Typing the tumor immune signatures in patients of Lynch syndrome facilitates predicting the responsiveness of immune checkpoint inhibition

Guoxing Zheng
Sun Yat-sen University

Yingsi Lu
Sun Yat-sen University

Zheng Yang
Sun Yat-sen University

Hong Chen
Sun Yat-sen University

Qian Liang
Sun Yat-sen University

Qingqing Zhu
Sun Yat-sen University

Yan Li
Sun Yat-sen University

Xing Xiao
Sun Yat-sen University

Zhuzhen He
Shenzhen Amcare Maternity Hospital

Yifan Zhu
Sun Yat-sen University

Bo Li
Sun Yat-sen University

Leilei Huang
Sun Yat-sen University

Nan Dong
Sun Yat-sen University

Shuang Hu
Sun Yat-sen University

Yihang Pan
Sun Yat-sen University

Changhua Zhang
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Abstract

Background: Although many efforts of predicting the responsiveness to immune checkpoint inhibition including expression of PD-L1 and MHC I, microsatellite instability (MSI), mismatch repair (MMR) defect, tumor mutation burden (TMB), tertiary lymphoid structures (TLSs) and several transcriptional signatures have been performed, the sensitivity remains to be further improved.

Methods and Results: Here, we integrated T cell spatial distribution and intratumor transcriptional signals in predicting the response to immune checkpoint therapy in Lynch Syndrome (LS) which is featured with MMR deficiency. In all three cohorts, LS patients displayed the personalized tumor immune signatures of inflamed, immune excluded, and immune desert, which were not only individual-specific but also organ-specific. Furthermore, the immune desert exhibited more malignant indicated by low differentiation adenocarcinoma, larger tumor sizes, and higher metastasis rate. Moreover, the tumor immune signatures associated with distinct populations of infiltrating immune cells were comparable to TLSs and more sensitive than transcriptional signature gene expression profiles (GEPs) in immunotherapy prediction. Surprisingly, the tumor immune signatures might arise from the somatic mutations. Notably, LS patients had benefited from the typing of immune signatures and later immune checkpoint inhibition.

Conclusions: Our findings suggest that compared to PD-L1 expression, MSI, MMR, TMB, and GEPs, characterization of the tumor immune signatures in Lynch syndrome improve the efficiency of predicting the responsiveness of immune checkpoint inhibition.

Background

In the past 2 decades, immunotherapy of cancer has made great advances, reflecting the crucial functions of the immune system against cancer progression. Although immunotherapy, especially through blockade of immune checkpoint such as programmed death-1 (PD1) and its ligand programmed death-ligand 1 (PD-L1), has been widely applied in treatment of a broad range of human cancers. The proportion of responsive patients experiencing immunologic eradication of cancer remains limited(1).

One important challenge is to predict the responsiveness before conducting checkpoint blockade immunotherapy. Many efforts have proved the possibility to anticipate the responses of cancer immunotherapy(2–4). The expression of PD-L1 arises as a valuable indicator(5), but PD-L1 alone is insufficient for selecting patients of immunotherapy(6). TMB displayed significant associations with immune checkpoint inhibitor responses in a variety of tumor types but not in particular cancers like Merkel cell carcinoma and rectal colon cancer (RCC)(7). TMB higher than 37.4 mutations/Mb appeared to hold very high success rate within MSI-H metastatic colorectal cancer to stratify patients for possibility of response to immune checkpoint inhibitors(8). However, chemotherapy contributed to the acquisition of hypermutated burden but not enhanced the response to PD-1 blockade in MMR-deficient gliomas(9), reflecting the suboptimal choices of TMB and MMR as predictive markers for immunotherapy. Furthermore, another study illustrated that Lynch Syndrome patients exhibited a striking immune
activation independent of mutation burden, neoantigen generation, and MMR status(10). These findings suggest that neither TMB nor MMR alone can be reliable indicators of immunotherapy. Other potential markers for the prediction consist of tumor heterogeneity(11), circulating tumor DNA(12), B cells(13), and distinct T cell subsets(14, 15).

One important step to improve the effectiveness and accuracy of response anticipation is the integration of various features including CD8 T cell infiltration pattern, expression of PD1/PD-L1, and assessment of signaling pathways like IFNγ, TGFβ, fatty acid metabolism(16). Accordingly, the tumor immune signatures(16) were defined as 3 subtypes: Inflamed, Immune excluded, and Immune desert, with their distinct features. Inflamed tumors are primarily associated with immune responses like IFNγ signaling, high PD-L1 level, the prevalence of tumor infiltration lymphocytes (TILs), B cells, and intact antigen presentation. Immune excluded tumors are defined by the reactive stromal biology – a physical barrier for exclusion of T cells(17), the signature of TGFβ signaling, and tumor angiogenesis. Immune desert tumors are devoid of lymphocyte infiltration and primarily characterized by increasing fatty acid metabolism and neuroendocrine features. The classification of tumor immune signatures has been proved effective in prediction of the response of anti-PD-L1 therapy across a range of cancers, especially in non-small cell lung cancer (NSCLC)(1) and metastatic urothelial cancer (mUC)(17), with that immune desert tumors are the poorest responders(18).

Lynch Syndrome (LS) is caused by autosomal dominant heterozygous germline mutations in one of the MMR genes mostly MLH1, MSH2, MSH6 or PMS2(19). The defect of DNA repair system always leads to MSI, increases risks for malignancies and accelerates neoplastic progression(19, 20). LS are found in a spectrum of cancers, mainly in the colorectal cancers and endometrial cancers(19–21). Here, we apply the integrated methods in predicting the response to immune checkpoint therapy in Lynch Syndrome through immunohistology and signaling pathway analysis of intratumor transcriptome, and find that the tumor immune signatures are featured with distinct immune cell populations and TLS status, and strongly correlated to tumor somatic mutations, cancer development, patient survival and the responding to immunotherapy. The findings provide references and guidance for successful immunologic elimination of cancer cells based on immune checkpoint inhibition.

Materials And Methods

Patients

The colon tumors and adjacent normal tissues from ten LS patients carrying germline mutation of the MMR genes MLH1 or MSH2, and endometrial tumor and adjacent normal tissues from one of these patients were collected at the Seventh Affiliated Hospital, Sun Yat-sen University. These samples were then analyzed using immunohistochemistry, whole-genome transcriptomic analysis, and whole exome sequencing. Patient consents were obtained following the guidelines approved by the Medical Ethics Committee of the Seventh Affiliated Hospital of Sun Yat-sen University. Formalin-fixed paraffin-embedded
tissue specimens of eighty-two colorectal cancer patients with MMR deficiency were used for immunohistochemistry analysis. Characteristics of these patients were provided on Table S1.

RNA-seq

The total RNA of tumors and adjacent normal tissues in Lynch syndrome patients was isolated with the RNeasy Mini kit (QIAGEN) after grinding of tissues in liquid nitrogen. The quality, integration, and quantity of obtained RNA were assessed via Nanodrop (Thermal), Qubit (Thermal), and agar gel running. Following the manufactory’s manual, qualified RNA was subjected to library construction with TruSeq® RNA LT Sample Prep Kit v2 (Illumina). The cDNA library was ligated to an adaptor and purified with AMPure XP Beads (Beckman). The second-generation sequencing of the obtained library on PE150 was conducted on Hiseq3000 (Illumina). Average number of raw bases per specimen was over 6GB. The percentage of bases having scores > Q30 for single and paired-end reads were higher than 90% in all the data obtained.

Immunohistology

The tumor and adjacent normal tissues from patients with Lynch syndrome were collected, fixed, and embedded in paraffin. Or the tumor specimens with MMR protein defects in colorectal patients were fixed and embedded with paraffin. The 8μm paraffin sections were stained with Abcam antibodies of CD3 (Ref# ab16669), CD8 (Ref# ab4055), PD1 (Ref# ab52587), PDL1 (Ref# ab213524), HLA I (Ref# ab23755), respectably, and counterstained with hematoxylin. The antibody-specific staining on the slides was captured with a Leica DM4B microscope system.

Tumor immune signature analysis

According to the summary by Priti Hegde1 and Daniel Chen in Immunity(16), the tumor immune signatures were defined as 3 subtypes: Inflamed, Immune excluded, and Immune desert, with their distinct features and the spatial localization of immune cells (CD8 or CD3 positive cells in this study) infiltrating in the tumor. The signaling pathways arising from GO, KEGG and Reactome function analysis of the target gene set were matched to the distinct tumor immune signatures, especially those dominant ones including IFNγ signaling, PD-L1 expression, the prevalence of TILs, B cells, and intact antigen presentation in Inflamed; TGFβ signaling, and tumor angiogenesis in Immune excluded; fatty acid metabolism and neuroendocrine in Immune desert tumors. The immune cells infiltration patterns were also defined with the defect of immune cells in the immune desert, enrichment of immune cells in the surrounding regions and highly dispersing immune cells in inflamed tumors.

Data mining in the GEO database

Based on the publication (10) of Kyle Chang et al., the raw data of serial GSE106500 was download from the NCBI-GEO database (https://www.ncbi.nlm.nih.gov/geo). The differential expression genes (DEGs) from adenomas of 11 patients with Lynch syndrome (Table S2) were analyzed with KEGG functional
enrichment. According to features of the signal pathways in each immune subtype, the patients were matched to one of the 3 tumor immune signatures: Inflamed, immune excluded, and immune deserts.

**GO, KEGG and Reactome function analysis of the target gene set**

Gene ontology (GO) function analysis on the differentially expressed genes (DEGs) or somatic mutation genes was performed based on TopGO software (http://www.bioconductor.org/packages/release/bioc/html/topGO.html), in which the target genes were selected from all the gene list. The online Kyoto encyclopedia of genes and genomes (KEGG) pathway database (http://www.genome.jp/kegg-bin) was applied for KEGG pathway functional enrichment analysis of the target gene set or somatic mutation genes. Additionally, the Reactome database that integrates the various reactions and biological pathways in human was utilized for functional analysis of the target gene set or somatic mutation genes. Whether the function set of GO, KEGG, and Reactome was significantly enriched in the target gene list was determined by the p-value calculated by Fisher’s exact test. The p-value was further corrected by Benjamini & Hochberg multiple tests to control the false discovery rate (FDR). The corrected P values less than 0.05 were annotated significant in all these three function analyses.

**Deconvocation Analysis of RNA seq data**

We used the Cell Fractions module of CIBERSORTx to estimate the percentage of the overall immune cells in each sample. The single-cell reference used was the colorectal cancer samples from Qian, J. et al. *A pan-cancer blueprint of the heterogeneous tumor microenvironment revealed by single-cell profiling*(22). The types of immune cells annotated in the reference are T cells, B cells, Mononuclear phagocytes, plasma cells and mast cells. The sum of all immune cell percentages estimated by CIBERSORTx for each sample was used as the overall percentage of immune cells in that sample, denoted as a. The CIBERSORT algorithm (23) is used to infer the relative proportion of 22 infiltrating immune cells from the normalized gene expression data. The relative percentage of each immune cell type in LM22 was estimated by CIBERSORT for each sample, denoted as b. The absolute percentage of each immune cell type in LM22 of each sample is equal to a * b.

**Immunofluorescence staining**

The tumor and adjacent normal tissues from patients with Lynch syndrome were collected, fixed, and embedded in paraffin. The 8μm paraffin sections were stained with TSAPLus fluorescent triple staining kit (Servicebio, Cat# G1236) following the manufacturer's protocol. Abcam antibodies of CD23 (Ref# ab92495), CD20 (Ref# ab64088), and DAPI were used. The antibody-specific staining on the slides was captured with a Zeiss LSM880 confocal microscope system.

**Whole exome sequencing**

The genomic DNA of tumors and adjacent normal tissues in Lynch syndrome patients was prepared with Axyprep genomic DNA miniprep kit (animal tissues and human tissues) following the manufactur
instructions. The quality and quantity of obtained DNA were evaluated via Qubit (Thermal), and electrophoresis on agar gel. The genome was subsequently submitted to library construction with TruSeq® DNA LT Sample Prep Kit v2 (Illumina). Then, the exon enrichment was performed via Nimblegen Exome Kit V4 (Roche). After quantification, the exon library was sequenced on Hiseq3000 (Illumina). The data with Q30 (The proportion of bases with 99.9% accuracy) higher than 80% was accepted and further analyzed in the following procedures.

**Somatic mutation detection**

MuTect2 [http://software.broadinstitute.org/cancer/cga/mutect](http://software.broadinstitute.org/cancer/cga/mutect) was utilized for somatic mutation detection. The filter criteria included (1) the sequencing depth of cancer and adjacent tissues is >= 10; (2) the number of reads supporting this variation in tumors is >= 3; (3) the allele frequency of this variation in tumors is >= 0.05; (4) the allele frequency of this mutation in adjacent normal tissues is <= 0.01; (5) and the filter is equal to pass.

**GEPIA Analysis of immune genes in the TCGA database**

Gene expression profiling interactive analysis (GEPIA, [http://gepia.cancer-pku.cn/](http://gepia.cancer-pku.cn/)) was applied to assay the expression of immune response genes including CD8, CD3, PDL1, and PD1 in colon adenocarcinoma and rectum adenocarcinoma. The level of different cancer stages was analyzed. And the overall survival of patients was compared in high expression and low expression groups of each gene.

**The combination therapy of patients**

One month after the tumors was surgically removed, the imaging assessment showed progressive disease in the patient. The immune signature has identified the tumor as immune excluded. The patient with colon cancer of Lynch syndrome received six cycles of combination therapy of anti-VEGF, FOLFIRI and anti-PD1 at each cycle of 3 weeks, then four cycles of combination therapy of anti-VEGF and anti-PD1 at each cycle of 4 weeks. Dosage of combination therapy of anti-VEGF, FOLFIRI and anti-PD1: anti-PD1 Sintilimab Injection (Xinda biopharmaceutical (Suzhou) Co., Ltd, Approval No. gyzz S20180016) 200mg intravenous (iv) + anti-VEGF Bevacizumab Injection (Roche Pharma (Switzerland) Ltd. Avastin, Approve No.: Import Drug Registration Certificate No.: S20170035) 200mg iv + Irinotecan Hydrochloride Injection (Jiangsu Hengrui Pharmaceutical Co., Ltd, Approval No.: gyzz H20061276) 130mg iv + 5-FU first dosage 0.56g iv, 3.3g iv maintain 46h. Dosage of combination therapy of anti-VEGF and anti-PD1: Sintilimab Injection 200mg iv + Bevacizumab Injection 200mg iv.

**Statistics**

Proportions for categorical variables were compared using the chi-square test, and Fisher's exact test was used when the data were limited. P < 0.05 was considered statistically significant.

**Results**
It is necessary to distinguish tumor immune signatures before immune checkpoint therapy

The spatial localization of T lymphocytes infiltrating in the tumor is a critical basis of categorization of tumor immune signature (16, 24). These were indicated as the following: Inflamed tumors - T lymphocytes dispersed in the whole tumor regions, immune excluded tumors - T lymphocytes rich at the surrounding stroma excluded from tumor regions, and immune deserts tumors - devoid of T lymphocytes. We collected the formalin-fixed paraffin-embedded tumor tissues of 82 colorectal cancer patients (Table S1), which exhibited defect of at least one of the 4 mismatch repair proteins indicated in LS, including MLH1, MSH2, MSH6, and PMS2. The spatial localization of infiltration T lymphocytes - one key feature of tumor immune signatures was distinguished by the staining of CD8, which has been applied in defining tumor immune signatures (1, 17). Among these, 27 specimens were referred to inflamed (Fig. 1A, Table S1), 28 specimens were dened as immune exclude (Fig. 1A, Table S1), and 27 tumors were the immune desert that displayed no or very few CD8 staining (Fig. 1A, Table S1). The proportion of inflamed (27/82), which yielded the best responses to anti-PD-L1 therapy (1, 16), is not dominant among these patients, suggesting the necessity of identifying tumor immune signatures before immune checkpoint therapy (ICT).

The chi-square test was performed to analyze the correlation between the immune signatures and pathologic features of Lynch Syndrome patients (Table 1). While no correlation was found in age and gender, the tumors of immune desert subtype were significantly associated with metastasis (p<0.0001) and large tumor sizes (diameter $\geq$3cm, p<0.0001). Furthermore, microsatellite instability high (MSI-H), the key feature of MMR deficiency cells used to anticipate the immune responses, was not associated with the tumor immune signatures (p=0.259). Therefore, the tumor immune signature is not always dependent on MSI and MSI may not be suitable for immune therapy anticipation in Lynch syndrome. Notably, the immune desert tumors markedly correlated with low differentiated adenocarcinomas (p=0.014). Together, the tumors of immune desert are more malignant, featured with low differentiation, increasing tumor sizes and higher metastasis ratios. This suggested that the application of tumor immune signatures in prediction the responsiveness of immune checkpoint therapy (ICT) is reasonable.

Table 1. The correlation between immune signature and pathologic features of Lynch Syndrome patients.
<table>
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<th>Features</th>
<th>N of cases</th>
<th>Immune Signature</th>
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MS microsatellite, MSI-H microsatellite instable high, MDA Middle differentiated adenocarcinoma, MHDA Middle high differentiated adenocarcinoma, WLIW Whole layer of intestinal wall

To apply the transcriptomic signaling - another feature for typing tumor immune signatures, the transcriptome profile of polyps in 11 LS patients reported by Kyle Chang et al. (10) were determined. Following the features annotated to the 3 subtypes, 3 patients featured with high PD-L1 expression and increasing immune responses were categorized as inflamed, 4 patients highly expressing TGF\(\beta\) and cytokines were identified as immune excluded, and the remaining 4 patients with increasing fatty acid metabolism and neuroendocrine were referred to immune deserts (Fig. 1B, Table S2). Surprisingly, the immune desert tumors - the immune signature associated with worse responsiveness of ICT had the 3 highest tumor mutation burdens (TMB, Table S2), indicating that TMB is not reliable of predicting the responsiveness of ICI in LS. Similar to NSCLC and mUC, Lynch syndrome could be categorized into all 3 types of tumor immune signatures which correlated to the cancer malignancy. Moreover, MSI, TMB and MMR defect are independent of immune signatures and may not suitable for the prediction of immune checkpoint inhibition (ICI) responses in LS patients. Together, it is feasible and necessary to type the tumor immune signatures via the pattern of T cell infiltration or intratumor transcriptome in the therapeutic prediction of immune checkpoint inhibition (ICI) in LS patients.

Two methods of typing tumor immune signatures from different individuals were consistent

We collected colon tumors and adjacent normal tissues from ten LS patients (Table S3), and endometrial tumor (30E) and adjacent normal tissues from one of these patients. The whole genome RNA-seq of these specimens were carried out. To confirm the applications of tumor immune signature categorization, we performed various functional enrichment analyses of DEGs including GO, KEGG, and Reactome. Matching the enriched pathways in each tumor (Fig. 2A right) to the features of immune signatures (Fig. 2A left) characterized 6 colon tumors including 38C, 8C, 41C, CC0518, CC0527, and CC124 as the inflamed subtype, 2 colon tumors 30C (colon tumor of #30 patient) and 2C as the immune excluded, and the remaining specimen 17C, CC0429 and 30E (endometrial tumor of #30 patient) as immune desert. The results suggested that the tumors from different individuals or organs of Lynch syndrome hold the personalized tumor immune signatures.

To assess the spatial distribution of T cells in LS specimens, the immunostaining of CD3 was conducted to label the T lymphocytes in tumors. As expected, the distribution of T cells matched to the tumor immune signatures derived from the assay of functional enrichment of transcriptome. The inflamed tumor was infiltrated with lymphocytes, immune excluded colon tumors associated with the surrounding
embedding of immune cells, and immune desert tumor was devoid of TILs (Fig. 2B). In addition, the expression of PD-L1 and MHC I are critical indicators in the classification of tumor immune signature(17), thus their immunohistochemistry (IHC) was also conducted. All the spatial distribution of PD-L1, its receptor PD1, and MHC I (Fig. S1) were similar to CD3 T lymphocytes. It seems that the spatial distribution of PD-L1 is more critical than the expression level itself in the prediction of ICT responses. Taken together, IHC of CD3, PD1, PDL1, and MHC I confirmed the personalized tumor immune signatures in different patients and organs. Additionally, this suggest that the typing of tumor immune signature via intratumor transcriptomic profile is consistent to the typing by the distribution of T lymphocytes.

**The tumors of various immune signatures were infiltrated by distinct immune cell populations**

To further dissect the constitution of immune cell types in the tumor microenvironment (TME), deconvolution analysis of RNA seq data was performed by the CIBERSORT algorithm. While inflamed tumors held the highest percentage of both CD8 T cells and plasma cells and relative higher level of activated CD4 T cells, resting Dendritic cells (DCs), and naïve B cells (Fig. 3A), immune excluded tumors were highlighted with highest level of M2 macrophage and medium level of both CD8 T cells and plasma cells (Fig. 3A), and immune desert tumors had the highest level of activated NK cells, neutrophils and activated mast cells (Fig. 3A). The deconvolution analysis illustrated that the subtypes of the tumor immune signatures were associated various infiltration of immune cell clusters in tumors. The results also suggest that the relative immune reactive activities marked by the level of CD8 T cells and plasma cells are highest in inflamed, medium in immune excluded and lowest in immune desert tumors, indicating the difference of responsiveness to immune checkpoint inhibition.

**The tumor immune signatures were comparable to tertiary lymphoid structures in ICT prediction**

Tertiary lymphoid structures (TLSs) are ectopic germinal center-like lymphoid organs that develop at sites different to lymphoid tissues like tumors(25). TLSs are consisted of a T cell-rich zone with follicular DCs juxtaposing a B cell follicle and surrounded by plasma cells. Recently, it is reported that tertiary lymphoid structures can be applied to predict the efficacy of immune checkpoint inhibitor in solid tumors(26-29). Especially, mature TLSs (mTLSs) with CD23⁺ follicular dendritic cells inside are favored by immune checkpoint inhibition, while immature TLSs (iTLSs) without CD23⁺ follicular dendritic cells are not(30).

From the deconvolution analysis of RNA seq data, we noticed that the lymphocytes related with TLSs like T cells, DCs and plasma cells were enriched in inflamed tumors. Thus, we asked whether the tumor immune signatures were related to tertiary lymphoid structures in Lynch syndrome. The 12-chemokine signature(31) in transcriptome for the detection of tertiary lymphoid structures in colorectal cancer were applied. As expected, the inflamed tumors in tumor immune signatures were rich of TLSs, indicated by the highest expression of most chemokines (Fig. 3B). Furthermore, multiplex immunofluorescence staining of the 2 typical marker of TLSs - CD20 (B cells) and CD23 (DCs) was performed to validate the presence of TLSs in LS tumors. The mTLSs (Fig. 3C top) were exclusively found in the inflamed tumors and immune excluded tumors, while the immune desert tumors only carried iTLSs (Fig. 3C bottom). The
data revealed that the tumor immune signatures are consistent to the status of tertiary lymphoid structures in prediction of the responsiveness to ICI in the tumors of Lynch syndrome.

**T cell–inflamed gene expression profiles (GEPs) could not distinguish the difference between immune excluded and immune desert**

In 2017, Mark Ayers, *et al.* developed a set of IFN-γ-related mRNA profile to predicts the clinical response to PD-1 blockade in melanoma(32). The final 18-gene T cell–inflamed gene expression profiles (GEPs) were more sensitive than PD-L1 IHC to detect responders to anti-PD-1 therapies with the area under the receiver operating characteristic (ROC) curve 0.75. Although the GEFs and TMB had a low correlation, their joint prediction could be utilized in identifying responders to the PD-1 antibody in pan-tumors(33). Importantly, the GEPs had a strong correlation (r > 0.9) with several previously published transcriptional signatures of TME including chemokine signature(31), Immunoscore(34), and cytolytic activity(35). In order to compare the sensitivity of the immune signatures to previously published transcriptional signatures, the analysis of T cell–inflamed GEPs were applied to our patients. The analysis (Fig. 4) distinguished the inflamed specimens from non-inflamed specimens very well. However, the differences between immune excluded and immune desert were not obviously determined (Fig. 4). Therefore, the tumor immune signatures may offer a more optimal choice than GEPs in identifying the responders to ICT.

**The tumors from different organs of the same patient had the unique immune signatures**

Interestingly, the tumors from different organs (colon tumors, 30C vs endometrial tumors, 30E) of #30 patient held different tumor immune signatures (30C immune excluded vs 30E immune desert, Fig. 2A). KEGG pathway enrichment of up regulated genes and down regulated genes showed distinct signaling pathways in the 2 tumors (Fig. 5A-D). The deconvolution analysis of RNA seq data revealed the 2 tumors had infiltration of different immune cell types (Fig. 3A). Moreover, detection of 12-chemokine signature of TLSs illustrated that the chemokines in the 2 tumors were totally different (Fig. 3B). Together, these results support that the tumors from different organs of the same patient may hold the unique tumor immune signatures, which may be due to the various tumor microenvironments in different organs. This effect should be taken into account in immune checkpoint therapy in future.

**The tumor somatic mutations contributed to the variation of the tumor immune signatures in Lynch syndrome**

Somatic mutations are defined as mutations specifically found in tumors but not in the surrounding normal tissues(36). We sought to elucidate the effect of somatic mutations via whole exome sequencing and relate these data to corresponding tumor immune signatures. The somatic mutations within exon regions showed radically differences in numbers of mutation (Table S4) and the proportion of Indel (insertion and deletion) and SNV (single nucleotide variation).
To decipher the influence of somatic mutations within exons, the functional enrichments of the mutation genes were performed. Mutations in a particular pathway usually means impairment in such a pathway. Interestingly, the signal pathways found in somatic mutations (Fig. 5E top) were similar to the down regulated signaling identified in transcriptome of the same specimen (Fig. 5E middle), indicating the dysfunction of signaling are raised from the somatic mutation. Collectively, the pathways of somatic mutation in the different colon tumors represented the distinct features, leading to the variation of the tumor immune signatures (Fig. 5E). In conclusion, somatic mutations leading to deficiency of certain signaling pathways, drive tumor development toward an opposite subtype.

The activity of immune responses was important for the development of colorectal cancer and patient survival

To detect the effect of immune activity in colorectal cancer, the relationship between immune gene level, cancer progression and patient survival were determined. According to GEPIA analysis of TCGA data, the expression of immune response genes including CD8, CD3, PD-L1, and PD1 all had a downward trend with the development of colorectal cancer from stage I through stage IV (Fig. 6A top). Furthermore, the overall survivals of patients with colorectal cancer were all enhanced in high expression groups of CD8, CD3, PDL1, and PD1 than those in low expression groups (Fig. 6A bottom). Collectively, the activity of immune responses indicated by the expression of CD3, CD8, PDL1, and PD1 genes was important in the predication of the progression of colorectal cancer and patient survival.

LS patients beneted from the prediction and therapy of immune checkpoint inhibition

The colon tumor in patient #2C was at stage of T4bN1a, and had the peritoneal metastases. Molecular analysis revealed that the patient carried PIK3CA H1047R cancer mutation, and POLE F699fs*11 mutation that is associated with high TMB and may benet from immune checkpoint inhibition(37, 38). And the patient had a TMB of 67Muts/Mb, which is much higher than the medium TMB level of 4.5Muts/Mb in colon cancer(39). One month after the tumors was surgically removed, the imaging assessment showed progressive disease in the patient. The mRNA profile of predicting clinical response to PD-1 blockade(32, 33) showed the strongest immune activation in inflamed tumors, the medium immune response in immune excluded tumors and the weakest immune response in immune desert tumors. Patient #2C was identified as immune excluded in tumor immune signatures and may effectively respond to therapy of PD-1 blockade. According to the Keynote177 study, this patient received six cycles of combination therapy of anti-VEGF, FOLFIRI and anti-PD1, then four cycles of combination therapy of anti-VEGF and anti-PD1 (Fig. 6B). While the imaging assessment showed partial response, evaluation of the three tumor markers CA125, CEA and CA19-9 suggested complete response after combination therapy (Fig. 6B). And till now, no cancer recurrence was detected in the patient for more than 1 year after the termination of treatment. The data indicated that typing the tumor immune signatures in Lynch syndrome are valuable in prediction of ICI response and can potentially be applied in clinicals.

Discussion
In this report, through histology and/or signal features the tumors of Lynch syndrome in 3 different cohorts all matched the categorization of the tumor immune signatures: inflamed, immune excluded, and immune desert. Furthermore, the categorization of the tumor immune signatures corresponded to unique infiltrating lymphocyte populations. Strikingly, the tumor immune signature had their specificities both in individuals and organs, suggesting the necessity of tumor immune signatures classification of LS tumor before immunotherapy. Tertiary lymphoid structures have been reported to promote immunotherapy responses and patient survival in multiple solid tumors(26–29) and are critical for ICI response prediction. Various tumor immune signatures showed distinct status of tertiary lymphoid structures through the analysis of 12-Chemokine signature(31) in transcriptome. In line with the status of TLSs in immune checkpoint inhibition(30), poorly responding desert tumors are associated with immature TLSs. Together, these results indicate the tumor immune signatures are same to TLSs in predicting the immunotherapy responses.

Though the exceptionally high burden of somatic mutations in LS were favored in the anti-PD1 treatment of patients across 12 different tumor types including colorectal cancer(40, 41), approximately half of the patients with MMR defect did not respond with 53% of objective radiographic responses, and 21% of complete responses(41). The findings in this study suggest that MSI, TMB and MMR defect are all independent of immune signatures and their sensitivity as reliable indicators of immunotherapy in LS needs to be further improved. The immune signature typing by bulk RNA-seq or T cell staining can easily distinguish the tumor responsiveness to immunotherapy in LS, comparable to the TLSs. Furthermore, the immune signature seems more sensitive than previously published transcriptional signatures - T cell - inflamed GEPs in identifying responders to ICT. The analysis of immunohistology of 82 patients with MMR defect in this study revealed 67.1% (27/82 of Inflamed pattern and 28/82 of Immune excluded) patients that may be responsive to blockade of immune checkpoint in cancer therapy. The tumor immune signatures represent one easy and robust tool in immunotherapy prediction.

A novel finding in this study is that functional enrichment of somatic mutation displayed opposite features of mRNA expression, indicating that the personalized tumor immune signatures raised from the different features of intratumorally somatic mutation (especially Indel). For instance, for patient 17C, which is immune desert type, we found those genes active in the immune excluded or inflamed were mutated. While MMR-d in LS greatly increased genomic mutation rate, the really oncogenic process is dependent on somatic mutations, which define tumorigenic properties, the oncogenic program as well as the host immune response. The activity of immune responses indicated by the expression of CD3, CD8, PDL1, and PD1 genes, which have similar distribution in tumors, exhibited strong correlation with the development of colorectal cancer and patient survival. Our data also reveal that patient with immune excluded can have a good responsiveness to the combination therapy of immune checkpoint inhibition. Importantly, the tumor immune signatures in LS patients are valuable to predict the effect of anti-PD1/PD-L1 therapy(1). Due to the limitation of patient number, the findings in this study need to be further validated in future.
Conclusions

We have shown that LS patients exhibited personalized tumor immune signatures, which were derived from intratumor somatic mutations. Category of tumor immune signatures based on histology and signal features likely represents a robust and novel strategy to distinguish the subset of patients with Lynch syndrome who may benefit from later immune checkpoint inhibition.

Abbreviations

MSI: microsatellite instability; MMR: mismatch repair; TMB: tumor mutation burden; TLSs: tertiary lymphoid structures; LS: Lynch syndrome; GEPs: gene expression profiles; PD1: programmed death-1; PD-L1: programmed death-ligand 1; RCC: rectal colon cancer; TILs: tumor infiltration lymphocytes; NSCLC: non-small cell lung cancer; mUC: metastatic urothelial cancer; DEGs: differential expression genes; GO: gene ontology; FDR: false discovery rate; GEPIA: gene expression profiling interactive analysis; ICT: immune checkpoint therapy; ICI: immune checkpoint inhibition; IHC: immunohistochemistry; TME: tumor microenvironment; DCs: Dendritic cells

Declarations

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Author Contributions

GZ and CZ jointly directed this work. Acquisition, analysis, or interpretation of data: All authors. Drafting of the manuscript: GZ and CZ. Statistical analysis: YSL, QZ, GZ. All authors read and approved the final manuscript.

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Availability of data and materials

The relevant data in the study can be obtained in the SRA database with Accession# PRJNA669198. https://www.ncbi.nlm.nih.gov/sra/PRJNA669198. Other data are available upon reasonable request.

Ethics approval and consent to participate
The study was conducted in accordance with the Declaration of Helsinki, and approved by the Medical Ethics Committee of the Seventh Affiliated Hospital of Sun Yat-sen University (#KY-2020-038-01, 2020-09-28). Informed consent was obtained from all subjects involved in the study.

**Consent for publication**

Informed consent form for publication was obtained from all participates.

**Competing interests**

The authors declare no potential conflicts of interest.

**References**


**Figures**

**Figure 1**


**Figure 2**

Typing the tumor immune signatures in tumors of Lynch syndrome by the integration of histology and transcriptome profile (A) Functional enrichment of mRNA expression profile suggests the basic features
of the three tumor immune signatures (B) Immunostaining of CD3, CD8 and hematoxylin-eosin staining exhibit the patterns of various tumor immune signatures in different LS patient specimens. Magnification 50X.

**Figure 3**

The tumor immune signatures were associated with the infiltration of distinct populations of immune cell and with the status of tertiary lymphoid structures. (A) Deconvolution analysis of RNA seq data reveals the infiltration of different populations of immune cell in various tumor immune signatures. The relative fraction of 22 immune cell types is inferred by CIBERSORT. (B) Analysis of the 12-chemokine transcriptional expression suggest that the presence of tertiary lymphoid structures are various in different tumor immune signatures. (C) Multiplex IF of CD20, CD23 shows that while mature TLSs (top) are found in the tumors of immune excluded and inflamed, immature TLSs (bottom) are related to the desert tumors.

![Heat map of gene expression profiles](image)

**Figure 4**

The GEPs could not distinguish immune excluded and immune desert tumors. The 18-gene T inflamed gene expression profiles (GEPs) were applied to the transcription profiles of 11 tumors, the cluster of GEPs was presented as heat map.
Figure 5

The tumors from different organs of the same patient display distinct tumor immune signatures. Scatterplot of enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of (A) down-regulated and (B) up-regulated DEGs in colon tumors -30C, indicating the activation of the cell cycle, proliferation, and inflammation. Scatterplot of enriched KEGG pathways of (C) down-regulated and (D) up-regulated DEGs in the endometrial tumor -30E, suggesting the activation of lipid metabolism. The vertical axis represents the KEGG pathway. The horizontal axis represents the percentage of DEGs in the total number of genes involved in certain KEGG pathways. The size of the bubble indicates the number of DEGs enriched in this item, and the color of the dots indicates the range of –log10 (FDR). (E) The tumor somatic mutations contributed to the variation of the tumor immune signatures in Lynch syndrome. The mRNA expression patterns, that are opposite to somatic mutation profiles, in different tumors are corresponding to various tumor immune signatures.

Figure 6

The tumor immune signatures may be potentially applied to predict the development of colorectal cancer, patient survival and the responsiveness of immune checkpoint inhibition. (A) According to GEPIA analysis of TCGA data, the expression of immune response genes including CD8, CD3, PDL1, and PD1 were gradually reduced with the development of colorectal cancer from stage I through stage IV. The overall survivals of patients with colorectal cancer are all elevated in high expression groups of CD8, CD3, PDL1, and PD1 than those in low expression groups. (B) The level change of 3 cancer markers during combination therapy of anti-PD1 on patient #2C of immune excluded indicated the complete response.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS1.pdf
- TableS1S4.docx