International Immunopharmacology 87 (2020) 106798

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Identification of the collagen family as prognostic biomarkers and immune-associated targets in gastric cancer

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ARTICLE INFO

Keywords:
Collagen
Prognosis
Methylation
Immune infiltration
Drug response

ABSTRACT

Background: Gastric cancer has extremely high morbidity and mortality. Currently, it is lack of effective biomarkers and therapeutic targets for guiding clinical treatment. In this study, we aimed to identify novel biomarkers and therapeutic targets for gastric cancer.

Methods: Differentially expressed genes (DEGs) between gastric cancer and normal tissues were obtained from Gene Expression Omnibus (GEO). Core genes were identified by constructing protein-protein interaction network of DEGs. The expression of core genes was verified in Gene Expression Profiling Interactive Analysis (GEPIA), UALCAN and clinical samples. Further, the mutation, DNA methylation, prognostic value, and immune infiltration of core genes were validated by cBioPortal, MethSurv, Kaplan-Meier plotter, and Tumor Immune Estimation Resource (TIMER) databases. Additionally, drug response analysis was performed by Cancer Therapy Response Portal (CTRP).

Results: A total of seven collagen family members were identified as core genes among upregulated genes. And copy number amplification may be involved in the upregulation of COL1A1 and COL1A2. Importantly, the collagen family was associated with the poor prognosis of patients with metastasis. Among them, COL1A1 had a higher hazard ratio (HR) for overall survival than other members (HR = 2.33). The correlation between DNA methylation levels at CpG sites of collagen family members and the prognosis was verified in gastric cancer. Besides, collagen family expression was positively correlated with macrophages infiltration and the expression of M2 macrophages markers. Further, collagen expression was related to the sensitivity and resistance of gastric cancer cell lines to certain drugs.

Conclusions: The collagen family, especially COL1A1, COL1A2, and COL12A1, may act as potential prognostic biomarkers and immune-associated therapeutic targets in gastric cancer.

1. Introduction

Gastric cancer is the sixth most common incident tumor and has the third-highest mortality rate of cancers worldwide [1]. The morbidity and mortality in Asian countries are high, especially in China, Japan, and South Korea [2,3]. Despite gastrectomy and chemotherapy, the five-year survival rate of patients is still less than 20% [4]. Currently, immunotherapy is also gradually playing a vital role in monotherapy and combination therapy of gastric cancer [5].

Inflammatory cell infiltration has a positive or negative effect on

Abbreviations: AUC, area under the curve; BET, extra-C terminal domain; BP, biological process; BRD, bromodomain; CC, cellular component; CCLE, Cancer Cell Line Encyclopedia; CIs, confidence intervals; CTRP, Cancer Therapy Response Portal; DAVID, Database for Annotation, Visualization, and Integrated Discovery; DEGs, differentially expressed genes; GEO, Gene Expression Omnibus; GEPIA, Gene Expression Profiling Interactive Analysis; GO, gene ontology; HRs, hazard ratios; KEGG, Kyoto Encyclopedia of Genes and Genomes; LR, likelihood ratio; MCODE, Molecular Complex Detection; MF, molecular function; NSCLC, non-small-cell lung cancer; OS, overall survival; PPI, protein-protein interaction; STAD, stomach adenocarcinoma; TAMs, tumor-associated macrophages; TCGA, The Cancer Genome Atlas; Tfh, follicular helper T; Th1, T-helper 1; Th2, T-helper 2; Th17, T-helper 17; TIMER, Tumor Immune Estimation Resource; TIICs, tumor-immune infiltrating cells; Tregs, regulatory T cells

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https://doi.org/10.1016/j.intimp.2020.106798
Received 8 May 2020; Received in revised form 3 July 2020; Accepted 7 July 2020
Available online 18 July 2020

tumor invasion, growth, metastasis, and prognosis [6]. Previous studies have identified that immune infiltration correlates with cancer prognosis [7,8]. In addition, assessing immune infiltration can provide biomarkers and predict therapeutic efficacy [9]. Currently, carcinoembryonic antigen and CA19-9 are the most commonly used biomarkers in the clinical application of gastric cancer, while they rarely provide guidance for better clinical outcomes [10]. Identifying new biomarkers of cancer will provide a basis for choosing appropriate treatments and monitoring these cancer patients. Recent studies have suggested circRNAs could be used as diagnostic biomarkers in gastric cancer [11,12]. Moreover, circulating microRNAs have turned to powerful candidates in the diagnosis of gastric cancer [13]. For example, miR-21 can be utilized as the potential diagnosis and prognosis biomarker in cancers [14]. However, the prognostic biomarkers relating to immune infiltration are still lacking. Thus, it is necessary to confirm valid prognostic biomarkers and immune-associated therapeutic targets in gastric cancer.

Collagen, which contains 28 different subtypes, is the main insoluble fibrin in human [15]. Previous studies have reported that collagen regulates cell growth, differentiation, and migration in gastric cancer [16,17]. The overexpression of COL11A1 promotes proliferation, migration, and invasion of gastric cancer [18]. A study has revealed that COL1A1 is a potential diagnostic marker and therapeutic target in hepatocellular carcinoma [19]. Besides, COL6A3 could promote epithelial-mesenchymal transition and serve as a biomarker in the development of bladder cancer [20]. COL12A1 has been confirmed to be linked to the prognosis in colorectal cancer [21]. Methylation of gene promoters can regulate transcription and determine the clinical value of biomarkers [22]. DNA methylation can also act as a prognostic biomarker for cancer [23]. There are some studies on collagen and clinical prognosis in gastric cancer, while the DNA methylation and mutations are less considered. Moreover, the correlations between collagen and immune infiltration, collagen and drug response are rarely reported. The role of collagen in gastric cancer has not been systematically elucidated.

In this study, a series of bioinformatics methods were performed to identify prognostic biomarkers and immune-associated therapeutic targets in gastric cancer. First, differentially expressed genes (DEGs) between gastric cancer and adjacent normal tissues were obtained from Gene Expression Omnibus (GEO) database. Then, collagen family members were identified as core upregulated genes through protein-protein interaction (PPI) network analysis. We estimated the prognostic value of collagen family members in terms of expression, mutations, and DNA methylation. More importantly, the correlations between collagen and immune infiltration, collagen and drug response were analyzed to explore the value as therapeutic targets and guiding clinical treatment. To the best of our knowledge, this is the first study on methylation, immune infiltration, and drug response of collagen family members in gastric cancer. We hope that this study may provide potential prognostic biomarkers and novel immune-associated therapeutic targets for gastric cancer patients.

2. Materials and methods

2.1. Microarray data information and data processing of DEGs

To identify biomarkers of gastric cancer, two gene expression profiles, GSE118916 and GSE79973, were downloaded from the GEO database on NCBI (www.ncbi.nlm.nih.gov/geo/) [24]. GSE118916 from the GPL15207 [Prime View] Affymetrix Human Gene Expression Array includes 15 pairs of gastric tumor and adjacent non-tumor tissues. GSE79973 from the GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array includes 10 pairs of gastric tumor and adjacent non-tumor mucosa tissues. The gene expression profiles were analyzed by the GEO2R tool. Genes with $|\log FC| \geq 2$ and adjusted $P < 0.05$ were confirmed as DEGs. We obtained volcanic plots of DEGs through the SangerBox tool. Common DEGs from the two profiles were identified via Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/).

2.2. PPI network analysis

The common DEGs were analyzed using the STRING website (https://string-db.org/) to obtain PPI networks [25]. Then, the Molecular Complex Detection (MCODE) plug-in in Cytoscape software was used to analyze the network diagram for screening core genes (node score cut-off: 0.2; haircut: true; fluff: false; k-core: 2; max. depth from seed: 100) [26,27].

2.3. Gene ontology and pathway enrichment analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/) provides functional annotation for numerous genes [28]. Through the DAVID website, we performed gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of core genes, including biological process (BP), molecular function (MF), cellular component (CC), and pathway.

2.4. Gene expression analysis

The expression levels of collagen family members in the Cancer Genome Atlas (TCGA) gastric cancer database were verified with the Gene Expression Profiling Interactive Analysis (GEPIA) website (http://gepia.cancer-pku.cn/) [29]. The cBioPortal for Cancer Genomics (https://www.cbioportal.org/) is an open-access resource for interactive exploration of multiple Cancer Genomics datasets [30]. The cBioPortal website currently stores DNA copy number data, mRNA and microRNA expression data, non-synonymous mutations, protein and phosphoprotein level data, DNA methylation data, and limited clinical data. In the study, the cBioPortal website was used to visualize somatic mutations and mRNA expression of collagen family members. In addition, we also analyzed their expression in different pathological stages of gastric cancer in UALCAN (http://ualcan.path.uab.edu/) [31].

2.5. Patient and specimens

Eight pairs of human gastric tumor and adjacent normal tissues were obtained from patients in Affiliated Cancer Hospital of Zhengzhou University (Zhengzhou, Henan, China). All specimens were stored in liquid nitrogen until used for western blotting analysis. All human specimens were obtained with the informed consent of patients. The study was approved by the ethics committee of Zhengzhou University and performed in accordance with the Declaration of Helsinki.

2.6. Western blotting

The tissues of patients were cleaved in RIPA buffer (Solarbio, Beijing, China) and quantified by BCA assay kit (Beyotime, Shanghai, China). Then, proteins were separated by 8% SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% non-fat milk at room temperature for 2 h and incubated overnight with primary antibody at 4 °C. Primary antibodies were Collagen I (1:1000, bs-10423R, Bioss, Beijing, China), Collagen X (1:1000, bs-0554R, Bioss, Beijing, China), COL11A1 (1:1000, ab166606, Abcam, Cambridge, UK), and β-actin (1:1000, sc-8432, Santa Cruz, Dallas, TX, USA). Collagen I antibody can detect COL1A1 and COL1A2. After that, we incubated the membranes with suitable secondary antibodies at room temperature for 2 h. Finally, protein bands were visualized using ECL reagent (Beyotime, Shanghai, China).

2.7. Kaplan-Meier plotter database analysis

The Kaplan-Meier plotter (http://kmplot.com/analysis/) can evaluate the impact of genes on survival in 21 types of cancer [32]. The correlation between collagen family expression and overall survival (OS) of gastric cancer was tested in Kaplan-Meier plotter. Moreover, the correlation between OS and collagen family expression in gastric cancer patients with clinical-pathological features was also estimated.

2.8. DNA methylation analysis in MethSurv

MethSurv is a web portal providing survival analysis and DNA methylation data using TCGA data (https://biit.cs.ut.ee/methsurv/). The stomach adenocarcinoma (STAD) data includes 395 patients, of whom 155 died. The covariates are age, sex, stage, and grade [33]. The MethSurv was used for analyzing prognostic value and obtaining the DNA methylation data of collagen family members in STAD.

2.9. TIMER database analysis

TIMER (https://cistrome.shinyapps.io/timer/) is a web tool for systematic analysis of immune infiltrates of diverse cancers [34]. We analyzed the relationship between immune infiltration levels and prognosis in gastric cancer patients. Then, the correlations between collagen family expression and tumor purity, collagen family expression and six immune-infiltrating cell types (B cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, macrophages, and dendritic cells) were estimated via TIMER. Tumor purity means the ratio of tumor cells in tumor tissues. Moreover, the correlation between collagen expression and gene markers of immune cells was also analyzed. These gene markers were chosen from prior studies [35,36]. The x-axis of the scatterplot was the expression level of gene markers; the y-axis was the expression level of collagen family members. The gene expression level was represented by log2 RSEM.

2.10. Correlation analysis between collagen expression and drug response

Drug response data and the expression data of collagen family members including COL1A1, COL1A2, and COL12A1 in different cancer cell lines were downloaded from the Cancer Cell Line Encyclopedia (CCLE; https://portals.broadinstitute.org/ccle/) and Cancer Therapy Response Portal (CTRP, https://portals.broadinstitute.org/ctrp.v2.2/), which contains 888 cell lines response profiles of 545 drugs [37]. Pearson correlation analysis between collagen expression and drug response area under the curve (AUC) was performed on each cancer cell type. We obtained the percentage of drugs significantly correlated with collagen expression in these cancer types. The histogram represented the ratio of drugs related to collagen expression in 10 different cancer cell types with at least 30 cell lines. Then, volcanic plots of correlation between collagen expression and 545 drugs response AUC in 32 stomach cancer cell lines were performed in SangerBox. Correlation coefficient greater than 0.3 and *P* value less than 0.05 was considered as the cut-off point.

2.11. Statistical analysis

The GEO2R software calculated the *P* value of the two gene expression profiles from the GEO database. Cox regression analysis of the collagen family was performed for OS with hazard ratios (HRs) and 95% confidence intervals (CIs). Spearman's correlation coefficient was used to measure the correlation of gene expression. And P value $<$ 0.05 was considered significant.

3. Results

3.1. Identification of DEGs in gastric cancer

In this study, we found 91 upregulated genes and 257 downregulated genes in GSE118916. There were 109 upregulated genes and 306 downregulated genes in GSE79973 (Fig. 1A, B). After that, the common DEGs of the two profiles were obtained through Venn diagram, which revealed 35 upregulated genes and 116 downregulated genes (Fig. 1C, D). The PPI network analysis showed the interaction of DEGs (Fig. S1). Then, we applied the MCODE plug-in in Cytoscape to obtain core genes. The results showed that there were fifteen upregulated core genes, including COL12A1, SERPINH1, TIMP1, SPP1, THBS1, FN1, COL10A1, COL6A3, SPARC, ASPN, COL1A1, THBS2, COL1A2, COL11A1, and COL8A1 (Fig. 1C). There were seven genes in the collagen family. Eleven downregulated core genes included CYP2C8, ATP4A, ALDH1A1, CHGA, CYP2C18, UGT2B15, CYP3A5, HDC, CYP2C9, GHRL, and CCKBR (Fig. 1D). Next, we performed GO and KEGG enrichment analysis on fifteen upregulated core genes (Fig. 1E–H). The biological processes involving collagen family members were extracellular matrix organization, cell adhesion, endodermal cell differentiation, response to cAMP, skeletal system development, and leukocyte migration (Fig. 1E). The main pathways involving collagen family members were ECM-receptor interaction, focal adhesion, protein digestion and absorption, and PI3K/Akt signalling pathway (Fig. 1H).

3.2. The expression levels and mutations of collagen family members

To verify the mRNA expression of collagen family members, 211 normal gastric mucosa and 408 gastric tumor samples were analyzed by GEPIA website. The results showed that COL1A1, COL1A2, COL6A3, COL8A1, COL10A1, COL11A1, and COL12A1 were highly expressed in gastric tumor samples (Fig. 2A). In order to explore the cause of their high expression, the mutations of collagen family members were analyzed through the cBioPortal. The mutation rate of COL12A1 was 14%, which was the highest among them. Compared to other members, COL1A1 and COL1A2 showed higher copy number amplification (Fig. 2B). We also analyzed the alteration frequency of the collagen family in different types of gastric cancer. The mRNA overexpression was more common among collagen family members in gastric cancer (Fig. 2C). Accordingly, the protein expression levels of Collagen I, Collagen X, and COL11A1 were detected in clinical specimens by western blotting. The expression levels of them increased in at least half of gastric cancer tissues, especially Collagen I (COL1A1 and COL1A2) (Fig. 2D).

3.3. Collagen family members differentially expressed in different pathological stages of gastric cancer

To explore the relationship between collagen expression and progression of gastric cancer, the expression levels of the collagen family in different pathological stages were analyzed via UALCAN. The results showed that the expression levels of COL1A1, COL1A2, COL6A3, COL8A1, COL10A1, COL11A1, and COL12A1 in stage 2, 3, 4 were higher than normal tissues. Besides, COL1A1 also displayed higher expression in stage 1 than normal tissues. Importantly, the expression levels of the collagen family in stage 2, 3, and 4 were significantly higher than those in stage 1 (Fig. 3A). Overall, the expression levels of collagen family members were correlated with pathological stages in gastric cancer.

3.4. High collagen expression predicted poor clinical prognosis in gastric cancer

Furthermore, to explore the potential prognostic value of collagen family members in gastric cancer, survival curves for collagen

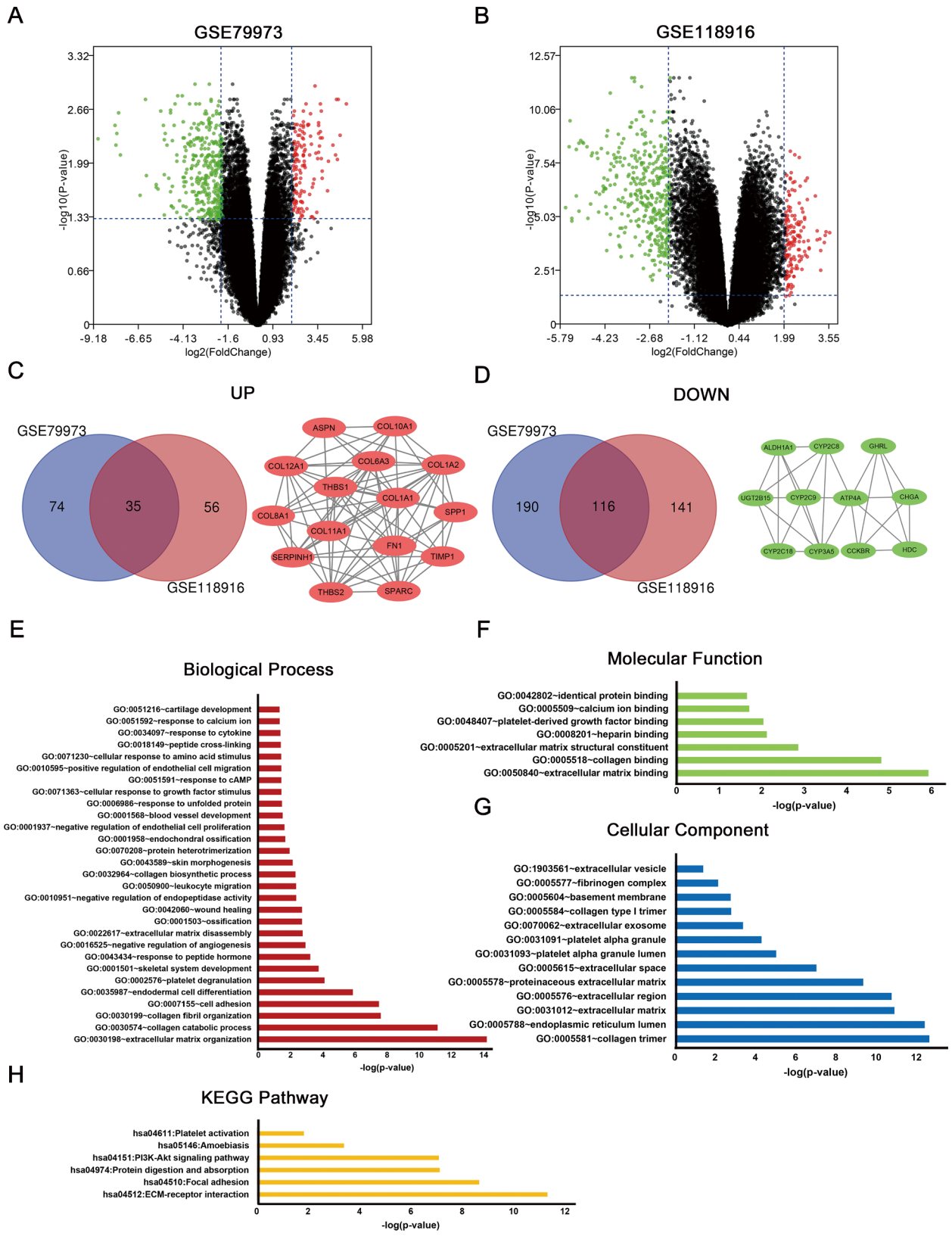

Fig. 1. Analysis of DEGs identified from two gene expression profiles. A, B. Volcano plots of upregulated (red) and downregulated (green) DEGs between gastric tumor tissue and adjacent non-tumor mucosa in GSE79973 (A) and GSE118916 (B). C. Venn diagram and module analysis of upregulated DEGs. D. Venn diagram and module analysis of downregulated DEGs. E-G. Gene ontology enrichment analysis based on upregulated core genes was performed for biological process (E), cellular component (F), and molecular function (G). H. KEGG pathway analysis based on upregulated core genes. DEGs: differentially expressed genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

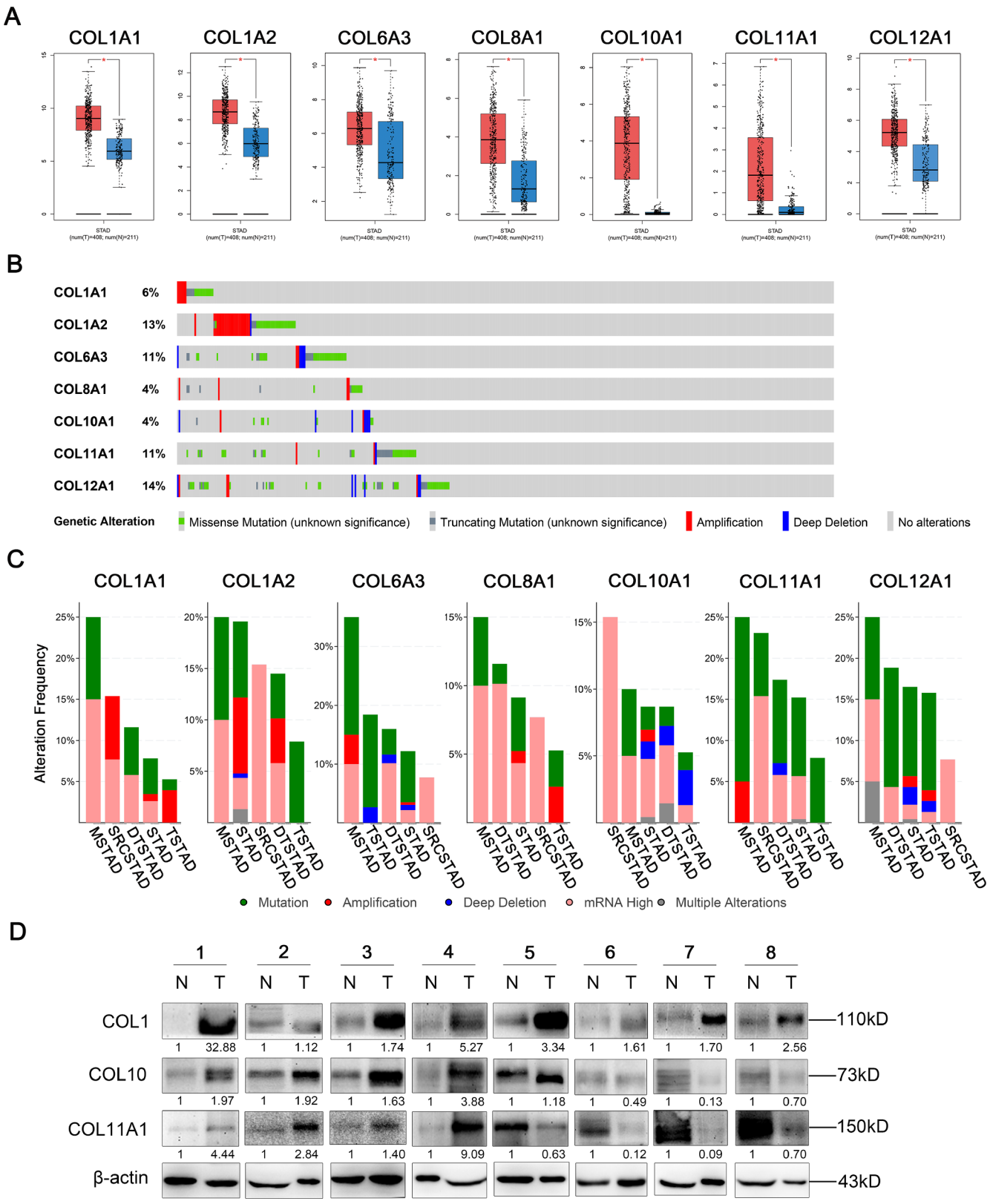

Fig. 2. Expression and mutations of the collagen family in gastric cancer. A. Expression of the collagen family in GEPIA; red indicated tumor, and blue indicated normal. B. Mutation ratio of the collagen family. C. Alteration frequency in distinct types of gastric cancer. D. Representative western blotting for Collagen I (COL1), Collagen X (COL10), COL11A1 protein in 8 paired gastric tumor and adjacent non-tumor tissues. N: normal; T: tumor. MSTAD: Mucinous stomach adenocarcinoma; SRCSTAD: Signet ring cell carcinoma of the stomach; DTSTAD: Diffuse type stomach adenocarcinoma; STAD: Stomach adenocarcinoma; TSTAD: Tubular stomach adenocarcinoma. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

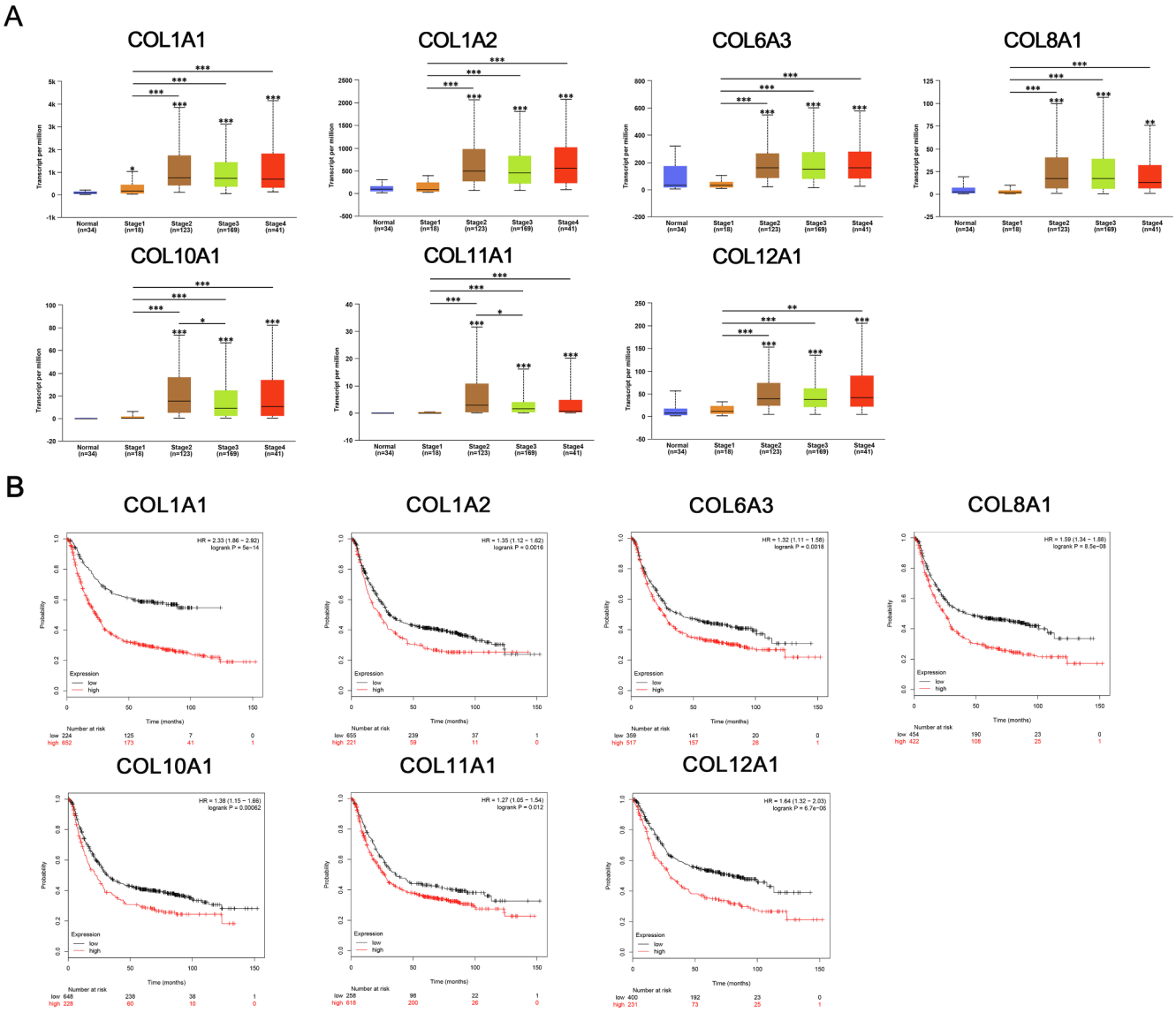

Fig. 3. Collagen expression was correlated with pathological stages and clinical prognosis. A. The collagen family expression in the pathological stages. The asterisk, which was directly above the error bar, represented the comparison between this stage and the normal group; the asterisk, which was above the horizontal line, represented the comparison of the corresponding groups at both ends of the line. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. B. OS curves comparing the high and low expression of the collagen family. OS: Overall survival.

expression were generated by Kaplan-Meier plotter (Fig. 3B). High expression of the collagen family suggested a poor prognosis. COL1A1, COL8A1, and COL12A1 were significantly related to OS in gastric cancer (log rank $P < 0.001$). Furthermore, COL10A1 had a strong correlation with OS in gastric cancer (HR = 1.38, log rank $P = 0.00062$). High expression of COL11A1 was slightly related to poor prognosis in patients (HR = 1.27, log rank $P = 0.012$). Importantly, COL1A1 had a higher HR for OS than other members (HR = 2.33). The above results indicated that the expression level of the collagen family was associated with OS in gastric cancer.

To better understand the relationship between collagen expression and prognosis, we investigated the correlation between collagen expression and OS in gastric cancer with different clinical-pathological features. The high expression of seven collagen family members predicted poor OS of male patients, HER2 positive patients, and patients with intestinal-type Lauren classification ($P < 0.05$). The high expression levels of COL1A1 and COL10A1 were significantly correlated with the prognosis of patients with different treatment methods, including surgery alone, 5 FU based adjuvant, and other adjuvant therapy. Moreover, the high expression levels of seven collagens were also correlated with the poor OS of gastric cancer patients in stage III, IV, and N1 + 2 + 3 (Table 1). COL8A1 had the highest HR for OS in stage N1 + 2 + 3 among them (HR = 2.35). Therefore, the high expression of COL8A1 predicted a worse prognosis of patients with lymph node metastasis in gastric cancer. Additionally, high expression of the collagen family members predicted a poor prognosis in patients with stage M1, except for COL6A3 ($P = 0.124$). Overall, these results revealed that high expression levels of the collagen family were closely linked to the poor OS, especially in patients with metastasis. Collagen family members may be effective prognostic biomarkers for gastric cancer.

3.5. DNA methylation of collagen family members correlated with prognosis in gastric cancer

DNA methylation highlighted in carcinogenesis was associated with cancer survival. DNA promoter hypermethylation often results in gene silencing [38]. To explore the correlation between prognosis and DNA

Table 1
Correlation between the expression of the collagen family and overall survival in gastric cancer with different clinical-pathological features by Kaplan-Meier plotter.

Clinical-pathological features		COL1A1		COL1A2		COL6A3	
	N	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Gender							
Female	236	2.53(1.62–3.95)	**2.40E−05**	1.91(1.34–2.72)	**2.40E−04**	1.54(1.04–2.28)	**0.03**
Male	545	2.15(1.71–2.7)	**1.40E−11**	1.6(1.29–1.98)	**1.40E−05**	1.38(1.12–1.71)	**0.003**
Treatment							
Surgery alone	380	1.58(1.17–2.15)	**0.003**	1.51(1.13–2.02)	**0.006**	1.69(1.25–2.29)	**5.10E−04**
5 FU based adjuvant	153	1.5(1.03–2.18)	**0.032**	0.71(0.48–1.05)	0.085	0.75(0.52–1.1)	0.142
Other adjuvant	76	2.65(1.1–6.4)	**0.025**	3.3(1.37–7.95)	**0.005**	2.75(1.14–6.64)	**0.019**
HER2 status							
Negative	532	2.22(1.69–2.93)	**6.20E−09**	1.64(1.31–2.05)	**1.20E−05**	1.35(1.06–1.72)	**0.016**
Positive	344	2.16(1.54–3.02)	**4.20E−06**	1.7(1.23–2.34)	**0.001**	1.38(1.06–1.79)	**0.015**
STAGE							
1	67	1.83(0.58–5.82)	0.297	0.42(0.13–1.39)	0.15	0.54(0.2–1.5)	0.23
2	140	1.86(1.02–3.42)	**0.041**	1.45(0.77–2.72)	0.25	2(1.1–3.65)	**0.021**
3	305	2.41(1.69–3.45)	**6.10E−07**	1.93(1.43–2.6)	**1.10E−05**	1.51(1.06–2.16)	**0.022**
4	148	1.67(1.14–2.46)	**0.008**	2.03(1.37–3)	**3.10E−04**	1.72(1.17–2.53)	**0.005**
STAGE T							
2	241	1.83(1.19–2.8)	**0.005**	1.75(1.14–2.68)	**0.009**	1.83(1.2–2.81)	**0.005**
3	204	1.74(1.22–2.48)	**0.002**	1.69(1.19–2.38)	**0.003**	1.28(0.89–1.84)	0.188
4	38	2(0.86–4.63)	0.1	2.34(1.01–5.4)	**0.041**	2.63(1.14–6.09)	**0.019**
STAGE N							
0	74	1.78(0.65–4.89)	0.26	1.92(0.57–6.51)	0.285	2.05(0.88–4.79)	0.09
1	225	2.65(1.67–4.2)	**1.60E−05**	2.4(1.59–3.62)	**1.60E−05**	2.28(1.5–3.45)	**6.70E−05**
2	121	3.17(1.99–5.05)	**3.20E−07**	2.71(1.68–4.37)	**2.40E−05**	1.84(1.11–3.07)	**0.017**
3	76	2.48(1.43–4.3)	**8.30E−04**	2.51(1.47–4.31)	**5.40E−04**	1.62(0.95–2.75)	0.075
1 + 2 + 3	422	2.23(1.69–2.95)	**6.90E−09**	2.21(1.7–2.88)	**1.40E−09**	1.9(1.45–2.51)	**3.00E−06**
STAGE M							
0	444	2.16(1.59–2.94)	**4.50E−07**	1.96(1.48–2.6)	**1.50E−06**	1.92(1.45–2.54)	**3.00E−06**
1	56	2.33(1.27–4.27)	**0.005**	2.08(1.09–3.94)	**0.022**	1.59(0.88–2.89)	0.124
Lauren Classification							
Intestinal	320	2.95(2.11–4.13)	**3.60E−11**	2.34(1.71–3.21)	**5.20E−08**	2.26(1.65–3.1)	**1.90E−07**
Diffuse	241	2.15(1.47–3.15)	**5.40E−05**	1.74(1.24–2.44)	**0.001**	1.43(1–2.03)	**0.048**
Mixed	32	1.85(0.67–5.12)	0.23	2.51(0.9–7)	0.07	1.77(0.62–5.03)	0.28
Differentiation							
Poor	165	1.31(0.82–2.09)	0.25	1.75(1.09–2.81)	**0.019**	0.75(0.48–1.18)	0.22
Moderate	67	1.88(0.94–3.76)	0.069	2.37(1.19–4.7)	**0.011**	1.79(0.93–3.43)	0.076

(*continued on next page*)

Table 1 (*continued*)

Clinical-pathological features	COL8A1		COL10A1		COL11A1			COL12A1	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	*N*	HR (95% CI)	*P* value
Gender									
Female	1.76(1.24–2.51)	**0.002**	1.91(1.32–2.77)	**4.70E − 04**	1.23(0.85–1.77)	0.276	187	1.59(1.02–2.48)	**0.04**
Male	1.58(1.28–1.96)	**2.20E − 05**	1.34(1.06–1.7)	**0.014**	1.31(1.03–1.65)	**0.025**	349	1.98(1.48–2.67)	**3.50E − 06**
Treatment									
Surgery alone	1.88(1.41–2.52)	**1.40E − 05**	1.55(1.14–2.09)	**0.005**	1.31(0.93–1.85)	0.117	380	1.42(1.06–1.9)	**0.019**
5 FU based adjuvant	0.66(0.46–0.93)	**0.019**	0.68(0.46–0.99)	**0.043**	1.36(0.96–1.93)	0.083	34	37652182.89(-0 − lnf)	**0.002**
Other adjuvant	2.3(0.95–5.55)	0.058	2.47(1.02–5.97)	**0.038**	2.82(1.03–7.77)	**0.036**	76	1.72(0.72–4.13)	0.22
HER2 status									
Negative	1.68(1.34–2.1)	**5.20E − 06**	1.22(0.96–1.56)	0.106	0.87(0.68–1.11)	0.271	429	1.59(1.22–2.08)	**5.70E − 04**
Positive	1.8(1.33–2.45)	**1.40E − 04**	1.73(1.31–2.28)	**1.00E − 04**	1.58(1.21–2.05)	**6.80E − 04**	202	2.13(1.36–3.33)	**6.80E − 04**
STAGE									
1	0.47(0.17–1.32)	0.144	0.11(0.02–0.49)	**4.60E − 04**	0.08(0.01–0.59)	**0.001**	62	0.27(0.07–0.99)	**0.035**
2	2.03(1.11–3.73)	**0.019**	0.65(0.34–1.23)	0.18	0.45(0.2–1.02)	0.05	135	1.86(0.98–3.54)	0.056
3	1.98(1.41–2.77)	**5.00E − 05**	1.9(1.41–2.57)	**1.70E − 05**	1.92(1.35–2.73)	**2.30E − 04**	197	1.83(1.26–2.66)	**0.001**
4	1.9(1.28–2.82)	**0.001**	1.82(1.23–2.69)	**0.002**	1.64(1.06–2.54)	**0.026**	140	1.62(1.08–2.42)	**0.018**
STAGE T									
2	1.79(1.15–2.77)	**0.008**	1.36(0.87–2.12)	0.18	0.68(0.4–1.14)	0.144	241	1.86(1.19–2.88)	**0.005**
3	1.78(1.26–2.51)	**8.60E − 04**	1.76(1.25–2.49)	**0.001**	1.65(1.13–2.41)	**0.009**	204	1.4(0.99–1.97)	0.055
4	3.48(1.31–9.25)	**0.008**	0.56(0.24–1.35)	0.192	0.57(0.23–1.4)	0.217	38	2(0.83–4.81)	0.117
STAGE N									
0	1.75(0.76–4.06)	0.185	0.49(0.21–1.12)	0.085	0.35(0.13–0.94)	**0.029**	74	1.75(0.76–4.06)	0.185
1	2.52(1.67–3.81)	**4.60E − 06**	1.97(1.3–3)	**0.001**	1.34(0.86–2.08)	0.192	225	2.32(1.53–3.52)	**4.10E − 05**
2	2.29(1.45–3.63)	**2.80E − 04**	2.78(1.6–4.85)	**1.60E − 04**	2.64(1.49–4.65)	**5.10E − 04**	121	2.01(1.27–3.17)	**0.002**
3	2.48(1.44–4.27)	**7.00E − 04**	1.79(1.03–3.09)	**0.035**	1.59(0.89–2.85)	0.117	76	1.73(0.99–3.02)	0.05
1 + 2 + 3	2.35(1.81–3.06)	**6.00E-11**	1.82(1.4–2.37)	**6.60E − 06**	1.57(1.17–2.09)	**0.002**	422	1.92(1.47–2.5)	**8.30E − 07**
STAGE M									
0	2.29(1.74–3.03)	**2.10E − 09**	1.74(1.3–2.33)	**1.50E − 04**	1.28(0.95–1.72)	0.1	444	1.84(1.39–2.43)	**1.50E − 05**
1	2.21(1.2–4.07)	**0.009**	1.96(1.05–3.67)	**0.033**	2.2(1.1–4.39)	**0.022**	56	1.97(1.07–3.62)	**0.028**
Lauren Classification									
Intestinal	2.5(1.82–3.44)	**5.50E − 09**	2.2(1.59–3.04)	**9.40E − 07**	1.66(1.19–2.31)	**0.002**	269	2.28(1.57–3.31)	**8.00E − 06**
Diffuse	1.57(1.12–2.2)	**0.009**	1.66(1.16–2.36)	**0.005**	1.83(1.18–2.86)	**0.007**	240	1.28(0.9–1.81)	0.168
Mixed	3.64(1.3–10.16)	**0.009**	0.59(0.2–1.76)	0.34	0.39(0.14–1.08)	0.06	29	2.49(0.82–7.49)	0.095
Differentiation									
Poor	1.68(1.11–2.54)	**0.013**	2.08(1.27–3.4)	**0.003**	1.69(1.08–2.65)	**0.021**	121	1.86(0.99–3.48)	**0.049**
Moderate	3.1(1.59–6.05)	**4.90E − 04**	2.34(1.21–4.53)	**0.01**	1.64(0.79–3.42)	0.184	67	1.67(0.86–3.23)	0.126

HR: hazards ratio; CI: confidence interval. The statistically significant results were marked in bold.

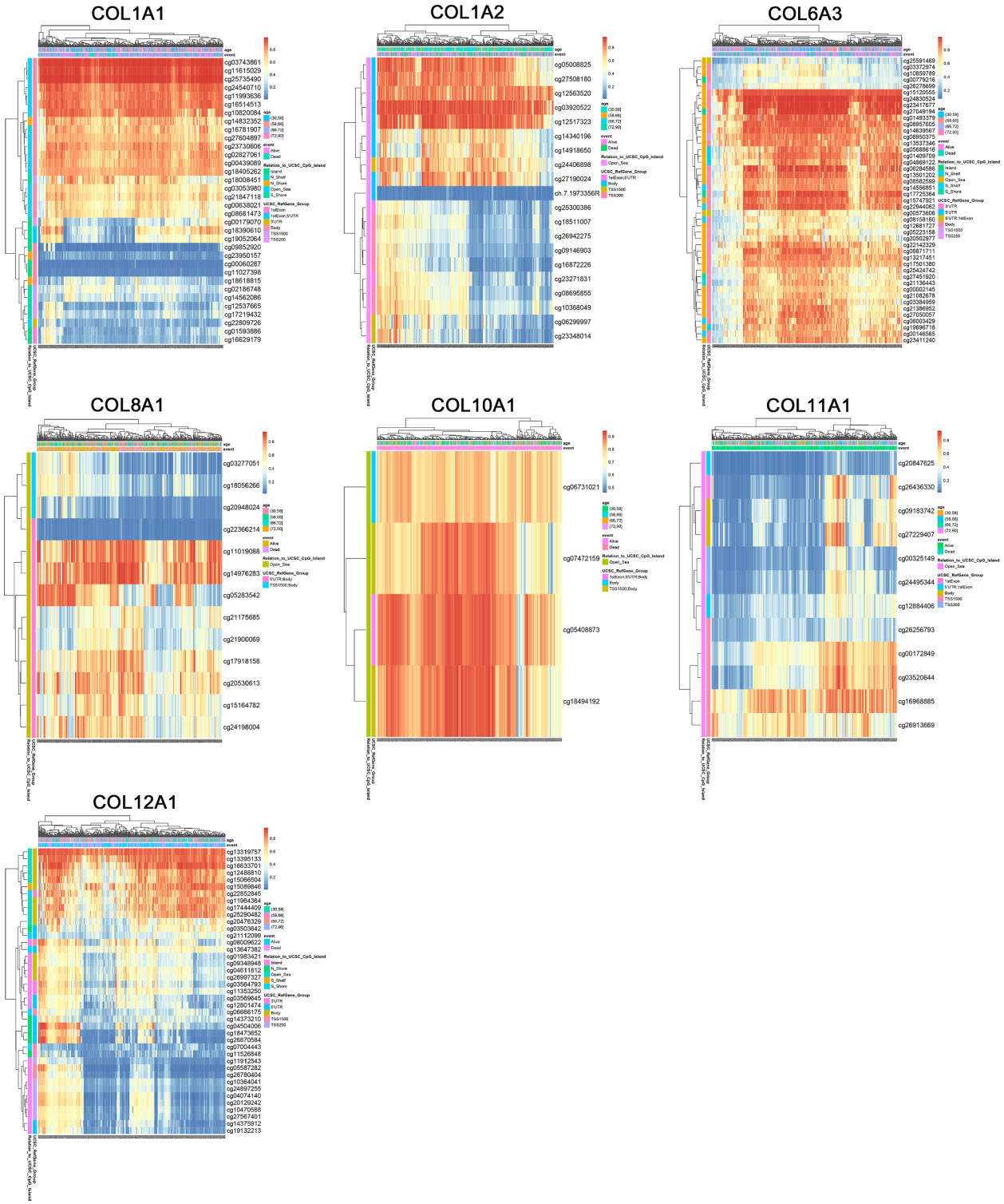

Fig. 4. DNA methylation of collagen family members in MethSurv.

methylation of collagen family members, the DNA methylation levels and survival analysis at each CpG site of collagen family members in STAD were analyzed via the MethSurv. COL6A3 and COL10A1 had higher methylation levels compared with other members. COL1A1, COL1A2, COL8A1, and COL12A1 had higher expression levels in STAD, and the DNA methylation levels of their promoter were lower (Fig. 4). Moreover, we showed the prognostic value of each CpG site with significant Likelihood-ratio (LR) test *P* value ($P < 0.05$). Totally 16 CpG sites of COL1A1, 6 CpG sites of COL1A2, 8 CpG sites of COL6A3, 3 CpG sites of COL8A1, 1 CpG sites of COL10A1, 8 CpG sites of COL11A1, and 15 CpG sites of COL12A1 were significantly correlated with the prognosis of STAD (Table 2). Additionally, the hypomethylation of CpG sites located on the CpG island indicated a poor prognosis, including cg18390610, cg19052064, and cg00179070 of COL1A1; cg12801474, cg03564793, cg14375912, and cg24897255 of COL12A1. Consistently, above CpG sites showed lower methylation levels in STAD, suggesting that the methylation levels of CpG sites were also associated with prognosis in gastric cancer.

Table 2
The significant prognostic values of CpG in the collagen family members.

Gene symbol	CpG Name	Hazard ratio	CI	LR test *P* value	UCSC RefGene Group	Relation to UCSC CpG Island
COL1A1						
	cg16781907	0.488	(0.313;0.762)	0.001	Body	N_Shelf
	cg03053980	0.582	(0.421;0.805)	0.001	Body	Open_Sea
	cg18390610	0.516	(0.333;0.799)	0.001	1stExon;5′UTR	Island
	cg14562086	1.841	(1.205;2.812)	0.003	TSS1500	S_Shore
	cg21847118	0.617	(0.446;0.854)	0.003	Body	Open_Sea
	cg11993636	0.608	(0.433;0.854)	0.005	Body	Open_Sea
	cg02827061	0.570	(0.373;0.87)	0.006	Body	Open_Sea
	cg00439089	0.650	(0.471;0.897)	0.009	Body	Open_Sea
	cg27604897	0.647	(0.466;0.9)	0.011	Body	Open_Sea
	cg23950157	1.517	(1.093;2.107)	0.012	Body	N_Shore
	cg18405262	0.634	(0.42;0.958)	0.024	Body	Open_Sea
	cg16514513	0.690	(0.499;0.954)	0.024	Body	Open_Sea
	cg19052064	0.639	(0.423;0.965)	0.026	1stExon;5′UTR	Island
	cg00638021	0.694	(0.503;0.958)	0.026	Body	Open_Sea
	cg23730606	0.666	(0.444;0.999)	0.041	Body	Open_Sea
	cg00179070	0.720	(0.52;0.996)	0.046	TSS200	Island
COL1A2						
	cg26942275	0.607	(0.432;0.854)	0.005	TSS200	Open_Sea
	cg08695855	0.645	(0.455;0.914)	0.017	TSS200	Open_Sea
	cg16872226	0.633	(0.422;0.951)	0.021	TSS200	Open_Sea
	cg18511007	0.656	(0.463;0.929)	0.021	TSS200	Open_Sea
	cg09146903	0.664	(0.471;0.936)	0.023	TSS200	Open_Sea
	cg23271831	0.641	(0.419;0.978)	0.031	1stExon;5′UTR	Open_Sea
COL6A3						
	cg01409709	0.606	(0.438;0.84)	0.003	5′UTR	Open_Sea
	cg04869122	0.561	(0.37;0.852)	0.004	5′UTR	Open_Sea
	cg12681727	0.653	(0.471;0.907)	0.010	Body	Open_Sea
	cg05688616	0.611	(0.405;0.922)	0.014	5′UTR	Open_Sea
	cg27050057	0.659	(0.465;0.935)	0.023	Body	Open_Sea
	cg00573606	0.640	(0.422;0.971)	0.028	5′UTR;1stExon	Open_Sea
	cg13217451	0.654	(0.436;0.981)	0.032	Body	Open_Sea
	cg14556851	0.715	(0.518;0.987)	0.041	Body	S_Shelf
COL8A1						
	cg03277051	0.594	(0.422;0.835)	0.002	TSS1500;Body	Open_Sea
	cg05283542	0.599	(0.434;0.827)	0.002	5′UTR;Body	Open_Sea
	cg21175685	1.430	(1.012;2.021)	0.048	5′UTR;Body	Open_Sea
COL10A1						
	cg05408873	0.716	(0.518;0.99)	0.045	1stExon;5′UTR;Body	Open_Sea
COL11A1						
	cg27229407	0.499	(0.317;0.785)	0.001	Body	Open_Sea
	cg09183742	0.518	(0.337;0.798)	0.001	Body	Open_Sea
	cg12884406	0.671	(0.485;0.93)	0.016	5′UTR;1stExon	Open_Sea
	cg20847625	0.681	(0.492;0.943)	0.020	5′UTR;1stExon	Open_Sea
	cg26436330	0.666	(0.465;0.954)	0.023	1stExon	Open_Sea
	cg03520644	0.643	(0.428;0.965)	0.026	TSS1500	Open_Sea
	cg26913669	1.458	(1.034;2.056)	0.036	TSS1500	Open_Sea
	cg00172849	0.673	(0.449;1.01)	0.047	TSS1500	Open_Sea
COL12A1						
	cg12801474	0.467	(0.297;0.735)	0.000	5′UTR	Island
	cg08009622	0.579	(0.374;0.897)	0.009	TSS1500	Island
	cg04611812	0.586	(0.375;0.915)	0.013	Body	Island
	cg11353250	0.596	(0.388;0.917)	0.013	3′UTR	Island
	cg03503642	1.645	(1.084;2.495)	0.014	5′UTR	N_Shore
	cg04504006	0.650	(0.462;0.916)	0.017	5′UTR	N_Shore
	cg03564793	0.620	(0.411;0.936)	0.017	3′UTR	Island
	cg26997327	0.677	(0.49;0.937)	0.018	Body	Island
	cg14375912	0.681	(0.491;0.944)	0.020	5′UTR	Island
	cg11526848	0.674	(0.477;0.952)	0.029	3′UTR	N_Shore
	cg12488810	0.674	(0.477;0.952)	0.029	Body	Open_Sea
	cg13395133	0.645	(0.427;0.975)	0.030	Body	Open_Sea
	cg24897255	0.653	(0.433;0.986)	0.034	TSS200	Island
	cg13319757	0.707	(0.511;0.977)	0.035	Body	Open_Sea
	cg15089846	1.384	(1.001;1.913)	0.048	Body	S_Shelf

LR: Likelihood ratio; CI: confidence interval.

3.6. The expression of collagen family members was positively correlated with immune infiltration

The infiltration levels of tumor-immune infiltrating cells (TIICs) are associated with patient prognosis. Therefore, we explored the relationship between collagen expression and the immune infiltration in gastric cancer by TIMER. The expression of collagen family members was negatively correlated with tumor purity and B cell infiltration levels, while positively correlated with $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells; however, some indicators were not statistically significant. The correlations between macrophage infiltration levels and the expression of COL1A1 (partial. Cor = 0.358, P = 1.21e − 12), COL1A2 (partial. Cor = 0.489, P = 1.34e − 23), COL6A3 (partial. Cor = 0.577, P = 2.91e − 34),

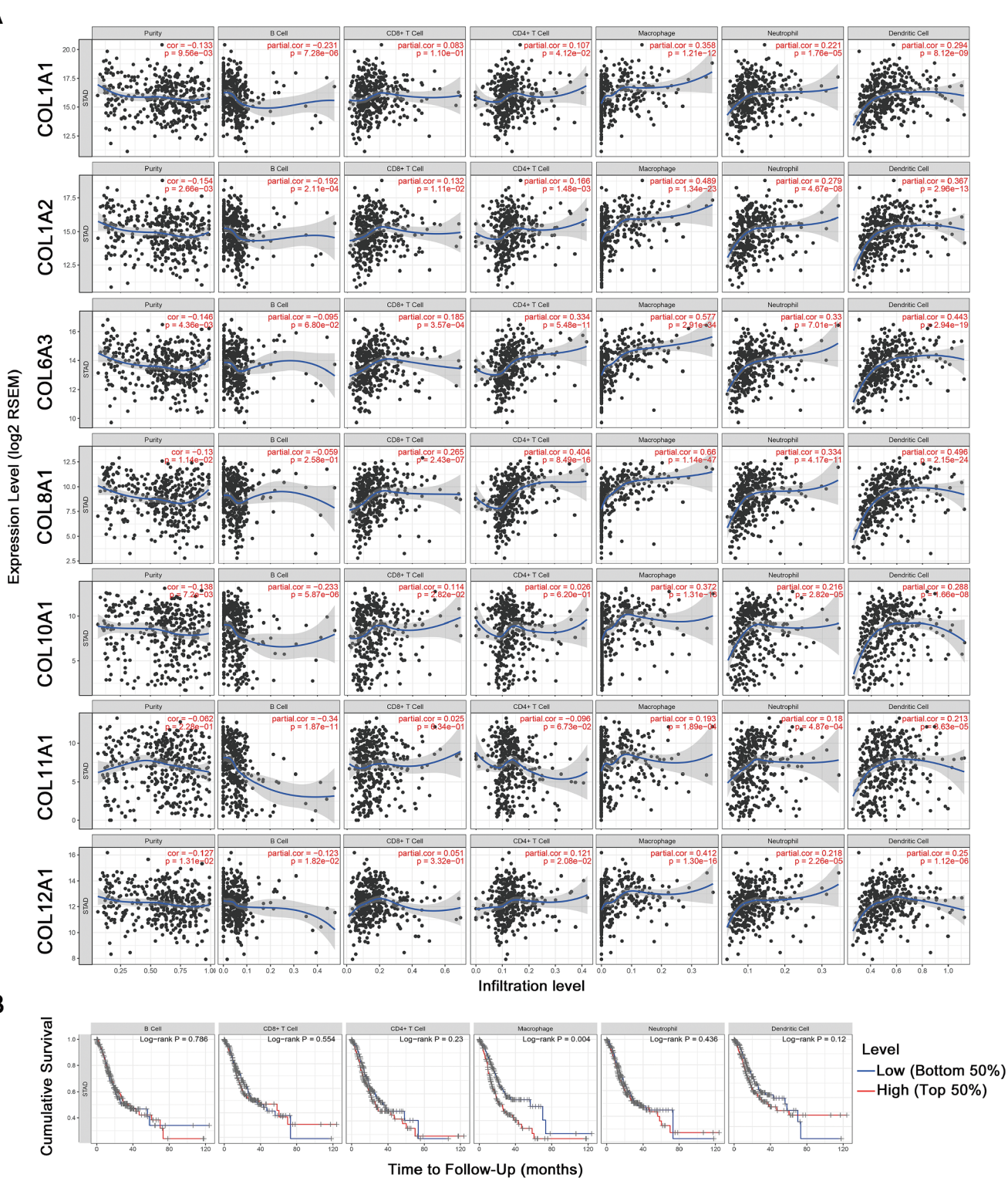

Fig. 5. Analysis of immune cell infiltration in STAD. A. Correlation between collagen family members expression and immune infiltration level in STAD. B. The relationship between the level of immune infiltration and the prognosis of STAD. STAD: stomach adenocarcinoma.

COL8A1 (partial. Cor = 0.660, P = 1.14e − 47), COL10A1 (partial. Cor = 0.372, P = 1.31e − 18), and COL12A1 (partial. Cor = 0.412, P = 1.30e − 16) were significant. Among them, COL6A3 and COL8A1 had a higher correlation (Fig. 5A). In addition, the higher infiltration level of macrophages predicted a worse prognosis of patients (Fig. 5B, log rank P = 0.004). These results indicated that the expression of collagen family members was correlated with the infiltration levels of TIICs in gastric cancer, particularly macrophages.

To further investigate the relationship between collagen family members and TIICs, we analyzed the correlations between collagen family members and immune gene marker sets of immune infiltrating cells in gastric cancer. We found that the expression of collagen family members was significantly associated with most immune gene markers (Table S1). In particular, collagen family members were significantly correlated with most of macrophages markers, so we presented scatterplots of the correlations between collagen family members and gene markers of monocytes, tumor-associated macrophages (TAMs), M1 macrophages, and M2 macrophages in gastric cancer (Fig. 6). Collagen family members all had higher correlations with gene markers of M2 macrophages. Apart from this, the expression levels of collagen family members were also correlated with neutrophils gene marker ITGAM; dendritic cells gene markers NRP1 and ITGAX; Th2 gene marker IL13; regulatory T cells (Tregs) gene markers CCR8 and TGFB1; exhausted T cell gene marker TIM-3. The correlations between PD-1 and the expression levels of COL1A1, COL6A3, and COL8A1 were statistically significant. These results revealed that the expression of collagen family members was correlated with TIICs infiltration in gastric cancer, suggesting that collagen family members might have a significant impact on tumor immunology.

3.7. Collagen expression and drug response AUC in stomach cancer cell lines

As presented in the previous results, COL1A1, COL1A2, COL8A1, and COL12A1 showed significant correlations in prognosis, methylation, and immune infiltration. To investigate the availability of them as therapeutic targets, we analyzed the relationship between expression and drug response AUC. However, the expression data of COL8A1 is not included in the CCLE database; therefore, we only show the data of the other three members. For cancer cell line types including more than 30 cell lines, the proportion of drugs significantly correlated with collagen expression was presented in a histogram (Fig. 7). Moreover, the percentage of drugs significantly associated with the expression of COL1A1, COL1A2, and COL12A1 in stomach cancer cell lines accounted for 12.1%, 10.5%, and 22.6% respectively. After that, volcanic plots showed the correlation between collagen expression and 545 drugs response AUC in stomach cancer cell lines (Fig. 7). Further, we showed the top 10 drugs associated with collagen expression in stomach cancer cell lines (Table 3). Positive correlation indicated that the high expression was related to drug resistance, while negative correlation represented better drug response. COL1A1 high expression was significantly related to a better response of PI3K inhibitors, including PI-103 and AZD6482. Besides, bendamustine and dasatinib, which related to COL1A1 expression, have been approved by FDA for the treatment of chronic leukemia [39,40]. COL1A1 high expression was correlated with drug resistance of GDC-0879, brefeldin A, cimetidine, and BRD-K29086754. In addition, tandutinib, MGCD-265, and quizartinib as inhibitors of VEGFR were confirmed to be correlated with COL1A2 expression, while COL1A1 was also sensitive to quizartinib. These

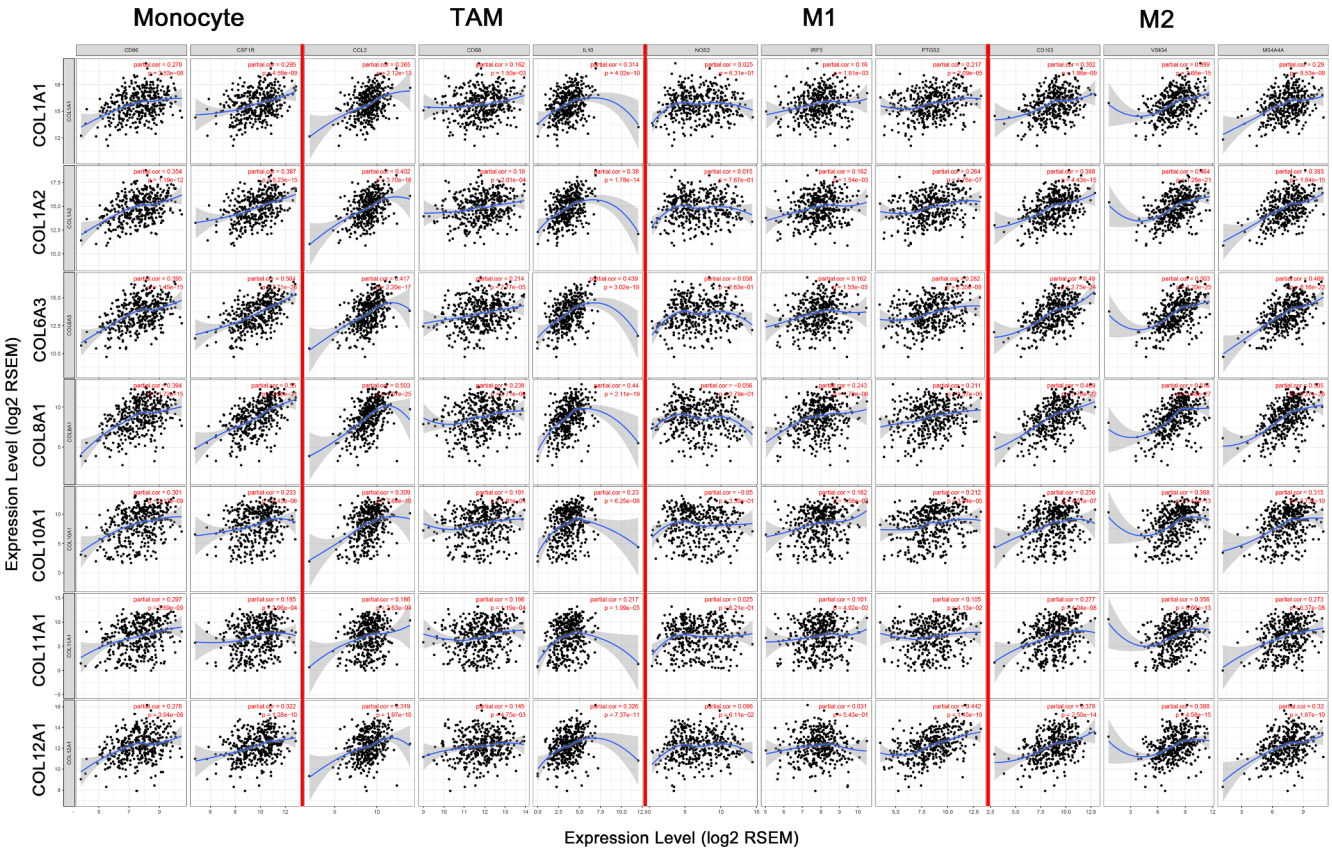

Fig. 6. Collagen family members expression was correlated with macrophage gene markers in gastric cancer. TAM: tumor-associated macrophage.

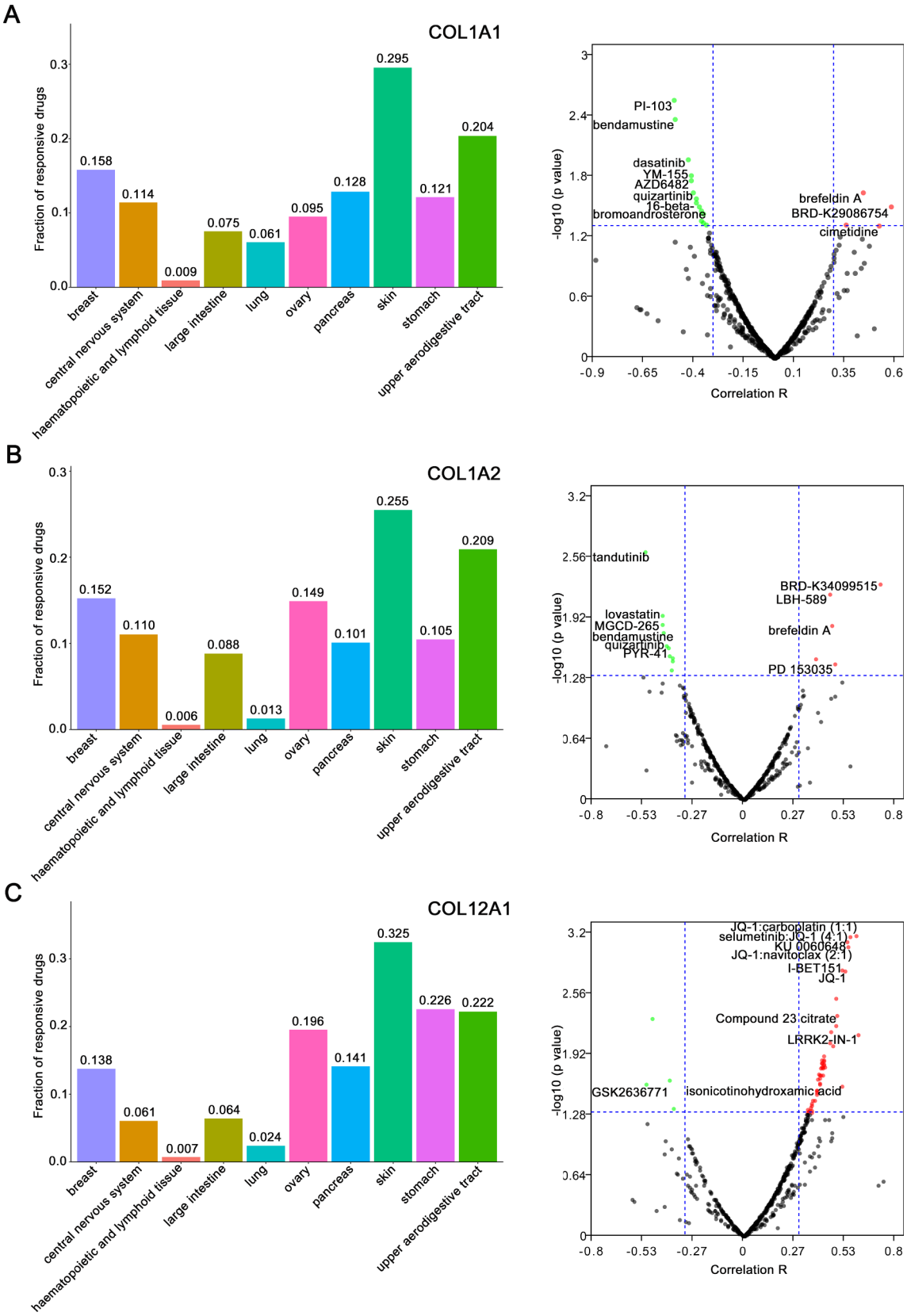

Fig. 7. Drug response analysis of COL1A1, COL1A2, and COL12A1. A. The ratio of drugs related to COL1A1 in 10 different cancer cell line types with at least 30 cell lines and volcano plot of correlation between COL1A1 expression and drug response in stomach cancer cell lines. B. The ratio of drugs related to COL1A2 and volcano plot of correlation between COL1A2 expression and drug response in stomach cancer cell lines. C. Ratio of drugs related to COL12A1 and volcano plot of correlation between COL12A1 expression and drug response in stomach cancer cell lines. Top 10 drugs were labelled.

Table 3
Drug response related to collagen expression in gastric cancer.

Gene symbol	Compound	*P* value	Correlation	Compound status	Target or activity of compound
COL1A1					
	PI-103	0.003	−0.505	probe	inhibitor of DNA-PK, PI3K p110 delta, mTORC1, and catalytic subunits of PI3K
	bendamustine	0.004	−0.499	FDA	DNA alkylator
	dasatinib	0.011	−0.432	FDA	inhibitor of SRC, YES1, EPHA2, c-KIT, and LCK
	YM-155	0.017	−0.419	clinical	inhibitor of survivin expression
	AZD6482	0.015	−0.418	clinical	inhibitor of PI3K catalytic subunits beta and delta
	quizartinib	0.022	−0.409	clinical	inhibitor of VEGFR3
	16-beta-bromoandrosterone	0.028	−0.394	probe	dehydroepiandrosterone (DHEA) analog
	brefeldin A	0.023	0.436	probe	modulator of ADP-ribosylation factor 1; inhibitor of protein translocation from ER to Golgi
	cimetidine	0.049	0.516	FDA	inhibitor of histidine receptor H2
	BRD-K29086754	0.031	0.576	GE-active	product of diversity-oriented synthesis
COL1A2					
	tandutinib	0.002	−0.518	clinical	inhibitor of c-KIT and VEGFR3
	MGCD-265	0.014	−0.430	clinical	inhibitor of c-MET and VEGFRs
	lovastatin	0.011	−0.429	FDA	inhibitor of HMG-CoA reductase
	bendamustine	0.017	−0.426	FDA	DNA alkylator
	quizartinib	0.024	−0.406	clinical	inhibitor of VEGFR3
	PYR-41	0.025	−0.396	probe	inhibitor of ubiquitin-activating enzyme in cells
	LBH-589	0.007	0.456	clinical	inhibitor of HDAC1, HDAC2, HDAC3, HDAC6, and HDAC8
	brefeldin A	0.014	0.466	probe	modulator of ADP-ribosylation factor 1; inhibitor of protein translocation from ER to Golgi
	PD 153035	0.037	0.482	probe	inhibitor of EGFR
	BRD-K34099515	0.005	0.722	probe	screening hit
COL12A1					
	GSK2636771	0.025	−0.513	clinical	inhibitor of PI3K catalytic subunit beta
	Compound 23 citrate	0.005	0.494	probe	analog of natural product cortistatin
	I-BET151	0.002	0.522	probe	inhibitor of bromodomain (BRD) and extra-C terminal domain (BET) proteins
	isonicotinohydroxamic acid	0.026	0.522	probe	inhibitor of HDAC6
	JQ-1	0.002	0.536	probe	inhibitor of bromodomain (BRD) and extra-C terminal domain (BET) proteins
	KU 0,060,648	0.001	0.549	probe	inhibitor of DNA-dependent protein kinase
	JQ-1: navitoclax (2:1 mol/mol)	0.001	0.552	probe	inhibitor of bromodomain (BRD) and extra-C terminal domain (BET) proteins; inhibitor of BCL2, BCL-xL, and BCL-W
	selumetinib: JQ-1 (4:1 mol/mol)	0.001	0.561	probe	inhibitor of MEK1 and MEK2; inhibitor of bromodomain (BRD) and extra-C terminal domain (BET) proteins
	JQ-1: carboplatin (1:1 mol/mol)	0.001	0.594	probe	inhibitor of bromodomain (BRD) and extra-C terminal domain (BET) proteins; inducer of DNA damage
	LRRK2-IN-1	0.007	0.608	probe	inhibitor of leucine-rich repeat kinase 2; inhibitor of doublecortin-like kinase

results suggested that the expression of COL1A1 and COL1A2 might be related to tumor angiogenesis. Moreover, COL1A2 high expression was not sensitive to PD 153035, the inhibitor of EGFR. Importantly, the data showed that COL12A1 was associated with the drug resistance of the probe JQ-1, an inhibitor of bromodomain (BRD) and extra-C terminal domain (BET) proteins. In addition, the stomach cancer cell lines with COL12A1 high expression were also sensitive to PI3K inhibitor, GSK2636771, which had been used for the clinical trial of advanced gastric adenocarcinoma treatment (NCT02615730). This is consistent with the results of COL1A1 and COL12A1 participating in the PI3K pathway. Overall, COL1A1, COL1A2, and COL12A1 may be feasible to act as therapeutic targets and provide guidance for clinical treatment.

4. Discussion

Gastric cancer is one of the most common malignant tumors worldwide and has extremely high morbidity and mortality. Although there are various treatment approaches, the prognosis of patients with gastric cancer is poor. The level of immune infiltration is closely related to the prognosis. However, the prognostic biomarkers related to immune infiltration are still few. Moreover, effective biomarkers and targets are currently lacking for guiding clinical treatment. Therefore, it is very important to identify novel prognostic biomarkers and immune-associated therapeutic targets in gastric cancer.

In this study, seven members of the collagen family were identified as core genes from two gastric cancer profiles. In addition, their high expression was verified by GEPIA. COL1A1 and COL1A2 showed higher copy number amplification and the high expression of collagen type I was verified in gastric cancer patient specimens. Accordingly, the results showed that the expression levels of collagen family in stage 2, 3, 4 were statistically different from those in stage 1 and normal tissues. Patients with higher stages had a worse prognosis. These results suggested that collagen expression might be related to patient prognosis.

Therefore, we estimated the prognostic value of seven collagen family members. Our results proved that the high expression of the collagen family as an unfavourable factor for predicting the prognosis of gastric cancer, especially in patients with metastasis. The expression levels of the collagen family were negatively associated with OS in gastric cancer. The correlation between the high expression level of collagen family members and prognosis in M1 patients was meaningful, apart from COL6A3. Some studies have shown the prognostic value of collagens. Jun Li found that COL1A1 and COL1A2 were correlated with poor clinical outcomes in gastric cancer [41]. The miRNA let-7i inhibited gastric cancer invasion and metastasis by targeting COL1A1 [42]. Furthermore, the co-expression of COL10A1 and SOX9 predicted poor prognosis and promoted metastasis in gastric cancer via epithelial-to-mesenchymal transition [43]. COL8A1 high expression predicted a worse prognosis of patients with lymph node metastasis. A study has shown that COL8A1 could promote invasion and metastasis in hepatocellular carcinoma [44]. Moreover, IDO1 and COL12A1 promoted gastric cancer cells migration via MAPK pathway [45]. These studies were concordant with our results. At present, it is widely believed that changes in DNA methylation contribute to the development of cancer. The whole blood DNA methylation has been considered as a risk marker

for gastric cancer [46]. The expression of COL1A1 and COL11A1 was regulated by DNA methylation [47]. Therefore, we estimated the DNA methylation status of collagen family members. COL1A1, COL1A2, COL8A1, and COL12A1 had lower DNA methylation levels in CpG sites of promoter. The hypomethylation of COL1A1 promoter predicted poor prognosis of gastric cancer. Consistent with this, it has been identified that COL1A1 with promoter hypomethylation is upregulated in esophageal cancer [48]. Besides, the data showed that the hypomethylation of COL1A2 in CpG sites was positively associated with poor survival in gastric cancer (HR $<$ 1). However, some studies revealed strong correlations of COL1A2 and COL12A1 hypermethylation and prognosis in cancer [21,49]. This contradiction may be caused by the different expression of COL1A2 and COL12A1 in different cancers. Above all, we predicted that the collagen family members might act as prognostic biomarkers for gastric cancer.

Currently, immunotherapy is an effective treatment. The intensity and cellular composition of immune infiltration are important for prognosis [50]. Consistent with our study, Zhang et al. suggested the negative prognostic effect of TAMs in gastric cancer [51]. Furthermore, the data showed that collagen type I involved in the GO BP of leukocyte migration. Accordingly, the correlation between the expression level of the collagen family and immune infiltration was verified in gastric cancer. Collagen family members were positively associated with the infiltration of dendritic cells. Schultz, H.S. suggested that collagen facilitated the maturation of dendritic cells derived from monocytes [52]. The correlation between T cell and collagen expression has also been confirmed. The data showed that COL1A1 was correlated with the expression of IL-13, which was a gene marker of Th1 cells. IL-13 significantly stimulated collagen type I production through MMP-2 and TGFB1 in airway fibroblasts of asthma patients [53]. Tregs inhibited the antitumor immune response in cancer [54]. TGFB1 as a gene marker of Tregs was strongly correlated with collagen family expression. TGFB1 can enhance Smad2 phosphorylation and the expression level of COL10A1 [43]. COL1A1, COL6A3, and COL8A1 were correlated with gene markers of exhausted T cells, PD-1 and TIM-3. PD-1 is an immune checkpoint, and PD-1/programmed cell death ligand 1 (PD-L1) interaction plays a predominant role in the suppression of T cell responses, especially in cancer [55]. TIM-3 is an activation-induced inhibitory molecule that induces T-cell exhaustion in cancers [56]. Targeting T cell inhibitory receptors, PD-1 and TIM-3, may be effective in reversing the immune escape of advanced gastric cancer [57]. Above results suggested that collagen might be associated with immunosuppression of gastric cancer.

Our results showed that the expression of collagen family members was correlated with the infiltration levels of TIICs in gastric cancer, particularly macrophages. In tumors, macrophages are important components, also known as TAMs. TAMs can direct the deposition, cross-linking, and linearization of collagen fibres during tumor development, especially in the area of tumor invasion [58]. Macrophages are generally divided into two subtypes, pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages [59]. M2 macrophages contribute to tumor proliferation and progression in gastric cancer [60]. The expression levels of collagen family members were correlated with most of macrophages markers, especially M2 macrophages. Actually, monocytes and macrophages could express all known collagens, such as types VI, VIII, X, XI, XII, XVIII, and XXIV collagen [61]. In addition, they would directly improve the survival of myofibroblasts and activate myofibroblasts to promote the production of the extracellular matrix as well [62]. In particular, M2 macrophages produced more COL6A3 than M1 macrophages [63]. Compared to M1 macrophages, COL6A3 had a strong correlation with M2 macrophage gene markers, CD163, VSIG4, and MS4A4A. Type VI collagen induced inflammatory responses by recruiting macrophages and promoted tumor development [63]. Qiu S., et al. suggested that TAMs regulated tumor growth through the integrin α2β1/PI3K/Akt signalling pathway induced by type I collagen [64]. Our results also indicated that the expression of COL1A1 and COL1A2 was associated with TAM gene markers, CCL2 and IL10. Collagen family members were significantly correlated with markers of M2 macrophages, which might be related to the secretion of collagen by M2 macrophages. The collagen family members may be potential immune-associated therapeutic targets.

In addition, the drug sensitivity analysis indicated that collagen expression was related to the response of gastric cancer cell lines to multiple drugs. For example, the high expression levels of COL1A1 and COL12A1 were significantly associated with a better response to PI3K inhibitors. Moreover, COL1A1 and COL12A1 involved in PI3K/Akt signalling pathway. PI3K/Akt/mTOR pathway had pro-proliferative and anti-apoptotic effects in gastric cancer cells and predicted worse prognosis [65]. Besides, downregulation of the phosphorylated PI3K/Akt cascade inhibited the expression of COL1A1, thereby inhibiting liver fibrosis [66]. Collagen I combined with β1-integrin to form positive feedback, thereby activating β1-integrin/mTOR/Akt and increasing the expression of collagen I [67]. Thus, we speculated that COL1A1 and COL12A1 could activate PI3K/Akt pathway, which in turn also increased the expression of collagen to promote the development of gastric cancer. Furthermore, COL1A1 and COL1A2 were sensitive to VEGFR inhibitors. Knockdown of VEGF reduced the production of collagen type 1 in fibroblasts [68]. Collagen type I promoted the metastasis and invasion of gastric cancer [17,42]. Tumor metastasis and angiogenesis process are inseparable [69]. Collagen type I might participate in the angiogenesis of gastric cancer. The specific mechanism needed further study. Importantly, we found a significant correlation of collagen expression and drug resistance. COL1A2 was associated with PD 153035 resistance, an inhibitor of EGFR. Consistent with our results, collagen type I induced the resistance of EGFR tyrosine kinase inhibitors in cancer cells through activating mTOR [70]. Moreover, COL12A1 was significantly correlated with the resistance of JQ-1, either single drug or combination medicine. JQ-1 inhibited cancer cell survival by activating autophagy [71,72]. We speculated that COL12A1 might be related to autophagy resistance. These results revealed the important value of collagen family members to act as therapeutic targets and guide clinical treatment.

5. Conclusions

In conclusion, we identified collagen family members as core genes in gastric cancer. Besides, the high expression levels and promoter hypomethylation of the collagen family predicted a poor prognosis in gastric cancer. Additionally, collagen expression was significantly associated with macrophages infiltration and drug response. More importantly, COL1A1, COL1A2, and COL12A1 were dominant prognostic biomarkers and immune-associated therapeutic targets in collagen family members. Therefore, our study may provide new insights into the role of collagen family members and clinical immunotherapy in gastric cancer.

Ethics approval

The study protocol was approved by the ethics committee of Zhengzhou University. The study was performed in accordance with the Declaration of Helsinki. All human specimens were obtained with informed consent of patients.

Data availability statement

The data supporting the conclusions of this article is included within this article and its additional files. Raw data are available from the corresponding author on reasonable request.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81572972) and the Supporting Plan of Scientific and Technological Innovation Team in Universities of Henan Province (No. 20IRTSTHN029).

CRediT authorship contribution statement

Yihuan Chen: Conceptualization, Methodology, Writing - original draft. **Wei Chen:** Investigation, Software. **Xiaoshuo Dai:** Methodology, Validation. **Chengjuan Zhang:** Resources, Visualization. **Qiushuang Zhang:** Software. **Jing Lu:** Funding acquisition, Supervision, Writing - review & editing.

Acknowledgements

We thank American Journal Experts for their professional English language editing.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2020.106798.

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