Journal of Gastrointestinal & Digestive System

Zhou X, et al. J Gastrointest Dig Syst 2021, 12:4

Research Article Open Access

The Impact of Genetic Mutation and Cytokines/Chemokines on Immune Response in Colorectal Cancer

Xinke Zhou[1], Jiachun Lu[2], Shenying Fang[3*]

[1]*The Institute for Chemical Carcinogenesis, Collaborative Innovation Center for Environmental Toxicity, Guangzhou Medical University, Guangzhou, China*
[2]*Department of Public Health and Emergency Management, Southern University of Science and Technology, Shenzhen, China*
[3]*Department of Public Health, Guangzhou Medical University, Guangzhou, China*

Abstract

Background: Systematically exploring the effect of tumor mutation and cytokines/chemokines on colon adenocarcinoma (COAD) immune response and outcome is very important and worth investigation.

Methods: We first estimated immune cell composition in 458 COAD tumors from TCGA, and then evaluated association between genetic mutation, expression of cytokine and chemokine, and immune cell subsets. Finally, we evaluated relationship between immune cell subsets, chemokines/cytokines, and patient survival.

Results: Compared to wild-type, samples with mutated tumor suppressor genes APC or TP53 had significantly lower CD8+, Neutrophil, DC, and NK cells infiltrates, while samples with mutated tumor promoter genes TTN, MUC16, or BRAF had significantly higher immune cell infiltrates. Gene expression of IFNG, TGFB1, TNF, IL6, IL10, CX3CL1, CXCL9, CXCL10 were all positively correlated with immune cell infiltrates, and inversely correlated with purity ($P<0.05$ after Bonferroni correction) in tumor specimens. In survival analysis, none of these chemokines or cytokines or CD8+ was significantly associated with overall survival (OS). Both increased CD4+ T and B cell subsets were associated with poorer OS in univariate analysis and in multivariable analysis after adjustment for age, sex, AJCC stage, and tumor purity (multivariable, CD4+: HR=23.49, 95% CI=1.55-356.92, P=0.023; B cell: HR=135.38, 95% CI=5.27-3480.28, P=0.003).

Conclusion: Our results suggest that genetic mutation and chemokines/cytokines were correlated with infiltration of immune killer cells and that the mutation status and inflammation biomarker expression levels could be used to select patients for immunotherapy and predict disease outcome.

Lay summary: Proportions of immune cell subsets were estimated in 458 COAD tumors from TCGA and the relationship between immune cell subsets, chemokines, and cytokines and patient survival was systematically assessed. Our study revealed significant biomarkers for tumor immune response and CRC progression.

Keywords: COAD; Genetic mutations; Cytokines/chemokines; Immune response; Outcomes

Introduction

Colon cancer is the third most common cancer with a high mortality worldwide. The prognosis of advanced patients is still very poor. The process of tumor development and progression is determined by two factors, genetic/epigenetic changes in the tumor cells and the interactions between the cell elements in the tumor microenvironment (TME). TME consists of different types of cells, including tumor, stromal, immune, and endothelial cells. Tumor-infiltrating lymphocytes (TILs) play a key role in anti-tumor immunity and therapy elicited response in patients with solid tumors including colon cancer and other cancers [1-4]. RNA sequencing (RNA-seq) deconvolution procedures can estimate cellular fractions and functions of infiltrating immune cells in TME and can help to evaluate their roles in patient progression [5-8].

Genetics has a key role in predisposition to colon adenocarcinoma (COAD) and in its initiation and progression. Neoantigens generated by somatic mutations in tumor cells can be recognized by host CD8+ and CD4+ T cells. Previous experimental studies used identified antigens in COAD cell lines to successfully induce downstream immune reactions [9,10]. High tumor mutation burden is an emerging biomarker for response to immunotherapy in several types of cancer [11,12]. In 2017, FDA approved pembrolizumab as the immune checkpoint therapy for a high mutation load COAD with DNA mismatch repair deficient or with elevated microsatellite instability. However, for those COAD tumors with low mutation load that had low response to immune checkpoint therapy, further evaluating the roles of germline and somatic mutation as potential determinants of immunogenicity in these subsets is essential.

Cytokines and chemokines play critical roles in regulating innate and adaptive immune responses and cell-cell interactions. Tumor neoantigens are recognized as foreigners to induce anti-tumor responses such as higher TIL density and increased expression of type II interferon (IFN-γ)(IFNG) related genes, for example PD-L1 and CTLA-4. A clinical study show that increased tumor IFNG gene expression predicts a better clinical outcome among multiple tumor types [13]. Furthermore, TGF-β led to enhanced activin secretion and a higher combined activin/TGF-β ligand expression score was associated with a shorter disease free survival in patients with

***Corresponding author:** Shenying Fang, Department of Public Health, Guangzhou Medical University, Guangzhou, China, E-mail: shenyingfang@hotmail.com

Citation: Zhou X, Lu J, Fang S (2022) The Impact of Genetic Mutation and Cytokines/Chemokines on Immune Response in Colorectal Cancer. J Gastrointest Dig Syst.12:681

Received: 23-March-2022, Manuscript No. JGDS-22-58147; **Editor assigned:** 25-March-2022, PreQC No. JGDS-22-58147(PQ); **Reviewed:** 08-April-2022, QC No. JGDS-22-58147; **Revised:** 13-April-2022, Manuscript No. JGDS-22-58147(R); **Published:** 20-April-2022, **DOI:** 10.4172/2161-069X.1000681

stage II CRC [14]. In the development of COAD cancer, increased levels of IL-6 induce hyperactivation of JAK/STAT3 signaling and often predict poorer patient outcomes [15]. In the TME, IL-6 and JAK/STAT3 signaling negatively regulate maturation and activation of effector T cells, NK cells, neutrophils, and dendritic cells (DCs), and promote a highly immunosuppressive TME in COAD [16-18].

Chemokines coordinate the migration of immune cells into the tumor. Several studies show that increased levels of CX3CL1, CXCL9 or CXCL10 are associated with higher tumor infiltrating CD8+ T cells, a lower tendency to metastasize and improved survival in colon cancer [19-22]. In addition, IFNs could stimulate the production of these chemokines in colon cancer and the released chemokine demonstrates anti-tumor effect in mouse models. An elevated level of these chemokines predicts better post-operative survival in colon cancer patients [23]. Therefore, documenting polarization in the cytokine repertoire or revealing distinct patterns of their production by immune cells in the COAD TME and relating cytokines/chemokines to patient outcomes is important because it would enable us to completely understand the mechanisms by which the host reacts against tumor cells.

In the current study, we used three approaches-Tumor Immune Estimation Resource (TIMER 2.0) [8], Estimating the Proportions of Immune and Cancer cells (EPIC) [6] and Cell type Identification By Estimating Relative Subsets Of known RNA Transcripts-Absolute (CIBERSORT-ABS)[7], to assess cell type compositions based on COAD RNA-seq data downloaded from The Cancer Genome Atlas (TCGA); we then investigated the associations of genetic mutations and cytokine/chemokine expression with immune cell fractions, and with patient outcomes.

Materials and Methods

TCGA dataset patients

We applied the TCGA dataset to estimate cellular fractions and discover biomarkers of immune response and COAD patient outcome. We downloaded demographic, clinical variables, RNA-sequencing data, and follow up data of 458 TCGA COAD patients (https://portal.gdc.cancer.gov/projects/TCGA-COAD). Clinical prognostic variables included 2009 American Joint Committee on Cancer stage (AJCC), grade, Breslow tumor thickness, gross tumor weight, greatest tumor dimension, lymph node site, lymph node positive status, mitosis, and tumor location. Complete clinical data were available for 458 patients: 243 male, 214 white, 254 stage I/II, 194 stage III/IV, and 11 with unknown stage. The median age at diagnosis was 67 years. Complete follow up data was only available for 277 patients when merged with mRNA sequence data; the median follow up time was 82 months, and 67 patients died during the follow up period. mRNA gene expression level was measured using the IlluminaHiSeq_RNASeqV2 platform.

Deconvolution analysis

We employed the widely validated deconvolution approach TIMER2.0 [8] to estimate fractions of immune cell subsets (CD8+ T cells, B cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells) based on RNA-seq data from bulk tumor tissue in TCGA samples. TIMER 2.0 has the built in gene expression signature matrix for six major immune cell types, which are generally used to assess the relative fractions of each cell type. Tissue specificity is carefully considered when estimating immune cell subsets in TIMER 2.0. We also applied EPIC [6] to estimate NK and cancer associated fibroblast cells and CIBERSORT-ABS [7] to estimate Tregs because those cell subsets only account for minor proportions. The deconvolution of cell fractions can be readily solved through a heuristic algorithm. Data measurements were first median centered and unit-normalized to change the variables into the same scale.

Statistical analysis

We first chose top 15 mutated genes APC, TP53, TTN, KRAS, PIK3CA, SYNE1, MUC16, CSMD3, FBXW7, ATM, BRAF, SMAD4, MUC4, PTEN, KIT for COAD [4] in 406 TCGA tumor samples and compared immune cell infiltrates between mutated and wild-type samples using nonparametric Wilcoxon test. We then chose several cytokines and chemokines that demonstrated association with tumor immune response or disease outcomes in previous studies, and assessed the association of these cytokines/chemokines with T-cell subsets and OS [13,22,24-27]. The relationship between expression levels of cytokine/chemokine and immune response was analyzed using Spearman correlation. Clinical variables and follow up data available from TCGA are shown above.

We performed the following survival analyses using TCGA data, for which only OS outcome was available. We first ran a univariate analysis to evaluate the effect of CD8+ T cell subset on survival using a Cox proportional hazards model. We then ran a multivariable analysis with adjustment for age at diagnosis, sex, race, AJCC stage, and tumor purity (the percentage of malignant cells in a tumor tissue specimen). Data analyses were run using R tools and the SAS Enterprise Guide 4.3 software program (SAS Institute Inc.). A two sided $P<0.05$ was considered statistically significant [28]. When considering the relationship between multiple chemokine and cytokine related genes and immune response or survival, P-values were adjusted for multiple comparisons using Bonferroni correction ($P<0.0042$ [=0.05/12] was considered significant).

Results

Immune cell fraction of COAD tumors

We evaluated RNA-seq data using TIMER 2.0 deconvolution to estimate the relative proportions of six major immune cell types in patients whose data are available in TCGA [8]. We also applied EPIC [6] to estimate NK and cancer associated fibroblast cells and CIBERSORT-ABS [7] to estimate Tregs because those cell subsets only account for minor proportions in the tumors. These approaches estimate the fractions of the cell types represented in the sample based on an input of reference gene expression signature matrix for each cell type. Our previous study demonstrated that immune cell subsets estimated through deconvolution approaches based on mRNA gene expression in TCGA melanoma are generally consistent with TIL scores determined by pathologists [29]. We further estimated immune cell subsets in TCGA COAD tumors in this study.

Tumor mutations related to immune cell infiltrates in COAD tumors

We selected top 15 mutated genes APC(70%), TP53(53%), TTN(51%), KRAS(43%), PIK3CA(32%), SYNE1(30%), MUC16(29%), CSMD3(18%), FBXW7(17%), ATM(14%), BRAF(14%), SMAD4(12%), MUC4(8%), PTEN(6%), KIT(5%) for COAD in 406 TCGA tumor samples and compared immune cell infiltrates between mutated and wild-type samples (Table 1). Samples with mutated tumor suppressor genes APC or TP53 had significantly lower immune scores, which includes CD8+, Neutrophil, DC, and NK that demonstrate function of cytotoxic T cell effect, antigen presentation, or cytotoxic effector lymphocytes, than wild-type tumors (Table 1, Figure S1); While samples with mutated tumor oncogenes TTN, MUC16, and BRAF had significantly higher immune scores than wild-type samples (Table 1, Figure S2); Samples with mutated genes such as CSMD3, SYNE1 and MUC4 had significantly higher one or more immune scores than wild-type samples(Table 1). Our data suggest that mutated tumor suppressor genes promote tumor immunosuppressive activity while mutated

oncogenes contribute to antitumor immune response. These mutated genes may be important biomarkers for tumor progression.

Chemokines/cytokines related to immune cell infiltrates in COAD tumors

We applied TIMER 2.0, EPIC and CIBERSORT-ABS deconvolution tools to estimate immune cell proportions and tumor purity based on RNA-seq

infiltrates	APC(mutated n=286)			TP53(mutated n=216)			TTN(mutated n=207)			KRAS(mutated n=174)		
	log2FC(Mutated/Wild)	p	adj.p	log2FC(Mutated/Wild)	p	adj.p	log2FC(Mutated/Wild)	p	adj.p	log2FC(Mutated/Wild)	p	adj.p
T cell CD8+_TIMER	-0.362034196	0.000732964	0.036543499	-0.133364512	0.075708612	0.289833979	0.255684781	0.001348312	0.043707779	-0.060916297	0.656740297	0.865703119
T cell CD4+_TIMER	-0.188952851	0.53517918	0.930530153	0.061777697	0.57129971	0.825363284	0.099183781	0.093355297	0.453545023	-0.087931747	0.82601536	0.963742483
B cell_TIMER	-0.498784644	0.001508677	0.263515627	-0.494472435	1.94E-05	0.000524711	0.089923945	0.310041945	0.788734871	-0.119652929	0.70069594	0.863152216
Macrophage_TIMER	-0.435130972	0.196181902	0.727681808	0.289000827	0.20835934	0.459777294	0.218942449	0.344572086	0.675886646	-0.410582862	0.06792074	0.374305793
Neutrophil_TIMER	-0.351072434	1.57E-05	0.003271979	-0.081931391	0.0937461	0.347321617	0.370302473	6.28E-06	0.001457548	-0.13832713	0.042298957	0.324081009
Myeloid dendritic cell_TIMER	-0.309708852	9.65E-06	0.002416206	-0.128536755	0.003945176	0.043977113	0.263905725	2.27E-06	0.000542758	-0.097088014	0.062894261	0.501581731
NK cell_EPIC	-1.897606437	0.000649749	0.045482462	-0.812336562	0.79122816	0.972448927	2.917046669	1.07E-05	0.001666583	-0.873366702	0.050809225	0.591234619
T cell regulatory (Tregs)_CIBERSORT-ABS	-0.374652343	0.21334308	0.756820951	-0.023123254	0.878709862	0.96089136	-0.000458455	0.45616116	0.910848778	-0.112744658	0.442608862	0.71713841
Cancer associated fibroblast_EPIC	-0.199800275	0.028016032	0.702430383	0.127298505	0.475868661	0.718561678	0.301034699	0.113390978	0.614917915	-0.018273315	0.505706188	0.892468317

infiltrates	PIK3CA(mutated n=128)			SYNE1(mutated n=121)			MUC16(mutated n=118)			CSMD3(mutated n=74)		
	log2FC(Mutated/Wild)	p	adj.p	log2FC(Mutated/Wild)	p	adj.p	log2FC(Mutated/Wild)	p	adj.p	log2FC(Mutated/Wild)	p	adj.p
T cell CD8+_TIMER	-0.043754015	0.858786558	0.924108371	0.255545074	0.017314451	0.373767246	0.339765619	0.002763784	0.094689839	0.181204794	0.016511933	0.42341314
T cell CD4+_TIMER	-0.055525769	0.79757987	0.985938958	0.046345861	0.743576225	0.97101168	0.247555574	0.012847124	0.220185427	-0.036068314	0.585899903	0.891463891
B cell_TIMER	0.144104289	0.054703975	0.619701504	0.133410759	0.68095179	0.908221673	0.321174876	0.491528873	0.997706811	-0.085637469	0.676203733	0.931243318
Macrophage_TIMER	-0.237053119	0.137506341	0.606704324	0.02955087	0.292346822	0.775948573	0.505728792	0.310310604	0.796486224	-0.033909506	0.175685201	0.725272231
Neutrophil_TIMER	0.085107442	0.377668478	0.867381337	0.311770152	5.80E-05	0.004370737	0.433816365	9.64E-07	0.000223585	0.381016079	6.73E-05	0.014400349
Myeloid dendritic cell_TIMER	0.074809253	0.169184879	0.651347391	0.182107685	0.003047914	0.143632942	0.266016701	3.31E-05	0.006404112	0.173589644	0.000898984	0.205566301
NK cell_EPIC	0.165167943	0.254258316	0.906264874	2.022280612	0.0010691	0.10833551	2.178173697	0.000149578	0.02333415	1.801734774	1.32E-05	0.003803888
T cell regulatory (Tregs)_CIBERSORT-ABS	0.240771656	0.071117683	0.451608907	-0.26658757	0.049483723	0.376400225	0.041848795	0.204015857	0.710906403	-0.504129317	0.061533469	0.632915682
Cancer associated fibroblast_EPIC	0.232350133	0.307330997	0.802342359	0.084202971	0.214815544	0.91256397	0.489666512	0.038267622	0.384433901	0.425793508	0.074194252	0.547974448

infiltrates	FBXW7(mutated n=67)			ATM(mutated n=56)			BRAF(mutated n=57)			SMAD4(mutated n=48)		
	log2FC(Mutated/Wild)	p	adj.p	log2FC(Mutated/Wild)	p	adj.p	log2FC(Mutated/Wild)	p	adj.p	log2FC(Mutated/Wild)	p	adj.p
T cell CD8+_TIMER	0.194254459	0.166644647	0.526485549	0.208446029	0.143291337	0.630650436	0.525204171	1.75E-05	0.00112104	0.249928502	0.030349828	0.650472875
T cell CD4+_TIMER	0.056671055	0.569479777	0.973445827	-0.073365639	0.336942527	0.877239889	0.199994184	0.415804561	0.873171705	0.144157174	0.05503314	0.833313193
B cell_TIMER	0.173685629	0.395258014	0.765666256	0.082882879	0.844774582	0.981530808	0.513131195	0.058621038	0.574246899	0.192400707	0.041393471	0.765936543
Macrophage_TIMER	-0.085562317	0.104168975	0.533548406	-0.252298335	0.53280286	0.881603406	0.166893044	0.543275598	0.852458164	0.230566535	0.129201674	0.752479817
Neutrophil_TIMER	0.33191966	0.00310371	0.10299188	0.304593192	0.000810201	0.057794367	0.65933089	1.10E-12	1.05E-10	0.102982659	0.099930561	0.577762938

Myeloid dendritic cell_TIMER	0.180946181	0.008527906	0.215523441	0.202528578	0.01046795	0.289076472	0.488521471	1.08E-08	4.92E-07	0.119136185	0.066703412	0.785281074
NK cell_EPIC	1.621902093	0.020230395	0.469425978	2.004444759	0.001105217	0.277039587	3.03449155	2.01E-07	1.71E-05	1.109534942	0.309605867	0.904572894
T cell regulatory (Tregs)_CIBERSORT-ABS	0.123376999	0.72002268	0.926642319	-0.289550309	0.115459223	0.587338472	0.447329901	0.510308218	0.816493149	-0.327211956	0.191497518	0.578266392
Cancer associated fibroblast_EPIC	0.115597525	0.899361379	0.979673156	-0.102649393	0.98562001	1	0.105023936	0.146607354	0.769483493	-0.071194645	0.898082018	0.938407549

infiltrates	MUC4(mutated n=32)			PTEN(mutated n=24)			KIT(mutated n=21)			TGFBR2(mutated n=18)		
	log2FC(Mutated/Wild)	p	adj.p	log2FC(Mutated/Wild)	p	adj.p	log2FC(Mutated/Wild)	p	adj.p	log2FC(Mutated/Wild)	p	adj.p
T cell CD8+_TIMER	0.355914318	0.016535124	0.373696527	0.059673921	0.387764399	0.914691574	0.229819551	0.085716668	0.466028279	-0.054053027	0.967633341	0.994289133
T cell CD4+_TIMER	0.084902884	0.668289024	0.958927201	-0.048628625	0.774819694	0.976425943	-0.195492049	0.484444119	0.864398632	0.04380454	0.344276675	0.858966887
B cell_TIMER	0.423691904	0.030211212	0.824603704	-0.056558203	0.993477939	1	-0.105958241	0.468241856	0.8947244	0.210082434	0.06506219	0.797021093
Macrophage_TIMER	0.198578541	0.276483446	0.745014245	-0.162106287	0.551003023	0.884553347	-0.235325637	0.501459631	0.811024963	0.297426054	0.940246646	0.99598644
Neutrophil_TIMER	0.511530945	0.000181411	0.021761463	0.224296257	0.099655109	0.514435834	0.348688419	0.008553766	0.338382301	0.12376846	0.142658417	0.903929021
Myeloid dendritic cell_TIMER	0.368077546	0.00182615	0.19984709	0.17092632	0.189412794	0.645287339	0.285201735	0.008700848	0.153032565	0.16844723	0.179884262	0.701708984
NK cell_EPIC	2.807536823	0.000102961	0.009609687	1.372131079	0.019358211	0.309731377	1.941173767	0.002780049	0.19569358	0.776073267	0.035857211	0.343607109
T cell regulatory (Tregs)_CIBERSORT-ABS	-0.39495076	0.021994248	0.864197531	-0.507070888	0.332911443	0.677549441	-0.470467518	0.163006362	0.889125609	0.511383638	0.063000052	0.625997312
Cancer associated fibroblast_EPIC	0.411751893	0.188808617	0.686258271	0.298542319	0.350975138	0.782619112	0.287829495	0.452416144	0.874838639	0.353225756	0.260715128	0.980263189

Table 1: Association between tumor mutation and immune cells among 404 COAD samples.

data for the COAD patients in TCGA[6-8]. We then evaluated correlations of chemokine and cytokine gene expression with T cell abundance and tumor purity (Table 2). We first focused mainly on the cytotoxic T lymphocytes because these cells can kill cancer cells directly and because the presence of cytotoxic T lymphocytes in TME associates with favorable clinical outcomes across multiple tumors [1-4]. We found that the CD8+ T cell subset was associated with both PDCD1 and CTLA4 gene expression levels (PDCD1: r^2=0.53, P=1.43E-21; CTLA4: r^2=0.42, P=5.96E-13; Table 2), Dendritic cell (PDCD1: r^2=0.64, P=1.62E-32; CTLA4: r^2=0.69, P=7.28E-40), Neutrophil cell (PDCD1: r^2=0.65, P=2.40E-34; CTLA4: r^2=0.71, P=5.96E-43), and NK cell (PDCD1: r^2=0.52, P=4.38E-20; CTLA4: r^2=0.45, P=2.62E-15) (Table 3) in COAD. Except for IL12A and IL23A, gene expression of chemokines and cytokines shown in Tables 2 and 3 was positively correlated with PDCD1 expression, CTLA4 expression, CD8+ T cell, Neutrophil cell, Dendritic cell, and NK cell in the tumor specimens, and inversely correlated with tumor purity (Bonferroni corrected P<0.05, Tables 2 and 3) in COAD patients. It is interesting to note that those chemokines and cytokines were also significantly associated with CAF, which was a negative tumor prognosis factor. However, those chemokines/cytokines were weakly associated with B cell, CD4+ cell, macrophages (r^2<0.5) or didn't reach statistical significance (P>0.05 after Bonferroni correction, Table 3). Our data indicate that these cytokines and chemokines play key roles in tumor immune response.

Mutations, immune response or inflammation that predicted COAD patient survival

We then evaluated the association between mutations, chemokines, cytokines, and immune cell subsets, and disease outcome, adjusting for clinical

Gene	PDCD1[1]		CTLA4[1]		Tumor purity[1]		CD8+ T cell subset[2]		Overall survival[3]		
	r^2	P	r^2	P	r^2	P	r^2	P	HR[4]	95% CI[4]	P
PDCD1	-	-	0.75	6.98E-65	-0.38	9.62E-16	0.53	1.43E-21	1.16	0.89-1.54	2.57E-01
CTLA4	0.75	6.98E-65	-	-	-0.4	3.31E-17	0.42	5.96E-13	0.88	0.66-1.16	3.63E-01
IFNG	0.66	2.07E-59	0.63	5.97E-53	-0.24	8.65E-07	0.49	2.69E-18	1.11	0.77-1.61	5.85E-01
TGFB1	0.59	9.38E-45	0.58	8.97E-42	-0.44	3.01E-20	0.36	1.12E-09	1.17	0.97-1.40	9.80E-02
TNF	0.3	6.83E-11	0.49	2.43E-29	-0.26	1.86E-07	0.3	3.66E-07	0.93	0.64-1.33	6.73E-01
IL6	0.41	1.43E-19	0.46	9.54E-25	-0.22	8.70E-06	0.22	3.09E-04	1.07	0.93-1.23	3.70E-01
IL10	0.54	7.17E-36	0.6	6.76E-47	-0.3	4.32E-10	0.4	9.85E-12	0.93	0.56-1.55	7.71E-01
IL12A	0.1	2.86E-02	0.16	4.79E-04	-0.1	4.82E-02	0.2	1.08E-03	1.18	0.82-1.71	3.71E-01
IL23A	0.15	1.43E-03	0.21	4.80E-06	-0.02	6.43E-01	-0.12	3.79E-02	0.92	0.76-1.12	4.12E-01

CX3CL1	0.49	1.58E-28	0.42	4.01E-21	-0.28	7.44E-09	0.35	3.64E-09	1.22	1.03-1.45	2.00E-02
CXCL9	0.71	9.98E-72	0.7	1.67E-69	-0.34	9.48E-13	0.5	6.20E-19	0.98	0.87-1.10	7.05E-01
CXCL10	0.65	2.23E-65	0.64	7.05E-55	-0.3	6.17E-10	0.47	8.58E-17	0.96	0.85-1.07	4.45E-01

[1]Spearman correlation test.

[2]Purity-corrected partial Spearman correlation.

[3]Univariate analysis.

[4]HR: hazards ratio; CI: confidence interval

Table 2: Relationship between cytokine or chemokine gene expression levels and tumor immune response or overall survival in 458 patients with CRC whose sequencing data were available in The Cancer Genome Atlas.

Gene	B cell subset[1]		CD4+ T cell[1]		Dendritic cell[1]		Macrophage[1]		Neutrophil[1]		NK cell[1,2]		Tregs[1,3]		CAF[1,2,4]	
	r^2	P	r^2	P	r^2	P	r^2	P	r^2	P	r^2	P	r^2	P	r^2	P
PDCD1	0.04	5.05E-01	0.22	2.02E-04	0.64	1.62E-32	0.19	1.51E-03	0.65	2.40E-34	0.52	4.38E-20	0.22	2.74E-04	0.41	7.74E-13
CTLA4	-0.05	4.35E-01	0.3	3.00E-07	0.69	7.28E-40	0.24	6.28E-05	0.71	1.04E-43	0.45	2.62E-15	0.32	9.45E-08	0.49	2.40E-18
IFNG	-0.02	7.09E-01	0.02	7.63E-01	0.52	1.19E-20	0.06	3.39E-01	0.58	9.02E-26	0.52	1.50E-20	0.04	5.20E-01	0.26	1.64E-05
TGFB1	-0.18	3.11E-03	0.35	2.48E-09	0.73	2.26E-46	0.5	1.36E-18	0.65	2.54E-34	0.54	1.12E-22	0.38	7.90E-11	0.79	4.16E-60
TNF	-0.14	1.77E-02	0.17	6.18E-03	0.54	2.17E-22	0.19	1.80E-03	0.61	1.25E-29	0.48	6.65E-17	0.21	5.12E-04	0.45	4.57E-15
IL6	-0.27	8.02E-06	0.06	3.94E-01	0.46	1.00E-15	0.14	1.70E-02	0.55	7.68E-23	0.29	8.87E-07	0.33	2.75E-08	0.48	1.20E-17
IL10	-0.21	5.37E-04	0.21	3.88E-04	0.7	9.39E-42	0.42	7.43E-13	0.74	5.13E-48	0.33	1.75E-08	0.08	2.16E-01	0.59	6.66E-27
IL23A	-0.12	5.15E-02	0.01	8.27E-01	0.2	9.77E-04	0.03	6.63E-01	0.27	4.84E-06	0.09	1.65E-01	0.23	1.61E-04	0.19	1.21E-03
IL12A	0.04	5.03E-01	0.16	8.96E-03	0.02	7.16E-01	-0.03	6.01E-01	-0.01	8.99E-01	0.01	8.23E-01	0.05	3.92E-01	-0.05	4.54E-01
CX3CL1	0.03	6.68E-01	0.26	1.10E-05	0.46	8.42E-16	0.27	6.57E-06	0.35	3.55E-09	0.31	9.93E-08	0.31	1.83E-07	0.45	5.21E-15
CXCL9	-0.07	2.68E-01	0.12	4.78E-02	0.65	1.02E-34	0.28	1.70E-06	0.67	1.94E-36	0.6	1.20E-28	0.14	1.98E-02	0.49	1.08E-17
CXCL10	-0.04	4.97E-01	0.08	1.82E-01	0.61	1.57E-29	0.21	4.30E-04	0.64	2.42E-33	0.61	1.81E-29	0.14	2.56E-02	0.45	3.20E-15

[1]Purity-corrected partial Spearman correlation.

[2]Estimated with EPIC, without adjustment for tumor purity

[3]Estimated with cibersort-ass

[4]CAF: cancer associated fibroblast

Table 3: Relationship between cytokine or chemokine gene expression levels and other immune cells estimated with TIMER in 458 patients with CRC whose sequencing data were available in The Cancer Genome Atlas.

covariates.

Genetic mutations

We assessed if APC, TP53, TTN, MUC16, or BRAF gene mutation had any effect on OS. We found that none of those gene mutations was significantly associated with OS in TCGA COAD samples (Figure S3).

Cytokines/chemokines

We discovered that immune response–related chemokines/cytokines were not corrected with OS in the COAD TCGA. Almost none of those factors significantly predicted OS (Bonferroni corrected P>0.05; Table 2). The non-significant results may be due to small sample size for survival outcome (only 277 patients with 67 dying available, 181 missing observations).

Inflammation or Immune cells

In TCGA patients, we detected that both elevated CD4+ T and B cell subsets were associated with poorer OS in a univariate analysis (CD4+: HR=11.10, 95% CI=1.13-109.14, P=0.039; B cell: HR=21.65, 95% CI=1.65-283.53, P=0.019; Tables 4 and 5). Both the CD4+ T and B cell subsets were still significant after adjustment for sex, age, stage, and tumor purity (CD4+: HR=23.49, 95% CI=1.55-356.92, P=0.023; B cell: HR=135.38, 95% CI=5.27-3480.28, P=0.003; Tables 4 and 5). However, we discovered that a CD8+ T cell population was not correlated with OS in univariate analysis (HR=1.60, 95% CI=0.23-11.44, P=0.638; Table S1) or in the multivariable analysis after adjustment for sex, age, stage, and tumor purity (HR=0.67, 95% CI=0.07-65, P=0.713; Table S1).

These results suggest that inflammation (CD4+ and B cell) in COAD tumor could negatively affect patient outcome, while tumor lymphocyte infiltration of tumor cell killer CD8+ was not found to be significantly associated with COAD patient outcome (Tables S2-S5).

Discussion

In the current investigation, we assessed immune cell populations in the COAD patients of TCGA using the TIMER 2.0 and other two algorithms based on RNA-seq data. We first discovered that compared to samples with wild-type genes, samples with mutated tumor suppressor genes had significantly lower immune scores, while samples with mutated tumor oncogenes had significantly higher immune scores. We found that expression of several chemokines/cytokines was correlated with immune response, as represented by the CD8+ T cell subset. We then detected an association of OS with inflammation B cell and CD4+ cell, but not with CD8+ cell or any effect T cell related chemokines/cytokines. These results show that systematic evaluation of genetic mutation and chemokines/cytokines contributing to lymphocyte infiltration may help select those COAD patients who may benefit from immune therapy and possibly suggest novel

Variable	Univariate: CD4+ T cell subset			Multivariable: CD4+ T cell subset		
	HR	95% CI	P	HR	95% CI	P
CD4+ T cell subset	21.65	1.65-283.53	**0.019**	23.49	1.55-356.92	**0.023**
Purity	-	-	-	1.01	0.26-3.94	0.987
Age	-	-	-	1.03	1.01-1.06	**0.013**
Male sex	-	-	-	1.42	0.80-2.49	0.228
White race	-	-	-	0.56	0.12-2.62	0.457
Stage 2	-	-	-	1.54	0.44-5.36	0.503
Stage 3	-	-	-	2.73	0.80-9.36	0.11
Stage 4	-	-	-	10.06	0.91-34.70	**<0.001**

[1]HR: hazards ratio; CI: confidence interval. Boldface type indicates statistical significance.

Table 4: CD4+T cell and other clinical variables associated with overall survival in 458 patients with CRC whose sequencing data were available in The Cancer Genome Atlas.[1]

Variable	Univariate: B cell subset			Multivariable: B cell subset		
	HR	95% CI	P	HR	95% CI	P
B cell subset	11.1	1.13-109.14	**0.039**	135.38	5.27-3480.28	**0.003**
Purity	-	-	-	0.81	0.22-2.96	0.749
Age	-	-	-	1.03	1.01-1.06	**0.012**
Male sex	-	-	-	1.34	0.76-2.35	0.31
White race	-	-	-	0.7	0.15-3.22	0.647
Stage 2	-	-	-	2.41	0.65-9.04	0.191
Stage 3	-	-	-	4.31	1.17-15.86	**0.028**
Stage 4	-	-	-	15.75	4.13-60.13	**<0.001**

[1]HR: hazards ratio; CI: confidence interval. Boldface type indicates statistical significance.

Table 5: B cell and other clinical variables associated with overall survival in 458 patients with CRC whose sequencing data were available in The Cancer Genome Atlas.[1"]

immunotherapeutic methods.

APC is a gene that suppresses tumor growth. It encodes protein that participates in several biological processes, such as cellular signaling, cell adhesion and migration, proliferation, differentiation, and apoptosis. Disruption of APC function leads to the uncontrolled activation of the WNT/β-catenin signal transduction pathway [30]. Germline mutation in APC gene accounts for the familial adenomatous polyposis and is also responsible for the majority of sporadic colorectal cancers [31]. We observed that mutated APC samples had lower immune scores than wild-type APC samples. However, the role of mutated APC in immunosuppressive activity remains unclear. APC gene mutation could possibly make tumor acquire selective advantage toward clonal expansion, and genetic instability that has been identified in several other genes results in colon and rectal cancers [30]. A previous study demonstrated that TP53 mutations are associated with less immune cell infiltration and decreased immunosuppressive activity in gastric cancer, colon cancer, and other cancer types. TP53 can activate tumor immunity in colon cancer and other tumor types. Mutated TP53 causes chromosomal instability and therefore depresses tumor immunity in TP53-mutated cancers [32]. The results from the above study are consistent with our findings that TP53 mutated tumors had lower immune scores than wild-type tumors. Our results suggest that TP53 may be involved in activating tumor immunity in colon cancer and TP53 mutation status could be used to select cancer patients who respond to a certain immunotherapy.

Somatic mutations in the tumor genome can cause tumors to express mutant peptides that are tumor specific and treated as foreigners or neoantigens that are recognized by the immune system. These neoantigens can be targeted to improve antitumor immunity for personalized cancer immunotherapy among patients who otherwise benefit little from conventional chemotherapy or radiotherapy. Our study demonstrated that tumors with mutated TTN, MUC16, and BRAF genes had higher immune score than the wild-type groups. The roles of mutated MUC16 and BRAF genes in activating tumor immunity were supported by previous evidence showing that the mutations of genes BRAF, PCLO, MUC16, and etc contributed a major part to elevated fraction of immune cells by promoting immune related gene expression in colon cancer [33]. The count of mutations in TTN gene is representative of TMB within a variety of tumor types and is used to predict the MSI status in colon cancer [34]. MUC16 plays key roles in inhibiting antitumor immune responses and MUC16 mutations possibly nullify its inhibitory immune effects [35]. Previous studies show that patients with MUC16 mutations had a higher mutational load in several cancer types [36,37]. It is also possible that the high mutation burden might boost antitumor immune responses.

Evaluating the role of modulatory chemokines and cytokines in lymphocyte infiltration or inflammation will help us understand the mechanisms of how immune response fights against tumor cells and demonstrate more specific T cell inhibition/activation data than those provided by pathologist reported TIL scale. Using TIMER deconvolution approach to assess

the fraction of immune cell subsets, Li et al discovered chemokine–receptor networks for lymphocyte infiltration in several tumors. In that research, CD8+ T cell levels were found to be correlated with abundance of chemokine–receptor pairs, including CCL3,4,5–CCR1,5 and XCL1,2–XCR1, and macrophage subset was associated with the CXCL12–CXCR4 pair in head and neck, thyroid, stomach, and colon cancers [4]. Among selected chemokines and cytokines related to tumor inflammation and immune response, we discovered that several cytokines were associated with CD8+ T cell enrichment, indicating that these biomarkers could be potentially targeted to boost CD8+ T cell responses, or to select patients more likely to respond to immunotherapy.

The current study has some limitations. TCGA database has limited information on clinical annotation, detailed pathology information, prior treatment data and sufficient survival outcome data in the patient cohort, which had prevented us from exploring potential roles of systemic therapy, including immunotherapy, in the patients used in the current study. In the current study, the association between tumor T-cell subsets and survival outcome was not significant in the COAD, and the association between CD4+ and B cell and COAD outcome was significant but had a wide confidence interval, which could be due to smaller sample size in the tumor cohort, or due to the smaller number of events among patients who provided tumors (277 samples with only 67 dying). Previous studies showed that elevated CD8+ T cell subsets predicted reduced recurrence in colorectal cancer [20,38]. Another study using TCGA data showed that CD4+ T cell related genes were correlated with OS, but no CD8+ T cell related genes were found to be associated with OS in COAD [39]. These findings in TCGA were consistent with our results. Another potential limitation of this study was the curse of data dimensionality problem the small number of samples with respect to the large features of gene expression data. Finally, we should recognize that the CD8+ T-cells population itself evolves over time and contains heterogeneous components; evaluating the relative roles of CD8+ T-cell subsets is essential but beyond the scope of the current study.

We assessed immune-cell populations based on RNA-seq from single time point tumor tissues using TIMER platform; the approach can't discern stromal or intra-tumor immune-cell localization or consider tumor heterogeneity or different metastatic tumor sites (for example, solid organ vs. lymph node). Further investigations that covered data with accurate spatial and temporal data could help resolve these problems. We recognize that the results reported herein, including those related to correlation between TME cytokines and COAD patient outcome; require further confirmation with independent validation cohort.

Conclusion

In summary, our results suggest that genetic mutations and expression of specific tumor cytokines represent important biomarkers of COAD immune response, and inflammation biomarkers contributes independently to COAD patient outcome.

Data Availability

Datasets related to this publication can be downloaded at [https://portal.gdc.cancer.gov/projects/TCGA-COAD], hosted at [National Cancer Institute GDC Data Portal]

Acknowledgement

Our study was supported by The National Natural Science Foundation of China 81973111.

Conflict of Interest

All authors declare no conflict of interest.

Author Contributions

Xinke Zhou: Conceptualization, data curation, writing– original draft, critical review and editing. Jiachun Lu: Conceptualization, methodology, and critical review and editing. Shenying Fang: Conceptualization, data curation, formal analysis, methodology, writing– original draft, and critical review and editing.

References

1. Pages F, Berger A, Camus M (2005) Effector memory T cells, early metastasis and survival in colorectal cancer. N Engl J Med 353:2654-2666.
2. Sato E, Olson SH, Ahn J (2005) Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A 102:18538-18543.
3. Schumacher K, Haensch W, Röefzaad C, Schlag PM (2001) Prognostic significance of activated CD8⁺ T cell infiltrations within esophageal carcinomas. Cancer Res 61:3932-3936.
4. Li B, Severson E, Pignon JC (2016) Comprehensive analyses of tumor immunity: Implications for cancer immunotherapy. Genome Biol 17:174.
5. Hunt GJ, Freytag S, Bahlo M, Gagnon-Bartsch JA (2019) dtangle: accurate and fast cell-type deconvolution. Bioinformatics 35(12):2093-2099.
6. Racle J, de Jonge K, Baumgaertner P, Speiser DE, Gfeller D (2017) Simultaneous enumeration of cancer and immune cell types from bulk tumor gene expression data. Elife 13:e26476.
7. Newman AM, Liu CL, Green MR (2015) Robust enumeration of cell subsets from tissue expression profiles. Nat Methods 12:453-457.
8. Li T, Fu J, Zeng Z (2020) TIMER2.0 for analysis of tumor-infiltrating immune cells. Nucleic Acids Res. 48:W509-w514.
9. Rospo G, Lorenzato A, Amirouchene-Angelozzi N (2019) Evolving neoantigen profiles in colorectal cancers with DNA repair defects. Genome Med 11:42.
10. Cafri G, Yossef R, Pasetto A (2019) Memory T cells targeting oncogenic mutations detected in peripheral blood of epithelial cancer patients. Nature Communications 10:449.
11. Samstein RM, Lee C-H, Shoushtari AN (2019) Tumor mutational load predicts survival after immunotherapy across multiple cancer types. Nat Genet 51:202-206.
12. Chan TA, Yarchoan M, Jaffee E (2019) Development of tumor mutation burden as an immunotherapy biomarker: Utility for the oncology clinic. Ann Oncol 30:44-56.
13. Fridman WH, Pages F, Sautes-Fridman C, Galon J (2012) The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 12:298-306.
14. Staudacher JJ, Bauer J, Jana A (2017) Activin signaling is an essential component of the TGF-β induced pro-metastatic phenotype in colorectal cancer. Scientific Reports 7:5569.

15. Kusaba T, Nakayama T, Yamazumi K (2006) Activation of STAT3 is a marker of poor prognosis in human colorectal cancer. Oncol Rep 15:1445-1451.

16. Kortylewski M, Kujawski M, Wang T (2005) Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. Nat Med 11:1314-1321.

17. Iwata-Kajihara T, Sumimoto H, Kawamura N (2011) Enhanced cancer immunotherapy using STAT3-depleted dendritic cells with high Th1-inducing ability and resistance to cancer cell-derived inhibitory factors. J Immunol 187:27-36.

18. Gotthardt D, Putz EM, Straka E (2014) Loss of STAT3 in murine NK cells enhances NK cell-dependent tumor surveillance. Blood 124:2370-2379.

19. Pagès F, Berger A, Camus M (2005) Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med 353:2654-2666.

20. Galon J, Costes A, Sanchez-Cabo F (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 313:1960-1964.

21. Siddiqui I, Erreni M, van Brakel M, Debets R, Allavena P (2016) Enhanced recruitment of genetically modified CX3CR1-positive human T cells into Fractalkine/CX3CL1 expressing tumors: importance of the chemokine gradient. J ImmunoTher Can 4:21.

22. Mlecnik B, Tosolini M, Charoentong P (2010) Biomolecular network reconstruction identifies T-cell homing factors associated with survival in colorectal cancer. Gastroenterology 138:1429-1440.

23. Kistner L, Doll D, Holtorf A, Nitsche U, Janssen K-P (2017) Interferon-inducible CXC-chemokines are crucial immune modulators and survival predictors in colorectal cancer. Oncotarget 8:89998-90012.

24. Vuoristo MS, Laine S, Huhtala H (2001) Serum adhesion molecules and interleukin-2 receptor as markers of tumour load and prognosis in advanced cutaneous melanoma. Eur J Cancer 37:1629-1634.

25. Taube JM, Anders RA, Young GD (2012) Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med 4:127-137.

26. Huang S, Ullrich SE, Bar-Eli M (1999) Regulation of tumor growth and metastasis by interleukin-10: the melanoma experience. J Interferon Cytokine Res 19:697-703.

27. Harlin H, Meng Y, Peterson AC (2009) Chemokine expression in melanoma metastases associated with CD8+ T-cell recruitment. Cancer Res 69:3077-3085.

28. Team R. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Viena, Áustria, 2014.

29. Fang S, Xu T, Xiong M (2019) Role of Immune Response, Inflammation, and Tumor Immune Response-Related Cytokines/Chemokines in Melanoma Progression. J Invest Dermatol 139:2352-2358. [Cross Ref] [Google Scholar] [Pubmed]

30. Fodde R, Smits R, Clevers H (2001) APC, Signal transduction and genetic instability in colorectal cancer. Nature Reviews Cancer 1:55-67.

31. Zajac V, Tomka M, Ilenciková D, Májek P, Stevurková V, et al. (2000) A double germline mutations in the APC and p53 genes. Neoplasma 47:335-341.

32. Jiang Z, Liu Z, Li M, Chen C, Wang X (2018) Immunogenomics Analysis Reveals that TP53 Mutations Inhibit Tumor Immunity in Gastric Cancer. Translational Oncology 11:1171-1187.

33. Yi T, Zhang Y, Ng DM (2021) Regulatory Network Analysis of Mutated Genes Based on Multi-Omics Data Reveals the Exclusive Features in Tumor Immune Microenvironment Between Left-Sided and Right-Sided Colon Cancer. Front Oncol 11:685515.

34. Oh J-H, Jang SJ, Kim J (2020) Spontaneous mutations in the single TTN gene represent high tumor mutation burden. npj Genomic Medicine 5:33

35. Belisle JA, Horibata S, Jennifer GA (2010) Identification of Siglec-9 as the receptor for MUC16 on human NK cells, B cells, and monocytes. Mol Cancer 9:118.

36. Hu J, Sun J (2018) MUC16 mutations improve patients' prognosis by enhancing the infiltration and antitumor immunity of cytotoxic T lymphocytes in the endometrial cancer microenvironment. Oncoimmunology 7:e1487914.

37. Ou Q, Lin D, Yu Y (2019) 142P - Association of MUC16 mutation with survival of immune checkpoint inhibitor in patients with cancer. Annals of Oncology 30:xi51-xi52.

38. Glaire MA, Domingo E, Sveen A (2019) Tumour-infiltrating CD8+ lymphocytes and colorectal cancer recurrence by tumour and nodal stage. Br J Can 121:474-482.

39. Yang X, Wu W, Pan Y, Zhou Q, Xu J, et al. (2020) Immune-related genes in tumor-specific CD4+ and CD8+ T cells in colon cancer. BMC Cancer 20:585.