

Nuclear Pore Glycoprotein 62 Genetic Variant rs9523 is Associated with Clinical Outcomes of Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors in Lung Adenocarcinoma Patients

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Introduction: Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have represented the prototype of targeted therapy in NSCLC. Patients with EGFR-mutant lung adenocarcinoma extract an extraordinary clinical benefit from EGFR-TKIs. However, the extent and duration of these responses are heterogeneous, suggesting the existence of genetic modifiers affecting an individual's response to TKIs. We investigated whether genetic variants in miRNA binding sites are associated with the clinical outcome of EGFR-TKIs in lung adenocarcinoma patients.

Methods: One hundred SNPs at miRNA binding sites in cancer-related genes were selected for the analysis using the crosslinking, ligation and sequencing of hybrids (CLASH) and CancerGenes database. qRT-PCR and luciferase assays were conducted to evaluate the functional relevance of the SNPs.

Results: *NUP62* rs9523A>G were significantly associated with worse response to EGFR-TKIs, overall survival (OS), and progression-free survival (PFS). The other three SNPs (*DVL2* rs2074216G>A, *ARF1* rs11541557G>T, and *UHRF1* rs2261988C>A) were significantly associated with worse OS and PFS. The rs9523A>G was significantly associated with decreased *NUP62* expression in tumor tissues. In addition, a significantly decreased luciferase activity was noted in *NUP62* rs9523 G allele compared to A allele.

Conclusion: Genetic variants in miRNA binding sites, especially *NUP62* rs9523A>G, may be useful in predicting the clinical outcomes of EGFR-mutant lung adenocarcinoma patients treated with EGFR-TKIs.

Keywords: lung adenocarcinoma, EGFR-TKI, clinical outcome, miRNA binding site, polymorphism

Introduction

During the last decades, pronounced development regarding cancer genomics and molecular biology has proposed a fundamental change in the paradigm of care in NSCLC, including targeted therapy and immunotherapy. Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have represented the prototype of targeted therapy in lung adenocarcinoma. EGFR-TKIs have an extraordinary effect in patients with EGFR-mutant NSCLC and prolong progression-free survival (PFS) significantly compared to conventional platinum-based chemotherapy.¹⁻⁵ However, those who initially respond to EGFR-TKIs will eventually develop

acquired resistance in approximately 12 months.^{1–5} Intensive researches have focused on the mechanisms of acquired resistance to EGFR-TKI to identify several mechanisms such as T790M gatekeeper mutation⁶ which explains the resistance in almost half of cases, mesenchymal–epithelial transition (MET) amplification,⁷ and transformation into small-cell lung cancer,⁸ among others.

Meanwhile, although most EGFR-mutant tumors exhibit dramatic initial response to EGFR-TKIs, the magnitude and duration of the responses varies considerably, suggesting the existence of genetic factors modifying an individual's response to EGFR-TKIs. Primary resistance occurs in approximately 20% of patients with EGFR-mutated NSCLC.^{1–5} Several coexisting genetic variations have been suggested for the mechanism of primary resistance to EGFR-TKIs, including *de novo* EGFR T790M mutation,⁹ MET amplification,¹⁰ PTEN loss,¹¹ KRAS mutations,¹² and germline variation such as BIM deletion polymorphism.¹³ In addition, even among the patients with EGFR mutation who achieve initial response to EGFR-TKIs, the duration of response varies widely. However, the underlying mechanism has been largely unknown.

MicroRNAs (miRNAs) play important roles in various biological functions, such as cell proliferation and survival, DNA repair, and immune response.^{14,15} Evidence indicates that miRNAs are critically involved in the development and progression of diverse human cancers.^{15,16} Studies have suggested that single nucleotide polymorphisms (SNPs) at miRNA target sites are associated with the risk and the prognosis of many types of cancer, including lung cancer.^{17–20} In contrast to the computational prediction methods for miRNA target recognition, which were developed to predict miRNA-mRNA binding based primarily on the complementarity to seed sequence, cross-linking, ligation, and sequencing of hybrids (CLASH) provided direct experimental observation of transcriptome-wide miRNA-target pairs, revealing that the interactions occurred more frequently in coding sequence than 3' UTR and the majority of miRNA-target bindings were noncanonical.²¹ Based on the important roles of miRNA network in carcinogenesis, we hypothesized that polymorphisms at miRNA target sites may influence miRNA-mRNA binding and consequently the expression of target genes, thereby influencing the clinical outcomes in EGFR-mutant lung adenocarcinoma patients who are treated EGFR-TKIs. To test this hypothesis, we selected SNPs at miRNA binding sites using CLASH data and evaluated

their association with the clinical outcome of EGFR-TKIs in EGFR-mutant lung adenocarcinoma patients.

Materials and Methods

Study Populations

In this study, 217 lung adenocarcinoma patients with available genomic DNA samples, who were treated with EGFR-TKI at Kyungpook National University Hospital (KNUH) in Daegu, Korea, between March 2007 and July 2015, were enrolled. The patients had stage III/IV or recurrent disease after surgery. Among 217 patients, 169 had positive EGFR mutation status. Since EGFR mutation analysis was not widely adopted in the early part of this period, 48 patients with unknown EGFR mutation status who had not progressed for longer than 6 months on EGFR-TKIs as a second- or further-line therapy were included in this study.²² Patients received either first-generation (erlotinib, gefitinib) or second-generation (afatinib) TKIs until disease progression, occurrence of major toxicity, or according to the patient's or physician's decision. Clinical data, including age at diagnosis, gender, smoking status, clinical staging, performance status, presence of weight loss, EGFR mutation status, were obtained retrospectively by reviewing medical records. Assessment of tumor response was performed by computed tomography, and responses were assessed using Response Evaluation Criteria in Solid Tumors.²³ The best overall response was reported and patients with a complete response (CR) or a partial response (PR) were defined as responders, and patients with stable disease (SD) or progressive disease (PD) were defined as nonresponders. Genomic DNA samples from the patients were provided by the National Biobank of Korea, KNUH, which is supported by the Ministry of Health, Welfare and Family Affairs. This study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines and was approved by the institutional review board (KNUH 2019-04-014). All patients provided written informed consent.

SNP Selection and Genotyping

Potentially functional polymorphisms at miRNA target sites were assessed using PolymiRTS database 3.0 (<http://compbio.uthsc.edu/miRSNP>),²⁴ and 24,027 SNPs at experimentally validated miRNA target sites were selected by downloading data from CLASH experiment, which has been integrated into PolymiRTS database 3.0. Among these,

1574 SNPs in cancer-related genes were selected using a list of cancer genes from the CancerGenes database (<http://cbio.mskcc.org/cancergenes>).²⁵ Finally, 100 SNPs with a minor allele frequency ≥ 0.05 in the HapMap JPT were collected after excluding those in linkage disequilibrium (LD, $r^2 \geq 0.8$). Genotyping was performed using the iPLEX[®] Assay and MassARRAY[®] System (Agena Bioscience, San Diego, CA, USA).

RNA Preparation and Quantitative Reverse Transcription-PCR (qRT-PCR)

Nucleoporin 62 (*NUP62*), disheveled 2 (*DVL2*), ADP-ribosylation factor 1 (*ARF1*), and ubiquitin-like with PHD and ring finger domains 1 (*UHRF1*) mRNA expression levels were measured by quantitative reverse transcription-PCR in tumor and corresponding normal lung tissues of lung adenocarcinoma patients who underwent surgical resection in Kyungpook National University Hospital (n = 82). Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). Real time-PCR was performed for each gene and beta-actin with QuantiFast SYBR[®] Green PCR Master Mix (Qiagen, Hilden, Germany) in a LightCycler 480 (Roche Applied Science, Mannheim, Germany) using the following primers: NUP62 forward, 5'-AGAAATCTTCCCAAGGC TGC-3'; NUP62 reverse, 5'-GTGCCTCCAAAATTAAAC CCG-3'; DVL2 forward, 5'-GCGAGTTCTTTGTGGATG TTATG-3'; DVL2 reverse, 5'-ACAATCTCCTGTATGGC AGC-3'; ARF1 forward, 5'-ACAGGAAGTGGTACATTC AGG-3'; ARF1 reverse, 5'-CACATGAGAGTAAAGCAG AGGG-3'; UHRF1 forward 5'-GAAACTCACCAACACC AACAG-3'; UHRF1 reverse, 5'-TGCTATTCTTGCCACC CTTG-3'; beta-actin forward, 5'-TTGTTACAGGAAGT CCCT.

TGCC-3'; beta-actin reverse, 5'-ATGCTATCACCTC CCCTGTGT-3'. The relative mRNA expression was normalized with beta-actin expression and then calculated by the $2^{-\Delta\Delta CT}$ method.

Cloning of the Luciferase Reports Gene and Dual Luciferase Assay

Luciferase report assay was performed to investigate whether rs9523A>G modulates the binding of miR-1914 and therefore changes the expression of *NUP62*. The psiCHECKTM-2 vector (Promega, Madison, WI, USA) was used to construct luciferase reporter plasmids. *NUP62* 3'-UTR sequence containing rs9523A or rs9523G was

synthesized by PCR from human genomic DNA and cloned into the psiCHECKTM-2 vector. The psiCHECKTM-2-*NUP62* constructs containing rs9523A>G were generated and co-transfected with miR-1914 into an *EGFR* mutant (PC9) cell line and an *EGFR* wild-type (H1299) cell line based on the manufacturer's instructions. The human lung carcinoma cell lines PC9 and H1299 were purchased from Korean Cell Line Bank (KCLB, Seoul, Korea). After the incubation period, relative *Renilla* luciferase values were measured using the firefly luciferase activities as a normalization control.

Statistical Analysis

Hardy-Weinberg equilibrium was tested using a goodness-of-fit χ^2 test with 1 degree of freedom. The genotypes for each SNP were analyzed as three-group categorical variable, and analyzed under dominant and recessive model. The association between clinical variables or genotypes and chemotherapy response was tested by odds ratio (OR) and 95% confidence intervals (CIs) using unconditional logistic regression analysis. For survival assessment, overall survival (OS) was defined as the interval between the first EGFR-TKI dose and the date of death, and progression-free survival (PFS) was defined as the duration between the initiation of EGFR-TKI and the date of objective disease progression or death. Kaplan-Meier method was used to calculate survival estimates, and the difference in OS and PFS according to different clinical variables or genotypes was compared using Log rank tests. Cox's proportional hazard regression model was used for the multivariate survival analyses. The hazard ratio (HR) and 95% CI were also estimated. A cut-off *P* value of 0.05 was adopted for all statistical analyses. Statistical data were obtained using the Statistical Analysis System for Windows, version 9.4 (SAS Institute, Cary, NC, USA).

Results

Clinical characteristics and the associations with clinical outcomes are shown in Table 1. The overall response rate of EGFR-TKIs was 84.8%, and median survival time (MST) was 35.4 months (95% CI = 30.8–39.7 months) for OS and 14.3 months (95% CI = 12.1–16.9 months). Response to EGFR-TKIs was not associated with clinical variables, such as age, gender, smoking status, stage, performance status, or weight loss. Compared with patients with *EGFR* mutation, response rate was significantly higher in those without the mutation test results, probably because only patients who experienced treatment

Table 1 Univariate Analysis for Response to Chemotherapy, Overall Survival, and Progression-Free Survival by Clinical Variables

Variables	No. of Cases	Response to Chemotherapy				Overall Survival				Progression-Free Survival			
		Responders ^a (CR+PR)	Nonresponders ^a (SD+PD)	OR (95% CI)	P	MST (months)	95% CI	HR (95% CI)	P	MST (months)	95% CI	HR (95% CI)	P
Overall	217	184 (84.8) ^a	33 (15.2)			35.4	30.8–39.7			14.3	12.1–16.9		
Age (years)													
< 64	106	92 (86.8)	14 (13.2)	1.00		36.5	30.8–41.4	1.00		12.4	11.2–15.5	1.00	
≥64	111	92 (82.8)	19 (17.1)	0.74 (0.35–1.56)	0.42	33.8	28.8–46.1	0.91 (0.64–1.29)	0.59	16.9	12.0–18.6	0.78 (0.57–1.06)	0.12
Gender													
Male	70	58 (82.9)	12 (17.1)	1.00		35.4	27.0–44.7	1.00		12.2	10.0–18.0	1.00	
Female	147	126 (85.7)	21 (14.3)	1.24 (0.57–2.69)	0.58	35.7	31.1–40.9	0.89 (0.59–1.32)	0.57	15.2	12.4–17.1	0.75 (0.53–1.04)	0.09
Smoking status													
Never	147	124 (84.4)	23 (15.7)	1.00		35.7	31.1–41.4	1.00		15.5	12.6–17.4	1.00	
Ever	70	60 (85.7)	10 (14.3)	1.11 (0.50–2.49)	0.79	35.4	26.3–45.2	1.11 (0.74–1.68)	0.61	12.1	9.6–17.0	1.30 (0.93–1.82)	0.13
Stage													
III+IV	176	150 (85.2)	26 (14.8)	1.00		34.5	29.4–38.3	1.00		12.7	11.3–15.6	1.00	
Recurred after surgery	41	34 (82.9)	7 (17.1)	0.84 (0.34–2.10)	0.71	44.7	29.3–69.2	0.58 (0.37–0.93)	0.02	20.4	14.0–24.6	0.55 (0.37–0.83)	0.005
PS ECOG													
0	61	52 (85.2)	9 (14.8)	1.00		37.5	29.3–69.2	1.00		18.3	12.9–20.4	1.00	
1–2	156	132 (84.6)	24 (15.4)	0.95 (0.42–2.18)	0.91	35.4	29.2–39.7	1.49 (0.99–2.24)	0.06	12.7	10.6–15.6	1.67 (1.17–2.39)	0.005
Wt-loss													
No	187	160 (85.6)	27 (14.4)	1.00		36.5	32.6–41.9	1.00		14.6	12.2–17.0	1.00	
Yes	30	24 (80.0)	6 (20.0)	0.68 (0.25–1.80)	0.43	24.8	19.8–39.7	2.01 (1.22–3.30)	0.006	11.2	7.4–17.4	1.32 (0.85–2.05)	0.22
mEGFR													
Not confirmed	48	46 (95.8)	2 (4.2)	1.00		36.5	29.3–42.2	1.00		10.6	9.1–12.9	1.00	
Positive	169	138 (81.7)	31 (18.3)	0.19 (0.05–0.84)	0.03	33.8	29.2–43.6	0.80 (0.56–1.16)	0.24	16.4	12.7–18.2	0.59 (0.42–0.83)	0.003
Ex 19	100	87 (87.0)	13 (13.0)	0.29 (0.06–1.35)	0.11	34.5	28.7–45.6	0.78 (0.52–1.18)	0.24	17.0	15.1–18.6	0.54 (0.37–0.78)	0.001
Ex 21	64	46 (71.9)	18 (28.1)	0.11 (0.02–0.51)	0.005	33.8	27.0–38.7	0.87 (0.55–1.39)	0.57	12.2	10.0–18.3	0.70 (0.46–1.06)	0.09
Others	5	5 (100)	0 (0.0)	-	0.98	-	-	0.00 (0.00–)	0.98	20.9	8.6–32.1	0.57 (0.18–1.84)	0.35
EGFR-TKIs type													
1st Generation ^b	176	148 (84.1)	28 (15.9)	1.00		35.4	30.7–39.4	1.00		12.9	11.4–16.3	1.00	
2nd Generation ^c	41	36 (87.8)	5 (12.2)	1.36 (0.49–3.77)	0.55	-	28.8-	0.74 (0.34–1.59)	0.44	18.2	12.2–24.1	0.77 (0.49–1.22)	0.26

Notes: ^aRow percentage. ^bCefitinib (142) + Erlotinib (34). ^cAfatinib (39) + Dacomitinib (2).

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; OR, odds ratio; MST, median survival time; CI, confidence interval; HR, hazard ratio; PS, performance status; ECOG, Eastern Cooperative Oncology Group; mEGFR, EGFR mutation; Ex 19, exon 19 deletion mutation; Ex 21, exon 21 missense mutation; TKI, tyrosine kinase inhibitor.

responses or stable disease for longer than 6 months comprised this subgroup. However, PFS was significantly better in patients with *EGFR* mutation than those without confirmed mutation status. Patients diagnosed with stage III/IV lung adenocarcinoma had worse OS and PFS compared with those with recurrent disease after surgery, suggesting tumor burden at the beginning of EGFR-TKI treatment may have affected the survival outcome.²⁶ Patients with ECOG 0 performance status had better OS and PFS than those with ECOG 1–2. Patients who experienced weight loss had worse OS than those who did not.

Among the 100 SNPs genotyped, 75 SNPs were further analyzed after excluding 2 SNPs with genotyping failure and 23 SNPs, which were deviated from the Hardy–Weinberg equilibrium ($P < 0.05$) or low call rates ($<95\%$) (Supplementary Table 1). Of the 75 SNPs analyzed, 6 SNPs were significantly associated with the response to EGFR-TKIs (Table 2), 19 SNPs with OS (Table 3), and 13 SNPs with PFS (Table 4), respectively. Among these SNPs, *NUP62* rs9523A>G were significantly associated with worse response to TKIs (adjusted odds ratio [aOR] = 0.26, 95% confidence interval [CI] = 0.11–0.64, $P = 0.003$), worse OS

Table 2 Summary of 6 SNPs and the Response to EGFR-TKIs

ID No. ^a	Target Gene	miRNA	Alleles	CR (%)	MAF	HWE-p	P for Response ^b		
							Dominant	Recessive	Codominant
rs9523	<i>NUP62</i>	hsa-miR-1914	AG	99	0.41	0.83	0.046	0.003	0.003
rs4705	<i>PDGFR</i>	hsa-miR-25	CT	100	0.47	0.62	0.028	0.046	0.010
rs11196251	<i>TCF7L2</i>	hsa-miR-324-5p	CT	99	0.26	0.10	0.371	0.045	0.112
rs1965024	<i>SALL1</i>	hsa-miR-423-5p	TC	99	0.35	0.90	0.044	0.950	0.129
rs3814026	<i>ANAPC1</i>	hsa-miR-744	TC	99	0.46	0.55	0.063	0.000	0.001
rs7091596	<i>PARD3</i>	hsa-miR-93*	AT	100	0.27	0.37	0.048	0.851	0.114

Notes: ^aInformation about SNPs and SNP ID were obtained from NCBI database (<http://ncbi.nih.gov>). ^bP values were calculated by multivariate regression analysis, adjusted for age, gender, smoking status, stage, ECOG performance status, and weight loss. *Passenger strand.

Abbreviations: CR, call rate; MAF, minor allele frequency; and HWE, Hardy–Weinberg equilibrium.

Table 3 Summary of 19 SNPs and Overall Survival

ID No. ^a	Target Gene	miRNA	Alleles	CR(%)	MAF	HWE-p	P for Overall Survival ^b		
							Dominant	Recessive	Codominant
rs9523	<i>NUP62</i>	hsa-miR-1914	AG	99	0.41	0.83	0.647	0.003	0.060
rs2074216	<i>DVL2</i>	has-miR-484	GA	96	0.36	0.54	0.211	0.003	0.016
rs11541557	<i>ARF1</i>	hsa-miR-92a	GT	100	0.08	0.18	0.004	-	0.004
rs2261988	<i>UHRF1</i>	has-miR-615-3p	CA	100	0.13	0.65	0.604	0.034	0.941
rs3212986	<i>CD3EAP</i>	hsa-miR-92a	GT	99	0.29	0.86	0.007	0.487	0.018
rs6934058	<i>CDC5L</i>	hsa-miR-505	TC	98	0.45	0.86	0.033	0.123	0.544
rs2297441	<i>RTEL1</i>	hsa-miR-615-3p	GA	98	0.31	0.86	0.795	0.001	0.138
rs7097	<i>POLR1D</i>	hsa-miR-374a*	AG	98	0.49	0.84	0.004	0.331	0.236
rs296888	<i>HNRNP</i>	hsa-miR-615-3p	CT	100	0.27	0.77	0.018	0.011	0.003
rs2295865	<i>SUPT16H</i>	hsa-miR-186	CA	98	0.13	0.31	0.025	0.976	0.015
rs3762158	<i>SUPT16H</i>	has-miR-484	GC	96	0.14	0.27	0.025	0.977	0.016
rs2228128	<i>POLR2A</i>	has-miR-744	TC	96	0.07	0.24	0.007	0.577	0.011
rs4074826	<i>HIPK2</i>	hsa-miR-423-5p	CT	99	0.17	0.21	0.027	0.649	0.047
rs3786362	<i>TYMS</i>	hsa-miR-615-3p	TC	96	0.17	0.39	0.172	0.033	0.098
rs1111667	<i>ERO1LB</i>	hsa-miR-106b*	AG	100	0.30	0.12	0.087	0.061	0.032
rs480727	<i>CDT1</i>	hsa-miR-20a	GA	100	0.28	0.12	0.017	0.651	0.157
rs1480153	<i>PPP2R2B</i>	hsa-miR-30e*	TC	96	0.46	0.86	0.100	0.018	0.017
rs12449580	<i>AIPL1</i>	has-miR-3615	CG	100	0.43	0.75	0.013	0.300	0.030
rs7081076	<i>SORBS1</i>	hsa-miR-320a	CA	100	0.12	0.57	0.283	0.048	0.166

Notes: ^aInformation about SNPs and SNP ID were obtained from NCBI database (<http://ncbi.nih.gov>). ^bP values were calculated using multivariate Cox proportional hazard models, adjusted for age, gender, smoking status, stage, ECOG performance status, and weight loss. *Passenger strand.

Abbreviations: CR, call rate; MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium.

Table 4 Summary of 13 SNPs and Progression-Free Survival

ID No. ^a	Target Gene	miRNA	Alleles	CR(%)	MAF	HWE-p	P for Progression Free Survival ^b		
							Dominant	Recessive	Codominant
rs9523	<i>NUP62</i>	hsa-miR-1914	AG	99	0.41	0.83	0.255	0.029	0.054
rs2074216	<i>DVL2</i>	has-miR-484	GA	96	0.36	0.54	0.085	0.025	0.017
rs11541557	<i>ARF1</i>	hsa-miR-92a	GT	100	0.08	0.18	<0.0001	-	<0.0001
rs2261988	<i>UHRF1</i>	has-miR-615-3p	CA	100	0.13	0.65	0.036	0.003	0.104
rs7091596	<i>PARD3</i>	hsa-miR-93*	AT	100	0.27	0.37	0.066	0.004	0.011
rs2297441	<i>RTEL1</i>	hsa-miR-615-3p	GA	98	0.31	0.86	0.033	0.143	0.021
rs1318648	<i>ESPL1</i>	hsa-miR-149	TG	100	0.28	0.24	0.236	0.011	0.048
rs40311	<i>GSPT1</i>	hsa-miR-183	GC	98	0.20	0.22	0.548	0.024	0.285
rs1569238	<i>REPS1</i>	hsa-miR-193b	GA	96	0.21	0.19	0.021	0.665	0.043
rs7195830	<i>CYBA</i>	hsa-miR-320a	GA	99	0.23	0.98	0.203	0.040	0.063
rs10467153	<i>DYRK2</i>	hsa-miR-378	TC	98	0.44	0.70	0.042	0.319	0.059
rs20554	<i>EP300</i>	hsa-miR-23b	GA	99	0.14	0.59	0.504	0.041	0.777
rs6573	<i>RAP1A</i>	hsa-let-7e	CA	97	0.05	0.49	0.046	0.372	0.071

Notes: ^aInformation about SNPs and SNP ID were obtained from NCBI database (<http://ncbi.nih.gov>). ^bP values were calculated using multivariate Cox proportional hazard models, adjusted for age, gender, smoking status, stage, ECOG performance status, and weight loss. *Passenger strand.

Abbreviations: CR, call rate; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium.

(adjusted hazard ratio [aHR] = 1.98, 95% confidence interval [CI] = 1.27–3.08, $P = 0.003$), and worse PFS (aHR = 1.59, 95% CI = 1.05–2.40, $P = 0.029$). Another three SNPs - *DVL2* rs2074216G>A, *ARF1* rs11541557G>T, and *UHRF1* rs2261988C>A - were significantly associated with worse OS (aHR = 2.19, 95% CI = 1.32–3.66, $P = 0.003$; aHR = 1.92, 95% CI = 1.24–2.97, $P = 0.004$; and aHR = 3.8, 95% CI = 1.11–13.04, $P = 0.034$, respectively) and worse PFS (aHR = 1.72, 95% CI = 1.07–2.76, $P = 0.025$; aHR = 2.5, 95% CI = 1.65–3.78, $P < 0.0001$; and aHR = 6.38, 95% CI = 1.85–22.06, $P = 0.003$, respectively) in multivariate analysis adjusted for age, gender, smoking status, stage, performance status, and weight loss (Table 5 and Figure 1). Next, we performed an exploratory analysis investigating the combined effects of the 4 SNPs. We considered the rs9523 GG, rs2074216 AA, rs11541557 GT+TT, and rs2261988 AA genotypes as bad genotypes and then evaluated their combined effects by grouping the patients based on the number of bad genotypes. Compared with the reference group that had no bad genotypes, OS and PFS decreased in a dose-dependent manner as the number of bad genotypes increased ($P_{trend} = <0.0001$ for both) and those with at least one bad genotype had HR of 2.38 for OS and 1.93 for PFS (Supplementary Table 2).

To evaluate the functional relevance of *NUP62* rs9523A>G, *DVL2* rs2074216G>A, *ARF1* rs11541557G>T, and *UHRF1* rs2261988C>A, we compared the relative expression level of *NUP62*, *DVL2*, *ARF1*, and *UHRF1* mRNA in

tumor and paired non-malignant lung tissues. The expression level of *NUP62*, *ARF1*, and *UHRF1* was significantly higher in tumor tissues than in non-malignant lung tissues ($P = 0.011$, $P = 0.044$, and $P = 2 \times 10^{-5}$, respectively), but there was no significant difference in *DVL2* expression level between tumor and normal lung tissues (Figure 2A). According to the genotypes, *NUP62* rs9523A>G was significantly related with decreased *NUP62* expression in tumor tissues ($P_{trend} = 0.016$, and $P = 0.043$ under recessive model; Figure 2B). When divided into the high and low *NUP62* expression groups based on the median expression level in tumor tissues, the survival outcome of the low *NUP62* mRNA expression group was worse than that of the high-expression group ($P = 0.019$, Figure 2C). Next, we evaluated the effect of rs9523A>G on miR-1914 binding and *NUP62* gene expression using a dual-luciferase reporter assay. psiCHECKTM-2-*NUP62* constructs containing rs9523A>G were generated and co-transfected with miR-1914 into PC9 and H1299 cells. As shown in Figure 3, the *Renilla* luciferase activity was significantly decreased in *NUP62* rs9523 G allele compared to A allele ($P = 0.004$, and $P = 0.04$, respectively). This result implicates that rs9523A>G in 3'UTR of *NUP62* gene modulates the miR-1914 binding and consequently suppresses the expression of *NUP62*.

Discussion

In the present study, we investigated whether polymorphisms in miRNA binding sites have an impact on clinical

Table 5 Genotypes of Polymorphisms and Their Associations with Clinical Outcomes of EGFR-TKIs

Polymorphism/ Genotype	Target Gene	miRNA	No. of cases (%) ^a	Response to Chemotherapy		OR (95% CI) ^c	P ^c	Overall Survival		Progression Free Survival	
				Responders n (%) ^b	Non-responders n (%) ^b			HR (95% CI) ^d	P ^d	HR (95% CI) ^d	P ^d
rs9523 ^f	NUP62 (UTR-3)	hsa-miR-1914	74(34.6) 105(49.1) 35(16.4)	68(91.9) 90(85.7) 24(68.6)	6(8.1) 15(14.3) 11(31.4)	1.00 0.51(0.18–1.46) 0.17(0.05–0.54) 0.36(0.14–0.98) 0.26(0.11–0.64) 0.40(0.22–0.73)	0.209 0.003 0.046 0.003 0.003 0.003	1.00 0.91(0.60–1.38) 1.87(1.13–3.09) 1.09(0.75–1.61) 1.98(1.27–3.08) 1.30(0.99–1.71)	0.653 0.015 0.647 0.003 0.060	1.00 1.1(0.77–1.57) 1.67(1.05–2.65) 1.22(0.87–1.71) 1.59(1.05–2.40) 1.26(1.00–1.59)	0.614 0.030 0.255 0.029 0.054
rs2074216 ^f	DVL2 (cds-synon)	hsa-miR-484	83(39.9) 100(48.1) 25(12.0)	73(88.0) 81(81.0) 21(84.0)	10(12.1) 19(19.0) 4(16.0)	1.00 0.57(0.25–1.33) 0.67(0.19–2.39) 0.59(0.26–1.33) 0.92(0.29–2.94) 0.75(0.43–1.31)	0.194 0.536 0.203 0.894 0.312	1.00 1.13(0.74–1.72) 2.36(1.33–4.19) 1.29(0.87–1.92) 2.19(1.32–3.66) 1.44(1.07–1.94)	0.578 0.003 0.211 0.003 0.016	1.00 1.25(0.87–1.78) 1.95(1.16–3.28) 1.35(0.96–1.91) 1.72(1.072.76) 1.36(1.06–1.75)	0.226 0.011 0.085 0.025 0.017
rs11541557 ^f	ARF1 (cds-non)	hsa-miR-92a	180(83.3) 36(16.7) 0(0.0)	155(86.1) 29(80.6) 0(0.0)	25(13.9) 7(19.4) 0(0.0)	1.00 0.61(0.23–1.62) - 0.61(0.23–1.62) - 0.61(0.23–1.62)	0.319 - 0.319 - 0.319	1.00 1.92(1.24–2.97) - 1.92(1.24–2.97) - 1.92(1.24–2.97)	0.004 - 0.004 - 0.004	1.00 2.5(1.65–3.78) - 2.5(1.65–3.78) - 2.5(1.65–3.78)	<0.0001 - <0.0001 - <0.0001
rs2261988 ^f	UHRF1 (cds-non)	hsa-miR-615-3p	162(75.0) 51(23.6) 3(1.4)	140(86.4) 42(82.4) 2(66.7)	22(13.6) 9(17.7) 1(33.3)	1.00 0.65(0.27–1.54) 0.35(0.03–4.27) 0.62(0.27–1.44) 0.39(0.03–4.72) 0.63(0.3–1.33)	0.325 0.411 0.262 0.461 0.225	1.00 0.82(0.53–1.27) 3.69(1.07–12.67) 0.9(0.59–1.36) 3.8(1.1–13.04) 0.99(0.67–1.46)	0.367 0.038 0.604 0.034 0.941	1.00 0.61(0.42–0.9) 5.98(1.73–20.68) 0.67(0.46–0.98) 6.38(1.85–22.06) 0.74(0.52–1.06)	0.013 0.005 0.036 0.003 0.104

Notes: ^aColumn percentage. ^bRow percentage. ^cOR, 95% CI, and their corresponding *P* values were calculated by multivariate regression analysis, adjusted for age, gender, smoking status, stage, ECOG performance status, and weight loss. ^dHRs, 95% CIs and their corresponding *P* values were calculated using multivariate Cox proportional hazard model, adjusted for age, gender, smoking status, stage, ECOG performance status, and weight loss. ^e*P*_{trend} for the additive model. ^fGenotype failure: 3 cases for rs9523, 9 for rs2074216, 1 for rs11541557, 1 for rs2261988.

Abbreviations: OR, odds ratio; CI, confidence interval; HR, hazard ratio.

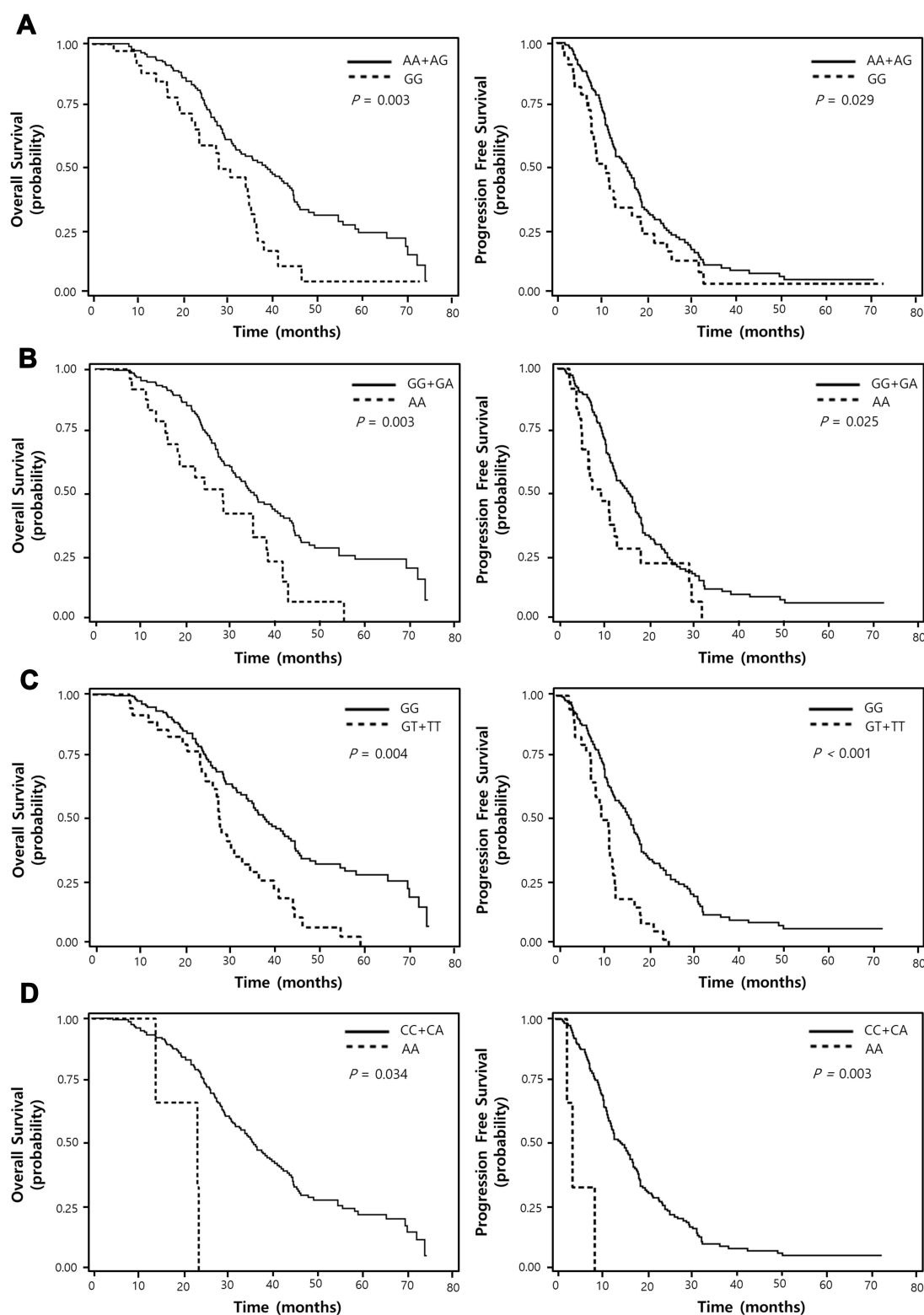


Figure 1 Overall survival and progression-free survival curves according to *NUP62* rs9523A>G (**A**), *DVL2* rs2074216 (**B**), *ARF1* rs11541557 (**C**), and *UHRF1* rs2261988 (**D**) genotypes. P values by multivariate Cox proportional hazard models.

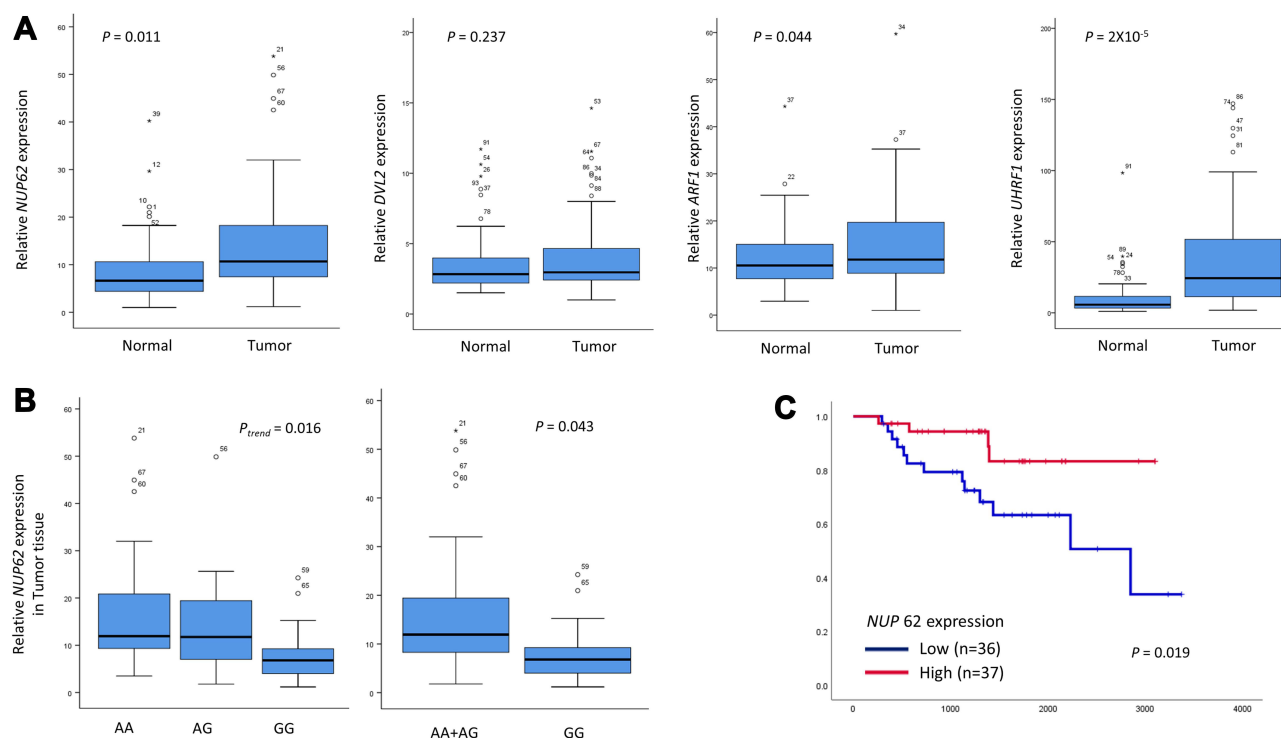


Figure 2 The mRNA expression levels of *NUP62*, *DVL2*, *ARF1*, and *UHRF1* in tumor and corresponding non-malignant lung tissues (**A**), *NUP62* mRNA expression level according to rs9523A>G genotypes (24AA, 30AG, and 14GG) in tumor tissues (**B**), and the Kaplan-Meier plot for overall survival according to the expression level of *NUP62* (**C**). The horizontal lines within the boxes represent median values; the upper and lower boundaries of the boxes represent 75th and 25th percentiles, respectively; the upper and lower bars represent the largest and smallest observed values, respectively, except outliers. Circles are the outliers, and asterisks are the extreme outliers. P values by Student's t -test, trend test, and Log rank test.

outcomes in lung adenocarcinoma patients who were treated with EGFR-TKIs. This study showed that *NUP62* rs9523A>G could predict worse response to EGFR-TKIs, PFS, and OS. In addition, *DVL2* rs2074216G>A, *ARF1* rs11541557G>T, and *UHRF1* rs2261988C>A were associated with PFS and OS. Functional analysis using clinical samples and in vitro assays supported the biological relevance of *NUP62* rs9523A>G. Those four SNPs, particularly *NUP62* rs9523A>G, may be useful in predicting the clinical outcomes in patients treated with EGFR-TKIs.

Nucleoporins are structural components of the nuclear pore complex (NPC), which regulates transport of a wide array of macromolecules including mRNA between nucleoplasm and cytoplasm. NPC also plays a role in transcriptional regulation, chromatin silencing, and DNA damage repair.²⁷ Export of mRNAs from the nucleus to the cytoplasm through NPC is a key regulatory step in protein expression.²⁸ It serves as a surveillance mechanism to sort out aberrant mRNAs, and controls translation and consequently the response to extracellular signals by permitting altered flow of specific mRNAs into the cytoplasm.²⁸ RNA export factors and NPC components

regulate the export of selected mRNAs involved in nearly all aspects of malignancy, such as survival, proliferation, metastases, and invasion.^{28,29} Aberrant mRNA export associated with altered nucleoporin expression or function has been linked to cancers.^{27,28} Nucleoporin 62 (NUP62), a protein complex that belongs to the class of nucleoporins, was highly expressed in squamous cell carcinomas, including head, neck and cervix, and was a key regulator of cell proliferation and differentiation via controlling the nuclear transport of p63.³⁰ The expression of NUP62 was notably increased in specimens of advanced prostate cancer by immunohistochemistry.³¹ However, a study showed that NUP62 expression was decreased in ovarian carcinomas and that the partial knockdown of *NUP62* confers cisplatin resistance in high-grade ovarian carcinoma cells,³² suggesting the dysregulation of nucleoporins may be cell type- and context-specific.

In this study, *NUP62* rs9523A>G was associated with worse response to EGFR-TKIs, PFS, and OS. The luciferase assay showed that rs9523A-to-G change in 3'UTR of *NUP62* led to altered binding efficiency of miR-1914, causing decreased *NUP62* mRNA expression.

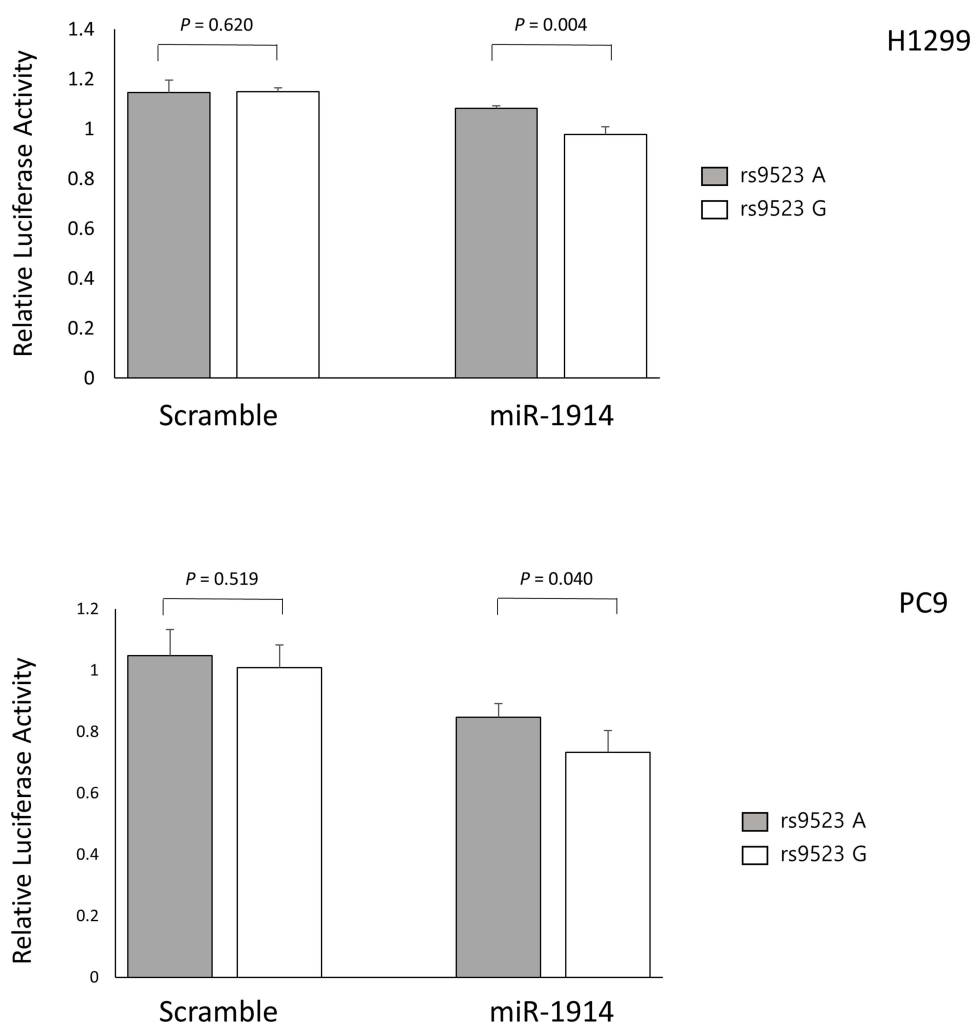


Figure 3 Functional analysis of *NUP62* rs9523A>G by dual luciferase reporter assay. *Renilla* luciferase assay for the effect of miRNA binding on rs9523A>G using H1299 and PC9 cells. *Renilla* luciferase activity was normalized to firefly luciferase activity and data are presented relative to the Mock control. Each bar represents mean \pm SE. P values by Student's *t*-test.

Consistently, *NUP62* rs9523A>G was significantly associated with decreased *NUP62* expression level in lung tumor tissues. Interestingly, the decreased *NUP62* expression in resected tumor samples is correlated with poor OS after surgery, collectively suggesting a potential tumor suppressor role of *NUP62*. Because export of mRNAs from the nucleus to the cytoplasm through NPC is a key regulatory step in protein expression, *NUP62*, a component of NPC, may play a role in regulating protein expression involved in survival, proliferation, metastases, and invasion of cancer cells in response to aberrant EGFR signals in lung cancer with activating *EGFR* mutation. Therefore, altered expression of *NUP62* may modulate the effect of EGFR-TKI. Based on our results, it can be speculated that decreased *NUP62* expression may have a negative impact on the effect of EGFR-TKI. Previous

studies suggested a potential mechanism for the association between NPC and resistance to EGFR-TKIs. It was reported that EGFR translocates from the cell surface to the nucleus through NPCs in response to EGF,^{33,34} and that nuclear localized EGFR was associated with increased resistance to anti-EGFR therapies.³⁵ Wang et al showed that down-regulation of *NUP62* expression inhibited EGF-dependent EGFR translocation,³⁴ suggesting that decreased *NUP62* expression may reduce resistance to EGFR-TKI mediated by nuclear translocation of EGFR, which seems to conflict with our results. Therefore, the molecular mechanism of the potential role of *NUP62* in the resistance to EGFR-TKIs is required to be further evaluated in the future studies.

Although most EGFR-mutant tumors exhibit dramatic initial response to EGFR-TKIs, the magnitude and

duration of the responses varies widely even among responders, leading to considerable variation in survival outcomes. The identification of patients who may experience early progression after the EGFR-TKI treatment is important for optimizing personalized therapeutic strategies. Possible mechanisms for these heterogeneous clinical outcomes include clinical characteristics,³⁶ tumor heterogeneity,³⁷ genetic variants,^{38–40} or various drug-resistance mechanisms.^{9–13} Studies reported that several genetic variants could predict clinical outcomes in patients treated with EGFR-TKIs, including polymorphisms in *EGFR* gene, TGF- β pathway genes, or BIM deletion polymorphism.^{13,38–40} Our result suggests that four genetic polymorphisms at miRNA binding sites, especially *NUP62* rs9523A>G, may be useful in predicting the PFS and OS after EGFR-TKIs. Because the duration of response to EGFR-TKIs is not predictable for individual patients even if the median PFS of 12 months from clinical trials is often referred to, the bad genotypes of those variants may be used as minor resistance factors helping the clinicians to predict the therapeutic course and to make a closer monitoring plan for disease progression. For potential clinical applicability, further studies are required to validate our findings.

Several limitations should be considered in this study. First, the EGFR mutation status was not assessed for all enrolled subjects because EGFR mutation test was not widely adopted in the early part of the enrollment period. Therefore, patients with unknown EGFR mutation status who experienced treatment responses or stable disease for longer than 6 months with EGFR-TKI as a second-line therapy were enrolled.²² However, there was no difference in genotype distribution between those with mutant EGFR and those with unknown EGFR status (data not shown). Second, because osimertinib was not available to many patients upon resistance to EGFR-TKIs, we could not analyze the role of osimertinib in patients who experienced disease progression after EGFR-TKI treatment, which could have had significant impact on overall survival.⁴¹ Third, we did not conduct experiments to confirm the difference in the efficacy of EGFR-TKIs according to the genotypes. Additional experiments such as CRISPR-Cas9 to generate PC9 cells with rs9523AA and PC9 cells with rs9523GG genotype may help reveal the different efficacy of EGFR-TKIs between A and G alleles.

In conclusion, this study shows that four SNPs in miRNA binding sites, especially *NUP62* rs9523A>G, may be useful for predicting the clinical outcomes of

EGFR-mutant lung adenocarcinoma patients treated with EGFR-TKIs. Further studies including larger population with various ethnicity are required to validate our findings.

Funding

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (NRF-2020R1A5A2017323), and by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2019R111A3A01061137).

Disclosure

The authors report no conflicts of interest in this work.

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