Characterization of cytokinome landscape for clinical responses in human cancers

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ABSTRACT
Dysfunctional intratumoral immune reactions are shaped by complex networks of cytokines (including chemokines), and how the cytokinome landscape coordinates with tumors has not been systematically investigated. Using high-dimensional datasets of cancer specimens, we explored the transcript abundance, biomarker potential, and prognostic impact of local cytokines across 19 tumor types. We found that many cytokines are highly locally dysregulated (p = 0.024), revealing spatiotemporal pattern of local cytokines in the development of cancers. In addition, we noted the significant downregulation of CCL14 and CXCL12 in 9 and 10 cancer types, respectively, implying their crucial roles in tumor pathogenesis. We also found that cytokines showed significantly higher specificity properties compared to other protein-coding genes (PCGs) in primary tumor specimens (p = 0.001), indicating that tissue context remains an issue when considering cancer cytokinomes. Finally, we linked concentrations of local cytokines to patient survival. Our results thus provide a panoramic view of pan-cancer cytokinomes, which highlights tumor type specificity of cancer-related cytokines and their impacts on disease prognosis.

Abbreviations: BLCA, urothelial bladder cancer; BMP, bone morphogenetic protein; BRCA, breast cancer; CESC, cervical cancer; COAD, colon adenocarcinoma; CSF, colony stimulating factor; DLBC, diffuse large B-cell lymphoma; FDR, false discovery rate; FoE, fold of enrichment; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; IFN, interferon; IL, interleukin; JS, Jensen–Shannon; KIRC, clear cell kidney carcinoma; KIRP, papillary kidney carcinoma; LGG, lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic ductal adenocarcinoma; PCG, protein-coding gene; PPI, protein–protein interaction; PRAD, prostate adenocarcinoma; READ, rectal adenocarcinoma; TCGA, The Cancer Genome Atlas; TGF, transforming growth factor; THCA, papillary thyroid carcinoma; TNF, tumor necrosis factor; TPM, transcript-per-million; TTD, Therapeutic Target Database; UCEC, uterine corpus endometrial carcinoma

Introduction

Cytokines (including chemokines) are some of the most important components within the tumor microenvironment, and are involved in the intricate crosstalk between tumor cells and tumor-infiltrating innate and adaptive immune cells.1,2 In particular, these genes participate in specific cellular processes and tumor-related responses including regulation of proliferation, differentiation, activation and inhibition of immune cells,3 recruitment of immune cells to local inflammatory sites,4 enhancement of tumor recognition by immune cells,5 cancer inhibition and eradication,6 infiltration of immune cells in local tumor masses,7 promotion of tumor immunoediting,8 and facilitation of tumor invasion and metastasis.9,10 Although the roles of cytokines and cytokine receptors have been widely investigated, a need remains for systematic analyses of the roles of cytokines across human cancers.

To comprehensively investigate the intricate expression profiles of cytokines across human cancers, integrative computational biology approaches are essential for depicting cytokinome transcription landscape. The Cancer Genome Atlas (TCGA) project has generated comprehensive genomic data sets of human cancers. Using the large-scale genomic datasets exemplified by the TCGA database, the interaction between complex networks of cytokines and cancer phenotypes can be explored.

In this study, we performed multiplatform integrative analysis of alterations of 261 cytokines and cytokine receptors across 19 human cancer types. Using these comprehensive analysis, we characterized the expression profiles of tumor local cytokines and identified the correlations between aberrations of intratumoral cytokines and clinical responses.

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Results

Summary of experimental design

In this study, we performed a comprehensive investigation of cytokines’ expression profiles (RNA-seq) across human cancers (Fig. 1A, urothelial bladder cancer (BLCA), breast cancer (BRCA), cervical cancer (CESC), colon adenocarcinoma (COAD), diffuse large B-cell lymphoma (DLBC), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), clear cell kidney sarcoma (KIRC), papillary kidney carcinoma (KIRP), lower grade glioma (LGG), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), ovarian serous cystadenocarcinoma (OV), pancreatic ductal adenocarcinoma (PAAD), prostate adenocarcinoma (PRAD), rectal adenocarcinoma (READ), papillary thyroid carcinoma (THCA), and uterine corpus endometrial carcinoma (UCEC)). We first characterized the local dysregulation (tumor vs. normal) and stage specificity.

Figure 1. Study design and cytokines included. (A) Schematic showing comprehensive analysis of cytokine gene dysregulations in human cancers. (B) Schematic showing 261 cytokines and cytokine receptors included in this study. (C) Selected cytokines for downstream analysis in a given cancer type.
(late-stage vs. early-stage) properties of 261 cytokines and cytokine-related genes in 19 cancer types. Then, we assessed the potential clinical implication of these cytokines from several facets including specific expression pattern across cancers, drug-target enrichment, and prognostic ability.

To study local cytokine expression profiles in human cancers, we focused on a gene set containing 261 cytokines (Fig. 1B, the interferon (IFN) family, interleukin (IL) family, transforming growth factor (TGF) superfamily, colony stimulating factor (CSF) family, tumor necrosis factor (TNF) family, and bone morphogenetic protein (BMP) family), chemokines (CC group, CXC group, C group, and CX3C group), and other related genes. For each cancer type, two filters were applied for downstream analyses; the first selected only cytokines that were annotated as PCGs according to the GENECODE V24 database; the second eliminated any transcript for which the transcripts-per-million (TPM) expression value was equal to 0 in the above 20% of specimens. As shown in Fig. 1C, 167 (63.99%) cytokines were commonly detected in all 19 cancer types, and 226 (86.59%) cytokines were detected in at least one cancer type (Fig. 1C).

**Characterization of local-dysregulated cytokines**

Using RNA-sequencing data from 7242 TCGA primary tumor specimens and 634 normal tissues (Fig. S1), a computational biology pipeline was established to characterize the spatiotemporal dynamics of these cytokines across cancers. We compared differences in cytokine levels in primary tumors and normal tissues by analyzing 12 cancer types for which the number of corresponding normal specimens was >15. As shown in Fig. 2A, we identified 300 local-dysregulated cytokines from 12 cancer types (Fig. 2A, Table S1). On average, 12.63% of cytokines were locally dysregulated compared to their counterparts (Fig. 2B). In comparison with other PCGs analyzed by the same approach, we found that percentages of local-dysregulated cytokines were significantly higher than that of other PCGs (6.60% on average, $p = 0.024$, Fig. 2B, Table S2). Based on these results, expressions of CCL14 and CXCL12 were significantly dysregulated in 9 and 10 cancer types, respectively. While further comparing local-dysregulated cytokines with other PCGs, we found no difference between sharing distributions of up- and down-regulated genes across different cancers (Fig. S2).

**Characterization of stage-specific cytokines**

We further characterized expression levels of cytokines in different pathological stages (early and late stages) in 12 cancers (Fig. S3). However, cytokines showed stage specificity in only seven cancers, including BLCA, COAD, KIRC, KIRP, LIHC, LUAD, and THCA (Fig. 3A, Table S3). We found no significantly higher or lower patterns of percentages of dysregulated cytokines than that of other PCGs (Fig. 3B, Table S4). In addition, the percentages of stage-specific cytokines were similar to those of other PCGs ($p = 0.724$). We noted that two cancers (KIRC (48.99%) and KIRP (34.97%)) contained high percentages of stage-specific cytokines and other PCGs. By comparing sharing patterns across cancers, we found that stage-specific cytokines were highly cancer-specific, with around 70% of cytokines being significantly up- or down-regulated in specific late-stage tumors (Fig. S4).

**Specificity score analysis revealing tissue specificity of cytokines across human cancers**

Aided by volcano plots for the roles of cytokines (Fig. 4A, tumor vs. normal), we found that 5.19% and 7.57% of cytokines were significantly up- and down-regulated in tumors, respectively, with log-multiples of change of $>1.5$. However, the perturbation of expression levels of stage-specific cytokines (Fig. 4B, late-stage vs. early-stage) was relatively small compared to locally dysregulated cytokines, with almost all log-multiples of change of stage-specific cytokines being $<1.5$ across cancers.

To further identify the value of cytokines as biomarkers in human cancers, we adopted Jensen–Shannon (JS) divergence scoring analysis for each gene to characterize the specificity of cytokine expression patterns across human cancers. Importantly, we found that expressions of cytokines demonstrated significantly higher specificity across primary tissues and normal tissues across cancers than other PCGs ($p < 2.2 \times 10^{-16}$, Fig. 4C, Table S5). Results also indicated that cytokines showed larger extents of expression variations not only across normal-tumor tissues, but also across different cancer types compared to other PCGs. In addition, as shown in Fig. 4D, JS scores of 0.45 ($p < 2.2 \times 10^{-16}$) can significantly discriminate the specificity of cytokines and other PCGs (Fig. 4D).

**Cytokine abundances are a specific biomarker in human cancers**

To verify the above observations, we then applied an unsupervised agglomerative hierarchical clustering approach based on cytokine gene. Our results indicated that expression signatures of cytokines were differentiated between primary tumors and normal tissues in most cancers, except for BLCA and PAAD (Fig. 5). In those cancer types, expressions of cytokines were homogeneous in adjacent normal tissues, compared to the relatively diverse expression patterns in primary tumors. The results suggested that local cytokine expression profiles were able to exploit specific and informative biomarkers to determine the tumor origin and to provide preliminary pathological diagnostic information.

**Clinical implications of cytokines across human cancers**

To assess the prognostic predictive value of local cytokines, we conducted retrospective survival analysis using a multivariate Cox proportional hazards model. For each cancer type, the prognostic value of each cytokine was quantified. Furthermore, we conducted a random effects meta-analysis to evaluate the pan-cancer prognostic value of the cytokine. We found that 22 cytokines were highly expressed in all cancers and showed independent significance in at least four cancer types (Table S6). In addition, 53 cytokines showed significant pan-cancer prognostic ability (Table 1).

Next, we sought to validate the prognostic ability of local cytokines by conducting meta-analysis of 261 cytokines from...
12 publically available BRCA data sets. As shown in the Table 2, 27 cytokines possessed significant prognostic potential for BRCA patients with distant metastasis (Table 2). Notably, TGFBI (effect size = 1.19 (1.11–1.27), p = 4.06 × 10⁻⁷), TNFAIP6 (effect size = 1.14 (1.05–1.24), p = 0.001), and BMP1 (effect size = 1.25 (1.08–1.47), p = 0.002) which reached pan-cancer significance were also identified to be significantly correlated with BRCA prognosis.

We then conducted a drug-target enrichment analysis¹¹ to evaluate the drug-targeting potential of prognostic-related...
cytokines. We selected cytokines that showed significant prognostic predictive ability in at least two cancer types. Then, the 157 (60.15%) prioritized cytokines were used to assess the fold of enrichment (FoE) by evaluating the significant overlap with 143 cancer-related drug-target genes annotated from DrugBank and the Therapeutic Target Database (TTD). Results demonstrated that a prioritized cytokine gene set significantly overlapped with cancer-related drug target genes, with a FoE of 1.34 ($p = 0.046$, Figs. 6A and B). Hence, we provided empirical evidence to support the idea that the human cancer cytokinome is a valuable resource for therapeutic development and drug repositioning (Fig. 6C).

Figure 3. Stage-specific cytokines. (A) Heatmaps of stage-specific cytokines in 12 cancer types. Genes were sorted according to log-multiples of change. (B) Bar plots showing percentages of all, upregulated, and downregulated stage-specific cytokines and other protein-coding genes (PCGs).
Figure 4. Potential of cytokines as specific biomarker in human cancers. (A) Volcano plot of local-dysregulated cytokines. Each dot represents one cytokine in a given tumor type. Cytokines with a false discovery rate (FDR) of <0.05 and log-multiple of change of >1.5 are colored red (downregulated) and blue (upregulated), respectively. (B) Volcano plot of stage-specific cytokines. (C) Distributions of maximal normal-tumor specificity scores calculated for each gene across TCGA cancer types for cytokines and other protein-coding genes (PCGs). (D) Bar plots depicting differences between percentages of genes with specificity scores of >0.45 for cytokines and other PCGs.

Figure 5. Dendrograms showing the hierarchical clustering effect of cytokines across primary tissues and normal tissues within each cancer type.
Inflammatory microenvironments are consistent components in human cancers, and tumor-associated cytokines (including chemokines) intricately participate in all immune responses. Interactions of local cytokines and chemokines with tumors are essential for comprehending the defense against tumors. Using pan-cancer data from TCGA, we characterized the spatiotemporal dynamic context of local cytokines in human cancers. Our results provided a perspective of the cytokinome landscape within tumors, and further revealed the value of the clinical utility of cytokines in cancer prognosis and therapeutics.

### Discussion

In this integrative study, we addressed several questions. First, what is the presence or absence of cytokine patterns across human cancers? Second, which local cytokines critically influence patient’s survival? Third, can information about the spatiotemporal patterns of local cytokines be applied to find new therapeutics in different cancers? We found that cytokine reactions in human cancers were individually described, regardless of the cytokine (including chemokines) or tumor type. However, a systematic model to depict cytokines in tumors is still lacking. This comprehensive analysis of local cytokines in tumors provides a panoramic view of cytokine interactions across human cancers.

By assessing spatiotemporal dynamics of cytokines across human cancers, we found that 51.1% of locally dysregulated cytokines were cancer-specific. In addition, cytokines that showed stage specificity were surprisingly cancer type-specific, with ~70% of these stage-specific cytokines only being found in one cancer type. Furthermore, the tumor specificity score analysis also revealed striking cancer type-specific properties of cytokines. In agreement with previous study, our results suggested that cytokine profiles were associated with tumor histology, and therefore provided an insight that tissue context must be considered when assessing the tumor cytokinome. In a systematic review, Lippsitz et al. reported a consistent cytokine reaction pattern (including IL1, IL2, IL4, IL5, IL6, IL8, IL10, IL12, IL15, IL17, IL18, TNFα, TGFβ, IFNG, MIF, and CXCR4) in tumor patients, which was independent of the tumor histology across 307 published clinical studies. In this present study, we disclosed distinct cytokine reaction patterns across tumor histology types using a TCGA pan-cancer transcriptomic study of 261 cytokine and cytokine receptor genes. We therefore noted that these integrative approaches can provide new insights into the unique characteristics of tumor cytokine patterns.
insights into cytokinome patterns across human cancers. Furthermore, our data also provided evidence that nearly half of cytokines (48.9%) were found to be commonly dysregulated in at least two cancer types, exemplified by downregulation of CCL4 and CXCL12 in primary tumor tissues. These findings are consistent with Lipcott’s work that there were some common cytokine perturbation features that were independent of cancer types.

Since local cytokine levels (at least some specific cytokine patterns) were reported to be significantly associated with patients’ prognosis, we retrospectively conducted a cancer-specific and pan-cancer survival analysis to identify correlations between cytokine concentrations and the overall survival of cancer patients. Furthermore, we showed that cytokines prioritized by their prognostic potential were enriched in drug-related genes.

There are some limitations that may confine our results. First, the genetic predisposition factors that regulate cytokine expression need to be further elucidated. In addition, the interaction between cytokine expression, immune infiltrates, and cancer cells was not characterized, which requires future research to clarify it.

In summary, we present the strength of correlations and relationships between local cytokines and tumors in a synthetic and sophisticated fashion. These integrative works across human cancers may overcome challenges caused by complex interactions among cytokines. Our data highlights the power of systems biology approaches to delineate the pan-cancer cytokine landscape, and represent an improvement on characterization of coordination among local cytokines and tumor cells in human cancers. Our works also suggest the validity of using a pan-cancer systems biology approach to prioritize cytokines for drug development and cancer immunotherapy. In light of the fact that cytokines are important mediators that have been linked to cancer therapy, our results identify histologic contexts and local cytokine profiles as rational therapeutic targets for drug discovery.

Materials and methods

Defining the cytokinome gene set

We investigated 261 genes including cytokines (26 IFN family and IFN-related molecules, 92 IL family and IL-related molecules, 10 TGF superfamily and TGF-related molecules, seven CSF family and CSF-related molecules, 42 TNF family and TNF-related molecules, and 16 BMP family and BMP-related molecules) and chemokines (40 CC chemokines and related molecules, 21 CXC chemokines and related molecules, 2 C chemokines and related molecules, and 2 CX3C chemokines and related molecules) and miscellaneous cytokines (i.e., EPO, EPOR, TPO).

Studied tumor types

We selected 19 tumor types from TCGA for this study, including 18 solid tumor types (BLCA, BRCA, CESC, COAD, GBM, HNSC, KIRC, KIRP, LGG, LIHC, LUAD, LUSC, OV, PAAD, PRAD, READ, THCA, and UCEC) and one hematologic tumor type (diffuse large-B cell lymphoma (DLBCL)). In several downstream analyses, we included as many tumor types as possible, limited by the availability of different data types.

Clinical information

Clinical data for each tumor type were downloaded using the “DownloadClinicalData” function in the TCGA-Assembler.
package. We further extracted the age, gender, and overall survival time of 19 tumor types, pathological clinical stage (which was only available for 12 cancer types, including BLCA, BRCA, COAD, HNSC, KIRC, KIRP, LIHC, LUAD, LUSC, PAAD, READ, and THCA), and BRCA molecular subtype information for downstream analyses.

**RNA-sequencing-based messenger (m)RNA transcript data and preprocessing**

Expression data of mRNA (TCGA RNA-sequencing vers. 2) for 19 tumor types were downloaded and processed using the “DownloadRNASeqData” and “ProcessRNASeqData” functions implemented in the TCGA-Assembler package, including only primary tumor (TP) and normal adjacent tissue (NT) data. For mRNA transcript data, expression values were multiplied by 1e6 to compute the TPM value for downstream analyses. For each cancer type, we further applied two filters for downstream analyses: the first selected only cytokines that were annotated as PCGs according to the GENECODE V24 database; the second eliminated any transcript for which the TPM expression value was equal to 0 in the above top 20% of specimens. We further identified outliers by calculating signed, weighted biweight midcorrelations across samples in each tumor type, and arbitrarily excluded samples that were more than five standard deviations above the mean connectivity.

**Annotation of protein-coding genes (PCGs)**

GENECODE PCG annotation vers. 24 information was downloaded from the official website: http://www.gencodegenes.org/. The PCG was defined as a gene that contained protein annotation, resulting in 18,960 unique genes.

**Identification of differentially expressed genes across normal-tumor parts and pathological stage**

After quality control and sample sanitization, protein-coding cytokines and other PCGs were subjected to differentially expressed gene (DEG) identification. For each tumor type, we used a moderated t-test implemented in the limma package\(^3\)\(^5\) to identify significant genes. In detail, we identified locally dysregulated cytokines by comparing expression levels of protein-coding cytokines across primary tumors and tumor-adjacent normal tissues. To ensure the power of detection, we included 12 cancer types which had more than 15 available normal samples, including BLCA, BRCA, COAD, HNSC, KIRC, KIRP, LIHC, LUAD, LUSC, PRAD, THCA, and UCEC. We defined cytokines that met the criteria of an FDR of <0.05 and log-multiple of change of >1.5 as a locally dysregulated cytokine. On the other hand, we identified stage-specific cytokines by comparing early-stage primary tumors to late-stage primary tumors. The stages of primary tumors were defined using pathological tumor stage clinical information; we defined pathological stages I and II as early stage and stages III and IV as late stage. We included 12 tumor types (BLCA, BRCA, COAD, HNSC, KIRC, KIRP, LIHC, LUAD, LUSC, PAAD, READ, and THCA) for which pathological tumor stage information was available. In consideration that the log-multiples of change of cytokines were relatively small across these cancers, we defined cytokines with an FDR of <0.05 as stage-specific cytokines. The heatmaps of significant genes were visualized using the ComplexHeatmap package.

**Specificity score analysis**

We quantified similarities among a gene’s expression patterns across tissues using a specificity score adopted from Yan et al.\(^3\)\(^6\) The specificity score of each gene was calculated as follows. First, we calculated the average value (as measured in TPM) of a gene from all specimens in each tumor type or subtype. Then, the value of these average values was divided by the sum across tumor types or subtypes, which could be used to measure the propensity of gene expression in the various cancer types or subtypes. Based on the idea of a complete cancer type-specific or subtype-specific expression, we further constructed matrices that contained a value of 1 in corresponding tissue order, while all other values were 0 to represent perfect tissue-specificity. The number of matrices was equal to the number of tumor types or subtypes. Then, distances between a gene’s expression profile across tumor types or subtypes and the perfect tumor-specific matrices were calculated using the JS divergence equation, by adding 10~300 to each value while calculating Shannon entropy to prevent logging 0. Finally, the specificity score of each gene was calculated by the minimum value of (1–JS) values. The calculated specificity score ranged 0~1, with larger values representing a higher degree of specificity of expression in one or more tumor types or subtypes. Distributions of JS specificity scores of cytokines and other PCGs were compared using the Kolmogorov–Smirnov test.

**Unsupervised agglomerative hierarchical clustering analysis**

Clusters were identified using the following parameters: distmethod = “euclidean” and hclustmethod = “average,” and dendrograms were visualized in polar type using the ape package\(^3\)\(^7\).

**Survival analysis and meta-analysis**

For each cancer type, correlations between 226 cytokines (that were expressed in at least one cancer type) and patients’ overall survival were calculated using a Cox-proportional hazard model implemented in the survival and rms packages by introducing age and gender as covariates. Hazard ratios (HRs) were calculated from Cox-regression coefficients, and 95% confidence intervals (CIs) of the HRs were also estimated. We further conducted a meta-analysis via a linear model as implemented in the metafor package. A random effects model was chosen, as it accounted for effect size heterogeneity among cancer types. In addition, the restricted maximum likelihood method was used to predict the random effect variance component (REV) as it limited the α-error to around 5%.
Validation of prognostic potential of 261 cytokines in breast cancer

We collected 12 primary BRCA datasets (including GSE2034, GSE2603, GSE5327, GSE6532 (GPL96), GSE6532 (GPL570), GSE7390, GSE1121, GSE12276, GSE46928, GSE17705, GSE20685, and GSE25066), which comprised of 2,593 samples with distant metastasis annotation. All data sets were normalized using GCMA algorithm. For each dataset with distant metastasis-free survival (DMFS) available, we conducted Cox-proportional hazard model by adjusting cancer grade to calculate prognostic significance of 261 cytokines. For each data set without DMFS data available, we conducted logistic regression test by adjusting cancer grade to assess the prognostic significance of 261 cytokines. The effect sizes of 261 cytokines were further meta-analyzed using random effects model to obtain combined statistics (effect size, 95% confidence intervals, and p value) of prognostic ability of each cytokine.

Drug-related target enrichment analysis

Information on drug target genes was obtained from the DrugBank database12 and TTD.13 We selected drug target genes based on several criteria: related to or targeted by approved drugs, and approved indications based on human orthologues. We further extracted cancer-related drugs manually by indications and combined data from the DrugBank and TTD using in-house R script. To assess the significance of enrichment of a defined gene set in the drug-related gene set, we conducted an enrichment analysis using a method proposed by Okada et al. Briefly, the FoE of overlapping defined gene sets to gene targets by drugs approved for cancer indications and all drug target genes was assessed by a 10,000-permutation procedure.

Statistical analysis

We used R (http://www.r-project.org/) and Bioconductor (http://www.bioconductor.org/) to perform all statistical analyses.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

H.S.C.W and W.C.C designed the experiments. H.S.C.W analyzed the data. H.S.C.W, W.C., and W.C.H wrote the manuscript. H.S.C.W, C.M.C, W.C.H, X.L, and W.C.C contributed to the manuscript preparation. W.C.C and W.C.H supervised the project.

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