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SHORT REPORT

Spatial transcriptomic analysis of tumour–immune cell interactions in melanoma arising from congenital melanocytic nevus

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Abstract

Background: Studies on the interaction between tumour-infiltrating immune cells (TIICs) and tumour cells in melanoma arising from congenital melanocytic nevus (CMN) are lacking.

Objective: The aim of this study was to determine the intratumoral immune landscape of TIICs and tumour cells during invasion and metastasis.

Methods: Tissue specimens were obtained from patients with melanoma originating from CMN. Differential gene expression in melanoma cells and TIICs during invasion and metastasis was determined using spatial transcriptomics.

Results: As invasion depth increased, the expression of *LGALS3*, known to induce tumour-driven immunosuppression, increased in melanoma cells. In T cells, the expression of genes that inhibit T-cell activation increased with increasing invasion depth. In macrophages, the expression of genes related to the anti-inflammatory M2 phenotype was upregulated with increasing invasion depth. Compared to primary tumour cells, melanoma cells in metastatic lesions showed upregulated expression of genes associated with cancer immune evasion, including *AXL* and *EPHA2*, which impede T-cell recruitment, and *BST2*, associated with M2 polarization. Furthermore, T cells showed increased expression of genes related to immunosuppression, and macrophages exhibited increased expression of genes associated with the M2 phenotype.

Conclusions: The interaction between melanomas arising from CMN and TIICs may be important for tumour progression and metastasis.

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Youngkyoung Lim and Beom Keun Cho contributed equally as co-first authors to this study.

INTRODUCTION

Congenital melanocytic nevus (CMN) is a benign proliferation of melanocytes, most commonly present at birth, and its prevalence is approximately 1% in newborns.[1] Although the risk of a single small CMN developing into melanoma is very low, the lifetime risk increases up to 10%–15% for a giant CMN accompanied by multiple satellite lesions.[1]

Intratumoral heterogeneity occurs during tumour progression in CMN-related melanoma.[2] Particularly, we previously reported that the expression of genes related to antigen processing and presentation via the major histocompatibility complex (MHC) decreased as the depth of invasion of melanoma cell clusters increased.[2] However, we only observed the gene expression profiles of melanoma cells, without focusing on tumour-infiltrating immune cells (TIICs).[2] Different TIIC types may play active roles in tumours, increasing or decreasing anti-tumour immunity in melanoma.[3,4] In the present study, we aimed to elucidate the interaction between TIICs and tumour cells and their role in the invasion and metastasis of melanoma arising from CMN using spatial transcriptomics.

PATIENTS AND METHODS

Tissue samples from primary and metastasized tumours were collected from patients diagnosed with malignant melanoma arising from CMN. The tissue section of an invasive melanoma sample of Patient 1 was divided into two areas according to the invasion depth, and the superficially and deeply invaded areas were annotated as 1(S) and 1(D), respectively (Figure 1a). In addition, the primary and skin metastasis lesions of Patient 2 were annotated as 2(P) and 2(M), respectively (Figure 1b). For spatial transcriptomic analysis, the sections were stained with four morphological markers to visualize the nuclei, T cells, macrophages and melanoma cells (Appendix S1). Regions of interest (ROIs) were selected (Appendix S2 and Figure S1). The ROIs of Patients 1 and 2 were segmented based on the fluorescent expression of CD3 and CD68. We analysed melanoma cells, T cells and macrophages, in each area to identify differentially expressed genes (DEGs) associated with invasion and metastasis. Immunohistochemical analysis was performed to confirm the results of spatial transcriptomic analysis (Appendix S3).

RESULTS

We analysed excised samples of primary and metastatic melanomas from two patients. Patient 1 was a 41-year-old man with a malignant melanoma arising from CMN on his lower back.[2] The melanoma was up to 4.4 mm thick with ulceration, with no lymph node involvement or distant metastasis. He was diagnosed with Stage IIC disease (T4bN0M0). *NRAS* Q61R mutation and wild-type *BRAF* were detected. 1(S) showed confluent nests of melanoma cells in the epidermis and upper dermis, and 1(D) showed pleomorphic melanoma cells with prominent vesicular nuclei and large nucleoli extending into the deeper dermis compared to 1(S) (Figure 2a–d). Lymphocyte infiltration decreased from 1(S) to 1(D), whereas macrophage infiltration increased.

CXCL9 and *CXCL10* expression decreased, whereas *LGALS3* expression increased in the deeply invaded area in Patient 1 compared with those in the superficially invaded area (Figure 2e,f). Heatmaps of significant DEGs revealed

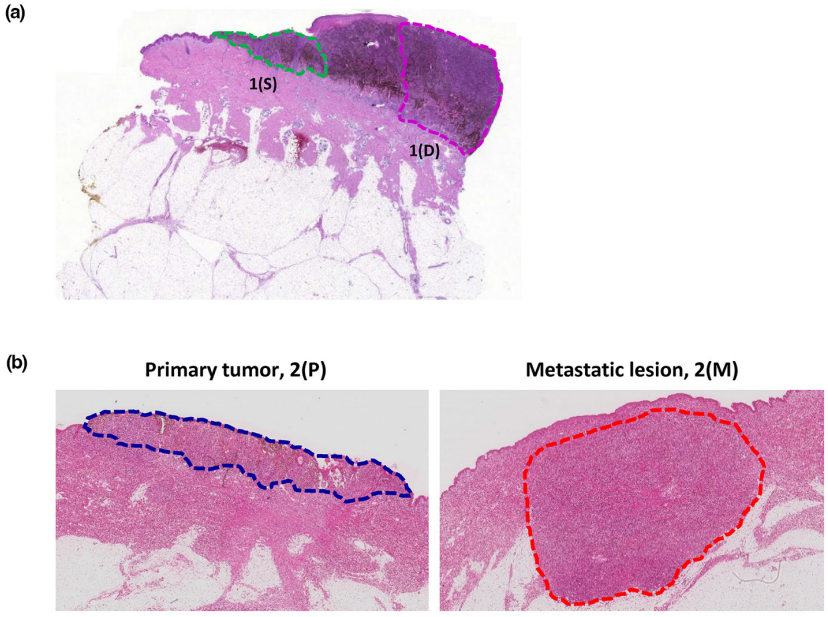

FIGURE 1 Tissue section annotations for patient samples. (a) The tissue section of the excised tumour of Patient 1 was divided into two areas, 1(S) and 1(D), according to the invasion depth (haematoxylin and eosin, 4× magnification). (b) Tissue samples of the excised primary and metastatic tumours of Patient 2 were obtained and annotated as 2(P) and 2(M) according to metastasis (haematoxylin and eosin, 10× magnification).

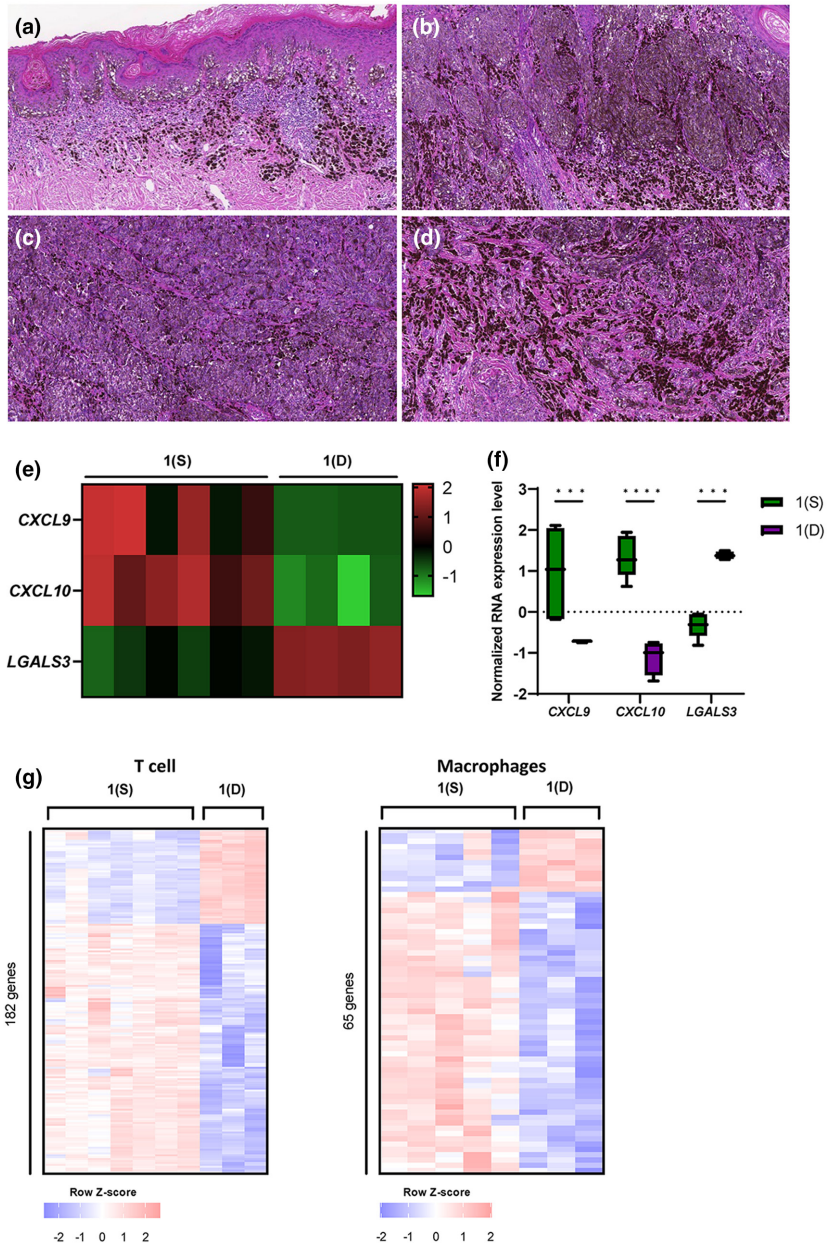

FIGURE 2 Histopathologic features of the melanoma sample of Patient 1 and differential gene expression profiles of tumour-infiltrating immune cells according to the depth of invasion. (a, b) 1(S) showed features of melanoma in situ and also showed confluent nests of melanoma cells in the epidermis and upper dermis. Prominent lymphocyte and macrophage infiltration around the tumour nests were observed in 1(S) (haematoxylin and eosin, 100× magnification). (c, d) Within 1(D), as the tumour thickness increased, infiltration increased more deeply into the dermis. Densely proliferating melanoma cells were observed in 1(D). The morphology of melanoma cells in 1(D) showed pleomorphic and bizarre melanoma cells with prominent vesicular nuclei and large nucleoli. Macrophage infiltration was predominant around the tumour cell nests and showed low levels of lymphocyte infiltration in 1(D) (haematoxylin and eosin, 100× magnification). (e) Heatmap showing the relative expression levels of *CXCL9*, *CXCL10*, and *LGALS3* in 1(S) and 1(D) melanoma cells. (f) Normalized RNA expression levels of *CXCL9*, *CXCL10*, and *LGALS3*. *CXCL9* and *CXCL10* were highly expressed in 1(S) but significantly decreased in 1(D). The expression of *LGALS3* increased from 1(S) to 1(D) with an increasing depth of invasion. (g) Heatmaps showing significant differentially expressed genes. Both columns and rows are clustered using the correlation distance, showing transcriptional heterogeneity of tumour-infiltrating T cells and macrophages in 1(S) and 1(D). Each column represents a ROI.

distinct gene expression patterns between 1(S) and 1(D), indicating heterogeneity in the expression of TIIC clusters between superficially and deeply invaded areas (Figure 2g). In 1(D) T cells, the expression of genes related with T-cell activation and tumour infiltration, such as *GZMK*, *CCL5*, *IL7R*, *TNFSF10*, *PYHIN1*, *JAML*, *GNLY*, *IL15RA*, *OAS2*, *GIMAP7*, *IL32*, *CXCR3*, *CCR4*, *IRF8* and *CST7*, was downregulated, whereas that of genes involved in T-cell inhibition, such as *GDF15* and *GPNMB*, was upregulated, compared with those in 1(S) T cells (Figure 3a). The expression of M1 macrophage marker genes, such as *CXCL9*, *GBP5*, *HLA-DQA2*, *HLA-DPA1*, *HLA-DPB1*, *ADAMDEC1* and *CCR7*, was downregulated in 1(D) macrophages compared with that in 1(S) macrophages. The expression of M2 macrophage genes such

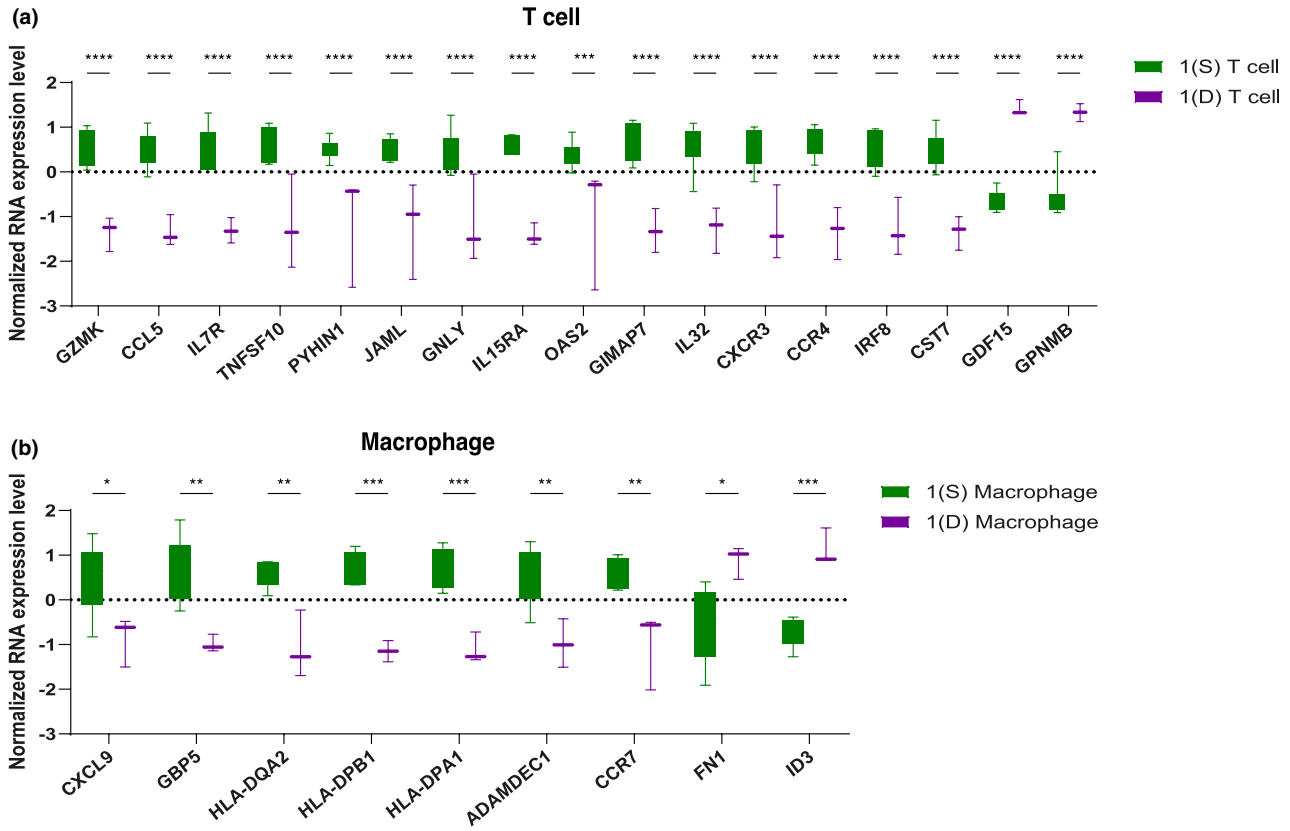

FIGURE 3 Gene expression signature of tumor infiltrating immune cells of Patient 1 according to the depth of invasion. (a, b) Box plots of normalized RNA expression levels of several differentially expressed genes between 1(S) and 1(D) T cells (a) and macrophages (b). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

as *FN1* and *ID3* was upregulated in 1(D) macrophages compared with that in 1(S) macrophages (Figure 3b). Consistent with the spatial transcriptomic results, galectin-3 protein expression in melanoma increased with the depth of invasion. Conversely, the number of tumour-infiltrating CD8+ T cells was lower in the deeply invaded area than in the superficially invaded area (Appendix S4 and Figure S2).

Patient 2 was a 68-year-old man with a malignant melanoma arising from CMN on the left buttock. The primary melanoma, 2(P), was 2.5 mm thick without ulceration. Regional lymph node metastases were observed without distant metastases. The patient was diagnosed with Stage IIIB disease (T3aN1bM0). *NRAS* Q61K mutation and wild-type *BRAF* were detected. During follow-up, a new skin metastasis, 2(M), was identified in the left thigh. 2(P) showed pleomorphic melanoma cells in the epidermis and upper dermis. 2(M) showed melanoma cells undergoing mitosis invading the deep dermis and subcutaneous fat, forming a nodular tumour mass without invasion of the overlying epidermis. Less lymphocyte infiltration was observed in melanoma cells in 2(M) compared with those in 2(P) (Figure 4a–d). Using heatmaps, we observed that the ROIs of 2(P) and 2(M) exhibited different gene expression patterns (Figure 4e). The upregulated genes in melanoma cells in the metastasized lesion of Patient 2 compared with those in the primary tumour were genes associated with immune evasion and cancer progression, including *AXL* and *EPHA2*, which impede T-cell recruitment, and *BST2*, associated with M2 polarization (Figure 4f). The expression of *ADGRG1*, associated with T-cell exhaustion, was upregulated in T cells of the metastasized lesion compared with those in the primary lesion. The levels of M2 macrophage-associated genes, such as *TSC22D3*, *MT2A* and *FKBP5*, were higher in 2(M) macrophages than in 2(P) macrophages (Figure 4f). Using immunohistochemistry, we observed increased AXL expression in the metastatic lesion compared with that in the primary tumour (Appendix S5 and Figure S3). Consistently, we observed a lower number of CD8+ T cells infiltrating the metastatic lesion tumour than the primary lesion (Appendix S5 and Figure S3).

DISCUSSION

Our results suggest that melanoma cells have evolved to adopt immune-evasion mechanisms during tumour invasion and metastasis. In the deeply invaded area, we observed an increase in the expression of *LGALS3* and a decrease in the expression of *CXCL9* and *CXCL10* compared with the superficially invaded area. In the metastatic lesion, the expression of *AXL*, *EPHA2* and *BST2* increased compared with that in the primary lesion. Galectin-3, encoded by *LGALS3*, induces tumour-driven immunosuppression by regulating immune cell function through the polarization to M2 macrophages,

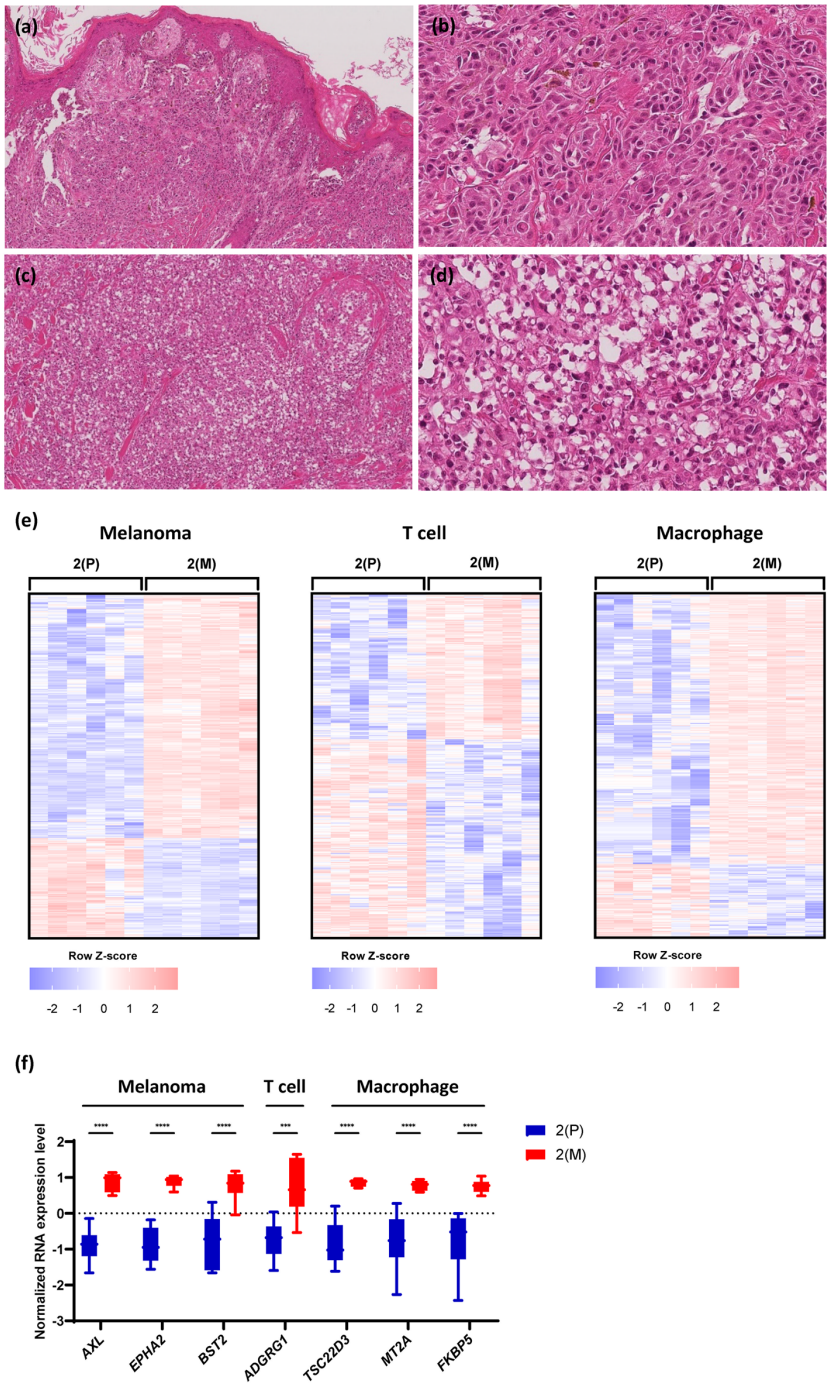

FIGURE 4 Histopathologic features of the primary and metastasized melanoma samples of Patient 2 and differential gene expression profiles of melanoma cells and tumour-infiltrating immune cells according to metastasis. (a, b) Haematoxylin and eosin staining of 2(P), showing the characteristic features of melanoma, including *Pagetoid* melanocytes and nests of pleomorphic melanoma cells in the epidermis and upper dermis. Lymphocytes and macrophages were observed in pleomorphic and atypical melanocytes. 100× (b) and 400× (c) magnification. (c, d) Haematoxylin and eosin staining of 2(M) showing haphazardly distributed atypical melanoma cells undergoing mitosis invading the deep dermis and subcutaneous fat, forming a nodular tumour mass without invasion of the overlying epidermis. Less lymphocyte infiltration was observed between melanoma cells in groups 2(M) and 2(P). 100× (d) and 400× (e) magnifications. (e) Heatmaps show significant differentially expressed genes in melanoma cells, tumour-infiltrating T cells and macrophages in 2(P) and 2(M). Both columns and rows are clustered using the correlation distance. Each column represents a ROI. (f) Normalized RNA expression levels of *AXL*, *BST2* and *EPHA2* in melanoma cells; *ADGRG1* and *TSC22D3* in T cells; and *MT2A* and *FKBP5* in macrophages are plotted. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

apoptosis of cytotoxic T lymphocytes and downregulation of T-cell receptor expression.[5] It has been reported that galectin-3-deficient mice are more resistant to metastatic malignant melanoma compared to wild-type mice.[5] CXCL9 and CXCL10 regulate the migration, differentiation and activation of immune cells by binding to CXCR3 of these cells,

leading to tumour suppression.[6] AXL signalling induces the secretion of immunosuppressive chemokines from cancer cells, inhibiting the recruitment of CD8+ T cells and promoting regulatory T-cell (Treg) activation, ultimately leading to an immune-suppressive effect.[7] The overexpression of EPHA2 in cancer cells reduces the recruitment of CD8+ T cells to tumours and regulates tumour invasion and progression.[8] In addition, overexpression of BST2 in cancer cells leads to an increase in the number of tumour-infiltrating M2 macrophages and results in enhanced proliferation.[8] M2 macrophages contribute to immune suppression and tumour progression by promoting fibroblast proliferation and angiogenesis through the expression of anti-inflammatory mediators and growth factors.[9] Collectively, these alterations in the gene expression of melanoma cells according to invasion depth and metastasis suggest that melanoma cells may have evolved to downregulate immune responses through T cells and to upregulate M2 macrophage polarization, as an immune-evasion strategy.

Changes in gene expression levels in TIICs, along with those in melanoma cells aforementioned, suggest a shift toward suppressing T-cell immune responses and inducing M2 polarization, during invasion and metastasis. T cells in the deeply infiltrated area of melanoma compared with those in the superficially infiltrated area showed low expression levels of genes related to CD8+ T-cell activation (*GZMK*, *GNLY*, *OAS2* and *IRF8*), tumour infiltration (*PYHIN1*, *GIMAP7*, *CXCR3* and *CCR4*) and antitumor activity (*JAML* and *IL32*).[6,10,11] The expression of genes suppressing immune responses was higher in T cells in the deeply invaded area than in the superficially invaded area; *GDF15*, which promotes the generation of Tregs, and *GPNMB*, which inhibits T-cell activation, were highly expressed.[12,13] T cells in metastatic lesions exhibited upregulation of genes associated with exhausted T cells, including *ADGRG1*, compared to T cells in the vicinity of the primary tumour.[14] Concomitant with these alterations of gene expression levels, the number of tumour-infiltrating cytotoxic T cells decreased during invasion and metastasis. Consistent with the upregulation of M2 polarization genes in melanoma cells during invasion and metastasis, tumour-infiltrating macrophages showed increased expression of *FN1*, *ID3* and *MT2A*, known inducers of M2 polarization and anti-inflammatory mediator genes (*TSC22D3* and *FKBP5*).[15–17]

In this study, differential gene expression profiles of melanoma cells and TIICs during invasion and metastasis were identified using spatial transcriptomics. Our results suggest that melanoma cells may interact with immune cells, including T cells and macrophages, to compromise anti-tumour immunity by T-cell immunosuppression and M2 macrophage polarization during invasion and metastasis. Further multicentre studies using a higher number of samples are needed to validate and extend our findings. This study paves way to a more comprehensive investigation into the dynamics of tumour–immune interactions and the immune escape mechanisms employed by advanced melanomas.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The study's supporting data can be obtained from the corresponding author on reasonable request.

ETHICS STATEMENT

This study was approved by the Institutional Review Boards of Asan Medical Center (approval no. 2020-1102) and Seoul National University Hospital (approval no. 2107-112-1235). Written informed consent was obtained from all included patients.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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