Prognostic significance of spatial immune profiles in human solid cancers

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Abstract
Immune-based tumor characteristics in the context of tumor heterogeneity are associated with suppression as well as promotion of cancer progression in various tumor types. As immunity typically functions based on intercellular contacts and short-distance cytokine communications, the location and spatial relationships of the tumor immune microenvironment can provide a framework to understand the biology and potential predictive biomarkers related to disease outcomes. Immune spatial analysis is a newly emerging form of cancer research based on recent methodological advances in in situ single-cell analysis, where cell-cell interaction and the tissue architecture can be analyzed in relation to phenotyping the tumor immune heterogeneity.

Spatial characteristics of tumors can be stratified into the tissue architecture level and the single-cell level. At the tissue architecture level, the prognostic significance of the density of immune cell lineages, particularly T cells, is leveraged by understanding longitudinal changes in cell distribution in the tissue architecture such as intra-tumoral and peri-tumoral regions, and invasive margins. At the single-cell level, the proximity of the tumor to the immune cells correlates with disease aggressiveness and therapeutic resistance, providing evidence to understand biological interactions and characteristics of the tumor immune microenvironment. In this review, we summarize recent findings regarding spatial information of the tumor immune microenvironment and review advances and challenges in spatial single-cell analysis toward developing tissue-based biomarkers rooted in the immune spatial landscape.

KEYWORDS
biomarker, multiplex immunohistochemistry, single-cell analysis, spatial analysis, tumor immune microenvironment

Abbreviations: FFPE, formalin-fixed paraffin-embedded; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; TLS, tertiary lymphoid structure.
INTRODUCTION

The immune system is involved in the process of cancer progression, from carcinogenesis to therapeutic resistance, in which cellular components such as immune infiltrates, the stroma, and vascular endothelial cells interact with each other to eliminate or promote tumors via the formation of the heterogeneous tumor microenvironment. Some studies have indicated that immune-based tumor characteristics in the context of tumor heterogeneity are deeply associated with therapeutic outcomes in a wide range of cancer types. Given that intercellular reactions are based on cell-cell contact and soluble factor gradients, spatial relationships in the multiple layers of the tumor immune microenvironment can provide a framework for understanding the biology of the tumor microenvironment, subsequently leading to the development of tissue-based predictive biomarkers for diseases outcomes. Recent advances have enabled adding spatial information to single-cell analysis in the context of tissues, phenotyping the tumor immune heterogeneity. In this review, we summarize recent findings regarding the prognostic significance of the spatial characteristics in the tumor immune microenvironment, and review the potential for developing tissue-based biomarkers based on immune spatial characteristics.

IMMUNE AND STROMAL CELL LINEAGES IN THE TUMOR MICROENVIRONMENT

Tumor cells are surrounded by the tumor microenvironment containing diverse cell types such as lymphoid and myeloid immune cell lineages, fibroblasts, endothelial cells, and a variety of tumor-associated tissue cells. Immune cells are a major component of the cellular milieu in the tumor microenvironment, where a combination of multiple lineage identification markers is utilized for classification of cell types, including CD8+ T cells, helper T cells, regulatory T cells (TREG), B cells, natural killer (NK) cells, macrophages and myelomonocytic populations, dendritic cells, mast cells, granulocytes, and other immune cells (Figure 2). In addition to the diversity of lineages, immune cells have functional and phenotypic heterogeneity. For example, macrophages are known to have functionally divergent phenotypes based on different polarization properties, in which tumor-associated macrophages show a typically M2-like phenotype, related to tumor progression via angiogenesis, immunosuppression, and activation of tumor cells. In non-hematopoietic lineages, cancer-associated fibroblasts and epithelial cells also exhibit heterogeneous profiles, which are associated with various stages of tumor progression via...
the formation of mechanical barriers, pro-inflammatory signals, recruitment of immune cells as well as immunosuppressive cascades.\textsuperscript{5,6} Notably, tumor cells and the surrounding cellular components have close relationships and intercommunications at the cellular and molecular levels, which provide a rationale for understanding cell-cell spatial relationships and tumor immune heterogeneity.

3 | IMMUNE SPATIAL RELATIONSHIPS AT THE TISSUE ARCHITECTURE LEVEL

The tissue architecture of established tumors can be classified historically into intratumor regions, and adjacent tissues, followed by functional characterization such as invasive margins, intratumoral stroma, TLS, and vascularization (Figure 1).

3.1 | Intratumoral regions

Early seminal studies revealed that immune cell density and distribution have prognostic implications in relation to the tumor tissue architecture, in which improved clinical outcomes are associated with the density of intratumoral T cells in colorectal\textsuperscript{7} and ovarian cancer.\textsuperscript{8} T cell infiltration in intratumor regions, but not in the peritumoral stroma, correlates with favorable prognosis in a wide range of tumor types such as colorectal cancer,\textsuperscript{7,9,10} ovarian cancer,\textsuperscript{8} urothelial carcinoma,\textsuperscript{11,12} non-small cell lung carcinoma,\textsuperscript{13} pancreatic ductal adenocarcinoma,\textsuperscript{14} triple negative breast cancer,\textsuperscript{15,16} and head and neck squamous cell carcinoma,\textsuperscript{17,18} providing the rationale for understanding cell-cell spatial relationships and tumor immune heterogeneity.

In papillary thyroid carcinoma, poor infiltration of T cells is associated with a predominance of myeloid lineages in the intratumoral regions, simultaneously correlating with pathological aggressiveness.\textsuperscript{23} As the degree of the intratumoral infiltration of immune cells is deeply associated with the profiles of the tumor microenvironment, the density and phenotypes of intratumoral immune infiltrates can provide clues to understanding the characteristics and potential prognostic factors for predicting therapeutic outcomes.

3.2 | Tissue area surrounding cancer cell nests

Beyond the significance of intratumor regions, phenotypes of the invasive margin and peritumoral regions also reflect the immune characteristics of tumors. The invasive margin of the tumor is typically defined as the border separating adjacent non-malignant area from the malignant tumor cell nests.\textsuperscript{24} Because the presence of T cells at the invasive margin independently correlates with prognosis, the invasive margin and central tumor are recommended to be evaluated separately in colorectal\textsuperscript{7} and breast cancer.\textsuperscript{24} As the invasive margin is formed by a physical mixture of cancer cells and host tissue, the invasive margin may have unique biological context from the whole tumor tissue. TLSs exhibit a structure analogous to follicles in lymphoid tissues, which are the results of lymphoid neogenesis in the course of chronic inflammation and tumor progression.\textsuperscript{25,26} In a wide range of tumor types, the presence of TLSs surrounding cancer cell nests is mostly associated with a favorable prognosis,\textsuperscript{25-29} although the degree of specificity of the recruited T cells in TLS is still unknown due to the extent of potential bystander activation.\textsuperscript{30} As the high density of T\textsubscript{REG} in TLS has a negative survival impact on patients with early-stage non-small-cell lung carcinoma,\textsuperscript{31} the characteristics of TLSs need to be stratified in the context of tissues. In fact, the quality of TLSs is reported to correlate with the degree of intratumoral
infiltration of CD8⁺ T cells in pancreatic cancer. Reported complexity of TLS highlights the functional and prognostic significance of peritumoral immune infiltrates and the formation of immune aggregates in the tumor microenvironment.

3.3 | Time and dynamics

In addition to the snapshot-based characterization of tumor immune spatial characteristics, the location and phenotypes of immune cells should be understood based on longitudinal changes during cancer progression and treatment. The distribution of immune cells at baseline frequently exhibits substantial changes during chemotherapy and immunotherapy, potentially contributing to therapeutic response or resistance. Responders for anti-PD1 blockade in melanoma showed longitudinal changes in T cell infiltration into the intratumoral regions with increased proliferation. In head and neck squamous cell carcinoma, differential tumor immune architectures were observed during the response to combination therapy using chemotherapy and cetuximab, where T cell exclusion at baseline (Figure 3A) was convergently changed into intratumoral infiltration at post-treatment (Figure 3B). Given that different sets of cancer treatments are applied either simultaneously or sequentially, understanding treatment-induced longitudinal changes in the tumor immune architecture can provide potential predictive information that guides therapeutic decision-making.

4 | SPATIAL RELATIONSHIPS AT THE SINGLE-CELL LEVEL

As immune cells typically communicate with each other via synaptic cell-cell contacts and cytokines, spatial configurations at the single-cell level can provide insights into the heterogeneous and heterotypic tumor microenvironment, where bidirectional interactions between cancer cells and immune cells can change the phenotypes and functional status of the cells surrounding the tumor cell (Figure 4A-C).

4.1 | Cancer cells vs anti-tumor immune cells

Because the close proximity between tumor cells and CD8⁺ T cells presumably means effective infiltration of tumor antigen-specific T cells, leading to the workability of anti-tumor immunity, a favorable prognostic significance has been reported in various types of cancer such as non-small-cell lung carcinoma, ovarian cancer, colorectal cancer, melanoma, and pan-cancer cohorts. Longitudinal analysis comparing matched primary and recurrent head and neck cancer tissues revealed that NK cells of recurrent tumors showed greater distance to tumor cells compared with those of primary tumors, which was potentially related to the mechanisms of recurrence.

4.2 | Cancer cells vs tumor-promoting immune cells

Cancer immunoediting is a process that attenuates anti-tumor immunity and promotes tumor development via immunosuppression. Recent reports have provided evidence of cancer immunoediting in the microregional spatial context. As TREG is a lineage of T cells that maintains tolerance to self-antigen and hinders anti-tumor immunity, a high density of TREG around tumor cells is associated with short overall survival in lung cancer. Similar findings have been observed in head and neck squamous cell carcinoma, in which recurrent tumor...
cells had close proximity to immunosuppressive ICOS$^+$ T$_{REG}$ and myeloid cells$^{42}$ (Figure 4A-C). Furthermore, proliferation and metastasis of cancer cells can be promoted by colocalization of tumor cells with neutrophils$^{42}$ and tumor-associated macrophages.$^{43}$

### 4.3 | Immune cells vs immune and other non-malignant cells

Spatial relationships between immune cells and non-malignant cells are also important factors determining the characteristics of the tumor microenvironment. T$_{H17}$ cells surrounding CD66$b^+$ granulocytes have Th2-like phenotypes identified by spatial relationship analysis in HPV-negative head and neck squamous cell carcinoma.$^{44}$ Colocalization of T cells with mature dendritic cells in draining lymph nodes is associated with negative lymph node metastasis.$^{45}$ A short distance between mast cells and CD8$^+$ T cells is negatively associated with chemotherapeutic response in breast cancer.$^{46}$ Because a huge number of combinations can be considered among various phenotypes of immune cell lineages, inter-immune cell spatial relationships might represent a potential gold mine of information such as cell functionality, regulation of metastasis and treatment outcomes.

As vascular and lymphatic endothelial cells are associated with recruitment and trafficking of immune cells, the proximity between vascular cells and immune cells can reflect the status of accessibility for intratumoral immune infiltration. In fact, the close proximity of T cells to lymphatic endothelial cells correlates with therapeutic response in melanoma.$^6$ Overall, spatial information at the single-cell level can provide a clue for understanding biological interactions and characteristics of the tumor immune microenvironment.

### 5 | NEW TECHNOLOGIES TARGETING SPATIAL RELATIONSHIP ANALYSES

Although imaging is the most efficient method for analyzing spatial information while preserving the tissue architecture, conventional immunohistochemistry (IHC) and immunofluorescence analyses on FFPE tissues have limitations determined by the number of species in which different antibodies are produced, as well as by the spectral overlap of fluorescence. These technical limitations are particularly disadvantageous for immune cell analyses where identification of cell types and phenotypes requires multiple lineage markers. Although traditional flow cytometry and recent mass cytometry technologies are capable of highly multiplexed evaluation of epitopes, these approaches also have restrictions, in that single-cell suspensions are required, thus the tissue architecture is lost. While traditional methods have technical limitations in the analysis of spatial information, innovative technologies are emerging that hold great promise as single-cell analysis platforms that preserve tissue integrity (Table 1).

### 5.1 | Spatially resolved transcriptomics

Single-cell RNA sequencing is a powerful platform for analyzing single-cell based genetic properties and heterogeneity within a tumor.$^{47}$ Because single-cell RNA sequencing is restricted in its ability to analyze spatial information due to lost tissue structure, several developments have been undertaken to reconstruct spatial information by combining in situ hybridization of landmark genes$^{48}$ and fluorescence reporter-based cell sorting.$^{49}$ Spatial detection of particular transcripts can be carried out using other

### TABLE 1 Emerging methodologies for multiplexed spatial relationship analyses

| Extending spatial capability to multiplexed analysis | Extending multiplex capability to imaging methods |
|-----------------------------------------------|-----------------------------------------------|
| **Transcriptomics**                           |                                               |
| Spatial mapping of scRNAseq                   | Multiplexed fluorescence in situ hybridization |
| In situ hybridization of landmark genes$^{46}$| SeqFISH$^{51}$                                |
| Reporter-based cell sorting (NICHE-seq)$^{47}$| MERTFISH$^{52}$                               |
| **Spatial transcriptome profiling**           |                                               |
| Spatial transcriptomics$^{48}$                 |                                               |
| Slide-seq                                     |                                               |
| Digital spatial profiling$^{50}$              |                                               |
| **Proteomics**                                |                                               |
| High parameter cytometry imaging              | Multiplex immunofluorescence                   |
| Imaging mass cytometry$^{53}$                 | Antibody stripping (Opal)$^{55}$               |
| Multiplexed ion beam imaging (MIBI)$^{54}$    | Bleaching$^{56}$                              |
|                                              | Oligonucleotide$^{57}$                        |
|                                              | Multiplex IHC                                  |
|                                              | Multiplex IHC and image cytometry$^3$          |
several approaches. DNA-barcode microbead-based spatial transcriptomics enables spatial detection of particular transcripts\textsuperscript{50,51} although those methodologies are applicable for fresh frozen tissue, but not for FFPE tissues that are widely available clinical specimens. Digital spatial profiler technology on FFPE tissues provides an up to 1000-plex assay based on oligonucleotide probes.
coupled to unique barcodes with a photocleavable linker, analyzed by a multiplexed probe detection system. A major technical limitation of those methodologies is the lack of sufficient resolution to enable single-cell analysis, thus further development of the methodologies is required. In view of fine resolution enabling single-cell analysis, multiplexed FISH has been established using iterations of probe stripping.

5.2 | Multiplexed proteomics

Using the multiplex ability of mass cytometry, imaging mass cytometry methods provide highly multiplexed data with subcellular spatial resolution for up to 100 protein markers on FFPE. Those methodologies can be promising platforms if the costs of reagents and instruments become economically feasible for large number of studies.

Multiplex immunofluorescence has been developed using antibody stripping and bleaching fluorophores. These technologies are accompanied by advancements in digital image analysis, enabling quantitative assessment of multiple biomarkers and cell phenotypes. Furthermore, antibodies conjugated to oligonucleotide sequences enables highly multiplexed imaging.

5.3 | Multiplex IHC and image cytometry

By revisiting traditional chromogen-based IHC, we previously reported a practical and cost-effective chromogenic sequential IHC method with iterative labeling, digital scanning, and subsequent antibody stripping of tissue sections. This approach enabled the simultaneous evaluation of 12+ biomarkers in a single FFPE tissue section (Figure 5A,B). Furthermore, quantitative evaluation of multiplex IHC images was optimized using image cytometry analysis, as flow cytometry in imaging allows single-cell analysis based on cell size, area, signal intensity, and location (Figure 5C,D). Simultaneously, PD-L1 expression on tumor and myeloid cells can be quantitatively and spatially evaluated in a single tissue slide (Figure 5E). Most recently, this method has been updated using a combination of heat and chemical stripping of antibodies and chromogen in between immunodetection cycles that enabled quantitative assessment of 29 biomarkers in a single FFPE tissue section. Importantly, this method is technically and economically equivalent to standard IHC, thus providing feasibility for large-scale studies without significant cost.

5.4 | Current challenges in spatial relationship analysis

There are several challenges for data analysis of immune spatial characteristics. As immune spatial analysis is an emerging approach for cancer research, there is a lack of standard statistical approaches for spatial relationships. A large number of reports analyze distance to the nearest neighboring cells, however this method can be affected by cell density when higher cell density simply correlates with closer proximity. A possible solution to this issue is to use Ripley’s K function and pair correlation function analyses, which have been developed through ecological studies of plant distribution, and can evaluate spatial randomness at several distance scales by taking into account all neighbors rather than only the nearest cells.

As image acquisition and processing for the whole tissue typically exceed capabilities of most current methodologies, selection of a region of interest is a standard practice in imaging studies, which is a process to identify small regions to be analyzed. This generates another challenge in terms of unbiased identification of region of interest in the morphological heterogeneity of tumor tissue structure. Possible solutions for this are whole tissue-based mapping analysis (Figure 3A,B) and application of machine learning algorithms. Seemlessly integrated microscopic and macroscopic image analyses provide comprehensive spatial information, enabling an understanding of the entire characteristics of the tumor immune microenvironment (Figure 1).

6 | CONCLUSION

Based on technical advancements, the tumor immune microenvironment has been intensively analyzed in view of spatial information. Overcoming current challenges in normalization and statistical considerations, and the accumulation of evidence based on integrative analysis of cell-cell proximity as well as tissue structure can provide potential breakthroughs in biomarker development.

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ETRICAL CONSIDERATIONS
The study was approved by the institutional review board at Kyoto Prefectural University of Medicine (ERB-C-43-4).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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