LETTER TO THE EDITOR

Intra-tumoral heterogeneity and immune escape of melanoma arising from congenital melanocytic nevus revealed by spatial gene expression profiling

Dear Editor,

Previous studies have revealed the presence of "inter-tumoral" heterogeneity of congenital melanocytic nevus (CMN), primary, and metastatic melanomas in patients using next-generation sequencing, suggesting that CMN cells evolve sporadically.[1,2] Furthermore, in practice, tissue slides of melanomas arising from CMNs often show several melanoma cell clusters with different morphologies, even within a single lesion. Here, we applied spatial transcriptomics to evaluate "intra-tumoral" heterogeneity and immune evasion processes during tumor progression in melanoma arising from CMN.

We obtained an excision specimen from a 41-year-old man who had malignant melanoma with $NRAS^{Q61R}$ mutation and wild-type BRAF/NF1 arising from a 4 cm CMN with several small satellites on his lower back (Fig. 1a). He had malignant melanoma up to 4.4 mm thick with ulceration and no lymph node involvement or distant metastasis. Therefore, he was diagnosed with stage IIC (T4bN0M0) disease. We divided the tissue slide of the patient's excised sample into four areas containing four different cell clusters with different histological features and annotated them as M1 to M4 (Fig. 1b,c). From M1 to M4, cell clusters gradually more deeply invaded, from 300 to 4200 μm, measured with Breslow thickness. Cytologic atypia of melanoma cell clusters increased from M1 to M4. Analysis of tumor-infiltrating immune cells showed that lymphocyte infiltration decreased from M1 to M4, whereas macrophage infiltration tended to increase.

We performed spatial transcriptomic analysis of the excised sample using the GeoMx DSP platform (NanoString Technologies, Seattle, WA, USA).[3–8] Four regions of interest (ROIs) were selected from each area, from M1 to M4 (Fig. 1d). A total of 16 ROIs were analyzed using the whole-transcriptome atlas.[3,5] Among the 18 677 genes collectively expressed in 16 ROIs, 2067 (11.07%) genes showed significant differences. Comparing M1 to M4, 839 out of 2067 (40.59%) genes were upregulated, while 1005 out of 2067 (48.62%) genes were downregulated ($P < 0.0005$, Fig. 2a). The heatmap of these significant differentially expressed genes revealed heterogeneity in the gene

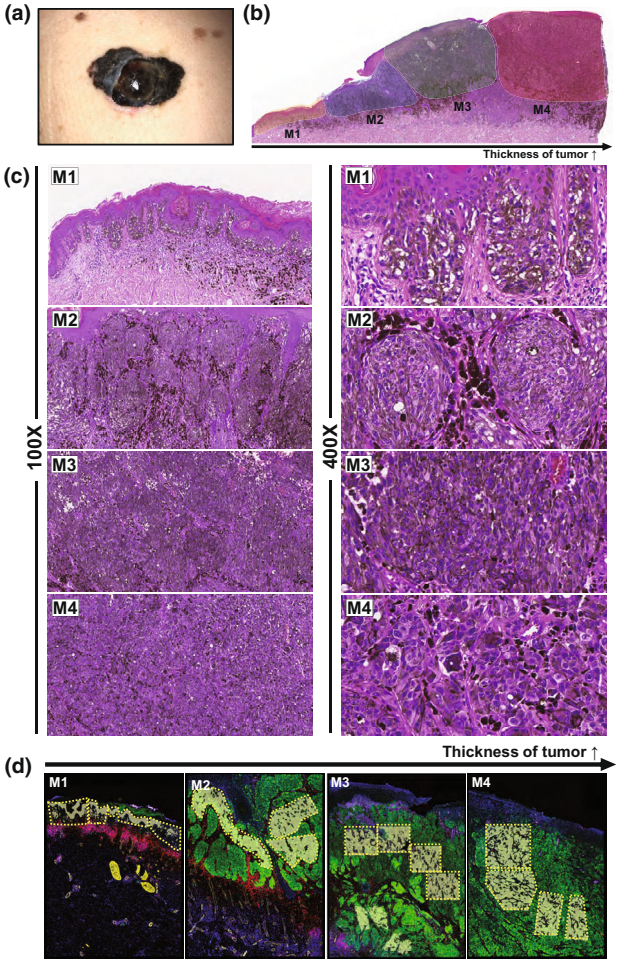
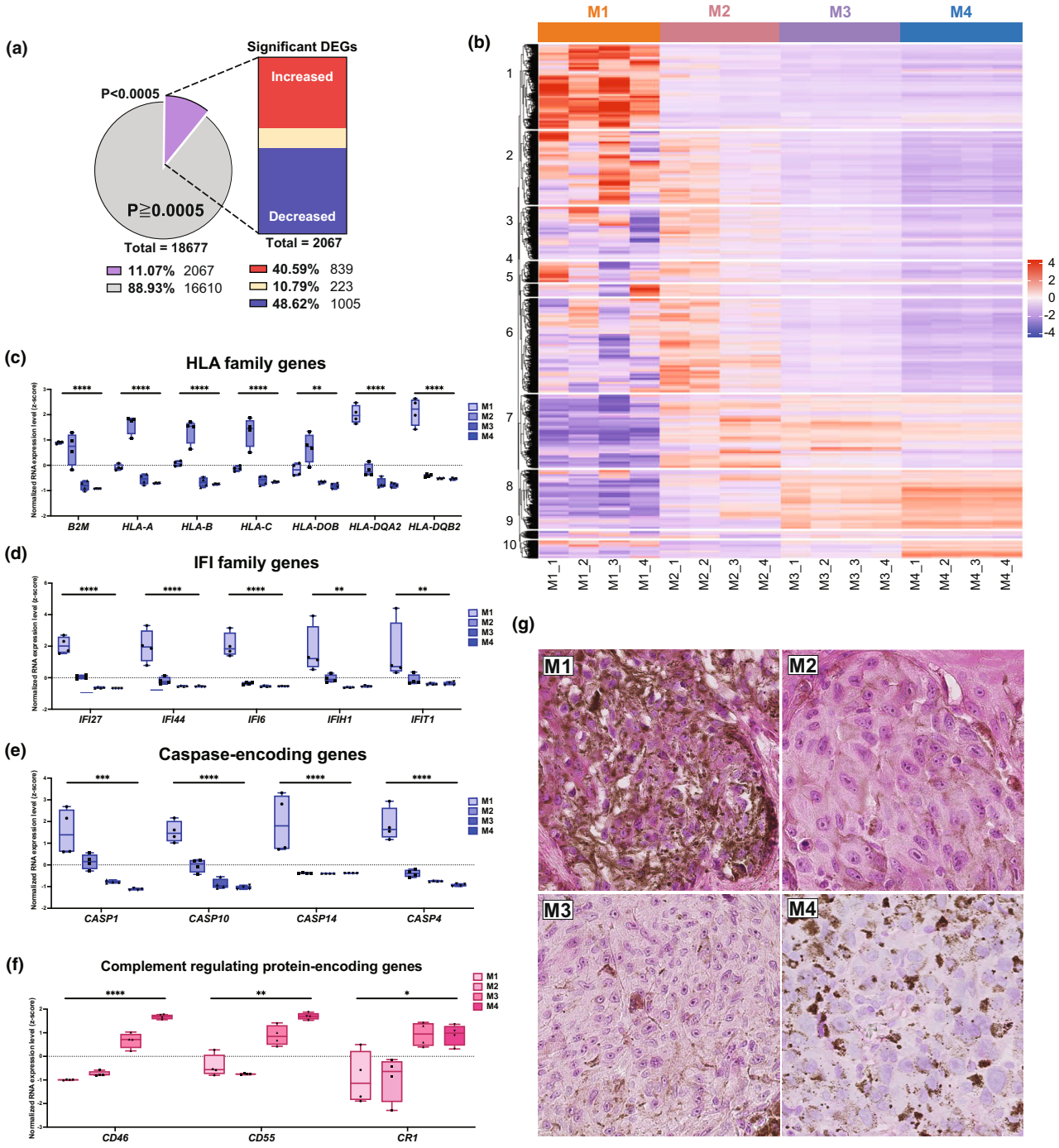


Figure 1 Region segmentation and histological features according to different cell clusters of melanomas arising from congenital melanocytic nevus. (a) Clinical photograph of the malignant melanoma on the left lower back of the patient. (b) Tissue slide of the excised sample was divided into four areas containing four different cell clusters showing different histological features and the clusters were annotated M1 to M4. The cell clusters more deeply invaded from 300 to 4200 μm from M1 to M4. (c) Representative magnified images of cell clusters of M1 to M4 (hematoxylin and eosin, 100 × in the upper row and 400 × in the lower row). Cytologic atypia of melanoma cell clusters increased from M1 to M4. For tumor-infiltrating immune cells, lymphocyte infiltration decreased from M1 to M4, whereas macrophage infiltration showed a tendency to increase. (d) Selected regions of interest (ROIs) for spatial transcriptomic analysis are indicated by polygonal boxes with yellow dotted lines and visualized using immunofluorescence staining; Blue, nucleus; green, S100B; red, CD3e. (200× magnification). Cells were segmented based on florescent expression of S100B+ (green) to capture RNA from each ROI.



expression of tumor cell clusters within each area (Fig. 2b). As cytologic atypia and the depth of invasion of melanoma cell clusters increased, significant differences in gene expression levels were observed between clusters, particularly for genes involved in immune evasion, such as antigen processing and presentation, interferon alpha-inducible proteins, caspases related to apoptosis, and complement-regulatory proteins (Fig. 2c–f). Our results showed a gradual loss of human leukocyte antigen (HLA) class I and II family molecules as cytologic atypia and tumor depth increased. The level of β2-microglobulin (B2M), a representative HLA-I molecule important for tumor antigen presentation, decreased with tumor progression (Fig. 2g). The decreased

Figure 2 Differential gene and protein expression in cell clusters showing different cell morphology revealed by spatial transcriptomics. (a) Analysis of differentially expressed genes (DEGs) revealed 2067 genes (11.07%) with significant differences ($P < 0.0005$) between M1 and M4. There were 839 genes of the 2067 (40.59%) upregulated in M4 compared to in M1, while 1005 of 2067 genes (48.62%) were downregulated in M4 compared to M1. Two hundred twenty two genes (10.79%) showed similar expression level between M1 and M4. (b) Heatmap of significant differentially expressed genes was constructed in R using the "ComplexHeatmap" package. Both columns and rows are clustered using the correlation distance, with transcriptional heterogeneity of tumor cell clusters within each area showing different histological features from M1 to M4. (c–f) Expression levels of (c) *HLA* family genes, (d) *IFI* family genes, and (e) caspase-encoding genes gradually decreased from M1 to M4, whereas those of (f) complement regulatory protein-encoding genes increased in M4 compared to M1. Significance levels are indicated by the number of asterisks (*). *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, and ****$P < 0.00005$. (g) Protein expression of B2M (red) of each melanoma cell cluster in M1 to M4 was confirmed using immunohistochemistry. M1 stained with intensity score 3 and percentage score 3; M2 stained with intensity score 2 and percentage score 3; M3 stained with intensity score 1 and percentage score 3; M4 stained with intensity score 1 and percentage score 1.

B2M expression in melanoma cells may have reduced antigenicity and decreased lymphocyte infiltration by inducing immune evasion as melanoma progressed. In melanoma, a decrease in HLA-I has been reported to be associated with poor prognosis,[9] and B2M loss is associated with a therapeutic response to immunotherapy such as checkpoint blockade.[10] Therefore, although further evaluation is needed to confirm our findings, examining the expression levels of HLA-I-related genes, including B2M, in heterogeneous progressive cell clusters in patients with melanoma may help predict the therapeutic response to immunotherapy and patient prognosis.

In conclusion, we identified the differential gene expression profiles during tumor progression of melanoma arising from CMN using spatial transcriptomics, providing deeper insights into the intra-tumoral heterogeneity and tumor evolutionary processes. Our results also suggest that a decrease in B2M is a key factor influencing immune escape mechanisms during the progression of CMN melanoma. Additional studies with a larger sample size are needed to further validate our findings. This study may facilitate the investigation of the evolutionary dynamics and immune evasion strategies of skin cancers.

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Ethics approval

This study was approved by the Institutional Review Board of Asan Medical Center (IRB No. 2020–1102).

Conflicts of interest

The authors have no conflicts of interest to declare.

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Data availability statement

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

Y. Lim,[1,*,†] S.-J. Kang,[2,3,†] B.K. Cho,[2] H.J. Kim,[3,4,5] J.-H. Mun,[1] M.R. Roh,[6] N. Gulati,[7] H.J. Yang,[8] J.H. Moon,[9] C.H. Won,[8,*] C.-G. Park[2,3,4,*]

[1]Department of Dermatology, Seoul National University Hospital, Seoul, South Korea, [2]Department of Biomedical Sciences, Seoul National University Graduate School, Seoul, South Korea, [3]Cancer Research Institute, Seoul National University College of Medicine, Seoul, South Korea, [4]Department of Microbiology and Immunology, Seoul National University College of Medicine, Seoul, South Korea, [5]Genome Medicine Institute, Seoul National University College of Medicine, Seoul, South Korea, [6]Department of Dermatology, Gangnam Severance Hospital, Cutaneous Biology Research Institute, Yonsei University College of Medicine, Seoul, South Korea, [7]Department of Dermatology, Icahn School of Medicine at Mount Sinai, New York, New York, USA, [8]Department of Dermatology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea, [9]Samsung Genome Institute, Samsung Medical Center, Seoul, South Korea

*Correspondence: Y. Lim, C.H. Won and C.-G. Park.

E-mail: ykwh01232@naver.com (YL); drwon@amc.seoul.kr (CHW); chgpark@snu.ac.kr (C-GP)

[†]These authors contributed equally as co-first authors to this study.

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