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Prognostic and theranostic impact of molecular subtypes and immune classifications in renal cell cancer (RCC) and colorectal cancer (CRC)

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Molecular and immune classifications powerfully predict cancer patient's survival and response to therapies. We herein describe the immune tumor microenvironment of molecular subgroups of colorectal and renal cell cancers, revealing a strong correlation between tumor subtypes and distinct immune profiles.

Keywords: Cancer molecular subtypes, colorectal cancer, immune classification, prognostic and theranostic markers, renal cell cancer, tumor microenvironment

Abbreviations: ccRCC, clear cell renal-cell carcinoma; CRC, colorectal cancer; MSI, microsatellite instable; MSS, microsatellite stable; CIN, chromosomal instability.

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During the last decade, 2 major prognostic classifications of human cancers have emerged based on the phenotype of tumor cells and the composition of the immune infiltrate. The first, molecular classification of cancer, stratifies patients according to genetic mutations, translocations, amplifications or deletions of chromosome fragments in malignant cells.[1] The second, immune classification, stratifies patients according to the location, quality and quantity of the tumor immune infiltrate.[2] To our knowledge and to date, no correlation between these 2 classifications has been performed.

Molecular classification has proven to be useful in the clinicopathological analyses of many cancer types, such as in the identification of patient subsets with distinct prognoses and in stratifying patients according to predicted responses to therapies. Thus, patients presenting mutations in particular driver oncogenes can be treated by specific inhibitors, such as vemurafenib that targets mutated BRAF in melanoma[3] or gefitinib and erlotinib that target EGFR mutations in lung cancer.[4,5] Patients afflicted with acute lymphocytic leukemia and chronic lymphoid leukemia harboring translocation of *BCR-ABL* genes are similarly known to be sensitive to imatinib.[6] Amplification of the *HER2/neu* gene in breast cancer cells manifest overexpression of the encoded HER2 protein, a therapeutic target for the monoclonal antibodies trastuzumab and pertuzumab in HER2-positive patients.[7] Conversely, patients with colorectal cancer (CRC) marked by the mutant oncogene *KRAS* are resistant to treatment with cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor (EGFR).[8]

Whole-transcriptome analyses of tumor cohorts also define molecular subgroups with prognostic and theranostic values. This principle was recently exemplified in a publication by our group in which we analyzed a cohort of patients with clear cell renal cell carcinoma (ccRCC)[9] who had developed metastatic disease and were treated with sunitinib, a tyrosine-kinase inhibitor (TKI) targeting tyrosine-kinase receptors. Sunitinib targets include the vascular endothelial growth factor (VEGF) receptors VEGFR1 (FLT1) and VEGFR2 (KDR), the proto-oncogenes RET and c-KIT (CD117), fms-related tyrosine kinase 3 (FLT3), and

the platelet derived growth factor receptor B (PDGFRB).[10] Transcriptome analyses were performed on resected primary ccRCCs from these patients and unsupervised consensus clustering approach identified 4 robust ccRCC subtypes (ccRCC1 to ccRCC4) that were associated with different responses to sunitinib treatment.[9] We found that ccRCC4 had the lowest response rate to sunitinib and the shortest progression-free survival (PFS) and most reduced overall survival (OS) in comparison to ccRCC2 and ccRCC3 (**Fig. 1A**). Of particular interest, ccRCC4 exhibited a stem-cell polycomb group (PcG) signature and a sarcomatoid differentiation profile.[9]

Six independent laboratories have reported transcriptomic molecular classifications of CRC.[11–16] They all agree on the identification of a patient subgroup with microsatellite instability (MSI) associated with longer PFS and OS, as well as on the identification of a mesenchymal subgroup associated with the worst prognosis and characterized by transforming growth factor ß (TGFß) activation, the presence of stromal cells, invasion and angiogenesis. This classification could also have a theranostic value since patients with tumors of the mesenchymal subgroup are more resistant to targeted therapies,[12] including cetuximab.[13] More recently, it was reported that CRC patients responding to anti-checkpoint PD-1-targeting antibodies (nivolumab) belonged to the MSI subgroup.[17] Among CRC classifications is a stratification method dividing CRC in 6 subgroups (C1 to C6).[16] These include: C1 displaying chromosomal instability (CIN) with a significant down regulation of immune pathways; C2 comprising the MSI tumors, which are known to be highly infiltrated by T lymphocytes; C3 enriched for tumors with *KRAS* mutations; C4 composed of CRCs exhibiting upregulation of cancer stem cell (CSC)-like phenotype signatures; C5 featuring CIN with activation of the Wnt-signaling pathway; and C6, which also display CIN but have a gene expression profile similar to normal tissues.[16] As expected, patients of the C2 subgroup had the best clinical outcome, in terms of PFS and OS, whereas patients from the C4 subgroup had the worst prognosis[16] (**Fig. 2A**).

In addition, these 2 molecular classifications of RCC and CRC were shown to correlate with immunological and inflammatory signatures.[9,16] For instance, pathway analyses revealed an overexpression and hypomethylation of genes involved in immune response and chemotaxis in the ccRCC4 group of tumors. In CRC, the "Hematopoietic cell lineage" pathway was overrepresented in C2 and C4, suggesting increased infiltration by immune cells.

However, in-depth analyses of the composition of the immune microenvironment in relation to molecular subgroups are still lacking. Such analyses appear mandatory since the immune classification of cancers is the other major prognostic factor that emerged during the last decade. The concept of an immunologic landscape affecting cancer patient outcome was initiated by the pioneering work of Zhang et al. in ovarian cancer[18] and subsequently extended by the work of Galon et al. in CRC[19] who showed that the density of intratumoral T cells, particularly memory $CD8^+$ T cells and a T helper type 1 (Th1) orientation was the strongest prognostic factor for PFS and OS. This notion was extended and confirmed to be functionally relevant to most cancer types and led to the concept of immune contexture, which proposes that the density, location, functional orientation and local education of memory T cells strongly impacts patient clinical outcome.[2] This breakthrough has allowed the establishment of a standardized, robust and reproducible immunoscore as a routine laboratory test being validated by a worldwide consortium.[20] The immune classification of human tumors also has theranostic value. For instance, the presence of $CD8^+$ T cells is necessary, although not always sufficient,[21] for response to therapy with anti-PD-1 antibodies in melanoma patients.[22] It also represents a theranostic marker for other immunotherapies, since high T-cell infiltration, in association with the presence of a high number of tertiary lymphoid structures,[23] accompanies the potential efficacy of therapeutic vaccines[24] or anti-checkpoint antibodies.[25] There are, however, exceptions to the beneficial effect of a high infiltration by $CD8^+$ T cells, as observed in head-and-neck cancer,[26] Hodgkin's lymphoma,[27] diffuse large B-cell lymphoma[28] and ccRCC.[2,29]

We have revisited the ccRCC case by studying the immune contexture of 135 primary ccRCC[30] and 51 lung metastases of patients with ccRCC.[30,31] We first reported an association between shortened patient survival and a high density of $CD8^+$ T cells in primary and metastatic sites.[31] Analysis of patient mRNA transcriptomes in The Cancer Genome Atlas[32] revealed that the expression of most of the genes associated with a $CD8^+$ T cell-oriented immune response, notably those including INFγ, correlated with a poor prognosis. More detailed analyses of the immune infiltrates revealed that many $CD8^+$ T cells co-expressed immune checkpoint inhibitors, such as programmed cell death 1 (PDCD1, or PD-1) and lymphocyte activation gene 3 (LAG3), and showed that high densities of PD-1 and/or LAG3 expressing T cells correlated with poor prognosis.[30] In some patients, neoplastic cells expressed PD-1 ligands, such as PD-L1 and PD-L2, while tumor-infiltrating T cells expressed PD-1. Strikingly, this coordinate expression was found to be associated with a higher risk of relapse and death.[30]

In contrast, high densities of $CD8^+$ T cells in CRC primary sites has been correlated with longer patient survival [19], a correlation also evinced to occur in association with CRC liver[33] or lung[31] metastatic sites. Thus, clear opposing prognostic impacts regarding the presence of $CD8^+$ T-cell infiltrates have been documented between ccRCC and CRC patient primary and metastatic tumors, suggesting that the clinical impact of the immune contexture depends primarily on the type of lesion rather than the tumor site.[34] These results prompted us to investigate the correlations between the molecular subgroups and the immune infiltrate. To this end, we set out to interrogate a large patient cohort and available transcriptome data to establish a robust and selective immunome, thus defining metagenes for all lymphocyte subsets (e.g., $CD3^+$, $CD4^+$, $CD8^+$, Th1, Th2, Th17, Treg, NK, B cells, etc.) monocyte-derived cells, mast cells, granulocytes[35] but also

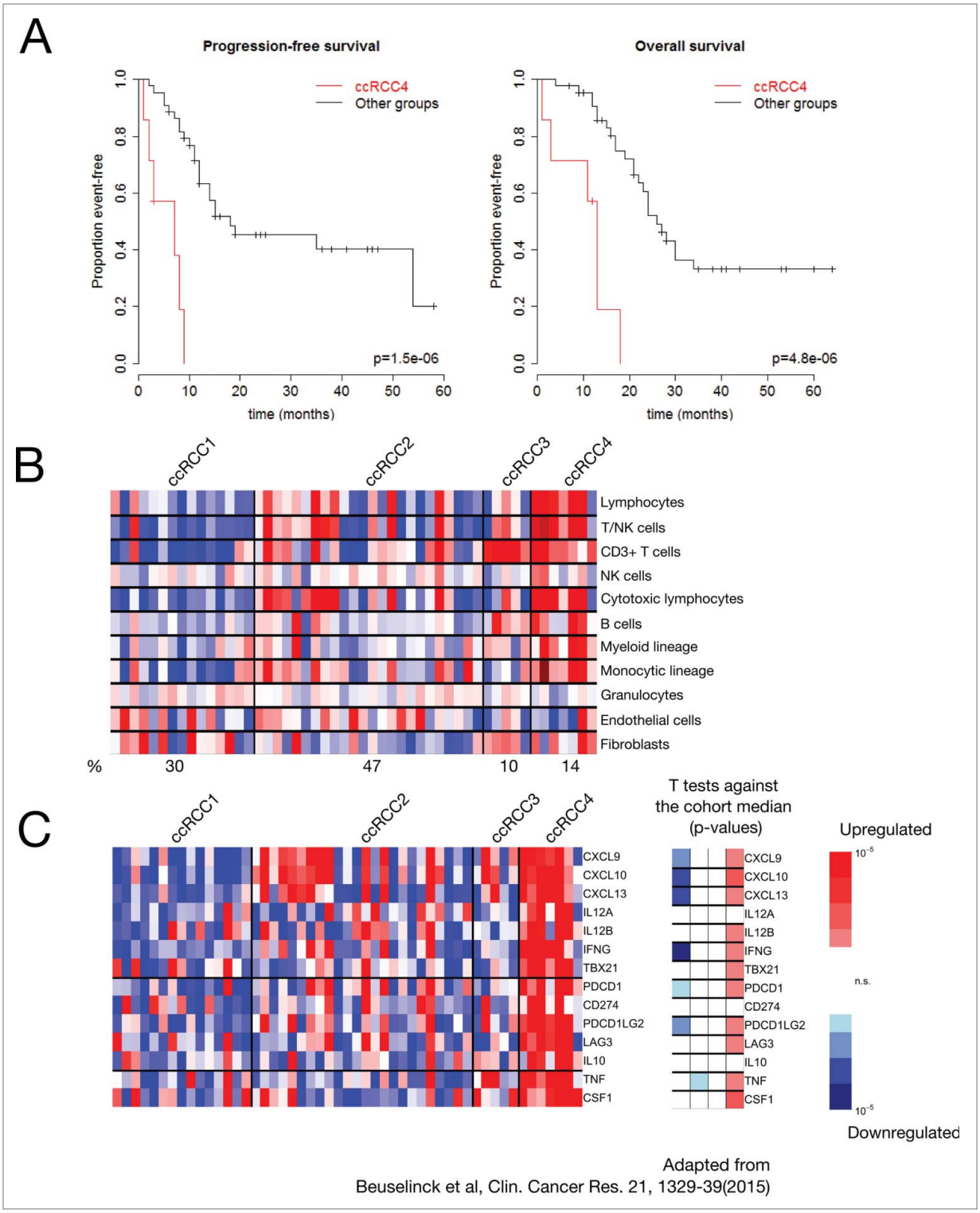

Figure 1. Correlation between clear cell renal cell carcinoma molecular subgroups and immune and inflammatory gene expression. (**A**) Kaplan-Meier curves representing the progression-free survival (PFS; left) and overall-survival (OS; right) of clear cell renal carcinoma type 4 (ccRCC4) patients compared to non-ccRCC4 patients (**B**) Relative expression of immune cell-specific markers in the 4 ccRCC subgroups (red: high expression, blue: low expression). Percentages indicate the frequency of each subgroups within the cohort. (**C**) Relative expression of functionally-relevant immune genes in the 4 ccRCC subgroups (red: high expression, blue: low expression). Dataset: ArrayExpress E-MTAB-3269.

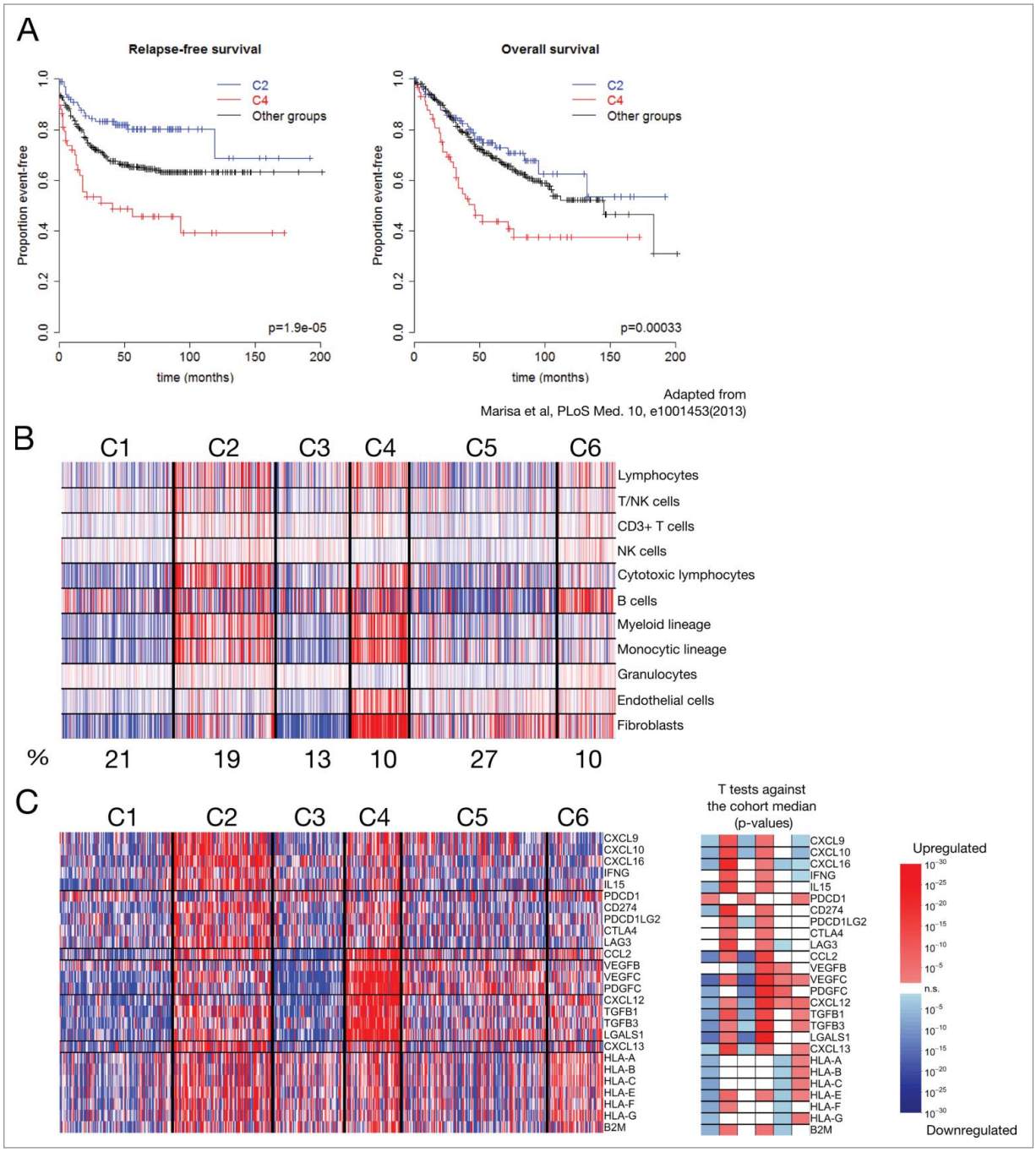

Figure 2. Correlation between colorectal cancer molecular subgroups and immune and inflammatory gene expression. (**A**) Kaplan-Meier curves representing the relapse-free survival (RFS; left) and overall-survival (OS; right) of C2, C4 and non-C2/C4 patients (**B**) Relative expression of immune cell-specific markers in the 6 CRC subgroups (red: high expression, blue: low expression). Percentages indicate the frequency of each subgroups within the cohort. (**C**) Relative expression of functionally-relevant immune genes in the 6 CRC subgroups (red: high expression, blue: low expression). Data set: Gene Expression Omnibus GSE39582.

endothelial cells and fibroblasts (Becht, submitted). The immunome was applied to the ccRCC and CRC molecular subgroup classifications presented above.[9,16]

In the ccRCC cohort, the immunome identified the ccRCC4 subgroup as exhibiting the highest expression of genes expressed overall in T and B cells, as well as specifically in cytotoxic cells and myeloid cells, whereas the ccRCC1 subgroup had the lowest expression of immune metagenes (**Fig. 1B**), confirming our previous observations.[9] Among the genes overexpressed in ccRCC4 –in addition to genes involved in Th1 polarization (*IFN*γ, *TBX21*), T cell activation (*IL12R*) and chemotaxis (*CXCL9, CXCL10*) – were genes governing T-cell inhibition,

including *PD-1, LAG3*, and *TGF*, as well as genes attracting (*CXCL12*) and activating (*CSF1*) myeloid cells[9] (**Fig. 1C**). Indeed, the ccRCC4 subgroup also exhibited hypomethylation of genes involved in the regulation of T-cell activation, regulation of the immune response, chemotaxis and apoptotic caspase cascades.[9] Finally, immunohistochemical analyses revealed that tumors of the ccRCC4 subgroup displayed the strongest $CD8^+$ T-cell infiltration, together with lymphocytic PD-1 expression and coincident PD-L1 expression on malignant cells.[9] The combined analyses of molecular subgroups of ccRCC and immune classifications therefore allowed the identification of an "immune high" and inflammatory subgroup, likely shaped by the sarcomatoid differentiated malignant cells producing chemokines and cytokines regulating the immune contexture, and inducing T-cell exhaustion (PD-1 expression) and immunosuppression (TGF). It identifies a poor-prognostic cohort, in which patient's tumor-infiltrating lymphocytes express immune checkpoint inhibitors (*e.g.*, PD-1 and LAG-3) and the corresponding ligands are expressed by tumor cells. With this in mind, we consequently propose that the ccRCC4 subgroup identifies patients that may respond to therapeutic immune checkpoint modulators.[9]

Application of the immunome stratification method to the CRC classification published by Marisa et al.[16] identified 2 "immune high" subgroups, as shown in **Figure 2B**. The expected MSI-enriched "C2" subgroup highly expressed T and NK cell metagenes and to a lesser extent the myeloid-cell metagene signature. The C2 subgroup displayed the highest expression of genes involved in Th1 orientation (*i.e., IFN*γ). Transcripts encoding immune checkpoint inhibitors, such as PD-1, T-cell attracting chemokines (*e.g.*, CXCL9, CXCL10), and the interleukin (IL) IL15 (which activates cytotoxic lymphocytes and promotes survival of memory $CD8^+$ T cells) were all differentially expressed,[36] as were molecules implicated in the formation of tertiary lymphoid structures (e.g., CXCL13), confirming prior observations by Bindea et al.[35] Surprisingly, C2 was not the only subgroup characterized by high immune-related metagene expression (**Fig. 2C**). The C4 subgroup, with a stem cell-like transcriptomic profile and expressing markers of epithelial-to-mesenchymal transition, comprised tumors with high T and NK metagene expression but in the context of a high myeloid cell metagene signature, and of endothelial and fibroblastic cells markers expression. Some tumors of this subgroup also differentially expressed the transcripts encoding the PD-1 ligands, CD274 and programmed cell death 1 ligand 2 (PDCD1LG2). In accordance with the high expression of a myeloid cell metagene signature, the C4 subgroup also exhibited a high expression of genes encoding myeloid cells attracting chemokines (*CCL2*), angiogenic factors (*VEGFA, VEGFC, PDGF*), and *TGFB1* (**Fig. 2C**). These observations are reminiscent of the fact that high *VEGF* gene expression impaired the beneficial clinical impact of high granulysin gene expression in CRC tumors.[37] On the contrary, the C1 and C5 subgroup metagene expression profiles were characterized by low immune- and inflammatory-associated profiles, associated with a low expression of MHC Class I genes, which may explain the low $CD8^+$ T lymphocyte infiltration of these subgroups (**Fig. 2B, C**). Altogether, the combined analysis of cancer molecular subgroups and immune classifications of CRC revealed unexpected immune and inflammatory associated heterogeneity in CRC tumors. The C2/MSI subgroup presents infiltration of canonical Th1 cells and cytotoxic memory $CD8^+$ T cells correlating with good prognosis, whereas the C4 subgroup exhibits a strong lymphocyte infiltration associated with myeloid cell infiltration, along with angiogenesis and high density of tumor-associated fibroblasts. These last 3 components most likely impair the immune reaction and are partly responsible for the poor prognosis of patients from this particular subgroup. Despite these deleterious elements in the microenvironment of C4 tumors, the presence of PD-1 and LAG-3 positive lymphocytes and PD-L1 expressing cells opens the possibility of targeted immunotherapies for the corresponding group of patients.

These results show similarities at the subgroup level between distinct tumor types such as ccRCC and CRC and allow us to define new groups of immune high patients that may be associated with distinct prognoses. They illustrate the high potential of combining the analyses of cancer molecular subgroups with immune classifications to define new groups of patients with similar tumoral and microenvironmental signatures independently of tumor types. By associating the mutational, differentiation or methylation status of the cancer cells together with the tumor microenvironments that they shape, these molecular and immune based classifications have a high prognostic value and may provide novel therapeutic targets and theranostic markers in the clinic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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