Association between polymorphisms in microRNA target sites and survival in early-stage non-small cell lung cancer

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Introduction

MicroRNAs (miRNAs) are short (21–23 bp) non-coding RNAs that regulate the expression of complementary messenger RNAs at the posttranscriptional level.¹ miRNAs are involved in a broad range of biological processes, including development, cell proliferation, and cell death.¹ In addition, miRNAs are frequently dysregulated in various human cancers and can play critical roles in carcinogenesis as oncogenes or suppressors.² Recent studies have also suggested that single nucleotide polymorphisms (SNPs) located in miRNA target sites can influence the prognosis of diverse human cancers, including lung cancer.³–⁵

The interactions between miRNAs and their target genes are complex. One miRNA can regulate multiple target genes, and one target gene can be influenced by multiple miRNAs.⁶ In general, miRNAs repress target genes via complementary binding between the seed region (6–8 bp) at the 5’ end of the miRNA and nucleotides at the 3’-untranslated region of target genes.⁶ Several computational tools, such as TargetScan and miRanda, which are based primarily on canonical seed sites, have been developed to predict miRNA targets.⁷ However, recent studies have demonstrated the prevalence and significant effects of non-canonical miRNA-target interactions.⁸ High-throughput mapping of RNA–RNA interactions via cross-linking, ligation, and sequencing of hybrids (CLASH)
serves as an alternative method for predicting miRNA target sites, as well as non-canonical interactions.\textsuperscript{10}

We hypothesized that SNPs in miRNA target sites can influence miRNA-target interactions by altering the expression of the target genes, thereby ultimately influencing the prognosis of non-small cell lung cancer (NSCLC). To test this hypothesis, we used CLASH data to investigate the associations between SNPs in miRNA target sites and the survival outcomes of early-stage NSCLC patients.

\section*{Methods}

\subsection*{Study populations}

A total of 782 stage I, II, or IIIA NSCLC patients were enrolled in the study. Of these, 354 patients underwent curative surgical resection at the Kyungpook National University Hospital from September 1998 to August 2007, and 428 patients underwent surgery at Seoul National University Hospital between September 2005 and October 2010. All patients were of Korean ethnicity. Written informed consent was obtained before surgery. Genomic DNA was extracted from normal lung tissues or whole blood samples using the QIAamp genomic DNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The institutional review boards of Kyungpook National University Hospital (no. KNUMCBIO_11-0001) and Seoul National University Hospital (no. B-1104-125-004) approved the study.

\subsection*{Single nucleotide polymorphism selection and genotyping}

The procedure used for SNP selection was described in our previous study.\textsuperscript{11} Briefly, we searched the PolymiRTS database 3.0 (http://compbio.uthsc.edu/miRSNP) for all potentially functional polymorphisms in the miRNA target sites.\textsuperscript{12} We selected a total of 24 027 SNPs in experimentally validated miRNA target sites using CLASH data. Among these, 1574 SNPs located in cancer-related genes were selected using the CancerGenes database (http://cbio.mskcc.org/cancergenes).\textsuperscript{13} Finally, 100 SNPs were included in the final set after excluding variants with minor allele frequency <0.05 in HapMap JPT data or in linkage disequilibrium ($r^2 \geq 0.8$). Sequenom MassARRAY iPLEX assay (Sequenom Inc., San Diego, CA, USA) was used for genotyping. Approximately 5% of the samples were randomly selected and subjected to another round of genotyping by a different investigator using a restriction fragment length polymorphism assay. The results obtained using the two methods were 100% concordant.

\section*{Statistical analysis}

Overall survival (OS) was defined from the date of surgery to the date of death or the last follow-up. Disease-free survival (DFS) was measured from the date of surgery to the date of recurrence or death from any cause. OS and DFS were assessed using the Kaplan–Meier test. Hazard ratios (HRs) and 95% confidence intervals (CIs) were analyzed using multivariate Cox proportional hazard models adjusted for the following categorical variables: age (<64 years vs. $\geq$64 years), gender (female vs. male), smoking status (never vs. ever), histological type (squamous cell carcinoma vs. adenocarcinoma), pathological stage (stage I vs. stage II or IIIA), and adjuvant therapy (yes vs. no). A cut-off $P$ value of 0.05 was adopted for all statistical analyses. All statistical analyses were calculated using SAS version 9.2 (SAS Institute, Cary, NC, USA).

\section*{Results}

The patients’ clinical and pathological characteristics and their association with OS and DFS are shown in Table 1. Pathologic stage was significantly associated with both OS and DFS (log-rank $P = 2 \times 10^{-11}$ and $6 \times 10^{-18}$, respectively). Patients of younger age, female gender, and with no history of smoking had favorable rates of OS (log-rank $P = 0.01, 4 \times 10^{-4}$ and $3 \times 10^{-4}$, respectively).

Among the 100 SNPs evaluated, five SNPs were significantly associated with OS or DFS based on univariate analysis (Table 2). Multivariate analysis identified two SNPs (rs2071504 and rs2288539) that showed a significant association with survival outcome (Table 3). The RNA polymerase II subunit A (POLR2A) rs2071504 C $>$ T showed significantly worse OS and DFS under a dominant model (HR 1.42, 95% CI 1.08–1.88; $P = 0.01$ and HR 1.34, 95% CI 1.08–1.67; $P = 0.01$, respectively) (Table 3, Fig 1). Patients carrying the nuclear receptor subfamily 2 group F member 6 (NR2F6) rs2288539 TT genotype had better OS than those with rs2288539 CC or CT genotypes (HR 0.13, 95% CI 0.02–0.90; $P = 0.04$) (Table 3, Fig 1).

\section*{Discussion}

We evaluated the associations between SNPs in miRNA target sites using CLASH data and the survival outcomes of surgically resected NSCLC patients. Two SNPs (rs2071504 and rs2288539) were found to be significantly associated with OS or DFS. The use of CLASH data for miRNA target site prediction offers several advantages over previous computational prediction models, because CLASH data provides not only canonical, but also non-canonical miRNA-target interactions and non-seed base pairings outside the 5’ ends of miRNAs.\textsuperscript{10,14}
The POLR2A gene encodes the largest subunit of RNA polymerase II, the enzyme responsible for synthesizing messenger RNA in eukaryotes. POLR2A is essential for cell survival, and inhibition of POLR2A has been reported to cause extensive cell death.\(^\text{15}\) The POLR2A gene is located within 200 kb of the TP53 gene and is frequently co-deleted with TP53 in colorectal cancer.\(^\text{16}\) Liu et al. found that colorectal cancer with partial loss of POLR2A was susceptible to low-dose α-amanitin treatment, which specifically represses POLR2A.\(^\text{16}\) POLR2A suppression is known to inhibit survival of colorectal cancer cells.\(^\text{16}\) In the present study, the POLR2A rs2071504 C > T polymorphism was found to be associated with lower survival outcomes in NSCLC patients. The POLR2A rs2071504 C > T polymorphism is likely to alter the expression of POLR2A; however, the biologic mechanisms underlying the effects of POLR2A rs2071504 C > T require full investigation.

The NR2F6 gene encodes the NR2F6 protein, also known as EAR2, which acts as a co-regulator of other nuclear receptors, including the thyroid hormone and renin.\(^\text{17,18}\) EAR2 is reported to be upregulated in colorectal cancer, and EAR2 knockdown has been shown to induce cell death in colorectal cancer cells.\(^\text{19}\) In this study, the NR2F6 rs2288539 C > T polymorphism was found to be associated with better OS. However, a limitation of the present study is

### Table 1

| Variables                        | No. of cases | No. of deaths (%)† | SY-OSR (%)‡ | Log-rank P | No. of event (%)† | SY-DFS (%)‡ | Log-rank P |
|----------------------------------|--------------|--------------------|-------------|------------|-------------------|-------------|------------|
| **Overall**                      | 782          | 208 (26.6)         | 62          |            | 340 (43.5)        | 45          |
| Age (years)                      |              |                    |             |            |                   |             |            |
| ≤64                              | 346          | 80 (23.1)          | 67          | 0.01       | 146 (42.2)        | 48          | 0.21       |
| >64                              | 436          | 128 (29.4)         | 58          |            | 194 (46.5)        | 42          |
| Gender                           |              |                    |             |            |                   |             |            |
| Male                             | 572          | 173 (30.2)         | 59          | 4 × 10\(^{-4}\) | 261 (45.6)        | 42          | 0.10       |
| Female                           | 210          | 35 (16.7)          | 71          |            | 79 (37.6)         | 52          |
| Smoking status                   |              |                    |             |            |                   |             |            |
| Never                            | 232          | 40 (17.2)          | 74          | 3 × 10\(^{-4}\) | 90 (38.8)         | 50          | 0.15       |
| Ever                             | 550          | 168 (30.5)         | 57          |            | 250 (45.4)        | 43          |
| Pack-years§                      |              |                    |             |            |                   |             |            |
| <40                              | 255          | 70 (27.4)          | 59          | 0.18       | 111 (43.5)        | 43          | 0.59       |
| ≥40                              | 295          | 98 (33.2)          | 56          |            | 139 (47.1)        | 42          |
| Histological type                |              |                    |             |            |                   |             |            |
| Squamous cell carcinoma          | 341          | 103 (30.2)         | 60          | 0.17       | 146 (42.8)        | 48          | 0.22       |
| Adenocarcinoma                   | 425          | 99 (23.3)          | 63          |            | 184 (43.3)        | 42          |
| Large cell carcinoma             | 16           | 6 (37.5)           | 59          |            | 10 (62.5)         | 35          |
| Pathologic stage                 |              |                    |             |            |                   |             |            |
| I                                | 378          | 59 (15.6)          | 76          | 2 × 10\(^{-11}\) | 107 (28.3)        | 60          | 6 × 10\(^{-18}\) |
| II or IIIA                       | 404          | 149 (36.9)         | 50          |            | 233 (57.7)        | 31          |
| Adjuvant therapy                 |              |                    |             |            |                   |             |            |
| No                               | 183          | 72 (39.3)          | 49          | 0.62       | 103 (56.3)        | 30          | 0.44       |
| Yes                              | 220          | 77 (35.0)          | 50          |            | 129 (58.6)        | 26          |

†Row percentage. ‡Five-year overall survival rate (SY-OSR) and five-year disease-free survival rate (SY-DFS), proportion of survival derived from Kaplan–Meier analysis. §In ever-smokers.

### Table 2

| ID No. | Target gene | miRNA | Base change | CR (%) | MAF | HWE-\(P\) | \(P\) for overall survival | \(P\) for disease free survival |
|--------|-------------|-------|-------------|--------|-----|-----------|---------------------------|-------------------------------|
|        |             |       |             |        |     |           | Global | Dominant | Recessive | Global | Dominant | Recessive |
| rs2071504 | POLR2A | hsa-miR-652 | C>T | 98.6 | 0.21 | 0.46 | 0.04 | 0.02 | 0.81 | 0.03 | 0.02 | 0.65 |
| rs2288539 | NR2F6 | hsa-miR-196a | C>T | 98.0 | 0.19 | 0.18 | 0.01 | 0.18 | 0.03 | 0.09 | 0.23 | 0.14 |
| rs1140034 | ADCK2 | hsa-let-7b | T>C | 99.0 | 0.09 | 0.07 | 0.04 | 0.02 | 0.54 | 0.27 | 0.39 | 0.27 |
| rs2229534 | ACADS | hsa-miR-92a | G>A | 98.6 | 0.20 | 0.82 | 0.04 | 0.01 | 0.62 | 0.43 | 0.21 | 0.93 |
| rs3212986 | CD3EAP | hsa-miR-92a | G>T | 98.1 | 0.26 | 0.66 | 0.10 | 0.03 | 0.47 | 0.03 | 0.01 | 0.24 |

\(P\) values were calculated using the Kaplan–Meier test. CR, call rate; DFS, disease-free survival; HWE-\(P\), \(P\) for Hardy–Weinberg equilibrium test; MAF, minor allele frequency; miR, micro RNA; OS, overall survival.
the lack of functional validation. Validation studies in other ethnic groups are also warranted. The different recruitment periods of the two hospitals may be another limitation.

In summary, we used CLASH data to investigate the influence of SNPs in miRNA target sites on the survival outcomes of NSCLC patients. Results revealed two SNPs (POLR2A rs2071504 C > T and NR2F6 rs2288539 C > T) that were associated with clinical outcome of NSCLC. Further studies are required to confirm our findings.

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Disclosure

No authors report any conflict of interest.

References

1 Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281–97.
2 Esquela-Kerscher A, Slack FJ. Oncomirs – microRNAs with a role in cancer. Nat Rev Cancer 2006; 6: 259–69.
3 Christensen BC, Moyer BJ, Avisar M et al. A let-7 microRNA-binding site polymorphism in the KRAS 3' UTR

Table 3 OS and DFS according to genotypes in patients with non-small cell lung cancer

| Gene/SNP | Genotype† | No. of cases (%)‡ | No. of deaths (%)§ | 5Y-OSR (%)¶ | HR (95% CI)†† | P†† | No. of events (%)§ | 5Y-DFSR (%)¶ | HR (95% CI)†† | P†† |
|----------|-----------|------------------|-------------------|------------|-------------|-----|------------------|------------|-------------|-----|
| POLR2A   | CC        | 480 (62.3)       | 115 (24.0)        | 66         | 1.00        | 192 (40.0) | 50 (1.00)          |           |             |     |
|          | CT        | 261 (33.9)       | 82 (31.4)         | 55         | 1.49 (1.12–1.99) | 0.01 | 131 (50.2) | 36 (1.41–1.76) | 0.003 |
|          | TT        | 30 (3.9)         | 8 (26.7)          | 56         | 0.96 (0.47–1.98) | 0.92 | 13 (43.3) | 39 (0.52–1.61) | 0.76 |
|          | Dominant  | —                 | —                 | —          | 1.42 (1.08–1.88) | 0.01 | —                | —          | 1.34 (1.08–1.67) | 0.01 |
|          | Recessive | —                 | —                 | —          | 0.83 (0.41–1.69) | 0.61 | —                | —          | 0.81 (0.46–1.41) | 0.45 |
|          | P dominant| —                 | —                 | —          | 1.24 (0.99–1.55) | 0.06 | —                | —          | 1.19 (1.00–1.42) | 0.06 |
| NR2F6    | CC        | 501 (65.4)       | 126 (25.1)        | 64         | 1.00        | 210 (41.9) | 46 (1.00)          |           |             |     |
|          | CT        | 244 (31.9)       | 76 (31.1)         | 57         | 1.23 (0.92–1.64) | 0.17 | 126 (48.4) | 41 (1.17–1.46) | 0.19 |
|          | TT        | 21 (2.7)         | 1 (4.8)           | 95         | 0.14 (0.02–0.97) | 0.05 | 6 (28.6) | 57 (0.54–1.24) | 0.14 |
|          | Dominant  | —                 | —                 | —          | 1.12 (0.84–1.49) | 0.46 | —                | —          | 1.10 (0.88–1.38) | 0.39 |
|          | Recessive | —                 | —                 | —          | 0.13 (0.02–0.90) | 0.04 | —                | —          | 0.51 (0.23–1.15) | 0.11 |
|          | P dominant| —                 | —                 | —          | 0.98 (0.76–1.26) | 0.86 | —                | —          | 1.02 (0.84–1.24) | 0.84 |

†Patients with missing genotype data (11 for the rs2071504 and 16 for the rs2288539) were not included in the analysis. ‡Column percentage. §Row percentage. ¶Five-year overall survival rate (5Y-OSR) and five-year disease-free survival rate (5Y-DFSR), proportion of survival derived from Kaplan–Meier analysis. ††Hazard ratios (HRs), 95% confidence intervals (CIs), and corresponding P values were calculated using multivariate Cox proportional hazard models adjusted for age, gender, smoking status, tumor histology, pathologic stage, and adjuvant therapy.
is associated with reduced survival in oral cancers. 
*Carcinogenesis* 2009; 30: 1003–7.

4 Ryan BM, Robles AI, Harris CC. Genetic variation in microRNA networks: The implications for cancer research. *Nat Rev Cancer* 2010; 10: 389–402.

5 Xu J, Yin Z, Gao W et al. Genetic variation in a microRNA-502 binding site in SET8 gene confers clinical outcome of non-small cell lung cancer in a Chinese population. *PLoS ONE* 2013; 8: e77024.

6 Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell* 2003; 115: 787–98.

7 John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA targets. (Published erratum appears in *PLoS Biol* 2005; 3: e264.) *PLoS Biol* 2004; 2: e363.

8 Seok H, Ham J, Jang ES, Chi SW. MicroRNA target recognition: Insights from transcriptome-wide non-canonical interactions. *Mol Cells* 2016; 39: 375–81.

9 Kim D, Sung YM, Park J et al. General rules for functional microRNA targeting. *Nat Genet* 2016; 48: 1517–26.

10 Helwak A, Kudla G, Dudnakova T, Tollervey D. Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. *Cell* 2013; 153: 654–65.

11 Hong MJ, Lee SY, Choi JE et al. A genetic variation in microRNA target site of ETS2 is associated with clinical outcomes of paclitaxel-cisplatin chemotherapy in non-small cell lung cancer. *Oncotarget* 2016; 7: 15948–58.

12 Bhattacharya A, Ziebarth JD, Cui Y. PolymiRTS database 3.0: Linking polymorphisms in microRNAs and their target sites with human diseases and biological pathways. *Nucleic Acids Res* 2014; 42: D86–91.

13 Higgins ME, Claremont M, Major JE, Sander C, Lash AE. CancerGenes: A gene selection resource for cancer genome projects. *Nucleic Acids Res* 2007; 35: D721–6.

14 Lu Y, Leslie CS. Learning to predict miRNA-mRNA interactions from AGO CLIP sequencing and CLASH data. *PLoS Comput Biol* 2016; 12: e1005026.

15 Lindell TJ, Weinberg F, Morris PW, Roeder RG, Rutter WJ. Specific inhibition of nuclear RNA polymerase II by alpha-amanitin. *Science* 1970; 170: 447–9.

16 Liu Y, Zhang X, Han C et al. TP53 loss creates therapeutic vulnerability in colorectal cancer. *Nature* 2015; 520: 697–701.

17 Zhu XG, Park KS, Kaneshige M et al. The orphan nuclear receptor Ear-2 is a negative coregulator for thyroid hormone nuclear receptor function. *Mol Cell Biol* 2000; 20: 2604–18.

18 Liu X, Huang X, Sigmund CD. Identification of a nuclear orphan receptor (Ear2) as a negative regulator of renin gene transcription. *Circ Res* 2003; 92: 1033–40.

19 Li XB, Jiao S, Sun H et al. The orphan nuclear receptor EAR2 is overexpressed in colorectal cancer and it regulates survivability of colon cancer cells. *Cancer Lett* 2011; 309: 137–44.