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Full Length Research Paper

TLR9 agonist enhances lung cancer invasiveness by alternating miRNA expression profile

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To investigate the role of miRNA expression profile in the increased invasiveness of non-small cell lung cancer (NSCLC) 95D cells upon TLR9 agonist stimulation, miRNA microarray assay was performed to detect the expression profile of miRNA in NSCLC 95D cells with or without treatment of CpG oligodeoxynucleotide (ODN). Real time PCR was performed in twenty NSCLC samples and corresponding normal tissues to verify the expressions of target miRNA. The miRNA microarray assay showed that CpG ODN stimulation alternated the miRNA expression profile in NSCLC 95D cells and the difference in the expressions of 23 miRNAs between the CpG ODN treated group and untreated group was at least two-fold, among which 20 miRNAs were down-regulated. The down-regulation of let-7a was the most significant, which was also confirmed by Real time PCR. We concluded that TLR9 agonist might raise the invasiveness of NSCLC 95D cells by alternating the expression profile of miRNA, especially the down-regulation of let-7a.

Key words: TLR9 agonist, CpG oligodeoxynucleotide, miRNA, lung cancer.

INTRODUCTION

Oligodeoxynucleotides with unmethylated CpG (CpG-ODN) have been regarded as the Toll-like receptor 9 (TLR9) agonists. CpG-ODN plays an important role as an adjuvant in the immunotherapy of cancers (Okamoto and Sato, 2003; Wooldridge and Weiner, 2003). However, study shows CpG ODN may promote the proliferation and invasiveness of cancer cells (Droemann et al., 2005). In our previous study (Ren et al., 2007), the direct effects of CpG ODN on non-small cell lung cancer (NSCLC) 95D cells (with high metastatic potential) were investigated, and results revealed the invasiveness of 95D cells was significantly enhanced after CpG ODN stimulation. However, the mechanism underlying the increased invasiveness of 95D cells following TLR9 agonist treatment remains poorly understood.

The microRNAs (miRNAs) are small noncoding RNA gene products about 22 nt in length and they work as gene regulatory molecules by targeting mRNAs and likely influencing the output of a variety of protein-coding genes (Bartel., 2004). The miRNAs have been shown to regulate some important cell functions including cell proliferation, apoptosis, development, differentiation and metabolism (Takamizawa et al., 2004). Recently, dysregulation of miRNA expression has been noted in patients with malignancies, including lung cancer. Increasing evidence shows that miRNAs can act as oncogenes or tumor suppressors. We speculate that CpG ODN treatment may alternate the expression profile of miRNAs, thereby promoting cell proliferation and invasiveness.

In this study, miRNA microarray assay was performed in the NSCLC 95D cells. Results showed CpG ODN stimulation alternated the expressions of 23 miRNAs by at least two-fold and the down-regulation of let-7a was the most significant. Real-time RT-PCR was then performed in twenty NSCLC samples of patients and corresponding normal tissues, which verified the significantly down-regulated expressions of let-7a in the NSCLC samples. Together with our previous study, we postulate that TLR9 agonist may raise the invasiveness of NSCLC 95D cells by alternating the expression profile of miRNAs, especially the down-regulation of let-7a.

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MATERIALS AND METHODS

miRNA-Microarray assay

The miRNA microarray assay was performed by Kangchen Bio-tech. The miRNA microarray was designed based on proprietary locked nucleic acids (LNA^{TM}) technology. The number of LNA capture probes is more than 2000, covering all the miRNAs in miRbase 9.2. Briefly, total RNA was extracted from treated and untreated 95D cell lines with Trizol reagent (Invitrogen, USA).The purity and concentration of total RNA were determined with Nanodrop ND 1000 and the integrity of total RNA was determined by gel electrophoresis. The total RNA was labeled with miRCURYTM Array Power and then hybridized to the miRNA microarray using a heat-shrank hybridization bag (Phalanx, USA). The microarray was washed and dried, followed by slide scanning, data extraction and preliminary analysis. The microarray was performed once in the experimental group (treated 95D cell line) and the control group (untreated 95D cell line) respectively.

Cell line and reagents

NSCLC 95D cell line which has high metastatic potential was provided by the Institute of Biochemistry and Cell Biology (Shanghai). The 95D cell line was maintained in RPMI 1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS) and 2 mM glutamine at 37℃ with 5% CO_2. The CpG ODN 2216 (5'-GGGGGACGATCGTCGGGGG-3') was purchased from Integrated DNA Technologies (Coralville, IO, USA).

Sample collection and Real time RT-PCR

Samples were collected from 20 pathologically proven NSCLC patients undergoing surgery at Zhongshan Hospital, Shanghai, China. Informed consent was obtained from all patients and this study was approved by the ethics committee of our hospital. All samples were snap-frozen in liquid nitrogen within 20 min and then stored at -80℃ until RNA extraction. At the same time, the corres-ponding normal lung tissues were also obtained and served as controls. The NSCLC was confirmed by pathological examination. Total RNA was isolated using the Trizol reagents [Sangon Biotech (shanghai) Co., Ltd, China]. RT-PCR was performed using TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA) Kit following manufacturer's instructions and the expressions were normalized to U6 levels assayed on the Rotor-Gene RG-3000 (CORBETT RESEARCH). The primer for hsa-let-7a is (UGAGGUAGUAGGUUGUAUAGUU). Data were presented as fold difference relative to U6 level based on the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was carried out with SPSS version 11.5 statistic software package. Student's t-test was done to compare the difference between two groups. A value of $P<0.05$ was considered statistically significant.

RESULTS

TLR9 agonist alternates the miRNA expression profile in 95D cells

Results showed that more than 800 mature miRNAs in the untreated 95D cells were normalized by the midvalue of all the non-control probes with allowance >50, and more than 200 miRNAs were considered as normalized. After normalization, the expressions of miRNAs of the treated group were compared to those of the untreated group. Results revealed the expression profile of miRNA was altered by CpG ODN treatment ($P<0.05$, 95% CI: -1.6085 to -0.4605). Among all the normalized miRNAs, most were down-regulated, and only a few up-regulated (data not shown).

Based on these findings, the miRNAs having at least two-fold alternation in the expression were selected and subjected to verification. A total of 23 miRNAs were found to have the difference of at least two folds in their expressions, among which 20 miRNAs were down-regulated and 3 up-regulated (Figure 1 and Table 1).

Expression of let-7a is the most significantly down-regulated in 95D cells and validated in NSCLC tissues

The expression of let-7a was the most significantly down-regulated in 95D cells. Then, the expression of let-7a was determined in 20 NSCLC samples of patients and the corresponding normal lung tissues. Down-regulation of let-7a was found in 70% (14/20) NSCLC samples and the expression of let-7a in the NSCLC samples was about 58% of that in normal tissues ($P<0.05$) (Figure 2).

DISCUSSION

In this study, the miRNA microarray assay was done and a series of miRNAs in 95D cells were found to be down-regulated following the treatment of CpG ODN. Further-more, the down-regulation of let-7a was the most striking, and its down-regulation was further validated in the NSCLC samples by Real time RT-PCR. Together with the results of our previous study (Ren et al., 2007), we speculate that CpG ODN (TLR9 agonist) may enhance the invasiveness of lung cancer cells by alternating the miRNA expression profile, especially by the down-regulation of let-7a.

In the present study, let-7a was down-regulated strikingly by almost 7 folds upon CpG ODN stimulation. Real-time RT-PCR indicated the expression of let-7a in the NSCLC tissues was significantly decreased when compared with that in the corresponding normal lung tissues, and the expression level of let-7a in the NSCLC tissues was about 58% of that in the corresponding normal tissues ($P<0.05$). Similarly, let-7i was also signify-cantly down-regulated. Let-7 family is one of the most extensively studied miRNAs which are highly correlated with lung cancer. Takamizawa et al. (2004) first reported the reduced expressions of let-7 in lung cancers both *in vitro* and *in vivo.* The down-regulation of let-7 were subsequently also noted in other cancer cell lines (Yu et al., 2007; Ibarra et al., 2007). Studies reveal the low expression of let-7 predicts a poor prognosis

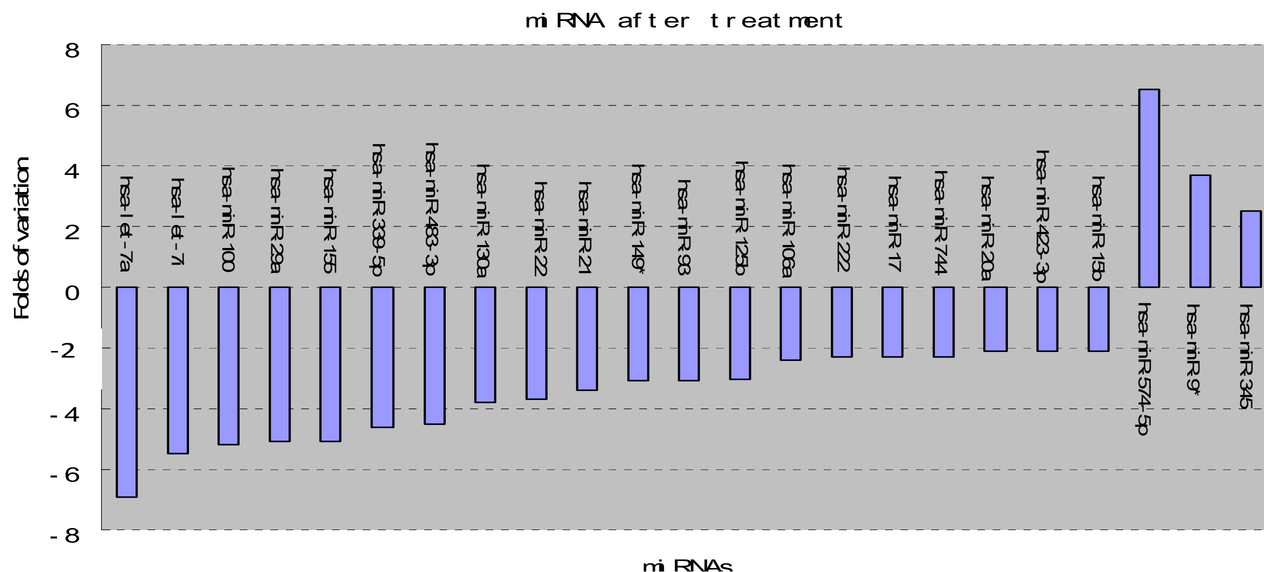

Figure 1. miRNA having at least two-fold alternation in the expression after CpG-ODN treatment.

Table 1. miRNA having at least two-fold alternation in the expression following CpG ODN treatment.

miRNAs	Alternation	Chromosome localization*	Expression in cancers #
hsa-let-7a	Down (6.9)	Not defined (ND)	Melanoma, lung cancer
hsa-miR-20a	Down (2.1)	13q31.3	
hsa-miR-423-3p	Down (2.1)	ND	
hsa-miR-222	Down (2.3)	X-P11.3	B-cell lymphoma, melanoma, prostate cancer, thyroid cancer
hsa-miR-149*	Down (3.1)	ND	
hsa-miR-17	Down (2.3)	13q31.3	
hsa-miR-155	Down (5.1)	21q21.3	Breast cancer, lung cancer, colon cancer, B-cell lymphoma, chronic lymphocytic leukemia, pancreatic cancer
hsa-miR-106a	Down (2.4)	X-q26.2	
hsa-miR-15b	Down (2.1)	3q26.1	
hsa-miR-93	Down (3.1)	7q22.1	Gastric cancer
hsa-miR-22	Down (3.7)	17p13.3	
hsa-miR-744	Down (2.3)	17p12	
hsa-miR-21	Down (3.4)	17q23.1	Breast cancer, colon cancer, lung cancer, prostate cancer, gastric cancer, brain cancer, hepatocellular cancer
hsa-miR-10 0	Down (5.2)	11q24.1	
hsa-miR-125b	Down (3.0)	ND	Breast cancer, prostate cancer, hepatocellular cancer
hsa-let-7i	Down (5.5)	12q14.2	Lung cancer
hsa-miR-29a	Down (5.1)	7q32.3	Prostate cancer, lung cancer
hsa-miR-130a	Down (3.8)	11q12.1	
hsa-miR-339-5p	Down (4.6)	ND	
hsa-miR-483-3p	Down (4.5)	ND	
hsa-miR-345	Up (2.5)	14q32.2	
hsa-miR-574-5p	Up (6.5)	ND	
hsa-miR-9*	Up (3.7)	ND	

* From miRBase Sequences (http://microrna.sanger.ac.uk); #(Rossi et al., 2007; Jay et al., 2007; Mocellin et al., 2009).

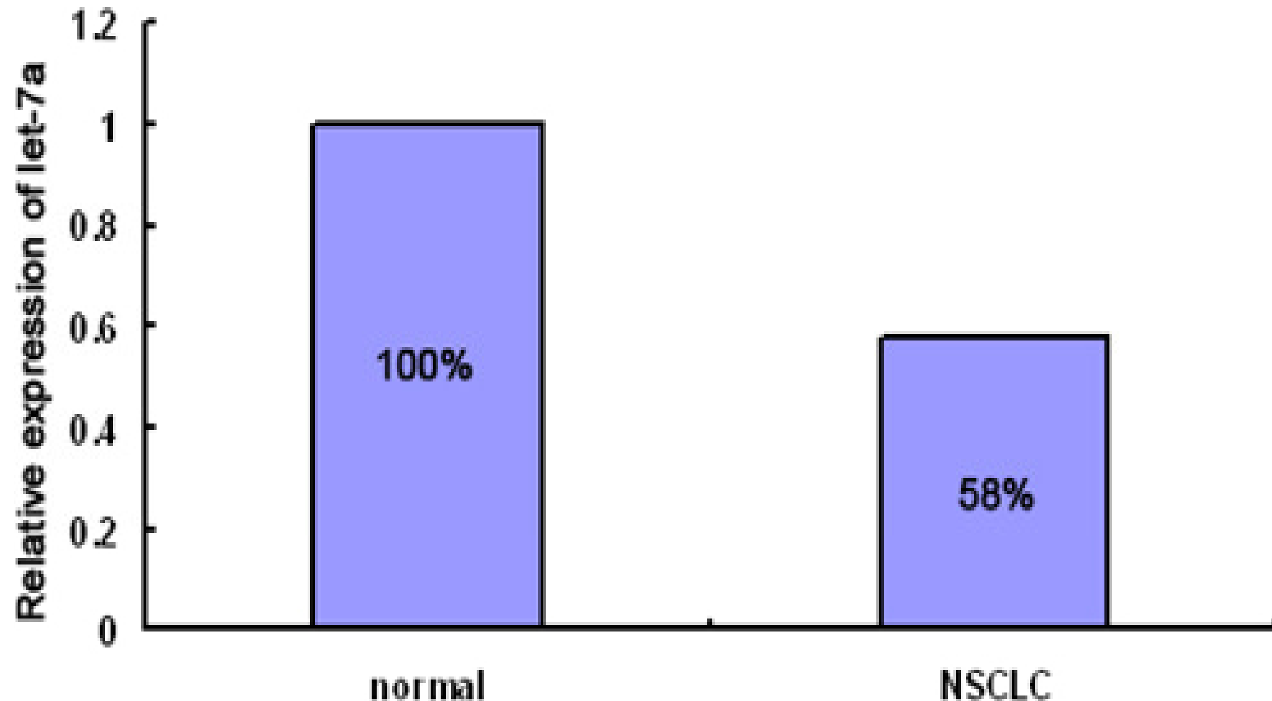

Figure 2. Relative expression of let-7a in the NSCLC samples. NSCLC samples and corresponding normal tissues were collected from 20 NSCLC patients. Down-regulation of let-7a was found in 70% (14/20) NSCLC samples and the expression of let-7a in the NSCLC samples was about 58% of that in normal tissues ($P<0.05$).

(Takamizawa et al., 2004; Yanaihara et al., 2006), while over-expression of let-7 can inhibit the cancer growth (Yu et al., 2008; Kumar et al., 2008; Esquela-Kerscher et al., 2008). Therefore, let-7 gene may serve as a tumor suppressor gene in NSCLC. At molecular level, members of let-7 family function through silencing RAS and high mobility group AT-hook 2 (HMGA2) oncogenes (Johnson et al., 2005; Mayr et al., 2007; Park et al., 2007; Lee et al., 2007). HMGA2 is a protein encoded by the HMGA2 gene in humans and its expression in adult tissues is closely associated with both malignant and benign tumor formation, as well as certain characteristic cancer-promoting mutations. Down-regulation of let-7 has been shown to result in up-regulation of RAS, and subsequent oncogenesis including formation of human lung cancer (Eder and Scherr, 2005), so, members of let-7 family have been proven to act as tumor suppressors on cell proliferation and metastasis. Our results also confirmed the reduced expression of let-7 which implies CpG ODN, as anti-cancer drug, has the potential risk for cancer metastasis.

Another important miRNA which acts as a tumor suppressor and is remarkably down-regulated is mir-29. It had been reported that the expressions of mir-29 family members were inversely correlated with expressions of DNA methyltransferase 3A (DNMT3A) and -3B in lung cancers, and with the down-regulations of both DNA methylated enzymes; Members of mir-29 family are shown to inhibit the cancerogenesis both *in vitro* and *in vivo* partly via reducing the global DNA methylation (Fabbri et al., 2007). Recently, the epigenetic alterations, especially the DNA methylation, have been shown to play an important role in the cancer metastasis (Lujambio et al., 2009). However, the mechanism of DNA methylation in the metastasis remains elusive. Study reveals over-expression of mir-29a suppresses the expression of tristetraprolin (TTP), a protein leading to epithelial-to-mesenchymal transition (EMT) and metastasis with the presence of oncogenic Ras signaling (Gebeshuber et al., 2009). In the present study, our results showed the expression of mir-29 was remarkably decreased (5.1 folds), suggesting the risk for cancerogenesis and metastasis following CpG ODN stimulation.

Of interest, mir-155, mir-20a, mir-222 and mir-21, which act as oncogenes, are also down-regulated following the CpG ODN stimulation. Mir-155 serves as a promoter of cancer metastasis and over-expression of mir-155 is highly correlated with a poor prognosis in lung adenocarcinoma patients (Jay et al., 2007). Additionally, down-regulation of mir-155 suppresses the EMT, tight junction dissolution, cell migration and invasion, while up-regulation of mir-155 were frequently detected in invasive breast cancer (Kong et al., 2008). Similarly, mir-20a and mir-222 functioned as anti-apoptotic factors (Matsubara et al., 2007; Garofalo et al., 2008). Aberrant up-regulation of mir-21 has been identified in the lung cancer (Yanaihara et al., 2006; Markou et al., 2008), and mir-21 also plays an important role in cancer invasion and

metastasis by inhibiting multiple metastasis suppressor genes, including TPM1, PDCD4, maspin, etc (Zhu et al., 2008; Asangani et al., 2008). It has also been shown that, over-expression of miR-21 is significantly associated with both high Ki-67 proliferation index and pancreatic liver metastasis (Roldo et al., 2006). Therefore, down-regulations of these miRNAs following CpG-ODN stimulation suggest the protective effects which are in contrast to those of let-7 and mir-29a. It appears paradoxical that both tumor suppressor and promoter are dysregulated. However, considering that each miRNA may exert distinct effects and the invasiveness is determined by the comprehensive effects of miRNAs, it is not difficult to draw the conclusion that miRNAs do not function mutually exclusively, but will potentially reconcile or impair their functions.

In addition, the dys-regulations of other miRNAs were also noted in the present study, but their functions are currently unknown. Ongoing studies will be focused on the role of these miRNAs in the formation of lung cancer.

Together with our previous work, our results show TLR9 agonist can enhance the invasiveness of NSCLC 95D cells and significantly alternate the expression profile of some miRNAs. We speculate that CpG ODN may induce the invasiveness of cancer cells by alternating the miRNA expression profile, especially the down-regulation of let-7a. Therefore, let-7a may play a key role in the lung cancer metastasis. Further studies should focus on the role of let-7a in the lung cancer metastasis in the future.

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