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Biomarker Discovery in Immunotherapy-treated Melanoma Patients with Imaging Mass Cytometry

Sandra Martinez-Morilla¹, Franz Villarroel-Espindola¹, Pok Fai Wong¹, Maria I. Toki¹, Thazin Nwe Aung¹, Vasiliki Pelekanou¹, Brian Bourke-Martin³, Kurt A Schalper¹, Harriet M. Kluger², David L. Rimm^{1,2}

¹Department of Pathology, Yale School of Medicine, New Haven, CT (USA)

²Section of Medical Oncology, Department of Internal Medicine, Yale School of Medicine, New Haven, CT 06510, (USA)

³Navigate BioPharma Services, Inc., Carlsbad, CA (USA)

Abstract

Purpose: Imaging Mass Cytometry (IMC) is among the first tools with the capacity for multiplex analysis of over 40 targets, which provides a novel approach to biomarker discovery. Here we used IMC to characterize the tumor microenvironment (TME) of patients with metastatic melanoma who received immunotherapy (ITx) in efforts to find indicative factors of treatment response. In spite of the new power of IMC, the image analysis aspects are still limited by the challenges of cell segmentation.

Experimental Design: Here, rather than segment we performed image analysis using a newly designed version of the AQUATM software to measure marker intensity in molecularly defined compartments: tumor cells, stroma, T cells, B cells, and macrophages. IMC data were compared to quantitative immunofluorescence (QIF) and Digital Spatial Profiling (DSP).

Results: Validation of IMC results for immune markers was confirmed by regression with additional multiplexing methods and outcome assessment. Multivariable analyses by each compartment revealed significant associations of 12 markers for progression-free survival (PFS),

*Corresponding Author: David L. Rimm M.D.-Ph.D., Professor of Pathology, Director, Yale Pathology Tissue Services, Dept. of Pathology, BML 116, Yale University School of Medicine, 310 Cedar St. PO Box 208023, New Haven, CT 06520-8023, Phone: 203-737-4204, FAX: 203-737-5089, david.rimm@yale.edu.

FVE currently works at Fundación Arturo López Pérez (Chile); PFW currently works at Verily Life Sciences, Alphabet Inc. (CA, USA); MIT is now doing her oncology residency in Greece; VP is now working for Sanofi (MA, USA).

Authors' Contributions

Conception and design: SMM, DLR

Development of methodology: SMM, FVE, BB, DLR

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): SMM, PFW, HMK, MIT, TNA

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): SMM, DLR

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and 7 markers for overall survival (OS). The most compelling indicative biomarker, beta2-microglobulin (B2M), was confirmed by correlation with overall survival by QIF in the discovery cohort and validated it in an independent published cohort profiled by mRNA expression.

Conclusions: Using digital image analysis based on pixel colocalization to assess IMC data allowed us to quantitatively measure 25 markers simultaneously on FFPE tissue microarray samples. In addition to show high concordance with other multiplexing technologies, we identified a series of potentially indicative biomarkers for immunotherapy in metastatic melanoma including B2M.

Keywords

melanoma; immunotherapy; biomarkers; IMC

Introduction

In the last decade, the development of immune checkpoint inhibitors (ICIs) has led to a dramatic change in immuno-oncology, especially in metastatic melanoma, where the median overall survival increased four times since 2011 (1–4). Among immune checkpoint molecules expressed in tumor-infiltrating lymphocytes (TILs), the most relevant for immunotherapy in melanoma are programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4), being the therapeutic target of the antibodies nivolumab or pembrolizumab and ipilimumab, respectively (5). Even though patients frequently respond to these drugs, reaching up to 60% with the combined therapy (6), there are still patients that do not benefit or develop adverse events related to immune resistance (1–4). Therefore, selecting the patients who will benefit prior to the treatment would improve overall patient outcome.

One of the ligands of PD-1 is programmed death ligand-1 (PD-L1), expressed in tumor and immune cells, which has been shown to predict response to immunotherapy (1,7). However, PD-L1 has limitations as a predictive factor due to the variability of performance and scoring requirements among the different immunohistochemistry (IHC) assays approved by the U.S. Food and Drug Administration (FDA) (8). Also, patients with absence or low expression of PD-L1 have shown clinical benefit to ICIs (4). Giving the complexity of the tumor microenvironment (TME), high-multiplexing technologies may be valuable to discover novel candidate biomarkers linked to response or resistance to ICIs and might uncover additional molecules targetable with new drug combinations. Ayers and colleagues developed an IFN- γ -related gene expression signature to predict clinical response to pembrolizumab in melanoma (9).

Given the limited number of fluorescent channels usable in quantitative immunofluorescence (QIF), new technologies are being used to study the complex interactions of tumor and host immune cells in the TME on a single FFPE tissue slide. IMC is an emerging high-plex technology that can address this problem as it allows the analysis of up over 40 protein markers simultaneously on tissue sections using metal-conjugated antibodies with subcellular resolution (10,11). Stained tissue is ablated using an argon-based laser followed by time-of-flight mass spectrometry. Afterwards, an image for each target (detected by a unique metal-conjugated antibody) is created and computational platforms, such as

Visiopharm or histoCAT (12), are applied for quantitative analysis on segmented images with subcellular resolution. As a result, molecular information at a single-cell level is obtained, allowing the characterization of the cell-cell interaction in the TME context. In this study, we used IMC in tissue from patients with melanoma who received immune checkpoint inhibitors to simultaneously assess the immune microenvironment and, for the first time, we applied a newly designed version of the AQUA software able to accommodate this kind of high-plex data, which maintains its characteristic approach to molecular compartmentalization in place of cell segmentation.

Materials and methods

Patient cohort and tissue microarray construction

- **Discovery cohort or immunotherapy-treated cohort TMA**
For the discovery cohort, pretreatment samples from 60 metastatic melanoma patients treated with immune checkpoint inhibitors (pembrolizumab, nivolumab, or ipilimumab plus nivolumab) from 2011–17 were collected from Yale Pathology archives, who were unresectable stage III or IV at the moment of the treatment. In order to partially avoid tumor heterogeneity, two blocks of this TMA were built by collecting tissue from two separate regions of the same tumor for each patient. Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 were used to classify best overall response as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD), and to determine PFS (13).
- **Historic cohort of non-immunotherapy-treated patients TMA**
To evaluate the prognostic value of beta2-microglobulin (B2M), a historic cohort of 131 untreated melanoma patients was used. The clinicopathological characteristics of the patients obtained from clinical records and pathology reports for both cohorts are included in Table S1.
- **Melanoma Index TMA**
For antibody validation, a Melanoma Index TMA was used, which consists of tumor tissue from 30 untreated melanoma patients from a historical cohort with no clinical information collected and controls cores including placenta, tonsil, and melanoma cell lines (Yugen8, MEL624 WT, MEL624 B7-H1, MEL1335). The total of cores was 40 and two replicates were placed in the TMA.
- **Orthogonal validation cohort from published data**
The validation cohort is a retrospective cohort of 121 advanced melanoma patients who received PD-1 blockade treatment. Complete details of this cohort may be found in the publication (14).
- **Tissue collection**
Representative tumor areas obtained from formalin-fixed, paraffin-embedded (FFPE) specimens from independent melanoma cohorts from Yale were prepared

in a tissue microarray (TMA) format as previously described (15). Tissues were collected with written-informed or waiver consent from patients under the approved Yale Human Investigation Committee protocol #9505008219 and conducted in accordance with the Declaration of Helsinki.

Antibodies and immunofluorescence staining for antibody validation for IMC experiments

Twenty-six commercial mAbs labelled with unique lanthanide metals were used, where six of them were conjugated in-house to specific metals using Maxpar[®] Metal Labeling Kits (Fluidigm) according to the manufacturer's instructions (Table S2). The six tagged antibodies were validated staining a Melanoma Index TMA by immunofluorescence, as described previously (16–18). The rest of the antibodies obtained from Fluidigm already labelled by the company. Briefly, TMA FFPE sections were deparaffinized and incubated in xylene and ethanol. Antigen retrieval was performed with EDTA buffer pH 8.0 at 97°C for 20 minutes in a pretreatment heating device (PT Module, Lab Vision, Thermo Fisher Scientific). Endogenous peroxidase activity was blocked with 2.5% hydroxyl peroxide in methanol, followed by blocking with 0.3% BSA in 0.1 mol/L of TBS-T (Tris-buffered saline, 0.05% Tween[®] 20 detergent), both for 30 minutes at room temperature (RT). TMA sections were incubated overnight (ON) at 4°C with each primary antibody and mouse anti-S100 (1:100, 15E2E, BioGenex) and HMB45 (1:100, BioGenex) antibodies. Sections were then incubated for 1 hour at RT with goat anti-mouse Alexa Fluor 488 secondary antibody (Molecular Probes) diluted 1:100 in rabbit EnVision Amplification Reagent (Agilent). Cyanine 5 directly conjugated to tyramide (PerkinElmer) at 1:50 dilution was used for target antibody detection. Slides were incubated with 4,6-diamidino- 2-phenylindole (DAPI) at 1:1000 to stain the nuclei and mounted with ProLong Mounting Medium (ProLong Gold, Molecular Probes) and coverslip.

Fluorescent measurement and scoring

Quantitative immunofluorescence (QIF) of each target was performed using the AQUA (Navigate BioPharma Inc.) method (10,19). This method allows us to objectively quantify the protein expression of a desired biomarker by assessing the signal intensity of the marker within a given molecular compartment. Briefly, the QIF scores were obtained by dividing the target compartment pixel intensities by the area of all cells (DAPI compartment) or tumor positivity (HMB45+S100). Additionally, QIF scores were normalized by exposure time and bit depth at which the images were captured. Besides visual evaluation, cases with staining artifacts or less than 3% tumor were excluded from the analysis.

Antibody titration and validation for IMC experiments

To optimize the titer for each in-house conjugated antibody, we used the same strategy published by our laboratory for IMC (20). Briefly, serial sections of the Melanoma Index TMAs were stained by immunofluorescence using a range of concentrations and quantified by AQUA platform. The optimal antibody concentration was defined as the one with the highest signal-to-noise ratio, by dividing the average QIF scores of the upper 10% of spots by the average QIF scores of the lower 10% of spots. Besides confirming specific and correct subcellular localization, the optimal concentration will provide the highest signal with the least background, allowing the detection of a dynamic range of target expression of

target expression (16,17). The rest of the antibodies were validated in a similar way using the metal-conjugated antibodies by IMC (21).

To confirm that the interaction with the antigen was not affected by metal conjugation, the metal-labeled antibody and the unlabeled antibody staining was compared with QIF on Melanoma Index TMAs.

Labeling FFPE slides for IMC

The same protocol as for QIF was used for IMC until the primary incubation step, where a cocktail of 26 antibodies conjugated with different metals were incubated ON at 4°C at the optimized concentration. The following day, samples were incubated with 1:2000 DNA intercalator Ir191/193 (Fluidigm) for 1 hr at RT for nuclei staining. Finally, slides were rinsed with distilled water and air-dried before IMC analysis. A cut for two independent TMA blocks were assessed.

IMC data acquisition, visualization and analysis

After staining, dried TMA was subjected to automated ablation using an argon-based laser in the Hyperion Imaging System (Fluidigm) at 200 Hz. The ablated tissue was totally vaporized and the aerosol containing the ion cloud of lanthanide metals was directly transported to Helios Mass Cytometer (Fluidigm, (11,12)). The metal isotopes associated with each spot are simultaneously measured and indexed against the location of each spot. Using the visualization software MCD Viewer (Fluidigm), the data were exported as ome.tiff and analyzed by AQUA platform to study marker intensity on the corresponding masks or compartments: all cells (DNA intercalator Ir191/193), tumor cells (HMB45/S100), stroma (tumor cells subtracted from all cells), T cells (CD3), B cells (CD20) and macrophages (CD68) (Fig. 1). We discarded B7-H4 from the analysis since the images obtained were mainly background based on total dual counts and visual assessment, as well as showing a signal-to-noise ratio lower than 1. From MCD Viewer, we obtained normalized Dual Counts (nDC) for each marker, collecting the total amount of dual counts for each marker divided by the background for each core, similar to initial publications describing IMC (11,20,22). The results obtained from IMC data analysis by AQUA platform were normalized by dividing by 1000 (AQUA score).

Gene expression data analysis

To validate IMC findings in an independent cohort, we used a study of advanced melanoma treated with PD-1 checkpoint blockade-based therapy, with available information for whole exome sequencing, response and RNA-seq data on 121 patients (14). Correlation plots of key markers were generated by GraphPad™ Prism® v7.0. After assessing the normality of the data set by D'Agostino & Pearson and Shapiro-Wilk tests, Spearman's correlation coefficients were calculated for not normally distributed data set, including statistical significance (*P* value).

Validation of selected indicative biomarkers identified by IMC using immunofluorescence staining and quantification

A section of one of the two TMA blocks of the discovery cohort was used to validate IMC results related to major histocompatibility complex class I (MHC-I), B2M and colony stimulating factor 1 receptor (CSF1R). Additionally, one slide of TMA76 as the control cohort was analyzed for B2M. Sections were subjected to the same deparaffinization, antigen retrieval, and blocking protocol mentioned above. For MHC-I and B2M, two separate sections were incubated with primary antibodies overnight at 4°C: one for MHC-I (mMs, EMR85, Abcam) and another one for B2M (mRb, D8P1H, Cell Signaling Technologies). Next, sections were incubated for 1 hour at RT with the mouse or rabbit EnVision Amplification Reagent (Agilent), respectively. Cyanine 5 directly conjugated to tyramide (PerkinElmer) at 1:50 dilution was used for target antibody detection. After, mouse anti-S100 (1:100, 15E2E, BioGenex) and HMB45 (1:100, BioGenex) antibodies were incubated during 1 hour at RT, followed by goat anti-mouse Alexa Fluor 488 secondary antibody incubation (1:100, Molecular Probes). For CSF1R, one section was used to multiplex CSF1R (mRb, E7S25, Cell Signaling Technologies) CD68 (mMs IgG3, PG-M1, Agilent) overnight at 4°C. Secondary antibodies and fluorescent reagents used were anti-Ms IgG3 (Abcam, 1:1000) with biotinylated tyramide/Streptavidine-Alexa750 conjugate (Perkin-Elmer), rabbit EnVision Amplification Reagent (Agilent) with Cy5-tyramide (Perkin-Elmer). Finally, sections were incubated with anti-S100 and HMB45 antibodies for 1 hour at RT and goat anti-mouse Alexa Fluor 488 secondary antibody incubation. Benzoic hydrazide solution was used to eliminate residual horseradish peroxidase activity between incubations with secondary antibodies. To stain nuclei, all slides were incubated with DAPI at 1:1000 and mounted with ProLong Mounting Medium and coverslip. QIF was assessed using the AQUA method explained above (10,19). Additionally, cell counts for B2M marker were assessed by inForm Tissue Finder (PerkinElmer) after image acquisition using a Vectra 3 system (PerkinElmer) as previously described (23). Our general approach to validation of antibodies has been recently published (18).

Statistical Analysis

Pearson's correlation coefficient (r) was used to assess the agreement between QIF scores, IMC scores and DSP counts from near serial sections of Yale melanoma discovery cohort. OS and PFS curves were constructed using Kaplan-Meier analysis and statistical significance was determined using the log-rank test. Multivariable Cox proportional hazards models included age, sex, mutation status, stage, treatment, and prior immune checkpoint blockade as covariates (24–26). For statistical analysis, the average AQUA scores from two available cores of each case was used. All statistical tests were two-sided, and P values below 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad™ Prism® v7.0 software for Windows (GraphPad Software, Inc., La Jolla, CA), and JMP Pro software (version Pro 13, SAS Institute Inc, Cary, NC). Based on the published definitions by Wong et al., we use the term “indicative biomarker” to classify a biomarker that is associated with treatment outcome, but, for ethical reasons, cannot be compared to an untreated control (27).

Results

IMC validation by multiplexing technologies on discovery cohort

Rather than use the suite of Cell Profiler, Ilastic and HistoCat, or work from the raw data as we did in our previous IMC effort (20), we collaborated with Navigate Biopharma to apply a modified version of the AQUA software, previously used extensively for QIF (10,19). The greatest difference between AQUA and other software is that AQUA works without cell segmentation, but instead uses molecularly defined, pixel-based compartments. After ablation, IMC data was exported and the distribution of 26 markers was analyzed simultaneously in different tissue and cellular compartments (Fig. 1 and images of each marker in Suppl. Fig. S1). The compartments were defined as follows: all cells (using DNA intercalator Ir191/193), tumor mask (HMB45/S100 positive cells), stroma ("tumor mask" subtracted from "all cells" compartment), macrophages (CD68+ pixels), T cells (CD3+pixels), and B cells (CD20+ pixels). Each patient case was represented by two non-adjacent TMA cores collected in separate experiments from two independent TMA blocks of the discovery cohort. The correlation between blocks is shown in Table S3 for IMC scores, obtained after AQUA platform analysis from IMC data, and normalized dual counts (nDC) from MCD Viewer. The degree of agreement between measurements from two analysis methods for each block was high and statistically significant for all the markers, being the lowest CSF1R ($r=0.309$, $p=0.017$) and IFNGR1 ($r=0.290$, $p=0.026$). When the comparison was done between blocks for each marker and each method of analysis (MCD Viewer or AQUA platform), the correlation decreased, mainly for the immune markers, which could be due to tissue heterogeneity rather than lack of analytic reproducibility in the case of highly expressed markers. It is important to notice that MCD Viewer is only a visualization tool, not useful for a reliable, reproducible quantification of biomarker signal. We used nDC only as an orientation.

Next, we sought to compare the analysis of IMC using molecular compartmentalization (AQUA) to the NanoString Digital Spatial Profiling (DSP) platform to a standard technique for 5-plex fluorescence (using AQUA software). The immunotherapy-treated melanoma cohort used here has been studied and published for both QIF (27) and DSP (28). Both methods assign compartments based on positive immunofluorescence signal to create where targets are measured. The difference between them is that while QIF measures target expression through fluorescence, DSP uses antibodies conjugated with DNA oligos specific for each marker. For comparison, we focused on T cell markers CD3, CD4, and CD8, only in the tumor mask (AQUA) or Area of Interest (AOI) by DSP, another molecular compartmentalization technology. Table S3 summarizes the correlation of the markers that overlapped only between DSP and IMC.

Correlation of IMC scores and both QIF scores and DSP counts for the T cell markers CD3, CD4, and CD8 showed a high concordance and were statistically significant when they were measured in tumor regions in near serial section TMAs (Fig. 2A–F). To confirm previous data published by our group by QIF (27,28), we investigated whether any of the above markers measured by IMC were associated with the response, PFS or OS, as this cohort includes melanoma patients treated with immunotherapy. By Mantel-Cox analysis,

expression of CD8 and CD3 was higher in patients that responded to therapy than in those who progressed ($P=0.0058$, $P=0.009$, respectively), confirming results previously published using QIF on the same cohort (Fig. 2G, I). The levels of CD4 (Fig. 2H) were similar among the three RECIST categories, as found by QIF and DSP (27,28). Upon patient stratification by median, significant survival advantage for PFS in melanoma patients was associated with high levels of CD8 ($P=0.036$ HR (H/L): 0.52, 95% CI: 0.27–0.97) and CD3 ($P=0.037$ HR (H/L): 0.52, 95% CI: 0.27–0.99) measured by IMC in all cells (Fig. 2J, L). However, as expected, CD4 was not associated with response to treatment (Fig. 2K). Multivariable analyses confirmed these results (CD8: $P=0.0008$ HR (H/L): 0.28, 95% CI: 0.12–0.59; CD3: $P=0.002$ HR (H/L): 0.29, 95% CI: 0.13–0.63). Thus, the IMC scores results are concordant with published data using QIF and DSP on the same cohort.

Discovery of markers indicative of response to immunotherapy in melanoma

To evaluate the potential of the IMC technology to discover novel immune-related biomarkers associated with response and survival, we next analyzed IMC results from twenty-five markers simultaneously with AQUA platform in the six compartments (Fig. 1), obtaining the average of two TMA cores collected in separate experiments from two different areas of the same tumor for each patient. Patients with only one core representation were excluded. Thus, from 25 markers in 6 compartments, we generate 150 variables that can be tested for association with outcome using a discovery format, considering variable one marker in one compartment. Any interesting variables are validated with an orthogonal method. After stratifying patients by median variable expression, we found 12 variables associated with longer PFS and 11 variables associated with longer OS (Table S4), as analyzed by Log-rank test. In Fig. 3, we show Kaplan-Meier plots of representative markers, such as beta-2-microglobulin (B2M) (Fig. 3A, D) and major histocompatibility complex class I (MHC-I) (Fig. 3B, E), where high levels of the corresponding proteins expressed either in tumor or stroma are associated with a good response to immunotherapy (Table S4).

When unadjusted univariable analyses were performed, we again detected 14 variables linked to longer PFS (Table S4). High levels of MHC-I, B2M, CD8, and LAG3 were associated with better response when their expression was analyzed both in tumor and stroma compartments. However, only colony stimulating factor 1 receptor (CSF1R) is associated with better outcome when measured in tumor, whereas TIM3 and PD-L2 were significant only when expressed at high levels in the stroma. In contrast, when cellular compartments were analyzed, only CD163 in the CD68 compartment and B7-H3 and Ki67 in the CD3 compartment were significantly associated with PFS (Table S4). For OS, the univariable analyses were summarized in Table S4. Multivariable Cox proportional hazard analyses for PFS and OS (Table 1, 2) by each compartment identified 19 and 12 candidate indicative markers, respectively, as statistically significant. Any of the markers analyzed on B cell compartment was associated with PFS or OS (data not shown). Note that this is a discovery study and hence the P values shown are not adjusted for multiple testing and all associations represent interesting candidates for secondary validation.

Validation of immunotherapy biomarkers identified with IMC

Based on the results obtained with IMC technology, the next step was to confirm the observations related to MHC-I, B2M and CSF1R by standard multiplexed immunofluorescence methods on the discovery cohort (Fig. S2). In the absence of other cohorts for protein validation, we further validated these observations by an orthogonal method using RNA-seq data from a study published by Schadendorf lab with an independent melanoma cohort treated with ICI (14). Since MHC-I and B2M are part of the same receptor (29), the expression of the two markers should be correlated, as shown in Fig. S3 at protein level by IMC ($r = 0.93$, $P < 0.0001$) and QIF ($r = 0.74$, $P < 0.0001$) in the discovery cohort, and at mRNA level in the validation cohort ($r = 0.72$ – 0.79 ; $P < 0.0001$). High levels of B2M protein were associated with better response to immunotherapy for OS in the discovery cohort, represented either as B2M expression in tumor mask (Fig. 4A) by a pixel-colocalization method (AQUA platform), or as percentage of B2M+ tumor cells (Fig. 4B) by cell segmentation analysis (inForm) (Table S5). We observed the same results at the mRNA level in an independent cohort, (Fig. 4C, Table S5). Furthermore, this relationship was confirmed by unadjusted univariable analyses for both protein, including AQUA and inForm analyses, and mRNA expression (Table S5). Multivariable analyses further demonstrated the significance of B2M expression at protein level by QIF assessment using AQUA platform in the discovery cohort [HR(95%CI): 0.20 (0.05–0.74), $P = 0.015$] but not for cell segmentation evaluation with inForm (Table S5), nearly reaching statistical significance in the validation cohort for mRNA expression [HR(95%CI): 0.60 (0.35–1.02), $P = 0.058$] (Table S5). Moreover, when we assessed outcome in a historic control of untreated melanoma patients, B2M protein levels were not associated with survival ($P = 0.26$ – 0.84) (Table S5). Both univariable and multivariable Cox regressions confirmed the lack of prognostic value of B2M when measured either in tumor or stroma (Table S5).

In contrast, there was no statistically significant benefit from anti-PD-1 therapy linked to either MHC-I or CSF1R protein expression levels determined by QIF, as observed by IMC (Table S5). Interestingly, high *CSF1R* mRNA levels were associated with a better outcome in the validation cohort, although that was not the case for any of the *MHC-I* genes analyzed (*HLA-A*, *HLA-B*, *HLA-C*) (Table S5).

Discussion

Although cancer immunotherapy has shown remarkable efficacy, especially in melanoma, the mechanisms underlying the response or resistance to therapy are not completely understood. High-plex technologies such as IMC are extraordinary tools to measure the expression of many variables simultaneously within the TME. It is hoped that this approach will help identify new selection tools to find patients who will benefit from ICI therapy. In this study, we applied IMC to characterize the immune-related protein expression patterns in a melanoma cohort prior to immune checkpoint blockade. We utilized the potential of this high-throughput multiplexing technology to uncover new candidate biomarkers that could improve patient stratification for immunotherapy treatment. Twelve immune markers were found to be correlated with PFS and OS. Furthermore, we evaluated 3 out of the 12 indicative biomarkers by QIF, but only B2M was validated. Then, we further validated this

finding using an independent cohort of ITx-treated melanoma patients (14) at the mRNA level. Furthermore, we compared these results to a non-immunotherapy treated cohort, revealing B2M protein was not associated with prognosis in melanoma. This was done since we cannot formally prove that B2M is a predictive biomarker without a placebo control clinical trial. It is important to point out that the control cohort, mainly low stage patients, is substantially different from the immunotherapy-treated cohort, where all the patients were unresectable stage III or IV at the moment of the treatment, which could be a limitation of the analysis. This is the best that can be done in the context of the clinical standard of care and in retrospective studies. We have previously addressed this issue and use the term “indicative” in previous publications (27) since the statistical test for interaction is required for proof of a predictive biomarker.

Loss of B2M expression has been described as a mechanism of intrinsic and acquired resistance to ICI therapy in patients with metastatic melanoma (30), where about 30% of patients with progressive disease harbored mutations or loss of heterozygosity (LOH) of B2M. Additionally, this may also lead to loss of surface expression of MHC-I (31), compromising adaptive response through CD8 cytotoxic T cells. While B2M forms a complex with MHC-I, we could not confirm MHC-I as an indicative biomarker by QIF, neither by reassessing the discovery cohort nor in the validation cohort by mRNA analysis. Perhaps, the reason for the lack of concordance between IMC and QIF results is that the antibody we used recognizes the products of all three genes of MHC-I complex (*HLA-A*, *HLA-B*, *HLA-C*). Chowell and colleagues found that melanoma patients with different supertypes for the 3 *HLA-I* molecules had diverse associations with the response (32). As noted by Rodig et al., low expression of *MHC-I* mRNA correlated with response to nivolumab but not ipilimumab (33). Interestingly, gene amplification and mRNA upregulation of *MHC-I* has been described in responders (n = 6) to anti-PD-1 treatments compared to non-responders (n = 11), both ipilimumab-naïve (14). Also, Lee and colleagues showed the association of downregulation of *MHC-I* by RNA-seq analysis and resistance to PD-1 inhibitors in melanoma (34). Furthermore, the central dogma of molecular biology “mRNA makes protein” might not always be directly proportionate, especially in cancer where the natural regulation of protein expression and metabolism may be altered (35–38).

Although our study is quantitative and includes outcome, it has some limitations. Firstly, patient samples were studied using the TMA format, which could be insufficient tissue to accurately represent the high heterogeneity of immune populations in the TME since each core corresponds to a very small portion of the standard tissue section. To compensate for this, we utilized two nonadjacent TMA cores for each patient. However, the high-plex capability of IMC gave us the opportunity to measure more than 20 markers simultaneously in a large number of patients. Another limitation of this work is inherent in the IMC method. Although the resolution of IMC technology (1 μm) (11) is higher than DSP (10 μm) (28), it is still lower than a light microscope (0.2 μm) and that might compromise accurate detection of targets at the subcellular level or in cells in close proximity. Additionally, there is not a standardized, broadly-accepted method for antibody validation in IMC, which introduces some limitations for interpreting IMC results for markers that differ from IF results. For example, the lack of signal amplification by a secondary antibody in IMC, as seen in traditional IF, limits the quantification of certain markers expressed at a low level, which is

often seen for immune-related biomarkers. As compensation for this limitation, our use of the molecular compartmentalizing AQUA software calculates the cumulative signal intensity per unit compartment area (10) and its accuracy relies on confluent tissue compartments. The molecular compartmentalization approach could be a weakness when studying low frequency events such as B and NK cells or other rare immune cells (27). Using other pipelines such as the combination of CellProfiler and histoCAT created by Bodenmiller lab (12), or Visiopharm, that allow you to segment cells and measure the expression of a biomarker at a single cell level may address this problem. Finally, only one cohort of immunotherapy-treated patients from a single institution was evaluated in this study. Validation of the biomarker results in an independent control cohort, ideally including patients from multiple institutions, will be required to confirm these findings.

In summary, we validated the AQUA platform for IMC analysis of multiple immune markers in several compartments on a metastatic melanoma cohort treated with immunotherapy. Moreover, we identified more than ten candidate indicative biomarkers of response to immunotherapy and we confirmed B2M as a target associated with survival by QIF and mRNA expression in a separate cohort. This study highlights the potential of IMC as a discovery tool to expand our understanding of tumor microenvironment and discover new biomarkers associated with better immunotherapy outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of translational relevance

Imaging mass cytometry (IMC) facilitates the high-plex measurement of more than 40 different proteins at a single cell level on a FFPE slide simultaneously. In this study, we simultaneously examined 25 targets on pretreatment biopsies from immunotherapy-treated melanoma. A modified AQUA software with molecular compartmentalization was used to analyze IMC data in tumor, stroma, T cells, B cells, and macrophages. First, IMC results for immune markers such as CD3 and CD8 were validated by fluorescent multiplexing and the high-plex technology digital spatial profiling on the NanoString platform. Then, 12 markers were found to be associated with response and survival. One of the identified proteins was beta2-microglobulin, which was associated with better outcome in patients expressing high levels in tumor cells. This finding was confirmed by quantitative immunofluorescence, raising the possibility of the use of this biomarker in the clinical setting after further study.

Figure 1.
Compartments created in AQUA to analyze IMC data.

Figure 2. IMC comparison to NanosString DSP and QIF in the discovery cohort for immune markers.

Regressions of QIF (A-C) and DSP (D-F) with IMC data on the same cohort for CD8 (A, D), CD4 (B, E), and CD3 (C, F) in tumor region. Quantification of CD8 (G), CD4 (H), and CD3 (I) expression by IMC per RECIST categories of best overall response, showing mean $\pm$ SEM. *: $P<0.05$; **: $P<0.01$, statistical analysis by Two-tailed Mann-Whitney U test. Below, Kaplan-Meier survival curves (J-L) showing PFS, stratified by median expression of corresponding markers in all cells by IMC. The markers analyzed were: CD8 (J), HR (H/L): 0.52, 95% CI: 0.27–0.97; CD4 (K), HR (H/L): 0.61, 95% CI: 0.33–1.16; and CD3 (L), HR (H/L): 0.52, 95% CI: 0.27–0.99. Survival analysis by log-rank (Mantel-Cox) test. HR (H/L): Hazard Ratio (High/Low).

Figure 3. Candidate indicative markers to immunotherapy in the discovery cohort by IMC.

Kaplan-Meier survival and progression free survival curves for representative immune markers in different compartments showing statistical significance. When looking at tumor mask, high levels of B2M (A, D) and MHC-I (B, E) were associated with better PFS and OS. In stroma compartment, patients with high levels of LAG3 (C) and TIM3 (F) showed a better PFS. Survival analysis by log-rank (Mantel-Cox) test was performed and results were included in Table S4.

Figure 4. Validation of B2M as an indicative immune marker to immunotherapy in a melanoma ITx cohort identified by IMC.

Kaplan-Meier survival curves showing overall survival for B2M protein measured in tumor by AQUA (A) and inForm (B) analysis, as well as *B2M* mRNA data from Schadendorf lab (C), where patients were stratified using the median as a cutpoint. Results from survival analysis by log-rank (Mantel-Cox) test were included in Table S5.

Table 1.
Multivariable (Cox regression) analyses of potential indicative markers for PFS.

The analyses included age, sex, mutation status, stage, treatment (pembrolizumab, nivolumab or nivolumab +ipilimumab), specimen category (lymph node, metastasis, or cutaneous specimen), and prior immune checkpoint blockade as covariates. HR=Hazard ratio; 95% CI=95% Confidence interval.

Indicative markers for PFS – Multivariable Analysis (cutpoint = median)											
Tumor			Stroma			CD68 compartment			CD3 compartment		
Variable (H/L)	HR (95% CI)	P value	Variable (H/L)	HR (95% CI)	P value	Variable (H/L)	HR (95% CI)	P value	Variable (H/L)	HR (95% CI)	P value
MHC-I	0.35 (0.14–0.83)	0.017	MHC-I	0.23 (0.09–0.57)	0.001	CD163	0.35 (0.14–0.81)	0.013	B7-H3	2.19 (1.05–4.74)	0.037
B2M	0.36 (0.15–0.85)	0.019	B2M	0.28 (0.11–0.69)	0.006						
CD8	0.21 (0.08–0.48)	0.002	CD8	0.44 (0.21–0.89)	0.022						
CSF1R	0.41 (0.19–0.84)	0.015	IRF1	0.28 (0.11–0.67)	0.004						
IRF1	0.42 (0.19–0.86)	0.019	LAG3	0.38 (0.15–0.95)	0.038						
LAG3	0.34 (0.14–0.79)	0.011	TIM3	0.45 (0.20–0.97)	0.041						
PD-1	0.41 (0.17–0.96)	0.039	PD-L2	0.26 (0.11–0.56)	0.0006						
PD-L2	0.36 (0.15–0.81)	0.013	MHC-II	0.38 (0.15–0.89)	0.026						
MHC-II	0.41 (0.16–0.97)	0.042									

Table 2.
Multivariable (Cox regression) analyses of potential indicative markers for OS.

The analyses included age, sex, mutation status, stage, treatment (pembrolizumab, nivolumab or nivolumab +ipilimumab), specimen category (lymph node, metastasis, or cutaneous specimen), and prior immune checkpoint blockade as covariates. HR=Hazard ration; 95% CI=95% Confidence interval.

Indicative markers for OS – Multivariable Analysis (cutpoint = median)										
Tumor			Stroma			CD68 compartment			CD3 compartment	
Variable (H/L)	HR (95% CI)	P value	Variable (H/L)	HR (95% CI)	P value	Variable (H/L)	HR (95% CI)	P value	Variable (H/L)	P value
MHC-I	0.28 (0.08–0.87)	0.027	PD-L2	0.28 (0.08–0.80)	0.017	MHC-I	0.29 (0.09–0.87)	0.028	B2M	0.33 (0.11–0.97)
B2M	0.31 (0.09–0.93)	0.036	B2M	0.28 (0.08–0.99)	0.048	CSF1R	4.01 (1.14–16.79)	0.029		
CD8	0.08 (0.02–0.27)	<0.0001	CD8	0.32 (0.10–0.87)	0.025					
CSF1R	0.31 (0.11–0.80)	0.015								
LAG3	0.33 (0.10–0.93)	0.035								
MHC-II	0.29 (0.09–0.88)	0.028								