Abstract. Background: Project HOPE (High-tech Omics-based Patient Evaluation) has been in progress since 2014 and uses whole-exome sequencing (WES) and gene expression profiling (GEP). Among a total of 1,685 patients with cancer, 13 with melanoma were registered and characterized using multi-omics analyses to investigate specific biomarkers in responders to programmed cell death-1 (PD-1) blockade. Materials and Methods: The patients with melanoma comprised of six males and seven females, and their mean age was 68 years. Five patients were treated with nivolumab, and two were responders. Results: GEP analysis demonstrated that PD-L1 expression was positive in for cases, and melanoma-associated antigens and tumor signaling-associated genes were up-regulated in tumor compared with normal tissues. Additionally, WES analysis indicated more single nucleotide variants (SNVs) per melanoma tumor compared to other tumor types. Remarkably, a case of complete remission after nivolumab therapy showed high expression of PD-L1 protein and the highest number of SNVs. Conclusion: The novel approach used in Project HOPE might be an efficient tool that facilitates identifying specific biomarkers predictive of good responders to anti-PD-1 therapy.

Because of the recently reported success of immune checkpoint antibodies, such as ipilimumab and nivolumab, against metastatic melanoma and other advanced types of solid cancers (1-3), the current research approach has been shifting to developing a method of evaluating patients with cancer to identify good responders who have few adverse responses to immune checkpoint blockade treatment (4-6). Therefore, novel and efficient biomarkers that could contribute to the accurate prediction of good responders have been eagerly studied. This research has been carried out for a promising combination therapy with ipilimumab and nivolumab, which resulted in a very high response rate and long-term survival benefit in patients with advanced cancer, including non-small cell lung cancer (7, 8).

Based on recent clinical research, programmed cell death 1 ligand-1 (PD-L1) expression and a high mutation load are currently considered possible biomarkers. Garon et al. reported that the PD-L1 expression level was closely associated with antitumor responses in pembrolizumab-treated patients with non-small cell lung cancer (9). Additionally, Snyder et al. demonstrated that metastatic melanomas with high mutation load showed good response to ipilimumab and efficient neoantigens (10). The more opportunities there are to use immune checkpoint blockade therapy for solid cancers, especially in combination with other chemotherapeutic agents, the more valuable immune checkpoint blockade therapies become. Thus, it is crucial that both the genetic status of cancer and the immunological status of the patient at the local tumor site are simultaneously evaluated. Project HOPE is an advanced and informative tool that represents a novel approach to genome-based patient evaluation that facilitates choosing an optimal chemotherapy or immunotherapy.

Here, we performed comprehensive genetic profiling of 13 Japanese patients with melanoma using whole-exome...
sequencing (WES) and gene expression profiling (GEP) in Project HOPE (High-tech Omics-based Patient Evaluation) and we investigated the association between immune checkpoint-related gene expression and the response to nivolumab and with markers of tumor-infiltrating lymphocytes (TILs).

Materials and Methods

Patient registration. This clinical research project used comprehensive whole-exome sequencing (WES) and gene expression profiling (GEP) of various tumor tissues; the project was called HOPE and was conducted in accordance with the “Ethical Guidelines for Human Genome and Genetic Analysis Research” revised in 2013 (11, 12). Informed consent was obtained from all patients participating in Project HOPE, and the study was approved by the Institutional Review Board of the Shizuoka Cancer Center, Japan (Approved No. 26-62-26-1-5). The tumor tissues, along with the surrounding normal tissues, were dissected from surgical specimens by trained pathologists. From 2014, a total of 1,685 patients with cancer were registered in Project HOPE. The characteristics of 13 patients with melanoma are shown in Table I.

![Table I. Melanoma patient list registered in Project HOPE.](image)

Comprehensive gene expression analysis using DNA microarray analysis. Total RNA was extracted from approximately 10 mg of tissue using the miRNaseasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Total RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and RNA with an RNA integrity number (RIN) of 6 or greater was used for DNA microarray analysis. Comprehensive gene expression analysis was performed using SurePrint G3 Human GE 8x60 K v2.0 arrays (Agilent Technologies) according to the manufacturer’s instructions and as previously reported (11). Briefly, signal data analysis was performed using GeneSpring software version 13.1.1 (Agilent Technologies). The ratio of the expression intensity between the tumor tissue (T) and the surrounding normal tissue (N) was calculated from the normalized values. A T/N ratio of more than 2 or less than 0.5 was scored as gene up-regulation or down-regulation, respectively. The ratio, which is shown on log scale, was used for heat map analysis. Differential expression of B7- and CD28-family genes was investigated between responders and non-responders to nivolumab therapy.

WES analysis of melanoma tissues using next-generation sequencing. WES and variant calling were performed using an Ion Proton AmpliSeq Exome kit and Ion Torrent server as previously reported (12). Briefly, all variants called by the variant caller were available. However, the data from the Shizuoka Cancer Center represent those variants considered to be of good quality, based on filtering: sequences with a quality <30, frequency <10% or coverage <20 were discarded. After filtering a second time to remove germline variants (using data from dbSNP, the 1,000 Genomes Project), the obtained sequence data were likely somatic. Single nucleotide variants (SNVs) of the total exonic mutations for each sequenced tumor included nonsynonymous, synonymous, and indels/frameshift mutations.

Immunohistochemistry. For immune checkpoint proteins staining, an antibody to PD-L1 was purchased (Cell signaling, Danvers, MA, USA). For staining of TILs, anti-CD4 and anti-CD8 (Thermo Fisher Scientific, Waltham, MA, USA) and antibody to programmed cell death 1 (PD-1) (Abcam, Cambridge, MA, USA) were purchased and used for immunohistochemical analysis. In each section stained with the different antibodies, more than 10 high-magnification (×200) fields were analyzed using Winroof image-analyzing software (Mitani Corporation, Tokyo, Japan).

Statistical analysis. Correlations between gene expression and pathological features were analyzed using unpaired two-tailed t-tests. Values of p<0.05 were considered significant.
Results

**Melanoma patient list in Project HOPE.** The patients with melanoma comprised of six males and seven females, with a mean age of 68 years. The number of patients in each clinical stage were as follows: stage I: one, II: two, III: nine and IV: one, respectively. Three patients were treated with chemotherapy and radiation therapy before HOPE registration. Five patients were treated with nivolumab, and two were responders. Eleven patients with primary and two with metastatic lesions were enrolled in Project HOPE. Nine patients were alive as of July 2016 (Table I). Genomic data derived from 11 evaluable melanomas from which both GEP and WES data were obtained were analyzed. WES analysis showed more SNVs per melanoma tumor compared with other tumor types (330 vs 183, respectively).

**WES and GEP analysis of 13 patients with melanoma.** Mutations in **BRAF** were identified in four cases; three had a V600E mutation, which was not found in a hypermutated case with more than 200 SNVs. Other mutations that are common in melanoma as well as Vogelstein driver mutations, were not detected (Figure 1A). GEP analysis showed that common melanoma antigen genes [tyrosinase (TYR), melanoma antigen family A1 (MageA1), baculoviral IAP repeat containing 5 (BIRC5)], stem cell-associated genes [ATP-binding cassette subfamily C member 2 (ABCC2), SRY-box 2 (SOX2)] and Vogelstein driver oncogenes [anaplastic lymphoma receptor tyrosine kinase (ALK), MET] were up-regulated. However, some Vogelstein driver genes [androgen receptor (AR), fibroblast growth factor 3 (FGF3), epidermal growth factor receptor (EGFR), GATA binding protein 3 (GATA3)] were down-regulated (Figure 1B).

**Expression of melanoma antigen genes.** Based on the GEP data obtained from Project HOPE, the expression of melanoma antigen genes, such as MageA1, MageA2, MageA6, MageA12, cancer/testis antigen 1B (CTAG1B, NY-ESO-1), TYR, melan-A (MLANA, MART1) and premelanosomal protein (PMEL, GP100) was investigated. With the exceptions of MEL-001 and MEL-013, most cases showed increased expression of several melanoma antigen...
Expression of B7 and CD28 family genes. The expression of B7 family genes [CD80, CD86, inducible T-cell co-stimulator ligand (ICOSLG, B7H2), programmed cell death 1 ligand 1 (PDCD1LG1, PD-L1), programmed cell death 1 ligand 2 (PDCD1LG2, PD-L2), CD276 (B7H3), V-set domain containing T-cell activation inhibitor 1 (VTCN1, B7H4), V-set immunoregulatory receptor (VSIR, B7H5), natural killer cell cytotoxicity receptor 3 ligand 1 (NCR3LG1, B7H6), HERV-H LTR-associating 2 (HHLA2,
and CD28 family genes [CD28, cytotoxic T-lymphocyte associated protein 4 (CTLA4), inducible T-cell costimulatory (ICOS), PD-1 (PDCD1), B- and T-lymphocyte-attenuator (BTLA)] was investigated. B7H3, and activated T-cell-associated markers such as CD28, CTLA4, ICOS and PD-1 were up-regulated in most melanomas. In contrast, CD86, ICOSLG, B7H4 and B7H5 were down-regulated (Figure 2B). The expression of PD-L1 was positive in four out of 11 cases (36.3%).

Comparison of expression of B7 and CD28 family genes between responders and non-responders to nivolumab therapy. Expression of B7 family and CD28 family genes was compared between two responders and two non-responders to nivolumab. The expression of PD-L1 was up-regulated in the responder group, but no other genes showed significantly altered expression (Figure 3).

Association of PD-L1 and PD-1 expression levels and high mutation load with T-cell markers from TILs. The correlation between PD-L1 and PD-1 mRNA levels in tumors, hypermutation and T-cell marker expression was investigated. T-cell marker genes, such as CD3E, CD4, CD8 and interferon γ (IFNG), were used for analysis. The SNV cut-off value for hypermutation was tentatively 100. Values of p<0.05 were considered significant. **p<0.01.
Discussion

As part of Project HOPE, comprehensive WES and GEP for 13 patients were performed. WES analysis detected BRAF mutations in four melanomas, three of which showed a hot spot (V600E). Many patients possessed the same mutations in the same genes, such as titin (TTN), mucin 16 (MUC16), and xin actin binding repeat containing 2 (XIRP2), which were not on the Vogelstein driver gene list but were apparently passenger mutations that are probably not linked to melanoma progression. The total number of exonic SNVs ranged from 15 to 2,712 (1,599 non-synonymous SNVs) with a mean of 330, which is a high value compared with the mean for other non-melanoma tumors (183 per tumor) (13). Metastatic melanomas in Caucasians have the most SNVs across various types of cancer (14); these SNVs are associated with good responses to immune checkpoint antibody therapy. (10,15).

The GEP analysis showed that the paired box 3 (PAX3), SOX10, TYR and melanogenesis-associated transcription factor (MITF) genes were overexpressed more than 10-fold in melanoma tissue compared with normal tissue. These transcription factors are known to be involved in melanoma progression and invasion by activating the MET signal (16-18). According to a recent genomic study and classification using 333 melanoma tumors, the most prevalent mutated genes were BRAF, RAS and neurofibromin 1 (NFI) (19). The ABCC2, NANO G and telomerase reverse transcriptase (TERT) genes are reported to be informative for identifying human melanoma stem cells (20).

Interestingly, Rambow et al. developed a similarity core analysis using a universal and unsupervised computational framework for extracting core molecular features common to melanomas; they identified MITF as a key molecule and determined a relevant network of transcription factors (PAX3, SOX10, TYR and BIRC5) that belong to a novel melanoma signature (21). Recently, MITF and PAX3 were reported to induce acquired resistance to BRAF or mitogen-activated protein kinases (MAPK) inhibitors and could be novel targets for BRAF inhibitor-resistant melanomas (22).

To investigate the association between melanoma antigen expression and immunological status inside the tumor, various melanoma antigens and the expression of B7- and CD28-family genes were evaluated. Most of the eight melanoma antigen genes were up-regulated in seven out of 13 cases. In contrast, all melanoma antigen genes were down-regulated in the metastatic melanoma lesion from MEL-001, as shown in Figure 2. The down-regulation of melanoma antigens in metastatic sites has been reported by several researchers (23, 24) and is considered a mechanism to escape the immuno-surveillance system (25, 26). Regarding B7 and CD28 gene expression, PD-L1 and B7H3 were up-regulated in more than half of the 13 melanomas; however, other immune checkpoint molecules, such as B7H4 and B7H5, were down-regulated, as shown in Figure 2. CD28-family genes, such as CD28, CTLA4, ICOS and PD-1, were up-regulated in most melanomas, which suggests the infiltration of activated T-cells into the tumor site.

Next-generation sequencing technology has enabled clinicians and researchers involved in cancer immunology to study the molecular events in the tumor microenvironment and has highlighted unresolved areas of tumor immunology (27, 28). Specifically, genomic analysis of the tumor-infiltrating T-cell receptor repertoire has been investigated using next-generation sequencing (29). The current study aimed to understand the association between immune checkpoint-related factors and melanoma antigen expression and immunological status by TILs and the co-relation...
between PD-L1, PD-1 and tumor-specific SNV number and TIL marker gene expression. PD-1 expression was strongly correlated with CD8 and IFMG gene expressions in the melanomas. These results might suggest that PD-1* activated effector T-cells infiltrated into the tumor site. PD-L1 and a high mutation load did not strongly correlate with TIL marker gene expression. The combination of PD-L1 and PD-1 is reported to be an efficient biomarker for immune checkpoint blockade at the tumor site (30,31).

Finally, a preliminary study comparing B7-family gene expression between responders and non-responders to nivolumab therapy demonstrated PD-L1 up-regulation in responding patients, as shown in Figure 3. However, the association between responses to nivolumab therapy, PD-L1 expression and the number of SNVs is not yet confirmed because of the small number of antibody-treated cases in the Japanese melanoma patient cohort. We expect to study the genomic signature of additional nivolumab-treated Japanese patients with melanoma in Project HOPE with the aim of identifying novel biomarkers predictive of good responses and few adverse effects.

Conflicts of Interest

The Authors declare that they have no conflict of interest.

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References


