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Transcriptomic analysis of the tumor microenvironment to guide prognosis and immunotherapies

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Abstract

Tumors are highly heterogeneous tissues where malignant cells are surrounded by and interact with a complex tumor microenvironment (TME), notably composed of a wide variety of immune cells, as well as vessels and fibroblasts. As the dialectical influence between tumor cells and their TME is known to be clinically crucial, we need tools that allow us to study the cellular composition of the microenvironment. In this focused research review, we report MCP-counter, a methodology based on transcriptomic markers that assesses the proportion of several immune and stromal cell populations in the TME from transcriptomic data, and we highlight how it can provide a way to decipher the complex mechanisms at play in tumors. In several malignancies, MCP-counter scores have been used to show various prognostic impacts of the TME, which we also show to be linked with the mutational burden of tumors. We also compared established molecular classifications of colorectal cancer and clear-cell renal cell carcinoma with the output of MCP-counter, and show that molecular subgroups have different TME profiles, and that these profiles are consistent within a given subgroup. Finally, we provide insights as to how knowing the TME composition may shape patient care in a near future.

Keywords: tumor microenvironment, immune cells, transcriptome, gene expression, MCP-Counter, NIBIT 2016

Précis

A newly available method can infer cellular composition of the tumor microenvironment from transcriptomic data, which can be linked to prognosis, mutational burden and immune classifications of several malignancies and can guide immunotherapies.

Abbreviations:

ccRCC	clear cell renal cell carcinoma
CIBERSORT	cell-type identification by estimating relative subsets of RNA transcripts
CMS	consensus molecular subgroups
CRC	colorectal cancer
GSEA	gene set enrichment analysis
MCP-counter	microenvironment cell population-counter
MSI	microsatellite instable
NGS	next-generation sequencing
NIBIT	Network Italiano per la Bioterapia dei Tumori (the Italian network for cancer biotherapy)
TCGA PANCAN	the cancer genome atlas pan-cancer cohort
Th17	T helper lymphocyte producing interleukin 17
TME	tumor microenvironment
Treg	regulatory T cell

Introduction

Tumors are living and dynamic tissues where malignant cells are intricately intertwined with host components recruited and modified by tumor cells. Either through direct interactions or through the chemokines and cytokines they produce, host cells influence tumor growth, migration and metastasis, and therefore patient clinical outcome [1, 2]. The composition of the tumor microenvironment (TME) is diverse, containing cancer cells- supporting partners such as matrix fibroblasts, feeding and routing elements such as blood and lymphatic vessels, and hematopoietic cells such as lymphocytes, granulocytes, macrophages, mast cells, dendritic cells (DC) and myeloid derived suppressor cells (MDSC)[3].

As a general rule, high densities of T and B lymphocytes, cytotoxic T and NK cells, M1 macrophages and mature DC correlate with patient's favorable prognosis, while high densities of M2 macrophages, granulocytes, mast cells, MDSC, immature DC, T_{reg} and T_{H17} lymphocytes are associated with poor prognosis [1, 2, 4]. Quantification of these cell populations in tumors provides a major insight in patient's outcome. The composition of the TME, at each stage of a cancer, untreated or even after chemo-, radio- or immunotherapy often represents the strongest predictor for disease control [5, 6]. Moreover, it has recently been established, and continuously updated, that the TME provides targets and tools for efficient immunotherapies such as checkpoint blockade [7] and bi-specific antibodies [8], tumor-infiltrating lymphocytes or chimeric antigen receptor expressing T cells [9]. It is therefore essential to quantify the numbers and the types of TME cells at diagnosis, and if possible following treatment, to guide efficient patient's care. Different methodologies have been successfully developed, including cell quantification by immunohistochemistry (IHC), or immunofluorescence (IF) [10–12]. These methods have the advantage of taking into account the architecture of the cancerous tissue. However, they are complex to interpret and time consuming. Transcriptome analyses require small amounts of RNA, allowing sequential analyses and the study of both malignant cells and TME characteristics in large series of

cancers. In addition, they enable the simultaneous analysis of a very large number of markers and, by measuring gene expression of chemokines, cytokines and inflammatory molecules, they inform about the functional orientation of tumor-host interactions. Moreover, transcriptomic studies allow for the use of Next-Generation Sequencing (NGS) technologies, with a high throughput. Finally, they can easily be integrated with other molecular analyses, such as the calling of molecular subgroups. We will review here several transcriptomic methodologies and provide examples of integration of TME composition analysis with the molecular subgroups of tumors.

Analysis of whole-tumor transcriptomic data to study the TME

Many efforts have been devoted to analyze tumor transcriptome and estimate the composition of the TME. The difficulty lies in the extremely high cellular heterogeneity within and/or around tumors, creating several sources of variability in the transcriptomic signal [13], notably the frequency of the cell populations and the plasticity of their phenotypes. All these aspects contribute to the transcriptomic measure for a given gene, which is an average of the single-cell signals for all cells present in the sample.

Several approaches have been used to solve this problem. In a pioneering work [14], a mathematical framework has been established to estimate cell types participation to heterogeneous samples gene expression. Another work [15] focused on immune cells, using four immune-originated transformed cell lines to establish distinct expression profiles of B cells, monocytes and T cells, hence allowing deconvolution of micro-array transcriptomic data for these 3 cell types. Gene expression signatures have been described for 28 subclasses of immune cells [16], including effector, memory and helper T lymphocytes. These signatures have been used for instance with Gene Set Enrichment Analysis (GSEA) to derive scores for each immune cell subset [17]. GSEA has also been used to define the

“immunophenoscore” [18], which interestingly associates with the response to anti-checkpoint therapies.

Recent methods aim at providing highly precise quantitative information about the cell content of heterogenous samples using deconvolution techniques. CIBERSORT, a method using support vector regression [19] allows to estimate relative proportions of 22 immune cell subtypes within heterogenous tumor samples. Our team has established extremely stringent and robust gene signatures for 8 immune cell types, as well as fibroblasts and vessels [20] and used them in a method called MCP-counter, which scores are proportional to the cell amounts within the samples. It has been validated against controlled RNA mixtures and correlates well with IHC measures, demonstrating that it can provide an interesting alternative. ISOpure [21] is a software that allows for identification of cancer profiles in tumor samples, quantification of the contribution of malignant and healthy cells to the transcriptomic signal from tumor data coupled with reference profiles. MCP-counter does not allow for the quantification of malignant cells, but does not need any reference “normal” profile. In some cases, such profile may be hard to obtain, for instance when working with biopsies.

In the context of the analysis of the TME, it is important to highlight that these methods are not intended for the same use. CIBERSORT allows to compare cell populations within a sample, whereas MCP-counter allows inter-sample comparison of immune and stromal cell populations. This is illustrated in figure 1. CIBERSORT estimates each screened population as a proportion within the total of the screened populations, but ignores unscreened populations. Therefore, it is a powerful tool to compare populations one with the other, but is limited when used to compare different samples. On the contrary, MCP-counter scores are expressed in arbitrary units specific for each population, and are proportional to the proportion of cells of this population within the whole sample, thus allowing comparisons between samples in a cohort.

We summarized in table 1 the availability, methodology, requirements and outputs provided by the MCP-counter, CIBERSORT and ISOpure.

One of the major hurdles of transcriptomic analyses of cell quantification is the potential discrepancies between quantification of cell population by gene signatures expression and the density of the corresponding cell type in a tissue, particularly in a tumor. We addressed this question recently. We have shown using in-vitro mRNA multi-populations spike-in experiments that MCP-counter scores strongly correlate with the abundance of the corresponding population (Pearson's r between 0.92 and 0.99). Moreover, although not as perfect, the metagene expression of T cells, cytotoxic cells and monocytes correlated ($r \geq 0.67$) with the tumoral density of CD3⁺, CD8⁺ and CD68⁺ cells quantified by IHC [20].

Applying MCP-counter to 25 different cancers, we confirmed that the inferred densities of lymphoid subsets, particularly T, B, NK and cytotoxic cells correlate with favorable prognosis in most cases whereas those of fibroblasts are markers of poor prognosis [20]. Endothelial cells metagene expression is variably correlated with prognosis [20], renal cell cancer being an interesting example. In this disease, high density T cells, including CD8⁺ lymphocytes [11] as well as the expression of their corresponding metagene correlate with shorter disease free survival while the metagene expression of endothelial cells correlates with longer survival [20]. We have recently shown that the deleterious impact of T cells on clear cell renal cell cancer (ccRCC) patients' clinical outcome correlates with a strong infiltration by T regulatory cells [22] which, in addition to PD-L1 and PD-L2 expression in malignant cells [11], potentially inhibit the anti-tumor immune reaction [22]. In addition it is surprising that a high endothelial cell density and subsequent angiogenesis positively correlates with patient's survival [20]. Whether it may be due to a good response rate of these patients to anti-angiogenic therapies [23] remains an open question [24].

It is of interest that, depending on the cancer type, combination of different metagene expression impedes patients' survival. Thus, combination of high T and B lymphocyte metagene expression, reflecting probably the density of tertiary lymphoid structures [25, 26], is the strongest favorable parameter for lung adenocarcinoma, whereas combination of high

T cells and low fibroblast gene signatures expression predict best survival in colorectal cancer; combination of high cytotoxic lymphocyte and low monocytic lineage signatures sign best prognosis in breast cancer [20]. It is therefore highly conceivable that the phenotypes of the malignant cells influence the composition and functional orientation of the TME. To address this question, we investigated the relationships between molecularly defined subgroups of cancers and TME composition.

Association between TME composition and mutational load of tumors

In parallel with the apparition and accumulation of somatic mutations, tumors tend to express a growing number of neoantigens that may trigger anti-tumoral immune response by the activation of lymphocytes directed against these neoantigens [27]. We therefore hypothesized that the immune infiltrate, and more generally the whole TME, may vary according to the mutational load of the tumors. Following Schumacher and Schreiber's classification of malignancies according to their propensity to generate neoantigens (as defined by the somatic mutation prevalence)[27], we analyzed the cohort-wide TME of 20 cancers from the TCGA PANCAN cohort, using MCP-counter (Figure 2). Malignancies are classified into 4 groups, referring to the formation of neoantigens: frequently, regularly, occasionally to regularly, and occasionally. We grouped them according to the TME composition in terms of T cells, CD8+ T cells, cytotoxic lymphocytes, NK cells, monocytic lineage, neutrophils, endothelial cells and fibroblasts (Figure 2a). Interestingly, this analysis grouped the cancers having the least amount of somatic mutations (thyroid, low grade glioma, glioblastoma and kidney chromophobe) in the same cluster characterized by low lymphocytic and fibroblastic infiltrates associated with heterogenous myeloid and vascularization contents. In particular, when comparing CD8+ T cells (Figure 2b) and fibroblasts (Figure 2c) with the mutational burden of malignancies, we found that the infiltration in the "occasionally mutated" tumors was significantly lower. However, of all the

other screened populations, only T cells exhibited a variation of their infiltrate between the cancer groups (Figure S1).

Integration of molecular subgroups and TME composition

Whole transcriptome analyses have been already used to classify different malignancies with prognostic-impacting molecular subgroups. The first reports were made in lymphomas [28, 29] and breast cancers [30, 31]. Many others have been published and are being refined (reviewed in [1]).

We have applied MCP-counter to characterize tumors that were previously classified into molecular subgroups: colorectal cancer (CRC) and ccRCC.

Efforts associating 6 laboratories have converged into a consensus classification of CRC in 4 molecular subgroups named “Consensus Molecular Subgroups” 1 to 4 [32]. CMS1 corresponds to highly mutated tumors, encompassing most microsatellite instable (MSI) tumors lacking DNA-repair genes; it is known to be highly infiltrated by lymphocytes. CMS2 contains canonical tumors with WNT / β -Catenin / MYC activation. CMS3 is rich in metabolically deregulated tumors, with some prevalence of KRAS mutated cancers. CMS4 has markers of epithelial-mesenchymal transition, with a TGF- β signature and high fibroblasts content. Using MCP-counter, we confirmed and extended the analysis of Guinney *et al.*, validating our approach, defining CMS1 as a sub-group highly enriched in cytotoxic T cells and moderate fibroblastic abundance, and we also showed that this subgroup had high immune checkpoint expression, IFN γ signature, high class 1 major histocompatibility complex (MHC1) expression, moderate inflammation and angiogenesis [33]. It is striking that it is indeed the best prognostic group for disease free survival [32] and that most if not all MSI patients from this group respond to anti-PD1 antibodies [34], which is consistent Guinney *et al.* findings showing PD-1 activation for CMS1 tumors. CMS2 and CMS3 are immune “deserts” with low MHC1 expression, no signs of immune and/or inflammatory cells. CMS4

has a high lymphocytic infiltration, with checkpoint inhibitors and high MHC1 expression but lacking IFN γ gene expression, together with high inflammatory, angiogenic and fibroblastic invasion. Strikingly, despite its high infiltration by lymphocytes, this subgroup, exhibits the worst clinical outcome. It illustrates the putative overcoming of a potential anti-tumor reaction by inflammatory, angiogenic, and immunosuppressive (via TGF β for instance) mechanisms. Therefore, the integration of TME and malignant cells classifications paves the way for different immunotherapeutics, combined and/or sequential, to control CRC. These ways are being explored [35, 36].

Metastatic ccRCC has also been recently stratified into 4 molecular subgroups [37] that we have subjected to MCP-counter analysis [38]. ccRCC1 is a poor prognosis group, with a low response rate to tyrosine kinase inhibitor (TKI) Sunitinib, with little to no immune infiltration; it also exhibits low MHC1 expression. Patients from ccRCC2 and ccRCC3 subgroups respond to Sunitinib and depict good prognosis; their tumors are heterogeneous in terms of TME composition. ccRCC4 is the worst prognostic group, very poorly responding to Sunitinib; it is highly enriched in T and NK lymphocytes in the context of high myeloid and fibroblastic infiltration. It probably corresponds to the subgroup recently being defined as being rich in Treg [39]. This subgroup, which also exhibits high MHC1 expression, checkpoint inhibitors on T cells and their ligands on malignant cells certainly might benefit from therapies associating immune checkpoint inhibition with other approaches.

Conclusions

The dialectic interactions of TME components with malignant cells depend on the organ where they happen, the oncogenic processes involved and their modification by treatments. The recent availability of high throughput methodologies to quantify the different elements of the TME and understand their functionality opens the time of the generalization of these approaches and the subsequent application of precision tailored therapies based on these

landscapes rather than on cancer subtypes only. The time of associating cancer targeted therapies with TME targeted agents is at its dawn (Figure 3). The main issue in the years to come will be to bring these powerful tools from the bench to the bedside. To use these high throughput methodologies in the clinical routine, two pre-requisites will be needed: first we will have to prove and validate their predictive value of response to tailored therapies rather than their overall prognostic value; second, we will have to validate these approaches using widely available tissues such as formalin-fixed and paraffin embedded tumor tissues. Once these major steps have been achieved, we will be ready to enter in a new era of a real precision and tailored medicine and there can be no doubt that we will see more patients cured.

Disclosure of potential conflicts of interest

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Conflict of Interest:

Yann A. Vano has received speaker honoraria from Novartis, Pfizer, Bristol-Myers-Squibb, Astellas Sanofi, and has received financial support for attending symposia from Bristol-Myers-Squibb and Novartis.

Aurélien de Reyniès holds intellectual property rights for patents related to immune cell population abundance estimation through transcriptomic analysis.

Wolf H. Fridman is a consultant for Pierre Fabre Medicament, Sanofi, Bristol-Myers-Squibb, Novartis, Curetech, Servier, Efranet, Efralys, Adaptimmune.

All other authors declare no conflicts of interest.

Figures captions

Fig.1 Comparison of CIBERSORT estimates and MCP-counter scores for different configurations of mixtures composition. The left panel is a schematic representation of 3 possible cell mixtures compositions, while the middle and right panels represent respectively the estimates that would be suggested by CIBERSORT and MCP-counter. We notice that the estimates of CIBERSORT for the first two mixes are similar, as they are expressed as percentages of cells among the screened populations only, regardless of the total infiltration in the sample. Conversely, MCP-counter scores are proportional to the amount of each cell population in the total sample, which allows inter-sample comparison for each population. However, these scores are expressed in a different arbitrary unit for each population, which forbids intra-sample comparison between population. CIBERSORT allows this type of comparison.

Fig.2 (a) Classification of 20 malignancies based on the TME composition assessed by MCP-counter, along with the estimation of the frequency of neoantigens proposed by Schumacher and Schreiber [27]. **(b)** and **(c)** Infiltration by CD8⁺ T cells **(b)** and fibroblasts **(c)** is significantly lower in tumors in which mutations occur only occasionally.

Fig.3 Main targeted therapies currently approved or in a late stage development in human solid tumors. Targeted therapies act directly on tumor cells or on populations of the tumor microenvironment (TME). **(a)** Tumor cells harbor activating DNA mutations or translocations which can be efficiently targeted by monoclonal antibodies (mAb) or TKI. **(b)** Endothelial cells involved in neoangiogenesis are efficiently targeted either by a VEGF or a VEGFR blocking mAb, a VEGF-trap or a VEGFR-TKI. Effector function of CD8 T cells can be restored by **(c)** antagonist mAb of co-inhibition signals such as PD-1, Tim-3, Lag-3 or CTLA-4, or by **(d)** agonist mAb of co-stimulation signals such as CD137, CD40L. Regulatory T cells (Treg) impede the anti-tumor immune response by a direct effect on CD8 T cells and by the blockade of the maturation of dendritic cells. Treg functions, as well as Myeloid-Derived Suppressor Cells (MDSC), may be suppressed by **(e)** IDO inhibitors or VEGF-VEGFR axis inhibitors. Fibroblasts control the trafficking of T cells from the invasive margin (IM) to the tumor stroma, hamper DC maturation, inhibit T-cell proliferation and sustain angiogenesis. Fibroblast might be targeted by TGF β blocking mAb.

Fig. S1 Infiltration of the different categories of cancers, based on their mutational burden, for **(a)** T cells, **(b)** Monocytic lineage, **(c)** Neutrophils, **(d)** Cytotoxic lymphocytes, **(e)** NK cells and **(f)** Endothelial cells.

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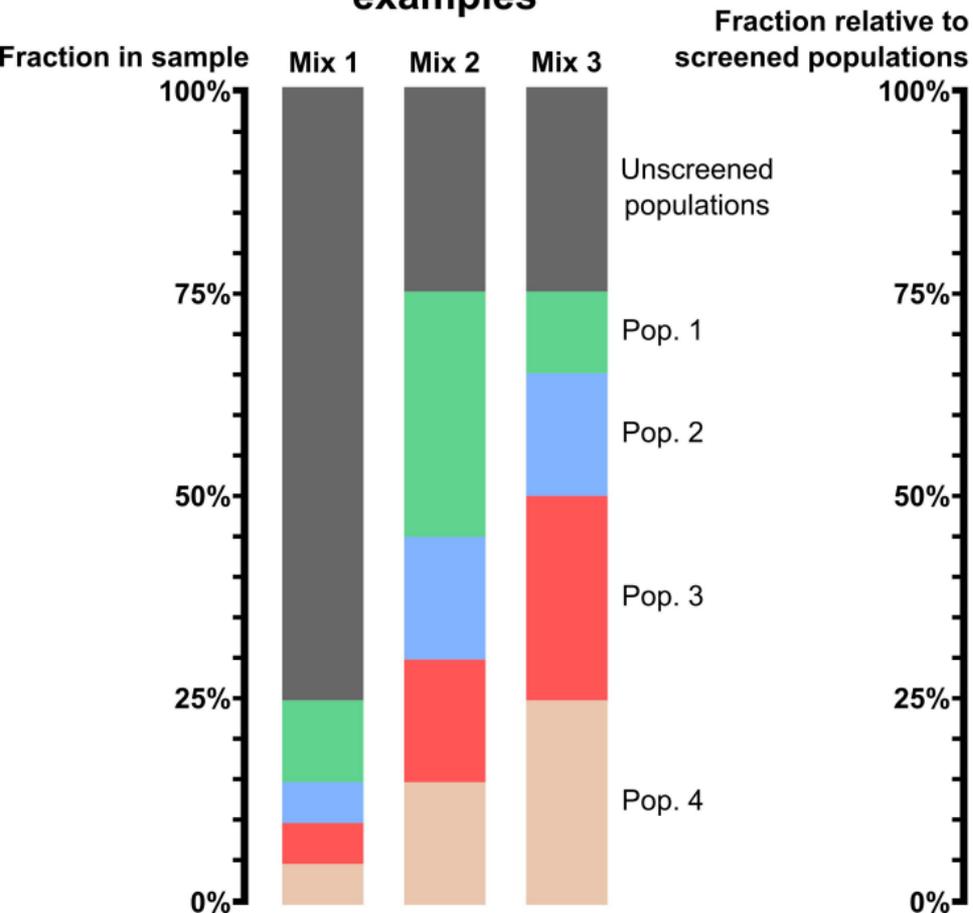
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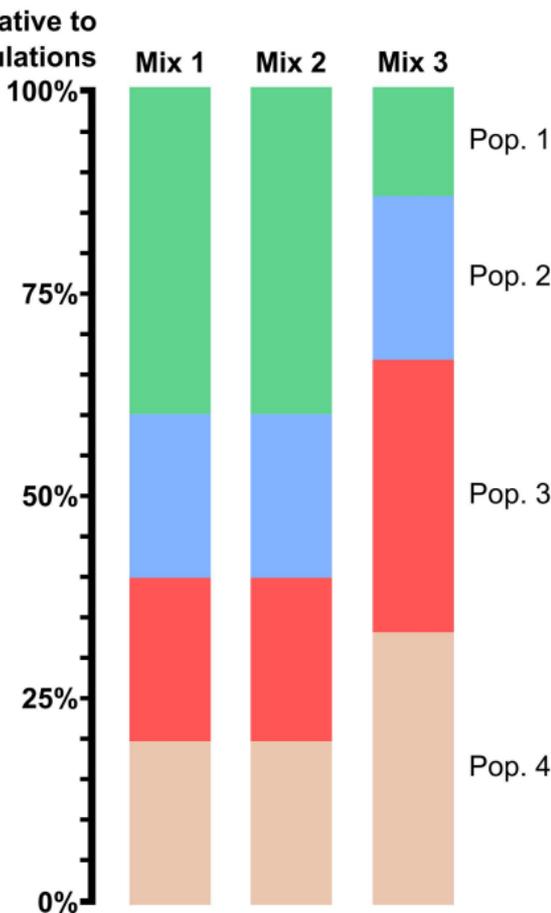
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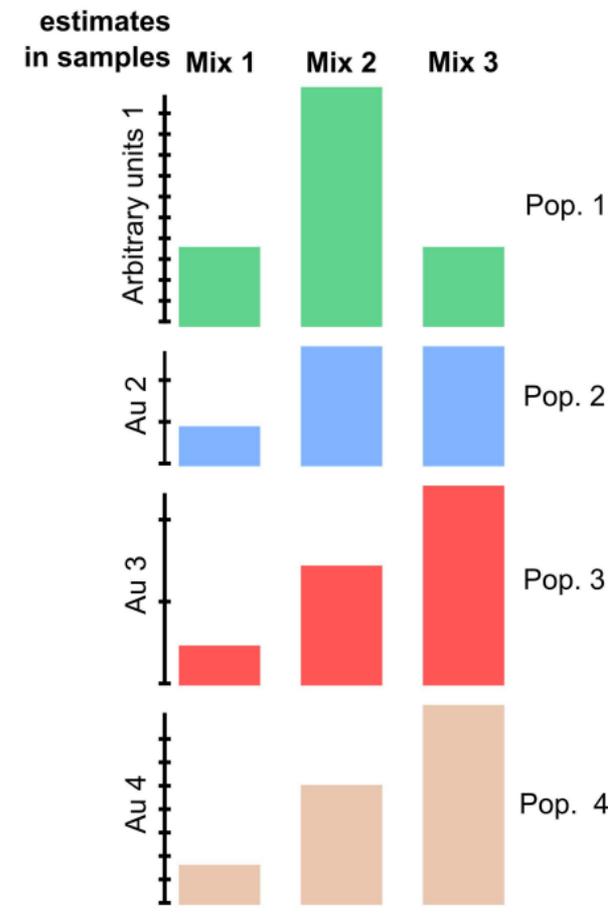
Cellular mixtures composition examples



CIBERSORT estimates



MCP-counter scores

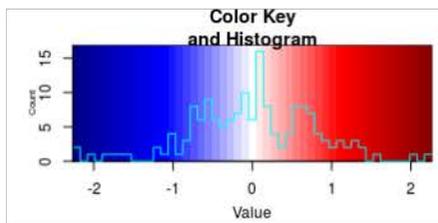
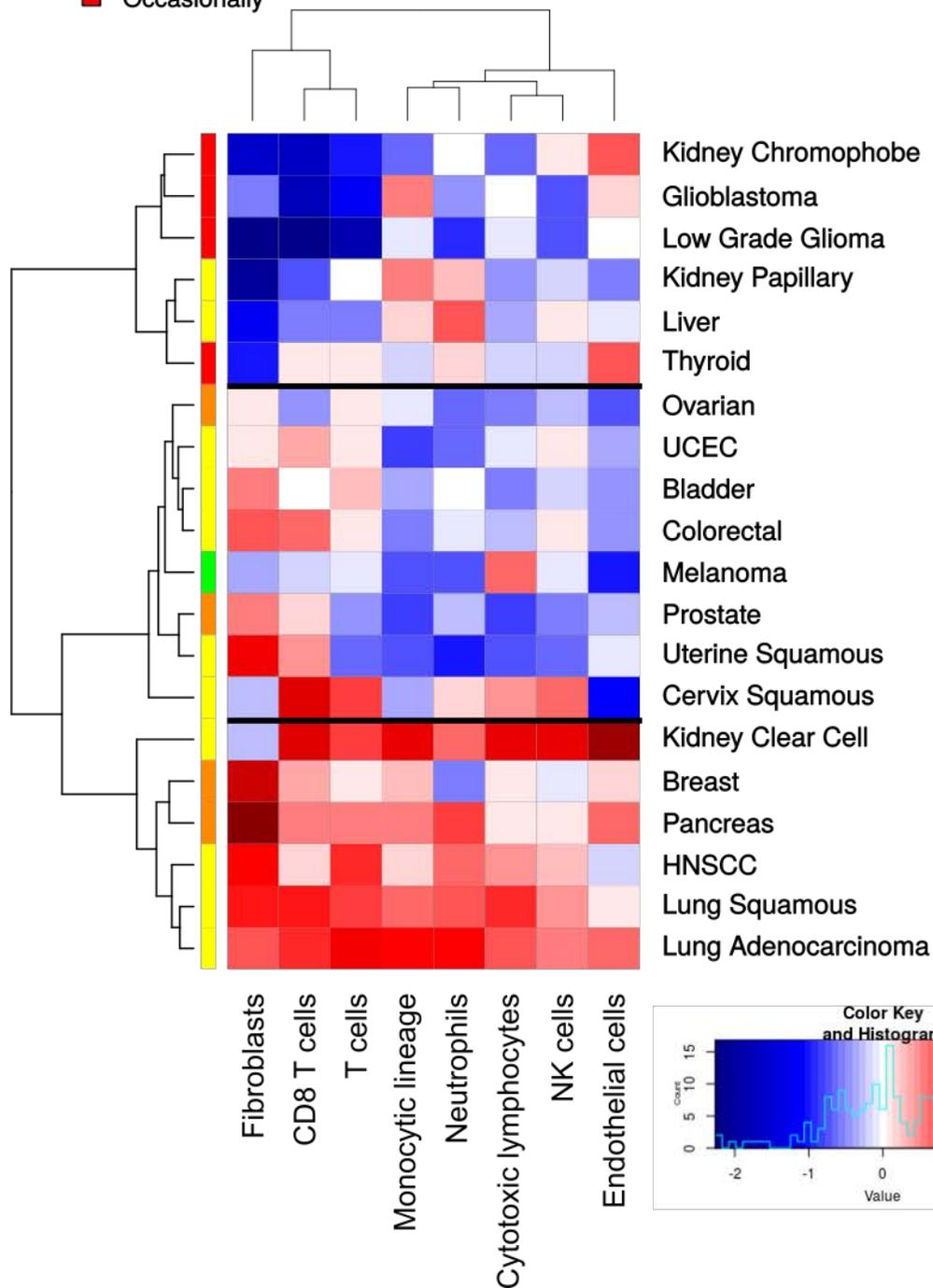
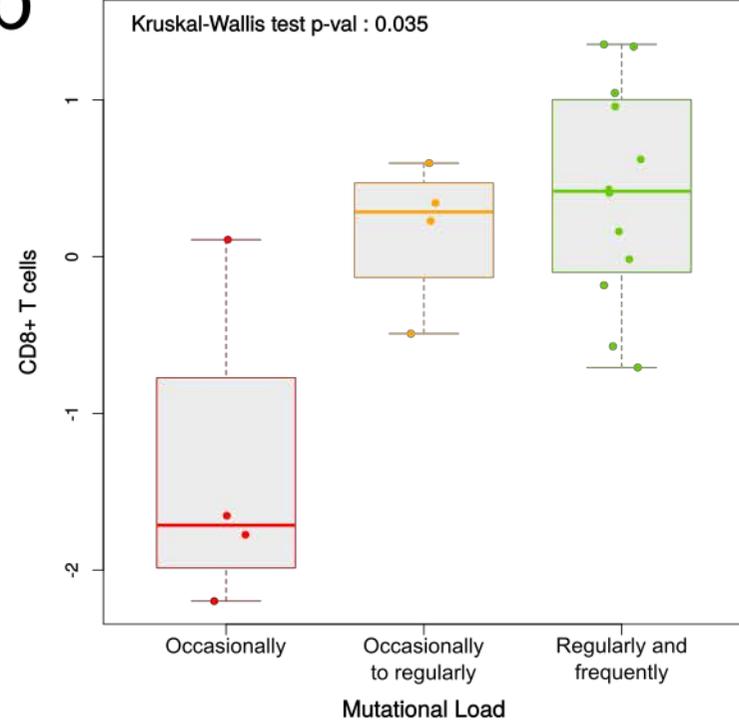


a

Formation of neoantigens
(Schumacher and Schreiber, 2015)

- Frequently
- Regularly
- Occasionally to regularly
- Occasionally

Fisher's exact test
p-value = 0.032

**b****c**