Identification of issue inhibitor of metalloproteinase 1 (TIMP1) as a potential biomarker for the diagnosis, pathogenesis and prognosis of colorectal cancer via integrated bioinformatic analysis

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Abstract

Background: Colorectal cancer (CRC) is a common malignant tumor of the digestive system. It is crucial to screen potential biomarkers for the diagnosis, pathogenesis, and prognosis of CRC because there are limited clinical symptoms associated with this cancer. Therefore, we attempted to identify biomarkers associated with the occurrence and progression of CRC by utilizing bioinformatic analysis and to elucidate a molecular mechanism for the diagnosis and treatment of CRC.

Methods: Two independent gene expression profile datasets of colonic neoplasms (GSE44076 and GSE37182) were collected from public GEO datasets, which included 182 tumor tissues and 236 normal tissues. Next, differentially expressed genes (DEGs) between CRC colonic samples and non-CRC colonic samples were obtained via GEO2R online tools. Subsequently, hub genes were selected by several analyses of DEGs, including GO pathway enrichment analysis, KEGG pathway enrichment analysis, and PPI network analysis. Finally, the correlation between the hub genes and the occurrence of CRC was tested by harnessing survival analysis and ROC curve analysis.

Results: Sixty-one shared DEGs were screened, including 44 high-expression genes and 17 low-expression genes, in CRC samples. Four genes (MYC, TIMP1, MMP7, and COL1A1) were considered to be hub genes because they exhibited higher connectivity degree scores through PPI network analysis. More importantly, there was a significant correlation between increased expression of TIMP1 and reduced survival time in patients with colorectal cancer.

Conclusion: By using bioinformatic analysis, this study suggested that Timp-1 may represent a potential biomarker for the diagnosis and prognosis of targeted molecular therapy for CRC.

KEYWORDS: TIMP1, prognostic signature, colorectal cancer, bioinformatic analysis,

Background

Colorectal cancer (CRC) is one of the most frequently diagnosed gastrointestinal malignant
tumors worldwide. CRC was ranked third in global morbidity and second in global mortality [1]. By 2020, approximately 147,950 people in the U.S. have been diagnosed with CRC, and 53,200 people have died because of CRC [2]. The incidence rate of CRC has recently increased in Asia and is highly correlated with such lifestyle factors as obesity, lack of exercise, drinking and smoking, and poor diet [1]. At present, the clinical screening and diagnosis of colorectal cancer primarily rely on colonoscopy, stool immunochemical testing, and stool occult blood testing. The conventional therapy for colorectal cancer is surgical treatment combined with chemotherapy and radiation therapy. However, 28% of patients with colorectal cancer still have unfavorable prognoses[3,4]. Also, the hospitalization of CRC patients places considerable economic burdens on families and society. Therefore, to effectively diagnose CRC, reduce its mortality, and improve its survival time, it is urgently important to identify new biomarkers and further explore the pathogenesis of this cancer.

Microarrays have been widely utilized to identify new potential biomarkers and perform molecular diagnosis of cancers[5], while bioinformatic analysis contributes to the identification of more precise biomarkers by collecting and integrating data from multiple projects[6]. Because these two types of technologies are able to integrate and analyze vast quantities of data, it is helpful for researchers to determine the differential expression between genes, identify hub genes comprehensively and discover new clues for the pathogenesis and diagnosis of colorectal cancer [7]. The purpose of this study was to identify potential biomarkers related to the diagnosis, pathogenesis, and prognosis of colonic neoplasms by integrating DEG analysis, GO and KEGG pathway analysis, survival analysis, and ROC curve analysis.

Methods

CRC gene expression profile data acquisition

The gene expression profile data were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) with the following research terms: (“Colon Cancer” [Description] OR “Colorectal Cancer” [Description] OR “CRC” [Description] OR “Colonic Neoplasms” [Description]) AND “Homo Sapiens” [Organism] And “Expression Profile by Array” [DataSet type]. The inclusion criteria of the included datasets were as follows: (1) gene expression from CRC human colonic samples compared with corresponding adjacent normal colonic samples or healthy human colonic samples, and (2) at least 40 samples in each of the groups.

In this study, two independent gene expression profile datasets of colonic neoplasms (GSE44076 and GSE37182) were downloaded, including 182 tumor tissues and 236 normal tissues (Table 1). Ethics review was not necessary for this study because we examined published data from public GEO datasets.

Table 1. Information for the Gene Expression datasets included in this study

| Dataset     | Platform                          | Samples Size (Tumor/Normal) | Last Update Date |
|-------------|-----------------------------------|----------------------------|-----------------|
| GSE44076    | GPL13667-[HG-U219] Affymetrix     | 98/148                     | Jul 1st, 2019   |
|             | Human Genome U219 Array           |                            |                 |
| GSE37182    | GPL6947-Illumina HumanHT-12       | 84/88                      | Aug 16th        |
Data Process and Identification of DEGs
We used GEO2R online tools (https://www.ncbi.nlm.nih.gov/geo/geo2r/) to identify the differentially expressed genes (DEGs) between CRC patients’ colonic samples and normal colonic samples. The genes with adjust P value < 0.01 and |logFC (fold change)| > 2 and were regarded as significance for DEGs.

GO and KEGG pathway enrichment analysis of DEGs in CRC
To obtain an in-depth understanding of DEGs, Database for Annotation, Visualization, and Integrated Discovery online software (DAVID, Version 6.8) (https://david.ncifcrf.gov/) was employed to perform Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The biological functions of genes can be analyzed via GO analysis, including biological process (BP), cellular component (CC), and molecular function (MF), while KEGG pathway enrichment analysis reflects the interaction of molecular biology and its signaling pathways. The visual results of these two analyses were performed via ggplot2 together with the GOplot package in R software (Version 4.0.2).

PPI network analysis
Protein-protein interaction (PPI) network analysis was conducted by submitting a common high-expression and low-expression DEGs list through an online biological database called the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING: https://string-db.org/). Subsequently, Cytoscape (Version 3.8.0) was utilized to determine the relevance among DEGs. To identify hub genes, CytoHubba, a plugin in Cytoscape, was employed to calculate the connectivity degree (the number of strings among nodes) of each protein node, and hub genes were recognized if the connectivity degree score was above 10.

Survival analysis of hub genes
Patients’ overall survival (OS) of each hub gene was calculated by assessing the RNA sequencing expression of selected hub genes in the TCGA database through a website tool, Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/). This method is used for identifying biomarkers in connection with survival time. Hazard ratios (HRs) with 95% confidence intervals were calculated, and the median was selected as the group cutoff. A P-value < 0.05 was considered to be significant.

ROC curve analysis
The predictive ability of hub genes in CRC was evaluated via receiver operating characteristic (ROC) curve analysis in R software (Version 4.0.2). The values of the area under the curve (AUC) were calculated, with > 80% being regarded as significant.

Results
Identification of DEGs in CRC
A total of 182 CRC colonic tissues and 236 normal colonic tissues were collected for bioinformatic analysis in this study. A total of 513 DEGs (153 high-expression and 360 low-expression genes) were recognized from GSE44076, and 140 DEGs (106 high-expression and 34 low-expression) were extracted from GSE37182 using the GEO2R online tool according to genes with $|\log\text{FC (fold change)}| > 2$ and adjusted $P$-values < 0.01. The distribution of DEGs was depicted by heatmap (Figure 1).

Next, the shared overexpressed and downregulated DEGs between these two datasets were illustrated by Venn diagrams. Sixty-one shared DEGs were identified, including 44 overexpressed items and 17 downregulated items in CRC samples (Figure 2 and Table 2).

![Heatmap of DEFs from GSE44076 (A) and GSE37182 (B). The green color indicates genes with low expression, while the red color indicates genes with high expression. Each row represents one gene probe, and each column represents one sample.](image)

![Venn diagrams of shared high-expression and low-expression DEGs in GSE44076 and GSE37182. (A) Forty-four shared upregulated DEGs in the two datasets and (B) seventeen shared downregulated DEGs in the two datasets were analyzed.](image)

| Shared DEGs | Gene Symbol |
|-------------|-------------|
| GSE44076    |             |
| GSE37182    |             |
GO and KEGG pathway enrichment analysis of DEGs in CRC

GO and KEGG pathway enrichment analyses were performed to obtain more information regarding the functions of the DEGs (Figure 3). In addition, we uploaded an entirely enriched GO and KEGG analysis table as supplementary material. Twenty-four significantly enriched GO terms were selected on the basis of having P-values < 0.05. Among the GO terms, there were 14 terms for biological process, which were mostly associated with one-carbon metabolism, the negative regulation of the canonical Wnt signaling pathway and collagen fibril organization; 7 items for cellular components, primarily in connection with the extracellular matrix and the extracellular exosome together with the lateral plasma membrane; and 3 items for molecular function, highly involved with guanylate cyclase activator activity, carbonate dehydratase activity, and metalloendopeptidase activity.

In the KEGG pathway enrichment analysis, 5 pathways were indicated based on having P-values < 0.05. Tight junctions, leukocyte transendothelial migration, the Wnt signaling pathway, cell adhesion molecules (CAMs), and transcriptional dysregulation in cancer were the main significantly enriched pathways among all types of KEGG pathways.

| Biological Process | Gene Ratio | GO terms |
|--------------------|------------|----------|
| One-carbon metabolic process | 4.0 | COL1A1, COL1A2, TIMP1, REG1A, GDF15, TACSTD2, PLAU, CDC25B, AXIN2, ETV4, TRIB3, TESC, SOX9, MMP11, PHLDA1, NFE2L3, FOXQ1, MMP3, UBE2C, MMP12, CLDN2, TOP1MT, DACH1, CLDN1, MTHFD1L, ARID3A, TRIP13, TGFB1, NKD2, SERPINB5, SLC7A5, CDH3, ASCL2, PSAT1, LY6G6D, CTHRC1, MMP7, MYC, AHCY, ENC1, TPX2, SCD, DPEP1, LCN2. |
| Extracellular matrix organization | 2.5 | DES, KCNMA1, SRPX, CXCL12, ZG16, AQP8, CLCA4, CA1, CLDN8, CA4, GUCA2A, PYY, MYH11, GUCA2B, FHL1, VIP, MAMDC2. |

| Cellular Component | Gene Count | KEGG terms |
|--------------------|------------|------------|
| Zymogen granule membrane | 4 | Tight junctions, leukocyte transendothelial migration, the Wnt signaling pathway, cell adhesion molecules (CAMs), and transcriptional dysregulation in cancer |
Figure 3 Go and KEGG pathway of the two datasets. (A) Biological process, (B) cellular components, (C) molecular function, and (D) KEGG pathway. The Y-axis reflects the GO and KEGG pathway terms, and the X-axis reflects the gene ratio. Different colors represent the degree of significance, and a redder color indicates higher significance. The size of the node indicates gene counts.

**PPI network analysis of DEGs in CRC**

To determine the relevance between the shared regulated DEGs in CRC, we analyzed the protein-protein interaction PPI network (Figure 4). According to the figure, there were 46 nodes and 68 edges. More importantly, four genes had higher connectivity degree scores (above ten) among all 46 nodes, including MYC, TIMP1, MMP7, and COL1A1, and all of them were upregulated genes. Hence, in this study, MYC, TIMP1, MMP7, and COL1A1 were considered to be hub genes. Notably, we observed that most upregulated genes were highly connected with one another. The file of the PPI network was uploaded with the supplementary materials.

Figure 4 Highly-expression DEGs are shown in red, while the DEGs with low expression are shown in blue. The shape of the node indicates the difference in connectivity degree. MYC, TIMP1, MMP7, and COL1A1 were considered to be hub genes due to a higher level of connectivity degree.

**Survival Analysis of Hub genes**

Because survival time information of selected datasets was scarce, the survival analysis of
Four hub genes was illustrated based on the GEPIA online tool, which shows the RNA sequencing expression difference between colon adenocarcinoma (COAD) samples and normal samples from the TCGA database. As shown in Figure 5, the results showed that overexpression of TIMP1 (HR = 1.7, P = 0.036) was significantly correlated with unfavorable overall survival for CRC patients. However, upregulation of COL1A1 (HR = 1.6, P = 0.075), MMP7 (HR = 1.1, P = 0.064), and MYC (HR = 1.1, P = 0.82) did not significantly affect the overall survival rate in colon cancer patients. From this aspect, we observed the importance of TIMP1 in CRC patients, which can be considered a biomarker to predict the survival time or survival rate of colonic neoplasm patients.

Figure 5 Overall survival analysis of TIMP1 (A), COL1A1 (B), MMP7 (C), and MYC (D). A

P value < 0.05 was considered to be significant.
To determine whether TIMP1 is valuable for diagnosing CRC, we used the GSE44076 and GSE37182 datasets to perform ROC analysis. There were two gene probes (11715359_a_at and 11715360_x_at) relating to TIMP1 in GSE44076 and one gene probe (ILMN_1711566) for TIMP1 in GSE37182. The AUC of 11715359_a_at was 94.44%, the AUC of 11715360_x_at was 96.54% (Figure 6) and the AUC of ILMN_1711566 was 99.11%, all of which were above 80%. Hence, we believe that TIMP1 could be regarded as a potential biomarker to diagnose or treat CRC in clinical research.

**Figure 6** ROC curve for gene expression in the GSE44076 (A) and GSE37182 (B) datasets.

**Discussion**

Colorectal cancer (CRC) is a common type of cancer in the gastrointestinal tract that also ranks third in global morbidity and second in global mortality [1]. In recent years, even though considerable progress has been made in the screening of colorectal cancer, no significant breakthroughs have been made in the early detection of tumors. We believe that the mortality rate of CRC would be significantly reduced if most patients with this cancer were diagnosed in the early period of the disease. Hence, it is pivotal to screen for potential tumor biomarkers and to improve the tumor detection rate.

There was a significant cluster of high-expression and low-expression genes in CRC samples, as depicted in heatmaps, which indicates differences in gene expression between tumor samples and normal samples. We identified 61 shared DEGs between CRC samples and normal samples from two independent gene profiles in the GEO database, including 44 highly expressed genes and 17 genes with low expression.

The functions of DEGs were investigated through GO enrichment analysis, including biological process (BP), cellular component (CC), and molecular function (MF). One-carbon metabolism, negative regulation of the canonical Wnt signaling pathway, and collagen fibril organization were the three main processes obtained in BP. The Wnt signaling pathway can regulate cell movement, and its function is inhibited in various cancers [15], such as breast...
cancer, thyroid cancer, and colon cancer \[16\]. The Wnt signaling pathway not only can regulate the tumor microenvironment (TME) but also can be an important target for inhibiting tumor growth \[17\]. The basement membrane is located between endothelial cells and epithelial cells and is composed of collagen and noncollagen. Collagen type IV is generally present in all kinds of basement membranes, and the lack of the collagen type IV α-chain is significantly related to tumor invasion, which is a sign of malignant tumors \[18\].

The extracellular matrix, extracellular exosomes, and lateral plasma membrane are three main components of CC. The extracellular matrix (ECM) is the framework that constitutes organs and tissues. Carcinoma-associated fibroblasts (CAFs) can achieve tumor cell multiplication and migration by degrading the structure of the ECM \[8\]. Therefore, remodeling of the structure of the ECM in tumor tissues can be utilized as a potential diagnostic and therapeutic target \[9,10\].

Guanylate cyclase activator activity, carbonate dehydratase activity, and metalloendopeptidase activity are highly involved in MF. Guanylin is the most commonly described downregulated gene product in sporadic CRC \[11\], and guanylate cyclase 2C (GUCY2C), a tumor suppressor, is a transmembrane receptor expressed in the lumen of the intestinal epithelium. Activated GUCY2C catalyzes the composition of cyclic guanosine monophosphate (cGMP) and induced cascades of intestinal epithelial homeostasis \[12\], which means that the lack of GUCY2C and cGMP could cause intestinal transport dysfunction and tumorigenesis \[13\]. The development of tumors is related to their microenvironments. The rapid growth of tumors increases the pressure on vascular function and causes an insufficient supply of oxygen the tumor area. Carbonic anhydrase IX (CAIX) (a type of carbonate dehydratase protein) is highly sensitive to cellular hypoxia. After CAIX is activated, it can promote cell migration and increase tumor cell infiltration. Various types of metalloendopeptidases have high expression levels in tumor tissues and play essential roles in the distribution and metastasis of tumor cells \[14\].

Furthermore, through PPI network analysis of shared regulated DEGs, four genes with high connectivity degree scores were considered to be hub genes, namely, MYC, TIMP1, MMP7, and COL1A1. Subsequently, by constructing a survival rate curve, we observed that upregulated TIMP1 has a significant correlation with the poor survival of patients with colorectal cancer, and TIMP1 had a high predictive ability to diagnose CRC through ROC curve analysis. Thus, we believe that TIMP1 could be a potential biomarker for diagnosing and treating early-stage colon cancer in clinical research.

There are four members in the tissue inhibitor of metalloproteinase (TIMPS) family, namely, TIMP1, TIMP2, TIMP3, and TIMP4. Among these genes, TIMP-1 is located on chromosome Xp11.3-p11.23 and is found in the plasma and intercellular matrix \[19,20\]. It has been reported that compared with the expression level of TIPM-1 in healthy people, the expression of this gene is significantly increased in the plasma of patients with primary rectal cancer and colon cancer \[20,21\]. Moreover, TIMP-1 not only has a high expression level in colorectal cancer \[23-25\] but also notably high expression in lung cancer \[24\], breast cancer \[26,27\], prostate cancer \[28\] and
some other types of tumors, which indicates that TIMP-1 could be a biomarker for patients with early-stage tumors. The expression level of TIMP-1 is related to tumor TNM stage, survival rate, distant metastasis rate, and recurrence rate. Compared with other tumor markers (MMP-9, CEA, and CA199), TIMP-1 has higher diagnostic sensitivity, and this diagnostic sensitivity could be improved if the gene were employed in combination with MMP-9 or CEA [20,29].

TIMP-1 can increase the risk of tumorigenesis by promoting excessive cell proliferation and chromosomal abnormalities. The activity of tissue gelatin and the stability of collagen fibrils in the tumor matrix are also affected by TIMP-1 [30]. Furthermore, this study observed that carcinoma-associated fibroblasts (CAFs) have an important function in the process of TIMP-1 overexpression and carcinogenesis. Overexpressed TIMP-1 promotes the accumulation of CAFs and promotes the migration and growth of tumor cells. In contrast, TIMP-1 inhibitors could achieve anticancer effects by blocking the function of CAFs [31].

The upregulated TIMP-1 in humans and cells may have different functions. Insufficiency of TIMP-1 could limit the growth and metastasis of CRC in vivo [20,32], while high expression of TIMP-1 stimulates cell apoptosis in vitro. TIMP-1 interacts with CD63 and integrin β1 (ITGβ1) on the cytomembrane to induce apoptosis in human breast epithelial MCF10A cells [33,34].

Glycosylated TIMP-1 has dual-directional regulation in cancer progression and metastasis, which may not only inhibit tumor progression [35] but also stimulate tumor cell growth and deterioration [36]. In the early stages of cancer, the expression level of glycosylated TIMP-1 is proportional to the tumor growth rate, while in the late-stage malignant tumors, the upregulation of glycosylated TIMP-1 delays the growth of tumor tissues [37]. These findings indicate that the degree of glycosylated TIMP-1 can also be used as a biomarker for judging tumor staging and prognosis.

Conclusion
In this study, employing bioinformatic analysis, we found that a hub gene (TIMP-1) correlates with unfavorable prognosis in patients with colorectal cancer. However, our current research analysis is only based on data analysis, and we need to perform more clinical trials and basic research regarding TIMP-1’s influence on CRC to determine the potential mechanism of TIMP-1 in the early diagnosis, pathogenesis, and prognosis of colorectal cancer.

Supplementary Information
We uploaded the search strategy as supplementary materials: file 1.

Abbreviations
TIMP1: Issue inhibitor of metalloproteinase 1; CRC: Colorectal cancer; DEGs: Differentially expressed genes; GEO: Gene Expression Omnibus; DAVID: Database for Annotation, Visualization, and Integrated Discovery online software; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; BP: Biological process; CC: cellular component; MF:
Molecular function; PPI: Protein-protein interaction; STRING: Search Tool for the Retrieval of Interacting Genes/Proteins; OS: Overall survival; GEPIA: Gene Expression Profiling Interactive Analysis; HRs: Hazard ratios; ROC: Receiver operating characteristic; AUC: Area under the curve; CAMs: Cell adhesion molecules; COAD: Colon adenocarcinoma; TME: Tumor microenvironment; ECM: Extracellular matrix; CAFs: Carcinoma-associated fibroblasts; GUCY2C: Guanylate cyclase 2C; cGMP: cyclic guanosine monophosphate; CAIX: Carbonic anhydrase IX; TIMPS: tissue inhibitor of metalloproteinase.

Authors’ contributions
Huanga Wu is guarantor of the article; Guona Li and Mengmeng Kang contributed the study conception and design; Siyuan Sheng, Ziyi Chen provided methodology; Kunshan Li and Luyi Wu contributed formal analysis and discussion; all authors wrote original draft preparation, reviewed and edited the manuscript; Yan Huang and Muen Gu contributed supervision; all authors approved the final version of the manuscript.

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Availability of data and materials
The data sets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Ethics review was not necessary for this study because we examined published data from public GEO datasets. It is bioinformatic analysis no consent was needed from patients.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no conflicts of interest.

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