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DATABASE ANALYSIS

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Prognostic Value of Enolase Gene Family in Colon Cancer

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Background: Colorectal cancer (CRC), the most common gastrointestinal cancer, is associated with high mortality rates. Enolase is a major enzyme present in the glycolytic pathway. However, the functional significance of the enolase (ENO) gene family in the pathogenesis of CRC has been unclear.

Material/Methods: The data associated with 438 CRC patients from The Cancer Genome Atlas database were extracted for analysis. Survival analyses with Cox regression was performed to construct a prognostic signature. We investigated the processes that underlies the correlation between ENO genes and overall survival (OS) using gene set enrichment analysis (GSEA). We then developed a connectivity map to identify candidate target drugs for CRC.

Results: The multivariate survival analysis showed that low expression of *ENO2* and *ENO3* had a significant correlation with longer OS. The joint-effects survival analysis indicated that the combined low expression of *ENO2* and *ENO3* was highly correlated with favorable OS. As indicated by the gene set enrichment analysis (GSEA), the ENO gene is involved in various biological pathways and has multiple roles. Potential pharmacological targets of *ENO2* and *ENO3* were constructed as well.

Conclusions: Low expression levels of both *ENO2* and *ENO3* were linked to a positive prognosis for CRC. Both *ENO2* and *ENO3* show promise as prognostic biomarkers for colon cancer patients.

MeSH Keywords: **Colorectal Neoplasms • Phosphopyruvate Hydratase • Prognosis • Survival Analysis**

Abbreviations: **ENO** – enolase; **NSE** – neuron-specific enolase; **CRC** – colorectal cancer; **OS** – overall survival; **TCGA** – The Cancer Genome Atlas

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Background

Colorectal cancer (CRC) is the most common gastrointestinal cancer and has high mortality. In the USA, the estimated incidence and mortality of CRC rank third among all cancers [1]. The 5-year relative survival rate for colorectal cancer patients is 65%. For patients with stage I or II disease, the 5-year relative survival rates are 91% and 82%, respectively, but the 5-year survival rate is only 12% for patients with stage IV disease. Moreover, the tumor stage has a strong association with CRC prognosis, and timely diagnosis and therapy improve overall survival (OS) rates [2].

Enolase is an important enzyme in the glycolytic pathway and is ubiquitous in organisms ranging from bacteria to mammals [3]. Enolase 1 (*ENO1*, α polypeptide, nonneuronal enolase), enolase 2 (*ENO2*, γ polypeptide, neuron-specific enolase), enolase 3 (*ENO3*, β polypeptide, muscle-specific enolase), and enolase 4 are members of the enolase gene family. *ENO1* is associated with many cancers, including bladder cancer, gastric cancer, and colorectal cancer [4–6]. *ENO2,* which is also a neuron-specific enolase (NSE), is overexpressed in the serum of patients with small cell lung cancer [7]. NSE is the tumor marker of first choice for use in patients with small cell lung cancer [8]. Downregulation of *ENO3* gene expression prevents the growth of cancer cells [9]. *ENO4* is reported to be particularly important in spermatozoa [10]. However, the functional importance of *ENO4* in the pathogenesis of cancer has been unclear. In the present study we assessed clinical data and ENO genes expressions of 438 CRC patients using publicly available data from the TCGA database.

Material and Methods

Data source

The medical data as well as the *ENO* levels of CRC patients were attained from The Cancer Genome Atlas (TCGA; *https://cancergenome.nih.gov/*). We created scatter plots of expression profiles of the ENO genes in CRC as well as normal colon tissue in the TCGA database.

ENO gene family correlation analysis and bioinformatics analysis

The co-expression analysis of ENO gene pathway and protein level expression was carried out with the use of GeneMANIA (*www.genemania.org*) [11], and functional bioinformatics analysis was performed in DAVID (*david.ncifcrf.gov/tools.jsp*) [12,13].

Survival analysis

We assessed the prognosis of patients with CRC based on OS. The associations between the expression of ENO genes in CRC and the OS of patients were assessed using Kaplan-Meier analysis, log-rank test, and the Cox proportional hazards regression after adjusting for not age, sex, and TNM stage.

Joint-effects survival analysis

Joint-effects analysis was used for the combination of significant ENO genes. The groups were developed by summarizing the chosen expression of ENO genes linked to better OS, worse OS, and other.

Table 1. Demographic and clinical data for 438 colon cancer patients.

Variables	Patients (n=438)	No. of events (%)	MST (days)	HR (95% CI)	Log-rank *P*
Age (years)					
<60	122	81.1	3039	Ref.	0.398
″60	316	76.3	2535	1.223 (0.766–1.952)	
Sex					
Female	204	78.4	2990	Ref.	0.545
Male	234	76.9	2320	1.131 (0.759–1.686)	
TNM stage					
I	73	94.5	3234	Ref.	<0.001
II	167	83.8	2838	2.24 (0.781–6.421)	
III	126	75.4	2856	4.068 (1.434–11.538)	
IV	61	49.2	1114	11.291 (3.980–32.026)	
Missing	11				

MST – median survival time; HR – hazard ratio; CI – confidence interval.

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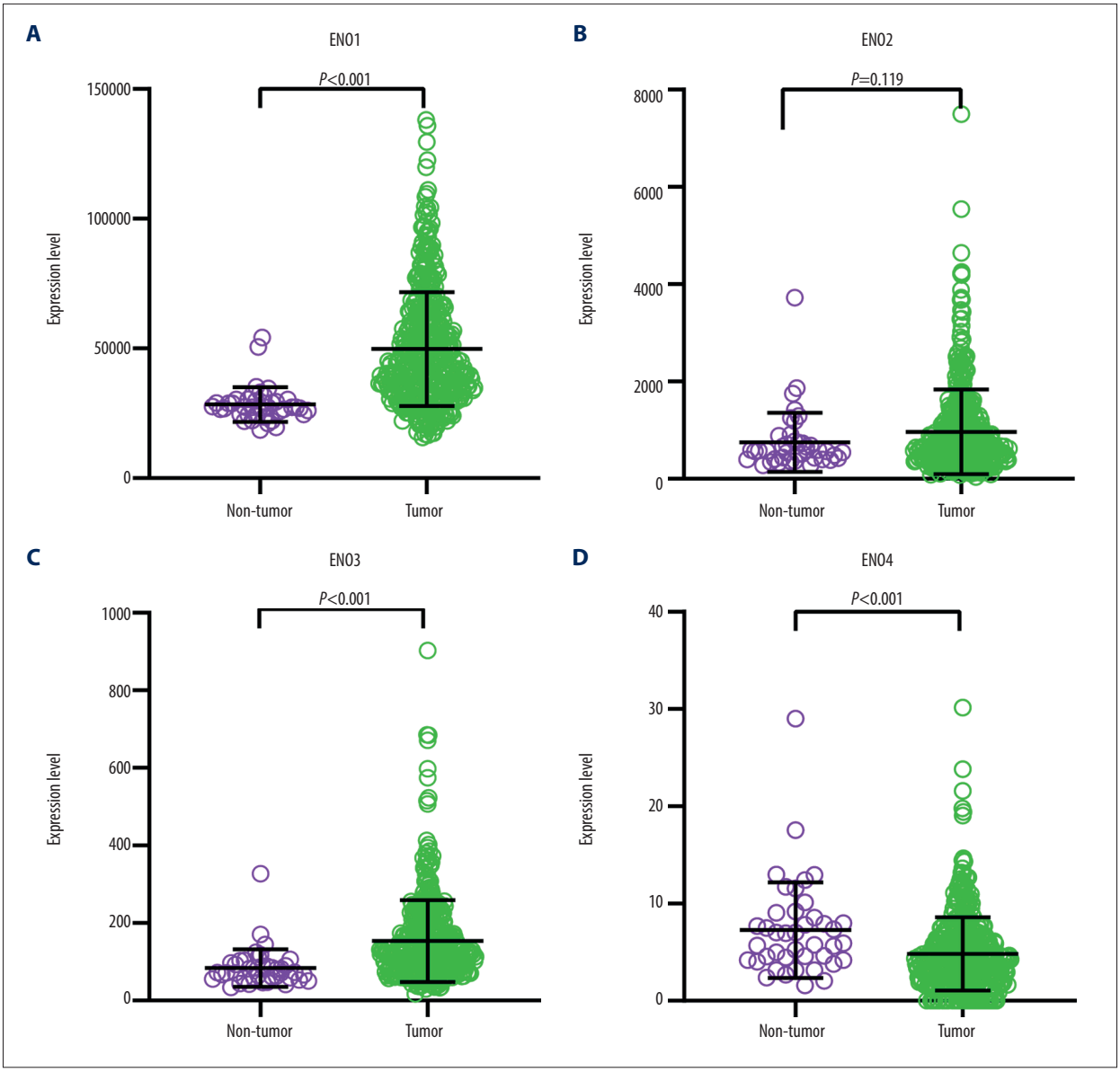

Figure 1. The scatter plots that show the expression profiles of ENO genes in CRC as well as normal colon tissue. (**A**) *ENO1*; (**B**) *ENO2*; (**C**) *ENO3*; (**D**) *ENO4*.

Prognostic risk score

We formulated a prognostic risk score for the ENO2 and ENO3 genes in CRC. We used nomograms to predict 1-, 3-, 5-, and 10-year survival to evaluate the correlation between ENO genes and OS in CRC patients [14].

Gene set enrichment analysis (GSEA)

The processes that underlie the correlation between the ENO genes and OS were investigated using GSEA. We performed biological pathway analysis in CRC with the ENO genes with the use of the reference c5 and c2 gene sets from the MSigDB, which made use of GSEA v.3.0 (*http://software.broadinstitute.org/gsea/msigdb/index.jsp*) [15]. The number of permutations was established at 1000. P<0.05 and FDR<0.25 were considered as having statistical significance.

Pharmacological targets

The genome-wide differentially expressed genes (DEGs), which include not just the upregulated but also the downregulated genes, together with the heatmaps and volcano plots, were attained with the use of edger [16]. Results with a fold change of >2 and P≤0.05 were used for analyses. Then, we chose target drugs from Connectivity Map (*https://portals.broadinstitute.org/cmap/*).

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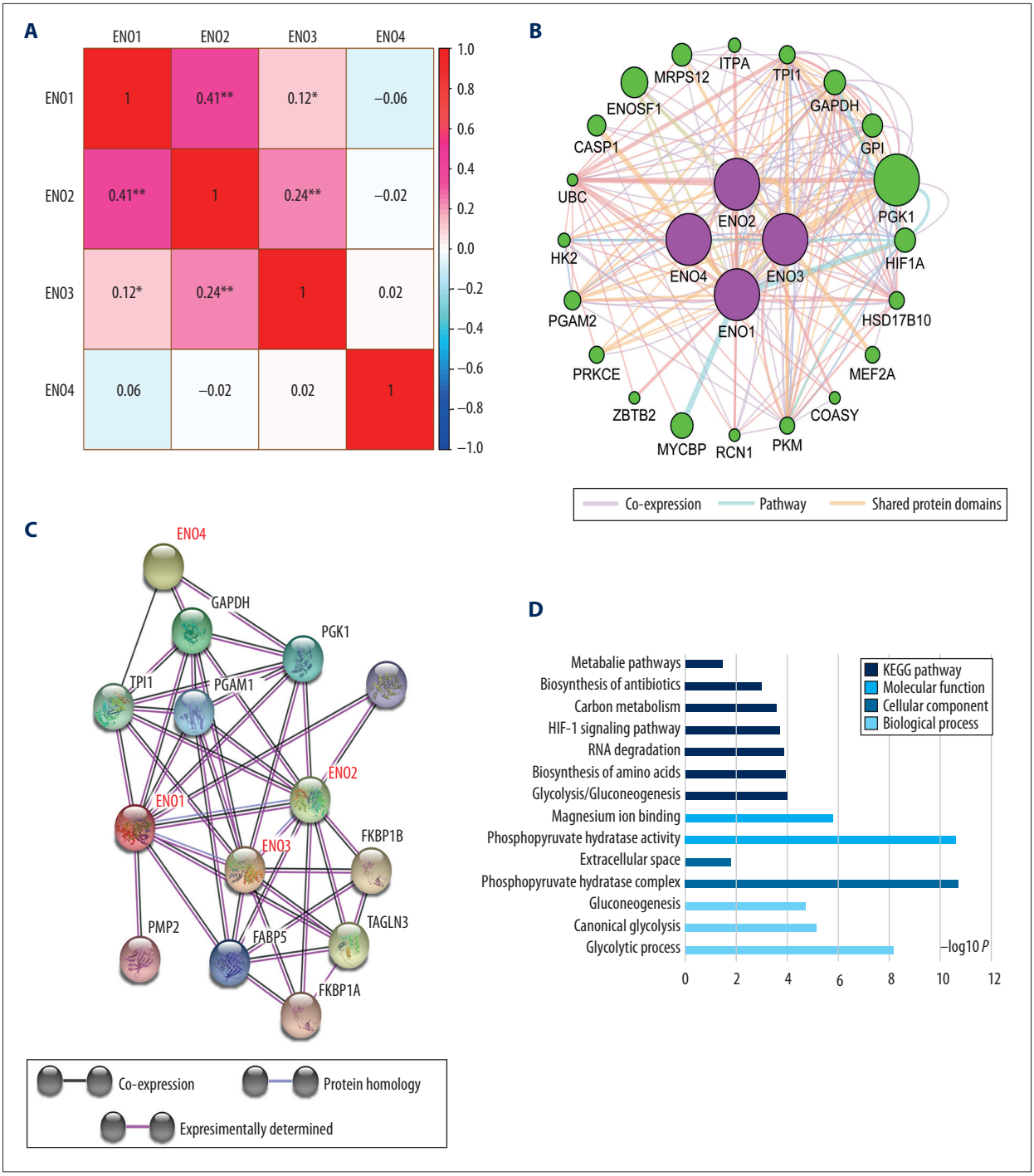

Figure 2. (**A**) Pearson's correlation coefficients for *ENO1*, *ENO2*, *ENO3* and *ENO4* expression; (**B**) The gene interaction networks among *ENO1*, *ENO2*, *ENO3* and *ENO4*; (**C**) The protein–protein interaction network of the ENO gene family; (**D**) GO pathway enrichment and KEGG pathway analysis carried out by DAVID.

In addition, the chemical compositions of related drugs were obtained from PubChem Compound (*https://www.ncbi.nlm.nih.gov/pccompound/*). The visualization of GO terms was done using DEGs with the use of BinGO. Thereafter, the enrichment analysis was carried on DEGs using DAVID.

Statistical analysis

SPSS v.25.0 software (IBM, Chicago, IL, USA) was used for statistical analyses. The calculation of OS was carried out using Kaplan-Meier analysis and log-rank test. The evaluation of the

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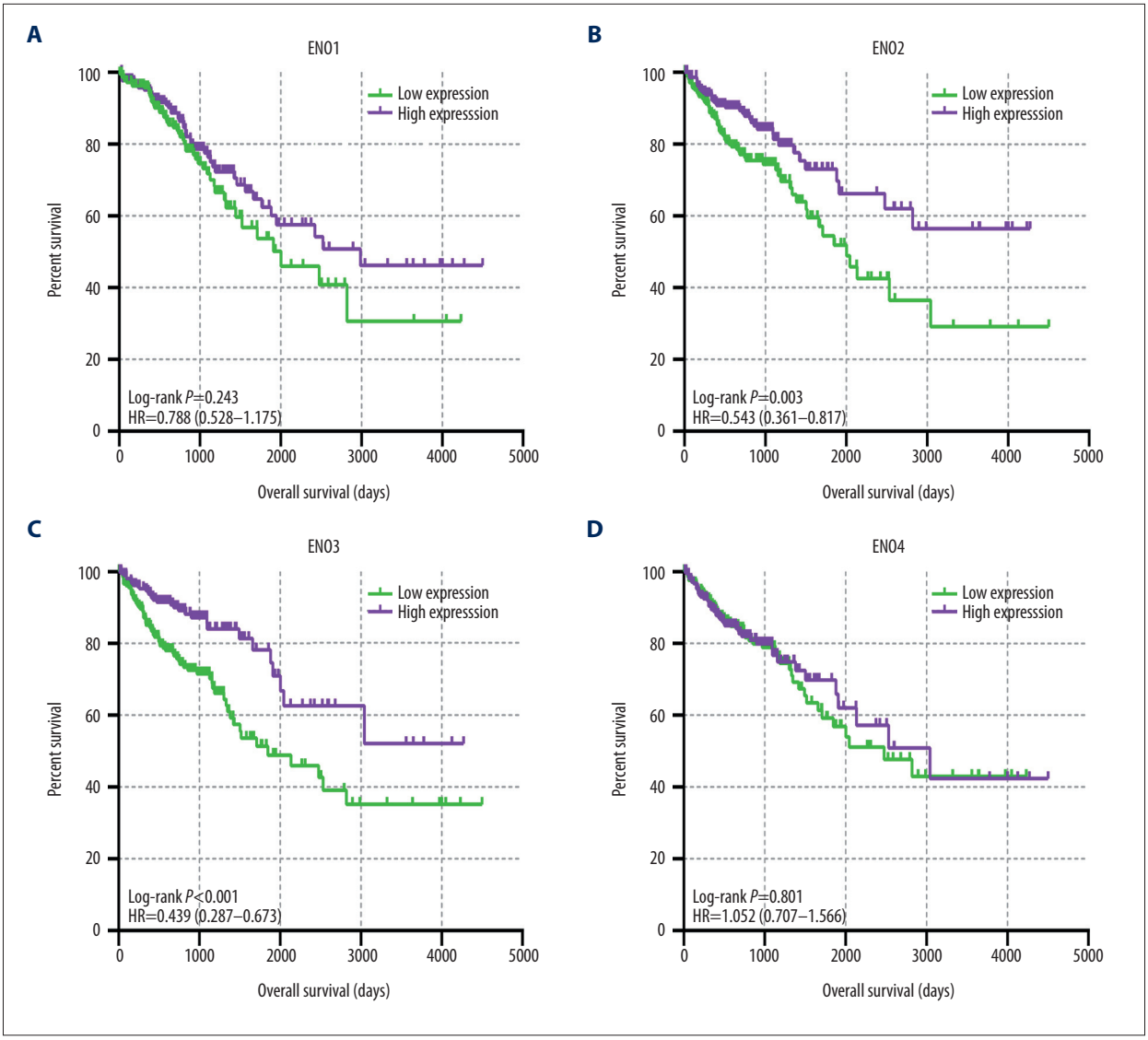

Figure 3. The prognostic significance of *ENO* expression for OS. (**A–D**) Kaplan-Meier survival curves concerning each of the colon cancer patients based on (**A**) *ENO1*, (**B**) *ENO2*, (**C**) *ENO3*, and (**D**) *ENO4* expression (n=438).

multivariate survival analysis was performed with log-rank P-values, hazard ratios (HR), and the calculation of the 95% percent confidence intervals (CIs) was done by Cox proportional hazards regression. P<0.05 was considered statistically significant.

Results

Patients' clinical features

The detailed clinical data attained from the TCGA concerning the 438 CRC patients are presented. Correlations between the clinical data and OS in the CRC patients are illustrated in Table 1 [17]. TNM stage had a significant association with OS (P<0.001; Table 1).

Scatter plots showing levels of ENO genes in CRC or normal colon tissue are shown in Figure 1. The median levels of *ENO1*, *ENO2,* and *ENO3* were higher in CRC tissue than in normal colon tissue.

ENO gene family correlation analysis and bioinformatics analysis

Associations between expression of ENO genes in CRC were assed using Pearson correlation coefficients (Figure 2A). Figure 2B shows the pathway and co-expression prediction for *ENO1*, *ENO2*, *ENO3,* and *ENO4*. ENO gene family co-expression was assessed at the protein level (Figure 2C). The biological roles of the ENO genes were assessed based on the biological process, together with the molecular function and cellular

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Table 2. Prognostic survival analysis of *ENO* family genes.

Gene expression	Patients (n=438)	No. of events (%)	MST (days)	Crude HR (95% CI)	Crude *P*	Adjusted HR (95% CI)	Adjusted *P**
ENO1							
Low	219	76.7	2309	Ref.	0.243	Ref.	0.840
High	219	78.5	2933	0.788 (0.528–1.175)		1.044 (0.689–1.580)	
ENO2							
Low	219	83.1	3016	Ref.	0.003	Ref.	0.020
High	219	72.1	2311	0.543 (0.361–0.817)		0.604 (0.395–0.923)	
ENO3							
Low	219	85.8	3033	Ref.	<0.001	Ref.	<0.001
High	219	69.4	2365	0.439 (0.287–0.673)		0.452 (0.292–0.699)	
ENO4							
Low	219	79.0	2784	Ref.	0.801	Ref.	0.685
High	219	76.3	2566	1.052 (0.707–1.566)		0.919 (0.611–1.382)	

* Adjusted for TNM stage. *ENO* – enolase; MST – median survival time; HR – hazard ratio; CI – confidence interval.

Table 3. Grouping according to *ENO* genes.

Group patients (n=438)	Composition
1 127	Low *ENO2*+low *ENO3*
2 184	Low *ENO2*+high *ENO3*
	High *ENO2*+low *ENO3*
3 127	High *ENO2*+high *ENO3*

ENO – enolase.

component in GO pathway analysis. Findings associated with the KEGG pathway analysis are demonstrated in Figure 2D.

Effect of differential ENO gene expression on survival

Figure 3 shows the major results of univariate survival analysis. Low expression levels of *ENO2* and *ENO3* were significantly correlated with enhanced OS in CRC patients (P=0.003 and P<0.001, correspondingly). TNM stage was correlated with the prognosis of CRC patients (Table 1). Furthermore, the low expression levels of *ENO2* (P=0.02) and *ENO3*(P<0.001) were associated with a longer OS (Table 2).

A joint-effects framework was constructed for the various cohorts based on the expression of *ENO2* and *ENO3* (Table 3). Low expression levels of *ENO2* and *ENO3* were significantly correlated with longer OS (P<0.001; Figure 4).

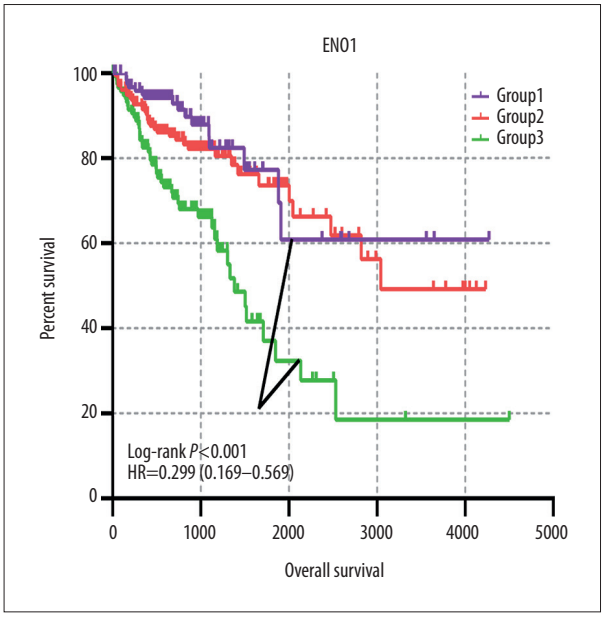

Figure 4. The joint-effects analysis of the influence of combined ENO gene expression on the OS with stratification on the basis of *ENO2* and *ENO3*.

Nomogram of CRC prognostic risk score model

The nomogram confirmed not that tumor stage and *ENO2* and *ENO3* expression in CRC predicted prognosis and contributed the majority of risk (range, 0–100 points) for poor OS. All of the variables were awarded points on the basis of Cox

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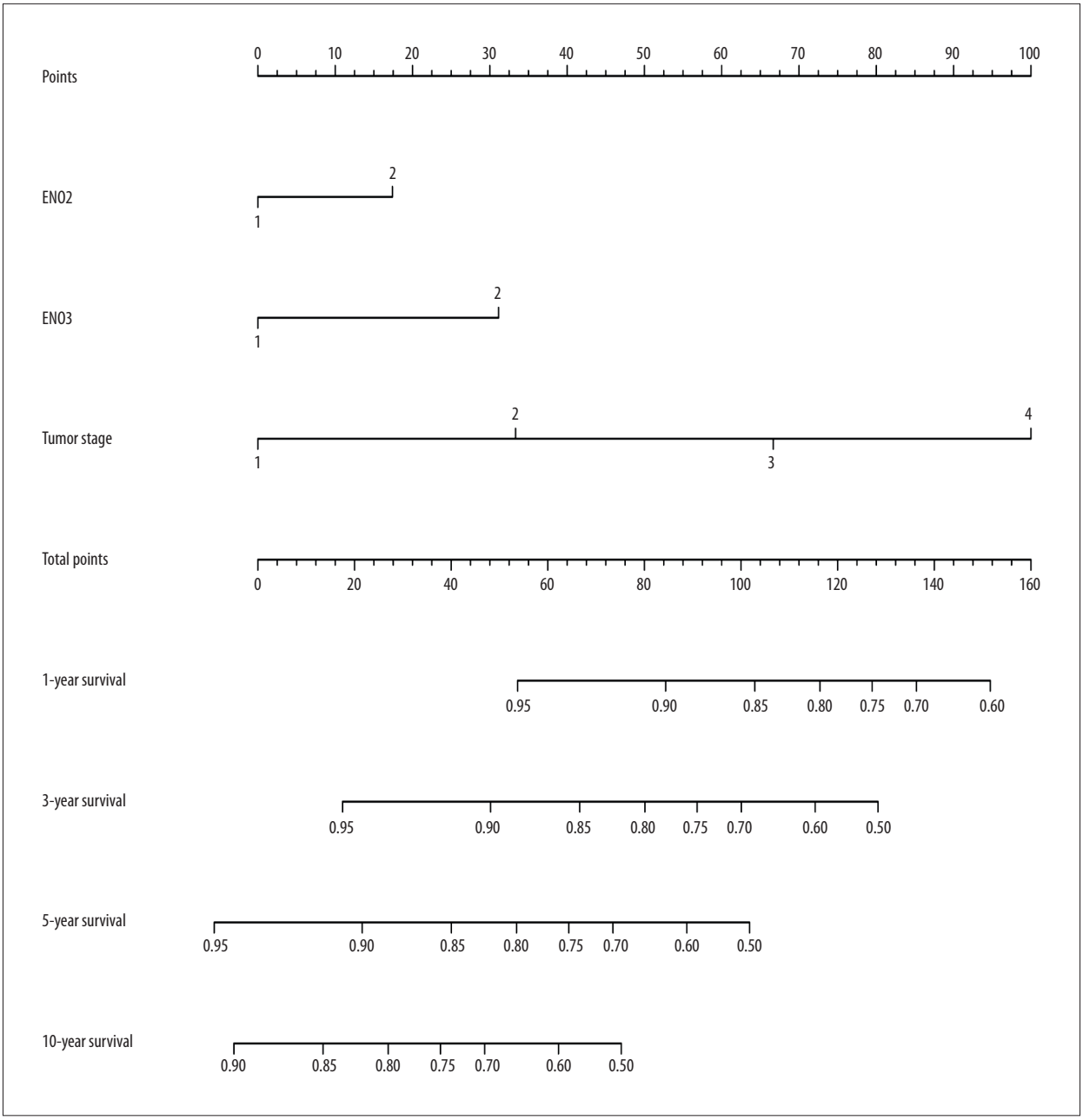

Figure 5. Nomogram for the prediction of OS in CRC with the use of *ENO2*, *ENO3,* and tumor stage.

regression coefficients. The points were totaled, and the estimation of probability of survival made by drawing a vertical line (Figure 5).

Gene set enrichment analysis (GSEA)

We performed the GSEA analysis to investigate the biological mechanisms underlying the effects of *ENO2* and *ENO3* overexpression. KEGG pathway analysis showed that overexpression of *ENO2* was positively correlated with cell adhesion (Figure 6A), focal adhesion (Figure 6B), natural killer cells (Figure 6C), MAPK signaling pathway (Figure 6D), VEGF signaling pathway (Figure 6E), and cancer pathways (Figure 6F). GO enrichment analysis showed that overexpression of *ENO2* had a positive correlation with cell adhesion (Figure 7A), as well as endothelial cell migration (Figure 7B), lymphocyte apoptotic process (Figure 7C), BMP signaling pathway (Figure 7D), ERK1 and ERK2 cascade (Figure 7E), and insulin-like growth factor receptor signaling pathway (Figure 7F).

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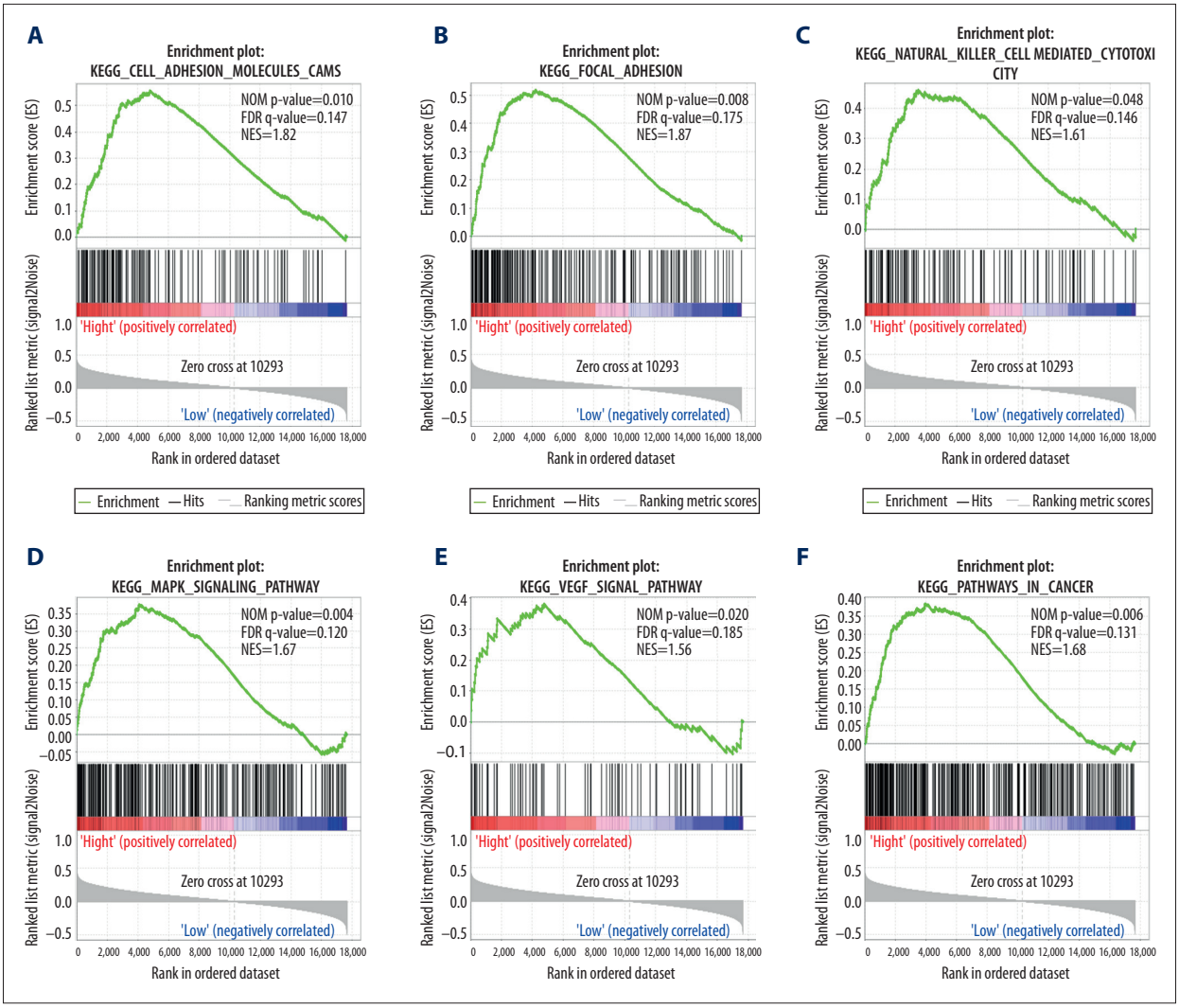

Figure 6. GSEA of *ENO2* expressed in the colon cancer patients by the KEGG pathway analysis (**A–F**).

Pharmacological targets and drugs

We obtained DEGs with the use of edgeR. Pharmacological targets and drugs were attained from the Connectivity Map using the DEGs. The negatively correlated drugs constitute the latent pharmacological targets for *ENO2* and *ENO3* (Tables 4, 5). The heatmaps and volcano plots of these DEGs are demonstrated in Supplementary Figures 1–4. Supplementary Figures 5 and 6 show the chemical composition and the 2D structure of these latent target drugs. We performed enrichment analysis of the DEGs in DAVID. Supplementary Figure 7 and 8 show GO terms visualized by BinGO.

Discussion

We used data from TCGA to investigate correlations between the ENO gene expression levels in CRC, together with developing a risk score, including the medical factors as well as the expression patterns of ENO genes for the prediction of prognosis in patients with CRC. We found that expression levels of *ENO2* and *ENO3* were higher in CRC compared to the normal colon tissue. Survival analysis suggested that low *ENO2* and *ENO3* expression levels were strongly associated with longer OS. The joint-effects analysis of these genes showed their diagnostic value was better when combined than alone. We developed a nomogram based on clinical data, and *ENO2* and *ENO3* were used for the prediction of 1-, 3-, 5-, and 10-year OS of CRC patients. In exploring the underlying molecular processes, GSEA showed that over expression of *ENO2* was positively correlated with cell adhesion, endothelial cell migration, focal adhesion, lymphocyte apoptotic process, natural killer cells, MAPK signaling pathway, VEGF signaling pathway, cancer pathways, BMP signaling pathway, ERK1 and ERK2 cascade, and insulin-like growth factor receptor signaling pathway. We assessed the pharmacological target drugs for *ENO2*

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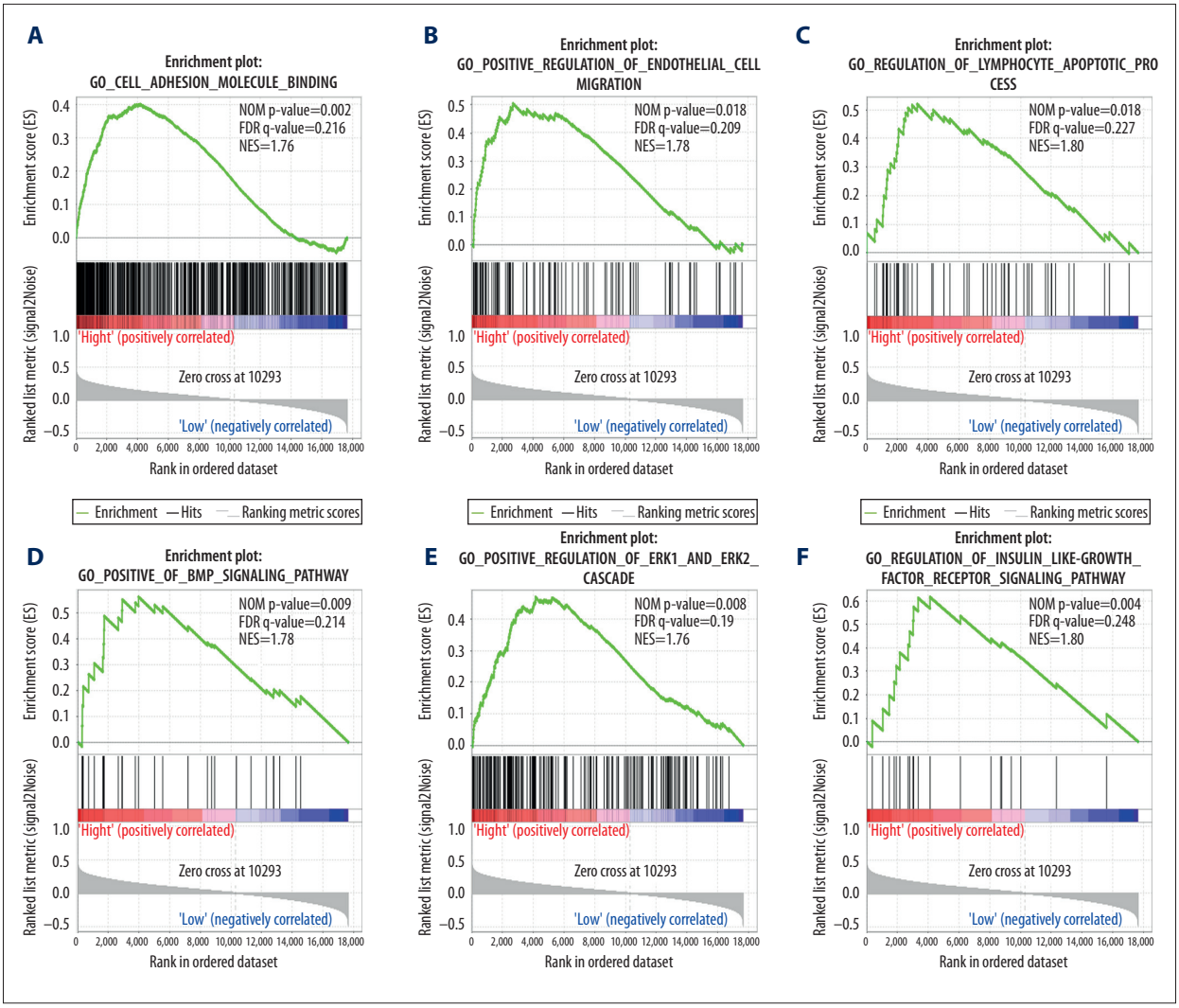

Figure 7. GSEA of *ENO2* expressed in the colon cancer patients in accordance with the GO enrichment analysis (**A–F**).

and found 10 drugs – canadine, isometheptene, amantadine, furazolidone, econazole, SR-95639A, vinburnine, Prestwick-857, quipazine, and N-acetylmuramic acid – that might be latent targets for ENO2 in CRC treatment. Pharmacological target drugs for *ENO3* were also determined, and 12 drugs – tetracycline, trimethobenzamide, cephaeline, rilmenidine, 0317956-0000, levobunolol, cefamandole, diethylstilbestrol, indoprofen, quipazine, tiaprofenic acid, and terazosin – were found that may serve as latent targets with regard to ENO3 for CRC treatment. Further research on these latent target drugs are likely to support the growth of innovative strategies to treat CRC.

Enolase was discovered in 1934 by Lohman and Mayerhof in the course of investigating the conversion of 3-phosphoglycerate to pyruvate in muscle extracts [18]. Enolase reaction has a major status in the metabolic pathway of fermentation generally, besides the glycolytic pathway, together with catalyzing the development of phosphoenolpyruvate from 2-phosphoglycerate, the second of the 2 high-power intermediates, generating the ATP in glycolysis [19. As indicated by the bioinformatics analysis of the current research work, the most evident molecular roles of *ENO* were phosphopyruvate hydratase activity, phosphopyruvate hydratase complex, and glycolytic process.

Enolase 2 (*ENO2*), which is also referred to as neuron-specific enolase (NSE), is a cell-specific isoenzyme of the glycolytic enzyme enolase, mainly expressed by mature neurons and cells of neuronal origin [8,20]. The major role of *ENO2* in cancer is accelerating glycolysis, thereby supporting the augmented tumor cell metabolic requirements and making their proliferation possible [21]. *ENO2* is a well-established tumor marker whose expression is modified in the development and progression of various cancers. *ENO2* controls neuronal survival, coupled with the differentiation and neurite regeneration by means of the activation of the PI3K/Akt, as well as the MAPK/ERK signaling pathways, resulting in downstream regulation of the molecular

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Table 4. Pharmacological targets and drug for *ENO2*.

Drug	PubChem CID	Mean	Enrichment	*P*-value
Canadine	34458	–0.332	–0.817	0.00209
Isometheptene	22297	–0.35	–0.743	0.00869
Amantadine	2130	–0.364	–0.742	0.00875
Furazolidone	5323714	–0.44	–0.735	0.00993
Econazole	3198	–0.363	–0.683	0.02206
Sr-95639a	195164	–0.384	–0.655	0.02586
Vinburnine	71203	–0.349	–0.653	0.02821
Prestwick-857	N/A*	–0.373	–0.646	0.03272
Quipazine	5011	–0.348	–0.642	0.03416
N-acetylmuramic acid	5462244	–0.4	–0.637	0.03686

N/A – not applicable. * Could not found CID in PubChem database.

Table 5. Pharmacological targets and drug for *ENO3*.

Drug	PubChem CID	Mean	Enrichment	*P*-value
Tetracycline	54675776	–0.4	–0.868	0.00008
Trimethobenzamide	5577	–0.432	–0.832	0.00032
Cephaeline	442195	–0.654	–0.794	0.00074
Rilmenidine	68712	–0.225	–0.851	0.00092
0317956-0000	N/A*	–0.311	–0.561	0.00646
Levobunolol	39468	–0.277	–0.763	0.00656
Cefamandole	456255	–0.314	–0.73	0.0107
Diethylstilbestrol	448537	–0.323	–0.61	0.01154
Indoprofen	3718	–0.412	–0.685	0.02152
Quipazine	5011	–0.321	–0.668	0.02733
Tiaprofenic acid	5468	–0.271	–0.642	0.03957
Terazosin	5401	–0.313	–0.637	0.04176

N/A – not applicable. * Could not found CID in PubChem database.

and cellular mechanisms of cytoskeleton reorganization, as well as cell remodeling, activation of transcriptional factors, and regulation of the cell cycle [22,23]. Previous research suggested that *ENO2* upregulates the glycolysis-related genes, together with enhancing the PI3K/Akt activity with the later glycogen synthase kinase3β (GSK-3β) phosphorylation, which induces cell proliferation and glycolysis in acute lymphoblastic leukemia [20]. In non-small cell lung cancer cells, an alternative splicing form of c-H-ras, $p19^{ras}$ was found to preferentially bind *ENO2* and inhibit its enzymatic activity, leading to reduced cell proliferation [24]. Furthermore, *ENO2* is overexpressed in breast epithelial cells exposed to the environmental contaminants arsenite and cadmium, strongly suggesting that the transformed cells are likely to attain the ability to express gamma-enolase to adapt to the increased metabolic requirements of a neoplastic state [25].

Similarity to other malignant neoplasms, CRC is characterized by changes in the cell signaling and metabolic pathways, including energy metabolism [26]. *ENO2* was reported to be overexpressed in CRC [27]. Moreover, *ENO2* was found to be significantly upregulated in a metastatic colon cancer cell line, which indicated a likely correlation with the metastatic mechanism *in vitro* and *in vivo* [28]. Some studies showed that a lncRNA (LOC285629) is involved in CRC pathogenesis through direct or indirect association with *ENO2* [29]. Other research indicated that *ENO2* combined with other known CRC markers can distinguish early-phase malignant colorectal tumors from benign tumors [30].

In contrast to *ENO2*, there is little information on the role of *ENO3* in cancer. Previous research demonstrated that downregulation of ENO3 gene expression and, subsequent to that,

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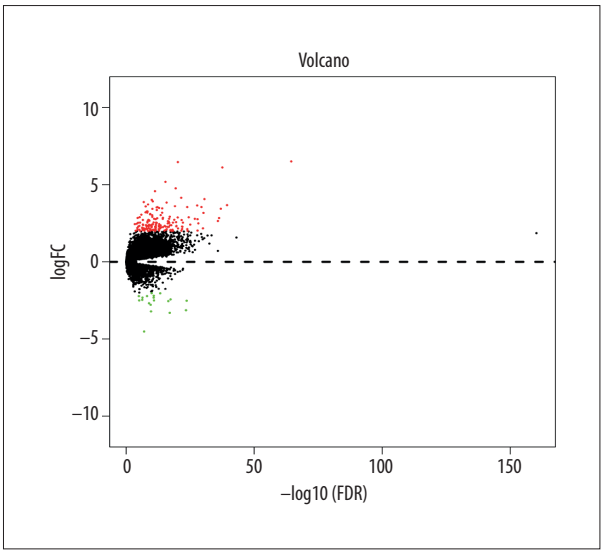
the encoded protein, are likely to inhibit the development of cancer cells [9]. The knockdown of *ENO3* expression exhibited a selective anticancer effect in STK11 mutant cells in comparison with the STK11 wild-type cells [31]. Nevertheless, some research indicated that the effect of *ENO3* varies among cancers. ENO3 protein levels were found to be lower in liver cancer tissues than in normal tissues [32].

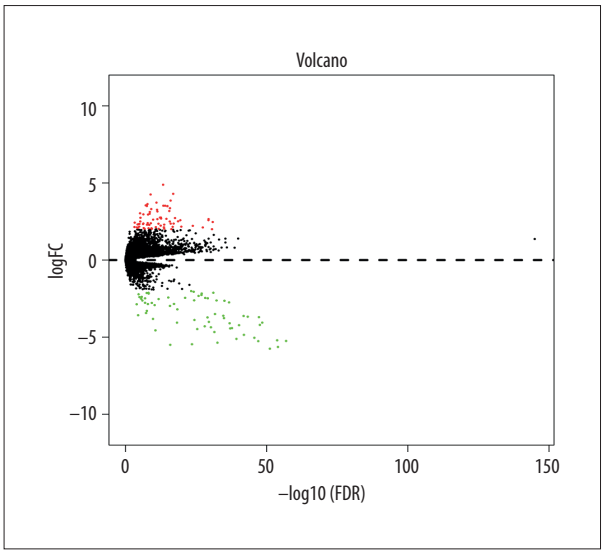
Our study has certain limitations. First, the clinical information in the public databases was not detailed. Second, the patient data were from a single source. It is imperative to validate the prognostic significance of ENO genes in CRC by independent data containing full medical information. This was a bioinformatics investigation, and the majority results were created from the public database and bioinformatics analysis, lacking confirmation by *in vitro* and *in vivo* experiments.

In spite of these constraints, this study is, to the best of our knowledge, the first to report that the downregulation of *ENO2* and *ENO3* in colon cancer is correlated with a favorable prognosis, and that *ENO2* and *ENO3* are the latent prognostic biomarkers for patients with colon cancer. Further research is warranted on these latent target drugs to support development of innovative strategies to treat CRC.

Supplementary Data



Supplementary Figure 2. Volcano plots of *ENO2* DEGs.



Supplementary Figure 4. Volcano plots of *ENO3* DEGs.

Conclusions

We found that low expression levels of *ENO2* and *ENO3,* individually and in combination, are correlated with a favorable prognosis in CRC. We also showed the various biological pathways and functions of the ENO gene, and potential pharmacological targets of ENO2 and ENO3 were constructed. Moreover, *ENO2* and *ENO3* show promise as prognostic biomarkers for patients with colon cancer.

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Conflict of interests

None.

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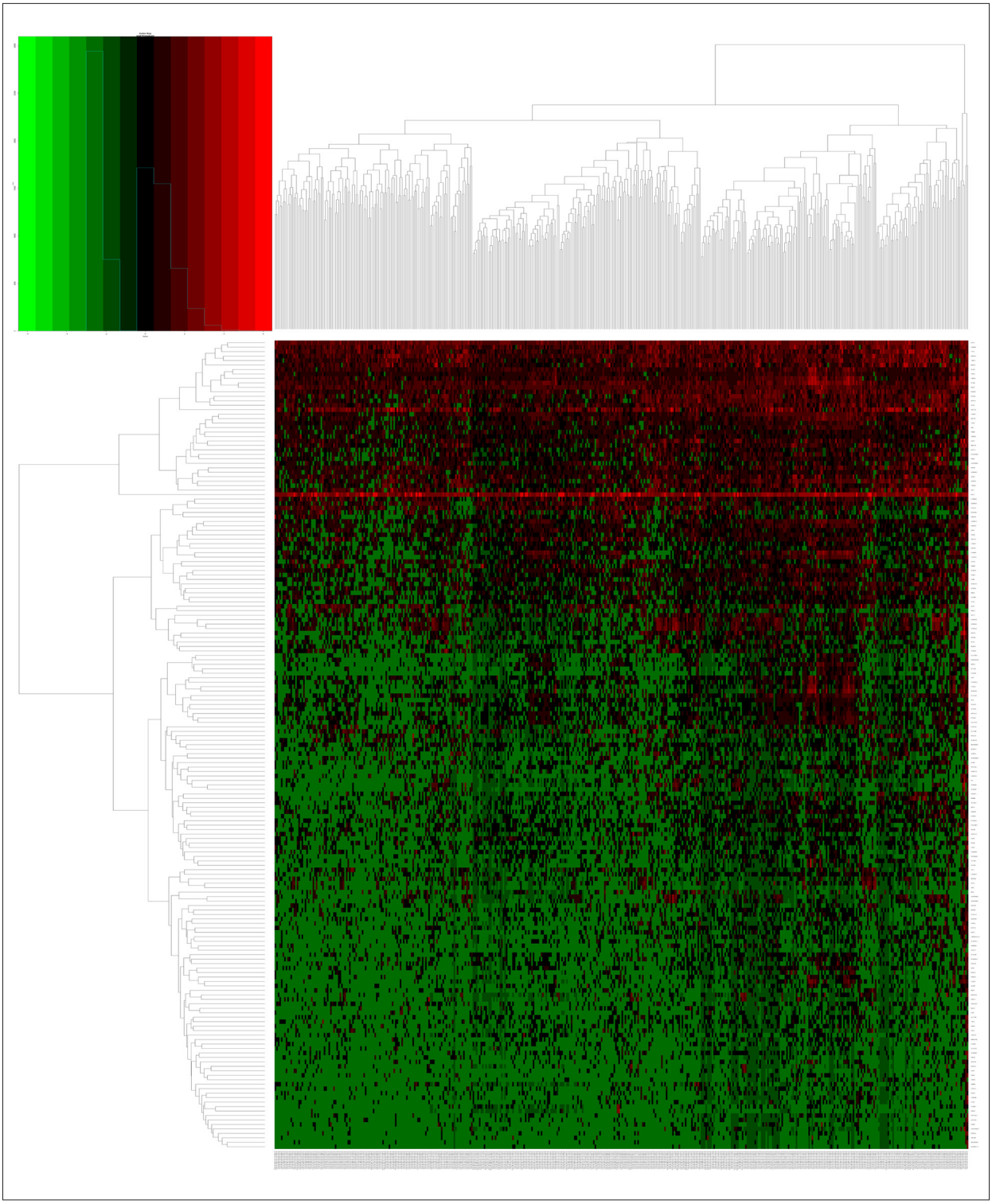

Supplementary Figure 1. Heatmaps of *ENO2* DEGs.

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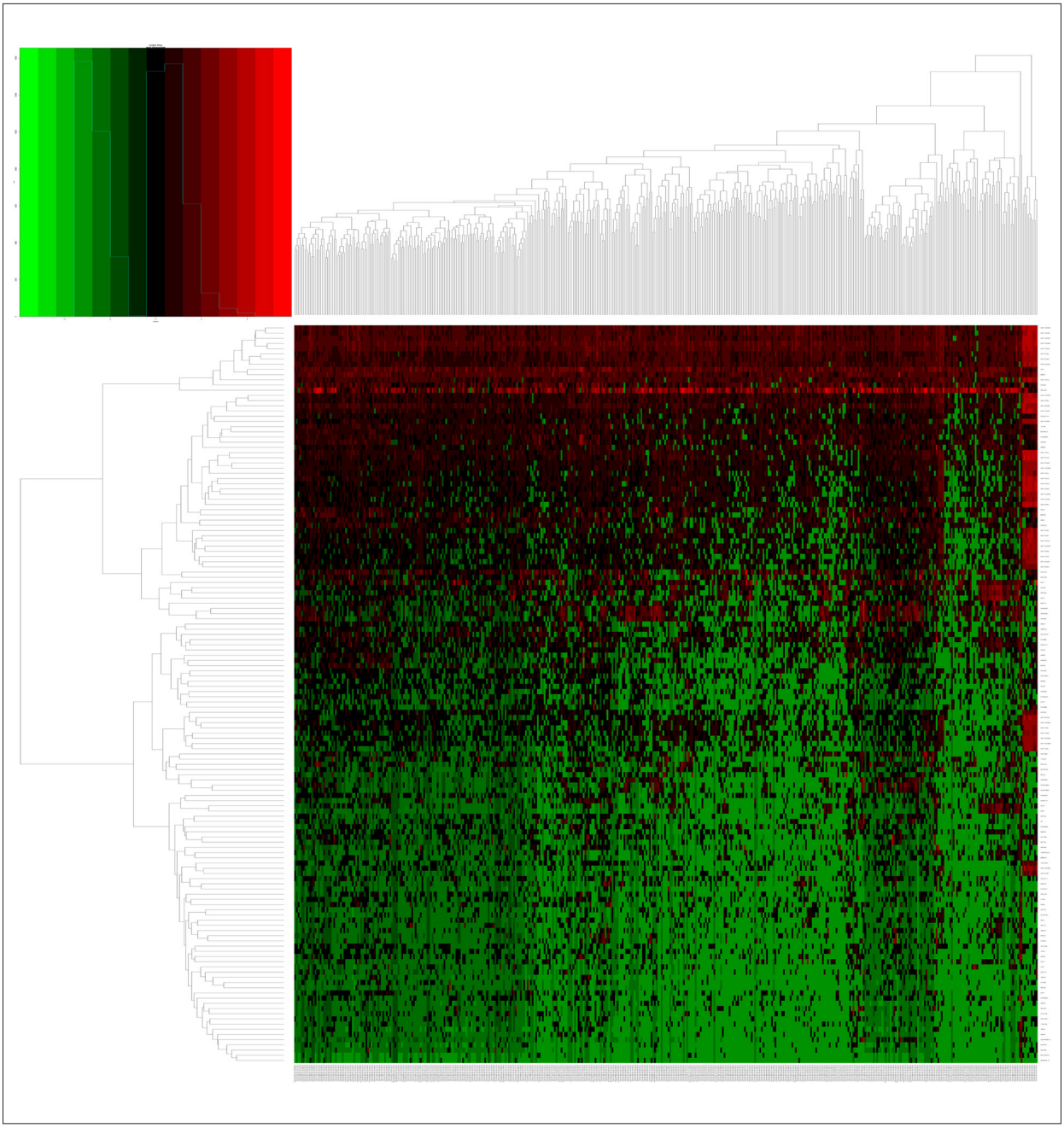

Supplementary Figure 3. Heatmaps of *ENO3* DEGs.

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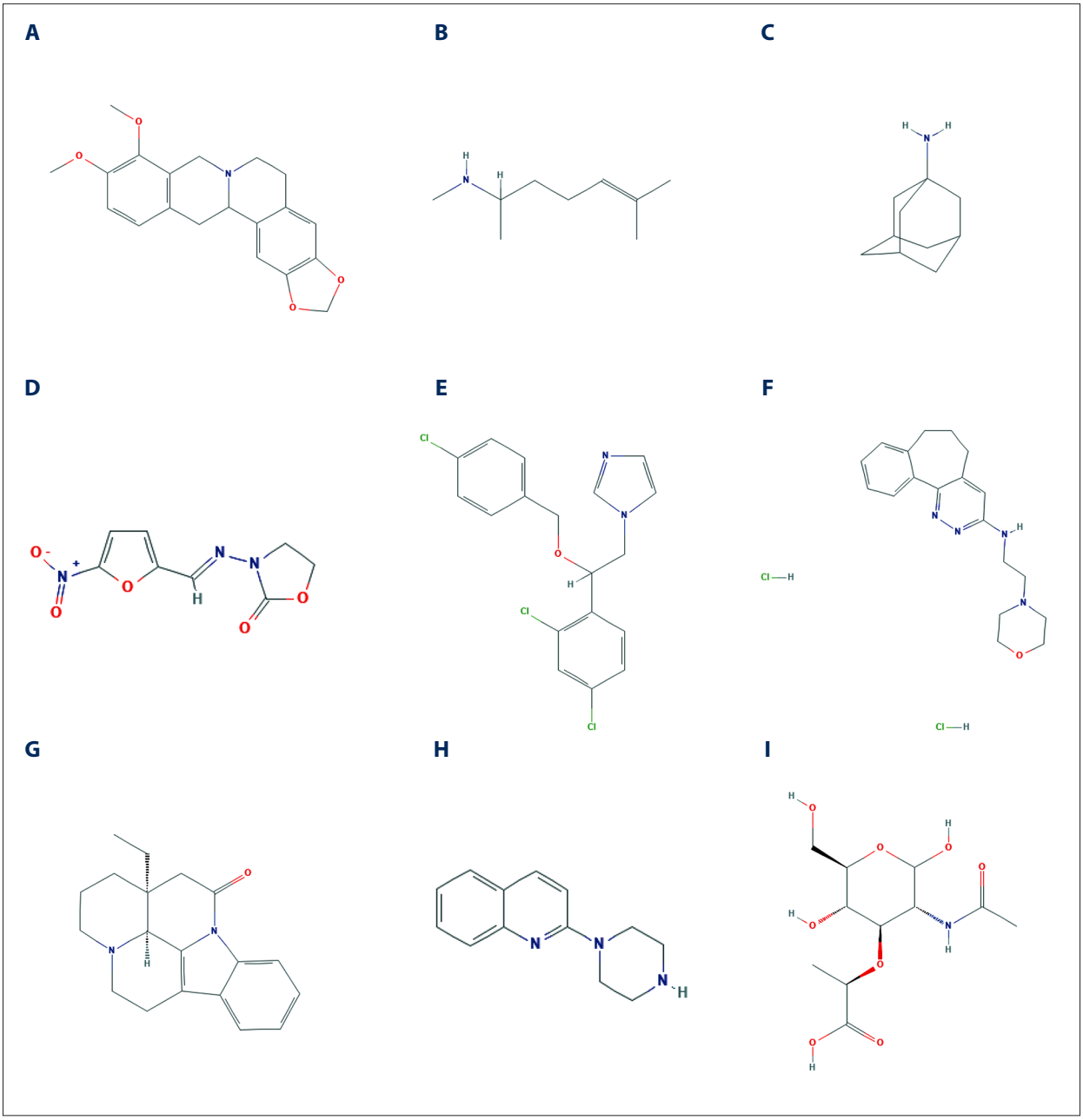

Supplementary Figure 5. The chemical composition and 2D structure of potential target drugs for *ENO2*. (**A**) Canadine; (**B**) Isometheptene; (**C**) Amantadine; (**D**) Furazolidone; (**E**) Econazole; (**F**) Sr-95639a; (**G**) Vinburnine; (**H**) Quipazine; (**I**) N-acetylmuramic acid.

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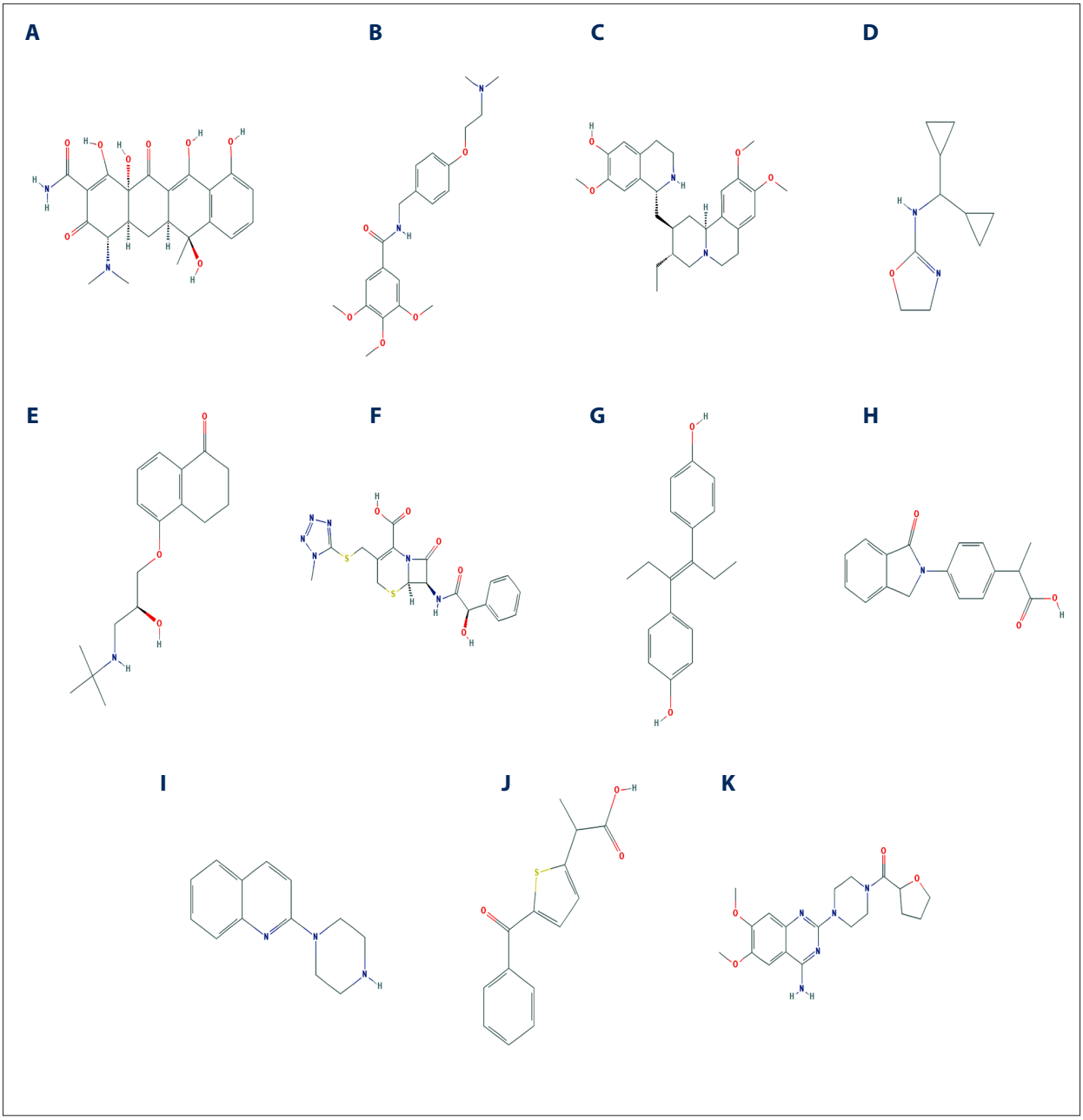

Supplementary Figure 6. The chemical composition and 2D structure of potential target drugs for *ENO3*. (**A**) Tetracycline; (**B**) Trimethobenzamide; (**C**) Cephaeline; (**D**) Rilmenidine; (**E**) Levobunolol; (**F**) Cefamandole; (**G**) Diethylstilbestrol; (**H**) Indoprofen; (**I**) Quipazine; (**J**) Tiaprofenic acid; (**K**) Terazosin.

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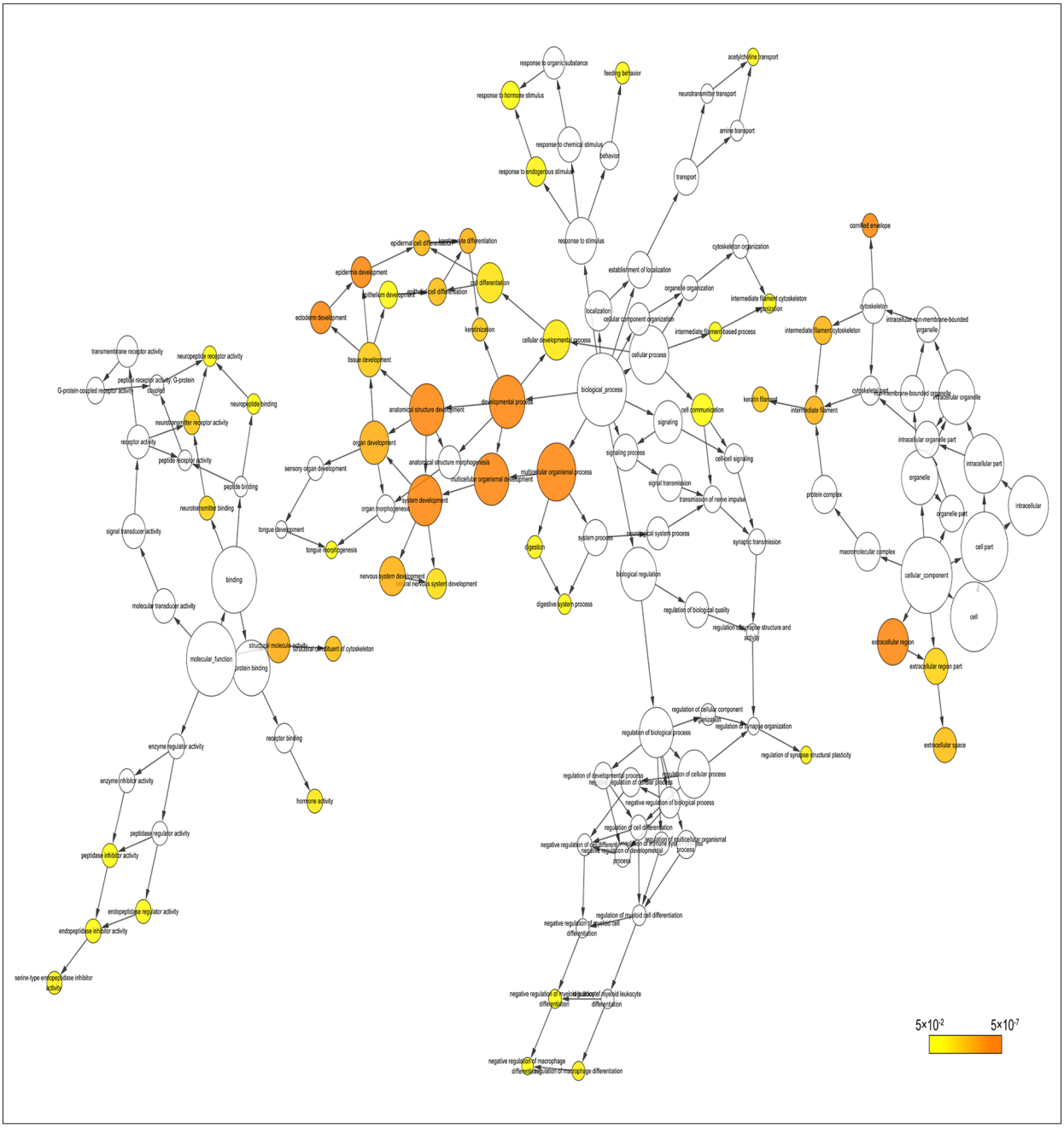

Supplementary Figure 7. The GO terms visualized by BinGO for *ENO2*.

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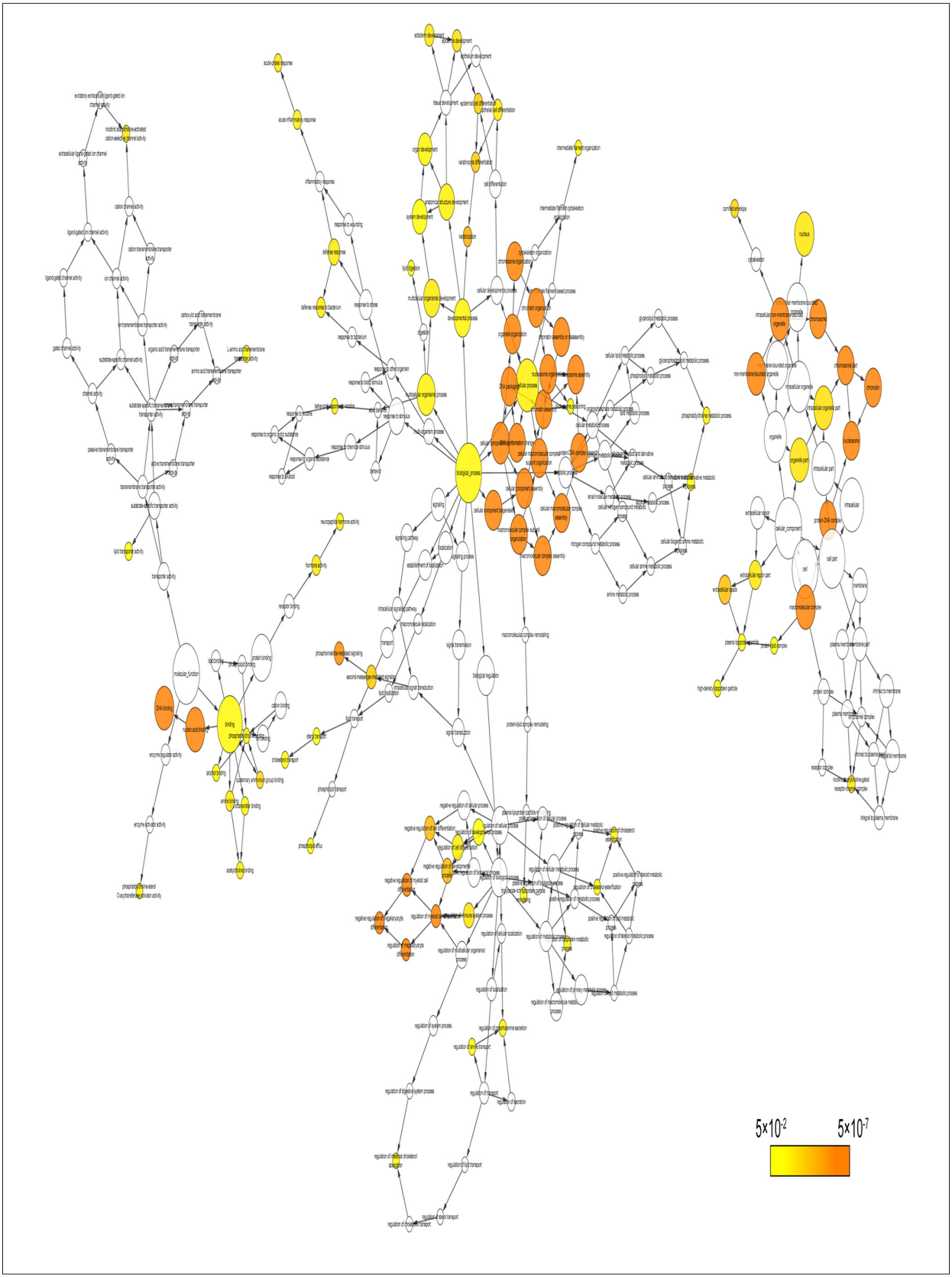

Supplementary Figure 8. The GO terms visualized by BinGO for *ENO3*.

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