Transcriptome-derived stromal and immune scores infer clinical outcomes of patients with cancer

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Abstract. The stromal and immune cells that form the tumor microenvironment serve a key role in the aggressiveness of tumors. Current tumor-centric interpretations of cancer transcriptome data ignore the roles of stromal and immune cells. The aim of the present study was to investigate the clinical utility of stromal and immune cells in tissue-based transcriptome data. The 'Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data' (ESTIMATE) algorithm was used to probe diverse cancer datasets and the fraction of stromal and immune cells in tumor tissues was scored. The association between the ESTIMATE scores and patient survival data was asessed; it was indicated that the two scores have implications for patient survival, metastasis and recurrence. Analysis of a colorectal cancer progression dataset revealed that decreased levels immune cells could serve an important role in cancer progression. The results of the present study indicated that trasncriptome-derived stromal and immune scores may be a useful indicator of cancer prognosis.

Introduction

Cancer is a genetic disease characterized by genomic abnormalities that alter the transcriptome and influence the pathways that control proliferation and survival (1). The application of next-generation sequencing technology and single-cell sequencing in oncology has provided evidence that cellular heterogeneity is common in cancer (2). In the majority of tumor studies, key information may be disregarded owing to the tissue-centric nature of research. Malignant solid tumor tissues consist mainly of tumor cells, but also contain tumor-associated stromal, immune and vascular cells. Although non-tumor cells constitute a relatively small proportion of the cancer tissue, their role as potent tumor promoters has been previously indicated (3). A previous study, using a network approach to identify the functional gene modules in cancer cells, verified the presence of immune, stromal and vascular gene modules in cancer tissues (4).

The majority of genomic and transcriptomic studies into cancer do not explicitly consider genetic heterogeneity, and the generated inferences usually refer to mixed cell populations (5). However, the experimental isolation of single cells from tissues is expensive and may affect cell physiology. Additionally, the single-cell sequencing of a large cohort is unrealistic. An efficient solution to this limitation may be the de-convolution of genomic data from heterogeneous samples. Publicly available transcriptome databases can provide resources that allow for this type of analysis. To date, only one method, referred to as 'Estimation of STromal and Immune cells in MAlignant Tumors using Expression data' (ESTIMATE) has been described that can be used to score the stromal and immune fraction in transcriptomic data of cancer tissue (5,6). However, to the best of our knowledge, the association of the proportion of immune and stromal fraction with patient survival has not been thoroughly investigated.

Several studies have examined the microenvironment-associated transcriptional tumor profile using transcriptomic data. Calon *et al* (7) identified transforming growth factor-β (TGF-β) response signatures in tumor-associated stromal cells that could predict disease relapse in colorectal cancer (CRC). Cheng *et al* (8) used principle component analysis and clustering methods to identify a signature of stromal activation that was associated with late recurrence in breast cancer. Teschendorff *et al* (9) described an immune response gene expression module associated with a good prognostic subtype in estrogen receptor negative breast cancer. Finak *et al* (10) used laser capture microdissection (LCM) to compare the gene expression profiles of tumor stroma from primary breast tumors and derived signatures that were strongly associated with the clinical outcome by clustering. Isella *et al* (11) used Gene Set Enrichment Analysis (GSEA) and examined the gene

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signatures of subtypes for expression in stromal cell subpopulations vs. CRC cells. Wu *et al* (12) identified a stromal gene super-module associated with gastric cancer patient survival using gene co-expression network analysis. Furthermore, extensive experimental research has indicated the role served by stromal and immune cells in breast cancer (8,10,13), CRC (7,11,14), lymphoma (15) and drug resistance (16,17).

Transcriptome-based subtyping of cancer identifies different subtypes by clustering; however, non-tumor components are usually ignored (18). The ESTIMATE algorithm scores stromal and immune cells that form the major non-tumor components of tumor samples. In the present study, the scoring of stromal and immune cells in healthy and cancerous tissues, as well as in disease prognosis and drug resistance was investigated. The scores were associated with the clinicopathological characteristics of various cancer types and chemotherapeutic drug resistance. The results of the present study indicated that ESTIMATE could be used as a metric for patient prognosis assessment.

Materials and methods

Microarray datasets of healthy and disease tissue. The normal tissue dataset GSE45878 and cancer tissue dataset GSE2109 were obtained from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/). A validation RNA-Seq dataset E-MTAB-2836 from 32 different normal tissues was downloaded from EBI ArrayExpress database (www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-2836/) (19).

ESTIMATE algorithm. Stromal and immune scores were calculated by the ESTIMATE package in R (version 2.15.3) (20). ESTIMATE algorithm exploits the unique properties of the transcriptional profiles of cancer samples to infer tumor cellularity and identify the infiltrating normal cells (6). Five rounds of gene filtering identified two distinct gene signatures: i) A 'stromal signature' that indicates the stroma, and ii) an 'immune signature' that represents the infiltration of immune cells in tumor tissue. ESTIMATE outputs stromal, immune and ESTIMATE scores by performing single-sample GSEA. For a given sample, gene expression values were rank-normalized and rank-ordered. The empirical cumulative distribution functions of the signature genes and the remaining genes were calculated. A value of statistical significance was calculated by integrating the difference between the empirical cumulative distribution function, which is similar to the one used in GSEA, but based on absolute expression rather than differential expression (6).

Survival analysis. The breast cancer (GSE31448), CRC (GSE17538, GSE41258, GSE39396), Ewing's sarcoma (GSE17679), glioma (GSE16011), hepatocellular carcinoma (GSE20140), leukemia (GSE12417), lung cancer (GSE3141), lymphoma (GSE10846), melanoma (GSE65904) and ovarian cancer (GSE32062) datasets, and the respective clinical information were obtained from the GEO repository.

For metastasis and relapse analysis, the sarcoma (GSE21050), breast cancer (GSE1456), hepatocellular carcinoma (GSE10140), gastric cancer (GSE26253) and prostate cancer (GSE46691) datasets were obtained from the GEO database. The Cancer Genome Atlas (TCGA) expression dataset was obtained from Firebrowse at Broad Institute of the Massachusetts Institute of Technology & Harvard (firebrowse.org/).

Statistical analysis. The ESTIMATE scores for each dataset were calculated and patients were divided into two equal groups of high or low ESTIMATE score by median split. The ESTIMATE scores were normalized prior to Cox proportional hazards multivariate analysis. Overall survival time curves were plotted using the Kaplan-Meier method. Distributions of overall survival were compared using the log-rank test. Metastasis-free survival curves were plotted similarly for the samples that metastasis information was available. Furthermore, TCGA datasets' scores were associated with clinical information using Cox proportional hazards multivariate analysis. All analyses were conducted using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). The concordance index was used to indicate the probability that a patient with decreased survival time is associated with a high value of a predictor. It was estimated using the rms R package (21). ESTIMATE score differences between two groups were assessed using unpaired two-tailed t-tests in Microsoft Excel. $P<0.05$ was considered to indicate a statistically significant difference. For multiple comparisons, Bonferroni corrections were applied following analysis of variance, and $P<0.05$/number of tests were used as significance threshold. The correlation between lymphoblastoid cell line immune score and 5-FU treatment response was calculation by Pearson's correlation analysis in SPSS.

Results

Stromal and immune scores in healthy and disease tissues. To investigate the difference in ESTIMATE scores between healthy and malignant tissues, two public microarray datasets, GSE45878 and GSE2109, were analyzed (Fig. 1). Among normal tissues, adipose had the highest stromal score, whereas brain had the lowest; lung had the highest immune score whereas brain had the lowest. The present findings are consistent with the results from an RNA-sequencing dataset (E-MTAB-2836). Regarding malignant tissues, almost all of them presented a positive average ESTIMATE score: Pancreas had the highest stromal score and prostate the lowest, whilst testis had the highest immune score and prostate the lowest. Notably, normal pancreas had a low stromal score and normal testis had a low immune score (data not shown), whereas cancerous pancreas had a high stromal score and cancerous testis had a high immune score. Leukemia presented an extremely narrow range of scores, indicating the robust performance of the ESTIMATE algorithm.

Stromal and immune scores as a potential prognostic marker for multiple types of cancer. We subsequently hypothesized that as the two scores represent common malignancy features, they may be used as potential markers for cancer prognosis. The prognostic efficiency of stromal and immune scores in breast cancer, CRC, Ewing's sarcoma, glioma, hepatocellular carcinoma, leukemia, lymphoma,

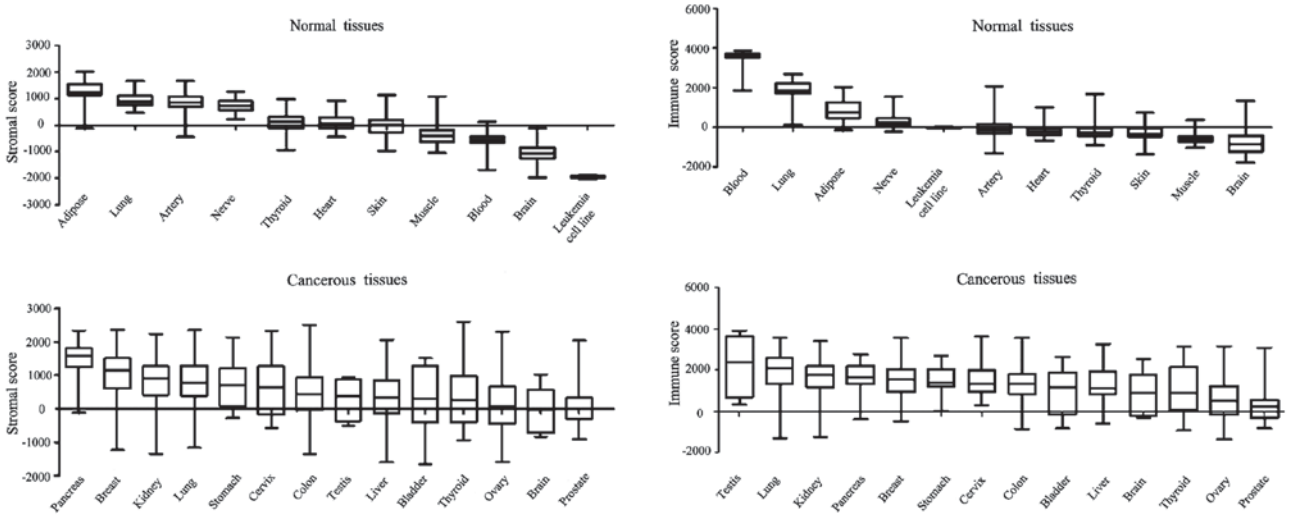

Figure 1. Stromal and immune scores in healthy and malignant tissues.

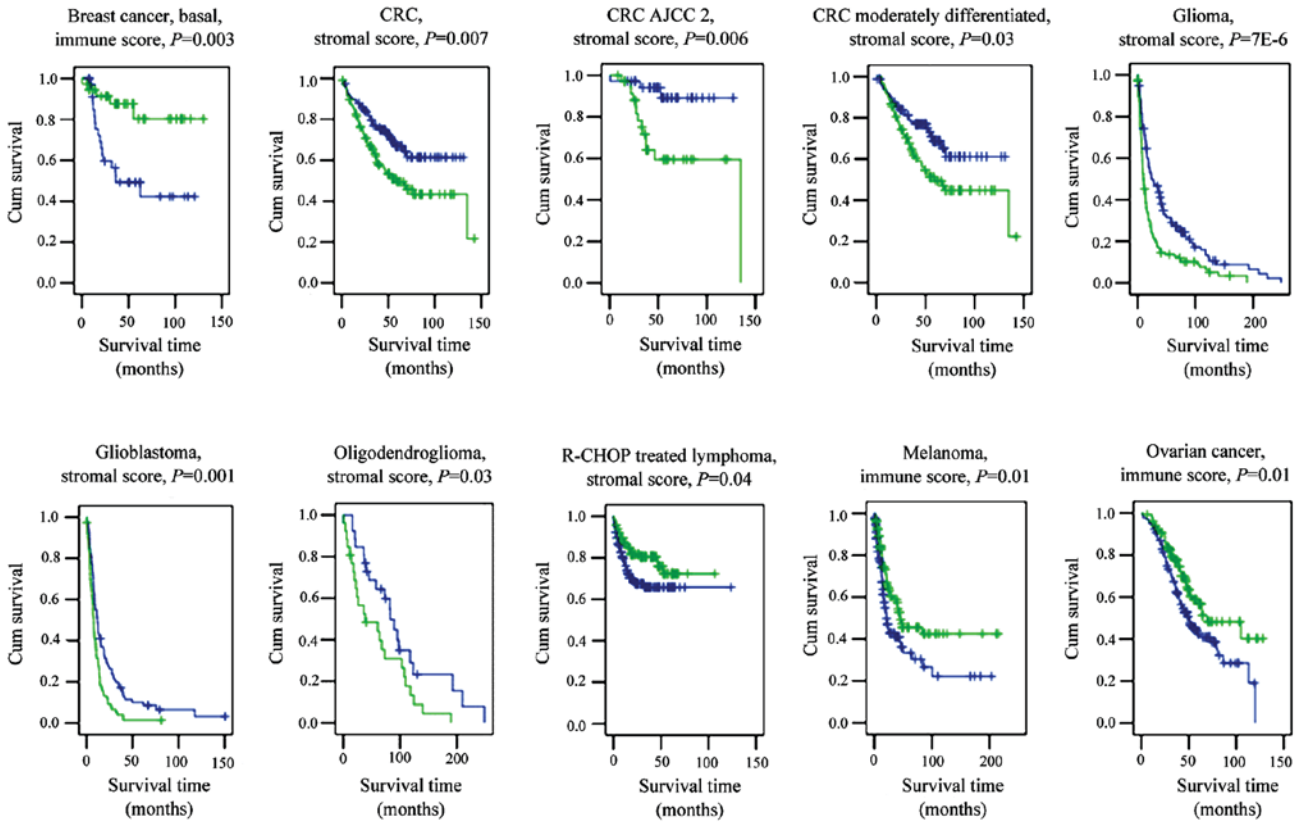

Figure 2. Stromal and immune scores are predictive of patient survival in several types of cancer. Kaplan-Meier curves of patients with breast cancer, CRC, glioma, lymphoma, melanoma and ovarian cancer. P-values were generated using the log-rank test. Blue lines indicate low scores; green lines indicate high scores. AJCC, American Joint Committee on Cancer; CRC, colorectal cancer; R-CHOP, rituximab, cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone; Cum, cumulative.

lung cancer, melanoma, ovarian cancer, prostate cancer and sarcoma was investigated. Survival curves were plotted following patient categorization in groups of high and low ESTIMATE scores. Notably, the two scores were indicative of patient survival in multiple types of cancer (Fig. 2). Indicatively, the immune score separated patients with long and short survival in ovarian cancer and melanoma. Additionally, the stromal score signified the survival time of patients with CRC and glioma. Furthemore, the scores indicated survival rates within specific subtypes of cancer. For instance, the stromal score indicated the survival rate of patients with stage 2 American Joint Committee on Cancer (AJCC) CRC (22), moderately differentiated CRC, oligodendroglioma, glioblastoma and served a prognostic role in patients with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone-treated lymphoma. The immune

Table I. Multivariate Cox regression of overall survival and metastasis data in several types of cancer.

Data source	Disease	Variable	P-value	Estimated hazard ratio
Gene Expression Omnibus	Colorectal cancer survival	Stromal score	0.008	1.487
	Breast cancer survival	Immune score	0.001	0.544
	Gastric cancer survival	Stromal score	0.002	1.314
	Glioma survival	Stromal score	0.009	1.437
	Melanoma survival	Immune score	0.000	0.541
	Ovarian cancer survival	Stromal	0.033	1.327
		Immune score	0.001	0.649
	Sarcoma metastasis	Stromal score	0.002	0.702
The Cancer Genome Atlas	Breast cancer survival	Stromal score	0.040	1.278
		Immune score	0.046	0.744
	Cervical squamous cell carcinoma survival	Stromal score	0.026	1.456
		Immune score	0.008	0.611
	Skin cutaneous melanoma survival	Immune score	0.002	0.685

score indicated the survival time of patients with basal breast cancer.

The stromal score separated patients with early relapse from those with late relapse in basal breast cancer, and differentiated between patients with early metastasis and late metastasis in sarcoma (Fig. 3). Notably, the immune score of hepatocellular carcinoma-adjacent hepatitis/cirrhotic liver tissue was found to be indicative of disease recurrence.

Certain GEO and TCGA datasets that contained additional clinical information were further analyzed by multivariate analysis. The immune score indicated the survival rate of patients with breast cancer, melanoma and ovarian cancer following adjustment for clinical parameters (Table I). Additionally, the stromal score indicated the survival rate of patients with CRC and glioma, and predicted cancer recurrence and metastasis in patients with sarcoma following adjustment for clinical parameters (Table I). Furthermore, the clinical implication of the immune score in breast cancer and melanoma was also validated in the TCGA dataset.

Stromal and immune scores predict CRC progression. The significance of the cancer microenvironment in tumor progression has been repeatedly indicated. Stromal and immune cells are major non-tumor components of cancer. Even though the stromal score is predictive of survival in patients with AJCC stage 2 CRC, the immune score indicated a progression from polyp to CRC. The average immune scores in normal colon mucosa, polyp, primary CRC and metastatic CRC were calculated as 1,203, 488, 887 and 500, respectively. However, no statistically significant difference between the normal colon mucosa and polyp tissue was observed. The immune score was significantly lower in primary and metastatic CRC than in normal colon or polyp tissue. An association between the downregulation of immune system-associated genes and metastasis in CRC has been previously reported (23). The immune score is significantly lower in p53 mutant patients (P=0.02) in the GSE41258 dataset. It has been demonstrated that p53 regulates immunological activities (24), indicating that p53 has a possible regulatory role in CRC progression.

Gene expression analysis of specific cellular populations isolated from CRC patients revealed that fibroblasts and leukocytes have the highest stromal and immune scores among endothelial cells, epithelial cells, fibroblasts and leukocytes (Fig. 4). These results indicate the robustness of the ESTIMATE algorithm.

Implication of drug resistance by immune score. The anticancer activity of fluorouracil (5-FU) involves the restoration of T-cell immunity (25). The GSE11582 dataset includes the response of lymphoblastoid cell lines treated with a range of 5-FU concentrations. Pearson's correlation analysis revealed that the immune score was positively correlated with 5-FU treatment response (R=0.2, P=0.008).

Discussion

To the best of our knowledge, this is the first study in which ESTIMATE scoring was used to differentiate between tissues. Stromal and immune scores were associated with the clinical outcome of the patient and chemotherapy drug resistance. The prognostic value of the two scores was validated using multiple microarray platforms. Either the stromal or the immune score was associated with patient survival, relapse and metastasis in multiple types of cancer. Furthermore, the two scores were associated with chemotherapeutic drug response. The present study has indicated a microenvironment view of tissue-based tumor transcriptomic data and highlighted the contribution of stromal and immune cells in carcinogenesis. However, the precise molecular mechanism underlying this phenomenon should be investigated in future studies.

Tissues are composed of a mixture of cell types. Currently, tissue-based transcriptomic data do not reflect the information from multiple cell types. Even though a number of technological approaches, including flow cytometry and LCM, have been developed, their application in cancer research is limited owing to the expensive cost. Therefore, it is crucial to study

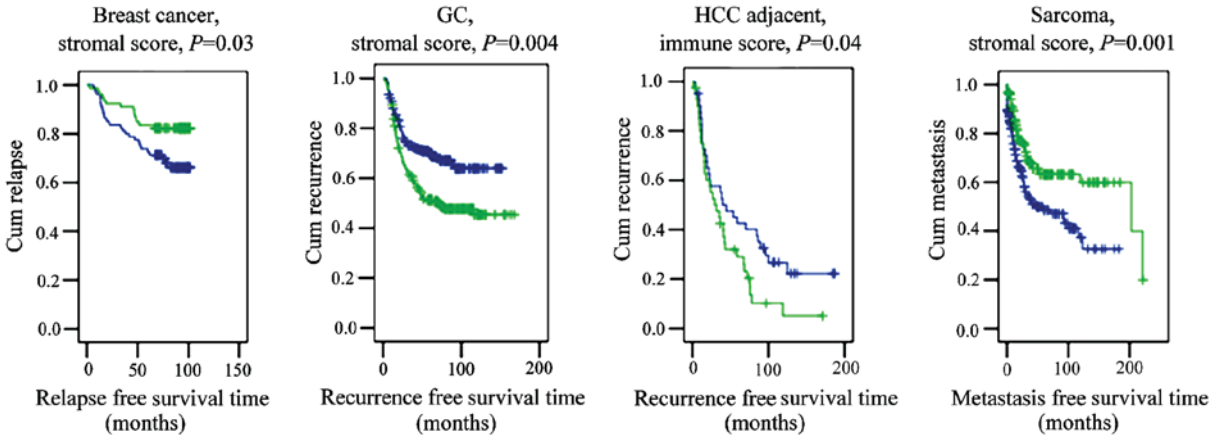

Figure 3. Stromal and immune scores are indicative of patient recurrence and metastasis. Kaplan-Meier curves of patients with breast cancer, gastric cancer, hepatocellular carcinoma and sarcoma. P-values were generated using the log-rank test. Blue lines indicate low scores; green lines indicate high scores. GC, gastric cancer; HCC, hepatocellular carcinoma; Cum, cumulative.

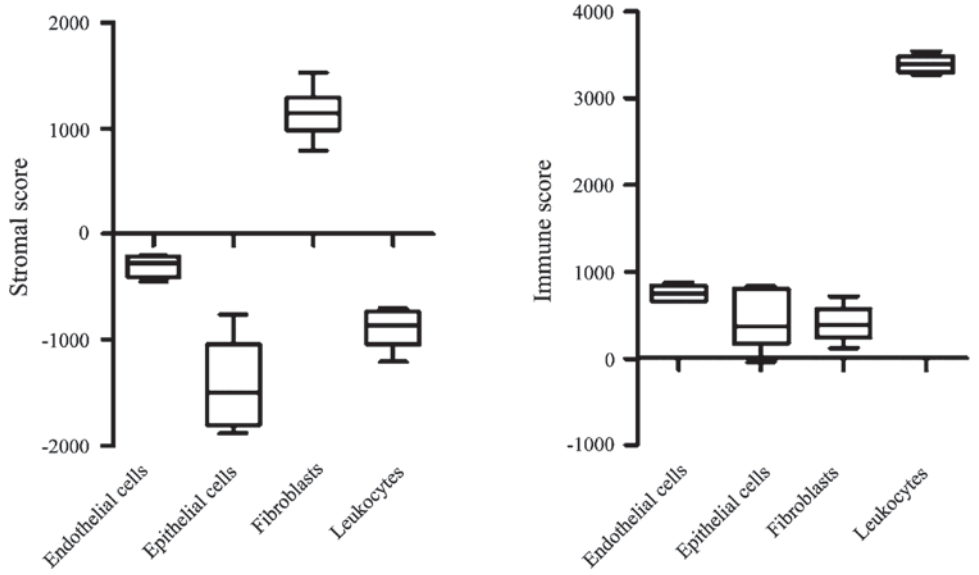

Figure 4. Stromal and immune scores in distinct cellular populations derived from patients with colorectal carcinoma.

the information hidden in available public datasets. Functional modules in cancer transcriptomic datasets have been previously identified by gene co-expression network analysis (4). In the present study, two major components of the cancer microenvironment, stromal and immune cells, were investigated, and it was demonstrated that the ESTIMATE scores for these components could predict patient clinical outcomes. For instance, the stroma score was identified as a predictor for survival in patients with gastric cancer (a lower stroma score was indicative of better patient prognosis). Notably, the ESTIMATE-derived stromal score was found to be more efficient than the recently described stromal super-module-based method (12). However, it should be mentioned that the stroma might serve distinct roles in different tissues. In the present study it was demonstrated that a higher stroma score indicated later relapse in breast cancer, which is consistent with a clustering-derived gene signature method (8,10). The immune score was also found to predict survival in basal breast cancer, in agreement with a previous study that used the Profile Analysis using the Clustering and Kurtosis method (9). Similarly, a higher immune score was associated with a longer survival time in melanoma and ovarian cancer, but earlier recurrence in hepatocellular carcinoma-adjacent tissue. The degree of tumour-infiltrating lymphocytes in particular activated CD8+ T-cells, within melanoma positively correlates with better prognosis (26). The decreased recruitment of tumor-infiltrating lymphocytes may lead to poor prognosis in high-risk ovarian cancer patients (27). It has been previously demonstrated that the poor prognostic signature in hepatocellular carcinoma-adjacent tissue involved genes associated with inflammation, including interferon signaling and activation of nuclear factor-κB and tumor necrosis factor (28). These results indicated that non-tumor liver tissue could serve a prognostic role in patients with early-stage disease. These results may suggest the dual host-protective and tumor-promoting roles of immune cells in different tumor types (29,30).

The results of the present study indicated that research into microenvironment-associated cells is warranted in

patients with cancer. Understanding the effect of the microenvironment on drug sensitivity may improve the efficiency of targeted therapies (31). For instance, an association has been demonstrated between the initiation of metastasis in CRC and the TGF-β stromal program (7). Even though the ESTIMATE algorithm is based on cancer tissue data, it was found to be effective in assessing cellular data as well (Fig. 4). In the present study, the ESTIMATE algorithm was used on cell line data, identifying a positive correlation between 5-FU treatment response and the immune score and indicating the potential mechanism of the drug (25). A lower immune score was observed in p53-mutant CRC patients, indicating that 5-FU may not be the optimal treatment choice for p53 mutant patients. Indeed, several clinical studies have reported that CRC patients with wild-type p53 benefit from 5-FU-based chemotherapy, but those with mutant TP53 do not (32,33). Thus, robust patient stratification using microenvironment data may aid the development and application of cancer therapies (34).

It is reasonable to apply the ESTIMATE scoring to a specific tissue-based transcriptomic dataset, as the sampling criteria are identical for every specimen within a study. To the best of our knowledge, the present study is the first to demonstrate that ESTIMATE scores are indicative of patient survival, relapse, metastasis and chemotherapeutic drug resistance. The two scores may have a prognostic value, indicating that stromal and immune cells contribute to tumor clinical outcome. It was further demonstrated that immune cells were associated with CRC development and that the ESTIMATE scores may become useful indicators of tissue-based patient prognosis.

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