

Clinical Application of Next-Generation Sequencing-Based Panel to *BRAF* Wild-Type Advanced Melanoma Identifies Key Oncogenic Alterations and Therapeutic Strategies



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ABSTRACT

Molecular profiling with next-generation sequencing (NGS) has been applied in multiple solid cancers to discover potential therapeutic targets. Here, we describe the results of a clinical NGS panel in patients with advanced melanoma. Thirty-six tumor tissues from patients with *BRAF* wild-type melanoma at Seoul National University Hospital (SNUH; Seoul, Republic of Korea) were collected and deep-sequenced using the SNUH FIRST-Cancer NGS panel to assess single-nucleotide variants, small insertions/deletions, copy number variations, and structural variations to estimate tumor mutation burden (TMB). We discovered 106 oncogenic alterations and most of the patients ($n = 33$, 92%) harbored at least one oncogenic alteration, including 2 patients who were initially diagnosed as *BRAF* V600E-negative but were later confirmed to be positive. Altogether, 36 samples were classified into *RAS/BRAF/*

NFI-mutant ($n = 14$, 39%) or triple wild-type ($n = 22$, 61%) melanoma subtypes. The estimated median TMB was 8.2 mutations per Mb, ranging from 0 to 146.67 mutations per Mb. Of the 36 patients, 25 (70%) had actionable alterations with currently developed drugs, and 7 (19.4%) were enrolled in clinical trials with an RAF inhibitor, multiple receptor tyrosine kinase inhibitor, and anti-programmed cell death-1 (PD-1) antibody. TMB tended to associate with progression-free survival (PFS) of treatment with anti-PD-1/PDL-1 antibody (HR, 0.96; 95% confidence interval, 0.92–1.00; $P = 0.07$). High-TMB (≥ 13) group was associated with longer PFS than the low-TMB group (median 34.0 vs. 11.0 weeks, $P = 0.04$). Overall, the clinical use of a NGS panel in patients with advanced melanoma shows association with clinical outcomes and several therapeutic strategies.

Introduction

Advanced malignant melanoma is an aggressive and incurable disease with limited expected survival (1). However, the introduction of immune checkpoint inhibitor ipilimumab and anti-programmed cell death-1 (PD-1) antibody therapeutics expanded the median survival by as much as 3 years, and those who responded to the immunotherapy are expected to benefit even more than the median survival (2, 3). Moreover, the discovery of several molecular pathways and the development of drugs targeting those pathways have meaningful clinical benefits for patients with melanoma (4–6). Combination treatment with *BRAF* kinase inhibitor and MEK inhibitor for previously untreated advanced melanoma with *BRAF* V600E mutation showed a 1-year overall survival rate of 72%~75.5% and objective response rate (ORR) of 64%~68% (7–9). Imatinib, the *KIT* inhibitor, produced ORR of 54% for patients with *KIT*-mutant melanoma in a

phase II trial (10). In the meantime, genomic analyses of melanoma had revealed not only genetic evolutions of key alterations in melanoma but also mutation landscapes defining disparate genomic subtypes, providing clinicians with various insights on pathogenesis and clinical features (11, 12). These encouraging results led to multiple therapeutic options for advanced melanoma and increased initiation of clinical trials with the new agents. As a result, there are increasing demands to evaluate multiple genomic profiles of melanoma efficiently.

Next-generation sequencing (NGS) is a prevalent technique that has been widely used in various cancer genomics researches (13). With the development and validation of various pipelines, NGS can identify multiple target alterations effectively with reduced clinical reporting time (13). Now the NGS is becoming to take a key role in current precision oncology and enables clinicians to identify even minor actionable targets in patients with cancer and to select those patients for appropriate clinical trials in ways such as umbrella trials and basket trials (14). Furthermore, recent reports show tumor mutation burden (TMB) calculated by a comprehensive targeted genomic profiling correlates with the TMB calculated by whole-exome sequencing, which typically takes far more resources and processes than the NGS-based panel (15). This implicates that NGS-based panel may be used to predict tumor response to anti-PD-1 antibodies with TMB (15–18). Altogether, an NGS-based panel harbors significant advantage as a tool for deciding the treatment strategies for advanced melanoma. Consequently, various NGS-based platforms have been developed for melanoma and described in earlier literature (19, 20).

There exist unmet clinical needs for such comprehensive genomic profiling of advanced melanoma especially in patients without *BRAF* mutation. For example, the demographics of patients with melanoma in the Korean population are quite different from those in the Western

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population. The incidence of melanoma is lower in the Korean population than in the Western population (21). The most common histologic subtype is not superficial spreading melanoma but acral lentiginous melanoma in the Korean population (22). Furthermore, *BRAF*-activating mutation frequency observed is 15% in the Korean patients with melanoma, which is lower compared with a frequency of 50% in the Western population (23, 24). As seen in Korean population, clinical application of NGS panel is essential to provide additional treatment options for these patients with *BRAF* wild-type melanoma.

In Seoul National University Hospital (Seoul, Republic of Korea), we used an NGS panel named SNUH FIRST-Cancer panel, which assesses 225 target genes for genomic profiling of melanoma. The panel includes not only most of the genes known to be associated with melanoma (*BRAF*, *NRAS*, *NF1*, and *KIT*; ref. 12) but also genes that are potentially actionable with currently developed drugs or new drugs under clinical trials. The panel is also capable of estimating TMB, which may be used to predict response to anti-PD-1/PD-L1 antibody. This study aims to describe the results of NGS panel in patients with *BRAF* wild-type melanoma along with clinical outcomes to evaluate their potential clinical applicability.

Materials and Methods

Sample collection

Patients with recurrent or metastatic melanoma who visited Seoul National University Hospital (Seoul, Republic of Korea) and who were initially *BRAF* V600E-negative by either IHC or Sanger sequencing were enrolled in the study. DNA from the archival formalin-fixed paraffin-embedded (FFPE) tumor tissues were collected and profiled using NGS. Demographic features at the time of tissue acquisition were reviewed. Treatment history, response, and survival data were also collected. This study was approved by institutional review board (H-1803-138-933) and in accordance with the declaration of Helsinki.

DNA sequencing

Genomic DNA was extracted from FFPE tissues using the ReliaPrep™ FFPE gDNA Miniprep System (Promega). Approximately 250 to 500 ng of DNA was fragmented by Covaris Sonicator (Covaris) and the amount of fragmented DNA was measured by qPCR. The libraries were constructed using Agilent's SureSelectXT Target Enrichment protocol for Illumina paired-end sequencing. The quality and quantity of libraries were assessed by Bioanalyzer 2100 and DNA 1000 Chips (Agilent Technology). Subsequently, paired-end sequencing (2 × 101bp) was performed on the Illumina HiSeq 2500 Platform (Illumina Inc.).

Sequencing data analysis

Technical sequences (adaptors) and low-quality reads were trimmed from the raw FASTQ data by Trimmomatic software (25). The cleaned reads were aligned to the human reference genome (GRCh 37) by BWA v0.7.12 aligner (26) and preprocessed following GATK best practices.

We detected genetic alterations and estimated TMB (mutation/Mb) in patients with melanoma from deep sequencing data of custom-targeted panel and three versions of SNUH FIRST-Cancer Panel (v2, v3, and v3.1) including 225 cancer-associated genes (Supplementary Table S1). Single-nucleotide variants (SNV) were called by an in-house script to combine MuTect v1.1.7 (27) and OxoG filter algorithm (28). Small insertions and deletions (INDEL) were called by IndelGenotyper v36.3336. For functional analysis, confident mutations were annotated by ANNOVAR (29) and further filtered out to

select rare nonsynonymous mutations only. Copy number variants (CNV) were detected by Z-score-based method and CNVkit software (30). Structural variants (SV) were called by DELLY (31) and manually reviewed with the Integrative Genomic Viewer (32, 33). We estimated TMB based on predicted somatic mutations by the modification of a method by Chalmers and colleagues (15). Details of the pipeline used to identify genomic alterations and predict TMB are described in Supplementary Methods.

Oncogenic/actionable alterations

We focused on genomic alterations with impact on the treatment plan and tumor biology using clinicians' knowledge and public database OncoKB (34) along with SNVs, CNVs, and SVs reported by SNUH FIRST-Cancer panel assay. The selected genomic alterations were separated into two classes: (i) oncogenic alterations evaluated as "oncogenic" or "likely oncogenic" in OncoKB and (ii) actionable alterations defined as oncogenic alterations to predict response or resistance against treatment. To investigate the feasibility of targeted panel sequencing for melanoma, properties of oncogenic alterations in this cohort were assessed on genomic subtypes of The Cancer Genome Atlas (TCGA; ref. 35); we also observed the incidence of oncogenic alterations in key genes and pathways that were reported recently from a large-scale landscape study (12).

Treatment response

We evaluated treatment responses using RECIST criteria v1.1 (36). ORR was defined as the percentage of patients with complete response (CR) and partial response (PR). Clinical benefit rate (CBR) was defined as the percentage of patients with CR, PR, and stable disease (SD) for 6 months. Progression-free survival (PFS) was defined as the time elapsed from treatment initiation and disease progression or death. Analysis of PFS was by the Kaplan-Meier method and the HR was calculated using the Cox-proportional hazard model. Comparison of continuous values between groups was with the Mann-Whitney test. To analyze the response to an anti-PD-1/PD-L1 antibody according to estimated TMB, patients were divided into two groups; high-TMB if estimated TMB is ≥ 13 , and low-TMB if estimated TMB is < 13 . A *P* value below 0.05 was considered statistically significant. All statistical analyses were performed with R 3.4.3 software (<https://cran.r-project.org/bin/windows/base/old/3.4.3/>).

Results

Patients

Tumor tissues were collected from 36 patients with recurrent or metastatic melanoma who were candidates for systemic treatment. The primary sites of melanoma were mainly cutaneous lesions ($n = 22$, 61%), 17 of which were acral (77.3%). A majority of tissues were obtained from noncutaneous sites ($n = 26$, 72.2%) including the liver, lymph node, lung, bone, mucosa of the conjunctiva, nasal cavity, gastrointestinal, and genital tract. Thirty-one patients underwent surgical measures to obtain biopsy samples while the other 5 patients received needle biopsy. Demographic features of 36 patients with melanoma are summarized in **Table 1**.

Distribution of oncogenic alterations

The SNUH FIRST-Cancer panel sequencing was performed on 36 collected tumors to an average median read depth of $539 \times$ (range 164–1138 \times). The panel assay detected 332 SNVs, 13 INDELS, 73 CNV genes, and 4 SVs. We evaluated the effect of these alterations and considered 106 oncogenic alterations including 46 SNVs, 4 INDELS,

Table 1. Demographic features of patients and tissues.

Demographics	N (%)
Age	41–83 (median, 63)
Gender	
Male	18 (50)
Female	18 (50)
Primary site	
Acral	17 (47.2)
Mucosal	10 (27.8)
Cutaneous	5 (13.9)
Others ^a	3 (8.3)
Unknown	1 (2.8)
Stage	
Locally advanced ^b	3 (8.3)
Metastatic	33 (91.7)
Biopsy site characteristic	
Skin	10 (27.8)
Non-skin ^c	26 (72.2)
Biopsy location type	
Primary	16 (44.4)
Metastatic	20 (55.6)
Tumor fraction	
≥80%	30 (83.3)
<80%	6 (16.7) ^d
Line of treatment for anti-PD-1/anti-PD-L1	
1	15
2	10
≥3	8
Not applicable	4

^aOther primary sites include dura, testicle, and conjunctiva.

^bDefinite staging was not available in the 3 patients because primary sites were atypical (dura, conjunctiva, and rectum) without definite distant metastasis. All 3 patients experienced local recur.

^cNon-skin biopsy sites include liver, lymph node, lung, bone, mucosa of conjunctiva, nasal cavity, gastrointestinal, and genital tract.

^dSamples of 2 patients had tumor fraction of less than 50%.

and 56 CNV genes for further analysis (Supplementary Table S2 and S3). We identified TCGA subtypes classified as *RAS*-mutated, *BRAF*-mutated, *NFI*-mutated, and triple-wild-type (WT) in the Korean patients with advanced melanoma and analyzed the distribution of oncogenic alterations across those subtypes (Fig. 1A–D; ref. 35).

Of the 36 patients with melanoma, 22% ($n = 8$) harbored *NRAS* mutations including Q61R/K/L ($n = 4$), G12D/S ($n = 2$), and G13D/R ($n = 2$). In another *RAS* family, *HRAS* Q61L mutation was observed in 2 patients. *NFI* was one of the most frequently mutated tumor suppressor genes ($n = 4$, 11%) along with *TP53* in this cohort (Fig. 1B). *NFI* mutations included three stop-gain SNVs and one missense SNV (p.Y489C) known to be associated with aberrant splicing. *KIT* mutations were found only and most frequently ($n = 4$, 11%) in triple-WT subtypes. Although we excluded *BRAF*-positive samples in tumor collection step, SNUH FIRST-Cancer panel detected *BRAF* V600E mutation in 2 patients that had been previously diagnosed as negative for *BRAF* V600E mutation by IHC and Sanger sequencing. Variant allele frequencies of *BRAF* V600E in the 2 patients were 7.6% and 7.8%, respectively. One of the patients diagnosed as *BRAF* V600E–negative by Sanger sequencing was confirmed positive for *BRAF* V600E mutation with additional validation experiments using peptide nucleic acid (PNA)-mediated PCR clamping method.

In CNVs, 38 amplified genes and 18 deleted genes were shown to be oncogenic alterations in 20 patients. Cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*, $n = 8$, 22%) and the adjacent gene *CDKN2B*

($n = 7$, 19%) were frequently deleted across all subtypes. Triple-WT–subtyped patients had a significantly higher frequency of amplified genes compared with the rest of the patients ($P < 0.01$). Half of the triple-WT subtypes harbored at least one copy number amplification of a gene, which included not only *TERT* amplification ($n = 6$), known to frequently occur in melanoma but also emerging therapeutic targets such as amplification of *KRAS* ($n = 2$) and *KDR* ($n = 2$).

Overall, all patients except three (92%) harbored at least one oncogenic alteration in key genes of melanoma reported by the recent landscape study (12). In pathway analyses of those genes, we found that MAPK, cell-cycle, receptor tyrosine kinases (RTK), p53 signaling, and PI3K pathways were altered in 53%, 39%, 22%, 17%, and 11% of tumors, respectively (Fig. 1C). Furthermore, about half of the patients (47%) had alterations in multiple pathways involved in the development of melanoma (Fig. 1D).

Estimated TMB

Overall, the median estimated TMB was 8.2 mutations per Mb, ranging from 0 to 146.67 mutations/Mb. *NFI*-mutated patients showed dominance in higher estimated TMB, with 3 patients exhibiting TMB over 40 mutations/Mb including the highest TMB among the whole samples (Fig. 1A). Patients with the second highest estimated TMB (74 mutations/Mb) harbored *MSH6* T955fs mutation, which is known to affect DNA mismatch-repair (MMR; ref. 37).

Clinical application of NGS results

Twenty-five patients with melanoma (70%) had at least one actionable alteration known to serve as a biomarker for the standard of care or as a potential target for newer investigational drugs (Table 2). Seven patients were enrolled in the clinical trial based on the profiling results from NGS. Data on target gene alterations and administered medications in these patients are summarized in Table 3. Among the 7 patients, 4 had SD and PD was seen in the other 2 patients. The response of 1 patient was not evaluable because the patient died 2 weeks after initiation of trial medication. Median PFS was 12.6 weeks (range 1.9–100.4 weeks, Supplementary Fig. S1). Patient 5 harboring *NRAS* Q61K mutation had early metabolic response, as revealed by PET-CT images, after 1-month treatment with the RAF inhibitor (Fig. 2).

The patient with *BRAF* V600E confirmed by NGS and PNA-PCR clamping received dabrafenib/trametinib combination after progression from anti-PD-1 inhibitor and cytotoxic chemotherapy and experienced PR. The patient's disease has not progressed for 10 months and is currently on follow-up at the time of analysis. The other *BRAF* V600E–positive patient had not received systemic treatment after NGS because the disease was stable.

Estimated TMB and anti-PD-1/PD-L1 antibody response

Thirty-three patients received anti-PD-1/PD-L1 antibody for the treatment of advanced melanoma. ORR was 12.1% (4 PRs) and CBR was 39.4% ($N = 13$). Median PFS was 15.9 weeks [95% confidence interval (CI), 11.0–35.0], ranging from 0.4 to 138.1 weeks. Estimated TMBs tended to associate with PFS of anti-PD-1/PD-L1 antibody (HR, 0.96; 95% CI, 0.92–1.00; $P = 0.07$). Estimated TMBs in samples of patients who responded to anti-PD-1/PD-L1 antibody tended to be higher than the others (median estimated TMB 12.5 vs. 8.2, $P = 0.33$). Also, estimated TMBs in samples of patients who experienced clinical benefit was higher than in others (median estimated TMB 12.0 vs. 8.1, $P = 0.26$). With the estimated TMB cutoff of 13, which was the best value to discriminate treatment response, the high-TMB group experienced significantly longer PFS (median 34.0 weeks, 95% CI,

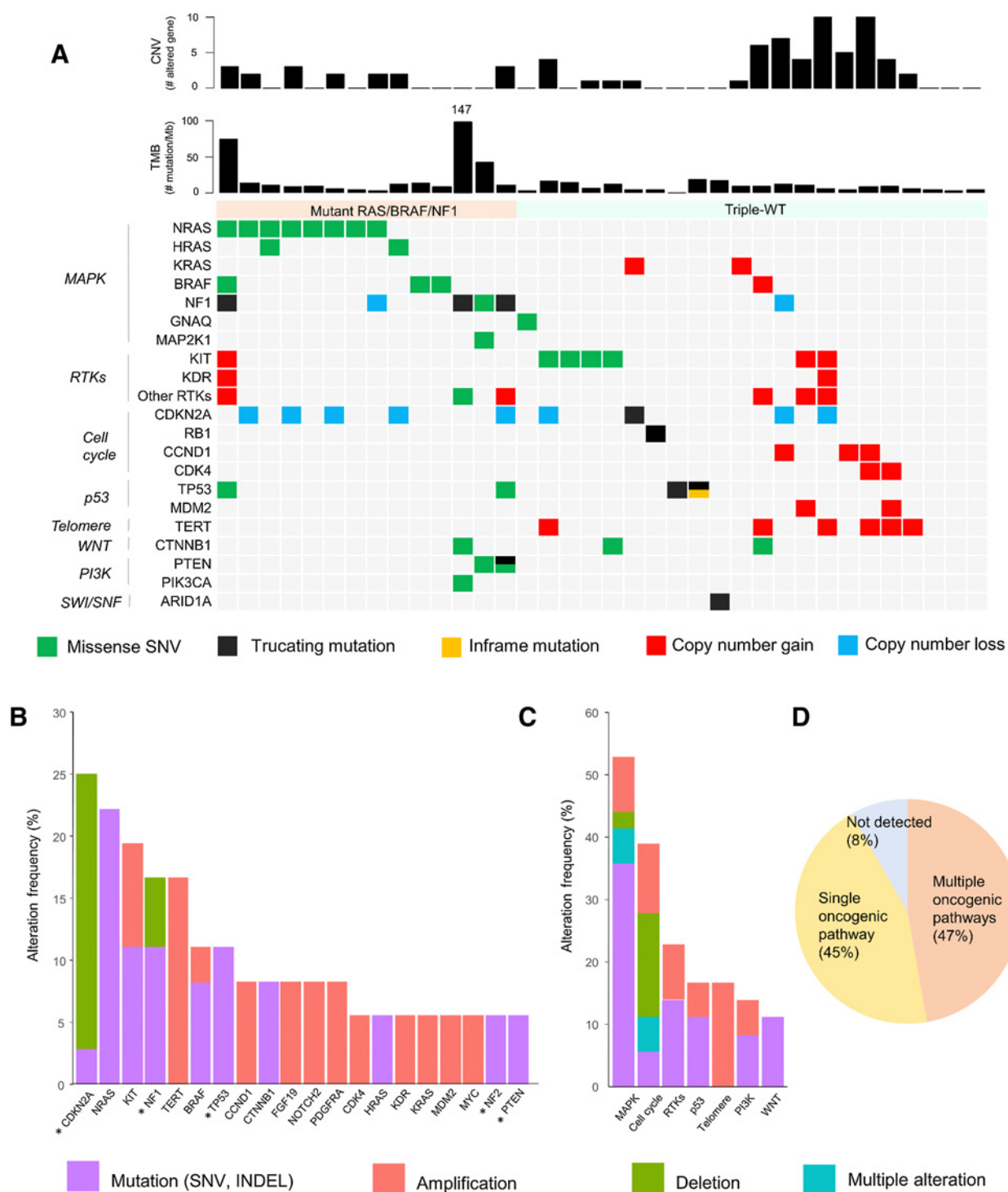


Figure 1. Distribution of oncogenic alterations in melanoma patient samples. **A**, Each column represents individual patient sample. The colors corresponding to the alterations are annotated at the bottom. **B**, Frequency of alterations in recurrently altered genes among patients (>5%). Tumor suppressor genes are indicated by asterisks. **C**, Frequency of altered oncogenic pathways in patients (>10%). **D**, Proportion of patients with melanoma altered in oncogenic pathways.

Table 2. Actionable alterations in 36 patients with melanoma.

Pathway	Gene	Alteration	# Cases
MEK (n = 16, 44%)	<i>BRAF</i>	V600E	2
	<i>NRAS</i>	Q61R	1
	<i>NRAS</i>	G12D	1
	<i>NRAS</i>	G12S	1
	<i>NRAS</i>	G13D	1
	<i>NRAS</i>	G13R	1
	<i>NRAS</i>	Q61K	2
	<i>NRAS</i>	Q61L	1
	<i>NRAS</i>	Q61L	2
	<i>KIT</i>	D816V	1
	<i>KIT</i>	K642E	3
	<i>KIT</i>	K693N	1
	<i>MAP2K1</i>	R49H	1
	<i>NF1</i>	Q1499X	1
	<i>NF1</i>	Q2213X	1
	<i>NF1</i>	Q236X	1
	<i>NF1</i>	Y489C	1
	<i>KRAS</i>	Amplification	2
	<i>MET</i>	Amplification	1
Cell cycle (n = 11, 30%)	<i>CDKN2A</i>	R80X	1
	<i>CDK4</i>	Amplification	2
	<i>CDKN2A</i>	Deletion	8
RTKs (n = 8, 22%)	<i>FGFR2</i>	E619K	1
	<i>KDR</i>	Amplification	2
	<i>PDGFRA</i>	Amplification	3
PI3K/mTOR (n = 3, 8%)	<i>PIK3CA</i>	H1047R	1
	<i>PTEN</i>	M1R	1
	<i>PTEN</i>	R173C	1
	<i>PTEN</i>	Splicing	1
p53 (n = 2, 6%)	<i>MDM2</i>	Amplification	2
DNA repair (n = 1, 3%)	<i>MSH6</i>	T955fs	1

15.1–NA) compared with low-TMB group (median 11.0 weeks, 95% CI, 8.1–35.0; $P = 0.04$; Fig. 3). The patient with the longest PFS (100.4 weeks) had an *MSH6* mutation.

Discussion

In this study, we analyzed molecular alterations across SNVs, INDELS, CNVs, and SVs, and also estimated TMBs in the patients with *BRAF* V600E–negative melanoma by using SNUH FIRST-Cancer panel. Although genomic features of melanoma development are well characterized in large-scale studies with whole-exome (35) and -genome (12) sequencing, clinical applications of FFPE sample sequencing without matched normal samples were not adequately

described. We found a prevalence of *NRAS* mutation and *CDKN2A* copy number deletion, as expected in our cohort diagnosed as *BRAF* WT. We classified 4 tumors into genomic subtypes described by TCGA. We observed high TMB with *NF1* mutation and prevalence of copy number amplification in triple-WT subtype in accordance with previous reports (35, 38). These results suggest that clinical NGS panel screen is feasible to discover various types of molecular alterations and to help estimate TMBs in melanoma.

We discovered that most of the patients (92%) harbored at least one oncogenic alteration in key genes of melanoma. Pathway analysis showed half the patients with oncogenic alterations in more than one signaling pathway of melanoma. These results are encouraging with respect to finding potential therapeutic targets for patients with *BRAF* WT, and also highlight the complexity of advanced melanoma and challenges in clinical treatment modalities.

Overall, 70% of patients had at least one actionable alteration, and seven of these patients were enrolled in clinical trials of targeted therapies. The observed clinical benefit was modest considering no observed objective response and median PFS of 12.6 weeks. Although there are many factors that may influence clinical outcomes, it is plausible that the modest benefit to patients from the implemented targeted therapies may be ascribed to redundancy of oncogenic signaling mechanisms and interaction of multiple pathways. Nevertheless, we observed metabolic response with RAF inhibitor in 1 patient showing promise of newer investigational drugs.

NGS enables identification of multiple oncogenic alterations to design effective treatments by massive parallel sequencing of numerous target genes instead of sequential testing by conventional methods. Thus, NGS panel enhances the potential to design relevant therapies in time and cost-effective manner for patients with advanced melanoma. As targeted therapies and combinatorial regimens are being increasingly developed, we expect that clinical NGS panel such as SNUH FIRST-Cancer panel will emerge as a powerful tool to guide clinical decisions for optimal outcomes in advanced melanoma.

We found 2 patients positive for *BRAF* V600E mutations with allele frequencies of 8%, who were initially diagnosed as *BRAF* V600E–negative using IHC assay and Sanger sequencing methods. This is consistent with the previous finding that although conventional molecular tests have high reliability, they also have drawbacks and limited sensitivities (39). One of the above 2 patients responded to dabrafenib/trametinib combination treatment, even though the allele frequency of *BRAF* V600E was not high. These results indicate that high-depth sequencing of clinical NGS panel allows to accurately detect mutations at low allele frequencies, which is likely to provide clinical benefits.

TMB estimated by the SNUH FIRST-Cancer panel also showed the ability to predict response to immunotherapy in patients with

Table 3. Genomic profiles and clinical information of patients who were enrolled into clinical trial.

Index	Gene	AA change	Allele frequency (%)	Copy number	TMB	Drugs in clinical trials	Best response	PFS (weeks)
Patient 1	<i>KRAS</i>	Amplification	—	14	4.0	RAF inhibitor	NE	1.9
Patient 2	<i>NRAS</i>	G12D	88.8	—	4.9	RAF inhibitor	PD	3.0
Patient 3	<i>NRAS</i>	G13D	89.2	—	2.0	RAF inhibitor	PD	28.1
Patient 4	<i>NRAS</i>	Q61L	75	—	8.0	RAF inhibitor	SD	15.3
Patient 5	<i>NRAS</i>	Q61K	45.8	—	3.3	RAF inhibitor	SD	7.3
Patient 6	<i>KIT/KDR/PDGFRA</i>	Amplification	—	133	5.0	Multiple receptor tyrosine kinase inhibitor	SD	12.6
Patient 7	<i>MSH6</i>	T955fs	31.8	—	74.0	Anti-PD-L1 antibody	SD	100.4

Abbreviation: NE, not evaluable.

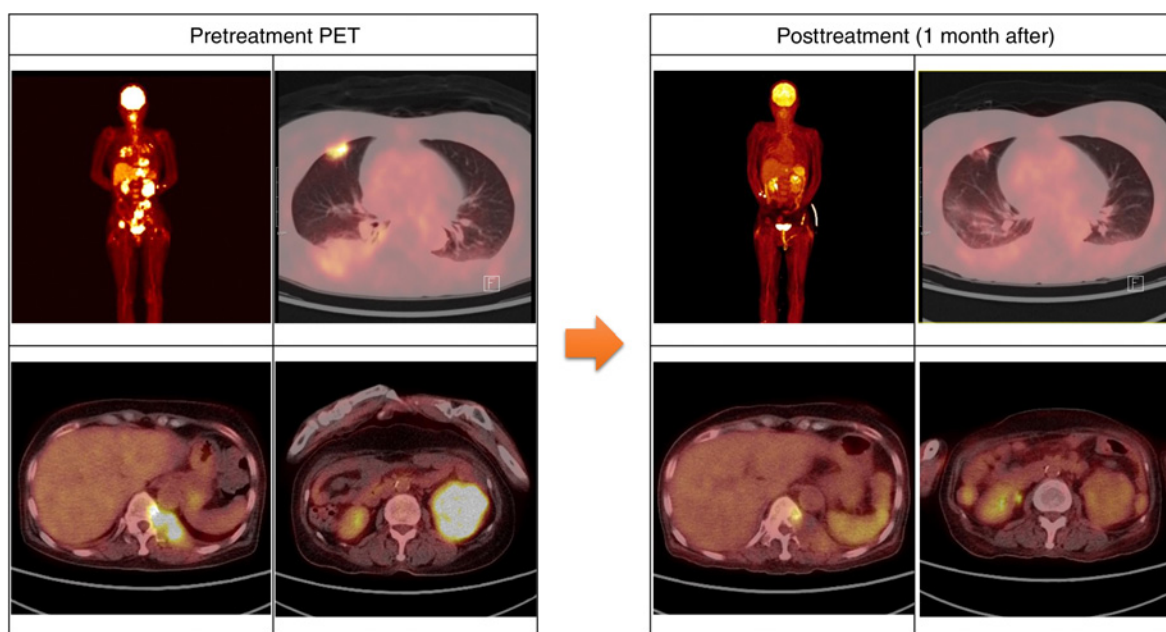


Figure 2. Metabolic response observed from PET images in Patient 5 who harbored NRAS Q61K mutation and received RAF inhibitor. The left panel shows pretreatment PET images: whole-body image in the left-upper, right middle lobe mass (SUV 9.3) in the right-upper, thoracic spine mass (SUV 13.3) in the left-lower, and left kidney mass (SUV 13.0) in the right-lower. The right panel shows PET images taken 1 month after treatment with RAF inhibitor: whole-body image in the left-upper, right middle lobe mass (SUV 2.0) in the right-upper, thoracic spine mass (SUV 2.8) in the left-lower, and left kidney mass (SUV 4.0) in the right lower. SUV, standardized uptake value.

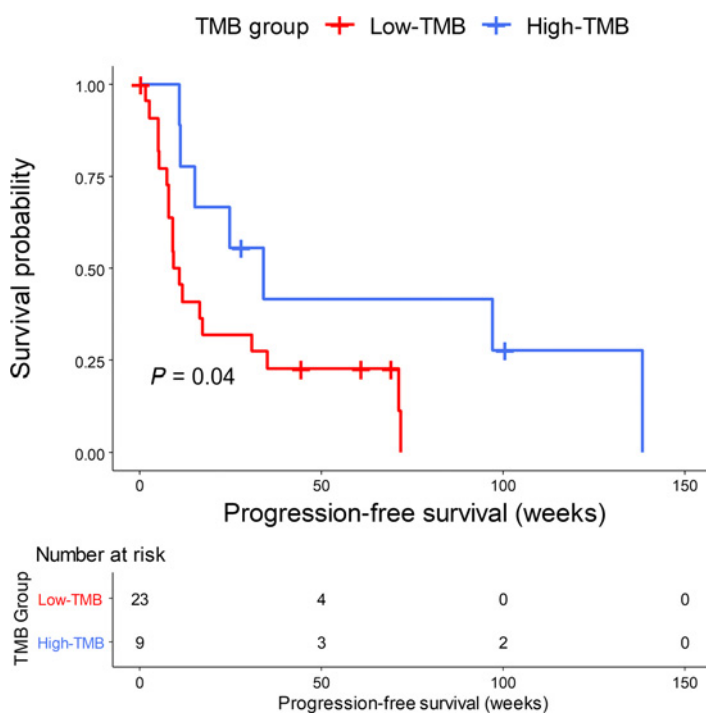


Figure 3. Progression-free survival of anti-PD-1/PD-L1-treated patients according to estimated TMB. Kaplan-Meier curve shows PFS of patients who received anti-PD-1/PD-L1 antibodies. Red line denotes the low-TMB group (estimated TMB < 13), and blue line denotes the high-TMB group (estimated TMB ≥ 13).

melanoma. Estimated TMB seems to associate with PFS of anti-PD-1/PD-L1 treatment, although statistically not significant, which might have been due to small sample size and diverse lines of treatments. Median PFS was 15.9 weeks, quite shorter than that reported previously (2). This result could be due to a higher proportion of acral lentiginous melanoma subtype prevalence in the Korean population, showing lower TMB than that of superficial-spreading melanoma (22). With the estimated TMB cutoff of 13 or above, the high-TMB group has longer PFS than the low-TMB group. The adopted cutoff of 13 is consistent with the TMB cutoffs in the range 7–20 reported in the literatures (18, 40, 41). However, our TMB estimation was done with somatic SNV prediction model rather than with matched germline genomic data and the cutoff we set is therefore arbitrary. Further independent validation of the estimated TMB is necessary to assess the response to immunotherapies.

It is remarkable that the patient with *MSH6* mutation had the second highest estimated TMB of 74 and experienced durable response (PFS 100.4 weeks) to immunotherapy. The patient harbored *MSH6* T955fs mutation, which is known to be associated with MMR defects (37). MMR defects are associated with genomic instability (microsatellite-high tumor) and commonly has a higher mutation burden, which is associated with favorable response to immunotherapy (15, 42, 43). We show supporting evidence to the above premise in 1 patient who harbored *MSH6* T955fs mutation and who responded favorably to immunotherapy. This implicates that using NGS for detecting MMR defects in patients with melanoma may be effective.

Because of the small sample size and exclusion of patients with *BRAF*-mutant melanoma, the results in this study may not reflect genomic demographics of patients with advanced melanoma. (21) Even though, we observed major genomic profiles and promising applicability of clinical NGS panel to these patients, which warrant further validation with larger number of patients in prospective clinical studies.

In summary, our NGS panel identified key oncogenic gene alterations and estimated TMB in patients with melanoma. These alterations showed association with clinical outcomes. Application of NGS panel

provides patients with melanoma with alternative therapeutic options including stratification of patients for clinical trials using the genetic analysis method described and tailored immunotherapy for better treatment outcomes.

Disclosure of Potential Conflicts of Interest

M. Kim has an unpaid consultant/advisory board relationship with AstraZeneca. T.M. Kim has an unpaid consultant/advisory board relationship with AstraZeneca, Novartis, and Takeda. D.-W. Kim has an unpaid consultant/advisory board relationship with AstraZeneca/MedImmune, Lilly, Yuhan, Boehringer Ingelheim, Chong Keun Dang, Pfizer, and Novartis. No potential conflicts of interest were disclosed by the other authors.

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