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Immune-based identification of cancer patients at high risk of progression

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Tumors are highly heterogeneous structures where malignant cells interact with a large variety of cell populations, including a clinically-relevant immune component. We review and compare the most recent methods designed to analyze and quantify the composition of immune and stromal microenvironment of tumors and discuss their use in identification of patients for high risk of progression. If the impact of the various immune components on patient's relapse share common rules in most malignancies, clear cell renal cell tumors behave differently with regards to immunity. We focus on this specific pathology to show how the tumor interacts with the host's immune system and how this intricate relationship shapes the clinical outcome.

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Introduction

Tumors are complex structures where malignant cells interact with a variety of different cell types that include cells from the adaptive and innate immune system, blood vessels, lymphatics, stromal cells and fibroblasts [1]. The immune and inflammatory cells contribute to a large extent to the tumor microenvironment (TME). Cells of the innate immune response including not only macrophages, NK cells and DC but also neutrophils, eosinophils and basophils can be found in tumors, together with cells of the adaptive immune response including cytotoxic $CD8^+$ T cells, Th1/Th2 skewed T cells, Th17 cells, Treg and B cells. Their relative densities, repartition and organization highly differ in different tumors and vary at each tumor stage [2]. Whereas macrophages are present both in the invasive margin (IM) and in the center of tumors (CT), lymphocytes are found in the IM preferentially. tertiary lymphoid structures (TLS) often develop in the IM and/or in the tumor stroma [3••]. They exhibit an organization similar to secondary lymphoid organs, including a T cell zone and a B cell follicular zone and are often surrounded by high endothelial venules. Plasma cells that produce antibodies are located at the vicinity of TLS [4]. The in depth analyses of the immune contexture of tumors in large cohorts of cancer patients using high throughput *in situ* detection methods and gene expression approaches have revealed a profound influence of the immune components of the TME and of their organization on patient's prognosis. Our group published two comprehensive reviews linking immune cell populations infiltrating tumors with prognosis [1,5••]. We reported that high densities of $CD3^+$ T cells, $CD8^+$ cytotoxic T cells and TLS were associated with a longer disease-free survival (DFS) and/or overall survival (OS) in most tumors. We noted that clear cell renal cell carcinoma (ccRCC) was one of the rare exceptions to the rule. Herein we briefly describe and compare the most recent tools for in depth analyses of the immune contexture of tumors, highlight the common features of the TME in patients at high risk of progression and then provide novel prognostic markers for ccRCC.

Tools to decipher the TME

Immunohistochemistry on sections of paraffin embedded tumors followed by quantification using image analysis software enables a precise estimation of the density of the cells in the invasive margin and the center of tumors. This was notably used to define an *Immunoscore* based on quantification of $CD3^+/CD8^+$ cell densities on both regions. The Immunoscore correlates with patients survival in colorectal cancer [6,7•]. Densities of other immune cell types can also be quantified and correlate

with patient's overall or progression free survival as reported in most if not all tumor types [5••,8]. The presence of TLS can be assessed using multiple labelings with CD3 and DC-Lamp markers to identify the T cell zone [9], CD20 allowing additional identification of the germinal center [4]. However, only a very limited number of markers can be assessed simultaneously using bright field IHC. The recent development of fluorescent-based multiplex technologies extending the number of simultaneous markers up to 8 or more and of imaging technologies providing cell proximity measurements will extend further our knowledge about cell organization and interactions within the TME [10–12].

With a larger set of parameters analyzed, cytometry methods also offer an analysis of the cellular content of tumors. Eleven colors flow cytometry data can be analyzed using unsupervised clustering [13••]. Recently, mass cytometry application for high dimensional single cell analysis has also produced spectacular results especially when coupled to single cell RNASeq [14]. Notably, Chevrier and colleagues used this technology to characterize macrophages and T cells infiltrating kidney tumors, identifying a number of subsets more important than ever before and deciphering their potential deleterious interactions correlating with patient's relapse [15••]. Lavin *et al.* applied a similar single-cell technology to lung tumors [16].

Several methods have been developed to estimate the quantity of various cell populations from the large amounts of transcriptomic data that can be extracted from The Cancer Genome Atlas (TCGA), Gene Expression Omnibus or ArrayExpress publicly available datasets. Moreover, as technology improves, Next Generation Sequencing RNA-Seq allows for high throughput procedures.

One of the most commonly used methods to analyze transcriptomic data is gene set enrichment analysis (GSEA) [17], which provides signatures for various cell types [2]. Recently, Charoentong and colleagues used GSEA on the TCGA data to design an immunophenoscore, related to survival and response to immunotherapies [18]. The other main class of methods consists of deconvolution methods.; their goal is to decipher the contribution of different cell populations to the overall transcriptomic signal of heterogeneous tissue samples [19,20]. A first step can be the identification of the signal originating from tumor cells, and its separation from that of the TME. This is the objective of ISOpure that uses a reference profile [21]. Another tool, CIBERSORT [22] uses a support vector regression to estimate the relative abundances of 22 cell subsets within all screened populations. Finally, MCP-Counter [23••] uses transcriptomic markers that are cell type specific and have significantly higher expression in each cell type as compared to the others. From these markers, MCP-Counter indicates scores proportional to the absolute amount of each cell population. A total of 10 cell populations (8 immune and 2 stromal) can be quantified using this method.

Common immune features of the TME in high-risk patients for progression

Using these tools, the prognostic value of each cell population has been analyzed for various cancers, in a large body of studies [5••]. As a general rule, $CD8^+$ T cells are associated with a favorable outcome in most cancers, such as gastric, colorectal, pancreatic and liver cancer as reviewed recently [24], breast cancer [25] and lung cancer [26], with the notable exception of renal cell carcinoma [27] and prostate cancer [28]. On the contrary, M2-polarized macrophages are linked to a shorter survival in all cancer types [5••]. Other cell types of the TME, such as Tregs exhibit a subtler relation to prognosis, being linked to a prolonged survival in some tumors, such as colorectal [24,29,30] and a shorter one in others, such as in liver tumors [24] or non small cell lung cancer [26,31]. Their role remains unclear in some tumors, including gastric cancer [24,32,33]. The application of MCP-Counter to transcriptomic TCGA datasets also reveals a generally favorable impact of cytotoxic lymphocytes, whereas fibroblasts are usually related with a poorer survival [23••]. The presence of TLS that delineates generation and maintenance of effector memory T and B cell responses correlates with prolonged survival in most tumor types [34], with the exception of liver cancer where they are associated with higher recurrence rate when present in the cirrhotic tissue surrounding tumor nodules [35,36,37••].

Immune-based identification of high-risk patients for progression in ccRCC

Renal cell carcinoma (RCC) is a group of heterogeneous tumors with distinct genetic abnormalities. The most common subgroup is clear cell (ccRCC) that accounts for more than 80% of RCC.

Localized ccRCC can be treated surgically by partial or radical kidney removal. However, 30% of the patients present with metastatic disease at time of diagnosis, and another 30% will eventually develop metastases during the course of the disease [38]. A better understanding of the molecular features and the main pathways involved in ccRCC tumor progression has led to the approval of 12 new systemic agents. Despite this huge progress, metastatic RCC (mRCC) is still not curable.

In the past decade, the rapid development of next-generation sequencing technologies permitted the discovery of the main genetic and epigenetic events driving tumor progression in ccRCC. Independent studies have consistently classified tumors into subgroups, associated with a distinct clinical outcome and different sensitivity to