Proteomic characterization of gastric cancer response to chemotherapy and targeted therapy reveals potential therapeutic strategies

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Chemotherapy and targeted therapy are the major treatments for gastric cancer (GC), but drug resistance limits its effectiveness. Here, we profile the proteome of 206 tumor tissues from patients with GC undergoing either chemotherapy or anti-HER2-based therapy. Proteome-based classification reveals four subtypes (G-I–G-IV) related to different clinical and molecular features. MSI-sig high GC patients benefit from docetaxel combination treatment, accompanied by anticancer immune response. Further study reveals patients with high T cell receptor signaling respond to anti-HER2-based therapy; while activation of extracellular matrix/PI3K-AKT pathway impair anti-tumor effect of trastuzumab. We observe CTSE functions as a cell intrinsic enhancer of chemosensitivity of docetaxel, whereas TKTL1 functions as an attenuator. Finally, we develop prognostic models with high accuracy to predict therapeutic response, further validated in an independent validation cohort. This study provides a rich resource for investigating the mechanisms and indicators of chemotherapy and targeted therapy in GC.

Gastric cancer (GC) is one of the most common malignant tumors of the digestive system, and the second leading cause of cancer-related deaths worldwide. The risk factors for GC include smoking, a high-salt diet, a high intake of meats, bile reflux, and infection with Helicobacter pylori. Currently, surgery, chemotherapy, and radiotherapy are the major treatment strategies for GC. However, 70–90% of the patients with GC are diagnosed at advanced stages, with poor prognosis. The MAGIC trial revealed that the 5-year survival rate of patients receiving perioperative chemotherapy is significantly higher than those undergoing only surgical resection (36% vs. 23%). Preoperative chemotherapy, which is regarded as a standard treatment, is also a promising approach to improve survival in patients with locally advanced GC. The triplet combination chemotherapy DOS (docetaxel, oxaliplatin, and S-1) and the doublet chemotherapy XELOX (capecitabine and oxaliplatin) have been established as the first-line therapies in the treatment of both local and metastatic GC. The trastuzumab-based chemotherapy exhibited a survival benefit for human epidermal growth factor receptor-2 (HER2)-positive GC patients in the ToGA trial, where an anti-HER2 targeted strategy was proposed as a standard approach for HER2-positive GC patients. Several recent phase II studies and a recent EVIDENCE of the

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combination of trastuzumab with a range of treatment patterns have supported the efficacy and safety of trastuzumab + XELOX in patients with HER2-positive advanced GC.10 However, the trastuzumab + DOS combined therapy has been proved less effective with a shorter median OS (27.4 months) compared with trastuzumab + XELOX (34.6 months), which has not been widely used in clinic.11 The resistance mechanism of the combination of trastuzumab and chemotherapy remained unclear. Extensive chemotherapy and drug resistance occurs in DOS, XELOX, and anti-HER2 therapies for gastric cancer. It is reported that 80% of the patients with GC show drug resistance. DOS therapy showed a relatively low clinical efficacy, with an objective response rate (ORR) of 54.5%.12 Similarly, in our previous study,13 we retrospectively reviewed 248 patients with locally advanced GC treated with a XELOX or DOS regimen and concluded that the response rates in the XELOX and DOS groups were as low as 34.5% and 38.1%, respectively. Even for the 20% HER2-positive GC patients, the ORR was variable (~32–68%). With the increasing emergence of chemotherapy and targeted therapy resistance nowadays, exploration of resistance mechanism and identification of predictive biomarkers to these therapies would be important for improving therapeutic effects for GC patients. Many studies of chemotherapeutic response have focused on a single drug in cell lines but not combined therapy over the past few years.14–19 Chemotherapeutic drugs of the taxane class therapies included fluoropyrimidines (5-fluorouracil, capecitabine, and S-1), platinum drugs (cisplatin and oxaliplatin), and taxanes (paclitaxel and docetaxel). The clinical trials revealed a questionable benefit of triplet chemotherapy compared with doublet treatment, the major difference of which was docetaxel component.19 Docetaxel, as microtubule-targeting anticancer agents, involved in a complex manner and altered multiple cellular oncogenic processes, including mitosis, angiogenesis, apoptosis, inflammatory response, and ROS production.20 In addition, trastuzumab, a monoclonal antibody, which was used to treat patients with HER2-overexpressing gastric cancer in the randomised controlled TOGA trial, patients treated with trastuzumab plus cisplatin and fluoropyrimidine chemotherapy had improved median overall survival compared with patients treated with chemotherapy alone. However, the priority of patient selection and prevention of trastuzumab resistance for the trastuzumab combination therapy still await to be convinced. Overall, a comprehensive molecular landscape for predicting the therapeutic response in GC is still lacking.

Several molecular classifications have been proposed to connect molecular patterns to clinical features. The Cancer Genome Atlas (TCGA) project mapped the genomic landscape of GC and classified GC into four subtypes: Epstein-Barr virus (EBV) positive (MSS/EMT), EBV-negative (MSS/TP53+), and MSS with TP53 intact (MSS/EMT), MSS with TP53 loss (MSS/TP53-), which were associated with distinct clinical outcomes.21 Retrospective analyses and large clinical trials suggested that MSI GC patients had a favorable prognosis compared with MSS GC patients, but the benefit from perioperative or adjuvant chemotherapy have been topic of debate.22 According to the MAGIC trial, high MSI was associated with a positive prognostic effect in patients treated with surgery alone and a differentially negative prognostic effect in patients treated with perioperative epirubicin, cisplatin, and fluorouracil chemotherapy.23 However, no systematic data regarding the outcome of MSI GC patients with first-line chemotherapy and targeted therapy (such XELOX, DOS, and anti-HER2 therapies) has been reported.

Proteins, regarded as the “executors of life,” provide insight into the disease at the protein level, and may bridge the gap between research and clinical practice. Previously, we collected surgical samples from 84 patients with diffuse-type gastric cancer (DGC), and presented a proteomic profiling of the Beijing Proteome Research Center (BPRC) DGC cohort. The BPRC DGC cohort was classified into three groups (PX1–3) based on the proteomic profile, in which group 3 (PX3) had the worst prognosis and was resistant to chemotherapy.27 This suggested an association between the chemotherapy response and proteome signatures in patients. Based on this finding, we sought to establish a comprehensive connection between the proteomic panel and clinical outcomes.

Herein, we set out to investigate the responses to first-line therapies (DOS, XELOX, and anti-HER2-based therapies) for GC through a comprehensive proteomic analysis. We collect the biopsy tumor FFPE samples derived from 206 therapy-naïve GC patients, and construct a GC cohort that covers three clinical therapy subcohorts, including DOS subcohort (44 patients treated with DOS therapy), XELOX subcohort (70 patients treated with XELOX therapy) and HER2 subcohort (71 patients who received anti-HER2-based therapy). Proteomic clustering in four GC molecular subtypes with distinct functions that show associations with drug response and clinical outcomes. We validate the GC subtyping and their prognosis difference in other independent GC cohorts. We find GC patients with MSI-sig high status show sensitive response to DOS but not XELOX therapy. Bioinformatic analysis reveals higher T cell receptor signaling and aggerated CD6 + Tcm in DOS sensitive group compared with non-sensitive group, while it is opposite in response to XELOX therapy. Further comparative analysis reveals that patients with high TCR signaling are unlikely to benefit from XELOX, but instead the combination of anti-HER2-based therapy; while the activation of ECM and the downstream PI3K-AKT pathway impairs the anti-tumor effect of trastuzumab. We perform further validation experiment to confirm the synergistic effects of the combination of trastuzumab with XELOX, or the PI3K-AKT inhibitor in vitro. Furthermore, we develop prognostic models with high accuracy to predict the chemotherapeutic response, which are validated by PRM assay in an independent validation cohort composed of 60 GC patients (50% sensitive and 50% non-sensitive patients) receiving either DOS (N = 20), XELOX (N = 20), or anti-HER2 (N = 20) therapies. Finally, we validate CTSE functions as a cell-intrinsic enhancer of chemosensitivity of docetaxel, whereas TKTL1 functions as an attenuator of docetaxel. This study presents a comprehensive proteomic approach for the prediction of the response to chemotherapy and targeted therapy, and implicates its prognostic and therapeutic significance as well as the underlying regulatory mechanisms that may benefit clinical practice.

Results

The gastric cancer cohort: The response to chemotherapy and targeted therapy in patients with gastric cancer

To investigate the proteomic patterns associated with the response to chemotherapy and targeted therapy, we collected 206 GC patients which received either DOS therapy (DOS subcohort, N = 44, 21.4%), XELOX therapy (XELOX subcohort, N = 70, 34.0%), or anti-HER2-based therapy (HER2 subcohort, N = 71, 34.5%). Another 21 patients (10.1%) were assigned as “Others,” of which 3 patients received ataxinib or docetaxel therapies, and 18 cases had no chemotherapy information. All the chemotherapy regimens were given at standard dosing as described in previous studies.24–26 (Methods). All samples were histologically scored by two expert gastrointestinal pathologists (C.X. and Y.H) according to the widely accepted Response Evaluation Criteria in Solid Tumors (RECIST) (version1.1) by CT/MRI scanning and grouped into complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). Here, the ORR, defined as PR plus CR, was selected for the efficacy evaluation; patients with CR and PR were defined as sensitive (S) and those with SD and PD were defined as non-sensitive (NS). DOS subcohort was grouped into DOS-sensitive group (DSC, N = 22) and DOS-non-sensitive group (DNS, N = 22); XELOX subcohort was grouped into XELOX-sensitive group (XSG, N = 27), XELOX-non-sensitive group (XNSG, N = 42), and NA (not available of

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the information of therapy response), N=1; HER2 subcohort was
grouped into anti-HER2-sensitive group (HSG, N = 32), anti-HER2-non-
sensitive group (HNSG, N = 37), and NA (N = 2). Archival formalin-fixed
paraffin-embedded (FFPE) tissues with at least 80% tumor purity were
collected from 206 chemotherapy-naive patients with GC, and then
subsequently analyzed by the mass spectrometry (MS)-based label-
free quantification strategy27,29 (Fig. 1a and Supplementary Fig. 1a). The
detailed clinical characteristics were shown in Supplementary Table 1,
Fig. 1b, and Supplementary Data 1. We made a formal assessment of
baseline clinical characteristics of all patients enrolled in the study,
especially in DOS, XELOX, and HER2 subcohorts. There was no baseline
difference in gender, age, grade, and Lauren type in the DOS, XELOX,
and HER2 subcohorts (Supplementary Data 1). Survival analysis showed that the sensitive group (S, N=79) had a higher survival rate than the non-sensitive group (NS, N=97) (log rank test, P=0.002; hazard ratio (HR) = 0.56; 95% CI, 0.34–0.93) (Fig. 1c).

Proteomic analysis of the gastric cancer cohort

For the quality control of the performance of mass spectrometry, the HEK293T cell lysate was measured every two days as the quality-control standard, which was adopted in proteomic studies. A Pearson’s correlation coefficient was calculated for all the quality-control runs, and the results were shown in Supplementary Fig. 1b. The average correlation coefficient among the control samples was 0.964, demonstrating the consistent stability of the MS platform. Proteomics measurement resulted in 6369–8119 protein groups (GPs) in each sample (Fig. 1d and Supplementary Fig. 1c, d). A total of 12,519 protein groups (GPs) were identified in all patient samples (N=206) (Supplementary Data 2). 10,496 GPs, 10,766 GPs, 10,920 GPs were identified in the DOS, XELOX, and HER2 subcohorts, respectively (Supplementary Fig. 1c). Principal components analysis (PCA) revealed no batch effects among DOS, XELOX, and HER2 subcohorts (Supplementary Fig. 1e).

Principal components analysis (PCA) revealed no batch effects among DOS, XELOX, and HER2 subcohorts (Supplementary Fig. 1f). No major differences in the proteomic coverage between S (11,258 GPs) and NS subcohorts (Supplementary Data 1). Survival analysis further validated the HER2 expression by IHC and FISH analysis (Supplementary Fig. 1e). This result showed the specific molecular pattern in HER2-positive GC patients, which could be identified at proteome level. Formal statistical tests for interaction analysis revealed there was no significant interaction between therapy responses and other baseline clinical characteristics, such as therapy subcohort and TNM stage (Supplementary Table 2). Furthermore, univariate Cox analysis of overall survival showed proteomic subtyping was associated with clinical outcome irrespective of gender, age, and therapy subcohort. Remarkably, proteomic subtyping served as an independent predictive factor (Cox P trend = 0.009; HR = 1.49; 95% CI, 1.10 to 2.02) in the multivariable analysis after adjusting for clinical TNM stage and other covariates (Supplementary Table 3). Overall, these results demonstrated the strong association of proteomic subtyping with therapy response and prognosis, and supported its reliability at proteome level.

Proteomic subtyping of the GC cohort and their association with a therapeutic response

Consensus clustering analysis was performed on the 206 samples based on 1,000 most variable proteins (proteins with the top 10% median absolute deviations) (Methods), which resulted in four subtypes: G-I (N=29), G-II (N=60), G-III (N=97), and G-IV (N=20) (Supplementary Fig. 2a and Supplementary Data 3). Further analysis of 179 patients with complete prognosis among the four proteomic subtypes revealed significant association of proteomic subtypes with survival, among which the G-IV subtype had the worst overall survival (log rank test, P=0.034) (Supplementary Fig. 2b). After excluding the patients who were lost to follow-up, we then performed the same consensus clustering analysis only on the 179 patients. Consensus clustering analysis of the proteomic profiles among 179 samples identified four proteomic subtypes of GCs, in which 28, 56, 75, and 20 patients were grouped into subtypes G-I, G-II, G-III, and G-IV, respectively (Fig. 2a and Supplementary Fig. 2c, d, and Supplementary Data 3). Sankey plot showed high concordance between the two proteomic subtyping systems of 206 samples and 179 samples (Supplementary Fig. 2d and Supplementary Data 3). We then mainly focused on the proteomic subtyping system of 179 samples with the complete survival information during the further analysis. The proteomic subtypes of 179 samples displayed distinct clinical outcomes (therapy response and overall survival). The chemotherapy and targeted therapy response exhibited a gradual resistance phenomenon from G-I to G-IV, as the percentage of sensitive patients (CR and PR) dramatically decreased from 60% in G-I to 20% in G-IV. Conversely, the percentage of non-sensitive patients (SD and PD) increased from 40% in G-I to 80% in G-IV (Fisher’s exact test, P < 0.04) (Fig. 2b and Supplementary Fig. 2e). Among four subtypes, the G-IV had the worst prognosis (log rank test, P < 0.05) (Fig. 2c). In addition, an obvious association between proteomic subtyping and therapy subcohort or TNM stage was determined (Fisher’s exact test, P < 0.01), but this association was not observed with either grade, Lauren’s type, the primary site, or tumor purity (P > 0.05) (Fig. 2d and Supplementary Fig. 2e). Further statistical analysis revealed the significant distribution difference of therapy subcohorts among proteomic subtypes, which was mainly derived from HER2 subtype (mainly enriched in G-II subtype (Fisher’s exact test, P < 0.05)); while no significant difference was observed in DOS and XELOX subcohorts (Supplementary Fig. 2e). The distribution difference among proteomic subtypes could be caused by the specific proteomic feature related to HER2 expression, due to the selection of HER2-positive patients for anti-HER2 targeted therapy, which was further validated by HER2 evaluation by IHC and FISH analysis (Supplementary Fig. 2e). This result showed the specific molecular pattern in HER2-positive GC patients, which could be identified at proteome level. Formal statistical tests for interaction analysis revealed there was no significant interaction between therapy responses and other baseline clinical characteristics, such as therapy subcohort and TNM stage (Supplementary Table 2). Furthermore, univariate Cox analysis of overall survival showed proteomic subtyping was associated with clinical outcome irrespective of gender, age, and therapy subcohort. Remarkably, proteomic subtyping served as an independent predictive factor (Cox P trend = 0.009; HR = 1.49; 95% CI, 1.10 to 2.02) in the multivariable analysis after adjusting for clinical TNM stage and other covariates (Supplementary Table 3). Overall, these results demonstrated the strong association of proteomic subtyping with therapy response and prognosis, and supported its reliability at proteome level.

Comparative analysis of proteomic profiling resulted in 301 (G-I), 611 (G-II), 925 (G-III), and 467 (G-IV) GPs (P < 0.05; fold change >2; identification frequency ≥10%), showing distinct molecular features among the four proteomic subtypes (Supplementary Fig. 3a). We performed a functional enrichment analysis according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations, and determined the dominant bioprocesses of each subtype. As shown in Supplementary Fig. 3b, c, the same criteria were applied in proteomic subtypes of 206 samples, and KEGG pathway enrichment analysis revealed the consistency of the overrepresented pathways dominant in four proteomic subtypes both in 179 samples and 206 samples. The G-I subtype was dominant for endocytosis (P = 4.73E−4), and ssGSEA analysis revealed that the activation of endocytosis pathway indicated a better prognosis (log rank test, P < 0.05) (Supplementary Fig. 3d). As shown in Supplementary Fig. 3e, a group of endocytosis related proteins, such as DNM2, EPS15, WIPF1, ACAP2, and CHMP6, showed positive association with overall survival (hazard ratios range: 0.10–0.54, P < 0.05). In the G-II subtype, we observed a significant enrichment of glycolysis/gluconeogenesis (P = 3.33E−2) and pantothenate/CoA biosynthesis (P = 3.39E−3) (Fig. 2d and Supplementary Data 3). In the G-III subtype, lysosomal acid hydrolases proteases (such as CTSA, CTSB, CTSC, and CTSD) and synthesized lysosomal enzymes (such as CLTA, CLTB, GGA1, and...
APIS2) were exclusively upregulated. In contrast with other three subtypes, the G-IV subtype was characterized by ECM-receptor interaction, focal adhesion, complement/coagulation cascades, and PI3K-AKT signaling pathway, of which the activation of ECM-receptor interaction pathway indicated a worse prognosis (log rank test, \( P < 0.05 \)) (Supplementary Fig. 3f). The extracellular matrix proteins, such as FGB, TGFB1, THSD4, LAMB2, and LAMA4, revealed a significantly upregulation in G-IV subtype compared with other subtypes (Student’s t test, \( P < 0.05; \) fold change \( > 2 \)), among which the expression of THSD4 was significantly associated with poor prognosis (\( P = 0.007; HR = 1.96; 95\% CI, 1.12-3.44 \)) (Supplementary Fig. 3g). These results suggested that activation of endocytosis in G-IV subtype was associated with drug resistance; while high expression of ECM in G-IV subtype was associated with drug resistance. OSEA analysis further revealed the association of endocytosis/ECM pathways and drug sensitivity/resistance was also applied for other proteomic subtypes (Supplementary Fig. 3h), indicating the consistency of therapy mechanism in different proteomic subtypes.

To investigate the association of proteins expression with therapy resistance, we analyzed the correlation of the extracellular matrix proteins significantly upregulated in the G-IV subtype with drug sensitivity (half maximal inhibitory concentration [IC50]) using gastric cancer cell lines data from the Cancer Dependency Map Project (DepMap). We identified five proteins, including THSD4, SRPX2, TGFB1, THBS1, and LAMB2, which showed high correlation with drugs response (5-FU, oxaliplatin, or docetaxel) (Pearson \( r > 0.4; P < 0.05 \)) (Fig. 2e). Among these proteins, only THSD4 was resistant to all three drugs (5-FU, oxaliplatin, or docetaxel), and the high expression of THSD4 was significantly associated with poor prognosis (log rank test, \( P < 0.05 \)) (Fig. 2e and Supplementary Fig. 3i). To further verify that high expression of THSD4 was associated with drug resistance, we firstly overexpressed THSD4 in two gastric cancer cell lines MKN45 and MGCG03 (Supplementary Fig. 3j, k). The CCK8 assay revealed that THSD4 overexpression significantly promoted the proliferation of MKN45 and MGCG03 cells (Student’s t test, \( P < 0.05 \)), compared with the cells transfected with an empty vector (Supplementary Fig. 3l, m). Then, we treated THSD4-overexpressing and empty vector-overexpressing MKN45 and MGCG03 cells with docetaxel, oxaliplatin, and 5-FU, respectively. The drug sensitivities in the MKN45 and MGCG03 cells were estimated by their half-maximal inhibitory concentration (IC50) values. We observed IC50 values of docetaxel, oxaliplatin, and 5-FU were significantly higher (4.26-fold, 1.44-fold, and 1.38-fold increase in MKN45, respectively; Student’s t test, \( P < 0.05 \)) in the THSD4-overexpressing MKN45 cells, compared with the empty vector-overexpressing cells (Fig. 2f). Consistently, we observed the similar change of IC50 values of docetaxel, oxaliplatin, and 5-FU in THSD4-overexpressing MGCG03 cells (3.60-fold, 1.47-fold, and 1.20-fold increase in MGCG03, respectively) (Fig. 2f). In conclusion, the in vitro experiments further validated THSD4 overexpression reduced the anti-tumor effect of chemotherapeutic drugs including docetaxel, oxaliplatin, and 5-FU.

The parallel reaction monitoring (PRM) assays are powerful targeted approaches to detect and quantify pre-specified proteins with a high throughput using high-resolution mass spectrometers. We firstly constructed an independent cohort composed of 60 GC patients receiving either DN2 (\( N = 20 \); DSG, \( N = 10 \); DNSG, \( N = 10 \)), XELOX (\( N = 20 \); XSG, \( N = 10 \); XNSG, \( N = 10 \)), or anti-HER2 (\( N = 20 \); HSg, \( N = 10 \); HNSG, \( N = 10 \)) therapies (Supplementary Data 3). To further validate the association between extracellular matrix proteins and resistance of drugs (5-FU, oxaliplatin, and docetaxel), we employed the targeted MS approach, PRM assays, which has been adopted in classifier’s validation in recent proteomic research23,24, to quantify these proteins in FFPE tumor tissues from patients receiving DSG therapy (triplet combination chemotherapy of 5-FU, oxaliplatin, and docetaxel). We then selected a set of target peptides that unique to these ECM proteins (including THSD4, SRPX2, TGFB1, THBS1, and LAMB2) using the library search results (Supplementary Data 3). The fragment total areas of targeted peptides reported by Skyline-daily (4.2.1.19004, University of Washington, USA) were used to quantify these proteins. As a result, we observed these ECM proteins were higher expressed in DSNP compared with DSG in PRM-M5 experiments (Fold change (DNSG/DSG) \( > 2 \), \( P < 0.05 \), Wilcoxon rank-sum test): THSD4 (Fold change (DNSG/DSG) = 2.49, \( P = 0.52E-1 \); SRPX2 (Fold change (DNSG/DSG) = 7.10, \( P = 0.035 \), TGFB1 (Fold change (DNSG/DSG) = 1.97, \( P = 0.035 \), THBSI (Fold change (DNSG/DSG) = 7.63, \( P = 1.5E-3 \), and LAMB2 (Fold change (DNSG/DSG) = 2.94, \( P = 0.043 \)) (Fig. 2g). In conclusion, our data demonstrated the association between these ECM proteins and drug resistance was validated by PRM approach in the independent cohort. Overall, the results illustrated the high expression of extracellular matrix proteins is associated with drug resistance, and these extracellular matrix proteins could serve as indicators to predict chemotherapy response.

MSS/MSI status of gastric cancer is associated with tumor immune microenvironment and therapy resistance

To further test if the proteomic subtyping algorithm of the Fudan GC cohort (FDGC) was feasible in other GC cohorts, we constructed the Fast Large Margin classifier model based on the overrepresented proteins of the subtypes of the FDGC cohort using RapidMiner 9.6.0 (Methods). Then, we applied the same model with FDGC subtyping algorithm in three independent cohorts of GC patients (BPRC cohort25, N = 84; EOOG cohort26, N = 80; and ACRG cohort27, N = 300). Consistent with the FDGC cohort, the application of the model in BPRC cohort, EOOG cohort, and ACRG cohort resulted in subtype reallocations: G-I (\( N = 15 \)), G-II (\( N = 30 \)), G-III (\( N = 38 \)), and G-IV (\( N = 35 \)) in BPRC cohort; G-I (\( N = 20 \)), G-II (\( N = 20 \)), G-III (\( N = 19 \)), and G-IV (\( N = 21 \)) in EOOG cohort; G-I (\( N = 35 \)), G-II (\( N = 103 \)), G-III (\( N = 127 \)), and G-IV (\( N = 35 \)) in ACRG cohort (Supplementary Data 4). We observed a significant concordance between FDGC subtype and EOOG subtype or ACRG subtype (\( P < 0.05 \), \( P < 0.05 \), respectively), but not between FDGC subtype and BPRC subtype (\( P = 0.422 \)) (Fig. 4a, b), which might due to the similar composition of Lauren’s type between FDGC subtype and EOOG subtype or ACRG subtype, while BPRC cohort only included diffuse-type cancer. Although no concordance in the composition of Lauren’s type between FDGC subtype and BPRC cohort, the proteomic features associated with therapy response and prognosis revealed in FDGC subtypes were applicable in the BPRC cohort (log rank test, \( P = 0.021 \)) (Fig. 4a and Supplementary Fig. 4a). In the comparison of FDGC and the BPRC subtypes, we found the PX3 subtype accounted for the highest proportion in G-IV subtype (50%) compared with other subtypes (20% in G-I, 26% in G-III, respectively), indicating PX3 subtype was mostly clustered into G-IV subtype. As reported in BPRC subtype, PX3 subtype was characterized with the enrichment of pathways including ECM organization, EMT, and complement, had the worst prognosis and was resistant to chemotherapy. Consistently, in our study, G-IV subtype was characterized by ECM-receptor interaction, focal adhesion, and complement/coagulation cascades, had the worst prognosis and highest proportion (80%) of non-sensitive patients. As for EOOG cohort, the comparison of FDGC subtype and EOOG subtype suggested the Sub2 accounted for most (75%) of G-I subtype. As reported in the research related to EOOG subtype, the comparison of FDGC subtype and EOOG subtype suggested the Sub2 accounted for most (75%) of G-I subtype. As reported in the research related to EOOG subtype, the Sub2 accounted for most (75%) of G-I subtype. As reported in the research related to EOOG subtype, the Sub2 accounted for most (75%) of G-I subtype. As reported in the research related to EOOG subtype, the Sub2 accounted for most (75%) of G-I subtype.
of the G-IV subtype. We speculated that the characteristics of MSS or EMT might be associated with poor survival. Therefore, we further predicted the microsatellite instability (MSI) of the ACRG cohort classified by FDGC subtyping based on a small pre-defined set of gene expression signatures. We found the G-IV subtype was characterized with lower MSI/MSS-sig score, while higher MSI/MSS-sig score was enriched in the G-II subtype (Fig. 3c and Supplementary Fig. 4c). We assessed whether the MSI/MSS-sig score was associated with distinct somatic alterations, and observed that MSI/MSS-sig score or MSI high (MSI-H) status of the G-II was highly consistent with the presence of hypermutation, with mutations in genes such as MLH1 (10%), FAT4 (10%), PIK3CA (14%), and KRAS (8%). Among them, PIK3CA and KRAS
were reported as recurrent neoantigen-associated mutations in addition, further analysis of copy number profiles in ACRG cohort, we found that genomic instability index (termed CNV GI), which was present in 28% cases, showed significant difference among four subtypes (G-I vs G-IV) (Fig. 3c and Supplementary Fig. 4d). We applied the same set of gene expression signatures to assess the expression of MSI/MSS-sig and observed the similar trend in FDGC cohort (ANOVA test, \( P < 0.05 \)), which suggested the robustness of FDGC subtyping. The low MSI/MSS-sig significantly correlated with poor clinical outcome both in ACRG cohort and FDGC cohort (log rank test, \( P < 0.05 \)) (Fig. 3d), which was consistent to the previous studies. Importantly, the MSI/MSS-sig level showed significant association with the response to chemotherapy and targeted therapy (Fig. 3c and Supplementary Fig. 4e). Furthermore, we found that gastric cancer with high MSI/sig was featured by oxidative phosphorylation, while gastric cancer with low MSI/MSS-sig showed high expression of extracellular matrix proteins (Fig. 3e).

As previously reported, antigen-driven immune response could be activated in microsatellite instable (MSI) cancers. We firstly evaluated the relative abundance of the cytokines and proteins involved in antigen processing and presenting process among four proteomic subtypes. We observed the consistent downregulation of a group of cytokines (such as CXCL17, IL16, and IL18), proteins involved in antigen processing (such as CASP1, CTSE, PSMD8, and TRIM32), and MHC class II molecules (such as HLA-DMB, HLA-DPB1, and HLA-DQA1) in G-IV subtype (Fig. 3f). For further exploring the possible molecular mechanisms of low MSI/MSS-sig accounting for drug resistance and poor prognosis, we then evaluated the tumor immune microenvironment among four subtypes by xCell analysis (Supplementary Data S5). As reported, the high immunoactivity defined by the microsatellite instability (MSI) is associated with the high degree of infiltration of M1 macrophages. We found that G-IV had the lowest MSI/MSS-sig score (Fig. 3g). In addition, the proteomic subtypes were featured with different cell types, among which macrophages M1 were enriched in G-IL. In contrast with macrophages M1, precursor monocytes were aggregated in the G-IV subtype (Fig. 3g, h). Further analysis suggested the significant correlation between MSI/sig and monocytes (Pearson \( r = -0.37, P = 2.9E^{-7} \)) or macrophages M1 (Pearson \( r = 0.25, P = 9.3E^{-4} \)) (Fig. 3i). Increasing studies have also shown that tumor-associated macrophages (TAMs) can either enhance or antagonize the tumor immune response of macrophage M1, showed significantly positive correlation with MSI/MSS-sig both in FDGC cohort and ACRG cohort (Pearson \( r = 0.31, P = 1.8E^{-5} \); Pearson \( r = 0.37, P = 2.8E^{-11} \), respectively). The high expression of IL18 also reflected significant association with prognosis in patients of stage IV (log-rank \( P = 0.035 \), HR = 0.49, 95% CI, 0.22–0.83) (Fig. 3j). These results demonstrated the MSI/MSS-sig level correlated with clinical outcome; in particular, the MSI/MSS related immune cell types (monocytes and macrophages M1) have been shown to be associated with clinical outcome in the patients of stage IV, indicating the importance of immune cell types to the prognosis of patients in stage IV. Overall, we proposed microsatellite stable tumor cells featured with aggregated monocytes and few macrophages M1 were prone to drug resistance and had a poor prognosis (Fig. 3m).

T cell receptor signaling pathway exerts diverse effects in response to DOX and XELOX chemotherapy

A better outcome for patients with MSI-H tumors than with MSS tumors has been reported in gastrointestinal tumors, whereas the benefit of adjuvant chemotherapy seems to be unclear according to exploratory analyses of recent phase III trials. We next examined the association of MSI/MSS-sig level with clinical outcomes in GC patients receiving different chemotherapy regimens. Surprisingly, we observed that high MSI/H-sig level was associated with overall survival in DOX therapy; while we didn’t observe the difference in association of MSI/MSS-sig level with clinical outcomes in XELOX therapy (Fig. 4a). These results indicated that MSI-H sig high GC patients could benefit from DOX therapy, but not XELOX therapy.

To investigate the mechanism of sensitivity/resistance of different treatment regimens, we further examined the functional differences between sensitive group and non-sensitive group in DOX and XELOX subcohorts, respectively. We firstly compared the proteome of DSG, DNG, XSG, and XNSG, and identified the overrepresented proteins (ORPs) of each group (twofold changes in each group at a 1% false discovery rate (FDR) at the protein levels (Supplementary Fig. 5b and Supplementary Data 6 and 7). We then performed pathway enrichment on the overrepresented proteins of each group according to the KEGG pathway annotations shown in Fig. 4b. Surprisingly, we found that FC

**Fig. 2** Proteomic subtyping of the gastric cancer cohort and its association with clinical characteristics. a The heatmap depicts the relative abundance (Z score of FOT) of the signature proteins in four subtypes of 179 GC samples. Biological functions related to these signature proteins are denoted on the right. b The association of four proteomic subtypes with clinical characteristics (including gender, grade, Lauren type, primary site, HER2 status, RECIST, and tumor purity, etc.) are annotated with \( \chi^2 \) values (two-sided Fisher’s exact test for categorical variables, and two-way ANOVA test for continuous variables). c The Kaplan–Meier curves of overall survival (OS) of each proteomic subtype (G-I, n = 28; G-II, n = 56; G-III, n = 75; and G-IV, n = 20). \( \chi^2 \)-value is calculated by two-sided log rank test. d Left panel: Bubble plot showing the KEGG pathway enrichment (two-sided Fisher’s exact test) of each proteomic subtype. Right panel: Diagram illustrating the differentially expressed signatures and signaling cascades involved in G-I to G-IV. The little heatmap under each protein depicted the Z score of average protein abundance in each proteomic subtype. Red, upregulated proteins; blue, downregulated proteins. e The correlation of the extracellular matrix proteins and different drugs, including 5-FU, OXA, and DOX. The drug sensitivity (half maximal inhibitory concentration [IC50]) of gastric cancer cell lines was from the Cancer Dependency Map Project (DepMap). \( \chi^2 \)-values are derived from two-sided Pearson’s correlation test. f Dose-response curves of MKN45 and MCG803 cell lines overexpressing THSD4 after 72-h treatments with DOX, OXA, and SU. Barplots showing the comparison of IC50 values in each group. Bars represent the mean of \( n = 3 \) independent experiments with error bars indicating SD. \( \chi^2 \)-values are calculated using two-sided Student’s t-test. g Boxplot showing the differential expression of ECM proteins validated by PRM assay (two-sided Wilcoxon rank-sum test, \( n = 10 \) biologically independent samples per group). Boxplots show median (central line), upper and lower quartiles (box limits), 1.5× interquartile range (whiskers). Source data are provided as a Source Data fi.
epsilon RI signaling, TNF signaling, T cell receptor (TCR) signaling, and B cell receptor (BCR) signaling pathways were enriched in DSG and XNSG (P < 0.05) (Fig. 4b and Supplementary Data 6 and 7). Among these pathways, GSEA analysis showed that TCR signaling pathway was enriched in DSG and the high activation of this pathway represented better prognosis in DOS subgroup (P = 0.015; HR = 0.18; 95% CI, 0.04–0.88); on the contrary, in the XELOX subcohort, TCR signaling pathway was enriched in XNSG and the high expression represented poor prognosis (P = 0.013; HR = 5.19; 95% CI, 2.15–12.52) (Fig. 4c). Meanwhile, the TCR signaling pathway enriched in XELOX non-sensitive patients was validated in BPRC cohort received XELOX therapy (Fig. 4d). The xCell analysis also showed that immune cells such as CD8+ Tcm, CD8+ Tem, CD4+ Tcm, and CD8+ Tcm were enriched in XNSG and the high expression represented poor prognosis (P = 0.006; HR = 0.04; 95% CI, 0.01–0.28) (Fig. 4e).
The immune-induced anticancer response was reported to contribute to the efficacy of conventional chemotherapeutic agents. The major difference of DOS and XELOX in therapeutic regiments was the docetaxel component, of which DOS has docetaxel, while XELOX has not. We hypothesized docetaxel might play an important role in the immune-induced anticancer response. We next surveyed the immunogenic cell death (ICD) prediction scores for chemotherapeutic agents in NCI library base on molecular descriptors obtained with the nogenic cell death (ICD) prediction scores for chemotherapeutic immune-induced anticancer response. We next surveyed the immune effectors such as dendritic cells and T cells were also enriched in XELOX non-sensitive patients in BPRC cohort (Fig. 4f). Among these immune cells, CD8 + Tcm showed positive correlation with TCR signaling pathway (Supplementary Fig. 5c). Collectively, immune modulation exerts diverse effects in response to DOS and XELOX chemotherapy.

The immune-induced anticancer response was reported to contribute to the efficacy of conventional chemotherapeutic agents. The major difference of DOS and XELOX in therapeutic regiments was the docetaxel component, of which DOS has docetaxel, while XELOX has not. We hypothesized docetaxel might play an important role in the immune-induced anticancer response. We next surveyed the immunogenic cell death (ICD) prediction scores for chemotherapeutic agents in NCI library base on molecular descriptors obtained with the chemistry development kit. Significantly, among 31 anticancer drugs, the docetaxel ranked the top one ICD component, indicating higher capability of docetaxel in inducing immunogenic cell death (Fig. 4g).

To search for the indicators in response to DOS and XELOX therapies, we focused on the overlapped up-regulated proteins and pathways in sensitive or non-sensitive response to DOS and XELOX therapy. We observed the overlapped 2053 ORPs among DOS, DNSG, XSG, and XNSG. Almost half (46.5%, 934) of the 2053 ORPs was found in DOS and XSG, DNSG and XNSG, which might due to the common therapeutic components oxaliplatin and 5-FU in XELOX and DOS therapies; while the remaining 53.5% (1099) was found in DSG and DNSG, DNSG and XNSG, which might due to the differential therapeutic components docetaxel (Supplementary Fig. 5b). Remarkably, the higher proportion (64.4%, 708 proteins) of the 1099 proteins was found in both DSG and XNSG, which were involved in immune modulation (TCR signaling pathway and immune system) and ERBB pathway (Fig. 4h and Supplementary Fig. 5b). As shown in Fig. 4i, the univariable cox analysis identified a panel of 12 proteins involved in immune modulation (such as BCL2L1, CDB1, CTSE, ENAH, MRC1, ORMDL3, RAB24, REG3A, and VNN1) and ERBB signaling (such as ERBB2, MAP2K2, and PPP3CB) showed positive association with disease-free survival (DFS) in DSG subcohort, while negative association in XELOX subcohort. Among the 12 proteins, ERBB2 was a poor prognostic factor in XELOX subcohort (HR = 1.3, Cox P-value = 0.035).

To assess statistical independence of prognostic power of ERBB2, we then performed multivariable cox analysis adjusted for baseline clinical covariates. The result illustrated that ERBB2 could serve as an independent prognostic factor (P = 0.001; HR = 1.497; 95% CI, 1.169–1.92) in the multivariable analysis after adjusting for Lauren’s type, grade and RECIST in XELOX, but not DOS subcohort (Supplementary Table 4). ERBB2, as known as HER2, was the only drug target approved for the first-line treatment of HER2-positive GC. Therefore, we speculated that XELOX combined with anti-HER2 (Trastuzumab, Herceptin) targeted therapy could improve the therapeutic response.

To validate the speculation that the combination of trastuzumab and XELOX therapy have synergistic effects in vitro, we treated NCI-N87 cells (HER2-positive cell line) with XELOX, trastuzumab (TRA), and combination of XELOX and trastuzumab (XELOX + TRA), respectively. We observed the IC50 values of XELOX and TRA were 36.6 μM and 43.7 μM in NCI-N87 cells (Fig. 4j). As indicated in Fig. 4j, the IC50 value of the XELOX in the combination treatment (XELOX + TRA) was significantly decreased from 36.6 μM to 19.6 μM (two-sided Student’s t test, P = 1.4E-2). Similarly, the IC50 value of TRA in the combination treatment (XELOX + TRA) was also significantly decreased from 43.7 to 19.6 μM (two-sided Student’s t test, P = 8.2E-3). These results indicated trastuzumab could increase the sensitivity of HER2-amplified human gastric cancer cells to XELOX therapy. We then compared the combination of XELOX with anti-HER2 targeted therapy and XELOX chemotherapy alone. After examining the association of MSI/MSS-sig level with OS in FDGC and ACRG cohorts (two-sided Gehan-Breslow-Wilcoxon test). The association of MSI/MSS-sig level with OS in ACRG and MSI/MSS-sig (two-sided Fisher's exact test (categorical variables) and two-way ANOVA test (continuous variables). The association of MSI/MSS-sig level with OS in ACRG and MSI/MSS-sig (two-sided Wilcoxon rank-sum test).

Fig. 3 | The application of FDGC subtyping algorithm in multiple independent cohorts, and the association of MSI/MSS characteristics with clinical outcomes. a Sankey diagram indicating the comparison of FDGC subtype and BPRC subtype or EO2G subtype. b Sankey diagram and barplot indicating the comparison of FDGC subtype and ACRG subtype. Survival analysis of ACRG cohort classified by FDGC subtyping (two-sided log rank test). c Heatmap of CNV GI (copy number variation genomic instability index), MS-H assay status, mutations, and MSI/MSS-sig (microsatellite instability/microsatellite stability gene expression signature) assessment of ACRG cohort classified by FDGC subtyping (upper). Heatmap of MSI/MSS-sig of FDGC subtype (bottom). P-values are calculated by two-sided Fisher’s exact test (categorical variables) and two-way ANOVA test (continuous variables). d The association of MSI/MSS-sig level with OS in ACRG and FDGC cohorts (two-sided log rank test). e Heatmap illustrating significantly differential expressed proteins in MSI/MSS-sig high and low group (two-sided Wilcoxon rank-sum test). f Heatmap illustrating down-regulated proteins in G-IV compared with other subtypes (Kruskal-Wallis test). g Heatmap illustrating the dominant cell type compositions of G-I, G-III, and G-IV (two-sided Student’s t-test). h Boxplot showing the xCell score of Macrophages M1 and monocytes among four proteomic subtypes (two-sided Student’s t-test), n (G-I) = 28, n (G-III) = 56, n (G-IV) = 75, n (G-IV) = 20 biologically independent samples examined. Boxplots show median (central line), upper and lower quartiles (box limits), 1.5x interquartile range (whiskers). i Correlation between MSI/MSS-sig with Macrophages M1 and monocytes (two-sided Pearson’s correlation test). j The association of Macrophages M1 and monocytes with OS in FDGC and ACRG cohorts (two-sided Gehan-Breslow-Wilcoxon or two-sided log rank test). k The abundance of Macrophages M1 markers in MSI/MSS-sig high and low groups (two-sided Wilcoxon rank-sum test). l The abundance of Macrophages M1 markers in MSI/MSS-sig high and low groups (two-sided Wilcoxon rank-sum test). m Diagram illustrating the potential association between MSS/MSI characteristics with drug response. *P < 0.05 is considered statistically significant. **P < 0.05. ***P < 0.001. ****P < 0.0001. Source data are provided as a Source Data file.
than XHNSG (3.2% and 2.9%, respectively) (P < 1E-4); while the percentage of CD4 positive cells and CD8 positive cells were higher in XHSG (43.7%) and CD8 positive cells (39.2%) compared with XSG (2.9% and 1.4%, respectively) (P < 0.05) (Fig. 4m). The result verified the findings that GC patients with high TCR signaling are unlikely to benefit from XELOX, but instead respond to XELOX + anti-HER2. Taken together, the combination of trastuzumab with XELOX therapy could resulted in a synergistic antitumor effect, and TCR signaling pathway and CD8 + Tcm emerged as a favorable response marker for the combination therapy (Fig. 4n).
Activation of ECM/PI3K-AKT pathway related to resistance to anti-HER2 targeted therapy

We then focused on the HER2 subcohort, and separated the subcohort as HER2 sensitive group (HSG) and non-sensitive group (HNSG). There was a significant difference of prognosis in OS \((P = 0.039; HR = 0.75; 95\% CI, 0.41–1.35)\) and DFS \((P < 1.0E–4; HR = 0.30; 95\% CI, 0.17–0.54)\) between HSG and HNSG as expected (Fig. 5a). We then hypothesized the therapy response of anti-HER2 targeted therapy was related to the expression of HER2. Therefore, we evaluated the expression of HER2 identified in HSG and HNSG by MS, and found no difference between HSG and HNSG. Cox analysis suggested that ERBB2 could not be regarded as an independent prognostic factor in HER2 subcohort. These results reflected the low association of HER2 expression level with trastuzumab resistance (Fig. 3b). For investigating functional characteristics and molecular markers in sensitive or non-sensitive response to anti-HER2 targeted therapy, we performed further comparative analysis of HSG and HNSG. The KEGG pathway enrichment analysis revealed that HSG was featured by TCR signaling pathway, etc.; while HNSG was featured by ECM-receptor interaction, PI3K-AKT pathway, etc. (Fig. 5c and Supplementary Data 9). In addition, ECM was negatively correlated with clinical outcome \((P = 0.046; HR = 2.6; 95\% CI, 1.19–5.67)\) (Fig. 5e).

To further validate the association of ECM proteins with the response to anti-HER2 targeted therapy, we compared gene expression profiles data from NCI-N87 cells with trastuzumab-resistant cell lines \((N87-TR1, N87-TR2, N87-TR3, N87-TR4)\) by microarray analysis. As a result, we found ECM proteins such as COL4A1, COL6A5, FNI, GPIBA, ITGA4, THBS3, and THBS4 were also overrepresented in trastuzumab-resistant cell lines \(^{17}\) (Fig. 5f). The PI3K-AKT signaling pathway could be activated by a range of signals, including hormones, growth factors and components of the extracellular matrix (ECM), subsequently regulating cell proliferation and apoptosis. We found PI3K-AKT signaling pathway, showed the highest positive correlation with ECM-receptor interaction pathway \((r = 0.72, P = 2.4E–12)\) (Fig. 5g and Supplementary Fig. 5h). In addition, we observed the subsequent downregulation of apoptosis-related proteins, including BCL2, BCL2L1, CASP3, CASP7, and CDKN2A, in HNSG (Fig. 5i). Based on these findings, we speculated there was a potential synergistic effect of PI3K-AKT inhibition in combination with anti-HER2 therapy.

To validate the hypothesis, we performed further in vitro validation experiment to confirm the synergistic effects of the combination of anti-HER2 and the PI3K-AKT inhibitors. As reported in the previous studies, buparlisib (BKM120) is a commonly used potent, pan-class I of anti-HER2 and the PI3K-AKT inhibitors. As reported in the previous experiment to confirm the synergistic effects of the combination of anti-HER2 and the PI3K-AKT inhibitors. As reported in the previous experiment to confirm the synergistic effects of the combination of anti-HER2 and the PI3K-AKT inhibitors. As reported in the previous experiment to confirm the synergistic effects of the combination of anti-HER2 and the PI3K-AKT inhibitors.
samples, the three predictive models based on DSG/DNSG-sig, XSG/ XNSG-sig, HSG/HNSG-sig separately achieved high accuracy of 89%, 93%, and 100% (Fig. 6b).

To evaluate the accuracy of the predictive signatures for the chemotherapeutic response, we designed PRM strategy to quantify these signature proteins in FFPE tumor tissues from the new independent cohort composed of 60 GC patients receiving either DOS (N = 20: DSG, N = 10; DNSG, N = 10), XELOX (N = 20: XSG, N = 10; XNSG, N = 10), or anti-HER2 (N = 20: HSG, N = 10; HNSG, N = 10) therapies (Supplementary Data 11). We selected a set of target peptides that unique to these proteins, including DSG/DNSG-sig (ATP5S, C11orf31, CDC42SE2, CHP2, and AHR), XSG/XNSG-sig (RFC2, NIT1, RAB32, FLG2, BCL2, BCL2L1, BCL7C, BCL9L, CASP3, CASP7, CASP10, CDKN2A, CDKN2AIPNL, CDKN2C, TP53BP1, AKT1, CDK2, GNG5, IKBKG, INSR, PIK3AP1, PPP2R1B, PPP2R2D, PPP2R5A, SOS1, TSC2), and HSG/HNSG-sig (COL4A1, COL6A5, FN1, GP1BA, ITGA4, THBS3, THBS4).

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**Figures**

- **a**: Survival analysis for HER2 subcohort showing OS and PFS.
- **b**: Heatmap and volcano plots showing enrichment of PI3K-AKT pathway.
- **c**: Heatmap showing expression of PI3K-AKT pathway.
- **d**: Heatmap showing expression of FoxO signaling pathway.
- **e**: Heatmap showing expression of Focal adhesion.
- **f**: Heatmap showing expression of ECM-receptor interaction.
- **g**: Heatmap showing expression of PI3K-Akt signaling pathway.
- **h**: Heatmap showing expression of PI3K-Akt signaling pathway.
- **i**: Heatmap showing expression of PI3K-Akt signaling pathway.
- **j**: Heatmap showing expression of PI3K-Akt signaling pathway.
- **k**: Heatmap showing expression of PI3K-Akt signaling pathway.

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**Table**

| Therapy | Sensitive | Non-sensitive |
|---------|-----------|--------------|
| DOS     | MSI       | TCR-high     |
| XELOX   | ERBB2     | TCR-high     |
| XELOX+HER2 | MSI   | TCR-high     |
| HER2    | MSI       | TCR-high     |

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**Supplementary Data**

- Supplementary Data 11: Details on the cohort composition and target peptide selection.
- Supplementary Data 12: Detailed analysis of the predictive models.

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**References**

- [1] ABC et al. (2022) Nature Commun. 13:5723. DOI: 10.1038/s41467-022-33282-0.
Overexpression of CTSE synergistically enhances sensitivity to docetaxel by stabilizing microtubules

Based on the proteomic subtyping analysis, we found that intracellular proteinase such as cathepsin E (CTSE) overexpressed in G-III subtype featured with response to DOS but not XELOX therapy; the result was further validated in the following differential analysis of DOS and XELOX subcohorts. Taken together, the expression level of CTSE positively correlated with the patient chemosensitivity to DOS (Supplementary Fig. 7a).

We first investigated how CTSE levels modulated the response of tumor cells to DOS and XELOX, in cultured MKN45 and MGC803 GC cell lines. Compared with the cells transfected with an empty vector, CTSE overexpression significantly promoted the proliferation of MKN45 and MGC803 (Student’s t test, \( P < 0.01 \)) (Supplementary Fig. 7b and Fig. 7a, b), consistent with previous reports \(^{21,22} \) and excluded the negative effect of CTSE overexpression on cell proliferation. Next, we conducted similar experiments using clinical combined patterns of DOS (docetaxel: oxaliplatin: 5-fluorouracil = 1:1:10) and XELOX (oxaliplatin: 5-fluorouracil = 1:7.7) to treat MKN45 and MGC803 cells \(^{16} \). The degree of DOS and XELOX sensitivities in the MKN45 and MGC803 cells were estimated by their half-maximal inhibitory concentration (IC50) values \(^{31,32} \). We treated CTSE-overexpressing and empty vector-overexpressing MKN45 and MGC803 cells with DOS and XELOX therapies, respectively; consistent results in the two GC cell lines were observed. The IC50 values of DOS were significantly lower (0.37-fold and 0.53-fold decrease in MKN45 and MGC803, respectively; Student’s t test, \( P < 0.01 \)) in the CTSE-overexpressing groups (IC50, 1.47 and 1.26 nM, respectively) compared with the control groups (IC50, 2.32 and 2.69 nM, respectively) (Fig. 7c), whereas there was no obvious change for XELOX (1.23 and 0.83 fold change, respectively; \( P > 0.05 \)) (Fig. 7d). Taking into consideration the differences between DOS and XELOX, we further treated each CTSE-overexpressing and empty vector-overexpressing MKN45 and MGC803 cells with docetaxel (DOC), 5-fluorouracil (5-FU), and oxaliplatin (OXA). The CTSE-overexpressing groups were shown to have lower IC50 values for DOC than the control groups (0.31-fold and 0.38-fold decrease in MKN45 and MGC803 cells, respectively) (Student’s t test, \( P < 0.01 \)) (Fig. 7e). This, together with the findings that the IC50s of 5-FU (0.91 and 1.03 fold change, respectively; \( P > 0.05 \)) and OXA (1.05 and 0.84 fold change, respectively; \( P > 0.05 \)) did not change (Supplementary Fig. 7c, d), suggested that CTSE overexpression is an important determinant of DOC, but not 5-FU or OXA, sensitivity.

To elucidate the molecular mechanism by which CTSE overexpression enhanced the cytotoxic effects of DOC, we performed proteomic analysis using a label-free technique and compared the proteome between the CTSE-overexpressing and control MKN45 cells treated with or without DOC. We performed three repeats in the CTSE-overexpressing and control MKN45 cells, and made a uniformed quality control, which resulted in the identification of 3371 and 5735 GPs, respectively, in MKN45 cells with and without DOC treatment at a 1% global protein FDR (Supplementary Data 12). There were no major differences in the coverage between the CTSE-overexpressing and control MKN45 cells with or without DOC treatment (Supplementary Fig. 7e–g). Differential proteomic analysis of the CTSE-overexpressing and control MKN45 cells without treatment showed that 971 upregulated GPs were enriched in basal transcription factors (\( P = 4.36E^{-3} \) and Wilcoxon rank–sum test).
Fig. 6 | The construction and validation of predictive classifiers. a Diagram describing a construction and validation of the predictive modeling for sensitive (S) and non-sensitive (NS) groups. b The discovery cohort: Classification error matrix using logistic regression classifier of 80% training set and 20% testing set in DOS, XELOX, and HER2 subcohorts. The number of samples identified is noted in each box. c Heatmap showing differential expression of DSG/DNSG-sig, XSG/XNSG-sig, and HSG/HNSG-sig based on PRM quantification in an independent cohort (validation cohort) composed of 60 GC patients (two-sided Wilcoxon rank-sum test). Source data are provided as a Source Data file.
mediated proteolysis (including TAF2, TAF3, TAF4, TAF13, GTF2H4, etc.), ubiquitin-mediated proteolysis ($P = 4.45 \times 10^{-3}$) (including UBE2C, UBE2G1, UBE2G2, CUL2, PIAS2, etc.), and p53 signaling pathway ($P = 3.52 \times 10^{-2}$) (including CCNB1, CASP3, TSC2, CDK6, IGFBP3, etc.), (Fig. 7f and Supplementary Data 12). The 969 downregulated GPs were mainly enriched processes involving endocytosis ($P = 2.29 \times 10^{-4}$) (including CHMP2A, CLTA, WASH1, SNX2, SNX6, etc.), lysosome ($P = 3.37 \times 10^{-3}$) (including CTSB, CTSC, GUSB, ATP6VOC, LAMPI, etc.), regulation of autophagy ($P = 7.76 \times 10^{-3}$) (including GABARAPL1, GABARAPL2, ATG5, ATG7, BECN1, etc.), and ribosome ($P = 9.47 \times 10^{-3}$) (including MRPL1, MRPL30, RPL17, MRPS11, RPLP1, etc.) (Fig. 7f and Supplementary Data 12), which potentiated CTSE's promotion of tumor growth.
Further differential proteomic analysis suggested that CTSE overexpression rendered MKN45 cells prone to necrosis with DOC treatment [e.g., the upregulation of the TNF pathway (P = 3.96E–2) (including MAPK7, AKT1, CEBPB, JUN, NFκB1, etc.) and the down-regulation of VEGF (P = 1.90E–2) (including MAPK3, MAPK14, PIK3CA, PKN, PIK3R2, etc.) and basal transcription factors (P = 2.68E–2) (including TAF8, TAF10, MNAT1, GTF2H5, GTF2B, etc.)], which suggested that CTSE has a positive response to DOC (Fig. 7g and Supplementary Data 13). Overexpression of CTSE did not alter the protein expression levels of MAP4, and TUBB3 (Wilcoxon rank-sum test, P > 0.05) (Supplementary Fig. 7h). Interestingly, overexpression of CTSE and DOC treatment synergistically reduced the expression levels of the proteins involved in the microtubule assembly bioprocesses [MAP4 (fold change = 0.15), and TUBB3 (fold change = 0.46) (Wilcoxon rank-sum test, P < 0.05)] (Supplementary Fig. 7h). According to previous research, microtubule-associated protein share the same binding site to microtubule as paclitaxel, and the increase of soluble intracellular tubulin is an indicator of microtubule stability16,40. Therefore, we speculated that the low expression of microtubule-associated proteins rendered the microtubules vulnerable to DOC and made the GC cell lines hypersensitive. We also performed immunoblot analysis to verify that DOC exposure in the CTSE group reduced the content of both soluble α- and β-tubulin subunits (Student’s t test, P < 0.001), indicating the enhanced polymerization of tubulin into microtubules to increase DOC sensitivity (Fig. 7h). The immunohistochemical staining showed significant differences in the CTSE expression between the sensitive and non-sensitive groups in patients treated with DOS and XELOX (Student’s t test, P < 0.0001) (Supplementary Fig. 7i–l), which verified the aforementioned observations that CTSE levels positively responded to DOS treatment.

Overexpression of TKTL1 decreases the sensitivity to docetaxel by inducing abnormal chromosome segregation

Transketolase-like-1 (TKTL1), a rate-limiting enzyme in the non-oxidative part of the pentose-phosphate pathway, has been demonstrated to promote carcinogenesis and cell proliferation1. In contrast to CTSE, the levels of TKTL1 negatively corresponded to DOS therapy (Supplementary Fig. 7a). A 6.9-fold increase of TKTL1 expression was observed in the DNG, but not DSG, suggesting that TKTL1 led to a decreased sensitivity to DOS. Consistent with previous findings17, we confirmed that the overexpression of TKTL1 promoted cell proliferation in both MKN45 and MGC803 cell lines (Student’s t test, P < 0.01) (Supplementary Fig. 8a and Fig. 7i, j). By comparing the sensitivities of TKTL1-overexpressing and control cells to DOS and XELOX, we showed that the IC50 values of DOS were significantly higher (3.76-fold and 2.50-fold increase in MKN45 and MGC803, respectively; Student’s t test, P < 0.001) in the TKTL1-overexpressing cells (IC50, 9.71 nM and 6.73 nM, respectively) compared with the control groups (IC50, 2.59 nM and 2.69 nM, respectively), whereas, no significant change in XELOX was observed (1.16 and 1.35 fold change respectively, P > 0.05) (Fig. 7k, l). Taking into consideration the differences between DOS and XELOX, we further treated each TKTL1-overexpressing and empty vector-overexpressing MKN45 and MGC803 cells with DOS, 5-FU, and OXA, and observed that the TKTL1-overexpressing groups had higher IC50 values for DOC than the control groups (3.57-fold and 3.18-fold increase, respectively; Student’s t test, P < 0.01) (Fig. 7m). This, together with findings that the IC50s of 5-FU (1.16 and 1.24 fold change, respectively; P > 0.05) and OXA (0.95 and 0.98 fold change, respectively; P > 0.05) did not change (Supplementary Fig. 8b, c), suggested that TKTL1-overexpressing cells were resistant to DOC. DOC is reported to cause cell cycle arrest in mitosis, and increases the apoptosis of cancer cells41. Here, we confirmed that DOC induced G2/M arrest and increased the apoptosis in MKN45 cell lines; however, the overexpression of TKTL1 alleviated the G2/M arrest and reduced cell apoptosis following treatment with DOC (Student’s t test, P < 0.01) (Supplementary Fig. 8d–g). Considering that increased TKTL1 promoted the release of a DOC-mediated mitosis block, we examined the chromosome status in TKTL1-overexpressing and control cells. Severe abnormal chromosome segregation phenotypes were observed in the DOC-treated TKTL1-overexpressing cells, including misaligned, multi-polar, and lagging chromosomes (Student’s t test, P < 0.05) (Fig. 7n). Moreover, increased aneuploidy was observed in DOC-treated TKTL1-overexpressing cells, compared with DOC-treated control cells (P < 0.05) (Fig. 7o). The immunohistochemical staining showed significant differences in TKTL1 expression between the sensitive and non-sensitive groups in patients treated with XELOX or DOS (Student’s t test, P < 0.0001) (Supplementary Fig. 8h–k), which agreed with the findings above that TKTL1 positively and negatively responds to XELOX and DOS, respectively. Taken together, these results indicated that TKTL1 promoted DOC-treated cell survival by causing abnormal chromosome segregation and DNA aneuploidy. Finally, we proposed a model in which CTSE functions as a cell intrinsic enhancer of the chemosensitivity of DOC via microtubule stabilizing effects, while TKTL1 functions as an attenuator by inducing abnormal chromosome segregation (Fig. 7p).

Discussion

GC is currently the fourth most common malignancy, and the second leading cause of cancer-related deaths worldwide. As it is most often diagnosed at an advanced stage, the preferred treatment for advanced GC is surgery42. However, for patients with no chance of surgical treatment, the ultimate goal of chemotherapy is to prolong survival and improve quality of life43. Recently, significant progress has been made by the iterative updates of first-line chemotherapeutic drugs44,45, and the integration of HER2-targeting drugs46,47. Meanwhile, significant challenges have been added as there are no indicators for their chemotherapeutic effectiveness, and the development of drug resistance remains unresolved. Therefore, it is imperative to make a preclinical diagnosis of tumor response to the first-line therapies XELOX and DOS. Herein, we collected FFPE tissues of 206 patients with GC before the
initiation of different treatments (including DOS, XELOX, and anti-HER2-based therapy), and presented an unprecedented large-scale clinical proteomic landscape, with the aim of identifying reliable predictive markers of the GC patient response to the diverse chemotherapies and targeted therapies.

In the study, the proteomic subtypes were identified with distinct molecular features and clinical outcomes, and associated with therapy subcohorts. Further analysis revealed that the significant distribution difference of therapy subcohorts among proteomic subtypes were mainly derived from HER2 subcohort, due to the selection of HER2-positive patients for anti-HER2 targeted therapy. In addition, HER2 expression detected by IHC and FISH analysis demonstrated the specific molecular pattern in HER2-positive GC patients, supporting the reliability of proteomic subtyping. We also surveyed the robust quantification of the signature proteins identified in four proteomic subtypes. We found 1679 signature proteins (74% of 2279 signature proteins) have immunohistochemistry (IHC) staining data as reported by The Human Protein Atlas (HPA), among which 1467 (87.4%) signature proteins showed medium to high tumor-specific staining in GC samples, demonstrating these signature proteins could be reused and validated in the future research community (Supplementary Fig. 3a).

Pathway enrichment analysis of the proteomic subtypes suggested that activation of endocytosis indicated drug sensitivity while high expression of ECM associated with drug resistance. These results suggested endocytosis activation or ECM inhibition could be applied in the combination of chemotherapy and targeted therapy, thus improving therapy sensitivity and alleviating therapy resistance. Further analysis revealed high expression of THSD4 was significantly associated with poor prognosis and highly correlated with responses of drugs (including 5FU, oxaliplatin, and docetaxel) in gastric cancer cell lines data from the DepMap. We also validated THSD4 over-expression reduced the anti-tumor effect of chemotherapeutic drugs in vitro. The association between ECM proteins and drug resistance was validated by PRM approach in an independent cohort, indicating these extracellular matrix proteins could serve as indicators to predict chemotherapy response in clinic.

In addition, the proteomic subtyping system have been applied in other gastric cancer cohorts (BPRC cohort, ACRG cohort, and EOGC cohort). The results demonstrated there was a significant concordance between FDGC subtype and EOGC subtype or ACRG subtype, but not between FDGC subtype and BPRC subtype. After our examination of the composition of clinical characteristics, we speculated the composition of Lauren’s type, not other clinical characteristics, was the possible reason for no concordance between the FDGC and the BPRC subtypes. Based on the unique features of our proteomic subtypes associated with therapeutic response, we could assess the possibility of response to specific therapy in other cohorts (although there was no exact therapy response). Overall, the result demonstrated the robustness of FDGC subtyping and their consistent and significant association with clinical outcomes despite the various sources of heterogeneity and cohort differences. Importantly, the FDGC subtyping in ACRG cohort provided a clue that GC patients with MSS status had an association with drug resistance and poor survival. We further explored the association of immune microenvironment and MSI/MSS characteristics among four proteomic subtypes. The results showed G-IV had the lowest MSI/MSS-sig level, and was featured by less cytotoxic and antigen processing and presentation, which made more monocytes aggregated and less macrophages M1 differentiated.

Whether MSI/MSS characteristics could be a predictive marker for response to chemotherapy and targeted therapy remain not clearly reported. Even though XELOX and DOS are two major first-line treatments\(^\text{17}\), there is no indication for patients to choose the appropriate treatment strategy. Surprisingly, we found MSS characteristics served as a chemo-resistance indicator and poor prognosis marker in the DOS subcohort, but not in the XELOX subcohort. Furthermore, we conducted a thorough bioinformatic analysis of the sensitive and non-sensitive patient groups to elucidate the potential mechanisms of drug response, thereby aiding the prediction of sensitivity to chemotherapy and the reversal of drug resistance to improve therapeutic efficacy. Interestingly, our results showed that patients with GC with predominant immune pathways (BCR, TCR, and FcεRI pathways) were more likely to be sensitive to DOS therapy while resistant to XELOX therapy. Among these pathways, TCR signaling pathway showed obvious association with drug response and clinical prognosis. The XCell analysis also revealed that immune cells such as CD8+ Tcm enriched in DSG and XNSG, indicating the distinct immunostimulatory effect in response to DOS and XELOX therapies. The ranked immunogenic cell death (ICD) prediction score of chemotherapeutic regimens showed docetaxel was the top one ICD drug. Surprisingly, we found ERBB pathway was enriched in XNSG and DSG, and unvariable and multivariable cox analysis validated ERBB2 was a predictive prognostic marker adjusted for baseline clinical covariates in XELOX subcohort, indicating the clinical implication of XELOX combined with anti-HER2 targeted therapy. The synergistic effects of the combination of anti-HER2 and XELOX therapy was confirmed by the in vitro validation experiment. Further differential analysis focused on the combination therapy and revealed the activation of TCR signaling pathway was associated with sensitive response to XELOX combined with anti-HER2 targeted therapy. These results indicated that TCR signaling pathway exerts diverse effects in response to DOS, XELOX, as well as XELOX combined with anti-HER2 targeted therapy, which assist to determine the appropriate chemotherapy and targeted therapeutic strategy for GC patients.

Clinically, the usage of the anti-HER2 targeted therapy was determined by the expression of HER2 detected by FISH (fluorescence in situ hybridization) and IHC (Immunohistochemistry). Although trastuzumab-based first-line treatments represent the standard approach for HER2-positive GC, not all HER2-positive patients with GC benefit from this treatment, and show variable ORR (~32–68%). Researchers have explored various approaches, such as circulating tumor DNA detection and gene expression analyses, to elucidate the possible mechanisms involved in trastuzumab resistance\(^\text{18}\). In this study, consensus clustering analysis identified four proteomic subtypes (G-I to G-IV), among which G-I subtype had the highest proportion of patients receiving anti-HER2 therapy, due to the selection of HER2-positive patients for anti-HER2 targeted therapy, indicating the specific proteome panel of HER2-positive patients. However, HSG and HNSG accounted nearly half in G-II subtype, suggesting glycolysis/gluconeogenesis and pantothenate/CoA biosynthesis (enriched in G-II subtype) were unrelated to anti-HER2 therapy response. On the contrary, G-IV subtype, featured by ECM pathway, had the highest proportion of non-sensitive patients of anti-HER2 therapy. Consistently, further comparative analysis of between sensitive and non-sensitive patients with anti-HER2 targeted therapy also revealed ECM-receptor interaction pathway were enriched in non-sensitive group, and negatively correlated with clinical outcome. Surprisingly, we found the low association of HER2 expression level with trastuzumab resistance. Importantly, apart from steric hindrance to anti-HER2 antibody binding through ECM components in tumor, we found a potential mechanism related to trastuzumab resistance that ECM could activate PI3K-AKT signaling pathway and inhibit the subsequent apoptosis signaling, thus impairing the anti-tumor effect of trastuzumab. Further experiments validated that PI3K-AKT inhibitor buparlisib (BKM120) could synergize with trastuzumab, resulting in an enhanced anti-tumor effect, and provided a promising therapeutic strategy for HER2-positive GC patients.

Current clinically used tumor markers for GC screening have achieved much progress, including HER2\(^\text{19}\), human epidermal growth factor receptor (EGFR)\(^\text{20}\), mammalian target of rapamycin (mTOR)\(^\text{21}\), PD-L1\(^\text{22}\), and TP53\(^\text{22}\). However, predictive markers for specific
therapeutic strategies in GC are still lacking. The identification of effective biomarkers for clinical diagnosis, prognosis, prediction, and therapy is a continuous effort. For clinical guidance in the response to therapy, in this study, we employed proteomic technology implemented with machine learning statistics to search for drug sensitive and non-sensitive predictive biomarkers in GC as an alternative to clinical therapeutic strategies. Statistical analysis was performed on the discovery set to identify potential chemosensitivity mediators for GC. Furthermore, these signature proteins identified in predictive models for DOS, XELOX, and HER2 subcohorts, were validated by parallel reaction monitoring (PRM) quantification with 100% expression frequency, in the new independent cohort, exhibiting a well distinguish between sensitive patients and non-sensitive patients and indicating the reliability of these results. In addition, the good diagnostic performance of prediction model for XELOX therapy was also confirmed by an external validation (BPRC DGC cohort, AUC = 0.95). Besides proteomic quantification, the expression of these signature proteins was also validated by the IHC staining data reported by HPA, (Supplementary Fig. 6f). Taken together, our study provides a panoramic view of the clinical proteomic landscape of GC which can be specifically coupled to the therapeutic response. Multicenter validation of predictive signatures demonstrated the robustness and accuracy of this prediction model: PRM quantification and HPA IHC staining data validated that these signature proteins in models could be reused and validated in the future research community.

Based on the proteomic analysis, we concluded that CTSE expression levels were sensitive to DOS therapy but non-sensitive to XELOX therapy. As recently reported, cathepsins have emerged as an important class of proteolytic enzymes in cancer development, and cysteine cathepsin inhibitors have been proposed as anticancer agents72–75. Data presented in this study confirmed the potential for CTSE to promote GC growth. More importantly, we further demonstrated that CTSE could enhance the cytotoxic response of GC cells to docetaxel, suggesting it as a candidate marker of the drug response. Differential proteomic analysis further revealed the downregulation of microtubule-associated proteins (MAP4 and MAP7), suggesting a potential chemoresistance mechanism by which CTSE overexpression can affect microtubule dynamics. CTSE is widely distributed in digestive cancers, such as esophageal cancer, GC, colorectal cancer, pancreatic cancer, and liver cancer. We speculate that CTSE also has an enhanced antimtumor effect on DOC-based chemotherapy in other digestive cancers.

In addition, increased TKTL1 expression76,77 attenuates the chemosensitivity of patients to DOS therapy, which is completely opposite to the response to DOC-based chemotherapy observed with CTSE. Our previous study revealed that TKTL1 gathered signals from the nutrition and cell cycles, and promoted cell cycle proceeding78. In the current study, we further found that high levels of TKTL1 promoted the escape of tumor cells from apoptosis induced by DOC, mediated by abnormal chromosome segregation and DNA aneuploidy. As a result, TKTL1 promoted DOC-treated cells’ survival. Taken together, we propose that CTSE functions as a cell intrinsic enhancer of chemosensitivity to DOC via microtubule stabilizing effects, whereas TKTL1 functions as an attenuator by inducing abnormal chromosome segregation.

In summary, our proteomic data described an atlas of chemotherapy and targeted therapy in GC, covering three first-line therapy subcohorts: the DOS, XELOX, and HER2 subcohorts. This study identified the proteomic subtyping correlated with clinical outcomes (overall survival and therapy response), the performance of which was further evaluated in multi-center GC cohorts. Importantly, we provided indicators for these chemotherapy and targeted therapy, including MSI/MSS, ERBB2 expression, the activation of ECM and TCR signals, to help develop rational treatment options for GC patients in clinic, thus providing them more effective strategy. Finally, we extracted S-overrepresented and NS-overrepresented proteomes and constructed predictive models, which could well distinguish sensitive GC patients from non-sensitive GC patients in response to DOS therapy, XELOX therapy, and anti-HER2 therapy. The construction and validation of proteomic-based predictive classifiers for these first-line therapies of GC contributed to personalized chemotherapy and targeted therapy, to a certain extent; thereby, moving us toward the era of proteomic-driven precision medicine. More importantly, we propose that CTSE coupled with TKTL1 can facilitate a more effective clinical decisions to determine the relative benefit of DOC-based therapy for patients. In summary, our study has potentially important clinical implications in GC, and we look forward to further developing potential therapeutic combinations for DOC-based therapy.

### Methods

#### Clinical sample acquisition

This study was approved by the Research Ethics Committee of Zhongshan Hospital (B2019-2008R). Written informed consent was received from all patients included in this study.

Archival formalin-fixed, paraffin-embedded (FFPE) tissues from patients with GC, from January 2002 to March 2020, were reviewed in the Department of Pathology, Zhongshan Hospital, Fudan University (Shanghai, R. P. China). The discovery cohort consisted of the DOS subcohort (44 cases treated with S-1 and oxaliplatin combined with docetaxel), the XELOX subcohort (70 cases treated with capecitabine and oxaliplatin), and the HER2 subcohort (71 cases treated with the anti-HER2-based therapy), according to the treatment profiles. Another 21 cases were assigned as “Others,” of which 3 cases received apatinib or docetaxel therapies, and 18 cases had no chemotherapy information. The independent validation cohort for PRM verification included 60 patients with GC, receiving either DOS (N = 20), XELOX (N = 20), or anti-HER2 (N = 20) therapies. All the chemotherapy regimens were given at standard dosing as described in previous studies. Briefly, the XELOX regimen was administered as follows: capicitabine (1000 mg/m², twice daily on days 1–14) and oxaliplatin (130 mg/m² on day 1). The DOS regimen was provided as S-1 (tegafur, gimeracil and oteracil potassium capsules; 40 mg/m² orally administered twice a day on days 1–14), oxaliplatin (100 mg/m² on day 1), and docetaxel (40 mg/m² on day 1). The two regimens were repeated every 3 weeks79. In the HER2 subcohort, patients generally received the anti-HER2-based therapy either as a XELOX combined anti-HER2 therapy or other chemotherapies combined anti-HER2 therapy, as reported in the previous two Phase II trials80. All the patients of the HER2 subcohort received trastuzumab with a dose of 6 mg/kg every 3 weeks after a first infusion of 5 mg/kg81. Medical records were reviewed to obtain the follow-up data. The treatment response was evaluated by CT/MRI scanning following the Response Evaluation Criteria in Solid Tumors (RECIST) (version1.1). Tumor response was assessed and categorized as a complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). In clinic, the ORR, generally defined by the Food and Drug Administration as the sum of PR plus CR, is a direct measurement of drug antitumor activity. Here, the ORR, defined as PR plus CR, was selected for the efficacy evaluation; patients with CR and PR were defined as sensitive (S) and those with SD and PD were defined as non-sensitive (NS). Major clinical parameters were collected including age, gender, grade, Lauren’s type, primary site, chemotherapy and targeted therapy, therapy cycle (3 weeks per cycle), RECIST, TNM stage (according to the eighth edition of the American Joint Committee on Cancer staging system), status of cancer recurrence or progression, and status of survival. Archival FFPE tissues with at least 80% tumor purity of GC cases were taken from chemotherapy naïve patients. The detailed clinical information of each patient is included in Supplementary Data 1.
HER2 evaluation

Immunohistochemistry (IHC) and in selected cases fluorescence in situ hybridization (FISH) diagnostics have been implemented to confirm the status of HER2 expression. For all patients, HER2 expression was detected by IHC. IHC staining was carried out using the rabbit monoclonal anti-HER-2/NEU (4B5) antibody (working solution with the concentration of 6 μg/mL for IHC; Ventana Medical Systems, Inc. Tucson, AZ, USA) as the primary antibody against HER2 on a Ventana Benchmark XT automatic staining system, according to the manufacturer’s instructions. The amended HER2 IHC scoring system for gastric cancer was used as the criteria for scoring the stained slides.

For scoring the IHC image, Histoscore (H-score) was calculated by multiplying the proportion of positive cells in the sample (0–100%) by the average intensity of the positive staining (1+, 2+, or 3+). A score ranging between 1 and 300 as previously described was applied for HER2 amplification levels. HER2 amplification levels were measured when the result of IHC was 2+. The PathVysion HER2 DNA Probe kit (Abbot Laboratories, Des Plaines, Illinois, USA) was used to perform FISH analysis, according to the manufacturer’s protocol. Any case with IHC 3+ or IHC 2+/FISH+ was considered to be HER2-positive, while cases with IHC 0 or IHC 1+ or IHC 2+/FISH− were considered as HER2-negative.

Cell line

Human HEK293T (Cat#: CRL-11268 from ATCC; RRID: CVCL_QWS4), MKN45 (Cat# JCRB0254 from Japanese Collection of Research Bioresources (JCRB) Cell Bank, RRID: CVCL_O434), MGC803 (Cat#: C6562 from Beyotime Biotechnology, RRID: CVCL_5334), and NCI-N87 (Cat# CRL-5822 from ATCC; RRID: CVCL_1603), were obtained and cultured in DMEM (GIBCO) with 10% FBS (GIBCO) in 5% CO2 at 37°C. Cells validation using short tandem repeat markers (STR) were performed by Meixuan Biological Science and Technology Ltd. (Shanghai). In detail, these cell lines were firstly tested cell species by PCR method using extracted total genomic DNA, and examined by STR profiling. Then, STR data were analyzed using the DSmZ (German Collection of Microorganisms and Cell Cultures) online STR database (http://www.dsmz.de/fg/cgi-bin/str.shtml). Cell lines were tested negative for mycoplasma contamination. All cells were grown according to the instruction.

Protein extraction and trypsin digestion of GC FFPE samples

The biopsy tumor FFPE samples derived from 206 therapy-naïve GC patients were collected, and the tumor regions were determined by pathological examination. For clinical sample preparation, sections (10 μm thick) from FFPE blocks were macro-dissected, deparaffinized with xylene, and washed with ethanol. The ethanol was removed completely and the sections were left to air-dry. For this purpose, a hematoxylin-stained section of the same tumor was used as reference. Areas containing 80% or more tumor were examined independently by two expert gastrointestinal pathologists (C.X. and Y.H.). Lysis buffer [0.1 M Tris-HCl (pH 8.0), 0.1 M DTT (Sigma, 43815), 1 mM PMSF (Amresco, MI45)] was added to the extracted tissues, and subsequently sonicated for 1 min (3 s on and 3 s off, amplitude 25%) on ice. The supernatants were collected, and the protein concentration was determined using the Bradford assay. The extracted tissues were then lysed with 4% sodium dodecyl sulfate (SDS) and kept for 2–2.5 h at 99°C with shaking at 1800 rpm. The solution was collected by centrifugation at 12,000 g for 5 min. A fourfold volume of acetone was added to the supernatant and kept in −20°C for a minimum of 4 h. Subsequently, the acetone-precipitated proteins were washed three times with cooled acetone. Filter-aided sample preparation (FASP) procedure was used for protein digestion. The proteins were resuspended in 200 μL 8 M urea (pH 8.0) and loaded in 30 kD Microcon filter tubes (Sartorius) and centrifuged at 12,800 g for 20 min. The precipitate in the filter was washed three times by adding 200 μL 50 mM NH4HCO3. The precipitate was resuspended in 50 μL 50 mM NH4HCO3. Protein samples underwent trypsin digestion (enzyme-to-substrate ratio of 1:50 at 37°C for 18–20 h) in the filter, and then were collected by centrifugation at 12,800 g for 15 min. Additional washing, twice with 200 μL of water, was essential to obtain greater yields. Finally, the centrifugate was pumped out using the AQ model Vacuum concentrator (Eppendorf, Germany).

LC-MS/MS

Peptide samples were analyzed on a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Rockford, IL, USA) coupled with a high-performance liquid chromatography system (EASY nLC 1200, Thermo Fisher Scientific). Peptides, re-dissolved in Solvent A (0.1% formic acid in water), were loaded onto a 2-cm self-packed trap column (100-μm inner diameter, 3-μm ReproSil-Pur C18-AQ beads, Dr. Maisch GmbH) using Solvent A, and separated on a 150-μm-inner-diameter column with a length of 15 cm (1.9-μm ReproSil-Pur C18-AQ beads, Dr. Maisch GmbH) over a 75 min gradient (Solvent A: 0.1% formic acid in water; Solvent B: 0.1% formic acid in 80% ACN) at a constant flow rate of 600 nL/min (0–75 min, 0 min, 4% B; 0–10 min, 4–15 B; 10–60 min, 15–30% B; 60–69 min, 30–50% B; 69–70 min, 50–100% B; 70–75 min, 100%). The eluted peptides were ionized under 2 kV and introduced into the mass spectrometer. MS was operated under a data-dependent acquisition mode. For the MS Spectrum full scan, ions with m/z ranging from 300 to 1400 were acquired by Orbitrap mass analyzer at a high resolution of 120,000. The automatic gain control (AGC) target value was set as 3E + 06. The maximal ion injection time was 80 ms. MS2 Spectra acquisition was performed in top-speed mode. Precursor ions were selected and fragmented with higher energy collision dissociation with a normalized collision energy of 27%. Fragment ions were analyzed using an ion trap mass analyzer with an AGC target value of 5E + 04, with a maximal ion injection time of 20 ms. Peptides that triggered MS/ MS scans were dynamically excluded from further MS/MS scans for 12 s. A single-run measurement was kept for 75 min. All data were acquired using Xcalibur software v2.2 (Thermo Fisher Scientific).

Peptide and protein identification

MS raw files were processed using the Firmiana proteomics workstation. Briefly, raw files were searched against the NCBI human Refseq protein database (released on 04-07-2013: 32,015 entries) using the Mascot search engine (version 2.4, Matrix Science Inc). The mass tolerances were: 20 ppm for precursor and 50 ppm for product ions collected by Q Exactive HF-X. Up to two missed cleavages were allowed. The database searching considered cysteine carbamidomethylation as a fixed modification, and N-acetylation, and oxidation of methionine as variable modifications. Precursor ion score charge were limited to +2, +3, and +4. For the quality control of protein identification, the target-decoy-based strategy was applied to confirm the FDR of both peptide and protein, which was lower than 1%. Percolator was used to obtain the quality value (q-value), validating the FDR (measured by the decoy hits) of every peptide-spectrum match (PSM), which was lower than 1%. Subsequently, all the peptides shorter than seven amino acids were removed. The cutoff ion score for peptide identification was 20. All the PSMs in all fractions were combined to comply with a stringent protein quality control strategy. We employed the parsimony principle and dynamically increased the q-values of both target and decoy peptide sequences until the corresponding protein FDR was <1%. Finally, to reduce the false positive rate, the proteins with at least one unique peptide were selected for further investigation.

Label-free-based MS quantification of proteins

The one-stop proteomic cloud platform “Firmiana” was further employed for protein quantification. Identification results and the raw...
data from the mzXML file were loaded. Then for each identified peptide, the extracted-ion chromatogram (XIC) was extracted by searching against the MSI based on its identification information, and the abundance was estimated by calculating the area under the extracted XIC curve. For protein abundance calculation, the nonredundant peptide list was used to assemble proteins following the parsimony principle. The protein abundance was estimated using a traditional label-free, intensity-based absolute quantification (iBAQ) algorithm, which divided the protein abundance (derived from identified peptides’ intensities) by the number of theoretically observable peptides. A match between runs was enabled to transfer the identification between separate LC-MS/MS runs based on their accurate mass and retention time after retention time alignment. We built a dynamic regression function based on the commonly identified peptides in tumor samples. According to correlation value R², Firmilana chose linear or quadratic functions for regression to calculate the retention time (RT) of corresponding hidden peptides, and to check the existence of the XIC based on the m/z and calculated RT. Subsequently, the fraction of total (FOT), a relative quantification value was defined as a protein’s iBAQ divided by the total iBAQ of all identified proteins in one experiment, and was calculated as the normalized abundance of a particular protein among experiments. Finally, the FOT values were further multiplied by 10⁵ for ease of presentation, and missing values were assigned 10⁻⁵;

Quality control of the mass spectrometry data
To quality control the MS performance, the HEK293T cell lysate was measured every three days as the quality control standard. The quality control standard was digested and analyzed using the same method and conditions as the GC samples. Pearson's correlation coefficient was calculated for all quality control runs using the R statistical analysis software v.3.5.1 (Supplementary Fig. 1b). The average correlation coefficient among the standards was 0.964, and the maximum and minimum values were 1 and 0.87, respectively. The log₁₀ transformed FOTs for each GC sample (Supplementary Fig. 1d) were plotted to show consistency of data quality. The dynamic range of protein identification of each sample was shown according to the descending sort of retention time (RT) of corresponding hidden peptides, to check the expression of the XIC based on the m/z and calculated RT. The dynamic range of protein identification (FOT), a relative quantification value was defined as a protein’s iBAQ divided by the total iBAQ of all identified proteins in one experiment, and was calculated as the normalized abundance of a particular protein among experiments. Finally, the FOT values were further multiplied by 10⁵ for ease of presentation, and missing values were assigned 10⁻⁵;

The proteomic subtypes generated by consensus clustering analysis and their biological function
The protein expression matrix of the gastric cancer cohort was used to identify the proteomic subtypes using the consensus clustering method implemented in the R package ConsensusClusterPlus v.3.8.89. All FOTs < 10⁻⁵ were replaced with 10⁻⁵. Prior to the consensus clustering analysis, we performed a log₁₀ transformation and median centered normalization to facilitate the interpretation of the expression data. Then, the top 1,000 proteins with the highest median absolute deviation were subjected to ConsensusClusterPlus in R v.3.5.1 for unsupervised consensus clustering. The cluster analysis was performed with the following setting: minK = 10, reps = 10,000, pthresh = 0.8, pfactor = 1, clusterAlg = “hc”, distance = “pearson” for the clustering runs. A preferred cluster result was selected by considering the profiles of the consensus cumulative distribution function (CDF) and delta area under the CDF curve for clustering solutions between 2 and 10 clusters. As shown in Supplementary Fig. 2a, c, the rank survey profiles of the consensus CDF and the delta area under the CDF curve, along with the consensus membership heat maps, indicated a four-subtype solution for 206 cases and 179 cases of GC using the proteomic data. This showed clear separation and the significant prognostic differences (OS) among 4 clusters (Fig. 2c and Supplementary Fig. 2b). To generate the abundance heatmap shown in Fig. 2a, the GC samples in each subtype were rearranged from G-I to G-IV, using the signature protein abundance matrix enriched in the signature pathways for each subtype. The signature proteins of each subtype defined here should meet the following criteria: (1) detected in at least 10% of the subtype, (2) differentially expressed of the subtype compared with other subtypes with fold change >2 and P < 0.05 (log10-transformed FOT, two-sided Student’s t test). KEGG pathway enrichment was performed to determine the biological function of proteomic subtypes.

Correlation between proteomic subtype and clinical features
The association between the clinical information and proteomic subtypes was examined using Fisher’s exact test for categorical data, including gender, grade, Lauren’s type, primary site, TNM stage, HER2 status, and response. A two-sided log rank test and Kaplan–Meier survival curves were applied to compare the prognosis among the four proteomic subtypes (G-I to G-IV) using GraphPad Prism 8 software. To evaluate the prognostic power of the proteomic subtypes, we applied univariable Cox analysis and multivariable Cox analysis of the subtypes with known clinical covariates (such as grade and Lauren’s type) (Supplementary Table 3). P-values < 0.05 were considered as significantly different. OS was used as primary endpoint. For proteomic subtype and clinical variables, hazard ratio was calculated from Cox proportional hazards regression analysis. P-values < 0.05 were considered as significantly different. OS was used as primary endpoint. Clinical variables analyzed with P-values < 0.05 using univariable analysis was chosen to enter Cox regression multivariate analysis. In the multivariate analysis, proteomics subtyping could also serve as an independent predictive factor after adjusting for clinical covariates.

Validation of the FDGC subtyping in other independent cohorts
We validated the FDGC subtyping identified in our cohort using other independent gastric cancer cohorts (BPRC cohort, EOGC, and ACRG cohort) using RapidMiner 9.6.0 (RapidMiner Inc, Boston, USA). Polynomial by Binominal Classification operator uses a binominal classifier and generates binominal classification models for different classes and then aggregates the responses of these binominal classification models for classification of polynomial label. The Fast large margin operator using logistic regression, applied in the subprocess of the Polynomial by Binominal Classification operator, was employed to build the prediction model based on signature proteins of each subtype in the FDGC cohort.

The evaluation of MSI/MSS characteristics
We calculated the gene expression signature scores using the average of log intensity (also known as the geometric average) of expression of genes in the signature. We compared the association of FDGC subtyping in our cohort and ACRG cohort with the pre-defined published gene expression signatures relevant for MSI/MSS examined by ANOVA test.

The single sample gene set enrichment analysis (ssGSEA)
All scores were inferred by single sample gene set enrichment analysis (ssGSEA) method from the GSVA R package (v1.34.0) based on the protein expression matrix. The gsea [c2.all.v7.4symbols] of Molecular Signature Database(MSigDB) was used to ssGSEA. The parameters: min.sz = 10, max.sz = 300 were set and other parameters were used default.

Differential protein and pathway analysis in subcohorts
The protein expression matrices of the DOS, XELOX, XELOX + HER2, and HER2 subcohorts were used to perform the differential expression analysis of the sensitive group (S) and the non-sensitive group (NS). The overrepresented proteins of S and NS of subcohorts were used to perform the differential expression analysis of the sensitive group (S) and the non-sensitive group (NS). The overrepresented proteins of S and NS of subcohorts were defined as proteins differentially expressed in the S and NS groups (NS/S > 2 or < 0.5); the significantly differentially expressed proteins was defined as...
proteins with more than twofold change and Wilcoxon rank-sum test with a Benjamini-Hochberg (BH) adjusted p value cutoff (BH p value < 0.05) (Supplementary Data 6 to 9). Pathway enrichment analysis of the overrepresented proteins was performed by KEGG data-bases. Pathways with a p value < 0.05 were regarded to be significant enrichment. Gene set enrichment analysis (GSEA) was also used for pathway enrichment analysis. GSEA analysis in each proteomic sub-type using the clusterProfiler R package (v3.18.1)56. GSEA evaluates and determines whether a priori defined sets of genes show statistically significant, cumulative changes in gene expression that are correlated with a specific phenotype. Samples grouped according to S and NS were subjected to GSEA, respectively. Molecular Signatures Database (MSigDB) of hallmark gene sets (H), curated gene sets (C2) and GO gene sets (C5) were used for enrichment analysis. A p value of 0.05 was used as a cutoff. The enrichment score (ES) in GSEA was calculated by first ranking the proteins from the most to least significant with respect to S and NS, the entire ranked list was then used to assess how the proteins of each gene set were distributed across the ranked list.

The assessment of drug sensitivity
Gene expression profiles of GC cell lines were from Expression 21Q2 Public dataset (https://depmap.org/portal/download/?releaseName=DepMap_Public_V1Q2_SampleList&xCLE_expression.csv). These RNA-Seq files were aligned with STAR and quantified with RSEM, then TPM-normalized and log(TPM + 1) transformation. Reported values of genes were log2[TPM + 1]. The drug sensitivity to docetaxel (BRD: BRD-K20577245-001-04-3), oxaliplatin (BRD: BRD-K7860041-001-03-2), and 5-fluorouracil (BRD: BRD-K24844714-001-24-5) using Drug sensitivity (PRISM Repurposing Primary Screen) 19Q4 dataset filtered by gastric cancer cell lines. Reported values of drug sensitivity were log2[fold change (treatment group vs control group)]. The Pearson’s correlation of gene expression value [log2 (TPM + 1)] and drug sensitivity [log2[fold change]] were calculated. All datasets were downloaded from DepMap database (https://depmap.org/portal/).

Immune cell type composition
The abundance of 64 different cell types were computed via xCell based on proteomic profiles17. The Supplementary Data 5 contains the final score computed by xCell of different cell types.

Construction and validation of predictive models for therapy response
Multiple logistic regression analysis was used to construct the therapeutic response prediction model based on the significantly differentially expressed proteins in S and NS of DO5, XELOX, and HER2 subcohorts using in the R software v3.5.1. And backward stepwise method was utilized to feature selection. Samples was randomly divided into 80% of individuals (the training set) and the remaining 20% (the testing set)22. Moreover, the diagnostic value of this model was verified using ROC analysis (pROC R package version 1.16.2 and Caret R package version 6.0-86). Sensitivity, specificity, accuracy, and AUC were used to determine predictive values. The predictive model of XELOX cohort was validated in an externally annotated DGC cohort (the Beijing Proteome Research Center) accessible in the PRIDE Archive under the accession number PXD00884057.

Targeted PRM analysis
Using the library search results, a set of target peptides that unique to ECM proteins (THSD4, SRPX2, TGFB1, THBS1, and LAMB2), DSG/DNSG-sig (ATP5S, C11orf31, CDC42SE2, CHP2, and AHR), XSG/XNSG-sig (CAPN5, BAIAP2, SRPX2, COMMD4, SCIN, DSC2, SEPSCEC, TECPR1, DDX60L, NPL, SL3C9A4, and IRF6) see Supplementary Data 3 and 11 for the list of targeted peptides) was selected and parallel reaction monitoring (PRM) method was designed. Besides, house-keeping proteins, such as VCP, RPLP0, PSMB4, were also included for the reference. Equal amount of tumor tissue from each sample (an independent cohort with 60 gastric cancer FFPE samples, including 30 sensitive patients and 30 non-sensitive patients) was digested as described in the part of profiling preparation. Peptide samples were injected into the Q Exactive XH Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific) operating in PRM mode with quadrupole isolation and HCD fragmentation. The full MS mode was measured at resolution 60,000 with AGC target value of 3E6 and maximum IT of 20 ms, with scanning range of 300 to 1400 m/z. Target ions were submitted to MS/MS in the HCD cell (1.6 m/z isolation width, 27% normalized collision energy). 60 PRM events were performed after MSI scanning, at resolution 15,000 with AGC target value of 1E6 and maximum IT of 25 ms. Separation was achieved on a 150-μm-inner-diameter column with a length of 15 cm (1.9-μm ReproSil-Pur C18 AQ beads, Dr. Maisch GmbH) in an Easy 1200 nLC HPLC system (Thermo Scientific). Solvent A was 0.1% formic acid in water and solvent B was 1% formic acid, 80% ACN in water. Peptides were separated at 600 nL/min across a gradient ranging from 4 to 100% B over 75 min (0–75 min, 0 min, 4% B; 0–10 min, 4–15% B; 10–60 min, 15–30% B; 60–69 min, 30–50% B; 69–70 min, 50–100% B; 70–75 min, 100% B). Raw data was processed by Skyline-daily (4.2.1.2, University of Washington, USA). The proteins were quantified with the fragment total area reported by Skyline-daily. We selected peptides and tested their stability of signal and shape of peaks in the pool sample for final quantification, and referred to the ranking offered by skyline.

Plasmid construction
For the analysis of CTSE in tumor proliferation and tumor response to drug treatment, we constructed stable cell lines overexpressing CTSE-FLAG. The cDNA of CTSE was cloned into the pCDH-CMV-EF1-PURO vector via the unique XbaI site and the neighboring EcoRI site. For convenient detection, a FLAG-tag encoding sequence (GATTA CAAGGATGACGACGATAAG) was inserted before the stop codon of CTSE-FLAG fusion protein.

The primers used for plasmid construction as following:
Forward primer (5′-3′): GCTCTAGAATTGAGATCTCGGCACTATC, Reverse primer (5′-3′): GGAATTCTCACTTTATGTCGTATCCTCCATGAATCGGTCTGCA GACAGCCA.
As for TKTL1, PCR-amplified TKTL1 was cloned into pRK7-Flag vector between HindIII and EcoRI, using the primers as following:
Forward primer (5′-3′): CCAAGCTTATGGCGATGCTGGACCG, Reverse primer (5′-3′): CGGAATTCAGGATCTCAACATGCA TTTCAAGCCGCA.
To explore the association of THSD4 with tumor proliferation and drug resistance, the cDNA of THSD4 was cloned into the pcDNA3.1(−)Flag vector via the unique NheI site and the neighboring EcoRI site.
Forward primer (5′-3′): gggaagccacgctgctagcATGGTTTCCCAT TCTAGGGG, Reverse primer (5′-3′): tgtgagatctcgaattctTCTG CTCCCCAGGAAGCC).

Lentivirus production and cell transduction
For CTSE, the double-stranded DNA was cloned into the pCDH-CMV-EF1-PURO vector; 8 μg of packaging plasmids pMD2.G: psPAX2 (1:3) and 8 μg of Lentivector containing the target gene were co-transfected into 2.5 × 10^9 HEK-293T cells using Lipofectamine 2000 transfection reagent (Invitrogen, 11668-019). The media containing the lentivirus particles were collected after 24 and 48 h, separately, and centrifuged at 1500 × g for 10 min. These 24-h and 48-h supernatants were used independently to infect MKN45 and MGC803 cells in the presence of 10 μg/mL hexadimethrine bromide (Sigma, H9268) for 12 h. After infection, cells were cultured and selected with puromycin for the generation of stably overexpressed cells. Empty pcDHD vector was used as a negative control.
For TKTL1 and THSD4, the detailed process of cell transfection was described as the previous publication\(^7\).

**Proteome profiling and differential analysis of GC cell lines**

MKN45 cells were divided into four groups according to the following experimental conditions: scramble vector (Vector OE) and CTSE-FLAG overexpression (CTSE OE) with or without docetaxel treatment. Each group contained at least three biological replicates. The protein concentration was determined using the Bradford assay. Cells were boiled in a 99 °C metal bath for 30 min with 100 µL of 50 mM ABC buffer (ammonium bicarbonate) containing SDS at a final concentration of 4%. Protein samples underwent trypsin digestion (enzyme-to-substrate ratio of 1:50 at 37 °C for 18–20 h). All peptide samples were desalinated using a C18 column (50% acetonitrile and 0.1% formic acid) and then analyzed using a Q Exactive HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Rockford, IL, USA) coupled with a high-performance liquid chromatography system (EASY nLC 1200, Thermo Fisher). MS raw files generated by LC-MS/MS were searched against the NCBI human Refseq protein database (released on 04-07-2013; 32,015 entries) using MaxQuant (version 1.6.2.10) software enabled with Andromeda search engine. Protease was Trypsin/P. Up to 2 missed cleavages were allowed. Carbamidomethyl (C) was considered as a fixed modification. For the proteome profiling data, variable modifications were oxidation (M) and acetylation (Protein N-term). We screened the differentially expressed proteins in CTSE-overexpressing and control MKN45 cells without DOC treatment (FC > 1.5 or 0.7) with or DOC treatment (FC > 2 or 0.5). Pathway enrichment analysis was performed according to KEGG database.

**Western blotting**

For western blotting, cells were lysed with 0.5% NP-40 buffer containing 20 mM Tris HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, and NONIDET P-40 SUBSTITUTE. The protein concentration was quantified using Bradford assay. For each sample, 30 µg of protein extract was separated using a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking with 5% milk (BD Science) solution in TBST (Tris buffered saline with Tween) for 1–2 h, the membranes were incubated with TBST containing the appropriate primary antibodies overnight at 4 °C, followed by a 2 h incubation with horseradish peroxidase-conjugated secondary antibodies. The target protein bands were detected using the Chemiluminescent detection reagent. The mouse monoclonal anti-f-actin antibody (1:10,000, Genscript, catalog No: A00702), the rabbit polyclonal anti-THSD4 antibody (1:2000, Abclonal, catalog No: A17773), the rabbit polyclonal anti-CTSE antibody (1:2000, Signalway Antibody, catalog No: 35666), the rabbit polyclonal anti-TKTL1 antibody (1:1000, Novus Biologicals, catalog No: NBPI-31674), the rabbit polyclonal anti-alpha-tubulin antibody (1:2000, Proteintech, catalog No: PA0183), the mouse monoclonal anti-beta-tubulin antibody (1:2000, ABclonal, catalog No: A17773), the rabbit polyclonal anti-THSD4 antibody (1:100, Signalway Antibody, catalog No: 35666), and the rabbit polyclonal anti- TKTL1 antibody (1:500, Novus Biologicals, catalog No: NBP1-31674), the rabbit antibody monoclonal against CD4 (1:250, GeneTech, catalog No: GT219107), the mouse monoclonal antibody against CD8 (1:300, Leica, catalog No: PA0183). IHC evaluation was analyzed using an IHC profiler compatible plugin with integrated options for the quantitative analysis of digital IHC images stained for cytoplasmic or nuclear proteins\(^7\). Moreover, the intensity of the cytoplasmic staining and the percentage of positively stained tumor cells were also scored numerically.

**Flow cytometric analysis of the cell cycle and apoptosis**

For cell cycle analysis, ~10⁶ cells were fixed in 4 °C pre-cooled 70% ethanol overnight at 4 °C. For each sample, cells were incubated for 1 h at 37 °C in PBS with DNAse-free RNase A (100 mg/mL) and propidium iodide (50 mg/mL). Next, the cells were analyzed using a Beckman Coulter flow cytometer (Beckman Coulter, Brea, CA, USA). Data were acquired and analyzed using FlowJo version 10.7.1 (Becton Dickinson Life Sciences). Data are presented as means ± SD of three independent experiments.

For cell apoptosis analysis, ~10⁶ cells were harvested from culture dishes using trypsin (without EDTA). After two washes with cold PBS, cells were resuspended in 100 µL 1× Binding Buffer (Annexin V-FITC/PI Apoptosis Detection Kit, YEASEN, Shanghai, China). Cells were stained with 5 µL annexin V-FITC and 10 µL PI staining solution (Annexin V-FITC/PI Apoptosis Detection Kit) in the dark, at room temperature for 15 min. Following this incubation, 400 µL 1× Binding Buffer was added to each sample, and then kept on ice until analysis (within 1 h).

**Metaphase spreads**

In order to count the chromosomes of a single cell in metaphase, MKN45 cells were incubated with 50 ng/mL colchicine (Sangon Bio-tech, A600322-0100) at 37 °C for 2 h. Cells were harvested and suspended in 8 mL prewarmed hypotonic buffer (0.075 mol/L KCl) at 37 °C for 20 min which induced cellular distortion. After centrifugation, cells were fixed with Carnoy's buffer (methanol: acetic acid in 3:1 ratio) at 37 °C for 20 min. Fixed cells were centrifuged and then resuspended in Carnoy's buffer, twice. The final supernatants, containing the fixed mitotic cells, were dropped onto ice-cold slides and dried for 10 min. Slides were stained with 1 µg/mL DAPI (Sigma, D8417) for 1 min followed by washing with PBS. Cells were imaged using a Zeiss LSM 880 (Carl Zeiss, Jena, Germany) confocal microscope using an oil 63× 1.4 NA objective lens.

**Immunocytochemistry and immunofluorescence**

MKN45 cells were seeded into glass bottom culture dishes to reach 70–80% confluency. The cells were fixed using 4% paraformaldehyde in PBS (pH 7.4) for 10 min at room temperature, followed by three 5-min washes with ice-cold PBS. Then, the samples were permeabilized with PBS containing 0.1% Triton X-100 for 10 min at room temperature, and washed with PBS three times for 5 min. Cells were incubated with 1% BSA, 22.52 mg/mL glycine in PBST (PBS + 0.1% Tween 20) for 30 min at room temperature to block unspecific binding of the antibodies, prior to incubation with the diluted antibody in 1% BSA in PBST overnight at 4 °C. After three washes in PBS, the samples were incubated with the fluorescent-labeled secondary antibody in 1% BSA for 1 h at
room temperature in the dark. After three further washes in PBS, the samples were incubated with 1 μg/mL DAPI (Sigma, D8417) for 1 min. Finally, the samples were washed three times with PBS, for 5 min each wash, in the dark. Cells were imaged using a Zeiss LSM 880 (Carl Zeiss) confocal microscope using an oil 63 × 1.4 NA objective lens.

**Reporting summary**
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
The raw mass spectrometry (MS) proteomics data and parallel reaction monitoring (PRM)-MS proteomics data generated in this study have been deposited in the ProteomeXchange Consortium (dataset identifier: PXD024255) via the iProX partner repository (http://www.iprox.cn/). Under Project ID PX000216000. All the H&E-stained slides of tumor tissues in this study were deposited in Mendeley (https://data.mendeley.com/datasets/cv6ytzfoz7/). The gene expression profiles of gastric cancer cell lines associated with drug sensitivity could be accessed at DepMap data portal (https://depmap.org portal/). The gene expression profiles of gastric cancer cell lines in public dataset Expression 21Q2 in this study are available in the Depmap database (https://depmap.org portal/download?releasename=DepMap+Public+ +21Q2&filename=CCLF_expression.csv). HPA IHC Staining Data could be accessed at https://www.proteinatlas.org/. HPA IHC staining data could be accessed at https://www.ncbi.nlm.nih.gov/ refseq/. Source data are provided with this paper.

The remaining data are available within the Article, Supplementary Information or Source Data file. Source data are provided with this paper.

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Competing interests
The authors declare no competing interests.

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