Evaluation of stromal cell infiltration in the tumor microenvironment enable prediction of treatment sensitivity and prognosis in colon cancer

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Current clinical factors for screening candidates that might benefit from adjuvant chemotherapy in colon cancer are inadequate. Tumor microenvironment, especially the stromal components, has the potential to determine treatment response. However, clinical translation of the tumor-associated stromal characterization into a practical biomarker for helping treatment decision has not been established. Using machine learning, we established a novel 31-gene signature, called stromal cell infiltration intensity score (SIIS), to distinguish patients characterized by the enrichment of abundant stromal cells in five colon cancer datasets from GEO (N = 990). Patients with high-SIIS were at higher risk for recurrence and mortality, and could not benefit from adjuvant chemotherapy due to their intrinsic drug resistance; however, the opposite was reported for patients with low-SIIS. The role of SIIS in detection of patients with high stromal cell infiltration and reduced drug efficiency was consistently validated in the TCGA-COAD cohort (N = 382), Sun Yat-sen University Cancer Center cohort (N = 30), and could also be observed in TCGA pan-cancer settings (N = 4898) and four independent immunotherapy cohorts (N = 467). Based on multi-omics data analysis and the CRISPR library screen, we reported that lack of gene mutation, hypomethylation in ADCY4 promoter region, activation of WNT-PCP pathway and SIAH2-GPX3 axis were potential mechanisms responsible for the chemoresistance of patients within high-SIIS group. Our findings demonstrated that SIIS provide an important reference for those making treatment decisions for such special patients.

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1. Introduction

Colon cancer is a disease characterized by considerable molecular heterogeneity that is related to the prognosis and treatment outcomes [1]. Although several molecular classification strategies have been proposed to characterize distinct biological properties [2], more effective and clinically accessible classifiers that could complement the TNM system to enable the personalization of fluorouracil (FU)-based postsurgical adjuvant chemotherapy (ADJC) in localized colon cancer remain to be explored.

The extensive literature has underscored the clinical importance of tumor microenvironment (TME) features of various cancer types [3]. For colon cancer, a consensus immunoscore, has been demonstrated to be a robust prognostic indicator independent of TNM staging, thereby making colon cancer a paradigmatic tumor for immune classification [4]. Besides its prognostic value, immunoscore was also revealed to be a reference for ADJC performance [5]. The consensus immunoscore only quantifies the densities of CD3\textsuperscript{+} and CD8\textsuperscript{+} T cells, therefore it mainly reflects degree of the local intratumoral adaptive immune reaction. However, in addition to T cells, some other cellular components in TME might also play an important role in tumorigenesis and progression. For example, our previous studies showed that the infiltration intensity of stromal cells is greater than that of T cells with respect to differentiating distinct TME subtypes and predicting the likelihood of the chemotherapeutic benefit of patients with colon cancer [6,7]. However, to date, a potential clinical translation of the stromal context-
ture into a marker for prognosis and therapeutic benefit prediction has not been established.

The consensus immunoscore is detected by immunohistochemical staining, a method that relies heavily on a limited repertoire of phenotypic markers and biopsy specimens of sufficient size. In other words, the immunohistochemical staining can only reflect a limited number of cell types so that most of the information regarding TME panoramic landscape features is ignored. Besides, immunohistochemical staining is typically applied to analyze a specific two-dimensional tissue section, so it cannot be used to estimate the TME content across three-dimensional tissue samples. To address these issues, several computational approaches have recently been developed to characterize TME compositions in a high-throughput manner directly from bulk tissue gene expression profiles [8]. Cell content data generated by these methods to establish clinical models has revealed that they are correlated with the clinical outcomes of tumor patients [9,10]. However, as the operation of these computational tools requires the whole transcriptome data as the input, they are not considered clinically practical. Hence, in this study, we first employed the enrichment methods single-sample gene set enrichment analysis (ssGSEA) to calculate the enrichment scores for 23 immune and stromal cells based on clinically annotated colon cancer gene expression profiles, and then identified a special patient population which is a robust prognostic biomarker for distinguishing patients who are at high risk of relapse and cannot benefit from ADJC simultaneously because of their intrinsic drug resistance.

2. Materials and methods

2.1. Patient cohorts used in this study

Public transcriptome data on colon cancer samples were retrospectively collected from the GEO (https://www.ncbi.nlm.nih.gov/geo) and TCGA-COAD (https://cancergenome.nih.gov/) datasets. For samples from the Sun Yat-sen University Cancer Center cohort, written informed consent was obtained from all patients. This study was approved by the Nanfang Hospital Ethics Review Board. RNA extraction and sequencing were performed as previously described [11]. All colon cancer samples included in the analysis in this study were obtained before ADJC performance. Details are provided in the Supplementary Materials and Methods.

2.2. Generation of the 31-gene-based quantitative indicator

A four-phase screening strategy was designed to filter out the informative genes to build the “stromal infiltration intensity score (SIIS)”. Briefly, a four-phase screening strategy was designed to filter out the informative genes to build the “stromal infiltration intensity score (SIIS)”, including differential gene expression analysis; robust prognostic gene identification; elastic net analysis (iteration = 1000); and Cox proportional hazards model (iteration = 1000) with the least absolute shrinkage and selection operator (LASSO) penalty analysis Differential expression gene (DEG) analysis among TMECS1 and TMECS2 clusters was conducted in the GSE39582 dataset using the “LIMMA” package. The significance criterion for DEGs was set as an absolute “log2FC” value >0.5 and an adjusted p value < 0.05. Then, robust prognostic DEGs were identified using the bootstrapping method (repeated 1000 times) as described previously, and DEGs that were incorporated in 70% of resample runs (achieved p < 0.05 in robustness testing) were considered as robust prognostic genes and selected for further analysis. The elastic net analysis (iteration = 1000) and LASSO-Cox analysis (iteration = 1000) were performed by utilizing a R package called “glmnet”. The elastic net regularization for generalized linear models is the linear combination of LASSO and ridge regularization methods. An elastic net model was developed to screen out the most informative genes for distinguishing patients within TMECS1 and TMECS2. The alpha parameter of the elastic net was set at 0.9.

The penalized Cox regression model with LASSO penalty was used to select the most useful prognostic markers among informative DEGs picked by the elastic net model, and the optimal values of the penalty parameter \( \lambda \) were determined by tenfold cross-validations. The expression levels of informative DEGs were analyzed as binary variables in LASSO-Cox regression; the optimal cut-off values were evaluated based on the association between relapse-free survival and gene expression in the GEO cohort using the “survminer” package. Among the 31 genes finally selected by repeated LASSO-Cox regression, 11 genes had positive Cox coefficients (named DEGs\(_{+}\)) and the other 20 genes had negative Cox coefficients (named DEGs\(_{-}\)). The final score was defined as, SIIS = the average expression of DEGs\(_{+}\) – the average expression of DEGs\(_{-}\).

2.3. The SubMap and nearest template prediction (NTP) analysis

The SubMap analysis and NTP analysis were used to explore the internal relationship between SIIS and TMECS algorithm. The implementation of these two algorithms is achieved through the online module of GenePattern website (https://cloud.genepattern.org/). Details are provided in the Supplementary Materials and Methods.

2.4. Prediction of the chemotherapeutic response

Chemoresponse was predicted using the R package “pRPropHetic” which implemented a built-in ridge regression model based on the CTRP database and GDSC database respectively. Details are provided in the Supplementary Materials and Methods.

2.5. Pathway, immune, and metabolic score estimation

The pathway, immune, and metabolic score estimation were achieved by different R packages. Details are provided in the Supplementary Materials and Methods.

2.6. Multi-omics analysis

The genomic mutation, DNA methylation, and proteomic data were download from TCGA-COAD dataset, details are provided in the Supplementary Materials and Methods.

2.7. CRISPR synergistic activation mediator library screen for identification of chemoresistance genes

The CRISPR synergistic activation mediator (SAM) library which contains 70,290 unique sgRNA sequences targeting 23,430 human genes (3 sgRNAs per gene) was used to generate a mutant cell pool for high-throughput screening. The count value of sgRNAs was transformed using the “voom” algorithm, and differentially enriched sgRNAs between chemotherapy drug- and vehicle-treated groups were analyzed using the “LIMMA” package. The adjusted p-value for multiple testing was calculated using the Benjamini–Hochberg correction. The criteria for screening candidate sgRNAs were: 1) the average count values of candidate sgRNAs in
both the chemotherapy drug-treated group and the vehicle group should be greater than 1,000; 2) the absolute “log2FC” value calculated by difference analysis of sgRNA level of chemotherapy drug-treated group versus vehicle group was more than 0.5. Details are provided in the Supplementary Materials and Methods.

2.8. Connectivity map analysis

Connectivity map (Cmap) analysis was performed using the 150 genes with the most significant fold changes (up-regulated and down-regulated) according to the instructions provided by the Cmap website (https://clue.io/).

2.9. In vitro and in vivo experiments

To address the role of GPX3 in mediating chemoresistance in colon cancer cells, several in vitro and in vivo experiments, including cell culture, cell transfection, Methylthiazolyl-tetrazolium (MTT) assay, clonogenic assay, western blotting, real-time quantitative reverse-transcription polymerase chain reaction, immunohistochemistry staining, and subcutaneous tumor formation assays, were performed as previously described [12].

2.10. Statistical analysis

All statistical tests were performed using R software (version 3.6.0) or SPSS software (version 25.0). Details are provided in the Supplementary Materials and Methods.

3. Results

3.1. Identification of distinct TME clusters associated with chemotherapeutic benefit

Using an unsupervised hierarchical cluster analysis (Fig. 1A), we identified three distinct TME clusters based on 990 samples (Tables S1-Tables S2) in the meta-GEO cohort, denoted as tumor microenvironment cell subtype1 (TMECS1; n = 369), tumor microenvironment cell subtype2 (TMECS2; n = 218), and tumor microenvironment cell subtype3 (TMECS3; n = 403). As shown in Fig. 1B and 1C, TMECS1 was characterized by enrichment of the highest level of stromal cells and innate immune cells with immunosuppressive properties (these cell types are classified into cell cluster 1), moderate level of cytotoxic lymphocytes and activated dendritic cell (these cell types were classified into cell cluster 2), and the lowest level of Th2 cells, central memory T cells, and total T helper cells (these cell types were classified into cell cluster 3), which was recognized as immune-excluded phenotype [13]. Compared with TMECS1, TMECS2 had significantly higher abundance of cell cluster 2 and 3 infiltration, while cell cluster 1 infiltration was significantly decreased, indicating that the TME feature of TMECS2 was recognized as immune-inflamed phenotype [13]. Unlike TMECS1 and TMECS2, TMECS3 exhibited lowest abundance estimates of both cell cluster 1 and 2, which were closely linked to previously reported immune-desert phenotypes [13]. While the infiltration of cell cluster 3 was similar with that in TMECS2. In terms of the correlation between TMECS and molecular features of colon cancer, we found that TMECS2 was significant enriched in dMMR, CIMP (+), BRAF mutation, CMS1, and C2 (CIT) tumors (Fig. 1D, E; Table S3), while CIN (+), CMS4, C4 (CIT), and C6 (CIT) tumors were more common in the TMECS1 cluster than in the other two clusters (Fig. 1D, E; Table S3). However, there was no obvious difference in the KRAS mutation or P53 mutation among TME clusters. The survival analysis revealed that patients with TMECS2 exhibited a prominent survival advantage for both relapse-free survival (RFS) and overall survival (OS) among the three TME clusters, whereas TMECS1 was associated with the worst prognosis (Fig. 1F). Next, we evaluated the association between TME clusters and the chemotherapeutic response. The administration of ADJC significantly reduced the mortality risk of patients only in the TMECS3 cluster; it did not confer survival benefits to patients in the TMECS1 or TMECS2 clusters (Fig. 1G). Considering the high risk for recurrence of patients with TMECS1, we speculated that the failure of ADJC to provide benefits to these patients might be attributable to the existence of primary drug resistance. To validate this hypothesis, we compared the fluorouracil response rates and the predicted IC50 values of several chemotherapeutic drugs between TMECS1 and TMECS2 clusters. The results showed that patients in the TMECS1 cluster exhibited the lowest fluorouracil response rate among all the clusters (Fig. 1H) and significantly higher estimated IC50 values for the majority of chemotherapy drugs as compared to patients in the TMECS2 cluster (Fig. 1I). Finally, the biological analysis revealed that the TMECS2 cluster had enriched pathways of DNA damage repair and immune activation, whereas the TMECS1 cluster was characterized by multiple pathways related to stromal activation, chemoresistance, and cancer progression, such as hedgehog signaling, epithelial-mesenchymal transition (EMT), cytochrome P450 metabolism, and transforming growth factor (TGF)-β signaling (Fig. 1J).

3.2. Generation of the gene signature to distinguish patients within TMECS1 in the meta-GEO cohort

Through two-step regressions (Fig. 2A, Fig. S1A-B), we generated a quantitative indicator consisting of 31 genes, termed the SIIS (Fig. 2B). Boxplots show that the median SIIS was highest in the TMECS1 cluster and lowest in the TMECS2 cluster (Fig. 2C), and the subsequent TME correlation analysis revealed a strong positive correlation between the SIIS and infiltration of cell cluster 1.
Univariate and multivariate survival analyses of SIIS model and clinical variables.

|               | UVA (RFS) | p     | UVA (OS) | p     | MVA (RFS) | p     | MVA (OS) | p     |
|---------------|-----------|-------|----------|-------|-----------|-------|----------|-------|
| Age           |           |       |          |       |           |       |          |       |
| Age (vs. 40-50)| 1.00      | 0.99  | 1.04     | 1.02  | <0.001    | 1.01  | 0.99     | 0.239 |
| Stage II      |           |       |          |       |           |       |          |       |
| Stage II      | 8.24      | 4.33  | 1.74     | 0.34  | 0.097     | 5.96  | 2.44     | 0.020 |
| Stage III     | 17.97     | 75.32 | 1.69     | 0.18  | 0.012     | 10.20 | 4.31     | 0.005 |
| CMS (vs. CMS4)|           |       |          |       |           |       |          |       |
| CMS1          | 0.55      | 0.86  | 0.96     | 1.45  | 1.48      | 0.66  | 0.26     | 0.129 |
| CMS2          | 0.59      | 0.84  | 0.63     | 0.92  | 0.15      | 1.33  | 2.25     | 0.291 |
| CMS3          | 0.42      | 0.73  | 0.38     | 0.27  | 0.003     | 1.22  | 2.53     | 0.392 |

*Continuous variable.

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For validation of the role of the SIIS signature in the TCGA-COAD cohort

The performance of the SIIS signature was further evaluated using the TCGA-COAD dataset (n = 382) (Table S2). Consistent with the results of the meta-GEO (Fig. S3A-B), SIIS value was also significantly elevated in predicted TMECS1 subtypes and the transcriptome characteristics of samples in high-SIIS group of TCGA-COAD dataset were closely linked to that of samples in TMECS1 subtypes of meta-GEO (adj p = 0.004). Moreover, the SIIS value also correlated positively with stromal component infiltration level in the TCGA-COAD cohort (Fig. 3A), and the high-SIIS group exhibited similar molecular traits and metabolic characteristics in the TCGA-COAD cohort as in the meta-GEO cohort (Fig. 3B, E and Fig. S4B). The pathway enrichment results at the transcriptome level were further confirmed at the protein level by analyzing the proteomic data of TCGA-COAD samples (Fig. 3C). We further tested the correlation of SIIS in the TCGA cohort using several known signatures obtained from the study by Thorsson et al [14]. Consistent with our previous findings, patients in the high-SIIS group exhibited significantly higher EMT, TGF-β, and intratumoral heterogeneity scores, whereas scores associated with tumor purity were all down-regulated (Fig. 3D). However, there were no significant differences in terms of tumor mutation burden or the number of neoantigens among the different SIIS groups (Fig. 3D). Clinically, higher SIIS indicated not only increased mortality risk but also diminished survival benefits of chemotherapy (Fig. 3F, G). Finally, the drug response analysis indicated that higher SIIS was also nota-

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For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.
bly linked to the fluorouracil nonresponse signature (Fig. 3H) and elevated estimated IC50 of multiple chemotherapy drugs (Fig. 3I) in analysis of the TCGA-COAD dataset. These results highlight the robustness of our SIIS signature to predict unfavorable prognoses for and chemoresistance in patients with colon cancer.

3.4. Validation of the role of the SIIS signature in the Sun Yat-sen University cancer Center (SYSUCC) cohort

In addition to the meta-GEO and TCGA-COAD cohorts, we also validated the role of SIIS in the SYSUCC cohort (Table S2). Consistent with the results of the meta-GEO and TCGA-COAD databases, the higher distribution of SIIS value in predicted TMECS1 (Fig. S3A), the transcriptome similarity between high-SIIS group and TMECS1 (Fig. S3B), the infiltration of cell cluster 1, activation of stromal-related and chemoresistant pathways, enrichment of fluorouracil nonresponse status and CMS54 subtypes in high-SIIS group could also be observed in this independent cohort (Fig. 3J-M). These data underscore the performance of the SIIS to reflect the infiltration intensity of stromal cells in colon cancer, thus indicating strong reproducibility.

3.5. Exploration of the role of the SIIS signature in pan cancer setting

Next, we evaluated the role of the SIIS across cancers. Transcriptome data of 11 TCGA cohorts (BLCA, BRCA, CESC, ESCA, HNSC, LIHC, LUAD, LUSC, READ, SKCM, STAD) (Table S4) were selected for pan-cancer analyses. In terms of the TME characteristics and transcriptome traits of SIIS value, we found that the SIIS value was positively associated with infiltration abundance of stromal cell (fibroblasts, endothelial cells) and mast cell, as well as the activation of TGF-β response scores and EMT signatures, but negatively associated with total T helper cells in all cancer cohorts we analyzed, (Fig. 4A; Table S5-S6). These results suggested that SIIS score can also be used as an effective quantitative tool of stromal component in pan cancer. However, different from the results of colon cancer cohort analysis, the SIIS value in BRCA, CESC, HNSC, LUAD, and SKCM was shown to be significantly negatively correlated with cytotoxic cell infiltration and IFN-γ signaling (Fig. 4A; Table S5-S6). As for the relationship between SIIS and chemodrug sensitivity, the results showed in the BLCA, BRCA, CESC, HNSC, LUAD, LUSC, SKCM, and STAD cohorts, the predicted IC50 values of more than 6 drugs increased significantly in the high-SIIS group. Notably, among these tumor types, the indication effect of SIIS model on chemoresistance may be the strongest in HNSC, because t-statistics of all chemotherapy drugs are greater than 5 (t-statistics of 7 drugs are greater than 10). Interestingly, the down-regulation of predicted IC50 of docetaxel in high-SIIS groups could only be observed in BRCA, ESCA and LIHC cohorts, indicating that the role of docetaxel as a potential drug choice for patients of high-SIIS group might be highly dependent on cancer type. We then explored the survival impact of the SIIS model in these cancers. As shown in Fig. 4B, ten cohorts (except ESCA) experienced an increase in the mortality risk when patients had higher SIIS; however, only three of them (BLCA, SKCM, and STAD) reached statistical significance (Fig. 4B). Finally, we compared the distribution of SIIS across different tumor types (Fig. 4C). Intriguingly, we found that colon and breast cancers were the tumor types with the lowest and highest SIIS distributions, respectively. We further obtained the PAM50 subtyping information documented in the TCGA-BRCA database. Patients with the luminal A subtype had the highest median SIIS, whereas the median SIIS for patients with basal-like disease (also known as triple-negative breast cancer) was the lowest (Fig. 4D). The basal-like subtype has been reported to be sensitive to chemotherapy; therefore, these results further suggest that the SIIS might be capable of predicting chemotherapy efficacy for patients with breast cancer.

3.6. SIIS signature in predicting immunotherapeutic benefits

Recent evidence has supported that the immune-excluded phenotype triggered by stromal cell enrichment, activation of TGF-β signaling, and the EMT pathway are associated with a lack of response to immunotherapy [15]. Considering the strong connection between the SIIS and stromal activation in pan-cancer cohorts, we next investigated whether the SIIS signature could predict immunotherapeutic benefits for solid tumors. Four independent immunotherapy cohorts with more than 50 samples in each cohort were analyzed (Table S8). In all cohorts, higher SIIS were significantly associated with lower treatment response rates (Fig. 4E-H). Moreover, the survival data showed that patients treated with immunotherapy in the low-SIIS group had significantly prolonged progression-free survival (GSE176307) and overall survival (Invigor210) than those in the high-SIIS group (Fig. S5). The comparison of the SIIS distribution of metastatic urothelial cancer patients with different molecular characteristics (Fig. 4I, J) showed that SIIS were significantly higher in the immune-excluded phenotype, low TMB, and low NEO groups than in the other groups. In terms of the TCGA subtype comparison (Fig. 4J), the SIIS was the highest for the “infiltrated” subtype patients and lowest for genomically unstable patients. However, although the SIIS tended to be decreased in the FGFR mutant subgroup (Fig. 4I) and in the subgroup with high PD-L1 expression on immune cells (Fig. 4J), statistical differences were not reached. Above all, the results of these four immunotherapy cohorts suggest that the SIIS is associated with the response to immunotherapies and can further predict the prognosis of patients.
Fig. 4. Validation of the SIIS model in the TCGA pan cancer cohorts and the immunotherapy-treated cohorts. (A) Heatmaps showing the associations between SIIS and TME cells (left), the associations between SIIS and other published signatures (middle), and the associations between SIIS and estimated IC50 values of chemotherapy drugs (right) in the TCGA pan cancer cohorts. (B) Forest plot showing the associations between SIIS and overall survival in TCGA pan cancer cohorts. (C) Violin plot of SIIS values in TCGA pan cancer cohorts. (D) Box plot of the comparison of SIIS values between the different PAM50 subtypes and between TNBC and non-TNBC group. (E-H) Box plot (left) of the comparison of SIIS values between the immunotherapy response and nonresponse group, and bar charts (right) summarizing the proportions of patients with immunotherapy response and those with non-response within and across low-SIIS and high-SIIS groups in the GSE35640 (E), GSE173839 (F), GSE176307 (G), and Imvigor210 (H) cohorts. (I) Box plot of the SIIS values in patients with different FGFR mutation and TMB status in the GSE176307 cohort. (J) Box plot of the SIIS values in patients with different immune phenotypes. SIIS, stromal cell infiltration intensity; Cor, correlation; Tcm, T central memory; Tem, T effector memory; Th, T helper; DC, dendritic cell; aDC, activated dendritic cell; iDC, immature dendritic cell; Tgd, T gamma delta; NK, natural killer; TFH, T follicular helper; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; ESCA, esophageal carcinoma; HNSC, head and neck squamous cell carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; READ, rectum adenocarcinoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; ITH, intratumor heterogeneity; HRD, homologous recombination deficiency; AS, aneuploidy score; MR, macrophage regulation; CI, confidence interval; R response; NR, nonresponse; MT, mutant type; WT, wild type; TNBC, triple negative breast cancer; NEO, neoantigen, TMB, tumor mutation burden; IC, immune cell; TC, tumour cell; IP, immune phenotypes; GU, genomically unstable; Uro, urobasal.
3.7. Multi-omic analysis

To uncover the potential mechanisms underlying the intrinsic chemoresistance in patients within high-SIIS group, the genomic analysis was performed in the TCGA-COAD cohort. There were 17 genes, the mutation frequencies of which were significantly different between patients in the high- and low-SIIS group, including 4 genes with higher mutation frequencies in the high-SIIS group and the mutaions of the remaining 13 genes were mainly enriched in the low-SIIS group (Fig. 5A). The co-occurrence status of these genes was shown in Fig. 5B. Through t-test analysis, we found that among these 13 low-SIIS related differentially mutated genes, the mutation status of 12 genes (except SMAD4) was significantly associated with fluorouracil sensitivity. We further constructed a combined mutation score based on the mutation states of these 12 genes: if the patient has mutation in any of these genes, 1 point will be counted. As shown in Fig. 5D-F, with the increase of mutation score, the SIIS value (Fig. 5D-E) and fluorouracil nonresponse rates (Fig. 5F) were both decreased. The following survival analysis showed that patients with non-mutation of these genes (the mutation score was 0) could not benefit from ADJC (Fig. 5G). Taken together, these results suggest that lack of genetic mutation is one of the mechanisms responsible for chemotherapy resistance in patients with high SIIS score.

In addition to genomic mutations, we also explored the correlations between gene promoter methylation level and SIIS values. Through a comprehensive investigation into the DNA promoter methylation landscape, we found a total of 125 probes with high variability, the β values detected by which were significantly correlated with the SIIS value (adjusted p value < 0.05, Table S9). Among them, we noted that there are 5 probes targeting the promoter region of ADCY4 gene, and the methylation level detected by cg14287235 showed the strongest correlation with the SIIS value (Fig. 5H). Thus, we focused on ADCY4 in the subsequent analysis. As shown in Fig. 5I and 5j, the correlation analysis revealed that ADCY4 methylation was significantly negatively correlated with the level of stromal cell infiltration and activation of EMT and TGFβ pathways, while its transcriptional expression was positively correlated with these stromal-related characteristics. However, the association between transcriptional expression and methylation of ADCY4 was weak, suggesting that the effect of ADCY4 methylation on biological behavior may not be achieved by affecting its transcription. Finally, the results of chemotherapy efficacy analysis also demonstrated that the methylation level of ADCY4 decreased significantly in patients who did not respond to fluorouracil (Fig. 5K), and only patients with high ADCY4 methylation (β value > 0.5) tended to benefit from ADJC (Fig. 5L). Collectively, we proposed that DNA methylation, such as hypomethylation of ADCY4, may offer a lens into the complexity and diversity of the TME, especially for stromal caused chemoresistance.

3.8. GPX3 and PRICKLE2 were identified as driver genes for chemotherapy resistance of patients in the high-SIIS group

The CRISPR screen provides an efficient high-throughput technique and a powerful opportunity to access novel therapeutic biomarkers. To identify critical genes involved in chemoresistance of patients with high SIIS, we performed gain-of-function screening of SW480 cells utilizing the human genome-scale CRISPR/CAS9 SAM2 pooled library with 40 μM of oxaliplatin, and 2 μM of 5-fluorouracil (5-FU) used as an effective selection pressure (see Materials and methods, Fig. 6A, B). From this screen, we identified a subset of 488 sgRNAs that were significantly enriched in the oxaliplatin-treated cells (Fig. 6C, upper), whereas 279 sgRNAs were significantly enriched in the 5-FU-treated cells (Fig. 6C, bottom) when compared to the vehicle control, indicating that the genes targeted by these sgRNAs might be potential drivers of fluorouracil or oxaliplatin resistance. Through a correlation analysis of the SIIS and expressions of these potential driver genes adjusted for tumor purity, a total of 31 genes that were significantly positively correlated with SIIS were identified in the GSE39582 and TCGA-COAD cohorts (Fig. 6E). Among these genes, GPX3 and PRICKLE2 attracted special attention because they are intersection genes whose sgRNAs were significantly increased in both oxaliplatin-treated and 5-FU-treated populations simultaneously, and whose expressions were increased in samples from the high-SIIS groups in the GSE39582 and TCGA-COAD cohorts (Fig. 6F). A clinical relevance analysis suggested that higher GPX3 and PRICKLE2 expressions reflected the adverse outcomes of RFS and OS, especially for patients who underwent ADJC (Fig. 6G, left). Moreover, ADJC increased the mortality risk for patients in the high GPX3 and high PRICKLE2 groups (Fig. 6G, right). Boxplots (Fig. 6H, I) show that mRNA expressions of GPX3 and PRICKLE2 were significantly elevated in the fluorouracil nonresponse and CMS4 subtype groups. Pathway and immune analyses confirmed that the infiltration abundance of members in cell cluster 1 and stroma pathway activation levels significantly increased as GPX3 and PRICKLE2 expressions increased in the GSE39582, TCGA-COAD, and SYSUCC cohorts (Fig. 6J).

PRICKLE2 is one of the components of the WNT/PCP pathway, a branch of noncanonical WNT pathways controlling tissue polarity and cell movement [16]. Because mounting evidence has uncovered the role of WNT/PCP signaling deregulation in malignant phenotypes, including chemoresistance in cancer, we assessed the association between SIIS and WNT/PCP activation. Interestingly, we found that the SIIS was significantly positively correlated with WNT/PCP activity levels in all three databases that we analyzed (Fig. 6K), whereas the correlations between the SIIS and β-catenin-dependent canonical WNT signaling were weak (TCGA-COAD) or even manifested significantly negative correlations (GSE39582 and SYSUCC) (Fig. 6K). We also observed a consistently
increased GSVa score of the WNT/PCP pathway in the fluorouracil nonresponse and CMS4 groups of the GSE39582, TCGA-COAD, and SYSUCC colon cancer cohorts (Fig. 6L, M). Collectively, these results suggest that the WNT/PCP pathway, but not the canonical WNT/β-catenin pathway, mainly contributed to the biological features of patients with high SIIS.

Because GPX3 was one of the members of the SIIS signature, we moved forward with GPX3 in the following study. To validate the results of our CRISPR library screen, we performed an in vitro study by transfecting exogenous GPX3 plasmid or siRNA targeting GPX3 into SW480 (GPX3 low-expressing cells [17]) and CACO2 (GPX3 high-expressing cells [17]) colon cancer cell lines, respectively. Consistently, exogenous GPX3 overexpression recapitulated the chemoresistance-promoting effect of sgRNA-mediated transcriptional activation in SW480 cells toward 5-FU and oxaliplatin treatment, as determined by MTI (Fig. 7A) and cell colony formation assays (Fig. 7C), whereas downregulation of GPX3 in CACO2 cells resulted in a dramatic increase in response to 5-FU and oxaliplatin during in vitro (Fig. 7B, C) and in vivo experiments (Fig. 7D, E). We detected the expression and clinical significance of GPX3 in colon cancer using a paraffin-embedded tissue microarray (Fig. 7G, Table S2). Based on the protein level, we also confirmed that high GPX3 expression independently identified patients with an inferior disease-free survival (Fig. 7F, Table S10). In terms of chemotherapy benefits, we found that ADJC, especially high-intensity chemotherapy schemes (oxaliplatin combination, 6 months of chemotherapy, or 8 cycles of Xeloda), tended to increase the risk of relapse or death (Fig. 7H, I) for patients with high GPX3 protein expression. Similar trends were also observed with the stratification of the tumor stage (Fig. S6A-S6B).

3.9. Identification of candidate molecular targets and compounds for chemosensitization of patients in the high-SIIS group

To identify candidate molecular targets and compounds that may be options for achieving chemosensitization in patients with high SIIS, we used Cmap tools. After filtering the results, we identified several enriched candidate molecular targets (Table S11) and compounds (Table S12) in the analysis performed in the GSE39582 and TCGA-COAD cohorts (Fig. 7J). The mechanisms of action of the compounds are shown in Fig. 7K. Among the intersection molecular targets, we noticed that SIAH2, an upstream regulator that is required for the maintenance of expression levels and activation of transcription activity of HIFα in cells under hypoxic conditions [18], had the highest enrichment score in both GSE39582 and TCGA-COAD cohorts. Coincidentally, hypoxia was identified as a strong transcriptional regulator of GPX3 expression through the presence of the HIFα-binding site on the promoter region of GPX3 [19]. Accordingly, we tested GPX3 expression in CACO2 cells transfected with siRNA targeting SIAH2 (siSIAH2) and in SW480 cells transfected with SIAH2 overexpression plasmid (oxSIAH2). We found that GPX3 mRNA and protein levels were obviously decreased or increased by siSIAH2 and oxSIAH2 transfection (Fig. 7L). Moreover, siSIAH2 transfection significantly increased the apoptotic cell population induced by 5-FU or oxaliplatin in CACO2 cells (Fig. 7M), further suggesting that SIAH2 regulated GPX3-mediated chemoresistance. Additionally, we tested the chemosensitization effects of the compounds with the highest enrichment scores and reported that several inhibitors, such as the MLN8054, LY317615 (a PKCθ inhibitor), and SJ-72550, possessed the capability for chemosensitization to both 5-FU and oxaliplatin in CACO2 cells (Fig. 7M).

4. Discussion

Given the significant heterogeneity in survival benefits observed among patients with colon cancer who underwent ADJC, developing an effective classifier with clinical practicability may be helpful for accelerating the application of precision medicine. The present guidelines for ADJC in patients with colon cancer are based on relapse risks indicated by clinicopathological factors [20], MMR status [21], or immunoscore proposed by Galon et al [5]. However, recent evidence has suggested that intrinsic drug sensitivity related to molecular heterogeneity is another fundamental factor determining the clinical benefits of chemotherapy, independent of relapse risk. For example, although CMS4 [22], stem-like [23], or CC3 subtype tumors [24] are associated with a high risk of relapse, no statistically significant benefits from oxaliplatin treatment could be observed in patients with these subtypes [25]. Similarly, during this study, we also identified a group of patients with colon cancer who were characterized by abundant stromal cell infiltration, had the shortest RFS and OS, lowest chemoresponse rate, and lacked the benefits of ADJC. These results suggest that formulating ADJC strategies according to the relapse risk alone is far from enough. Moreover, most molecular classifications are constructed based on global gene expression profiles derived from microarray or RNA sequencing platforms, which are impractical in actual clinical settings because of their exorbitant cost, long turnaround time, and reliance on bioinformatics expertise. In view of these facts, we designed and focused on the development of a quantitative indicator that closely associates with major cell types, especially stromal cells, within the TMECS1 group and can help provide a more individualized chemotherapeutic benefit assessment for patients with colon cancer. Our efforts allowed us to establish a novel 31-gene SIIS signature, capable of identifying colon cancer patients with both poor prognoses and intrinsic chemotherapeutic resistance, that was successful independently validated in multiple public datasets and in the cohort of patients.
Fig. 7. Validation of the role of GPX3 in driving chemotherapy resistance in colon cancer. (A) Dose–response curves of SW480 cells transfected with empty vectors or GPX3 plasmid after treatment with 5-FU (left) or oxaliplatin (right) in different concentration for 24 h. (B) Dose–response curves of CACO2 cells transfected with empty vectors or GPX3 siRNA after treatment with 5-FU (left) or oxaliplatin (right) in different concentration for 24 h. (C) Colony formation ability of shGPX3, oxGPX3 and corresponding control cells with or without chemotherapy drug treatment. (D) Xenograft tumors of killed mice in different groups. (E) Growth curves of subcutaneous xenograft tumors in different groups. (F) The survival curve of for disease-free survival in the tissue microarray analysis. (G) Representative micrographs of GPX3 protein expression in colon cancer patients, as detected by immunohistochemistry. (H-I) The survival curve of ADJC performance, oxaliplatin combination, chemotherapy duration, and Xeloda cycles for disease-free survival in the low-GPX3 (H) and high-GPX3 (I) patients. (J) Heatmaps showing the significantly enriched molecular targets (upper, enrichment score < 96) and compounds (down, enrichment score < 90) based on the connectivity map analysis. (K) Heatmap showing the mechanisms of the action (rows) of each compound revealed by the connectivity map analysis. (L) The mRNA (upper) and protein levels (down) of GPX3 expression in cells transfected with SIAH2 siRNA or oxSIAH2 cells as compared to the vehicle control. (M) Dose–response curves of CACO2 cells transfected with SIAH2 siRNA or in treatment groups based on different regimens. (N) Summary of the current study. OXA, oxaliplatin; ADJC, adjuvant chemotherapy; SIS, stromal cell infiltration intensity; ES, enrichment score.
from SYSUCC. Intriguingly, through the GDSC analysis, we found that although the estimated IC50 values of the majority of chemotherapeutic drugs were significantly increased in the high-SIIS group, higher SIIS were consistently associated with improved efficiency of docetaxel. Docetaxel is mainly used to treat patients with platinum-resistant tumors in the clinic [26]. Our results suggest that docetaxel may be a potential treatment option for patients within high-SIIS group (also harboring platinum resistance). Collectively, the SIIS model has great potential to become an effective supplement to the current TNM staging system and could help to improve treatment decision for patients with colon cancer. More importantly, the SIIS structure consisted of only 31 genes, thereby offering a smaller panel of genes that can be easily translated into a simple, inexpensive, and quantitative PCR-based assay for routine clinical testing. Besides, the SIIS value is very easy to be calculated since it is just defined as difference between the average expression values of the two groups of genes.

During this research, we also evaluated the application value of the SIIS model at the pan-cancer level. The consistent positive correlations of SIIS value with level of stromal cell infiltration, stromal pathway activation, and predicted IC50 values of multiple chemotherapeutic drugs across a variety of tumors indicated that SIIS score is an universal tool for effective identification of patients whose tumors were characterized by high stromal components and chemoresistance. It is also encouraging to find that the SIIS model could predict the efficacy of immunotherapy outcomes in solid tumors, and this may be partly due to the correlation between SIIS value and TGF-β pathway activation [15]. Therefore, trials to determine whether patients in the high-SIIS group could benefit from the combination of TGF-β inhibition and immune checkpoint blockade may have clinical significance.

Mounting evidence has shown that genomic alterations and DNA methylation status can reform TME and affect therapeutic efficacy. For example, the germline or somatic mutations in DNA damage repair genes usually confer improved long-term survival for patients treated with platinum-containing chemotherapy [27–29]. Similarly, the DNA methyltransferase inhibitors have also been shown to enhance chemosensitivity in multiple solid tumors [30]. In this study, we reported for the first time that the lack of multiple genetic mutations and the ADCV4 promoter hypomethylation were two potential mechanisms mediating chemoresistance in the background of stromal cell infiltration, and they could serve as novel biomarkers for predicting ADJC benefit in patients with colon cancer.

Another interesting aspect of our work was the identification of GPX3, one of the 31 genes that comprise the SIIS panel, as the driver gene contributing to chemotherapy drug resistance in the background of stromal cell infiltration using the CRISPR screen. GPX3 functions as a plasma antioxidant enzyme that protects cells against oxidative damage by inactivating reactive oxygen species [31]. Because inducing reactive oxygen species accumulation and oxidative stress is one of the main mechanisms by which chemotherapeutic agents exert cytotoxic effects [32], the antioxidant role has led to the exploration of GPX3 as a determinant of chemotherapeutic activities. For example, Saga et al. reported that high GPX3 expression was linked with cisplatin resistance in ovarian cancer cells [33]. Similarly, Pelosof et al. discovered that GPX3 promoter hypermethylation, which leads to reduced GPX3 mRNA and protein expression, increased oxaliplatin and cisplatin sensitivity in colon cancer cell lines [17]. Consistent with the findings of Pelosof et al., we also found that GPX3 is a core gene that mediates 5-FU and oxaliplatin resistance in colon cancer cells simultaneously. Moreover, using a tissue microarray analysis, we found that the indication for GPX3 in chemotherapy resistance was also maintained at the protein level, and that patients with high GPX3 protein expression experienced more adverse prognostic events when receiving ADJC, oxaliplatin combination, and longer durations of chemotherapy. These results are encouraging and highlight the potential clinical significance of using GPX3 as a simple biomarker for the identification of patients in the high-SIIS group and those who would suffer from more aggressive adjuvant treatment. However, expanding the sample size and prospective clinical studies are required to confirm these findings. Additionally, although our study revealed in vitro that some compounds, such as aurora kinase inhibitors, could increase the chemosensitivity of GPX3 highly-expressed colon cancer cells, whether these drugs could be used as effective combined treatment strategies is also warranted further investigations.

There were some limitations to the present study. First, although the comprehensive analysis in this study has yielded several important conclusions, it is still insufficient for clinical transformation of SIIS model because this study is based on retrospective datasets. Therefore, the results of our study should be further validated in prospective clinical trials. Moreover, an appropriate cutoff value of the SIIS model also needs to be determined for consequent clinical practice.

5. Conclusion

In conclusion, we developed and validated a novel individual scoring system based on quantifying the tumor-associated stromal components in the TME, that may serve as a practical and robust biomarker for the prediction of survival and providing more precise therapeutic options in colon cancer.

Availability of data and materials

The public data used in this study are available at: GSE17536 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17536); GSE33113 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33113); GSE37892 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37892); GSE38832 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38832); GSE39582 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39582); GSE188711 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE188711); GSE35640 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35640); GSE173839 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE173839); GSE176307 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE176307); TCGA datasets (https://xenabrowser.net/datapages/); IMvigor210 R package “IMvigor210”.

The SYSUCC colon cancer dataset generated and analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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CRedit authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.04.037.

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