The infiltration pattern of microenvironmental cells and different immune escape mechanisms in colorectal cancer

1 Yihao Mao^{1, †}, Qingyang Feng^{1, 2, †}, Wenju Chang^{1, 2, †}, Yang Lv^{1, †}, Yuqiu Xu¹, Jiang
2 Chang¹, Peng Zheng¹, Shanchao Yu¹, Zhiyuan Zhang¹, Zhiqiang Li³, Qi Lin^{1, 2}, Wentao
3 Tang^{1, 2}, Dexiang Zhu^{1, 2}, Meiling Ji^{1, 2}, Li Ren^{1, 2}, Ye Wei^{1, 2}, Guodong He^{1, 2, *} and
4 Jianmin Xu^{1, 2, *}

5 ¹ Department of General Surgery, Zhongshan Hospital, Fudan University, Shanghai, 200030, China

6 ² Shanghai Engineering Research Center of Colorectal Cancer Minimally Invasive, Shanghai,
7 200030, China

8 ³ Department of General Surgery, No.2 Hospital, Nanping, 353000, China

9 [†] These authors contributed equally to this work.

* Correspondence:

11 Guodong He and Jianmin Xu

12 he.guodong@zs-hospital.sh.cn and xujmin@aliyun.com

13 **Keywords:** colorectal cancer, tumour microenvironment, immune escape, infiltration pattern,
14 immune subtypes.

Abstract

16 **Background:** The tumour microenvironment (TME) plays a crucial role in tumour progression and
17 metastasis. However, the infiltration pattern of TME cells in CRC samples and the immune escape
18 mechanism underneath have not been studied sufficiently.

19 **Methods:** Transcriptomic data from public datasets were retrieved online. In total, 1802 samples
20 from the microarray dataset and 619 samples from the TCGA dataset were enrolled. The ssGSEA
21 algorithm and unsupervised clustering were used for TME cells infiltration speculation and
22 infiltration pattern recognition.

Results: CRC samples can be classified into three distinct TME cell subtypes. Subtype 1, the immune-active subtype, was characterised by high infiltration of activated adaptive immune cells. Subtype 2, the immune-desert subtype, featured high tumour purity and low infiltration of immune and stromal cells. And subtype 3, stroma-rich subtype, had high infiltration of stromal cells. The stroma-rich subtype conferred a significantly worse prognosis. Three subtypes had different immune escape mechanisms. The immune-active subtype has the highest immune checkpoint expression level. In comparison, the immune-desert subtype had the lowest immunogenicity and the defective antigen presentation. And the stroma-rich subtype lacked activated immune cells.

Conclusions: Distinct TME cell subtypes and immune escape mechanisms may provide inspiration and direction for further researches on CRC immunotherapy.

1 Introduction

Colorectal cancer (CRC) is estimated to be the third most commonly diagnosed and cause of death in both men and women in the USA in 2021[1]. Tumour microenvironment (TME), defined as the surrounding environment of a tumour, comprises tumour cells, immune cells, stromal cells, blood vessels, and other mesenchymal cells. TME plays a crucial role in tumour progression and metastasis[2].

Researchers have focused on CRC TME from multiple perspectives. Multiple TME cells were found to have prognostic value and predict therapeutic benefit [3, 4]. As a classic example, Immunoscore, which quantifies the in situ T cell infiltration in CRC tissue, was validated to be a valuable prognostic factor[5] and was recommended in the European Society for Medical Oncology (ESMO) guidelines[6]. However, numerous studies only focused on one or few types of TME cells. Moreover, most of them were based on pathology level with a limited sample size. Confusing, even

contradictory results were gotten. As CRC was a highly heterogeneous cancer, we assumed that the same type of TME cell might play different roles in different CRC tissues.

Recently, emerging papers explored the CRC TME using the bioinformatic based method, such as CIBERSORT[7], MCPcounter[8], TIMER[9], et al. However, because of the limitation of the above methods, like only relative infiltration results provided or limited predictable cell types, the landscape of TME cell infiltration pattern in CRC samples has not been systematically clarified to data, nor did the immune escape mechanism underneath elucidated.

In this study, we comprehensively explored the TME cell infiltration pattern in two large independent cohorts. CRC samples can be divided into three subtypes with distinct TME cell infiltration patterns and their immune escape mechanism underneath. Also, the different clinical, prognostic, and genomic characteristics were described.

2 Materials and methods

2.1 Study population

Public datasets selection criterion was described as follows: (1) transcriptomic data, including microarray data using Affymetrix HG-U133A (GEO accession number GPL96) or HG-U133 Plus 2.0 (GEO accession number GPL570) platforms or RNA-Seq data, were available; (2) the basic clinicopathological information, including AJCC/UICC TNM stage and survival information (overall survival (OS) or disease-free survival (DFS)) was available; (3) the sample size was larger than 50. After systematically searching, GSE14333[10], GSE17538[11], GSE33113[12], GSE37892[13], GSE38832[14], GSE39084[15], GSE39582[16], KFSYSCC, TCGA-COAD and TCGA-READ datasets[17] were included in our research. Finally, 1802 samples from the microarray dataset and 619 samples from the TCGA dataset were enrolled for subsequent analysis. The composition of each

dataset was illustrated in Fig. S1. The microarray dataset was utilised to discover the infiltration pattern of TME cells and perform survival-related analysis. Subsequent pattern validation and mechanism exploration were conducted in the TCGA dataset. Due to the relatively shorter follow-up time of the TCGA dataset (median, 21.9 months), survival-related analysis was only performed in the microarray dataset.

2.2 Data acquisition and preprocessing

The transcriptome and clinical data of GSE14333, GSE17538, GSE33113, GSE37892, GSE38832, GSE39084, and GSE39582 were downloaded from Gene Expression Omnibus (GEO) repository (<https://www.ncbi.nlm.nih.gov/gds/>). The expression matrix and clinical information of KFSYSCC were acquired from the supplementary material of a previous publication[18]. The RNA-Seq (RSEM normalised), copy number variation (CNV), mutation, images of pathology slides, and clinical data of TCGA-COAD and TCGA-READ datasets were downloaded from Genomic Data Commons (GDC, <https://portal.gdc.cancer.gov/>) in Dec. 2019.

We used the voom function (in R package "limma" [19]) to transform RSEM normalised TCGA RNA-Seq expression matrix. The ComBat algorithm ("ComBat" function in R package "sva" [20]) was applied to calibrate the heterogeneity among different datasets in microarray and TCGA datasets, respectively. The PCA plots illustrating the effect of calibration were shown in Fig. S2.

2.3 Microenvironmental cell infiltration speculation and validation

We used the ssGSEA algorithm ("gsva" function in R package "GSVA" [21]) for infiltration speculation of 31 microenvironmental cells based on transcriptomic data as previously described[22, 23]. Gene signature used in our study was mainly based on signature described in CHAROENTONG P et al. [24], XIAO Y et al.[22] (which was a modification of LM22 signature of CIBERSORT) and MCPcounter[25]. The detailed gene signature was described in Table S1.

To validate the accuracy of our methods, we compared our calculation results with the estimation of the cell abundances by CIBERSORT and MCPcounter (Table S2 and S3). The comparison results showed high accordance between methods. These format

2.4 Unsupervised clustering based on TME cell infiltration pattern

For TME cell infiltration pattern discovery and validation, a nonnegative matrix factorisation algorithm (NMF) ("nmf" function in R package "NMF" [26]) was performed for unsupervised clustering on the scaled ssGSEA results. We used the "nmfEstimateRank" function (30 runs) to choose the optimal number of clusters based on the Cophenetic correlation coefficient changing. The highest clustering number before the Cophenetic correlation coefficient dropping most was selected as the optimal clustering number.

2.5 Slide Image analysis

Hematoxylin and eosin (HE) stained diagnostic slides of the TCGA dataset were retrieved from GDC Data Portal. The infiltration of tumour-infiltrating lymphocytes (TILs) was recorded as the mean number of lymphocytes in tumour tissue from three random high power fields (HPF, 200×). Furthermore, the infiltration of stromal cells was evaluated as the mean proportion of stromal cells in tumour tissue from three randomised HPFs. Tumour necrosis in HE slides was characterised by degraded tumour cells, presented as amorphous coagulum with nuclear debris (Fig 3G)[27]. Pathologists annotated the necrosis area in each diagnostic slide and the proportion of necrosis was calculated by ImageJ (US National Institutes of Health, Bethesda, MD, USA). The above results were all assessed by two independent pathologists who were blinded to the clinical data and the results were averaged.

2.6 Genomic analysis

TCGA level 3 mutation data were downloaded from GDC Data Portal. The mutation load was defined as $\log_2(\text{non-silent mutation number} + 1)$. The neoantigen data was downloaded from The Cancer Immunome Atlas (TCIA, <https://tcia.at/>)[28]. The cancer-testis antigen (CTA) scores, homologous recombination deficiency (HRD) scores were downloaded from the supplementary material of a previous publication[23]. The intratumor heterogeneity (ITH), defined as the subclonal genome fraction measured by ABSOLUTE, was retrieved from an earlier publication[23]. The GISTIC2.0 results of In silico Admixture Removal (ISAR) calibrated SCN data (minus germline CNV) were obtained from a previous publication[23]. The GISTIC2.0 thresholded result of 2 or -2 was defined as deep CN alterations called amplifications and depletions. While the result of 1 or -1 was defined as shallow CN alterations, which were called gains and losses, respectively. The SCN load was calculated as the percentage of altered cytoband in each sample. T cell & B cell receptor (TCR&BCR) diversity scores (Shannon Entropy, Evenness, and Richness) were obtained from a previous publication[23]. Immune cytolytic activity score (CYT), which highly correlated with CD8+ T cell activation, was defined as the log-average (geometric mean) of GZMA and PRF1 expression as previously described[29].

2.7 Mutations and CNVs comparison between subtypes

Mutations and CNVs were compared between every two subtypes. To adjust the difference of mutation burden between subtypes, we adopted the method based on logistic regression as described in a previous publication[22]. Specifically, the subtype was modelled as a logistic function as $\text{subtype} \sim \text{mutation burden} + \text{gene mutation status}$. If the P-value of a specific gene mutation status adjusted for mutation burden less than 0.05, such gene was defined as differentially mutated between subtypes. Notably, gene mutations with an overall mutation rate of less than 2.5% were excluded from the comparison. CNVs were compared similarly. Gains/amplifications and losses/deletions were compared between every two subtypes separately. For gains/amplifications comparison,

losses/deletions events were set to zero. The subtype was modelled as a logistic function as subtype ~ CNV burden + gene gain/amplification status. If the P-value of a specific gene gain/amplifications adjusted for CNV burden less than 0.05, such gain/amplification was defined as differentially distributed between subtypes. The losses/deletions comparisons were performed similarly. The above calculations were performed using the "glm" function in R.

2.8 Statistical analysis

All statistical analyses were performed in R software, version 3.6.1 (The R Foundation for Statistical Computing, <http://www.rproject.org/>). Continuous and ordered categorical variables were compared using Student's t-tests or Kruskal–Wallis test with post hoc pairwise Dunn's-tests. Unordered categorical variables were compared by Pearson χ^2 test or Fisher exact test. Spearman correlation analyses were employed for evaluating correlations between continuous variables. Survival analyses were performed using Kaplan–Meier analyses and survival differences between groups were compared by log-rank tests. Prognostic factors were identified by univariate and multivariate Cox regression analyses. Factors with univariate regression $P < 0.1$ were further enrolled in multivariate analyses. A two-sided $P < 0.05$ was considered statistically significant unless additionally stated.

3 Results

3.1 Microenvironmental cell infiltration pattern in CRC

We used the ssGSEA algorithm for estimating the absolute infiltration abundance of 31 different TME cells in 1802 samples of the microarray dataset and 619 samples of the TCGA dataset. After normalisation, a nonnegative matrix factorisation (NMF) algorithm, an unsupervised clustering method, was applied to classify CRC samples into different subtypes based on TME cell infiltration. The optimal cluster number of 3 was selected by the Cophenetic correlation coefficient in both datasets (Figs. S3 and S4). Thus, we classified 1802 samples into three distinct TME cell (TMEC)

159 subtypes (Fig. 1A). Subtype 1, named as immune-active subtype (marked by red), was characterised
 160 by high infiltration of adaptive and innate immune cells, especially activated adaptive immune cells,
 161 such as activated CD4 T cells, activated CD8 T cells and activated B cells, and low infiltration of
 162 stromal cells. Subtype 2, named as immune-desert subtype (marked by blue), featured low infiltration
 163 of most immune and stromal cells. And subtype 3, named as stroma-rich subtype (marked by
 164 yellow), had high infiltration of adaptive and innate immune cells, as well as stromal cells but low
 165 infiltration of activated adaptive immune cells compared with subtype 1. The above infiltration
 166 pattern was also validated in the TCGA dataset (Fig. 1B).

167 To further validate the above subtypes, we evaluated the pathological presentation of three distinct
 168 subtypes in diagnostic slides of 499 TCGA samples. The representative pathology features of each
 169 subtype were illustrated in Fig. 1C. Tumour tissue in subtype 1 featured median stromal proportion
 170 with high tumor infiltrating leukocytes (TILs) infiltration. Subtype 2 had high tumour purity and low
 171 TME cell infiltration. And mesenchymal cells infiltrated extensively in subtype 3. Then we evaluated
 172 the TILs and stromal cell infiltration pathologically in TCGA diagnostic slides. TILs infiltrated most
 173 in subtype 1 and least in subtype 2 (median, 47 vs. 8 vs. 29, $P < 0.001$), while subtype 3 had
 174 significantly more stromal cell proportion (median, 0.047 vs. 0.037 vs. 0.098, $P < 0.001$).

175 Further, we explored other characteristics of each subtype. We used the ESTIMATE algorithm to
 176 calculate Immune Score, Stromal Score, and tumour purity for each sample. As shown in Fig. 1F,
 177 Subtype 1 had the highest Immune Score, median Stromal Score, and relatively low tumour purity.
 178 And subtype 2 featured the lowest Immune Score and Stromal Score but the highest tumour purity.
 179 Subtype 3 had a median Immune Score, highest Stromal Score, and relatively low tumour purity (all
 180 $P < 0.001$). Also, The macrophage M1/M2 ratio was the highest in subtype 1 ($P < 0.001$), while
 181 Th17/Treg ratio was the highest in subtype 2 ($P < 0.001$). Samples in subtype 3 own the highest

Th1/Th2 ratio ($P < 0.001$) and the proportion of activated adaptive immune cells was the highest in subtype 1 ($P < 0.001$). The above characteristics were all validated in the TCGA dataset (Fig. S6).

3.2 TMEC Subtypes and clinicopathological features

The correlation between TMEC subtypes and clinicopathological features of microarray and TCGA datasets was presented in Table S4 and S5, respectively. Patients in subtype 1 were relatively older (microarray dataset: mean $\pm$ SD, 66.83 ± 14.48 vs. 65.87 ± 13.70 vs. 64.22 ± 12.99 , $P = 0.005$; TCGA dataset: mean $\pm$ SD, 68.37 ± 13.51 vs. 65.25 ± 10.98 vs. 65.31 ± 12.63 , $P = 0.019$). Subtype 3 had more samples with advanced stages ($P < 0.001$ in both datasets). Subtype 1 was associated with more right colon cancer ($P < 0.001$ in both datasets). Also, the dMMR/MSI-H samples were significantly enriched in subtype 1 ($P < 0.001$ in both datasets). Moreover, we compared the distribution of Consensus Molecular Subgroups (CMS)[30] and TMEC subtypes, illustrated as Sankey plots in Fig. 1G and Fig. S7. Subtype 1 was mainly composed of CMS1 (MSI immune), CMS2 (Canonical), and CMS3 (Metabolic) samples. The majority of subtype 2 samples were from CMS2. And samples of CMS2 and CMS4 (Mesenchymal) constituted most of subtype 3. The above features were presented in both microarray and TCGA datasets.

3.3 TMEC Subtypes and CRC prognosis

TMEC subtypes also plays an important role in CRC prognosis. The analyses related to overall survival (OS) were performed in 1281 samples from GSE17538/GSE38832/GSE39084/GSE39582/KFSYSCC with OS data available, while the analyses related to disease-free survival (DFS) were performed in 1107 samples from GSE14333/GSE17538/GSE33113/GSE38832/GSE39084/GSE39582 with DFS data available. Subtype 3 conferred significantly worse OS and DFS, while subtype 1 and subtype 2 had similar prognoses (Fig. 2A and 2B). Next, we conducted univariate and multivariate Cox regression of

clinicopathological factors and TMEC subtypes for OS and DFS (Table S6 and Fig. 2C, respectively). TMEC SUBTYPE of subtype 3 was a significant prognostic indicator in univariate analyses of both OS ($P < 0.001$, HR = 1.449, 95% CI = 1.174 -1.787) and DFS ($P = 0.002$, HR=1.456, 95% CI = 1.153-1.839). Multivariate analyses showed subtype 2 and subtype 3 were independent prognostic factors of DFS ($P = 0.020$, HR=1.769, 95% CI=1.096-2.855 and $P = 0.013$, HR=1.722, 95% CI=1.121-2.646, respectively) (Fig. 2C) but not OS (Table S6). Subgroup analyses of OS and DFS revealed that subtype 3 indicated impaired survival in most scenarios (Fig. S8). Furthermore, we analysed the prognostic value of different TME cells in all samples and each subtype in the microarray dataset for OS (Fig. 2D) and DFS (Fig. 2E). Interestingly, the same cell type may play converse prognostic roles in different TMEC subtypes. In subtype 2, the majority of immune and stromal cells presented as protective factors of survival, even immunoinhibitory cells like MDSCs and M2 macrophages, which indicated heterogeneous immune networks in different TMEC subtypes.

3.4 Possible immune escape mechanism of distinct TMEC subtypes

The distinct characteristics of TMEC subtypes made us wonder whether they had different immune escape mechanisms. Previous studies summarised vital factors leading to tumour immune escape as follows: (1) defective antigen presentation; (2) tolerance and immune deviation; (3) infiltration of immune-suppressive cells; (4) immune-suppressive mediators secretion [31, 32]. As multi-omics data was available for the TCGA dataset, we explored the possible immune escape mechanism of three TMEC subtypes.

3.4.1 Defective antigen presentation

Defective antigen presentation was composed of at least two aspects: alteration of tumour immunogenicity and down-regulation of antigen presentation pathway. For tumour immunogenicity

evaluation, subtype 1 had the highest mutation burden (Fig. 3A) and neoantigen load (Fig. 3B) (both $P < 0.001$), which could be easily speculated for the highest proportion of dMMR/MSI-H samples. While subtype 3 featured the highest SCNv load (Fig. 3C). The CTA and HRD scores of subtype 3 were higher than subtype 1 ($P = 0.008$ and $P < 0.001$, respectively) but not subtype 2 (both $P > 0.05$) (Fig. 3D, Fig. 3E, respectively). Also, subtype 3 had the highest ITH level ($P < 0.001$) (Fig 3F). The necrosis level between subtypes was not significantly differentiated ($P = 0.167$) (Fig. 3G). Subtype 2 showed the lowest BCR&TCR richness diversity (both $P < 0.01$) (Fig. 3H), which were positively correlated with cytolytic activity (Fig. 3I). As for antigen presentation pathway-related gene expression, subtype 1 had higher expression of most MHC-related genes, while the expression of subtype 2 was the lowest (all $P < 0.01$) (Fig. 3J). Overall, three subtypes all had impaired antigen presentation to some extent, but subtype 2 had the lowest immunogenicity and antigen presentation gene expression level.

3.4.2 Tolerance and immune deviation

Tumours were also known to induce immune tolerance by upregulating immune inhibitors and downregulating immune stimulators. The relative expression levels and mean log2(copy number) value of immune inhibitors and immune stimulators were illustrated in Fig. 3J. Subtype 1 had the highest expression level of most immune inhibitors and part of immune stimulators, which indicated pro-tumour and anti-tumour immunity activation. The activation level of most above genes was the lowest in subtype 2. Subtype 3 highly expressed TNF/TNF-receptors. Some differentially expressed genes, such as SLAMF7, LAG3, CTLA4, BTLA, TNFSF8, et al. in two modules might attribute to SCNv. Correlation analyses found that immune cytolytic activity was positively associated with most immune checkpoint expression (Fig 3I). Generally, subtype 1 had a high expression of both immune stimulators and immune inhibitors, whose expression level was the lowest in subtype 2.

3.4.3 Infiltration of immune-suppressive cells

Next, we compared the infiltration of each cell type between subtypes, illustration as volcano plots (Fig.4A). Comparing to subtype 2 and subtype 3, subtype 1 had significantly more activated CD4 and CD8 T cells infiltration. Subtype 2, as an immune-cold subtype, had the least adaptive and innate immune cells infiltration among three subtypes. For subtype 3, it had enriched fibroblasts and endothelial cells than the other two subtypes. Immune-suppressive cells, such as Tregs, MDSCs, M2 macrophages, were more abundant in subtype 1 and 3. Also, we used the gene signature from a high-quality publication[33] for exhausted T cells infiltration speculation and compared the infiltration of exhausted T cells in each subtype (Fig. 4B). Subtype 1 had a significantly higher exhausted T cell proportion than the other two subtypes ($P < 0.001$), as well as the ratio of exhausted T cells to CD8 T cells ($P < 0.001$).

3.4.4 Immune suppressive mediators secretion

Furthermore, we investigated the fourth aspect of the immune escape mechanism. The relative expression levels to the median value and mean $\log_2(\text{copy number})$ value of chemokines and receptors, interferons and receptors, interleukins and receptors, and other cytokines in three subtypes were compared, and cytokines with $P < 0.01$ was illustrated in Fig. 4E. Most cytokines and receptors expression were negatively correlated between subtype 1 and subtype 3, and the expression level of subtype 2 was the lowest on most occasions. Subtype 1 expressed higher pro-immune chemokines like CXCL9, CXCL10, CXCL11, which could recruit effector T cells and NK cells[34]. The IFN- γ , which robustly stimulated anti-tumour immunity, was expressed higher in subtype 1. Pro-immunity IL-2 and IL-15 were accumulated in subtype 1. IL-34, CCL2, CCL-3, CXCL8, and some of their receptor CCR5, CXCR1, CXCR2, which promoted homeostasis of myeloid cells[35], were expressed extensively in subtype 3. Mesenchymal development-related PDGFs and PDGFRs[36] and cell adhesion-related SELP, ICAM1, ITGB2 were enriched in subtype 3. However, IDO, which

contributed to peripheral tolerance, was significantly highly expressed in subtype 1 ($P < 0.001$) (Fig. 4C), indicating the existence of inhibitory cytokines in the inflamed microenvironment. Notably, subtype 2 expressed a significantly higher level of VEGFA. Moreover, STING, a crucial factor in the cGAS–Sting pathway, which contributes to the initiation of innate immunity and recognition of the tumour, is highly expressed in subtype 1 ($P < 0.001$) (Fig. 4D), suggesting the impaired immunity initiation in subtype 2 and 3.

3.5 TMEC subtype-specific genomic alterations

Other than the heterogeneous immune escape mechanism among the TMEC subtypes, we further explored the subtype-specific genomic alterations. First, we explored the activation of each subtype in a comprehensive cancer development-related pathways collection [37, 38]. As presented in Fig. 5A, TP53, NRF2, PI3K pathways were upregulated in subtype 1, while MYC, PI3K pathways were enriched in subtype 2. Hippo, NOTCH, Hedgehog, TGF- β , Wnt, RAS pathways were activated in subtype 3. As for other cancer development-related pathways, cell cycle, genomic repair, protein expression, metabolism, and immunity pathways were generally upregulated in subtype 1. The immunity and stromal pathways, which were highly enriched in subtype 3, were relatively suppressed in subtype 2.

Next, we compared the subtype-specific gene mutations. Due to the high percentage of MSI-H patients in subtype 1, it is easy to speculate that most mutations were enriched in subtype 1. However, most mutations in MSI-H patients were random and irregular. Therefore, we focused on the comparison among MSS/MSI-L patients. As shown in Fig. 5B, after adjusting for mutation burden, KRAS, ZFHX4, SCN1A, MXRA5, WBSCR17, WDR87, COL5A2, KMT2B, DSCAML1, KCNT2, PCDHGA3, CECR2, AKAP3, and GNAS were highly mutated in subtype 1. And the

297 mutation frequency of HECW1, MYO5A, and ADAM29 was higher in subtype 2, While TP53
 298 mutated more in subtype 3 (all adjusted $P < 0.05$).

299 As the CNV load of subtype 1 was significantly lower than the other two subtypes, the proportion of
 300 alteration in most cytoband was the lowest among subtypes (Fig 5C). However, after adjusting for
 301 CNV burden, most cytobands in Chr12 were more frequently amplified in subtype 1. Also,
 302 18p11.31-18q12.2 amplification was enriched in subtype 1. Subtype 2 exhibited more frequent
 303 17p11.2-17q24.3 gain/amplification. Moreover, chr18 loss/deletion was more frequent in subtype 2
 304 and 3.

305 **4 Discussion**

306 The development of personalised cancer medicine has garnered the concern of CRC subtyping. The
 307 CRC subtyping system developed from pathological staging, mutation-based subtyping to omic-
 308 based subtyping[30]. However, the large-scale comprehensive presentation of CRC TME cell
 309 infiltration pattern and subsequent subtyping research was rare and insufficient. This study
 310 systematically showed the infiltration pattern of primary TME cells in two large independent cohorts,
 311 the microarray dataset and the TCGA dataset for cross-validation. The CRC samples can be mainly
 312 divided into three subtypes, immune-active, immune-desert, and stroma-rich. The major
 313 characteristics of each subtype were concluded in Fig. 6. The immune-active subtype features high
 314 activated adaptive immune cells infiltrate, more right colon, more dMMR/MSI-H, and more CMS1
 315 samples. The immune-desert subtype was characterised by low infiltration of most TME cells, more
 316 left colon, and more CMS2. And the stroma-rich subtype presents the distinctiveness of high
 317 infiltration of both immune cells and stromal cells, advanced stages, poor prognosis, and more CMS4
 318 samples. Similar TME subtypes can also be observed in ovarian cancer[39] and TNBC[22].

319 The potential immune escape mechanism of each subtype may have important clinical implications.

320 The immune-active subtype has increased immune-suppressive cells, high immune-suppressive

321 cytokine levels, and upregulating immune inhibitors, implying both intense tumour-suppressing and

322 tumour-promoting immune responses existing. Theoretically, such subtype may benefit most from

323 immune checkpoint inhibitors (ICI), the most studied immunotherapy targeting TME in CRC, and

324 MSI status is a solid predictive marker for ICI efficacy[40]. However, not all dMMR/MSI-H patients

325 respond to anti-PD1/anti-PD-L1 therapy. Previous researches reported an ORR of 31% in refractory

326 mCRC[31] and 44% in the first-line setting[41]. Most dMMR/MSI-H CRC is of immune-active

327 subtype, targeting the potential immune escape mechanism may be a novel strategy to improve the

328 efficacy. Strategies such as erasing the infiltration of immunosuppressive cells, neutralising

329 immunosuppressive cytokines, and combining other checkpoint inhibitors can be considered to

330 activate tumour immunity further.

331 The immune-desert subtype has decreased immune-promotive cells, low immune-promotive cytokine

332 level, downregulating immune inhibitors, and defective antigen presentation. Nevertheless, the OS is

333 similar to the immune-active subtype, implying the survival impairment of stromal cells has

334 neutralised the survival benefit of high infiltration of activated adaptive immune cells. Even the

335 infiltration of immunosuppressive cells, such as MDSC and Treg, may improve prognosis, which

336 shows the complexity and heterogeneity of CRC TME. The major difference between the immune-

337 desert subtype ("cold tumour") to other two subtypes ("hot tumour") is the antigen presentation

338 defect. Therefore, increasing tumour immunogenicity may induce the immune cell chemotaxis and

339 transform a "cold tumour" into a "hot tumour". Chemotherapy and radiotherapy can cause tumour

340 cell death with subsequent release of cellular fragmentation and cancer-associated neoantigens,

341 which are presented to APCs to increase tumour immunogenicity[42]. The use of pembrolizumab

342 plus FOLFOX achieved ORR 55% in the first-line setting[43]. However, the synergistic effect of

343 pembrolizumab and radiotherapy was not as expected, which only achieved an ORR of 4.5% in 22
344 pMMR/MSS mCRC patients[44]. Further researches of a dual checkpoint inhibitor (anti-PD-L1 +
345 anti-CTLA-4) following radiotherapy are underway (NCT02701400, NCT03122509). Other novel
346 approaches like tumour vaccine, and oncolytic virus are under early-phase research.

347 The stroma-rich subtype, which takes up nearly half the samples, has a similar immune escape
348 mechanism with the immune-active subtype but decreased immune-promotive cells. This subtype is
349 "hot" but owns the worst survival. The primary reason may be the pro-tumoural effect of excessive
350 stromal cell infiltration, excluding activated adaptive immune cells from the tumour. Anti-fibroblast,
351 anti-TGF- β pathway, anti-angiogenesis, anti-immunosuppressive cytokines, or the combination of the
352 above therapy may be hoping to transform it into an immune-active subtype. Dual antagonising of
353 TGF- β and PD-1/PD-L1 showed promising results in preclinical researches[45]. The combination of
354 vactosertib (a small-molecule inhibitor of TGF- β) and pembrolizumab showed an ORR of 15.2% in
355 previously treated MSS mCRC patients[46]. Furthermore, REGONIVO (regorafenib + nivolumab)
356 trial reported inspiring 36% ORR in unselected mCRC patients[47], which implies the potential of
357 ICI and anti-angiogenesis. Previous studies showed that cancer-associated fibroblasts (CAFs) could
358 secrete tumourigenic factors, modulate immunosuppressive TME, and create an ECM barrier to block
359 CD8 T cells accessing the tumour[48]. Multiple strategies targeting CAFs are under research, such as
360 normalising the phenotype of CAFs, inhibiting CAF generation and activation, reducing CAF
361 secretome[49], et al., and awaiting the results.

362 Also, the limitation of this study cannot be ignored. The major one is that the subtyping was based on
363 transcriptomic data, which needed to be further validated pathologically. Moreover, the immune
364 escape mechanism speculation was mainly based on in silico analysis, and numerous results only
365 reflected the correlation but not the causation. Further in vitro and in vivo experiments were needed.

366 In conclusion, we systematically presented the infiltration pattern of CRC TME cells. CRC samples
367 can be divided into three subtypes with distinct TME cell infiltration patterns, clinical features,
368 genomic characteristics, as well as their immune escape mechanism underneath. The results may
369 provide inspiration and direction for further researches on CRC immunotherapy.

370 **5 Declarations**

371 **5.1 Ethics approval and consent to participate**

372 Not applicable.

373 **5.2 Consent for publication**

374 All authors gave consent for publication.

375 **5.3 Availability of data and materials**

376 All datasets mentioned in this article were publicly available.

377 **5.4 Competing interests**

378 The authors declare that they have no competing interests.

379 **5.5 Funding**

380 This work was supported by the National Natural Science Foundation of China (No. 82002517,
381 82072678), Shanghai Sailing Program (20YF1407100), Clinical Research Plan of SHDC
382 (SHDC2020CR1033B, SHDC2020CR5006) and Shanghai Science and Technology Committee
383 Project (18140903200).

384 **5.6 Authors' contributions**

YM, GH and JX contributed to the planning of the study. YM, QF and YX collected the data and performed most of the bioinformatics analysis. YL and WC verified the numerical results by an independent implementation. YM, QF, YX, and YL drafted and revised the manuscript. All authors contributed to the interpretation of data and review of the manuscript. All authors reviewed and approved the final manuscript.

5.7 Acknowledgements

Not applicable.

6 References

1. Siegel RL, Miller KD, Fuchs HE, Jemal A: **Cancer Statistics, 2021**. *CA: A Cancer Journal for Clinicians* 2021, **71**(1):7-33.
2. Quail DF, Joyce JA: **Microenvironmental regulation of tumor progression and metastasis**. *Nat Med* 2013, **19**(11):1423-1437.
3. Feng Q, Chang W, Mao Y, He G, Zheng P, Tang W, Wei Y, Ren L, Zhu D, Ji M *et al*: **Tumor-associated Macrophages as Prognostic and Predictive Biomarkers for Postoperative Adjuvant Chemotherapy in Patients with Stage II Colon Cancer**. *Clinical Cancer Research* 2019, **25**(13):3896-3907.
4. Bruni D, Angell HK, Galon J: **The immune contexture and Immunoscore in cancer prognosis and therapeutic efficacy**. *Nat Rev Cancer* 2020.
5. Pages F, Mlecnik B, Marliot F, Bindea G, Ou FS, Bifulco C, Lugli A, Zlobec I, Rau TT, Berger MD *et al*: **International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study**. *Lancet* 2018, **391**(10135):2128-2139.
6. Argiles G, Tabernero J, Labianca R, Hochhauser D, Salazar R, Iveson T, Laurent-Puig P, Quirke P, Yoshino T, Taieb J *et al*: **Localised colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up**. *Ann Oncol* 2020.
7. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, Hoang CD, Diehn M, Alizadeh AA: **Robust enumeration of cell subsets from tissue expression profiles**. *Nat Methods* 2015, **12**(5):453-457.
8. Becht E, Giraldo NA, Lacroix L, Buttard B, Elarouci N, Petitprez F, Selves J, Laurent-Puig P, Sautes-Fridman C, Fridman WH *et al*: **Estimating the population abundance of tissue-infiltrating immune and stromal cell populations using gene expression**. *Genome Biol* 2016, **17**(1):218.
9. Li T, Fan J, Wang B, Traugh N, Chen Q, Liu JS, Li B, Liu XS: **TIMER: A Web Server for Comprehensive Analysis of Tumor-Infiltrating Immune Cells**. *Cancer Res* 2017, **77**(21):e108-e110.

- 420 10. Jorissen RN, Gibbs P, Christie M, Prakash S, Lipton L, Desai J, Kerr D, Aaltonen LA,
421 Arango D, Kruhøffer M *et al*: **Metastasis-Associated Gene Expression Changes Predict**
422 **Poor Outcomes in Patients with Dukes Stage B and C Colorectal Cancer.** *Clin Cancer*
423 *Res* 2009, **15**(24):7642-7651.
- 424 11. Smith JJ, Deane NG, Wu F, Merchant NB, Zhang B, Jiang A, Lu P, Johnson JC, Schmidt C,
425 Bailey CE *et al*: **Experimentally derived metastasis gene expression profile predicts**
426 **recurrence and death in patients with colon cancer.** *Gastroenterology* 2010, **138**(3):958-
427 968.
- 428 12. de Sousa E Melo F, Colak S, Buikhuisen J, Koster J, Cameron K, de Jong JH, Tuynman JB,
429 Prasetyanti PR, Fessler E, van den Bergh SP *et al*: **Methylation of cancer-stem-cell-**
430 **associated Wnt target genes predicts poor prognosis in colorectal cancer patients.** *Cell*
431 *Stem Cell* 2011, **9**(5):476-485.
- 432 13. Laibe S, Lagarde A, Ferrari A, Monges G, Birnbaum D, Olschwang S: **A seven-gene**
433 **signature aggregates a subgroup of stage II colon cancers with stage III.** *OMICS* 2012,
434 **16**(10):560-565.
- 435 14. Tripathi MK, Deane NG, Zhu J, An H, Mima S, Wang X, Padmanabhan S, Shi Z, Prodduturi
436 N, Ciombor KK *et al*: **Nuclear factor of activated T-cell activity is associated with**
437 **metastatic capacity in colon cancer.** *Cancer Res* 2014, **74**(23):6947-6957.
- 438 15. Kirzin S, Marisa L, Guimbaud R, De Reynies A, Legrain M, Laurent-Puig P, Cordelier P,
439 Pradère B, Bonnet D, Meggetto F *et al*: **Sporadic early-onset colorectal cancer is a specific**
440 **sub-type of cancer: a morphological, molecular and genetics study.** *PLoS ONE* 2014,
441 **9**(8):e103159.
- 442 16. Marisa L, de Reyniès A, Duval A, Selves J, Gaub MP, Vescovo L, Etienne-Grimaldi M-C,
443 Schiappa R, Guenot D, Ayadi M *et al*: **Gene expression classification of colon cancer into**
444 **molecular subtypes: characterization, validation, and prognostic value.** *PLoS Med* 2013,
445 **10**(5):e1001453.
- 446 17. Cancer Genome Atlas N: **Comprehensive molecular characterization of human colon and**
447 **rectal cancer.** *Nature* 2012, **487**(7407):330-337.
- 448 18. Guinney J, Ferté C, Dry J, McEwen R, Manceau G, Kao KJ, Chang K-M, Bendtsen C,
449 Hudson K, Huang E *et al*: **Modeling RAS phenotype in colorectal cancer uncovers novel**
450 **molecular traits of RAS dependency and improves prediction of response to targeted**
451 **agents in patients.** *Clin Cancer Res* 2014, **20**(1):265-272.
- 452 19. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK: **limma powers**
453 **differential expression analyses for RNA-sequencing and microarray studies.** *Nucleic*
454 *Acids Res* 2015, **43**(7):e47.
- 455 20. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD: **The sva package for removing**
456 **batch effects and other unwanted variation in high-throughput experiments.**
457 *Bioinformatics* 2012, **28**(6):882-883.
- 458 21. Hänzelmann S, Castelo R, Guinney J: **GSVA: gene set variation analysis for microarray**
459 **and RNA-seq data.** *BMC Bioinformatics* 2013, **14**:7.
- 460 22. Xiao Y, Ma D, Zhao S, Suo C, Shi J, Xue M-Z, Ruan M, Wang H, Zhao J, Li Q *et al*: **Multi-**
461 **Omics Profiling Reveals Distinct Microenvironment Characterization and Suggests**

- 462 **Immune Escape Mechanisms of Triple-Negative Breast Cancer.** *Clin Cancer Res* 2019,
463 **25**(16):5002-5014.
- 464 23. Thorsson V, Gibbs DL, Brown SD, Wolf D, Bortone DS, Ou Yang T-H, Porta-Pardo E, Gao
465 GF, Plaisier CL, Eddy JA *et al*: **The Immune Landscape of Cancer.** *Immunity* 2018, **48**(4).
- 466 24. Charoentong P, Finotello F, Angelova M, Mayer C, Efremova M, Rieder D, Hackl H,
467 Trajanoski Z: **Pan-cancer Immunogenomic Analyses Reveal Genotype-**
468 **Immunophenotype Relationships and Predictors of Response to Checkpoint Blockade.**
469 *Cell Rep* 2017, **18**(1):248-262.
- 470 25. Becht E, Giraldo NA, Lacroix L, Buttard B, Elarouci N, Petitprez F, Selves J, Laurent-Puig P,
471 Sautès-Fridman C, Fridman WH *et al*: **Estimating the population abundance of tissue-**
472 **infiltrating immune and stromal cell populations using gene expression.** *Genome Biol*
473 2016, **17**(1):218.
- 474 26. Gaujoux R, Seoighe C: **A flexible R package for nonnegative matrix factorization.** *BMC*
475 *Bioinformatics* 2010, **11**:367.
- 476 27. Vayrynen SA, Vayrynen JP, Klintrup K, Makela J, Karttunen TJ, Tuomisto A, Makinen MJ:
477 **Clinical impact and network of determinants of tumour necrosis in colorectal cancer.** *Br*
478 *J Cancer* 2016, **114**(12):1334-1342.
- 479 28. Charoentong P, Finotello F, Angelova M, Mayer C, Efremova M, Rieder D, Hackl H,
480 Trajanoski Z: **Pan-cancer Immunogenomic Analyses Reveal Genotype-**
481 **Immunophenotype Relationships and Predictors of Response to Checkpoint Blockade.**
482 *Cell Rep* 2017, **18**(1):248-262.
- 483 29. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N: **Molecular and genetic properties of**
484 **tumors associated with local immune cytolytic activity.** *Cell* 2015, **160**(1-2):48-61.
- 485 30. Guinney J, Dienstmann R, Wang X, de Reynies A, Schlicker A, Soneson C, Marisa L,
486 Roepman P, Nyamundanda G, Angelino P *et al*: **The consensus molecular subtypes of**
487 **colorectal cancer.** *Nat Med* 2015, **21**(11):1350-1356.
- 488 31. O'Donnell JS, Teng MWL, Smyth MJ: **Cancer immunoediting and resistance to T cell-**
489 **based immunotherapy.** *Nat Rev Clin Oncol* 2019, **16**(3):151-167.
- 490 32. Vinay DS, Ryan EP, Pawelec G, Talib WH, Stagg J, Elkord E, Lichter T, Decker WK,
491 Whelan RL, Kumara H *et al*: **Immune evasion in cancer: Mechanistic basis and**
492 **therapeutic strategies.** *Semin Cancer Biol* 2015, **35** Suppl:S185-S198.
- 493 33. Newman AM, Steen CB, Liu CL, Gentles AJ, Chaudhuri AA, Scherer F, Khodadoust MS,
494 Esfahani MS, Luca BA, Steiner D *et al*: **Determining cell type abundance and expression**
495 **from bulk tissues with digital cytometry.** *Nat Biotechnol* 2019, **37**(7):773-782.
- 496 34. Nagarsheth N, Wicha MS, Zou W: **Chemokines in the cancer microenvironment and their**
497 **relevance in cancer immunotherapy.** *Nat Rev Immunol* 2017, **17**(9):559-572.
- 498 35. Berraondo P, Sanmamed MF, Ochoa MC, Etxeberria I, Aznar MA, Pérez-Gracia JL,
499 Rodríguez-Ruiz ME, Ponz-Sarvisé M, Castañón E, Melero I: **Cytokines in clinical cancer**
500 **immunotherapy.** *Br J Cancer* 2019, **120**(1).
- 501 36. Manzat Saplacan RM, Balacescu L, Gherman C, Chira RI, Craiu A, Mircea PA, Lisencu C,
502 Balacescu O: **The Role of PDGFs and PDGFRs in Colorectal Cancer.** *Mediators Inflamm*
503 2017, **2017**:4708076.

- 504 37. Sanchez-Vega F, Mina M, Armenia J, Chatila WK, Luna A, La KC, Dimitriadou S, Liu DL,
505 Kantheti HS, Saghafeina S *et al*: **Oncogenic Signaling Pathways in The Cancer Genome**
506 **Atlas**. *Cell* 2018, **173**(2):321-337 e310.
- 507 38. Tamborero D, Rubio-Perez C, Muiños F, Sabarinathan R, Piulats JM, Muntasell A,
508 Dienstmann R, Lopez-Bigas N, Gonzalez-Perez A: **A Pan-cancer Landscape of**
509 **Interactions between Solid Tumors and Infiltrating Immune Cell Populations**. *Clin*
510 *Cancer Res* 2018, **24**(15):3717-3728.
- 511 39. Desbois M, Udyavar AR, Ryner L, Kozlowski C, Guan Y, Dürrbaum M, Lu S, Fortin JP,
512 Koeppen H, Ziai J *et al*: **Integrated digital pathology and transcriptome analysis**
513 **identifies molecular mediators of T-cell exclusion in ovarian cancer**. *Nat Commun* 2020,
514 **11**(1):5583.
- 515 40. Sahin IH, Akce M, Alese O, Shaib W, Lesinski GB, El-Rayes B, Wu C: **Immune checkpoint**
516 **inhibitors for the treatment of MSI-H/MMR-D colorectal cancer and a perspective on**
517 **resistance mechanisms**. *Br J Cancer* 2019, **121**(10):809-818.
- 518 41. Shiu K-K, Andre T, Kim TW, Jensen BV, Jensen LH, Punt CJA, Smith DM, Garcia-
519 Carbonero R, Benavides M, Gibbs P *et al*: **KEYNOTE-177: Phase III randomized study of**
520 **pembrolizumab versus chemotherapy for microsatellite instability-high advanced**
521 **colorectal cancer**. *Journal of Clinical Oncology* 2021, **39**(3_suppl):6-6.
- 522 42. Franke AJ, Skelton WP, Starr JS, Parekh H, Lee JJ, Overman MJ, Allegra C, George TJ:
523 **Immunotherapy for Colorectal Cancer: A Review of Current and Novel Therapeutic**
524 **Approaches**. *J Natl Cancer Inst* 2019, **111**(11):1131-1141.
- 525 43. Shahda S, Noonan AM, Bekaii-Saab TS, O'Neil BH, Sehdev A, Shaib WL, Helft PR, Loehrer
526 PJ, Tong Y, Liu Z *et al*: **A phase II study of pembrolizumab in combination with**
527 **mFOLFOX6 for patients with advanced colorectal cancer**. *Journal of Clinical Oncology*
528 2017, **35**(15_suppl):3541-3541.
- 529 44. Segal NH, Kemeny NE, Cercek A, Reidy DL, Raasch PJ, Warren P, Hrabovsky AE,
530 Campbell N, Shia J, Goodman KA *et al*: **Non-randomized phase II study to assess the**
531 **efficacy of pembrolizumab (Pem) plus radiotherapy (RT) or ablation in mismatch**
532 **repair proficient (pMMR) metastatic colorectal cancer (mCRC) patients**. *Journal of*
533 *Clinical Oncology* 2016, **34**(15_suppl):3539-3539.
- 534 45. Liu S, Ren J, Ten Dijke P: **Targeting TGFβ signal transduction for cancer therapy**. *Signal*
535 *Transduct Target Ther* 2021, **6**(1):8.
- 536 46. Kim TW, Lee KW, Ahn JB, Lee J, Ryu J, Oh B, Ock C-Y, Hwang S, Hahm KB, Kim S-J *et*
537 *al*: **Efficacy and safety of vactosertib and pembrolizumab combination in patients with**
538 **previously treated microsatellite stable metastatic colorectal cancer**. *Journal of Clinical*
539 *Oncology* 2021, **39**(15_suppl):3573-3573.
- 540 47. Fukuoka S, Hara H, Takahashi N, Kojima T, Kawazoe A, Asayama M, Yoshii T, Kotani D,
541 Tamura H, Mikamoto Y *et al*: **Regorafenib Plus Nivolumab in Patients With Advanced**
542 **Gastric or Colorectal Cancer: An Open-Label, Dose-Escalation, and Dose-Expansion**
543 **Phase Ib Trial (REGONIVO, EPOC1603)**. *J Clin Oncol* 2020, **38**(18):2053-2061.
- 544 48. Ford K, Hanley CJ, Mellone M, Szyndralewicz C, Heitz F, Wiesel P, Wood O, Machado M,
545 Lopez M-A, Ganesan A-P *et al*: **NOX4 Inhibition Potentiates Immunotherapy by**
546 **Overcoming Cancer-Associated Fibroblast-Mediated CD8 T-cell Exclusion from**
547 **Tumors**. *Cancer Res* 2020, **80**(9):1846-1860.

49. Domen A, Quatannens D, Zanivan S, Deben C, Van Audenaerde J, Smits E, Wouters A, Lardon F, Roeyen G, Verhoeven Y *et al*: **Cancer-Associated Fibroblasts as a Common Orchestrator of Therapy Resistance in Lung and Pancreatic Cancer.** *Cancers (Basel)* 2021, **13**(5).

7 Captions

Figure 1. The landscape of TME cell infiltration pattern in CRC. (A-B) Consensus heatmap illustrating the infiltration of 31 TME cells in the microarray dataset (A) and the TCGA dataset (B). Both of them were divided into three subtypes. (C) The representative pathology presentation of each subtype in the TCGA dataset (red, subtype 1; blue, subtype 2; yellow, subtype3; from top to bottom). (D) The number of TILs among three subtypes per high power field (HPF, 200×) in the TCGA dataset. (E) The proportion of stromal cells among three subtypes per high power field (HPF, 200×) in the TCGA dataset. (F) The distribution of Immune Score, Stromal Score, tumour purity, macrophage M1/M2 ratio, Th17/Treg ratio, Th1/Th2 ratio, adaptive immune cells, activated adaptive immune cells, innate immune cells, and stromal cells among subtypes in the microarray dataset. (G) Sankey plot illustrating the distribution of CMS subtypes and TMEC subtypes in the microarray dataset.

Figure 2. (A-B) Kaplan-Meier analysis of OS (A) and DFS (B) in the microarray dataset. (C) Univariate and multivariate Cox regression of clinicopathological factors and TMEC subtypes for DFS in the microarray dataset. (D-E) The prognostic value of different TME cells in all samples and each subtype in the microarray dataset for OS (D) and DFS (E).

Figure 3. (A-F) The mutation load (A), neoantigen load (B), SCNv load (C), CTA (D), HRD (E) and ITH (F) among TMEC subtypes. (G) The representative pathology image of tumor necrosis (left) and the distribution of necrosis percentage of each subtype. (H) The BCR and TCR richness of different subtypes. (I) The correlation between immune cytolytic activity and tumour immunogenicity factors, TILs, stromal cells, and immune checkpoint molecules. (J) The relative expression levels to the median value and mean log₂(copy number) value of immune inhibitors and immune stimulators among three subtypes. ***: $P < 0.001$; **: $0.001 < P < 0.01$; *: $0.01 < P < 0.05$; ns: $P > 0.05$.

Figure 4. (A) The comparison of each cell type between subtypes. (B) The distribution of exhausted T cells infiltration and the ratio of exhausted T cells to CD8 T cells infiltration among each subtype. (C-D) The expression of IDO and STING among each subtype. (E) The relative expression levels to the median value and mean log₂(copy number) value of chemokines, interleukins, other cytokines and their receptors among three subtypes. ***: $P < 0.001$; **: $0.001 < P < 0.01$; *: $0.01 < P < 0.05$; ns: $P > 0.05$.

Figure 5. TMEC subtype-specific genomic alterations. (A) Heatmap illustrating activation of comprehensive cancer development-related pathways among three TMEC subtypes. (B) Subtype-specific gene mutation frequency among MSS/MSI-L samples in three TMEC subtypes. (C) Subtype-specific SCNVs of each TMEC subtype (top three plots) and comparison of SCNVs between every two subtypes.

Figure 6. Summary of clinical features, immune escape mechanisms and subtype-specific genomic alterations of three TMEC subtypes.

A Microarray dataset

B TCGA dataset

F

C

Univariate and multivariate Cox regression analysis for DFS

Factors	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)	P
Age (years)				
≤ 60	1 (reference)			
> 60	1.106 (0.881 - 1.388)	0.386		
Gender				
Male	1 (reference)			
Female	0.887 (0.724 - 1.087)	0.246		
Primary site				
Right colon	1 (reference)			
Left colon	1.089 (0.799 - 1.484)	0.591		
Rectum	/	/		
TNM Stage				
I	1 (reference)		1 (reference)	
II	1.027 (0.734 - 1.440)	0.874	3.957 (0.962 - 16.270)	0.057
III	1.501 (1.071 - 2.104)	0.019	7.286 (1.786 - 29.718)	0.006
MMR status				
pMMR	1 (reference)		1 (reference)	
dMMR	0.514 (0.290 - 0.910)	0.022	0.675 (0.374 - 1.218)	0.192
TMEC subtype				
Subtype 1	1 (reference)			
Subtype 2	1.220 (0.923 - 1.613)	0.163	1.769 (1.096 - 2.855)	0.020
Subtype 3	1.456 (1.153 - 1.839)	0.002	1.722 (1.121 - 2.646)	0.013

A**Subtype 1 vs. Subtype 2** **Subtype 1 vs. Subtype 3** **Subtype 2 vs. Subtype 3** **B** **C** **D** **E**