COL4A family: potential prognostic biomarkers and therapeutic targets for gastric cancer

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Background: The type IV collagen alpha chain (COL4A) family is a major component of the basement membrane (BM) that has recently been found to be involved in tumor angiogenesis and progression. However, the expression levels and the exact roles of distinct COL4A family members in gastric cancer (GC) have not been completely understood.

Methods: Here, the expression levels of COL4As in GC and normal gastric tissues were calculated by using TCGA datasets and the predicted prognostic values by the GEPIA tool. Furthermore, the cBioPortal and Metascape tools were integrated to analyze the genetic alterations, correlations and potential functions of COL4As, and their frequently altered neighboring genes in GC.

Results: Notably, the expression levels of COL4A1/2/4 in GC were higher to those in normal gastric tissues, while the expression levels of COL4A3/5/6 were lower in GC than normal. Survival analysis revealed that lower expression levels of COL4A1/5 led to higher overall survival (OS) rate. Multivariate analysis using the Cox proportional-hazards model indicated that age, gender, pathological grade, metastasis and COL4A5 expression, are independent prognostic factors for OS. However, TNM stage, lymph node metastasis, Lauren’s classification, COL4A1-4 and COL4A6 were associated with poor OS but not independent prognostic factors. Function-enriched analysis of COL4As and their frequently altered neighboring genes was involved in tumor proliferation and metastasis in GC.

Conclusions: These results implied that COL4A1/2 were potential therapeutic targets for GC. COL4A3/4/6 might have an impact on gastric carcinogenesis and subsequent progression, whereas COL4A5 was an independent prognostic marker for GC.

Keywords: COL4As; gastric cancer (GC); prognostic value; Kaplan-Meier plot; bioinformatics analysis

Submitted Jan 13, 2020. Accepted for publication Jul 14, 2020.
doi: 10.21037/tcr-20-517

View this article at: http://dx.doi.org/10.21037/tcr-20-517

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Introduction

Gastric cancer (GC) is the fourth most common malignancy and remains the second leading cause of cancer-related deaths (1). GC is a multifactorial disease, including environmental and genetic factors (2,3). Despite considerable advancements in prevention, diagnosis and treatment, the disease is still a great threat to human health with a five-year overall survival (OS) rate of less than 30% (4,5). Therefore, potential targets for treatments and new biomarkers for the prognosis of GC should be identified.

Conventional biomarkers (CEA, CA19-9, AFP and CA125) have been applied in diagnosis and prediction of prognosis for GC in clinical practice. Then the first molecular biomarker, HER2, is also available to improve recurrence and the efficacy of treatments. The fibroblast growth factor receptor 2 (FGFR2), vascular endothelial growth factor, E-cadherin, and TP53, etc. are recognized as metastasis related genes and could be biomarkers for recurrence forecast and metastasis assessment in GC patients (6). Besides, Immune checkpoint receptors ligands (PD-L1/2) and microsatellite-high (MSI-High) may serve as prognostic biomarkers for treatment response for GC (7). Recently, with the progression of liquid biopsy, more and more researches focus on using body fluids to detect GC biomarkers. Circulating tumor cells (CTCs), circulating cell-free DNA (cfDNA) such as EBV DNA, microRNAs such as miR-21 and miR-23a, long noncoding RNAs such as ncRuPAR, GACAT1, and GACAT2, and exosomes may provide prognostic and predictive markers for GC (8,9). With the development of knowledge of novel approach, such as TCGA Research Network, high specific and sensitive markers will continue to be tested.

The basement membrane (BM) acts as a physical barrier for prohibiting invasion and metastasis of tumors. The type IV collagen alpha chain (COL4A) family is a major component of BM that may be involved in tumor angiogenesis and progression. COL4A family constitutes six genetically different alpha chains, α1(IV) to α6(IV), also known as COL4A1 to COL4A6 (10). COL4A1 and COL4A2 are ubiquitous, whereas COL4A3 to COL4A6 are tissue-specific. Six COL4A proteins have been found in mammalian cells, numbered according to the abundance and tissue distribution (11). COL4A1 and COL4A2 are major types and COL4A3 to COL4A6 are minor types. Mutations of COL4As genes have been confirmed to result in defective BM synthesis diseases like Goodpasture Syndrome, Alport Syndrome, and thin BM nephropathy (12-14). However, abnormal expressions of COL4As proteins have been reported involved in not only proliferation and malignant transformation but also migration and invasion of cancers by several studies (15-20).

It has been observed that up-regulated COL4A1 was closely associated with tumor growth and metastasis in papillary thyroid carcinoma (16) and promoted the proliferation of the invasive ductal carcinoma cells in breast (15). Inhibition of miR-29c could upregulation the expression of COL4A1 and increase proliferation of endometrial cancer cells (21). The suppression of COL4A2 could also significantly inhibit the migration and proliferation of triple-negative breast cancer cells (19). Compared with patients with extrahepatic bile duct carcinoma of positive COL4A2 and COL4A6, loss of COL4A2 and COL4A6 had significantly poorer prognosis (22). Nie et al. (20) reported that aberrant expression of COL4A3 might play a role in the malignant transformation of gastric epithelial cells, which is a key step in the progression of gastric carcinogenesis. COL4A4 has been observed to be downregulated in esophageal cancer (17). COL4A5 may promote lung cancer progression through discoidin domain receptor-1 (23). Ikeda et al. (18) showed that COL4A5 and COL4A6 were under-expressed in colorectal cancer as compared to normal colorectal tissues and that might remodel the epithelial BM during cancer cell invasion. Baba et al. (24) demonstrated that the expressions of COL4A5 and COL4A6 were closely related to the grade of histological atypia and tumor cell growth activity in gastric intramucosal carcinoma.

Thus, it can be inferred that COL4As family are closely related to the progression of many kinds of cancers, including gastrointestinal cancers. COL4A2 and COL4A6 may be prognostic biomarkers in extrahepatic bile duct carcinoma whereas COL4A5 and COL4A6 may be prognostic biomarkers in gastric intramucosal carcinoma. Although COL4As family are considered to be GC-related factors (20,25), the underlying mechanisms by which the COL4A factors are activated or suppressed, and their separate function in GC have not been elucidated so far.

In this study, the relationship between COL4A factors and GC was further explored. With the development of microarray technology, RNA and DNA research has been revolutionized as an essential method of biological and biomedical studies (26). The expression levels and alterations of different COL4A factors in GC patients were analyzed to identify their expression patterns, the potential functions and distinct prognostic values in GC based on the thousands of gene expression or copy number variation.
analysis published online.

**Methods**

**ONCOMINE analysis**

ONCOMINE gene expression array datasets (www.oncomine.org), an online cancer microarray database, was used to analyze the mRNA expression levels of COL4As in different cancers. The mRNA expressions of COL4As in clinical cancer specimens were compared with that in normal controls, using a Students’ *t*-test to generate a *P* value. The cut-off of *P* value and fold change was defined as 1E-4 and 2, respectively.

**Gene Expression Profiling Interactive Analysis (GEPIA) dataset**

GEPIA (http://gepia.cancer-pku.cn/) is a developed interactive web server for analyzing the mRNA expression data. It consists of 9,736 tumors and 8,587 normal samples from the TCGA and the GTEx projects, using a standard processing pipeline. GEPIA provides customizable functions such as tumor/normal differential expression analysis, profiling according to the cancer types or pathological stages, patient survival analysis, similar gene detection, correlation analysis and dimensionality reduction analysis (27).

**XENA analysis**

The clinical and pathological data of 380 cases of GC patients and 37 adjacent normal tissues as well as the relative expression levels of COL4As were downloaded from TCGA 2015 RNA sequencing database from UCSC Xena (http://xena.ucsc.edu/). The mRNA expressions of COL4As in clinical cancer specimens were compared with that in normal controls. The clinical and pathological data were compared between high expression of COL4As in GC patients and low expression of that in GC patients. Statistical analyses were carried out by using SPSS 22.0 (IBM, SPSS, Chicago, IL, USA) and GraphPad Prism 7. Student’s *t*-test or Chi-square test was used to assess the statistical significance for comparisons of two groups (28). *P* value <0.05 was considered as significant.

**OncoLnc tool**

OncoLnc (http://www.oncolnc.org) is a tool that contains survival data for 8,647 patients from 21 cancer studies performed by The Cancer Genome Atlas (TCGA), along with RNA-SEQ expression for mRNAs and miRNAs from TCGA, and lncRNA expression from MiTranscriptome beta. It can be used to interactively explore survival correlations and to download clinical data coupled to expression data for mRNAs, miRNAs, or lncRNA. Users can investigate the range of expression of the gene at the Kaplan-Meier plotting page (29).

**TCGA and CBioPortal analysis**

The frequency of the COL4A family gene alterations (amplification, deep deletion, and missense mutations) and copy number variance were obtained from the cBioPortal (http://www.cbioportal.org/) (30). Besides, according to the online instructions of cBioPortal, we performed co-expression and network analyses.

**Functional enrichment analysis**

In this study, Metascape (http://metascape.org) was used to conduct pathway and process enrichment analysis of COL4A family members and neighboring genes significantly associated with COL4As alterations (31). The Gene Ontology (GO) terms as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were enriched based on the Metascape online tool. PPI enrichment analysis was performed. Further, MCODE (Molecular Complex Detection) algorithm was applied to identify densely connected network components.

**Results**

**The mRNA expression levels of COL4As in patients with GC**

ONCOMINE database was used in this study to compare the mRNA expression levels of COL4As in cancers to those in normal tissues (Figure 1). It was found that the mRNA expression level of COL4A1 was significantly upregulated in GC patients in six datasets. In Chen’s dataset (32), the expression level of COL4A1 was significantly up-regulated in all types of GC as compared to that in normal tissues (in diffuse gastric adenocarcinoma with a fold change of 5.045, gastric intestinal-type adenocarcinoma of 4.104, and gastric mixed adenocarcinoma of 6.23, Table 1). COL4A2 was also overexpressed with a fold change of 10.501, 2.113, and
Figure 1 The mRNA expression levels of COL4A factors in different types of cancers (ONCOMINE). The best gene rank percentile for the analyses within the cell determines cell color. The numbers in cells refer to numbers that met the threshold (P<1E-4, fold change ≥2).

3.82 in diffuse gastric adenocarcinoma, gastric intestinal-type adenocarcinoma, and gastric mixed adenocarcinoma respectively in Chen’s dataset as shown in Table 1. D-Errico (33) showed another increased expression of factor, COL4A4 in diffuse gastric adenocarcinoma with a fold change of 2.739 compared with normal samples (Table 1).

On the contrary, in the same dataset of D-Errico, the expression levels of COL4A3 and COL4A6 were significantly decreased in gastric intestinal-type adenocarcinoma with a fold change of −2.526 and −2.748 respectively (Table 1). As shown in Table 1 for COL4A5, there was down-regulation of mRNA expressions in diffuse gastric adenocarcinoma and gastric intestinal-type adenocarcinoma in comparison with normal patients with −2.585 and −4.467 fold changes separately according to Cho’s dataset (34).

The relationship between mRNA levels of COL4A4s and clinicopathological parameters of GC patients

By using GEPIA dataset, a comparison of the mRNA expression of COL4A factors between GC and gastric tissues. It was indicated from the results that COL4A1
Table 1 The significant changes of COL4As expression in transcription level between different types of gastric cancer and gastric tissues (ONCOMINE database)

| Types of GC vs. gastric                                     | Fold change | P value       | t-test    | Ref  |
|------------------------------------------------------------|-------------|---------------|-----------|------|
| COL4A1 Diffuse gastric adenocarcinoma vs. normal            | 5.045       | 4.54E-13      | 14.254    | Chen |
| Gastric mixed adenocarcinoma vs. normal                    | 6.23        | 6.43E-07      | 10.438    | Chen |
| Gastric intestinal type adenocarcinoma vs. normal           | 4.104       | 6.04E-18      | 15.779    | Chen |
| Total gastric cancer vs. normal                             | 2.276       | 5.67E-06      | 5.853     | Wang |
| COL4A2 Diffuse gastric adenocarcinoma vs. normal            | 10.501      | 1.63E-09      | 10.501    | Chen |
| Gastric mixed adenocarcinoma vs. normal                    | 2.133       | 2.38E-17      | 10.669    | Chen |
| Gastric intestinal type adenocarcinoma vs. normal           | 3.82        | 4.23E-06      | 9.131     | Chen |
| Total gastric cancer vs. normal                             | 2.483       | 1.85E-06      | 5.93      | Wang |
| COL4A3 Gastric intestinal type adenocarcinoma vs. normal    | –2.526      | 1.03E-05      | –4.775    | DErrico |
| COL4A4 Diffuse gastric adenocarcinoma vs. normal            | 2.739       | 1.26E-05      | 5.168     | DErrico |
| COL4A5 Diffuse gastric adenocarcinoma vs. normal            | –2.585      | 9.71E-08      | –6.25     | Cho   |
| Gastric intestinal type adenocarcinoma vs. normal           | –4.467      | 7.41E-05      | –4.467    | Cho   |
| COL4A6 Gastric intestinal type adenocarcinoma vs. normal    | –2.748      | 2.26E-08      | –6.522    | DErrico |

and COL4A2 expressions were up-regulated in gastric adenocarcinoma compared with gastric tissues, whereas the expression levels of COL4A3 and COL4A5 were lower in gastric adenocarcinoma than gastric tissues (Figure 2).

In order to further validate the relationship between mRNA levels of COL4As and clinopathological parameters of GC patients, we downloaded the clinopathological data of 380 cases of GC patients and 37 adjacent normal tissues as well as the relative mRNA expression levels of COL4As from UCSC Xena database (28). The clinopathological data of 380 cases of GC patients is shown in Table S1. By using TCGA sequencing data, we further validated that there was the increased mRNA expressions of COL4A1, COL4A2 and COL4A4 and the decreased mRNA expressions of COL4A5 and COL4A6 in human gastric adenocarcinoma tissues (n=380), adjacent normal tissues (n=37), and in paired GC tissues (n=34) (Figure 3).

To evaluate whether the mRNA expression levels of COL4As were associated with clinical and pathological characteristics and prognosis of GC patients, 380 GC patients were divided into two groups based on the mean of mRNA expressions of each one of COL4As. There were COL4As high expression (the value > the median) and COL4As low expression (the value ≤ the median). As indicated in Table 2, COL4A2 expression positively correlated with classification of the grade (P=0.044). High expressions of both COL4A3 and COL4A4 were linked to poor TNM stage, pathological grade, lymph node metastasis, and Lauren’s classification (P<0.05). The expression of COL4A5 was high in patients aged less than 60 years. COL4A6 harbored no association with clinical parameters (P>0.05). Multivariate analysis using the Cox proportional hazards model indicated that age, gender, pathological grade, metastasis and COL4A5 expression are independent prognostic factors for OS. However, TNM stage, lymph node metastasis, Lauren’s classification, COL4A1-4 and COL4A6 were associated with poor OS but not independent prognostic factors (Figure S1).

To determine the COL4As protein expressions, they were analyzed using clinical specimens retrieved from the Human Protein Atlas (the Human Protein Atlas available from www.proteinatlas.org). It showed that COL4A1 and COL4A2 had strong expressions in GC and weak expressions in normal tissues (Figure S2). Whereas COL4A3 had the inverse expression (Figure S2). Unfortunately, related results of other COL4As have not been uploaded till date; hence, they could not be presented here.
Figure 2  The mRNA expression of COL4As in GC (GEPIA). (A) The differences of gene expression profiles between stomach adenocarcinoma (STAD) and normal tissues (the red, green, and black “STAD” in the top represent that the expressions of related genes were increased, decreased or not significant in STAD as compared to normal tissues); (B) the differences in gene expression on box plots between STAD and normal tissues. *, P<0.01. N, normal gastric tissues, T, tumor tissues.
Increased mRNA expressions of COL4A1/2/5 were associated with OS of GC patients and increased mRNA expressions of COL4A3/4 were associated with poor disease-free survival (DFS) of GC patients (Figure 4).

COL4A1 and COL4A5 high mRNA expression levels were found to be associated with poor OS of patients in GC by using both GEPIA and OncoLnc tools (29) (Figure 4A,E, Figure S3). The high mRNA expression of COL4A2 was correlated with poor OS of patients in GC by using OncoLnc. However, results not same with GEPIA tool (Figure 4B, Figure S3). Different results between GEPIA and OncoLnc tools may derived from different cut-off values. The high expressions of COL4A3 and COL4A4 were found to be associated with poor DFS of patients in GC (Figure 4C,D). Other factors had no links with OS or
Table 2 Correlation of COL4A expression with clinicopathologic features of gastric cancer (GC) patients

| Clinicopathologic features [cases of data available*] | COL4A1 | COL4A2 | COL4A3 |
|-------------------------------------------------------|--------|--------|--------|
|                                                       | Low    | High   | P      | Low    | High   | P      | Low    | High   | P      |
| Age [375]                                              |        |        |        |        |        |        |        |        |        |
| ≥60                                                    | 258    | 127    | 131    | 132    | 126    | 0.181  | 135    | 123    | 0.374  |
| <60                                                    | 117    | 62     | 55     | 0.506  | 57     | 60     | 0.738  | 52     | 65     |
| Gender [380]                                           |        |        |        |        |        |        |        |        |        |
| Female                                                | 131    | 67     | 64     | 68     | 63     | 0.666  | 68     | 63     | 0.666  |
| Male                                                  | 249    | 123    | 126    | 0.829  | 122    | 127    | 0.666  | 122    | 127    |
| TNM stage [376]                                        |        |        |        |        |        |        |        |        |        |
| I                                                      | 53     | 30     | 23     | 33     | 20     | 0.003  | 37     | 16     | 0.003  |
| II–IV                                                  | 323    | 159    | 164    | 0.374  | 155    | 168    | 0.075  | 151    | 172    |
| Pathological grade [371]                               |        |        |        |        |        |        |        |        |        |
| G1–2                                                   | 148    | 76     | 69     | 85     | 63     | 0.003  | 88     | 60     | 0.003  |
| G3                                                     | 223    | 110    | 113    | 0.460  | 104    | 119    | 0.044  | 97     | 126    |
| Lymph node metastasis [370]                            |        |        |        |        |        |        |        |        |        |
| N0                                                    | 117    | 57     | 60     | 64     | 53     | 0.004  | 72     | 45     | 0.004  |
| N1–N3                                                 | 253    | 129    | 124    | 0.738  | 122    | 131    | 0.265  | 113    | 140    |
| Metastasis [361]                                       |        |        |        |        |        |        |        |        |        |
| M0                                                    | 341    | 173    | 168    | 174    | 167    | 1      | 173    | 168    | 1      |
| M1                                                     | 20     | 10     | 10     | 1.000  | 8      | 12     | 0.366  | 10     | 10     |
| Lauren classification [241]                            |        |        |        |        |        |        |        |        |        |
| Intestinal type                                        | 165    | 83     | 82     | 83     | 82     | 0.004  | 92     | 73     | 0.004  |
| Diffuse type                                           | 76     | 32     | 44     | 0.268  | 31     | 45     | 0.211  | 27     | 49     |

* there are some data missed in clinicopathological data of GC patients from TCGA database.

DFS of patients in GC (Figure 4F).

Predicted functions and pathways of the changes in COL4A factors and their frequently altered neighboring genes in patients with GC

The cBioPortal online tool (30,35) was used to analyze the COL4A alterations, correlations, and networks for GC (TCGA, Provisional). There were 152 samples out of 478 patients of COL4A altering with GC (32%). In almost half of the samples (72 samples), two or more alterations were detected (Figure 5A). The percentages of genetic alterations in individual genes of COL4A family members for GC varied from 6% to 13% (COL4A1, 13%; COL4A2, 11%; COL4A3, 7%; COL4A4, 7%; COL4A5, 8%; COL4A6, 6%; Figure 5A). The correlations of COL4As with each other were calculated by analyzing their mRNA expressions (RNA Seq V2 RSEM) via the online tool mentioned above. Pearson’s correction was included. The results indicated significant and positive correlations in the following COL4As: COL4A1 with COL4A2, COL4A3 with COL4A4, and COL4A5 with COL4A6 (Figure 5A). Then we performed network analysis for COL4A and the 50 most frequently altered neighboring genes (Figure 5B). The names, abbreviations, and functions for these genes are shown in Table S2. The results showed that the integrin family of protein-coding genes (ITGA2B/3/4/6/7/E/L/V/X, ITGB4/5/7/8/L1) and the laminin family of protein-coding genes (LAM1/2/3/4/5, LAMB1/2/3/4, LAMC1/3) were closely associated with COL4As alterations.
Functional gene and pathway enrichment analysis of COL4A factors and their frequently altered neighboring genes in patients with GC

The GO functions and KEGG pathway enrichment analysis of candidate COL4As and their frequently altered neighboring genes was performed based on the Metascape databases (31). As shown in Figure 6A, there were the top 20 GO and KEGG enrichment items (17 terms and 3 pathways) involved. It was indicated that these COL4As and their frequently altered neighboring genes were mainly enriched in extracellular matrix organization, integrin-mediated signaling pathway, endoplasmic reticulum lumen, endodermal cell differentiation, cell morphogenesis involved in differentiation, cell junction assembly, positive regulation of cell migration, blood vessel morphogenesis, platelet degranulation, laminin-5 complex, and laminin-1 complex, etc. Three significantly enriched pathways: focal adhesion, toxoplasmosis, and proteoglycans in cancer were identified in correlations with COL4As and their frequently altered neighboring genes. Furthermore, these enriched terms were closely connected with each other and clustered into intact networks (Figure 6B).

For a better understanding, the relationship between COL4A family members and GC, the Metascape database was used to perform protein-protein interaction (PPI) enrichment analysis. The PPI network is shown in Figure 6C,D. The five most significant MCODE components were extracted from the PPI network. Each MCODE component was applied by pathway and process enrichment analysis independently, and the three best-scoring terms of the corresponding components by P value were retained as the functional description shown in Table 3. The results suggested that ECM-receptor interaction, PID integrin pathway, extracellular matrix organization, focal...
adhesion, laminin interactions, and cell junction assembly were mainly associated with COL4A family members.

Discussion

In this study, the mRNA expression levels, prognostic values, genetic alterations, correlations, and potential functions of different COL4As in GC, were systematically explored by bioinformatics analysis.

COL4A1, the most classic member of the COL4A family is found to play a pivotal role in proliferation, metastasis, and invasion in most cancers (15,16,36). Zhang et al. (37) reported that miRNA-29c-3p represses proliferation of gastric adenocarcinoma BGC-823 cells by directly targeting COL4A1. Huang et al. (38) identified that COL4A1 is upregulated in trastuzumab resistance in GC cells and may induce trastuzumab resistant in GC in silico. In the present study, it was revealed that the mRNA expression of COL4A1 was upregulated in human GC as compared to normal tissues. High mRNA expression level of COL4A1 was found to be associated with poor OS by using both GEPIA and OncoLnc tool. However, expression of COL4A1 did not correlate with the DFS and clinical characteristics of the patients with GC. These phenomena indicate COL4A1 may serve as a new biomarker for the prognosis and a potential target of GC. As demonstrated in Figure 1, COL4A1 is also upregulated in lymphoma and sarcoma. Moreover, Chida et al. (39) identified that COL4A1 is located predominantly in cancer stroma. Immunohistochemistry (Figure S2) also showed COL4A1 is expressed in stromal tissue but not in tumor cells. In addition, COL4A1 may be derived from stromal reaction during the tumor progression and not from tumor cells themselves. Miyake et al. (36) found that the formation of tumor budding is involved in the carcinogenesis of COL4A1 in human urothelial cancer of the bladder. However, in papillary thyroid cancer, exosomal miR-21-5p can increase endothelial tube formation by inhibiting COL4A1, consequently promoting angiogenesis (40). Angiogenesis may be involved in the effects of COL4A1 on
Figure 6 The enrichment analysis of COL4A family members and neighboring genes in OC (Metascape). (A) Heatmap of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enriched terms colored according to P values; (B) enriched terms or pathways are distinguished by nodes. Colors and node sizes indicate number of genes involved; (C) and (D) Protein-protein interaction (PPI) network and five most significant MCODE components form the PPI network.

In triple-negative breast cancer, knockdown of COL4A2 could inhibit the proliferation and migration of cancer cells (19). COL4A2 is identified as a methylation marker with high accuracy for the detection of colorectal cancer (41). Notch3 can upregulate COL4A2 and promote anoikis resistance in ovarian cancer (42). In our study, use of ONCOMINE, GEPIA, and UCSC Xena datasets also revealed the mRNA expression level of COL4A2 was up-regulated in GC. By using the GEPIA tool, it was analyzed that COL4A2 was not related to OS and DFS of patients in GC. High transcriptional expression level of COL4A2 was associated with poor OS in OncoLnc. Moreover, COL4A2 high expression positively correlated with pathological grade (P=0.044). These findings suggested that COL4A2 may be a promising therapeutic target and could predict the prognosis of patients in GC as a biomarker.

Metodieva et al. (43) found that COL4A3 is downregulated in early-stage non-small cell lung cancer by real-time PCR. COL4A3 expressed to a lesser extent in GC than in normal mucosa at both mRNA and protein levels but its expression positively related to poor prognosis and worse clinicopathologic features of GC (20). A decreased mRNA expression of COL4A3 in GC is also shown in our study. Both GEPIA and OncoLnc results showed COL4A3 was not related to OS of patients in GC. However, to our surprise, high COL4A3 mRNA expression was significantly associated with poor DFS and the high mRNA expressions of COL4A3 was correlated with poor TNM stage, pathological grade, lymph node metastasis, and Lauren's classification (P<0.05). It seemed COL4A3 might serve as a biomarker to indicate a worse prognosis of patients in GC.

COL4A4 was confirmed to be down-regulated in esophageal cancer (17). However, in the present study, excluding GEPIA datasets, ONCOMINE and UCSC Xena showed an increased mRNA expression of COL4A4 in GC. High COL4A4 mRNA expression was associated with poor DFS and poor TNM stage, pathological grade, lymph node...
metastasis and Lauren classification (P<0.05). There was no association between COL4A4 expression level and OS of patients in GC. COL4A4 may play a role of an oncogene and a potential prognostic biomarker for GC.

ONCOMINE, GEPIA and UCSC Xena datasets all revealed that the mRNA expression of COL4A5 was downregulated in GC. Except for GEPIA dataset, ONCOMINE, and XENA datasets showed low mRNA expression of COL4A6 in GC compared with normal samples. COL4A5 was not related to DFS. We found high mRNA expression of COL4A5 was correlated with poor OS. Multivariate analysis indicated that age, gender, pathological grade, metastasis, and COL4A5 expression are independent prognostic factors. COL4A6 harbored no link with OS, DFS and clinical characteristics. Loss of expressions of COL4A5 and COL4A6 were reported in colorectal cancer and might be involved in the remodeling of the epithelial BM during cancer cell invasion (18). COL4A5 may be an indicator of a worse prognosis of GC. Further research is needed to prove whether COL4A6 plays a role in GC.

The percentages of genetic alterations in COL4A family members for GC were calculated to further illustrate the genetic alterations, potential functions, and carcinogenic mechanisms of the same. The percentages of genetic alterations ranged from 6% to 13% for individual genes based on TCGA Provisional dataset. In addition, we predicted COL4As alterations related 50 genes and constructed a network. COL4As alterations were closely associated with the integrin family of protein-coding genes, including ITGA2B/3/4/6/7/E/L/V, ITGB4/5/7/8/L1, and the laminin family of protein-coding genes, including LAM1/2/3/4/5, LAMB1/2/3/4, LAMC1/3. The GO and KEGG pathway analysis indicated they were enriched in pathways between ECM and the adhesion process. Adhesion-related pathways, such as extracellular matrix organization, focal adhesion and integrin-mediated signaling pathways were associated with the processes of proliferation, migration and invasion of GC (44-46). Combined with the results above, we hypothesized that COL4As may have impacts on adhesion-related pathways and integrin-mediated signaling pathways, thereby regulating the downstream of Akt pathway. Activation of Akt pathway could promote the proliferation and invasion of GC.

This study is a descriptive research using bioinformatics analysis. In future, a large sample sizes with high quality and experimental studies in our hospital are needed to further elucidate and verify our research.

**Table 3** Independent functional enrichment analysis of three Molecular Complex Detection (MCODE) components.

| MCODE   | GO             | Description                                           | Log$_{10}$(P) |
|---------|----------------|-------------------------------------------------------|---------------|
| MCODE_1 | hsa04512       | ECM-receptor interaction                              | -23.9         |
| MCODE_1 | M18            | PID INTEGRIN1 PATHWAY                                 | -21.6         |
| MCODE_1 | R-HSA-1474244  | Extracellular matrix organization                     | -21           |
| MCODE_2 | hsa04510       | Focal adhesion                                        | -18.8         |
| MCODE_2 | R-HSA-3000157  | Laminin interactions                                  | -12.6         |
| MCODE_2 | GO:0034329     | Cell junction assembly                                 | -12.5         |
| MCODE_3 | R-HSA-186797   | Signaling by PDGF                                    | -10.5         |
| MCODE_3 | R-HSA-3000171  | Non-integrin membrane-ECM interactions                | -10.5         |
| MCODE_3 | R-HSA-2214320  | Anchoring fibril formation                            | -9.1          |
| MCODE_4 | CORUM:6990     | THSD1-FAK-talin-vinculin complex                       | -11.8         |
| MCODE_4 | CORUM:5177     | Polycystin-1 multiprotein complex (ACTN1, CDH1, SRC, JUP, CTNNB1, PXN, BCR1, PKD1, PTK2, TNL1) | -10.2         |
| MCODE_4 | M281           | PID FAK PATHWAY                                       | -7.9          |
| MCODE_5 | R-HSA-1650814  | Collagen biosynthesis and modifying enzymes           | -7.7          |
| MCODE_5 | R-HSA-1474290  | Collagen formation                                    | -7.3          |
| MCODE_5 | R-HSA-1474244  | Extracellular matrix organization                     | -5.7          |
Conclusions

In this study, we used the GEPIA tool, cBioPortal, and Metascape tool to explore the expression and prognostic value of COL4As in GC from which we could have a further understanding of the molecular biological properties of GC. Our findings suggested that COL4A1/2 are potential therapeutic targets for GC, COL4A3/4/6 may have an impact on gastric carcinogenesis and subsequent progression and COL4A5 is found to be an independent prognostic marker for GC.

Acknowledgments

Funding: This study was supported by the National Natural Science Foundation of China (81570783), National Key R&D Program of China (2016YFC1302201 and 2017YFC0908302), Key Research and Development Program of Gansu Province, China (18YF1FA110), the Key Program of the Natural Science Foundation of Gansu Province, China (18JR3RA366), the Foundation of The First Hospital of Lanzhou University, China (ldyyyn2018-54), and Excellence Program of the First Clinical Medical College of Lanzhou University, China.

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr-20-517). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved. All the datasets were retrieved from the published literature and publicly available datasets. Datasets links were attached in the Methods and Results. The written permissions are not required. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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Cite this article as: Zeng X, Wang HY, Wang YP, Bai SY, Pu K, Zheng Y, Guo QH, Guan QL, Ji R, Zhou YN. COL4A family: potential prognostic biomarkers and therapeutic targets for gastric cancer. Transl Cancer Res 2020;9(9):5218-5232. doi: 10.21037/tcr-20-517
### Table S1 Clinicopathological data of gastric cancer (GC) patients from TCGA database

| Parameters [cases of data available*] | Cases (n=380) |
|---------------------------------------|---------------|
| **Age [375]**                         |               |
| ≥60                                   | 258 (68.8)    |
| <60                                   | 117 (31.2)    |
| **Gender [380]**                      |               |
| Female                                | 131 (34.5)    |
| Male                                  | 249 (65.5)    |
| **Pathological stage [368]**          |               |
| I/II                                  | 171 (46.5)    |
| III/IV                                | 197 (53.5)    |
| **T classification [377]**            |               |
| T1/T2                                 | 98 (26.0)     |
| T3/T4                                 | 279 (74.0)    |
| **N classification [370]**            |               |
| N0/N1                                 | 218 (58.9)    |
| N2/N3                                 | 152 (41.1)    |
| **Metastasis and[or] recurrence [380]** |             |
| Negative                              | 315 (82.9)    |
| Positive                              | 65 (17.1)     |

* there are some data missed in clinicopathological data of GC patients from TCGA database. Data present as n (%).
| Clinicopathologic feature | Relative risk (95% CI) | P  |
|---------------------------|------------------------|----|
| Age (<60 y)               | 0.463 (0.284, 0.756)   | 0.002 |
| Gender (female)           | 0.599 (0.367, 0.976)   | 0.040 |
| TNM stage (II-IV)         | 1.650 (0.566, 4.813)   | 0.359 |
| Pathological grade (G3)   | 1.738 (1.034, 2.919)   | 0.037 |
| Lymph node metastasis (N1-N3) | 1.569 (0.797, 3.088) | 0.192 |
| Metastasis (M1)           | 2.448 (1.119, 5.358)   | 0.025 |
| Lauren class (diffuse)    | 0.992 (0.587, 1.676)   | 0.977 |
| COL4A1 expression (high)  | 1.275 (0.828, 1.962)   | 0.270 |

| Clinicopathologic feature | Relative risk (95% CI) | P  |
|---------------------------|------------------------|----|
| Age (<60 y)               | 0.456 (0.280, 0.745)   | 0.002 |
| Gender (female)           | 0.598 (0.367, 0.975)   | 0.039 |
| TNM stage (II-IV)         | 1.672 (0.574, 4.873)   | 0.346 |
| Pathological grade (G3)   | 1.734 (1.032, 2.914)   | 0.038 |
| Lymph node metastasis (N1-N3) | 1.545 (0.786, 3.036) | 0.207 |
| Metastasis (M1)           | 2.433 (1.111, 5.327)   | 0.026 |
| Lauren class (diffuse)    | 0.994 (0.589, 1.678)   | 0.981 |
| COL4A2 expression (high)  | 1.278 (0.833, 1.961)   | 0.260 |

| Clinicopathologic feature | Relative risk (95% CI) | P  |
|---------------------------|------------------------|----|
| Age (<60 y)               | 0.460 (0.282, 0.751)   | 0.002 |
| Gender (female)           | 0.588 (0.361, 0.959)   | 0.033 |
| TNM stage (II-IV)         | 1.752 (0.604, 5.078)   | 0.302 |
| Pathological grade (G3)   | 1.773 (1.053, 2.984)   | 0.031 |
| Lymph node metastasis (N1-N3) | 1.502 (0.766, 2.945) | 0.236 |
| Metastasis (M1)           | 2.501 (1.123, 5.569)   | 0.025 |
| Lauren class (diffuse)    | 0.979 (0.579, 1.656)   | 0.937 |
| COL4A3 expression (high)  | 1.012 (0.652, 1.571)   | 0.957 |

| Clinicopathologic feature | Relative risk (95% CI) | P  |
|---------------------------|------------------------|----|
| Age (<60 y)               | 0.463 (0.282, 0.761)   | 0.002 |
| Gender (female)           | 0.590 (0.362, 0.963)   | 0.035 |
| TNM stage (II-IV)         | 1.749 (0.604, 5.065)   | 0.303 |
| Pathological grade (G3)   | 1.763 (1.042, 2.985)   | 0.035 |
| Lymph node metastasis (N1-N3) | 1.497 (0.762, 2.941) | 0.242 |
| Metastasis (M1)           | 2.507 (1.137, 5.529)   | 0.023 |
| Lauren class (diffuse)    | 0.978 (0.578, 1.653)   | 0.932 |
| COL4A4 expression (high)  | 1.033 (0.656, 1.626)   | 0.889 |

| Clinicopathologic feature | Relative risk (95% CI) | P  |
|---------------------------|------------------------|----|
| Age (<60 y)               | 0.420 (0.255, 0.692)   | 0.001 |
| Gender (female)           | 0.573 (0.352, 0.934)   | 0.025 |
| TNM stage (II-IV)         | 1.778 (0.608, 4.198)   | 0.293 |
| Pathological grade (G3)   | 1.808 (1.075, 3.043)   | 0.026 |
| Lymph node metastasis (N1-N3) | 1.386 (0.701, 2.742) | 0.242 |
| Metastasis (M1)           | 3.028 (1.345, 6.815)   | 0.007 |
| Lauren class (diffuse)    | 0.911 (0.538, 1.544)   | 0.730 |
| COL4A5 expression (high)  | 1.593 (1.022, 2.482)   | 0.040 |

| Clinicopathologic feature | Relative risk (95% CI) | P  |
|---------------------------|------------------------|----|
| Age (<60 y)               | 0.433 (0.261, 0.717)   | 0.001 |
| Gender (female)           | 0.585 (0.359, 0.953)   | 0.031 |
| TNM stage (II-IV)         | 1.785 (0.618, 5.162)   | 0.285 |
| Pathological grade (G3)   | 1.762 (1.048, 2.954)   | 0.032 |
| Lymph node metastasis (N1-N3) | 1.454 (0.740, 2.857) | 0.277 |
| Metastasis (M1)           | 2.847 (1.237, 6.554)   | 0.014 |
| Lauren class (diffuse)    | 0.988 (0.585, 1.670)   | 0.965 |
| COL4A6 expression (high)  | 1.245 (0.792, 1.957)   | 0.342 |
Figure S2 COL4A4s expressions in normal gastric tissues and gastric carcinoma specimens. Images were taken from the Human Protein Atlas (http://www.proteinatlas.org) online database. N, normal gastric tissues; T, tumor tissues.

Figure S3 Kaplan Meier analysis of the correlation of COL4A4s expression levels with the overall survival (OS) by OncoLnc tool analysis. P<0.05 was considered statistically significant.
| NO. | Gene symbol | Full name | Function |
|-----|-------------|-----------|----------|
| 1   | ACTB        | Actin beta| Cell mobility, structure, integrity, and intercellular signaling |
| 2   | ACTN2       | Actinin alpha 2 | Bind actin to the membrane, anchor the myofibrillar actin filaments |
| 3   | ACTN4       | Actinin alpha 4 | Bind actin to the membrane, anchor the myofibrillar actin filaments |
| 4   | CASP8       | Caspase 8 | Be involved in the programmed cell death induced by Fas and various apoptotic stimuli |
| 5   | COL18A1     | Collagen type XVII alpha 1 chain | Inhibit angiogenesis and tumor growth |
| 6   | COL21A1     | Collagen type XXI alpha 1 chain | Maintain the integrity of the extracellular matrix |
| 7   | COL22A1     | Collagen type XXII alpha 1 chain | Contribute to the stabilization of myoendinous junctions and strengthen skeletal muscle attachments during contractile activity |
| 8   | COL26A1     | Collagen type XXVI alpha 1 chain | Be associated with aspirin-inhibitor asthma |
| 9   | COL26A1     | Collagen type XXVI alpha 1 chain | Be associated with aspirin-inhibitor asthma |
| 10  | COL7A1      | Collagen type VII alpha 1 chain | Function as an anchoring fibril between the external epithelia and the underlying stroma |
| 11  | FBN4        | Fibulin A | Be involved in remodeling the cytoskeleton to effect changes in cell shape and migration; interact with integrins, transmembrane receptor complexes, and second messengers |
| 12  | FBN2        | Fibulin B | Repair vascular injuries |
| 13  | FN1         | Fibronectin 1 | Be involved in cell adhesion and migration processes including embryogenesis, wound healing, blood coagulation, host defense, and metastasis |
| 14  | ITGA2B      | Integrin subunit alpha 2b | Blood coagulation |
| 15  | ITGA3       | Integrin subunit alpha 3 | Form an integrin that interacts with extracellular matrix proteins including members of the laminin family; be correlated with breast cancer metastasis |
| 16  | ITGA4       | Integrin subunit alpha 4 | May play a role in cell motility and migration |
| 17  | ITGA6       | Integrin subunit alpha 6 | Function in cell surface adhesion and signaling |
| 18  | ITGA7       | Integrin subunit alpha 7 | Play a role in cell migration, morphogenetic development, differentiation, and metastasis |
| 19  | ITGA8       | Integrin subunit alpha E | Adhesion; serve as an accessory molecule for human intestinal intraepithelial lymphocytes activation |
| 20  | ITGD4L      | Integrin subunit alpha L | Leukocyte intercellular adhesion; function in lymphocyte costimulatory signaling |
| 21  | ITGD4V      | Integrin subunit alpha V | Regulate angiogenesis and cancer progression |
| 22  | ITGD4X      | Integrin subunit alpha X | Adherence of neutrophils and monocytes to stimulated endothelium cells, and phagocytosis of complement coated particles |
| 23  | ITGB4       | Integrin subunit beta 4 | Play a pivotal role in the biology of invasive carcinoma |
| 24  | ITGB5       | Integrin subunit beta 5 | Participate in cell adhesion as well as cell-surface mediated signaling |
| 25  | ITGB6       | Integrin subunit beta 7 | Play a role in leukocyte adhesion |
| 26  | ITGB8       | Integrin subunit beta 8 | Play a role in human airway epithelial proliferation |
| 27  | ITGB6L      | Integrin subunit beta like 1 | Contain integrin-like cysteine-rich repeats |
| 28  | LAMA1       | Laminin subunit alpha 1 | Cell adhesion, differentiation, migration, signaling, neurite outgrowth and metastasis |
| 29  | LAMA2       | Laminin subunit alpha 2 | Mediate the attachment, migration, and organization of cells into tissues during embryonic development |
| 30  | LAMA3       | Laminin subunit alpha 3 | Be essential for formation and function of the basement membrane and have additional functions in regulating cell migration and mechanical signal transduction |
| 31  | LAMB4       | Laminin subunit alpha 4 | The exact function of laminin, alpha 4 is not known |
| 32  | LAMA5       | Laminin subunit alpha 5 | The major noncollagenous constituent of basement membranes |
| 33  | LAMB1       | Laminin subunit beta 1 | Inhibit metastasis |
| 34  | LAMB2       | Laminin subunit beta 2 | The maturation of neuromuscular junctions and maintain glomerular filtration |
| 35  | LAMB3       | Laminin subunit beta 3 | Belong to a family of basement membrane proteins |
| 36  | LAMB4       | Laminin subunit beta 4 | Cell adhesion, differentiation, migration, signaling, neurite outgrowth and metastasis |
| 37  | LAMC1       | Laminin subunit gamma 1 | Cell adhesion, differentiation, migration, signaling, neurite outgrowth and metastasis |
| 38  | LAMC3       | Laminin subunit gamma 3 | Cell adhesion, differentiation, migration, signaling, neurite outgrowth and metastasis |
| 39  | MSR1        | Macrophage scavenger receptor 1 | Mediate the endocytosis of modified low-density lipoproteins; regulation of scavenger receptor activity in macrophages |
| 40  | P3H2        | Prolyl 3-hydroxylase 2 | Collagen chain assembly, stability and cross-linking |
| 41  | P4HB        | Prolyl 4-hydroxylase subunit beta | A highly abundant multifunctional enzyme |
| 42  | PDGFA       | Platelet derived growth factor subunit A | Be critical for the stability of intermembranous crosslinks |
| 43  | PLOD2       | Procollagen- lysine,2-oxoglutarate 5-dioxygenase 2 | Be critical for the stability of intermembranous crosslinks |
| 44  | PLOD3       | Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3 | Be critical for the stability of intermembranous crosslinks |
| 45  | PTX2        | Protein tyrosine kinase 2 | Cell growth and intracellular signal transduction pathways |
| 46  | SERPINF1    | Serpin family H member 1 | A marker for cancer |
| 47  | THBS2       | Thrombospondin 2 | A potent inhibitor of tumor growth and angiogenesis |
| 48  | THBS4       | Thrombospondin 4 | Be activated during the stromal response to invasive breast cancer; play a role in inflammatory responses in Alzheimer’s disease |
| 49  | TNK1        | Talin 1 | Assist in the attachment of adherent cells to extracellular matrices and of lymphocytes to other cells |
| 50  | VCL         | Vinculin | Anchor F-actin to the membrane |