

Single nucleotide polymorphisms of the haptoglobin gene in non-small cell lung cancer treated with personalized peptide vaccination

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Abstract. The present study analyzed polymorphisms of the 5' flanking region (from nt -840 to +151) of the haptoglobin gene in 120 patients with advanced non-small cell lung cancer (NSCLC) receiving personalized peptide vaccinations. In the region, six single nucleotide polymorphisms (SNPs) were confirmed, of which two, rs5472 and rs9927981, were completely linked to each other. The minor allele frequencies of rs5472/rs9927981 and rs4788458 were higher than those of the other three SNPs. The genotype frequencies of rs5472 or rs9927981 were A/A or C/C (42.5%, n=51), A/G or C/T (40.8%, n=49), and G/G or T/T (16.7%, n=20), respectively; and those of rs4788458 were T/T (34.2%, n=41), T/C (40.0%, n=48), and C/C (25.8%, n=31). The association between polymorphism rs5472/rs9927981 and prognosis, or between rs4788458 and prognosis, was analyzed further. However, no correlation was found between these SNPs and overall survival, regardless of subgroup analysis of gender, histology or concurrent therapy. These results suggest that the polymorphisms rs5472/rs9927981 and rs4788458 are not useful prognostic tools for patients with NSCLC treated with personalized peptide vaccination.

Introduction

Lung cancer is the most commonly diagnosed cancer in the world and a leading cause of cancer-associated mortality (1). Diagnoses of non-small cell lung cancer (NSCLC) make up ~80% of all lung cancer cases (2). Surgery is the standard treatment modality for patients with early stage NSCLC, however, this may not be appropriate for the majority of patients with NSCLC (>65%) who have already progressed to the advanced stages (stages IIIb/IV) at the time of diagnosis (3).

Improved knowledge of tumor biology has facilitated the development of molecular targeted therapies, including those against epidermal cell growth factor receptor mutations and the abnormal fusion of anaplastic lymphoma kinase, which has improved prognoses for patients with advanced NSCLC (4,5). However, prognosis remains inadequate and new treatment modalities are required.

Cancer immunotherapies have attracted great interest as promising novel therapeutic modalities for NSCLC. The personalized peptide vaccine (PPV), which was developed by the authors of the current study, is one such approach. PPV is designed to boost the patient's anti-cancer immunity by administering vaccine peptides matched to the patient's human leukocyte antigen (HLA)-A types and selected based on the pre-existing immunity of the patient prior to vaccination (6). PPV vaccine peptides are cytotoxic T-lymphocyte (CTL) epitopes derived from a number of tumor-associated antigens. The current list of 31 vaccine peptides consists of 12, 9, 14, and 4 epitopes for HLA-A2, -A3 super-type (A3, A11, A31, or A33), -A24 and -A26, respectively (several epitopes are recognized by >1 type of HLA allele) (6,7). Patients were subcutaneously injected weekly or bi-weekly with ISA51VG along with ≤4 peptides selected from the 31 peptide candidates. Clinical studies of small cell and non-small cell lung cancers have indicated that PPV does not cause severe side effects but demonstrates potential immunological efficacy to some extent (8,9). However, only a proportion of patients experience clinical benefits from such vaccines. Therefore, it is crucial to identify appropriate biomarkers to predict overall

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Abbreviations: CTL, cytotoxic T-lymphocyte; Hp, haptoglobin; MST, median survival times; NSCLC, non-small cell lung cancer; OS, overall survival; PPV, personalized peptide vaccine; SNPs, single nucleotide polymorphisms

Key words: haptoglobin, SNPs, lung cancer, peptide vaccine, prognosis

Table I. Characteristics of 120 patients with advanced non-small cell lung cancer treated with personalized peptide vaccines.

Characteristic	Total	rs5472 (rs9927981)			rs4788458		
		A/A (C/C) n=51	A/G (C/T) n=49	G/G (T/T) n=20	T/T n=41	T/C n=48	C/C n=31
OS, days	120						
Median	674	667	646	1182	692	639	753
(95% CI)	(452-870)	(357-870)	(361-997)	(231-NR)	(411-1200)	(302-996)	(234-NR)
Age, years	120						
Median	63	63	66	61.5	63	66.5	62
(Range)	(29-84)	(29-84)	(42-84)	(37-82)	(29-84)	(42-84)	(37-82)
Gender							
Female/male	59/61	27/24	24/25	8/12	21/20	23/25	15/16
Stage							
IIIB	12	5	4	3	4	5	3
IV	66	27	28	11	22	24	20
Recurrence	42	19	17	6	15	19	8
Histology							
Adeno	100	44	41	15	38	39	23
SCC	15	6	6	3	3	6	6
AdSq	2	1	1	0	0	2	0
LCC	2	0	1	1	0	1	1
Pleomorph	1	0	0	1	0	0	1
Pre-vaccination regimen							
0-2	66	28	26	12	23	25	18
≥3	54	23	23	8	18	23	13

OS, overall survival; CI, confidence interval; Adeno, adenocarcinoma; SCC, squamous cell carcinoma; AdSq, adenosquamous carcinoma; LCC, large cell carcinoma; Pleomorph, pleomorphic carcinoma; NR, not reached.

survival (OS). It has been suggested that PD-1⁺ T-cell subsets in the peripheral blood may be developed as a prognostic marker for NSCLC patients treated with PPV (10). However, its prediction of patient prognosis remains insufficient and novel effective biomarkers are required.

Haptoglobin (Hp) is a plasma protein able to bind with high affinity to the free hemoglobin released from destroyed erythrocytes (10,11). It contributes to the salvaging and recycling of hemoglobin and to antioxidation against hemoglobin-stimulated lipid peroxidation (11,12). High levels of plasma Hp have been observed in a number of malignancies (13-18). In patients with NSCLC, plasma Hp levels are higher than those of healthy donors and correlate with aspects of disease progression, including clinical stage (19,20). The presence of single nucleotide polymorphisms (SNPs) in the 5' flanking region of the Hp gene has been reported and some SNPs may affect plasma Hp levels (21,22). However, to the best of our knowledge, an association between SNPs and the prognosis of patients with malignant diseases has not yet been reported.

The present study aimed to analyze the allele/genome frequency of six SNPs of the 5' flanking region of Hp genes in patients with NSCLC and investigate any associations Hp SNPs and patient prognosis.

Materials and methods

Clinical samples. A total of 120 patients with advanced NSCLC were subjected to analyses in the current study, which was conducted with the approval of the Kurume University Ethics Committee (Kurume, Japan). Patients were enrolled in phase II studies of PPV between December 2008 and January 2014 (clinical trial registry ID: UMIN1839 and UMIN2984) at Cancer Vaccine Center at Kurume University Medical Center; Department of Surgery, Kurume University Hospital; and Department of Pulmonary Medicine, Sendai Kousei Hospital. The designs of the clinical trials have been described in a previous report (9). Peripheral blood samples were obtained prior to and following one vaccination cycle that consisted of 6 or 8 peptide vaccinations. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient separation, frozen and stored in the liquid nitrogen until later use.

Single nucleotide polymorphism analysis. The Hp polymorphism was determined by sequencing polymerase chain reaction (PCR) products of genomic DNA. Genomic DNA was extracted from PBMCs of 120 patients enrolled with a NucleoSpin® Blood kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. The target template was amplified

Table II. SNPs of the 5' flanking region of the haptoglobin gene.

SNPs	Position from transcription start	Alleles	Allele frequency	Genotypes	n (Total=120)	Genotype frequency
rs5467	-242	C	0.9167	C/C	102	0.85
		T	0.0833	C/T	16	0.1333
				T/T	2	0.0167
rs5468	-191	T	0.9708	T/T	115	0.9583
		G	0.0292	T/G	3	0.025
				G/G	2	0.0167
rs190482744	-102	A	0.9917	A/A	118	0.9833
		G	0.0083	A/G	2	0.0167
				G/G	0	0
rs5472	-55	A	0.6292	A/A	51	0.425
		G	0.3708	A/G	49	0.4083
				G/G	20	0.1667
rs9927981	+131	C	0.6292	C/C	51	0.425
		T	0.3708	C/T	49	0.4083
				T/T	20	0.1667
rs4788458	+154	T	0.5417	T/T	41	0.3417
		C	0.4583	T/C	48	0.4
				C/C	31	0.2583

SNPs, small nuclear polymorphisms.

using KOD-Plus (TOYOBO, Osaka, Japan) with the following thermocycling conditions: Initial denaturation at 94°C for 2 mins, followed by 35 cycles of 15 sec denaturation at 94°C, the 30 sec annealing at 56°C, and the one min elongation at 68°C, and the 7 min final extension at 68°C. A set of primers, forward 5'-TCAGTGTCACTGATTATCCA-3' and reverse, 5'-GAT TTAACACACTAAGCCCTTG-3', amplified a 991 bp DNA fragment, consisting of the 5'-flanking region [840 bp upstream from the transcriptional start site (+1)], the starting codon, and the first intron ≤151 bp downstream from the transcriptional start site of the Hp gene. The PCR products were purified by the NucleoSpin Gel PCR Clean-up kit (Macherey-Nagel) and subjected to sequence analyses performed by Eurofins Genomics (Tokyo, Japan). In addition to the PCR primers named above, a third forward primer, 5'-GCAATAGGGAGATGCCACA-3' was used for the sequence analyses. The section of the current study regarding human genome analysis was approved by the Ethics Committee of Kurume University.

Statistical analysis. Possible associations between patient prognoses and the SNPs were examined using survival curves constructed by the Kaplan-Meier method and evaluated by the log-rank test. JMP software ver. 11 (SAS Institute, NC) was used for all analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristics. The characteristics of the 120 patients with advanced (stages IIIb and V) or recurrent NSCLC

receiving PPV treatment are presented in Table I. There were 59 females and 61 males, with a median age of 63 years (range, 29-84 years) at the time of enrollment. The clinical stages of the patients were determined according to the TNM classification of malignant tumors (7th ed.) provided by Union for International Cancer Control, and were as follows: 12 cases in stage IIIb, 66 in stage IV, and 42 with recurrence. Tumor histology consisted of adenocarcinoma (n=100), squamous cell carcinoma (n=15), adenosquamous carcinoma (n=2), large cell carcinoma (n=2) and pleomorphic carcinoma (n=1). By the time PPV treatment began, 111 of the 120 patients had failed ≥1 chemotherapy regimen: 25, 32, 19, and 35 patients had failed 1, 2, 3, or 4-12 regimens, respectively. A total of 9 patients were naïve to any chemotherapy regimens prior to treatment with PPV.

Polymorphism of 5' flanking region of haptoglobin gene. A total of six SNPs were identified from the genomic DNA samples of NSCLC patients: rs5467 (-242C>T), rs5468 (-191T>G), rs190482744 (-102A>G), rs5472 (-55A>G), rs9927981 (+131C>T) and rs4788458 (+154T>C). Minor alleles of rs5467, rs5468, and rs190482744 were rarely detected. By contrast, those of rs5472, rs9927981, and rs4788458 were abundant. The detected number and frequency of each allele and genotype of the SNPs are presented in Table II. Allele frequencies of rs5472A>G and rs9927981C>T were the same and all patients with rs5472A possessed the rs9927981C allele. These results suggest that the rs5472 and rs9927981 are completely linked to each other. By contrast, no such linkage was observed between rs4788458 and rs5472/rs9927981.

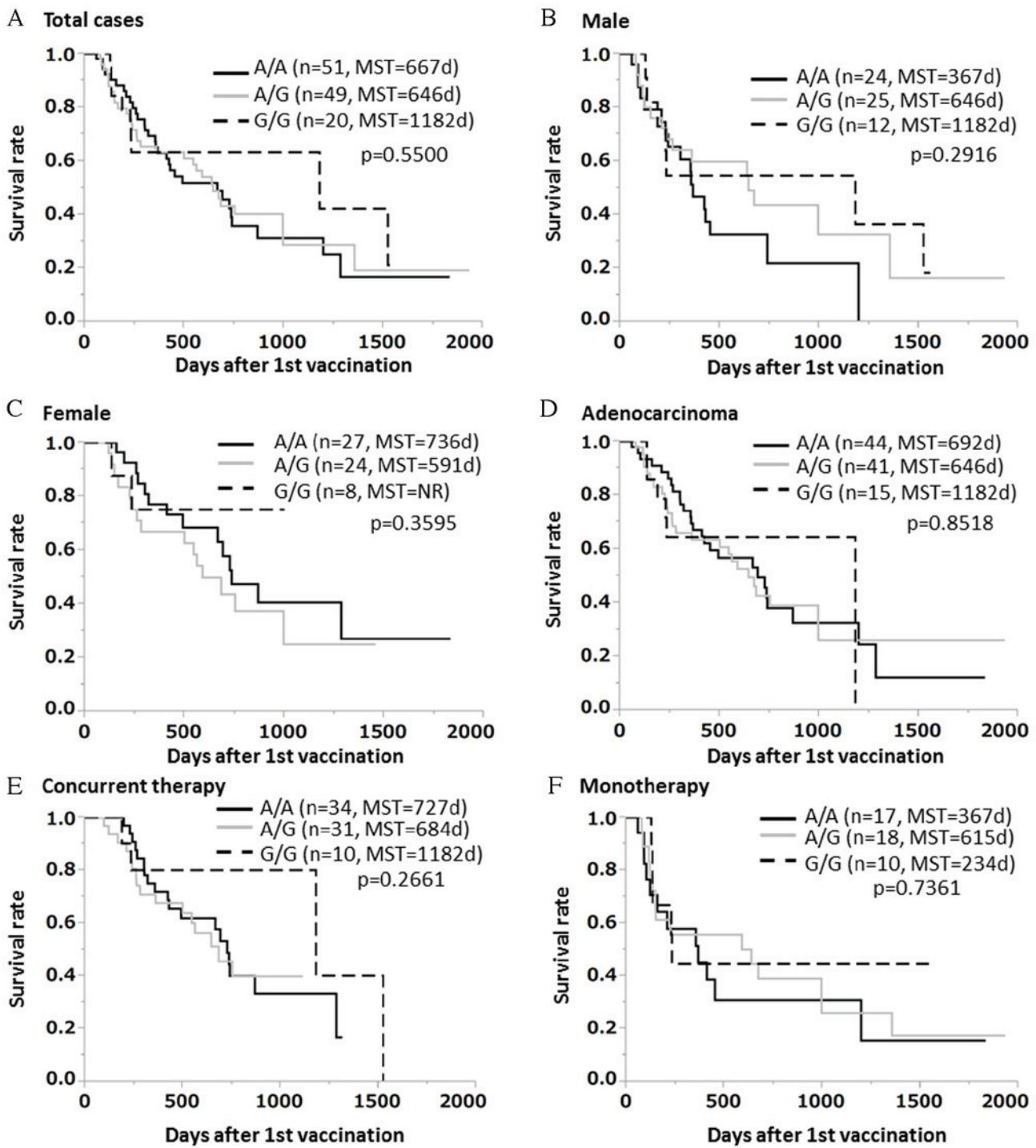


Figure 1. Hp SNP rs5472/rs9927981 and prognosis in NSCLC. Genotypes A/A, A/G, and G/G of rs5472 are compatible with C/C, C/T, and T/T of rs9927981 and only the genotypes of rs5472 are presented in the figures. (A) The association between SNP rs5472/rs9927981 and OS rates of the cases was analyzed by constructing a Kaplan-Meier plot using the log-rank test. Associations between SNP 5472/rs9927981 and OS were analyzed among (B) male (n=61) and (C) female (n=59) patients, (D) patients with adenocarcinoma (n=100) and patients undergoing (E) combination therapy (n=75) or (F) monotherapy (n=45). Hp, haptoglobin; SNP, small nuclear polymorphisms; NSCLC, non-small cell lung cancer; OS, overall survival.

The minor allele frequencies of rs5472/rs9927981 and rs4788458 were higher than those of the other three SNPs. The genotype frequencies of rs5472 or rs9927981 were A/A or C/C (42.5%, n=51), A/G or C/T (40.8%, n=49), and G/G or T/T (16.7%, n=20), respectively, and those of rs4788458 were T/T (34.2%, n=41), T/C (40.0%, n=48), and C/C (25.8%, n=31), respectively. The genotype frequencies of rs5472 observed in the current study are similar to those documented in a

previous report on a Japanese population (140/385: 36.4%, 197/385: 51.2%, and 48/385: 12.4%, respectively) (22), with no significant differences.

Hp SNP rs5472/rs9927981 and prognosis in NSCLC. Associations between the Hp SNPs rs5472/rs9927981 and the OS of NSCLC patients were assessed by a Kaplan-Meier plot (Fig. 1). Genotypes A/A, A/G, and G/G of rs5472 are compatible with

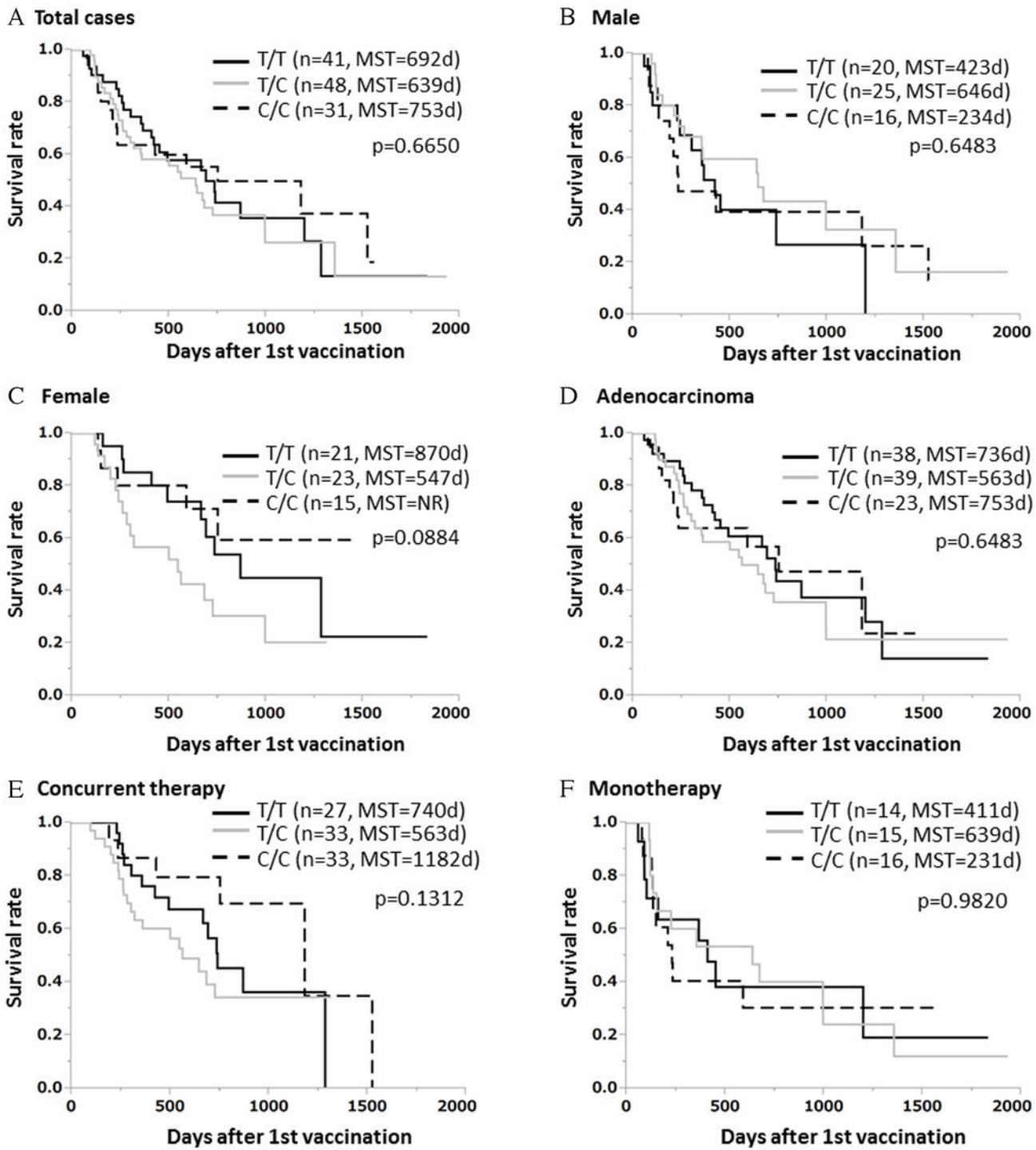


Figure 2. Hp SNP rs4788458 and prognosis in NSCLC. (A) The associations between SNP rs4788458 and OS of the total cases was analyzed by the Kaplan-Meier plot with the log-rank test. Associations between SNP rs4788458 and OS were analyzed among (B) male (n=61) and (C) female (n=59) patients, among patients with (D) adenocarcinoma (n=100) and patients undergoing (E) combination therapy (n=75) or (F) monotherapy (n=45). Hp, haptoglobin; SNP, small nuclear polymorphisms; NSCLC, non-small cell lung cancer; OS, overall survival.

C/C, C/T, and T/T of rs9927981 therefore, only results from SNP rs5472 genotype are presented in Fig. 1. The median survival times (MST) from the date of the first vaccination in the A/A, A/G, and G/G groups of rs5472 were 667, 646, and 1182 days, respectively (Fig. 1A). No significant differences in MST were observed among the genotype groups.

Subgroup analyses by gender and tumor histology type were subsequently performed. The histology type of the

majority of patients was adenocarcinoma, thus only the adenocarcinoma group (n=100) was subjected to subgroup analysis by tumor histology type. Kaplan-Meier plots for male, female, and all adenocarcinoma patients were constructed (Fig. 1B-D). Statistical analyses of the subgroups revealed no correlations between SNP rs5472/rs9927981 and OS among male, female, or adenocarcinoma patients. In addition, possible associations between rs5472/rs9927981 genotypes and OS in the

patients with bone metastasis were examined. No significant correlations, however, were observed (data not shown).

Previous studies have suggested that patients concurrently treated with PPV and chemotherapy (concurrent therapy) exhibit better prognosis compared to those treated with PPV alone (monotherapy), in castration-resistant prostate cancer, pancreatic cancer, colorectal cancer, and NSCLC (23-26). Similar results were observed in the present study; patients treated with concurrent therapy had a significantly longer MST ($n=75$, 736 days) than patients treated with PPV monotherapy ($n=45$, 367 days; $P=0.038$; data not shown). However, the OS did not differ among patients with SNP rs5472/rs9927981 treated with either concurrent therapy or PPV monotherapy (Fig. 1E and F, respectively).

Hp SNP rs4788458 and prognosis in NSCLC. The possible association between Hp SNP rs4788458 and OS was assessed (Fig. 2A). The MST of OS in the T/T, T/C, and C/C groups of rs4788458 were 692, 639, and 753 days, respectively. Similar to the case with rs5472/rs9927981, there were no significant differences among the genotype groups of rs4788458. In addition, subgroup analyses of gender, histology, or concurrent therapy were performed (Fig. 2B-F). Again, however, there were no significant differences among the genotype groups of rs4788458.

Discussion

A number of studies have investigated possible associations between the SNPs of various genes and disease susceptibility or prognosis. Correlations have previously been observed between the SNPs of the TNF- α gene, and susceptibility to human T-cell lymphotropic virus 1 uveitis (27) and rheumatoid arthritis (28). Furthermore, possible associations between SNPs and the prognosis of cancer patients have been previously investigated. In NSCLC, such correlations were demonstrated between prognosis and the SNPs of DNA excision repair 1/2 endonuclease non-catalytic subunit (29), xeroderma pigmentosum group D (30) and vesicular epidermal cell growth factor (31).

Patients with NSCLC exhibit higher plasma Hp protein levels than healthy donors and those levels correlate with aspects of disease progression, including clinical stage (19,20). Additionally, correlations between plasma Hp levels and patient prognosis have been reported in other types of cancer (32). The 5' flanking regions of genes generally control gene transcription through the binding of transcription factors. Therefore, in the current study, the SNPs of the 5' flanking region of the Hp gene were investigated, and six SNPs of the Hp gene in NSCLC were identified. Subsequently, possible correlations between the SNPs and OS in patients with NSCLC treated with PPV were investigated, but none were observed. Another research group have obtained different results in patients with castration-resistant prostate cancer treated with PPV; they observed a certain degree of correlation between Hp SNP rs5472 and OS (Miyazaki *et al.*, manuscript submitted for publication). Therefore, in the current study, further subgroup analyses of gender, histology or concurrent therapy were performed, however, no correlations were observed. At present, it is unclear why such different results were obtained in patients with NSCLC and castration-resistant

prostate cancer. It is noted that Motifmap analysis, an *in silico* analysis of transcription factor binding sites (33,34), of the Hp gene suggested that there were seven binding sites (-691, -432, -159, -148, -84, +9, and +328) for transcription factors in the 5' flanking region of the Hp gene. These sites do not include the six SNPs (-242, -191, -102, -55, +131, and +154) identified in the present study.

The present study investigated possible correlations between the SNPs of the Hp gene and immune responses to the vaccinated peptides: responses to immunoglobulin G and CTL. Again, however, no correlation was observed (data not shown).

In conclusion, the current study investigated possible correlations between Hp polymorphisms and the prognosis of advanced NSCLC patients treated with PPV. However, no correlations between the Hp SNPs and prognosis in advanced NSCLC were identified. This suggests that the SNPs of the Hp gene are not suitable biomarkers for NSCLC.

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