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BRIEF REPORT

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

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Abstract

A high-throughput mapping method of RNA–RNA interactions by crosslinking, ligation, and sequencing of hybrids (CLASH) can not only provide information about canonical but also non-canonical interactions. We evaluated the associations between variants in microRNA target sites using CLASH data and survival outcomes of 782 early-stage non-small cell lung cancer (NSCLC) patients who underwent curative surgical resection. Among the 100 variants studied, two variants showed significant association with survival outcomes. The *POLR2A* rs2071504 C > T variant was associated with poor overall and disease-free survival under a dominant model (hazard ratio [HR] 1.42, 95% confidence interval [CI] 1.08–1.88; $P = 0.01$ and HR 1.34, 95% CI 1.08–1.67; $P = 0.01$, respectively). Patients carrying the *NR2F6* rs2288539 TT genotype showed significantly better overall survival than those with the *NR2F6* rs2288539 CC or CT genotypes (HR 0.13, 95% CI 0.02–0.90; $P = 0.04$). These findings suggest that *POLR2A* rs2071504 C > T and *NR2F6* rs2288539 C > T can influence prognosis in early-stage NSCLC patients.

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.[6,7] However, recent studies have demonstrated the prevalence and significant effects of non-canonical miRNA-target interactions.[8,9] High-throughput mapping of RNA–RNA interactions via crosslinking, ligation, and sequencing of hybrids (CLASH)

serves as an alternative method for predicting miRNA target sites, as well as non-canonical interactions.[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.

Methods

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.

Single nucleotide polymorphism selection and genotyping

The procedure used for SNP selection was described in our previous study.[11] Briefly, we searched the PolymiRTS database 3.0 (http://compbio.uthsc.edu/miRSNP) for all potentially functional polymorphisms in the miRNA target sites.[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).[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.

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. ≥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).

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 6×10^{-18}, respectively). Patients of younger age, female gender, and with no history of smoking had favorable rates of OS (log-rank $P = 0.01$, 4×10^{-4} and 3×10^{-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).

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.[10,14]

Table 1 Univariate analysis for OS and DFS by age, gender, smoking status, histological type, pathologic stage, and adjuvant therapy

		Overall survival			Disease-free survival		
Variables	No. of cases	No. of deaths (%)†	5Y-OSR (%)‡	Log-rank *P*	No. of event (%)†	5Y-DFSR (%)‡	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 (5Y-OSR) and five-year disease-free survival rate (5Y-DFSR), proportion of survival derived from Kaplan–Meier analysis. §In ever-smokers.

Table 2 Five polymorphisms significantly associated with OS or DFS based on univariate analysis

							P for overall survival			*P* for disease free survival		
ID No.	Target gene	miRNA	Base change	CR (%)	MAF	HWE-*P*	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	has-miR-196a	C>T	98.0	0.19	0.18	0.01	0.18	0.03	0.09	0.23	0.14
rs1140034	ADCK2	has-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 *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.[15] The *POLR2A* gene is located within 200 kb of the *TP53* gene and is frequently co-deleted with *TP53* in colorectal cancer.[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.[16] POLR2A suppression is known to inhibit survival of colorectal cancer cells.[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.[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.[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 3 OS and DFS according to genotypes in patients with non-small cell lung cancer

			Overall survival				Disease-free survival			
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	—
rs2071504	CT	261 (33.9)	82 (31.4)	55	1.49 (1.12–1.99)	0.01	131 (50.2)	36	1.41 (1.12–1.76)	0.003
	TT	30 (3.9)	8 (26.7)	56	0.96 (0.47–1.98)	0.92	13 (43.3)	39	0.91 (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_{trend}	—	—	—	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	—
rs2288539	CT	244 (31.9)	76 (31.1)	57	1.23 (0.92–1.64)	0.17	126 (48.4)	41	1.17 (0.93–1.46)	0.19
	TT	21 (2.7)	1 (4.8)	95	0.14 (0.02–0.97)	0.05	6 (28.6)	57	0.54 (0.24–1.22)	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_{trend}	—	—	—	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.

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.

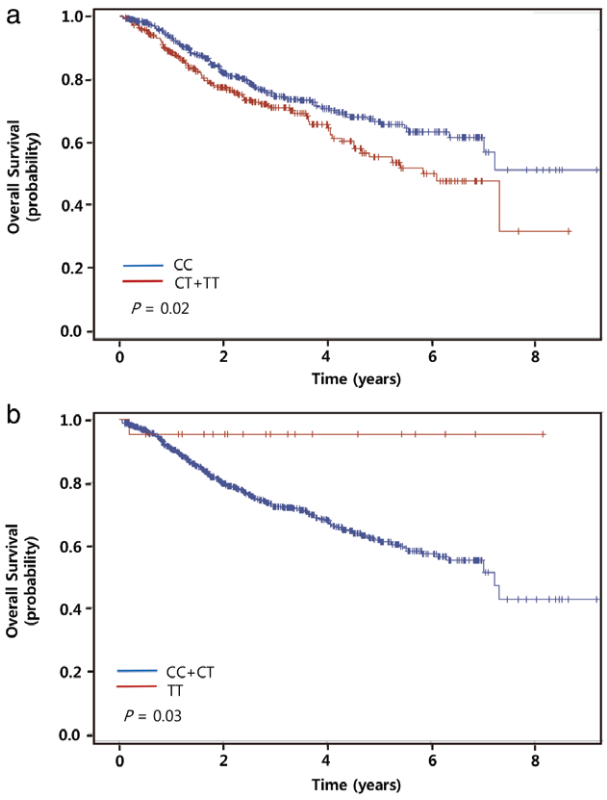

Figure 1 Kaplan–Meier plot of overall survival according to (**a**) *POLR2A* rs2071504 C > T and (**b**) *NR2F6* rs2288539 C > T genotypes. Log-rank *P* in univariate analysis.

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.

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