MicroRNA-205 Promotes Cell Proliferation of Non-Small Cell Lung Cancer by Targeting Smad4

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Abstract

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer and the leading cause of cancer-related death worldwide. Despite recent advances in cancer diagnosis and treatment, the survival rate of NSCLC patients remains dismally low. There is an urgent need for better mechanistic understanding and development of novel diagnostic and treatment strategies against this malignant disease. MicroRNAs (miRNAs), a class of 19- to 24-base pair non-coding RNAs, have increasingly been implicated as crucial regulators of NSCLC malignancy, and promising biomarkers and/or therapeutic targets for such aggressive disease. Here, we report clinical, molecular, and functional studies of miR-205 in NSCLC. Our analyses of an NSCLC patient cohort showed that expression of miR-205 in primary tumors was 7-fold higher than their corresponding adjacent noncancerous tissues. However, miR-205 was not associated with tumor stage, smoking status, age, or gender, implying a functional link to early-stage tumorigenesis of NSCLC. To test this possibility, we turned our attention to Smad4, a putative target of miR-205 and a widely recognized tumor suppressor. As expected, the expression of Smad4 mRNA in primary tumors of our patient cohort was lower than their normal counterparts. Importantly, we detected a strong negative association between miR-205 and Smad4 in patient tumor tissues. With these clinical leads, we next evaluated the molecular and functional links between these two distinct types of molecules. Our initial mutagenesis analyses showed that miR-205 repressed the expression of Smad4 by directly targeting the 3'-UTR region of its mRNA. Subsequently, we found that overexpression of miR-205 enhanced the proliferation of cultured NSCLC cells. Conversely, siRNA-directed knockdown of Smad4 markedly suppressed tumor cell proliferation. Moreover, our MassARRAY technology-based analyses showed that the DNA methylation of the -77CpG site in the promoter region of miR-205 was significantly impaired in patient tumor tissues. Taken together, our study for the first time provides clinical, molecular and functional evidence on the critical roles of miR-205 in human NSCLC. In particular, our analyses demonstrate that miR-205 drives tumor cell proliferation of NSCLC by directly downregulating Smad4 expression. As such, our findings strongly support the potential of miR-205 as a candidate biomarker and therapeutic target for the diagnosis and treatment of NSCLC.

Keywords: Non-small cell lung cancer; Smad4; miR-205; cell proliferation; TGF-β signaling

Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related death worldwide [1]. Despite recent advances in cancer diagnosis and treatment, the five-year survival rate of NSCLC patients remains below 10%. Patients with NSCLC frequently develop rapid proliferation; TGF-β signaling.

MicroRNAs(miRNAs), a class of small non-coding RNAs with 19- to 24-base pair, have recently emerged as crucial regulators of NSCLC malignancy via accelerating mRNA degradation of their target genes [3,4]. It is estimated that about 30% of human protein-coding genes are regulated by miRNAs [5]. Thus, miRNAs represent a group of important players in diverse biological and pathological processes, including tumor cell proliferation, differentiation, and survival [6-8]. There is also evidence that the expression of microRNAs appear to be tissue- or tumor type-specific [9]. As a result, they have been strongly implicated as candidate biomarkers for clinical diagnosis,
including identification of cancer type or tumor subtype [10,11]. Thus, a better understanding of these unique small RNAs is of clinical importance in human NSCLC, particularly regarding detection and diagnosis.

Aberrant expression of miR-205 has been reported for multiple cancer types, including lung cancer [12]. This miRNA is localized at 1q32.2, a region strongly associated with lung cancer-related gene amplifications [10]. How this miRNA is regulated at the genomic level, however, remains largely unknown. Recently, DNA methylation in the promoter region of miRNAs has been suggested as a critical means for the regulation of miRNA expression [13,14]. In fact, comprehensive bioinformatic analyses have shown that expression of approximately 50% of miRNAs is associated with DNA methylation status in CpG-islands region of their promoters [15]. Thus, DNA methylation may represent an important avenue for the regulation of miR-205 expression and functions in human NSCLC.

Aside from miR-205, members of the transforming growth factor β (TGF-β) superfamily have been widely recognized for their importance in tumor cell proliferation, apoptosis, differentiation, and angiogenesis during NSCLC carcinogenesis and progression [16,17]. In particular, TGF-β-mediated signaling is markedly altered in NSCLC, and functionally associated with the tumorigenic and metastatic processes of this disease [18,19]. Upon ligand stimulation, TGF-β receptor R1 and R2 heterodimerize to become activated and in turn phosphorylate SMAD2 and SMAD3 proteins. These phosphorylated proteins in turn form tight protein complexes with SMAD4, and subsequently translocate to the nucleus, where they interact with other transcription activator or repressors to control gene expression or cellular functions or processes [20]. Notably, the Smad2/Smad3/Smad4 complexes interact with transcription factor FoxO and Sp1 to activate the expression of p21Cip1 and p15Ink4b [21,22]. As a result, the activities of cyclin-CDK complexes are impaired and tumor cell proliferation is inhibited [23]. Importantly, such tumor-suppressing function of TGF-β signaling pathway is strongly dependent on the status of Smad4, also known as DPC4. Despite such functional importance, how Smad4 is regulated at transcriptional level in human NSCLC remains largely unclear, particularly via the expression of microRNAs.

Here, we studied the role of miR-205 in NSCLC with respect to its clinical relevance, cellular functions and molecular regulation. The primary tumors and adjacent normal tissues in a cohort of 52 patients were analyzed by qRT-PCR for the expression of miR-205 and Smad4. The functional roles of miR-205 and its molecular link to Smad4 were also investigated with cultured NSCLC cell lines. In addition, we examined the methylation status of CpG sites in the promoter region of miR-205. Data from our analyses indicate that miR-205 regulates tumor cell proliferation in human NSCLC by suppressing the expression of Smad4. Our study provides crucial molecular and cellular bases of miR-205 as a promising biomarker and therapeutic target for highly aggressive NSCLC.

Materials and Methods

Collection of patient tissue samples and cell culture

Fifty-two paired NSCLC tumor and adjacent non-cancerous lung tissues were collected after informed consent from patients in the First Affiliated Hospital of Soochow University between 2009 and 2013. Histological and pathological diagnostics of NSCLC patients were determined according to the Revised International System for Staging Lung Cancer. The NSCLC patients received neither chemotherapy nor radiotherapy prior to the removal of tissue samples. All patient samples were snap-frozen and stored at -80°C. This study was approved to be performed by the Ethics Committee of the First Affiliated Hospital of Soochow University.

Human lung adenocarcinoma cell lines A549 and SPC-A1 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium (HyClone, South Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum and L-glutamine and antibiotics (Invitrogen, Carlsbad, CA, USA). All cell culture was conducted in a humidified incubator containing 5% CO₂ at 37°C.

RNA extraction, CDNA synthesis, and quantitative real-time PCR (qRT-PCR)

Total RNA in tissues and cultured cells was extracted with RNAiso Plus kit (TaKaRa, Osaka, Japan) by following the manufacturer’s protocol. CDNA was synthesized using the Reverse Transcribe M-MLV kit (TaKaRa, Osaka, Japan). The sequences of qRT-PCR primers for Smad4 were: Forward: 5'-CAGCCCATGTTGTCACCT-3'; Reverse: 5'-GTGGGTTGGCCTGATTC-3'. For β-actin were: Forward: 5'-CACAGAGC-CTCAGCCTTGCC-3'; Reverse: 5'-ACCCATGCCCAACCATCAG-3'.

All qRT-PCR analyses were conducted using SYBR Premix ExTaq ™ (TaKaRa, Osaka, Japan) on an ABI StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The PCR program was 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. The mRNA values of Smad4 and miR-205 were respectively normalized to internal controls β-actin and U6. Relative expression was calculated using the Ct method.

Construction of luciferase reporter plasmids, transient transfection, and luciferase assay

The psiCHECK2 dual luciferase vector (Promega, Madison, WI, USA) was selected for cloning of the Smad4 3'-UTR region fused with a luciferase reporter gene. The stretch of 215 base pair DNA fragments in the 3'-UTR region of Smad4 gene(position 262-269) were initially identified as putative targeting site of the miR-205, according to bioinformatic analyses. This stretch of DNA fragments was synthesized in an intact or mutated from, and subsequently subcloned into the psiCHECK2 vector. Transient transfection of lung cancer A549 cells were conducted using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). After 48 h of incubation, transfected cells were collected, and analyzed for luciferase activities by using the Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI, USA).

Cell proliferation assay

To measure cell proliferation, tumor cells were transiently transfected for 48 h. Then, the transfected tumor cells were detached and seeded into 96-well plates (2 × 10³ cells/well) for subsequent analyses of cell viability. The cell viability assay was performed using the Cell Counting Kit-8 assay kit (CCK-8, Boster, Wuhan, China).
DNA quantitative methylation analysis

Quantitative DNA methylation analysis was conducted using MassArray EpiTYPER assays (Sequenom, San Diego, CA). In brief, this system used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in combination with RNA base-specific cleavage. After initial bisulfite modification, genomic DNA was amplified with MassArray primers. The resulted PCR products were cloned under a T7 promoter sequence for in vitro production of RNA transcript. After T-base-specific cleavage, stretches of small RNA fragments were obtained. Flight mass spectrometry (MALDI-TOF) was used to detect the molecular weights of individual fragments. The methylation data were outputted with EpiTyper software. DNA methylation standards (0%, 20%, 40%, 60%, 80% and 100%) were used to control the bias of PCR amplification. Correction algorithms based on the R statistical computing environment were used for data normalization.

Cell lyses and immunoblotting

Human NSCLC cells were grown to 80%–90% and lysed in a RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with protease inhibitor and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Protein samples were subjected to SDS-PAGE electrophoresis and transferred to nitrocellulose membranes prior to immunoblotting. Detection was performed using the ECL kit (Pierce, Rockford, IL, USA). The band intensity was quantified using Quantity One 4.6 software. Primary antibodies against Smad4 and β-actin, along with the HRP-conjugated secondary antibodies, were obtained from Cell Signaling Technology (Danvers, MA, USA).

Statistical analysis

All experiments were independently repeated three times. The differences in the expression of miR-205 or Smad4 between NSCLC tissues (T) and adjacent noncancerous lung tissues (N) were evaluated by using a paired, two-tailed t-test. A similar analysis was also adopted for cell line-based studies. The correlation between the expression of miR-205 or Smad4 and clinicopathologic parameters in NSCLC patient cohort were assessed with nonparametric tests (Unpaired t-test for 2 groups, Kruskall-Wallis test for 3 or more groups). Values were calculated as mean ± SE. All statistical analyses were performed using GraphPad Prism 5.02 (GraphPad, San Diego, CA, USA) and SPSS 16.0 software (SPSS, Chicago, IL, USA).

Results

Expression of miR-205 and Smad4 in NSCLC

To evaluate the clinical significance of miR-205 in NSCLC, expression of miR-205 in the paired primary tumor and adjacent noncancerous tissues was examined with a cohort of 52 patients across different age groups, genders and smoking status as well as tumor subtypes and stages. As shown in Figure 1A, the expression of miR-205 was much more higher in tumors than their corresponding adjacent noncancerous tissues or normal counterparts (p<0.05) (Figure 1A). The expression of miR-205 was strongly associated with tumor subtypes, including the highest expression in the squamous carcinoma (SC) subtype (Table 1). However, no significant correlation was found between miR-205 and patient age, gender or clinical stage or smoking status (Table 1). Together, these data indicate a strong elevation of miR-205 expression in the primary tumor tissues of human NSCLC, and imply a potential functional role in the early-staged tumorigenesis of the disease.

Figure 1: Expression of miR-205 and Smad4 in primary tumors and adjacent noncancerous tissues in a cohort of 52 NSCLC patients. Values: mean ± SEM. T: primary tumors. N: adjacent noncancerous tissues. A Expression of miR-205 level. B Expression of Smad4 mRNA level. C Correlational analyses of miR-205 and Smad4. *p<0.05; **p<0.001; ***p<0.001.

Next, we sought to understand how miR-205 might contribute to the malignancy of human NSCLC. Our TargetScan 6.2 analysis indicated that Smad4, a critical regulator of TGF-β-mediated signaling, was a putative target of miR-205. To test this possibility, we subsequently examined the expression of Smad4 in our NSCLC patient
cohorts. As shown in Fig. 1B, the expression of Smad4 mRNA in the primary tumor tissues was 1.8-fold lower than their normal counterparts (Figure 1B). Furthermore, a negative association between miR-205 and Smad4 mRNA was detected in our patient cohort (Figure 1C). A close association was found between Smad4 and tumor subtype (Table 1). However, there was a minimal association between Smad4 and patient age, smoking status or tumor stage (Table 1). Together, these data implicate that miR-205 is a potential master regulator of tumorigenesis in NSCLC, and part of its function is likely conveyed via the regulation of Smad4.

<table>
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<th>Characteristics</th>
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<th>miR-205 expression</th>
<th>Smad4 mRNA expression</th>
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<td>≤ 65</td>
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<td>Squamous cell carcinomas</td>
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<td>Others</td>
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<td>14(26.9%)</td>
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Table 1: Clinicopathological parameters and expression of miR-205 and Smad4 mRNA in NSCLC tissues (mean ± SE). Date presented as mean ± SE. Unpaired t-test for comparison of 2 groups. Kruskal-Wallis test for comparison of 3 or more groups. n: number of patients.

miR-205 reduces Smad4 expression by targeting its 3′-UTR in NSCLC cells

Next, we tested the possibility that miR-205 controlled the expression of Smad4 mRNA by directly targeting its 3′-UTR region. For such purpose, the intact and mutated stretches of putative miR-205 binding site in the Smad4 3′-UTR region were subcloned into psiCHECK-2 vector (Figure 2A). The resulting plasmids, together with a reporter construct containing miR-205 mimics or control, were transiently transfected into A549 cells. Our subsequent analyses showed that only miR-205 mimics led to an inhibition in the luciferase activities in A549 cells expressing intact Smad4 3′-UTR region only, supporting the specificity of miR-205 targeting (Figure 2B). Such effect of miR-205 was consolidated by our observation that a marked inhibition of endogenous Smad4 mRNA occurred in both A549 cells and SPC-A1 cells upon over expression of miR-205(Figures 2C and 2D). Together, these data provide strong evidence that Smad4 is a direct target of miR-205 in NSCLC cells.
Figure 2: Identification of Smad4 as a direct target of miR-205 in NSCLC cells. A Schematic illustration of the predicted miR-205 binding site in the 3'-UTR region of the Smad4 gene. Predicted duplex formation between miR-205 and the wild-type/mutant of miR-205 binding site is indicated. B Molecular targeting of 3'-UTR region of Smad4 gene by miR-205. The luciferase activities in A549 cells transiently transfected with different combinations of plasmids containing the wild-type or mutant Smad4 3'-UTR fragment and miRNAs were analyzed. Scrambled sequence was used as miR-NC. Relative Renilla luciferase activity is obtained after normalizing to the firefly luciferase activity. C Impact of miR-205 on the mRNA expression of endogenous Smad4 in NSCLC A549 and SPC-A1 cell lines. Tumor cells were transiently transfected with miR-NC or miR-205 mimics, followed by real-time PCR analysis to evaluate expression of Smad4 mRNA. D Downregulation of endogenous Smad4 protein by miR-205. A549 cells and SPC-A1 cells were transiently transfected with miR-205 mimics or miR-NC, followed by immunoblotting of Smad4 and β-actin.

Functional roles of Smad4 and miR-205 in NSCLC cells

Smad4 has been extensively implicated as a negative regulator of tumorigenesis across multiple cancer types, particularly regarding tumor cell proliferation [24]. Thus, we reasoned that miR-205 may contribute to the malignancy of NSCLC by promoting tumor cell proliferation. Our knockdown analyses indicated that when Smad4 was knocked down by 60-80% at both mRNA and protein levels (Figures 3A and 3B), the proliferation of A549 cells was significantly upregulated (Figure 3C). A similar effect of miR-205 overexpression on the cell proliferation was also detected (Figure 3D). These effects were also independently confirmed by our colony- formations assay (Figures 3E and 3F). Together, these results indicate that miR-205 likely drives the proliferation of NSCLC cells by impairing the tumor-suppressing function of Smad4.

Regulation of miR-205 expression by DNA methylation

DNA methylation is increasingly implicated as a crucial mean for controlling mRNA expression in tissues or cells under normal or pathological condition. Hence, we sought to determine if this was the case for miR-205 in NSCLC, particularly in the context of hypomethylation. Our initial scanning of the promoter region of miR-205 with Methyl Primer Express software suggested that the 12 CpG sites within -522~+271 region of miR-205 promoter were prone to DNA hypomethylation (Figure 4A). A similar indication was also obtained with our use of TFSEARCH. Hence, we subsequently applied the MassARRAY technology to examine the methylation status of these candidate sites in twenty NSCLC patient samples. Our data showed that among 12 putative sites analyzed, only the -77CpG site (Site 5), exhibited a significant downregulation in DNA methylation (p=0.0093) (Figure 4B). Furthermore, there was a trend of a negative association between miR-205 and the methylation status of its promoter region (Figure 4C). Together, these data provide evidence that the aberrant expression of miR-205 in NSCLC is likely attributed to the methylation of its promoter region.
Figure 3: Effect of SMAD4 knockdown or miR-205 overexpression on the proliferation of NSCLC cells. A Effect of siRNA-based knockdown on mRNA expression of Smad4. B Validation of siRNA-based knockdown effect in Smad4 in NSCLC A549 cells and SPC-A1 cell lines. The knockdown effect of Smad4 was assessed by immunoblotting analyses. C-D Effect of siRNA-based Smad4 knockdown or miR-205 overexpression on the proliferation of A549 cells. Tumor cells were transiently transfected with siRNA against Smad4 or miR-205, followed by harvesting cells at indicated times for assay of cell proliferation. *p<0.05; **p<0.001; ***p<0.001.

Discussion

Data from our clinical and functional analyses consistently demonstrate that miR-205 is a crucial driver of NSCLC tumorigenesis. In particular, this miRNA is markedly upregulated in the primary tumors, and exhibited a strong association with tumor subtype. In line with these observations, there is a strong negative correlation between miR-205 and Smad4 expression in tumor tissues. Moreover, our data show that the promoter of miR-205 in NSCLC is prone to DNA methylation, particularly the -77CpG site. The P value of negative association between miR-205 and the methylation level of -77CpG site in its promoter is bigger than 0.05 because of using 10 data points only. In addition, our results provide strong evidence for the first time that miR-205 promotes tumorigenesis of NSCLC by repressing mRNA expression of Smad4.

Clinical significance of miR-205 in NSCLC cancer

Recent profiling analyses of miRNA have revealed a close link of these small RNAs to the neoplastic status of tumor tissues and patient prognosis in lung cancer [10,25-27]. Here, our study demonstrates a significant upregulation of miR-205 in NSCLC, particularly adenocarcinoma and squamous subtypes (Table 1). Intriguingly, the level of this miRNA in squamous NSCLC subtype was about 20-fold higher than adenocarcinomas (Table 1). Also, the expression of this miRNA was nearly 8-fold higher in smoking patients than the non-smokers. A similar trend in the expression of miR-205 was found for the male and female patients. These observations raise the possibility that the strong elevation of miR-205 may characterize the group of smoking male patients in our cohort. As such, our study implicates a utility of miR-205 as a biomarker for the diagnosis of such cancer patients. Despite this strong implication, additional analyses of miR-205 and a larger patient cohort at molecular, genetic, epigenetic...
and histological levels will be needed to validate these clinical connections [28-31].

Figure 4: DNA Methylation site in the promote region of miR-205 in human NSCLC tumor tissues. **A.** Schematic illustration of the -522~+271 region in miR-205 promotor. **B.** Identification of CpG islands in the promoter region of miR-205. A total of 22 pairs of NSCLC patient primary tumors and their normal counterparts were examined for the methylation status of miR-205 promoter region. **C.** Correlational analysis of promoter methylation and RNA expression for miR-205 in the paired patient tumor and normal tissue samples (T/N). X and Y axes represent log10 transformed fold change in methylation level and RNA expression, respectively.

**Molecular and functional links of miR-205 to Smad4**

One of the major finding of current study is the identification of Smad4 as a molecular target of miR-205 in human NSCLC. Several lines of our observations support this notion. Firstly, we detected a strong negative association between miR-205 level and Smad4 mRNA in our NSCLC patient cohort (Figure 1). Secondly, our cell line-based analyses indicate a direct repression of Smad4 by miR-205 (Figure 2). Thirdly, our study reveals a tumor-promoting role of miR-205 overexpression in NSCLC cells (Figure 3). To a large extent, these observations are in line with prior reports on the cellular and signaling roles of Smad4 [32,33]. In particular, the suppression of NSCLC cell proliferation by Smad4 is consistent with its pro-apoptotic and G1 growth arrest functions as well as its loss during tumor lymph node-associated metastasis across multiple cancer types [24,32,33]. Hence, besides cell proliferation, miR-205 may drive cell cycle progression or tumor metastasis in human NSCLC by directly repressing the expression of Smad4. As such, our study reveals important functional links between miR-205 and TGF-β signaling pathway for such aggressive disease.

It is worth noting that the TGF-β-mediated signaling and function are highly complex in human cancer. Notably, Smad4 knockdown decreases VEGF expression in A549 cells, while increasing the expression of TSPA protein and other miRNAs [34]. This line of observations established the relationship between Smad4 with tumor angiogenesis. On the other hand, Smad4 may confer a dual role in certain cancer types, including being a driver of late-staged tumor progression and metastasis. In fact, its tumor-promoting role has been reported for pancreatic cancer [35]. At present time, the precise mechanisms underlying the dual roles of Smad4 still remain largely unclear. Nonetheless, data from current study suggest that the complex roles of Smad4 in human NSCLC, at least in part, are attributable to the aberrant expression of miR-205.
Other putative molecular targets of miR-205 in NSCLC

While our data support a close link between miRNA-205 and Smad4 in NSCLC, we cannot rule out that this miRNA may impact other disease through other molecular mechanisms. In fact, the oncogenic function of miR-205 has recently been linked to its targeting of SHIP2, a phosphatase being shown to suppress tumor cell survival and signaling in head and neck squamous cancer [35]. Also, the expression of miR-205 has been associated with the attenuation of PTEN and PHLP2, two crucial regulators of PI3K/AKT signaling and cell proliferation in NSCLC [12]. Furthermore, there is evidence that miR-205 may have a dual role in tumorigenesis and progression. For instance, it has been reported that miR-205 suppresses cell migration/invasion by impacting the epithelial-to-mesenchymal transition (EMT) process in both human prostate and breast cancer cells [36,37]. Collectively, these observations suggest that like Smad4, miR-205 may convey multifaceted functions at different stages of NSCLC tumorigenesis and progression.

In conclusion, our clinical, molecular and functional studies demonstrate that miR-205 is up-regulated in NSCLC and promotes tumor cell proliferation via repressing the expression and function of Smad4. Also, our observations indicate that the aberrant expression of this miRNA is strongly connected to the DNA methylation of the -77 CpG site in its promoter region. In addition, our data implicate a potential connection between miR-205 and a unique subgroup of NSCLC patients, particularly the smoking male patient with squamous carcinomas. As such, our study for the first time provides key clinical and molecular roles of miR-205 as a valuable target and diagnostic biomarker for human NSCLC.

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References


