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BRAF and *NRAS* mutations and antitumor immunity in Korean malignant melanomas and their prognostic relevance: Gene set enrichment analysis and CIBERSORT analysis

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ABSTRACT

Cutaneous malignant melanomas (CMMs) are rare but are the cause of the highest skin cancer-related mortality in Korea. Very few studies have investigated the associations between *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* mutations and TICs, as well as their prognostic impact on Korean CMMs. Peptide nucleic acid-mediated polymerase chain reaction clamping and Mutyper and immunohistochemistry were used to detect these mutations in 47 paraffinized CMMs. *BRAF* and *NRAS* mutations were detected in 21.3% and 12.8% of CMMs, respectively. No *KRAS* or *PIK3CA* mutations were identified. *NRAS* mutations correlated with low FOXP3 (regulatory T lymphocyte marker) and indoleamine 2,3-dioxygenase (IDO) (activated dendritic cell marker) TICs in CMMs, which is consistent with the negative correlation of regulatory T cells with *NRAS* mutations in TCGA data, while *BRAF* mutations were not associated with any TICs. In gene set enrichment analysis, *BRAF* and *NRAS* mutations were enriched in decreased CD8 (suppressor/cytotoxic T lymphocyte marker) T cell-linked and increased CD4 (helper/inducer T lymphocyte marker) T cell-linked gene signatures, respectively, confirming the trend in our cohort of associations only with *NRAS*. *BRAF* or *NRAS* mutations alone did not affect any prognosis. In the subgroup analyses, *BRAF* mutations, as well as high CD4, CD8, FOXP3, and IDO TICs, caused worse overall survival in *NRAS*-mutated melanoma. No correlation of CD163 (monocyte–macrophage-specific marker) was detected.

We found that approximately one-third of our cohort had *BRAF* and *NRAS* mutations, none had *KRAS* or *PIK3CA* mutations, and most displayed decreased anti-tumor immunity. These findings may warrant further study on combined immunotherapeutic and molecular targeted therapy in Korean CMMs. Subgroup analyses according to TICs and *BRAF/NRAS* mutations may help to identify high-risk patients with worse prognoses.

1. Introduction

Cutaneous malignant melanoma (CMM) is the most aggressive skin cancer, arises in melanocytes located throughout the lower part of the epidermis [1], and is notorious for a dismal prognosis, with a 5-year survival rate of less than 5% in advanced stages [7]. While CMM is the

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fifth most common malignancy and has shown the tenth highest cancer-related mortality in the United States, with an increasing incidence worldwide [1,12], its incidence has been low (0.02%) and stable in Korea [19,33]. Nevertheless, CMM is responsible for the majority of skin cancer-related deaths in Korea [19], which highlights the need for studies on CMM in Korea despite its rarity.

Alterations in critical genes from the RAS-RAF-MAPK and the PI3K/Akt pathways have been identified as playing driving roles in the development of melanoma [35]. Consequently, small molecules that target the oncogenic MAPK pathway, specifically the tyrosine kinase BRAF and its downstream signaling partner MEK, have been found to promote improved overall survival and progression-free survival for *BRAF*-mutant melanoma [4]. *BRAF* and *NRAS* mutations are the most common and important oncogenic mutations in the RAS-RAF-MAPK pathway, and are found in over 80% of primary melanomas [35]. Although *KRAS* and *PIK3CA* mutations are rarely reported in melanomas [10], these mutations provide potential mechanisms of resistance during treatment with BRAF inhibitors [4]. Nearly 20% of patients do not respond to therapy due to intrinsic or acquired resistance mediated by hyperactivation of MAPK and PI3K/Akt pathway signaling and interactions with the tumor microenvironment [4]. There is also a rising awareness that various tumor-infiltrating immune cells (TICs) can interact with melanoma cells, reform the tumor microenvironment, and even affect chemotherapeutic efficacy [26].

Melanoma has been previously recognized as a highly immunogenic tumor and a good target for immunotherapy [26]. Several studies have demonstrated that high densities of TICs within CMMs are related to more favorable clinical outcomes [6,47]. However, a contrast report suggested that TICs in thin CMMs with regression phenomena may promote progression and metastasis [32]. However, TICs are composed of a heterogenous population that includes helper/inducer T lymphocyte, suppressor/cytotoxic T lymphocyte, regulatory T lymphocyte, activated dendritic cells, and monocytes [11,26]. It has been reported that a high number of CD4-positive (+) and CD8 + TICs in metastatic CMMs is associated with better survival, whereas high density of regulatory T cells is related to worse survival [11]. However, most previous studies have been conducted in Caucasian populations, and very few studies have investigated the associations of *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* mutations with TICs in Korean patients with CMM. Thus, the prognostic values of TICs and *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* mutations need to be further investigated.

In this study, we investigated the spectrum of *BRAF*, *NRAS*, *KRAS*, and *PIK3CA* mutations, along with TICs, and their clinical, pathological, and prognostic relevance in Korean CMMs. We also used gene set enrichment analysis to identify potential enriched gene sets involved in antitumor immunity that are associated with the above mutations.

2. Materials and methods

2.1. Patients and histologic evaluation

We retrospectively analyzed the clinical records of 47 patients who had been treated for pathologically-proven melanoma at Hallym University Sacred Heart Hospital consecutively from 2005 to 2015. All the patients were chemotherapy- and targeted drug therapy-naive at the time of the excision or biopsy. The patients did not receive targeted therapy or immune checkpoint inhibitor after recurrence, because they had been approved for clinical use of the treatment of refractory or metastatic melanoma in Korea since 2017. Clinical data, including age, sex, tumor site, tumor location, clinical stage, ulceration, recurrence or metastasis of disease after initial diagnosis, surgical therapy, adjuvant therapy, and survival, were obtained from medical records, radiologic examination, and pathology report files. The samples were approved according to the standard operating protocol of the Hallym University Sacred Heart Hospital institutional review board (IRB 2016-I040).

Two investigators (MJ Kwon and HK Lee) independently reviewed all hematoxylin and eosin (H&E) slides and evaluated tumor characteristics. Diagnoses and histological subtypes were classified based on the World Health Organization classification criteria. Staging was performed based on the American Joint Committee on Cancer staging system (8th edition) [13].

2.2. Mutation analysis

Genomic DNA was extracted from 10-μm-thick sections of 10% neutral formalin-fixed paraffin embedded (FFPE) tumor tissue blocks using the Maxwell® 16 FFPE Purification Kit for DNA (Promega, USA) according to the manufacturer's instructions. Detection of *BRAF* V600 and *PIK3CA* (exons 9 and 20) mutations was performed using the PNA Clamp™*BRAF* Mutation Detection kit and the PNA Clamp™*PIK3CA* Mutation Detection kit (PANAGENE, Daejeon, Korea), respectively, as previously shown [22,23]. Detection of mutations in exon 2 (at codons 12 and 13), exon 3 (at codon 61), and exon 4 (at codon 117 and codon 146) of *KRAS* and *NRAS* was achieved through PNA clamping-assisted fluorescence melting curve analysis using the PANAMutyper™ *KRAS* kit and the PANAMutyper™ *NRAS* kit (PANAGENE, Daejeon, Korea), respectively, as previously described [21].

2.3. Immunohistochemistry and scoring interpretation

Immunohistochemical staining was performed on 2-μm-thick paraffin-embedded block sections using the BenchMark Ultra automated immunohistochemistry system (Ventana Medical Systems, Inc., Tucson, AZ, USA) according to the manufacturer's instructions, as described previously [24]. Sections were incubated with primary antibodies against CD4 (helper/inducer T lymphocyte marker; pre-diluted, Ventana Medical Systems), CD8 (suppressor/cytotoxic T lymphocyte marker; 1:100, Cell Marque, Rocklin, CA, USA), FOXP3 (regulatory T lymphocyte marker; clone SP97, 1:100, AbCam, Cambridge, UK), IDO (activated dendritic cell marker; 1:1000, AbCam), and CD163 (monocyte–macrophage-specific marker; 1:100, AbCam) for 20 min, and UltraView Red Universal Multimer (UltraView Universal Alkaline Phosphatase Red Detection Kit; Ventana Medical Systems) for 12 min. The specimens were counterstained with hematoxylin for 4 min and post-counterstained with bluing reagent for 8 min. Sections of tonsils were used as a positive control.

TIC scoring was performed semi-quantitatively by measuring the cell densities of CD4, CD8, IDO, FOXP3, and CD163, which are modified from the grades of tumor-infiltrating lymphocytes [31,47]. The density of TICs was scored semi-quantitatively on a three-tiered scale on low-powered view (x100): 1+, indicating no or sporadic TICs; 2+, indicating moderate numbers of TICs (tumor and stromal cells predominating over TICs); 3+, indicating abundant occurrence of TICs (TICs predominating over tumor and stromal cells) (Supplementary Fig. 1). A score of 2+ or 3+ was considered to represent a high density of immune cell infiltration, whereas 1+ was considered to indicate a low density of immune cell infiltration.

Three investigators (MJ Kwon, HK Lee, and JY Choe) independently scored, and cases with discrepant scores were reevaluated to achieve a consensus score. Differences in interpretation were resolved using a multi-head microscope by consensus.

2.4. Gene Set Enrichment Analysis and CIBERSORT analysis using TCGA data

Gene expression data from the Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/abouttcga) was downloaded from the public domain training data (www.cbioportal.org/). Exome capture was performed using the Agilent SureSelect Human All Exon V2 44 Mb kit, followed by 2 × 76 bp paired-end sequencing on the Illumina HiSeq platform. Gene set enrichment analysis was performed using GSEA version 3.0 from the Broad Institute at MIT

Table 1
Clinical and pathological features of patient with cutaneous melanoma and their correlations with *BRAF* and *NRAS* mutation.

		BRAF mutation status			*NRAS* mutation status		
	Total N = 47(%)	Mutant n = 10 (21.3%)	Wild n = 37 (78.7%)	*P*	Mutant n = 6 (12.8%)	Wild n = 41 (87.2%)	*P*
Gender				0.276			0.609
Male	19 (40.4)	6 (60.0)	13 (35.1)		3 (50.0)	16 (39.0)	
Female	28 (59.6)	4 (40.0)	24 (64.9)		3 (50.0)	25 (61.0)	
Age(y)				0.306			0.678
≤60	21 (44.7)	6 (60.0)	15 (40.5)		2 (33.3)	19 (46.3)	
> 60	26 (55.3)	4 (40.0)	22 (59.5)		4 (66.7)	22 (53.7)	
Site				0.309			0.499
Head	11 (23.4)	4 (40.0)	7 (18.9)		2 (33.3)	9 (21.9)	
Trunk	2 (4.3)	0 (0.0)	2 (5.4)		0 (0.0)	2 (4.9)	
Extremities	10 (21.3)	3 (30.0)	7 (18.9)		0 (0.0)	10 (24.4)	
Hand & foot	24 (51.1)	3 (30.0)	21 (56.8)		4 (66.7)	20 (48.8)	
Histologic type				0.044*			0.836
ALM	24 (51.1)	3 (30.0)	21 (56.8)		4 (66.6)	20 (48.8)	
SSM	12 (25.5)	2 (20.0)	10 (27.0)		1 (16.7)	11 (26.8)	
NM	9 (19.1)	5 (50.0)	4 (10.8)		1 (16.7)	8 (19.5)	
LMM/DM	2 (4.3)	0 (0.0)	2 (5.4)		0 (0.0)	2 (4.9)	
Breslow thickness (mm)				0.911			0.985
0.01–1.00	16 (34.0)	4 (40.0)	12 (32.5)		2 (33.3)	14 (34.2)	
1.01–2.00	13 (27.7)	3 (30.0)	10 (27.0)		2 (33.3)	11 (26.8)	
2.01–4.00	8 (17.0)	1 (10.0)	7 (18.9)		1 (16.7)	7 (17.1)	
> 4.00	10 (21.3)	2 (20.0)	8 (21.6)		1 (16.7)	9 (21.9)	
Ulceration				0.237			0.141
Yes	12 (25.5)	4 (40.0)	8 (21.6)		3 (50.0)	9 (21.9)	
No	35 (74.5)	6 (60.0)	29 (78.4)		3 (50.0)	32 (78.1)	
cAJCC stage				0.873			0.863
I	11 (23.4)	2 (20.0)	9 (24.4)		3 (50.0)	21 (51.2)	
II	10 (21.3)	3 (30.0)	7 (18.9)		3 (50.0)	16 (39.0)	
III	3 (6.4)	1 (10.0)	2 (5.4)		0 (0.0)	3 (7.3)	
IV	1 (2.1)	0 (0.0)	1 (2.7)		0 (0.0)	1 (2.5)	
Distant metastasis				0.046*			1.000
Present	12 (25.5)	5 (50.0)	7 (18.9)		1 (16.7)	11 (26.8)	
Absent	35 (74.5)	5 (50.)	30 (81.1)		5 (83.3)	30 (73.2)	
$CD8^+$ TIC				0.573			0.655
1+	30 (63.8)	5 (50.0)	25 (67.6)		4 (66.7)	26 (63.4)	
2+	13 (27.7)	4 (40.0)	9 (24.4)		1 (16.7)	12 (29.3)	
3+	4 (8.5)	1 (10.0)	3 (8.0)		1 (16.7)	3 (7.3)	
$CD4^+$ TIC				0.834			0.296
1+	27 (57.4)	5 (50.0)	22 (59.5)		4 (66.7)	23 (56.1)	
2+	11 (23.4)	3 (30.0)	8 (21.6)		0 (0.0)	11 (26.8)	
3+	9 (19.2)	2 (20.0)	7 (18.9)		2 (33.3)	7 (17.1)	
$FOXP3^+$ TIC				0.107			0.030*
1+	36 (76.6)	8 (80.0)	28 (75.7)		4 (66.7)	32 (78.0)	
2+	10 (21.3)	1 (10.0)	9 (24.3)		1 (16.7)	9 (22.0)	
3+	1 (2.1)	1 (10.0)	0 (0.0)		1 (16.7)	0 (0.0)	
IDO^+ TIC				0.107			0.030*
1+	36 (76.6)	8 (80.0)	28 (75.7)		4 (66.7)	32 (78.0)	
2+	10 (21.3)	1 (10.0)	9 (24.3)		1 (16.7)	9 (22.0)	
3+	1 (2.1)	1 (10.0)	0 (0.0)		1 (16.7)	0 (0.0)	
$CD163^+$ TIC				0.545			0.388
1+	16 (34.0)	2 (20.0)	14 (37.8)		1 (16.7)	15 (36.6)	
2+	18 (38.3)	5 (50. 0)	13 (35.1)		2 (33.3)	16 (39.0)	
3+	13 (27.7)	3 (30.0)	10 (27.0)		3 (50.0)	10 (24.4)	

ALM, acral lentiginous melanoma; NM, nodular melanoma; SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; DM, desmoplastic melanoma; TIC, tumor-infiltrating immune cells.

* Statistically significant, $P < 0.05$.

(software.broadinstitute.org/gsea/index.jsp/) to identify the immunologic signatures that are enriched in genes associated with *BRAF*, *NRAS*, *KRAS*, and *PIK3CA* mutational status in CMMs. The gene set database (c7.all.v6.1symbols.gmt including 4872 gene set) from the Molecular Signature Database (MSigDB) was used for gene set enrichment analysis, 1000 permutations were used to calculate the *P* value, and the permutation type was set to phenotype. We defined a meaningful gene set as having a false discovery rate (FDR) < 25% and $P < 0.05$ and the top 100 ranked genes within the identified gene set as significant.

We applied an established computational approach (CIBERSORT) (https://cibersort.stanford.edu/) in order to estimate the relative proportions of 22 immune cell types. CIBERSORT analyses associated with recruitment of CD8+ cells, resting CD4+ memory T cells, and regulatory T cells were done with 100 permutations, enabling quantile normalization and default statistical parameters. The results were filtered by setting the maximum *P*-value to 0.05. Comparisons of relative fractions were performed using the Mann–Whitney U test.

2.5. Statistical analysis

A chi-squared test or two-tailed Fisher's exact test was used to analyze possible associations between qualitative clinicopathological variables and *BRAF* or *NRAS* mutations. Disease-free survival (DFS) was

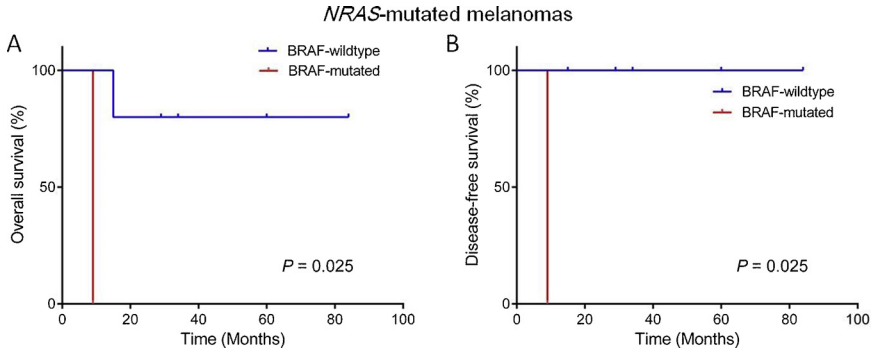

Fig. 1. In *NRAS*-mutated melanomas, *BRAF* mutations are associated with poorer overall survival (A) and disease-free survival (B).

defined as the time from the first surgery until a documented relapse, including locoregional recurrence and distant metastasis. Overall survival (OS) was defined as the time from the first surgery until death. Overall and disease-free survival rates were evaluated until August 2016. Survival differences among groups were calculated using the Kaplan-Meier method with a log-rank test. Univariate and multivariate analyses were performed using the Cox proportional-hazards regression model to determine the hazard ratio (HR) and 95% confidence interval (CI) for specific variables with respect to OS and DFS. For subgroup survival analysis, a log-rank test with the Bonferroni correction was used. SPSS statistical software (version 18, Statistical Package for the Social Sciences) was used for all statistical analyses. P values < 0.05 were considered statistically significant.

3. Results

3.1. Clinicopathologic demographics

A total of 47 patients were recruited, consisting of 19 men (40.4%) and 28 women (59.6%); their median age was 67 years (range: 26–87 years). The most common melanoma tumor locations were hands and feet (24/47, 51.1%), followed by the head (11/47, 23.4%), extremities (10/47, 21.3%), and trunk (2/47, 4.3%).

The most predominant histologic subtype of melanomas was acral lentiginous melanoma (ALM) (24/47, 51.1%), followed by superficial spreading melanoma (SSM) (12/47, 25.5%), nodular melanoma (NM) (9/47, 19.1%), desmoplastic melanoma (DM) (1/47, 2.1%), and lentigo maligna melanoma (LMM) (1/47, 2.1%). The mean Breslow thickness in tumors was 3.16 ± 4 mm. Ulceration accompanied 12 (25.5%) tumors.

A total of 11 (23.4%) patients were diagnosed as clinical stage IA, 13 (27.7%) as stage IB, 10 (21.3%) as stage IIA, 5 (10.6%) as stage IIB, 4 (8.5%) as stage IIC, 3 (6.4%) as stage III, and 1 (2.1%) as stage IV.

Thirty-eight patients including refusing further adjuvant therapy underwent wide excision only, 5 patients received adjuvant chemotherapy, and 4 were treated with interferon-alpha after surgery. Two of 47 patients showed microscopically positive margins (R1) after initial surgery. Twelve patients received a neck dissection.

The median duration of follow-up was 65 months (range: 3–126 months). Twelve of 47 patients (25.5%) developed tumor progression or recurrences. Two of the 12 recurrent CMM patients had local recurrence and 10 developed locoregional or distant metastases. Six patients with tumor progression were treated solely by surgery, 2 received adjuvant interferon-alpha, and 4 were treated with adjuvant chemotherapy. Overall, fifteen patients died of disease, and there are currently 32 patients alive (68.1%).

3.2. Clinicopathological correlation with BRAF, NRAS, KRAS, and PIK3CA mutations

BRAF mutations were detected in 10 (21.3%) tumors (all in codon 600), while *NRAS* mutations were detected in 6 (12.8%) tumors: 3 in codon 61 (50%), 2 in codon 12 (33.3%), and 1 in codon 13 (16.7%). In contrast, no mutations in *KRAS* or *PIK3CA* were found.

BRAF mutations were associated with distant metastases during the follow-up period and histologic types ($P = 0.046$ and $P = 0.044$, respectively) (Table 1). *BRAF* mutation was the lowest detected in ALM among histologic subtypes. *NRAS* mutations were more frequently detected in melanomas with lower FOXP3+ or IDO + TICs ($P = 0.030$ and $P = 0.030$, respectively).

In ALMs, *BRAF* mutations were more frequently detected in younger patients (≤60 years) ($P = 0.017$). In SSMs, *BRAF* mutation was frequently detected in melanomas with lower CD8+, FOXP3+, or IDO + TICs ($P = 0.028$, $P = 0.020$, and $P = 0.020$, respectively). *NRAS* mutation was only associated with ulceration in SSMs ($P = 0.020$).

Only 1 tumor had coexisting *BRAF* and *NRAS* mutations. The patient carrying both *BRAF* and *NRAS* mutations was a 68-year-old male with stage IIC nodular melanoma, with ulceration in the head region and distant metastasis, but without lymph node metastasis. The H&E slide review from this patient showed a large, well-circumscribed tumor (2 × 2 × 1.5 cm) with atypical epithelioid melanocytes with occasional prominent nucleoli and a brisk mitotic activity. The patient died 9 months after surgery because of disease dissemination.

3.3. Prognostic significance of BRAF and NRAS mutations and TICs

BRAF or *NRAS* mutations did not affect OS or DFS in CMMs. However, *BRAF* mutation was associated with worse OS and DFS in *NRAS*-mutated tumors ($P = 0.025$ and $P = 0.025$, respectively) (Fig. 1). Higher CD8+, CD4+, FOXP3+, or IDO + TICs also tended to correlate with worse OS in *NRAS*-mutated tumors (all, $P = 0.018$).

In overall CMMs, univariate analysis revealed that older age (> 60 years), lymph node metastases, ulcerations, higher stages (III-IV), and high CD8 + TICs were significantly associated with worse OS ($P = 0.034$, $P = 0.005$, $P = 0.009$, $P < 0.001$, and $P = 0.046$, respectively). Tumor thickness, lymph node metastases, and higher stages (III-IV) were associated with shorter DFS ($P = 0.020$, $P = 0.004$, and $P = 0.036$, respectively).

In multivariate analyses, ulceration was an independent prognostic factor for OS (HR 9.73, 95% CI 2.18–43.35, $P = 0.003$). Older age and higher stages exhibited borderline statistically significant correlations with OS ($P = 0.061$ and $P = 0.077$, respectively). There were no independent prognostic factors for DFS (Table 2).

Table 2
Clinicopathological and biological factors affecting overall and disease-free survival rates.

	Overall survival				Disease-free survival			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Male sex	0.56 (0.20–1.57)	0.267			1.01(0.39–2.62)	0.992		
Age (> 60 yrs)	3.94 (1.11–13.98)	0.034*	3.60 (0.94–13.75)	0.061	2.51 (0.74–8.44)	0.138		
Thickness (> 4 mm)	2.03 (0.72–5.70)	0.182			3.87 (1.23–12.11)	0.020*	2.51 (0.65–9.76)	0.183
LN metastasis	5.37 (1.68–17.16)	0.005*	2.58 (0.31–21.30)	0.380	8.21 (1.94–34.82)	0.004*	5.72 (0.55–59.49)	0.144
Ulceration	4.38 (1.45–13.23)	0.009*	9.73 (2.18–43.35)	0.003*	1.59 (0.42–5.95)	0.494		
Stages III-IV	11.17(3.21–38.83)	< 0.001*	7.06 (0.81–61.73)	0.077	5.61 (1.12–28.20)	0.036*	0.68 (0.61–7.51)	0.751
BRAF mutation	1.67 (0.53–5.31)	0.384			0.74 (0.26–2.15)	0.584		
NRAS mutation	1.37 (0.30–6.15)	0.684			1.25 (0.29–5.50)	0.766		
High $CD8^+$ TICs	2.83 (1.02–7.85)	0.046*	1.55 (0.47–5.05)	0.471	1.72 (0.54–5.45)	0.357		
High $CD4^+$ TICs	1.73 (0.63–4.79)	0.288			2.27 (0.72–7.16)	0.163		
High $FOXP3^+$ TICs	2.47 (0.84–7.29)	0.102			0.37 (0.05–2.89)	0.345		
High IDO^+ TICs	2.47 (0.84–7.29)	0.102			0.37 (0.05–2.89)	0.345		
High $CD163^+$ TICs	2.61 (0.73–9.36)	0.141			1.39 (0.41–4.68)	0.592		

HR, hazard ratio; CI, confidence interval; yrs, years-old.
* Statistically significant, $P < 0.05$.

3.4. Identification of significant genes related to BRAF, NRAS, KRAS and PIK3CA mutations via gene set enrichment analysis

In order to validate and compare our results, we conducted gene set enrichment analysis to find gene sets associated with *BRAF*, *NRAS*, *KRAS*, and *PIK3CA* mutations in CMMs using TCGA data (Table 3). The TCGA included 443 patients who were treated with cutaneous melanoma. The mean age of the patients was 57 (range: 15–90) years. The median duration of follow-up was 64.4 months (range: 1–370 months). The *BRAF* mutation rate was the most common (229, 51.7%) followed by *NRAS* (118, 26.6%), *PIK3CA* (22, 5%) and *KRAS* (10, 2.3%). AJCC staging information was available for only 403 patients. Seven (1.6%) patients were diagnosed as stage 0, 89 (20.1%) as stage I, 119 (26.9%) as stage II, 165 (37.2%) as stage III and 23 (5.2%) as stage IV. Two hundred thirty-eight patients (53.7%) died. These TCGA data showed that *BRAF*, *NRAS*, *KRAS*, or *PIK3CA* mutation was not associated with OS ($P = 0.232$, $P = 0.083$, $P = 0.406$, and $P = 0.412$, respectively). CD8 + T cell-linked gene sets were enriched in CMMs with *BRAF* mutations. *NRAS* mutations correlated with gene signatures identifying CD4 + T cells. *KRAS* mutations were associated with gene signatures of activated dendritic cells. *PIK3CA* mutations showed a correlation with monocyte-linked gene sets (Supplementary Fig. 2).

Using CIBERSORT analysis, we evaluated the relative abundances of immune cells according to these genetic mutational statuses (Fig. 2). Recruitment of CD8 + T cells was higher in *BRAF* wild-type and *PIK3CA* wild-type melanomas compared to those with *BRAF* and *PIK3CA* mutations ($P = 0.003$ and $P = 0.002$, respectively). Higher recruitment of memory resting CD4+ T cells was identified in melanomas with *BRAF*, *NRAS*, *KRAS*, and *PIK3CA* mutations ($P < 0.001$, $P < 0.001$, $P = 0.001$, and $P = 0.002$, respectively). Recruitment of regulatory T cells was higher in *NRAS* wild-type, *KRAS* wild-type, and *PIK3CA* wild-type melanomas than in those with *NRAS*, *KRAS*, and *PIK3CA* mutations ($P < 0.001$, $P = 0.005$, and $P < 0.001$, respectively).

4. Discussion

Because of the rarity of Korean CMMs, studies on *BRAF*, *NRAS*, *KRAS*, or *PIK3CA* alterations and immune cell infiltration and their prognostic relevance for patients with CMMs have been limited in Korea. The enrolled number of 47 patients, while objectively small, is not small sample size compared to Asian previous molecular studies of CMMs [2,25,29,38]. In the present study, we have shown that *BRAF* is the most common mutation (21.3%), followed by *NRAS* (12.8%) in CMMs, with only one (2.1%) coexisting *BRAF* and *NRAS* mutation showing poor clinical outcome. We have also found that all CMMs harbored wild-type *KRAS* or *PIK3CA* genes. *BRAF* or *NRAS* mutations did not predict the survival of patients with overall CMMs, consistent with TCGA data. Decreased anti-tumor immunity was associated with *BRAF* or *NRAS*-mutated CMMs, and it might contribute to poor survival outcome.

There have been conflicting reports regarding *BRAF* or *NRAS* mutation status and prognosis in Asian patients with CMMs (Table 4). Three studies reported no correlation between *BRAF* mutations and patient survival [25,29,42]. Four other studies found a significant association between *BRAF* mutations and poor prognosis [18,27,38,44]. Interestingly, two of the four studies that found positive correlations between *BRAF* mutations and adverse prognoses were conducted in Korean populations [27,38]. This prognostic correlation suggests that the MAPK signaling pathway is constitutively activated in the majority of ALMs, and that ALM is likely to be a good candidate for treatment with BRAF inhibitors because of the alternative pathway [46]. Other mutations including *NRAS*and *KIT* and autocrine growth factor stimulation may constitute alternative routes for *BRAF* activation [5]. This raises the possibility that treatments targeting BRAF might be beneficial in Korean CMMs, independent of *BRAF* mutational status. Unfortunately, the patients in our cohort did not receive BRAF inhibitor or immune checkpoint inhibitor after recurrence, because the medication was approved for clinical use since 2017 in Korea.

We found that one tumor had coexisting mutations in both *BRAF* and *NRAS*, of which case showed highly aggressive behavior and the patient died of disease dissemination less than one year after surgery. Combined analyses showed that concurrent *BRAF* and *NRAS* mutations had a significant prognostic impact compared to melanomas without them. Although *NRAS* mutations and *BRAF* mutations were reported to be mutually exclusive in earlier studies [15], *BRAF* and *NRAS* proved to be the most common concurrent mutations in melanomas [48]. Several studies detected rare but evident simultaneous *BRAF* and *NRAS* mutations in CMMs that occurred preferentially in chronic sun exposure areas (back and neck) at a frequency of up to 1.7%–8.5% of CMMs [14,41,42,48]. These cases seem to be aggressive, resulting in regional nodal or distant metastases, and causing death within several weeks to 7.7 years later [41,42]. Patients with concomitant *BRAF* and *NRAS* mutations (2.1%) developed distant metastases and disease-related death later on [41,42]. The coexistence of *BRAF* and *NRAS* mutations may contribute to synergistic oncogenic effects on the aggressive behavior and metastatic potential of melanoma, and ultimately influences the clinical outcomes of CMM patients. This observation is supported by

Table 3
Top 11-ranked gene sets related to mutations of *BRAF*, *NRAS*, *KRAS*, and *PIK3CA*, respectively.

Gene set	Size	ES	NOM (p-value)	FDR (q-value)	FWER (p-value)
BRAF mutation					
GSE32423_MEMORY_VS_NAIVE_CD8_TCELL_IL7_IL4_DN	194	0.626	2.366	0	0.002
GSE9601_UNTREATED_VS_NFKB_INHIBITOR_TREATED_HCMV_INF_MONOCYTE_DN	171	0.624	2.320	0	0.009
GSE39820_CTRL_VS_TGFBETA3_IL6_IL23A_CD4_TCELL_UP	193	0.664	2.294	0	0.010
GSE10239_KLRG1INT_VS_KLRG1HIGH_EFF_CD8_TCELL_DN	192	0.639	2.263	0	0.012
GSE39820_CTRL_VS_TGFBETA1_IL6_IL23A_CD4_TCELL_UP	196	0.645	2.256	0	0.011
GSE44649_NAIVE_VS_ACTIVATED_CD8_TCELL_MIR155_KO_DN	193	0.687	2.249	0	0.011
GSE40493_BCL6_KO_VS_WT_TREG_UP	175	0.554	2.244	0	0.010
GSE6674_CPG_VS_PL2_3_STIM_BCELL_DN	193	0.542	2.235	0	0.010
GSE41867_NAIVE_VS_DAY15_LCMV_CONE13_EFFECTOR_CD8_TCELL_DN	187	0.540	2.233	0	0.009
GSE21033_1H_VS_12H_POLYIC_STIM_DC_DN	133	0.630	2.223	0	0.010
GSE32423_MEMORY_VS_NAIVE_CD8_TCELL_IL7_IL4_DN	194	0.626	2.366	0	0.002
NRAS mutation					
GSE39820_CTRL_VS_TGFBETA3_IL6_IL23A_CD4_TCELL_UP	193	0.683	2.392	0	0.005
GSE10239_MEMORY_VS_DAY4.5_EFF_CD8_TCELL_DN	193	0.645	2.367	0	0.005
GSE7460_CD8_TCELL_VS_TREG_ACT_DN	195	0.596	2.360	0	0.004
GSE44649_NAIVE_VS_ACTIVATED_CD8_TCELL_MIR155_KO_DN	193	0.717	2.350	0	0.003
GSE13485_CTRL_VS_DAY1_YF17D_VACCINE_PBMC_UP	189	0.662	2.343	0	0.003
GSE10240_IL22_VS_IL17_STIM_PRIMARY_BRONCHIAL_EPITHELIAL_CELLS_UP	198	0.577	2.333	0	0.002
GSE28737_WT_VS_BCL6_KO_MARGINAL_ZONE_BCELL_DN	191	0.617	2.331	0	0.002
GSE29164_UNTREATED_VS_CD8_TCELL_AND_IL12_TREATED_MELANOMA_DAY3_DN	195	0.545	2.330	0	0.002
GSE10239_MEMORY_VS_KLRG1INT_EFF_CD8_TCELL_DN	194	0.591	2.330	0	0.001
GSE37534_UNTREATED_VS_GW1929_TREATED_CD4_TCELL_PPARG1_AND_FOXP3_TRASDUCED_DN	198	0.563	2.328	0	0.001
GSE39820_CTRL_VS_TGFBETA3_IL6_IL23A_CD4_TCELL_UP	193	0.683	2.392	0	0.005
KRAS mutation					
GSE5503_LIVER_DC_VS_PLN_DC_ACTIVATED_ALLOGENIC_TCELL_DN	197	0.575	2.230	0	0.044
GSE6674_CPG_VS_PL2_3_STIM_BCELL_DN	194	0.545	2.208	0	0.039
GSE32423_MEMORY_VS_NAIVE_CD8_TCELL_IL7_IL4_DN	194	0.594	2.207	0	0.026
GSE10239_KLRG1INT_VS_KLRG1HIGH_EFF_CD8_TCELL_DN	192	0.593	2.174	0	0.032
GSE39820_CTRL_VS_TGFBETA1_IL6_IL23A_CD4_TCELL_UP	196	0.623	2.173	0	0.026
GSE39820_TGFBETA1_IL6_VS_TGFBETA1_IL6_IL23A_TREATED_CD4_TCELL_UP	196	0.547	2.171	0	0.022
GSE9960_HEALTHY_VS_GRAM_NEG_AND_POS_SEPSIS_PBMC_DN	185	0.628	2.161	0	0.022
GSE37532_TREG_VS_TCONV_CD4_TCELL_FROM_VISCERAL_ADIPOSE_TISSUE_DN	161	0.556	2.160	0	0.019
GSE32901_NAIVE_VS_TH17_ENRICHED_CD4_TCELL_DN	169	0.523	2.155	0	0.018
GSE24142_DN2_VS_DN3_THYMOCYTE_ADULT_DN	194	0.509	2.143	0	0.020
GSE5503_LIVER_DC_VS_PLN_DC_ACTIVATED_ALLOGENIC_TCELL_DN	197	0.575	2.230	0	0.044
PIK3CA mutation					
GSE34515_CD16_POS_MONOCYTE_VS_DC_UP	192	0.608	2.303	0	0.006
GSE21033_1H_VS_12H_POLYIC_STIM_DC_DN	132	0.642	2.299	0	0.003
GSE17721_0.5H_VS_4H_GARDIQUIMOD_BMDC_DN	195	0.579	2.293	0	0.002
GSE21380_NON_TFH_VS_GERMINAL_CENTER_TFH_CD4_TCELL_DN	192	0.660	2.270	0	0.002
GSE9601_UNTREATED_VS_NFKB_INHIBITOR_TREATED_HCMV_INF_MONOCYTE_DN	171	0.618	2.267	0	0.002
GSE10273_HIGH_VS_LOW_IL7_TREATED_IRF4_8_NULL_PRE_BCELL_UP	198	0.545	2.252	0	0.002
GSE10240_IL22_VS_IL17_STIM_PRIMARY_BRONCHIAL_EPITHELIAL_CELLS_UP	198	0.564	2.244	0	0.002
GSE10239_KLRG1INT_VS_KLRG1HIGH_EFF_CD8_TCELL_DN	192	0.625	2.223	0	0.002
GSE40493_BCL6_KO_VS_WT_TREG_UP	175	0.548	2.221	0	0.002
GSE32423_MEMORY_VS_NAIVE_CD8_TCELL_IL7_IL4_DN	194	0.612	2.221	0	0.002
GSE34515_CD16_POS_MONOCYTE_VS_DC_UP	192	0.608	2.303	0	0.006

ES, Enrichment Score; NES, Normalized Enrichment Score; FDR, False Discovery Rate; NOM, Nominal p Value; FDR, False discovery rate; FWER, Family wise-error rate.

Mann et al. [30] showing that co-existing *BRAF* mutation, *NRAS* mutation and absence of immune-related expressed genes are associated with adverse prognosis in CMM. Because the prognostic relevance of concurrent *BRAF* and *NRAS* mutations had a limitation of only one patient in our study, the role of coincident mutations as the poor survival affection in Korean CMMs is not conclusive.

In the present study, both *BRAF* and *NRAS* mutation was negatively correlated with FOXP3+ or IDO + TICs, which is consistent with the negative correlation between regulatory T cells and *NRAS* mutations found in TCGA data. *BRAF* mutation was negatively correlated with additionally CD8 + TICs. Subgroup analyses revealed that high CD4+, CD8+, FOXP3+, and IDO + TICs had worse OS in *NRAS*-mutated melanomas. And high CD8 + TICs showed poorer OS in *BRAF* wild-type melanomas. It seems to be contradictory to an early study reporting a favorable prognosis of high CD8 + TICs in CMMs [11]. However, that study did not consider the prognostic impact of TICs depending on *BRAF* status. *BRAF*, but not *NRAS*, may reflect genetic characteristics based on ethnic differences. In addition, higher pre-treatment CD4+/FoxP3+ Tregs has been associated with favorable survival [45]. T cells are the effector cells of immune check inhibitor treatment. Regulatory T cells might be one of the main targets of ipilimumab [45]. Nevertheless, there has been few information regarding prognostic contributions of TICs according to *BRAF* or *NRAS* mutation in melanomas, and this is the first study evaluating those relevance specifically in Korean CMMs. Decreased anti-tumor immunity in *BRAF* or *NRAS* mutation may suggest that Korean CMMs may be suitable for combined immunotherapeutic strategies and molecular targeted therapy. Using TCGA and gene set enrichment analysis, we also identified significant correlations between *BRAF*, *NRAS*, *KRAS*, or *PIK3CA* mutation signatures and specific decreased anti-tumor immunity in the clinical database of melanoma. *BRAF* mutations were enriched in CD8 + T cell-linked gene sets, and consequently downregulated CD8 + T cells and

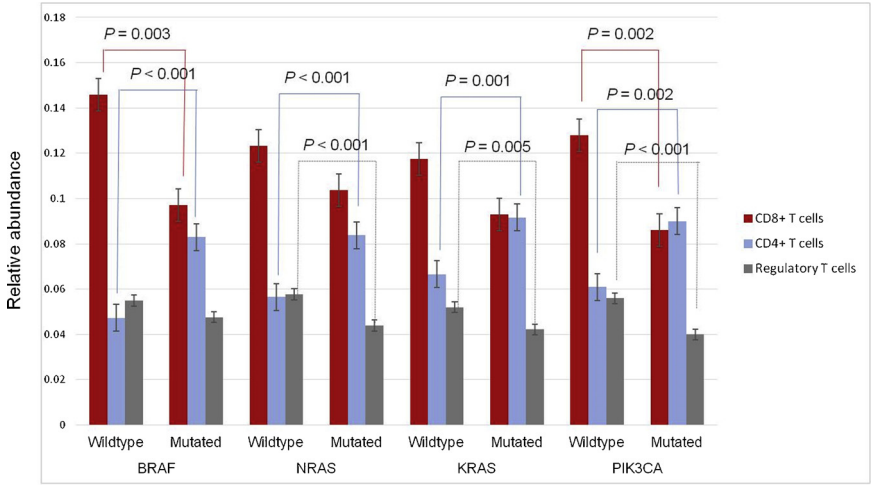

Fig. 2. Relative abundance of CD8 + T cells, CD4 + T cells, and regulatory T cells, evaluated by CIBERSORT in TCGA skin melanoma datasets based on *BRAF*, *NRAS*, *KRAS*, and *PIK3CA* mutations.

upregulated resting memory CD4 + T cells. On the other hand, *NRAS* mutations correlated positively with CD4 + T cell-linked gene signatures, resulting in significantly increased high memory resting CD4 + T cell infiltration and low regulatory T cells. Previously, it has been reported that the immune response activation and MAPK activation via *NRAS* mutation are common features of all signatures of alterations of those pathways [40]. Our results show more detailed information of TICs related to respective *BRAF*, *NRAS*, *KRAS*, and *PIK3CA* mutations in CMMs.

We detected *BRAF* and *NRAS* mutations in 21.3% and 12.8% of our cohort, respectively. Our results are within the previously reported ranges of *BRAF* mutations (15.9%–35.9%) and *NRAS* mutations (11.4%–12.2%) in other Korean-based CMM studies [2,25,27,38]. The frequency of *BRAF* mutations in our cohort is comparable to that observed in Chinese (14.3%–31%) [17,29,42,44] and Japanese (31.3%–45.3%) populations [3,18,39], but is considerably lower than the 30–66% frequency calculated by studies done in Western Caucasian populations [8,9,12,15,28,34,37]. The discrepancy in the rate of *BRAF* mutation between Asian and Caucasian populations may be because the most common histologic subtype composition in Asian CMMs is ALM, which paradoxically harbors a low frequency of *BRAF* mutations [20]. Likewise in our study, although ALM was the most common histologic subtype (46.8%), ALM harbored the lowest *BRAF* mutation rate (13.6%). Furthermore, the *BRAF* mutation rate within the same histologic subtypes like ALM is much lower in Asian CMMs than in Caucasian CMMs [20]. In contrast, the frequency of *NRAS* mutations in our cohort is similar to that reported in Caucasian populations (5.2%–18%) [12,14,15,36] as well as in the same Far East Asian populations (Chinese, 6%–10.1%; Japanese, 12.3%) [39,42,44].

BRAF mutations were related to distant metastasis of melanoma, histologic subtypes, and younger age of onset in ALM patients (≤60 years). The strong association of *BRAF* mutation in young ALM patients has been previously reported in Korean ALMs [16]. However, there were no associations of *NRAS* mutations with adverse clinical or histopathologic factors in CMMs overall, except to association with ulceration in SSMs. The clinical or pathological significance of *BRAF* or *NRAS* mutations in CMMs in our cohort does not seem to correspond with reported western data [39], possibly due to the aforementioned differences in epidemiologic and ethnic characteristics.

In contrast, *BRAF* mutations were not associated with any TICs in CMMs overall in our cohort. And high CD8 + TICs showed poorer OS in *BRAF* wild-type melanomas. Our result It seems to be contradictory to an early study reporting a favorable prognosis of high CD8 + TICs in CMMs [11]. However, that study did not consider the prognostic impact of TICs depending on *BRAF* status. *BRAF*, but not *NRAS*, may reflect genetic characteristics based on ethnic differences. Although *BRAF* and *NRAS* mutations tend to correlate with decreased anti-tumor immune activation in patients with CMM overall, thereby worsening prognostic outcomes in patients with CMM, subgroup analyses based on TICs and mutational statuses might provide more detailed prognosis prediction

Table 4
Studies examined for *BRAF* or *NRAS* mutations in Asian cutaneous malignant melanomas.

	Country	*BRAF*(+) (positive/total)	*NRAS*(+) (positive/total)	Method	Survival relation
Lee et al (2012) [25]	Korea	17.2% (10/58)	ND	RT PCR, DS	No
Ahn et al (2013) [2]	Korea	35.9% (23/64)	ND	DS,DPO-PCR,RT PCR	ND
Roh et al (2017) [38]	Korea	15.9% (14/88)	11.4% (10/88)	DS	Yes (U), No (M)
Lee et al (2018) [27]	Korea	19.8% (26/131)[a]	12.6% (16/131)[a]	DS	Yes (*BRAF*), No (*NRAS*)
Si et al (2012) [44]	China	31% (83/268)[a]	6% (16/268)[a]	DS	Yes (*BRAF*, *NRAS*)
Liu et al (2014) [29]	China	23.3% (10/43)[a]	ND	DS	No
Sheen et al (2016) [42]	Taiwan	14.3% (17/119)	10.1% (12/119)	DS	No
Huang et al(2016) [17]	China	25.4% (18/71)[a]	ND	DS	ND
Ashida et al (2012) [3]	Japan	31.3% (25/80)[a]	ND	DS	ND
Sakaizawa et al(2015) [39]	Japan	39.8% (49/123)[a]	12.3% (NA)	DS	ND
Ide et al (2017) [18]	Japan	45.3% (48/106)[a]	ND	DS, RT PCR	Yes (U, M)
Present study (2019)	Korea	21.3% (10/47)	12.8% (6/47)	RT PCR, DS	Yes (in NRAS mutant)

RT PCR, real-time polymerase chain reaction; DS, direct sequencing; DPO-PCR, dual-priming oligonucleotide- polymerase chain reaction; U, Univariate analuysis; M, Multivariate analysis; ND, not done; NA, not accessible in detail.

[a] Case numbers and frequencies of *BRAF* and *NRAS* mutation included only cutaneous malignant melanomas.

in Korean CMMs to identify high-risk patients with worse clinical outcomes.

There have been no molecular studies investigating the frequency of *KRAS* and *PIK3CA* mutation Korean CMMs. *KRAS* and *PIK3CA* mutations have been registered at very low frequencies in previous studies, found in 4.7%–13.6% and 0.9% of CMMs, respectively [43,48]. To the best of our knowledge, currently this is the first study evaluating *KRAS* and *PIK3CA* mutation specifically in Korean CMMs, and confirmed no mutations in *KRAS* or *PIK3CA*.

The small numbers of cases and the possible selection bias of current study may make definitive conclusions difficult. Nevertheless, some messages emerged from our results. The presence of *BRAF* and *NRAS* mutations in approximately one third of subjects, the lack of *KRAS* or *PIK3CA* mutations, and their decreased anti-tumor immunity suggest that Korean CMMs may be beneficial for combined immunotherapeutic strategies and molecular targeted therapy. Because the prognostic impact of *BRAF* or *NRAS* mutations alone is limited, subgroup analyses based on TICs and *BRAF* and *NRAS* mutations may help to identify high-risk patients with worse clinical outcomes.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152671.

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