

**Results**

**M1 macrophages demonstrate greater expression of miR-130a compared to M2 macrophages.** We investigated the levels of miR-130a in the proinflammatory M1 subset and immunosuppressive M2 subset. These subsets were induced in vitro and characterized based on their phenotypic characteristics. As shown in Fig. 1A, the M2 macrophages expressed lower levels of TNF-α, IL-1β and iNOS compared to the M1 macrophages but higher levels of CD163, CCL17 and CCL22 mRNA. Furthermore, the shift in macrophages was detected using ELISA analysis. As shown in Fig. 1B, M1 enhanced the levels of TNF-α and IL-1β, whereas M2 reduced the levels of CCL17 and CCL22.

The expression of miR-130a was detected by RT-qPCR. We found that M1 macrophages exhibited a considerably higher level of miR-130a compared to the M2 macrophages (Fig. 1C). The initial findings suggested that miR-130a participates in macrophage polarization. To examine whether miR-130a contributes to the plasticity of macrophage polarization, we converted one population into another by culturing M1 macrophages with M-CSF and M2 macrophages with IFN-γ. As shown in Fig. 1D, M1 to M2 macrophages conversion resulted in decreased miR-130a, whereas M2 to M1 conversion led to increased miR-130a expression.

Overexpression of miR-130a reduces M2-polarized THP-1 macrophages. To determine whether miR-130a participates in macrophage polarization, we transfected M2 macrophage, which have lower levels of miR-130a compared to M1 macrophages. We found that the overexpression of miR-130a with miR-130a mimics (Fig. 2A) in M2 macrophages enhanced the mRNA and protein levels of TNF-α and iNOS, and reduced the expression of IL-10 and CCL22 (Fig. 2B and C). Additionally, the flow cytometric analysis revealed that miR-130a overexpression significantly enhanced CD80 (M1 marker), but inhibited the expression of CD206 (M2 marker) on M2 macrophages (Fig. 2D).
Figure 2. Overexpression of miR-130a reduces M2-polarized THP-1 macrophages. M2 macrophages were transfected with 20 nM control mimics or miR-130a mimics. Forty-eight hours after transfection, the cells were harvested and RNA and protein isolated. (A) RT-qPCR showing the relative expression of miR-130a in untreated M2 macrophages (blank control, BC), and cells treated with miR-130a mimics and negative controls (NC). Relative (B) mRNA and (C) protein levels of proinflammatory cytokines were assayed by qPCR or ELISA, respectively. (D) Expression of CD80 and CD206 was detected by flow cytometry. Representative histograms and average relative mean positive percentage. The results are from at least three independent experiments and presented as mean ± SD. *P<0.05, **P<0.01 vs. negative controls.

Figure 3. Inhibition of miR-130a results in M2 polarization of macrophages. (A) RT-qPCR showing the relative miR-181a expression in untreated M1 macrophages (BC), and cells treated with miR-130a inhibitors and the negative control (NC). Relative (B) mRNA and (C) protein levels of proinflammatory cytokines were assayed by qPCR or ELISA, respectively. The results are from at least three independent experiments and presented as mean ± SD. *P<0.05, **P<0.01 vs. negative controls.
Inhibition of miR-130a results in M2 polarization of macrophages. Earlier, we showed that miR-130a suppresses the expression of the M2 phenotype. Therefore, we determined whether miR-130a inhibition in M1 macrophages, which have higher levels of miR-130a compared to M2 macrophages, demonstrated an effect opposite to that observed in M2 macrophages transfected with miR-130a mimics. To verify this, we simulated the THP-1 cells with IFN-γ for 48 h. As shown in Fig. 3B and C, miR-130a knockdown decreased the IFN-γ-induced expression of TNF-α and iNOS, and enhanced the expression of M2-associated genes IL-10 and CCL22. Given our findings that M2 macrophages with overexpression of miR-130a decreased the anti-inflammatory response to M-CSF, the results showed that miR-130a has a suppressive role in M2 macrophage polarization.

3'UTR was identified (Fig. 4A). To verify that PPARγ is a functional target of miR-130a, we cloned a reporter plasmid containing the wide-type 3'UTR of PPARγ at the 3' position of the firefly luciferase reporter gene. In parallel, we constructed reporter plasmids in which the observed target sequences were mutated individually or in combination, and transfected 293T cells with these constructs with miR-130a mimic and NC. Luciferase activity was markedly reduced in cells transfected with miR-130a mimics and wild-type PPARγ-3'UTR reporter plasmid-transfected cells, compared to the cells transfected with NC mimics, but had no effect on the mutant 3'UTR of PPARγ (Fig. 4B), indicating that miR-130a can regulate gene expression through the putative binding site in the 3'UTR of PPARγ mRNA.

To confirm that miR-130a represses PPARγ expression in THP-1 cells, we performed RT-qPCR analysis and found that the transfection of miR-130a mimic led to a significant decrease in the PPARγ mRNA level, while the transfection of miR-130a inhibitor led to a significant increase in the PPARγ mRNA level compared to the respective controls (Fig. 4C). In addition, western blot analysis showed that transfection of the miR-130a mimic led to a significant decrease in the PPARγ protein level (Fig. 4D) and miR-130a inhibitor resulted in an
increased protein expression of PPARγ. Taken together, these results provide evidence that miR-130a inhibits the expression of PPARγ by directly targeting the 3’UTR of PPARγ.

Expression of miR-130a is inversely associated with advanced stage and lymph node metastasis of NSCLC. We examined miR-130a expression in 75 NSCLC patients samples. miR-130a was significantly downregulated in NSCLC tissues, compared to that in non-tumor tissues (P<0.05, Fig. 5A). We also investigated the association between miR-130a expression and established clinicopathological characteristics. As indicated in Table I, miR-130a was significantly associated with the metastasis and TNM of NSCLC, and the expression level of miR-130a in tumor tissues decreased statistically with the increasing stage of NSCLC (P<0.05) (Fig. 5B). In addition, miR-130a expression was significantly reduced in NSCLC, exhibiting lymph node metastasis compared to NSCLC that did not exhibit lymph node metastasis (Fig. 5C). No significant association to age, gender, smoking history or histological subtype was identified. Therefore, the low miR-130a expression was closely associated with the progression and metastasis of NSCLC.

miR-130a downregulation predicts poor overall survival in NSCLC patients. To evaluate the potential clinical relevance of the downregulated miR-130a with regard to prognosis, the Kaplan-Meier survival analysis was performed using overall
The results indicated that miR-130a was significantly associated with patient survival (Fig. 6). Patients with a high fold change of miR-130a survived longer (n=43; median survival of 65 months) than the patients with a low fold change of miR-130a (n=32; median survival of 49 months) (P=0.003).

miR-130a expression is associated with CD163 and PPARγ expression in NSCLC tissues. We investigated the association between miR-130a and CD163, an M2 macrophage marker. As shown in Fig. 7, the Spearman's correlation analysis revealed a direct correlation between CD163 and miR-130a. The lower CD163 mRNA expression tumors had a substantially higher miR-130a content compared to the reduction in miR-130a levels with increasing CD163 macrophage contents indicating an inverse association between miR-130a expression and macrophages. In addition, the inverse association was observed between PPARγ mRNA expression and miR-130a level.

Discussion

One of the hallmarks of malignancy is the polarization of TAMs from a pro-immune (M1-like) phenotype to an immunosuppressive (M2-like) phenotype. The two distinct subsets, which coexist in tumors, adapt to the changing tumor microenvironment, and can be re-educated by immunoregulatory cues (23,24). This event has primed interest in developing therapies, with the aim of skewing TAMs to an M1-like phenotype (25). Nonetheless, only a few molecules have been identified to orchestrate this process thus far. Evidence has shown that miRNAs are relevant in macrophage activation and function. For example, miR-155, -146, -147, -9 and -21 are induced by TLR ligands (26,27). However, the potential of miRNAs to alter macrophage phenotype and function has been rarely studied. Our investigation provides insight into the role of miR-130a in the control of macrophage polarization.

The biological role of miR-130a in the macrophage has yet to be reported. In the present study, we demonstrate that miR-130a is at a higher level (M1 macrophages) compared to M2 macrophages. The transfection of macrophages with miR-130a mimic resulted in the downregulation of markers and cytokines associated with the phenotype of the classically activated (M2) macrophages CD206, IL-10 and CCL22, whereas cytokines and markers associated with the phenotype of alternatively activated (M1) regulatory macrophages CD80, iNOS and TNF-α, were upregulated. These results suggest that miR-130a skews their polarization from an M2 towards an M1 phenotype. Our results therefore reveal a critical role of miR-130a in the induction of pathogenic M1 macrophage activation and the transition between the pro-and anti-inflammatory phenotypes, which is believed to provide novel insight into the molecular regulation of the functional shaping of macrophages and associated inflammatory disorders.

PPARγ is a ligand-activated transcription factor belonging to the nuclear receptor superfamily. It plays a pivotal role in the control of lipid metabolism and maintenance of energetic homeostasis. PPARγ has been known to inhibit pro-inflammatory gene expression through several mechanisms, including the transrepression of NF-κB (28). For macrophage programming towards M2 polarization, the activation of PPARγ is considered to be critical (29). It has been demonstrated that IL-4 and IL-13 induce the expression and activation of PPARγ (30,31). The present study provides evidence that miR-130a regulates inflammatory cytokine production via PPARγ targeting. The results of four sets of experiments from the present study support this conclusion. First, the bioinformatics analysis reveal that PPARγ is a potential target of miR-130a. Second, the results from the luciferase reporter

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**Figure 6.** Recurrence-free survival among NSCLC patients according to miR-130a expression. Overall survival curves for two groups defined by a low and high expression of miR-130a in patients with NSCLC.

**Figure 7.** miR-130a was inversely correlated with CD163 and PPARγ expression in NSCLC tissues. Expression of miR-130a, CD163 and PPARγ in 75 NSCLC tissues were detected by RT-qPCR. (A) Correlation analysis between miR-130a and CD163 mRNA level in NSCLC tissues (Spearman's correlation analysis, r=-0.340; P<0.01). (B) Correlation analysis between miR-130a and PPARγ mRNA level in NSCLC tissues (Spearman's correlation analysis, r=-0.426; P<0.01).
assay demonstrate that miR-130a may regulate PPARγ protein expression through the conserved miR-130a binding site in the 3’UTR of the PPARγ mRNA. Third, the level of PPARγ protein is downregulated by the ectopic expression of miR-130a, but is upregulated by the inhibition of endogenous miR-130a with the synthetic inhibitor. Fourth, an inverse association was observed between PPARγ mRNA expression and the miR-130a level in NSCLC tissues. These results clearly indicate that PPARγ is a target for miR-130a and that miR-130a controls cytokine production in THP-1 cells by releasing its translational inhibition of PPARγ.

TAMs are abundant components of NSCLC and play a key role in the progression of NSCLC (32). Aberrant miRNAs have been observed in different types of cancer and their expression signatures can be extremely informative for the diagnosis of cancer (33-35). The above results show that miR-130a is a key factor in M1/M2 modulation, raising the question of whether the evaluation of miR-130a expression has a prognostic role in NSCLC patients. Therefore, we examined the role of miR-130a in NSCLC. To the best of our knowledge, we report for the first time that miR-130a expression was downregulated in NSCLC samples compared with the adjacent tissues. Tumors with low miR-130a levels were associated with high tumor stage and poor recurrence-free survival suggesting that miR-130a is a potential marker for tumor progression. Therefore, miR-130a may be a novel tumor-suppressor miRNA, and its downregulation may contribute to lung cancer progression and metastasis. Few reports have shown the involvement of miR-130a in tumorigenesis. Pan et al. (36) have demonstrated that mRNA-130a inhibits cell proliferation, invasion and migration in human breast cancer by targeting the RAB5A. Chen et al. (37) have reported that miR-130a can predict response to temozolomide in patients with glioblastoma multiforme. Acunzo et al. (38) showed that miR-130a targets MET and induces TRAIL sensitivity in NSCLC by downregulating miR-221 and -222. In addition to the link to tumor prognosis, miR-130a was strongly and inversely correlated with CD163 expression. CD163, a marker of M2 macrophages, has been studied in several aggressive tumors, and the increased expression of CD163 was significantly associated with a poor overall survival in various types of cancer (39-41). Our results are in accordance with those obtained from THP-1 cells, suggesting that miR-130a is an important factor in macrophage polarization.

In conclusion, in the present study, we have identified an unknown role for miR-130a in macrophages, providing further insight into the complexities of macrophage plasticity, suggesting that targeting miR-130a may have unforeseen effects on macrophage function. Additionally, miR-130a is frequently downregulated in NSCLC and correlates with tumor stage and poorer patients' prognosis. These results suggest that miR-130a functions as a tumor suppressor in NSCLC and is a potential molecular target for NSCLC therapy.

References