Differential MicroRNA Expression between *EGFR* T790M and L858R Mutated Lung Cancer

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Background: MicroRNAs (miRNAs) are short, non-coding RNAs that mediate post-transcriptional gene regulation. They are commonly deregulated in human malignancies, including non-small cell lung cancer (NSCLC). The aim of this study is to investigate miRNA expression in T790M-mutated NSCLC resistant to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors. **Methods:** Six cases of resected NSCLC harboring the T790M mutation were examined. We performed miRNA time polymerase chain reaction (PCR) array profiling using *EGFR* T790M-mutated NSCLC and L858R-mutated NSCLC. Once identified, miRNAs that were differentially expressed between the two groups were validated by quantitative real-time polymerase chain reaction (qRT-PCR). **Results:** miRNA PCR array profiling revealed three up-regulated miRNAs whose expression levels were altered 4.0-fold or more in the *EGFR* T790M mutation group than in the L858R group: miR-1 (fold change, 4.384), miR-196a (fold change, 4.138), and miR-124 (fold change, 4.132). The three differentially expressed miRNAs were validated by qRT-PCR, and they were found to be overexpressed in the T790M group relative to L858R group. In particular, expression levels of miR-1 and miR-124 were significantly higher in the T790M group (p-value of miR-1 = .004, miR-124 = .007, miR-196a = .096). **Conclusions:** MiR-1, miR-124, and miR-196a are overexpressed in *EGFR* T790M mutated NSCLC.

Key Words: MicroRNAs; Lung neoplasms; EGFR T790M mutation

Lung cancer is the most common cause of cancer death worldwide, and advanced-stage non-small cell lung cancer (NSCLC) is considered an incurable disease.[1] Recently, the identification of genetic alterations present in lung cancer has resulted in the development of new treatment options for NSCLC and improved survival of NSCLC patients.[2] Among these genetic alterations, the epidermal growth factor receptor (*EGFR*) activating mutation is the most widely used target for therapy. However, most patients who initially benefit from the *EGFR* tyrosine kinase inhibitor (TKI) experience subsequent disease progression, and in more than 50% of cases, the mechanism of resistance is the T790M point mutation in the *EGFR* gene.[3-5] As treatment with EGFR-TKIs has become routine for advanced lung cancer, the need to better understand the *EGFR* T790M mutation has increased.

MicroRNAs (miRNAs) are a class of short, non-coding RNAs that mediate post-transcriptional gene regulation. They are involved in nearly all biologic processes, and deregulation of miRNA is correlated with many diseases, including cancer.[6] Multiple previous studies have observed distinct patterns of miRNA expression across tumor types and revealed that up- or down-regulation of miRNA expression is indicative of a specific cancer.[7,8] Moreover, a growing number of evidence indicates that certain miRNA profiles distinguish poor-prognosis cancers, and specific miRNA signatures can predict the clinical outcomes of tumors.[9-11] Recent research has suggested that miRNAs have therapeutic capacities and can be used in cancer treatment.[12-14] All things considered, miRNAs are clinically useful biomarkers and potential therapeutic agents.

The aim of this study is to investigate the expression of miRNAs in EGFR-TKI resistant T790M mutation-positive lung cancer. For this purpose, we performed miRNA array profiling and compared miRNA expression between (1) NSCLC with the EGFR-TKI sensitive mutation (L858R) and (2) NSCLC with

the EGFR-TKI resistant mutation coexisting with the EGFR-TKI sensitive mutation (T790M/L858R). Using this method, we identified three differentially expressed miRNAs between the two groups. In this paper, we report on these differentially expressed miRNAs with a prediction of common target genes and discuss the possible role of each miRNA in the biology of lung cancer containing the *EGFR* T790M mutation.

MATERIALS AND METHODS

Sample collection

Pathology files from three institutions (Pusan National University Hospital, Pusan National University Yangsan Hospital, and Inje University Haeundae Paik Hospital) and from the time period between January 2011 and June 2016 were reviewed to identify NSCLC harboring the *EGFR* T790M mutation. After the exclusion of biopsy samples due to insufficient tumor material, six out of 1,445 lung cancer patients who had undergone surgical resection were enrolled (Pusan National University Hospital, 4 cases; Pusan National University Yangsan Hospital, 1 case; and Inje University Haeundae Paik Hospital, 1 case). All the included cases were adenocarcinomas harboring pre-existing *EGFR* T790M mutations before exposure to EGFR-TKI. All patients had coexisting TKI sensitive *EGFR* L858R point mutations, and no patients were known to have coexisting exon 19 deletion mutations. All patients underwent curative resection as their first treatment and did not have a history of *EGFR*-targeted therapy, neo-adjuvant chemotherapy, or radiation therapy. *EGFR* mutation testing was conducted as follows: Direct sequencing of the *EGFR* gene was performed in three patients (Pusan National University Hospital, 1 case; Pusan National University Yangsan Hospital, 1 case; Inje University Haeundae Paik Hospital, 1 case). At the Pusan National University Hospital, pyrosequencing was used in the case of one patient, and the peptide nucleic acid–mediated polymerase chain reaction (PCR) clamping method was used to detect the mutation in two patients. All six patients had an EGFR-TKI resistant T790M mutation and a coexisting TKI sensitive L858R mutation. For the control group, *EGFR* L858R mutant adenocarcinoma tissues from eight patients who underwent lung resection surgery were randomly selected. Additionally, four cases of *EGFR* wild-type adenocarcinoma and three non-neoplastic lung tissues were randomly selected and used as control in the miRNA array profiling. This study was approved by the institutional review board of Pusan National University Hospital (C1608-003-001), and informed consent from patients was waived.

miRNA extraction and cDNA synthesis

Hematoxylin and eosin (H&E) stained slides were prepared from routinely processed tissue sections using 10% buffered formalin and then reviewed to confirm the diagnosis. Five 10-μm sections were cut from a representative paraffin block of each tumor and mounted on glass slides. The tumor area intended for tissue dissection was marked on the unstained slides using the matched H&E stained slide; therefore, only the tumor portion was subjected to miRNA analysis. Manually dissected tissue samples were placed in 1.5 mL microcentrifuge tubes and deparaffinized in xylene. After washing with ethanol, the total miRNA of the tissue was extracted and purified using a miRNeasy formalin-fixed paraffin-embedded (FFPE) kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The concentration of each isolated miRNA was measured by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the samples that showed optimal purity and quantities were subjected to further analysis. Then, miRNA was processed using an miScript II reverse transcription kit (Qiagen) and cDNA was synthesized.

miRNA array profiling

The synthesized cDNA was used as a template for subsequent real-time PCR. The reaction mix was prepared using a miScript SYBR Green PCR Kit (Qiagen). The miRNA PCR array method was employed for comprehensive miRNA expression profiling. We used the miScript miRNA PCR Array Human Cancer Pathway Finder (MIHS-102Z, Qiagen), which is composed of a 96-well plate containing 84 human cancer specific miRNA primers and 12 controls. The selected miRNAs are known to be associated with the diagnosis, staging, progression, or prognosis of various cancers. The primers used for miRNA array profiling are described in Supplementary Table S1. Using the RT^2 PCR array loading reservoir (Qiagen), equal amounts of the reaction mix were added to each well of the array plate. Then, real-time PCR was performed in the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), and 84 miRNAs were simultaneously quantified in each sample. For miRNA PCR array profiling, we used four cases of *EGFR* T790M- and L858R-mutated adenocarcinoma as test samples and five cases of *EGFR* L858R-mutated adenocarcinoma as control samples. Additionally, four cases of *EGFR* wild-type adenocarcinoma and three non-neoplastic lung tissues were also used as controls in this array profiling.

Data analysis

The miRNA profiling data were analyzed by web-based miRNA PCR Array Data Analysis software (http://pcrdata-analysis.sabiosciences.com/mirna/arrayanalysis.php) to identify differentially expressed miRNAs between the groups. This software automatically performs fold-change calculations from the user's uploaded Ct values obtained in miRNA PCR.

Validation of differentially expressed miRNAs

We validated the differentially expressed miRNAs selected from miRNA array profiling results with a larger sample group that included six cases of T790M/L858R-mutated adenocarcinoma and eight cases of L858R-mutated adenocarcinoma. In the T790M-positive group, four out of six cases were the same cases included in the microarray profiling. In the control group without the T790M mutation, all eight cases were different from those of microarray profiling. miRNA was processed using the miRNeasy FFPE (Qiagen) and TaqMan MicroRNA reverse transcription (Applied Biosystems) kits. For the targeted quantification of up- or down-regulated miRNAs, preformulated primers included in the TaqMan MicroRNA Assays (Applied Biosystems) were used. The PCR reaction mix was prepared with TaqMan Universal Master Mix II (Applied Biosystems) and the ABI 7500 Real-Time PCR system (Applied Biosystems) was used for quantitative real-time PCR (qRT-PCR) analysis. The miRNA expression level was normalized to endogenous control gene RNU48. Following the calculation of the ΔCt values, miRNA expressions of T790M/L858R-mutated and L858R-mutated samples were compared by t-test using the statistical program R. A $p < .05$ was considered statistically significant.

Target gene prediction and gene set enrichment analysis

We evaluated common target genes of differentially expressed miRNAs using MicroRNA.org (http://www.microrna.org).[15] Gene set enrichment analysis was done with the top 10%-ranked genes in the common target gene list using MSigDB v6.0.[16]

RESULTS

Clinicopathologic factors of the study population

Six cases of *EGFR* T790M-positive and eight cases of T790M-negative pulmonary adenocarcinoma were included in the study. The clinicopathologic information of the two patient groups are described in Supplementary Tables S2 and S3. In the T790M-positive group, all patients were female (age range, 47 to 72; mean, 63). Tumor size ranged from 2.0 to 3.6 cm (mean, 2.78). Five adenocarcinomas exhibited an acinar pattern, while 1 was an adenocarcinoma with a papillary pattern. In the control group without the *EGFR* T790M mutation, six patients were female and two were male. The patients' ages ranged from 44 to 72 years (mean, 61). Mean tumor size was 2.29 cm (range, 1.4 to 4.0 cm). The predominant histologic patterns were acinar (5 cases), lepidic (2 cases), and papillary (1 case). A representative microphotograph of T790M mutated adenocarcinoma (case 5) is shown in Fig. 1.

miRNA array profiling analysis

We identified three up-regulated miRNAs whose expression levels were altered by 4.0-fold or more in *EGFR* T790M/L858R mutated lung cancer tissues compared to L858R mutated tissues (Table 1). These were miR-1 (fold change, 4.3841), miR-196a (fold change, 4.1380), and miR-124 (fold change, 4.1321). There were no down-regulated miRNAs in the T790M-positive lung cancer group. Compared to *EGFR* wild-type lung cancer, T790M/L858R mutated lung cancer was found to have 20 up-regulated miRNAs, including miR-1 and miR-196a (Table 2). No significantly differentially expressed miRNAs were identified between the L858R and *EGFR* wild-type groups. When non-neoplastic lung tissue was used as a control sample, there were seven overexpressed miRNAs in the T790M/L858R group, one of which was miR-196a (Table 3).

Comparison of miRNA expression between the T790M/L858R and L858R groups (miR-1, miR-196a, miR-124)

The expression of miR-1, miR-196a, and miR-124, which
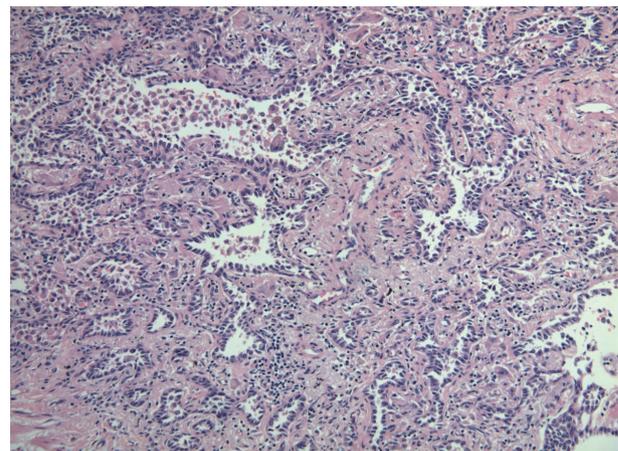
Fig. 1. Representative microphotograph of *EGFR* T790M mutated adenocarcinoma (predominantly acinar pattern of case 5). EGFR, epidermal growth factor receptor.

were found to be differentially expressed in array profiling, was validated by qRT-PCR. The three miRNAs were overexpressed in the T790M/L858R group when compared to the L858R group. In particular, the expression of miR-1 and miR-124 was significantly higher in the T790M group (p-value of miR-1 = .004, miR-124 = .007) (Fig. 2A, B). Expression of miR-196a was higher in the T790M group than in the L858R group, but not significantly so (p = .096) (Fig. 2C).

Prediction of common target genes of miR-1, miR-124, and miR-196a

We investigated putative common target genes of these three miRNAs using microRNA.org, and found 2,010 candidate genes. Then, the MSigDB web tool was used to conduct gene set enrichment analysis of the top 10%-ranked genes in the common target gene list. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that 18 gene sets were significantly enriched (false discovery rate < 0.05), including the sets of several cancer-related and signaling pathways (Supplementary Table S4).

DISCUSSION

Lung cancer is the leading cause of cancer mortality worldwide. Despite extensive efforts to improve the survival rate, the 5-year survival for NSCLC is only 21%.[1] Moreover, 57% of

Table 1. miRNAs overexpressed in *EGFR* T790M/L858R mutated lung cancer (vs L858R mutation)
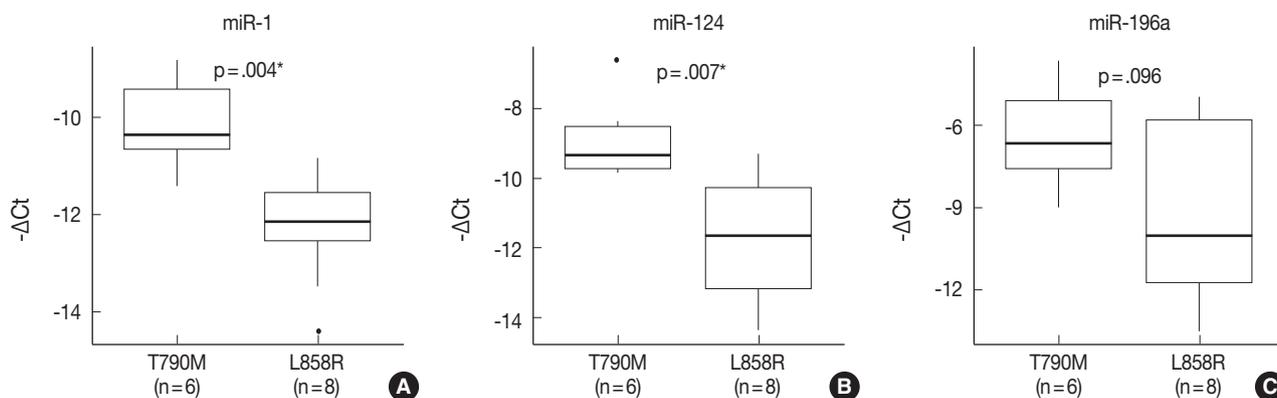
miRNA	Fold change
miR-196a	4.1380
miR-124	4.1321
miR-1	4.3841

EGFR, epidermal growth factor receptor.

Table 2. miRNAs overexpressed in *EGFR* T790M/L858R mutated lung cancer (vs *EGFR* wild type)

miRNA	Fold change
miR-196a	5.9922
miR-148b	5.0038
miR-146b	5.0653
miR-183	7.0982
miR-148a	5.7195
miR-218	6.0127
miR-29b	4.2675
miR-135b	8.4407
miR-21	7.1416
miR-10b	4.9795
miR-155	4.2678
miR-1	7.1204
miR-27b	5.0205
miR-7	6.0372
miR-9	7.0287
let-7f	5.9577
miR-10a	5.8620
miR-16	4.2105
miR-98	5.0532
miR-126	5.0032

EGFR, epidermal growth factor receptor.

Table 3. miRNAs overexpressed in *EGFR* T790M/L858R mutated lung cancer (vs non-neoplastic lung tissue)

miRNA	Fold change
miR-196a	17.5784
miR-96	4.4977
miR-183	7.3777
miR-135b	9.3675
miR-21	6.1286
miR-9	9.7106
miR-210	4.2718

EGFR, epidermal growth factor receptor.

Fig. 2. Expression of miRNAs (A, miR-1; B, miR-124; and C, miR-196a) in *EGFR* T790M and L858R groups. EGFR, epidermal growth factor receptor.

lung cancers are diagnosed at an advanced stage, with a 5-year survival rate of 4%.[1] Over the last few decades, the development of targeted agents has provided marked survival benefits to lung cancer patients whose tumors harbor specific genomic alterations.[17] In particular, agents targeting *EGFR*-activating mutations are the most widely used targeted therapy, and *EGFR* mutation analysis of NSCLC is now routine in standard clinical practice. It is well known that patients carrying a point mutation in exon 21 (L858R) or a deletion in exon 19, which account for approximately 90% of *EGFR*-activating mutations, have significant survival benefit when treated with EGFR-TKI.[18] However, the majority of patients who initially respond to EGFR-TKIs acquire resistance to the drug, with the most important mechanism of resistance being a T790M gatekeeper mutation in the *EGFR* gene.[4] This mutation leads to decreased drug binding through steric hindrance and increased binding affinity with ATP.[19,20] The *EGFR* T790M mutation is a current focus of study for many investigators attempting to overcome EGFR-TKI resistance.[21,22] Osimertinib, which was recently developed to treat patients with *EGFR* T790M mutation-positive NSCLC, is being tested in clinical trials.[23] However, the complex biology of EGFR-TKI resistance is not thoroughly understood, and new approaches are being explored.[24] We speculated that altered miRNA expression of T790M-mutated NSCLC might provide us with new insights into EGFR-TKI resistance.

miR-1 is known to be a muscle-specific miRNA that is preferentially involved in muscle development. However, many studies have also demonstrated that miR-1 functions as a tumor suppressor, regulating several oncogenic pathways in human cancers.[25,26] According to previous research, the expression of this miRNA was significantly down-regulated in several cancers, and restoration of miR-1 in cancer cells resulted in inhibition of proliferation, migration, and invasion, indicating its therapeutic potential.[27] miR-124 has also been reported to act as a tumor suppressor in human cancers. Several studies have demonstrated that this miRNA is frequently silenced by DNA methylation, and thus, plays a pivotal role in carcinogenesis. Moreover, down-regulation of miR-124 was correlated with tumor progression and its reinstatement was suggested to be a novel therapeutic strategy.[28-30] In the present study, these tumor suppressive miRNAs (miR-1, miR-124) were overexpressed in the T790M/L858R mutation group relative to the L858R mutation group. This result corresponds with previous studies indicating that the natural clinical behavior of the T790M mutated tumor is more indolent except for the poor response to EGFR-TKI therapy.[31] Some studies revealed that patients who acquired the

EGFR T790M mutation after TKI therapy had longer survival, suggesting that the T790M mutation may be related to indolent biology and slow tumor growth.[32,33] In the EURTAC (EURopean TArceva vs Chemotherapy) study, the survival of patients with a pre-existing T790M mutation was superior to that of patients without it.[34] Fujita *et al.*[35] found that presence of the T790M mutation correlated with better outcomes even in patients treated with TKI. Taken together, these data suggest that T790M mutant tumors may have indolent clinical course and the tumor suppressive miRNAs identified in the present study may contribute to this.

Most studies of miR-196a have reported that this miRNA is overexpressed in a variety of human malignancies, especially in pancreatic cancer.[36,37] It has a dominant effect on the inhibition of tumor suppressor genes, finally acting as oncogenes.[38] In this study, we found that miR-196a was up-regulated in T790M/L858R-positive tumors when compared to tumors that were only L858R-positive. We also observed that miR-196a was highly overexpressed in T790M-positive tumor tissue compared to non-neoplastic lung tissue, a finding that is consistent with the previous reports suggesting that miR-196a has an oncogenic function.

In our investigation, we also analyzed the common target genes of 3 miRNAs (miR-1, miR-124, and miR-196a) by gene set enrichment analysis. KEGG pathway enrichment analysis showed that the predicted targets were mainly enriched in cancer-related gene sets that included renal cell carcinoma, chronic myeloid leukemia, endometrial cancer, and non-small cell lung cancer, as well as in signaling pathway gene sets that included JAK-STAT, ErbB, and RIG-I-like receptor signaling pathways. Four genes (*SOS1*, *PIK3CG*, *NRAS*, and *CRKL*) were common to most of these gene sets. A review of previous studies revealed that these pathways or genes were reported to be associated with EGFR-TKI resistance. In a study using T790M-mutated NSCLC cell lines, the authors demonstrated that activation of the IL-6R/JAK1/STAT3 pathway induced *de novo* resistance to irreversible EGFR-TKIs.[39] In another study, oncogenic CRKL was shown to activate the SOS1-RAS-RAF-ERK and SRC-C3G-RAP1 pathways, and overexpression of CRKL in *EGFR*-mutant cells induced resistance to EGFR-TKI.[40] Wu *et al.*[41] reported that targeted regulation existed between miR-124 and STAT3, and up-regulation of miR-124 suppresses STAT3. It is also noteworthy that miR-124 was observed to directly target the 3'-untranslated regions of SOS1 mRNA and regulate the behavior of tumor cells.[42] These findings indicate an association between resistance to EGFR-TKI and the up-regulated miRNAs

examined in this study, and we conjecture that miR-124 regulates the expression of STAT3 or SOS1 and ultimately targets the EGFR pathway. However, miRNAs target numerous genes, and their mechanism of action is extremely complicated. Therefore, further study is needed to validate the function of these miRNAs.

In this study, we used cases in which the T790M mutation pre-existing, rather than acquired. Acquired *EGFR* T790M mutations occur in 50%–60% of patients who initially benefited from EGFR-TKIs and are the primary mechanism of resistance to therapy. In some patients, however, *EGFR* T790M mutations may exist before EGFR-TKI exposure, and these pre-existing mutations are associated with *de novo* resistance to the drugs.[43] We used samples with pre-existing T790M mutations because repeat biopsies were rarely performed for lung cancer patients whose disease progressed after treatment with EGFR-TKIs. In cases where the biopsy was repeated, only small tissue fragments or cytology specimens were included, so even after confirming the presence of an acquired T790M mutation, the biopsy tissue was inadequate for further miRNA analysis. Therefore, all the available tissues for this study were of pre-existing T790M mutants before EGFR-TKI therapy. We assumed that the fundamental action of the *EGFR* T790M mutation does not differ between cases of acquired and pre-existing mutations. According to one previous study, a minor clone with a pre-existing T790M mutation is present at a low frequency in NSCLC, and selective pressure from EGFR-TKI therapy causes expansion of these mutant cells.[44] This selection results in the emergence of a resistant phenotype in the entire tumor cell population. Therefore, the acquired T790M mutation can be considered an enriched pre-existing T790M mutation. In a certain point of view, an investigation with a *de novo* T790M mutation sample might be better than with the samples of acquired T790M mutation, especially when the researcher wants to know the innate nature of *EGFR* mutation types, since the acquired T790M mutation may be accompanied by additionally accumulated genetic alterations during EGFR-TKI therapy.

We performed miRNA PCR array profiling using NSCLC tissue, and the results showed that 3 miRNAs (miR-1, miR-124, and miR-196a) were up-regulated in the T790M mutation group compared to the L858R mutation group. The miRNA expression levels obtained with qRT-PCR were in accordance with the array profiling results. The results from this study may yield insights into the mechanisms of EGFR-TKI resistance.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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