

Profiles of differentially expressed genes and overexpression of NEBL indicates a positive prognosis in patients with colorectal cancer

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Abstract. The present study aimed to identify differentially expressed genes (DEGs) in colorectal cancer (CRC) and provide novel prognostic biomarkers for CRC. The microarray dataset GSE41258 was used to screen DEGs of CRC. Subsequently, a protein-protein interaction network of DEGs and Gene Ontology analysis were performed to identify hub genes and associated biological processes. Nebulette (NEBL) and complement C1q like 1 (C1QL1) were validated using reverse transcription-quantitative polymerase chain reaction in patients with CRC. Survival analysis was performed for the two hub genes. GSE41258 dataset included 182 CRC samples and 54 normal tissues. A total of 759 DEGs, including 279 upregulated and 480 downregulated were screened between both groups. NEBL and C1QL1 were identified as the two hub genes and upregulated genes involved in various biological processes, including 'regulation of biological quality' and 'response to stimulus', respectively. Additionally, the overexpression of NEBL and C1QL1 in experimental validation was consistent with the aforementioned bioinformatics analysis results. Survival analysis suggested that overexpressed NEBL in patients with CRC was associated with a positive prognosis for overall survival. In conclusion, CRC was associated with a large group of DEGs. From the upregulated genes, overexpressed NEBL in patients CRC indicated a positive prognosis

for overall survival and may be used as a prognostic biomarker for patients with CRC.

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies with an estimated 1.4 million cases and 693,900 mortalities by GLOBOCAN 2012 and a leading cause of cancer-associated mortality worldwide (1). The pathogenesis of CRC involving genetic and environmental factors, and their interactions has been investigated previously (2-4), and numerous risk factors of CRC, such as tobacco use, unhealthy diet, obesity and physical inactivity, have been identified (1). It is widely recognized that loss of genomic stability and alterations in tumor suppressor genes and oncogenes have a key role in occurrence and progression of CRC (2,5,6). Preventive measures, such as maintaining a healthy body weight, being physically active, minimizing consumption of red, processed meat and alcohol, and avoidance of smoking, screening options (fecal test, colonoscopy, stool DNA test, computed tomography), and improved treatments at an early stage of the disease are most likely attributed to reducing the CRC mortality rate observed in a large number of countries worldwide (1,7). Additionally, advances in prognostic biomarkers that may allow personalized treatments could have also contributed to improvements in overall survival of patients with CRC. In addition, various biomarkers that may be used to assist in the diagnosis of CRC have been identified (8,9). However, survival outcomes of CRC, particularly predictive and prognostic biomarkers, remain to be elucidated (10,11). Therefore, valid prognostic biomarkers are urgently required to assist the prediction of the outcomes of CRC, which would result in earlier performances of preventative interventions or surgery that would improve survival rates (12).

The advent of high-throughput transcriptomic profiling, has allowed biomarker identification to be taken to the genomic level (13,14). Additionally, it is important to understand the mRNA expression profiles involved in CRC and identify reliable biomarkers that may predict the survival of patients with CRC. The development of gene microarray and RNA sequencing has allowed for the use of gene expression

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Abbreviations: CRC, colorectal cancer; DEG, differentially expressed gene

Key words: colorectal cancer, differentially expressed genes, bioinformatics analysis, nebulette, prognosis

profiling to identify genes associated with the carcinogenesis and development of CRC (13). Therefore, screening differentially expressed genes (DEGs) based on microarray analysis is an important novel way to investigate the pathogenesis of CRC, additionally it is a quicker and more effective method of identification of the gene transcripts involved in energy metabolism in CRC and the mRNA isoforms used for diagnosis (15). Additionally, various databases, such as The Cancer Genome Atlas (TCGA) and PROGeneV2 also help identify additional, valuable information using the data mining method.

In the current study, microarray analysis combined with a protein-protein interaction network was performed to identify the DEGs associated with CRC. Additionally, nebulin (NEBL) and complement C1q like 1 (CIQL1) were identified as hub genes of interest among DEGs using bioinformatics analysis. Subsequently, NEBL and CIQL1 were validated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in 3 CRC cell lines and 30 CRC tissues with paired adjacent non-cancerous controls from patients. In order to investigate their prognostic values, the overall survival of patients with NEBL and CIQL1 in patients with CRC was determined using PROGeneV2. Overall, the present study aimed to identify prognostic biomarkers among DEGs for CRC and allow for earlier treatment strategy intervention.

Materials and methods

Data preprocessing and DEGs screening. The mRNA expression profile dataset GSE41258 was downloaded from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/), which was performed on the platform of Affymetrix Human Genome U133A Array. GSE41258 dataset contained 182 samples isolated from patients with CRC and 54 normal colorectal tissues (16). Expression profiling microarrays were firstly preprocessed by background correction and normalization, subsequently, the DEGs were statistically analyzed in both groups. All analyses were conducted using the limma package in R (17). The following thresholds were used to identify DEGs: $P < 0.01$ and $|\log_2 \text{fold-change (FC)}| > 1$.

Construction of PPI network of DEGs and identification of hub genes. Protein-protein interactions (PPIs) are indirectly reflected as reciprocal interactions among DEGs. In the present study, the online server Search Tool for the Retrieval of Interacting Genes (STRING v10.5) was used to investigate these interactions (string-db.org). Interacting DEGs were subsequently visualized using Cytoscape v3.4.0 software (18) to identify the hub genes, which were identified as highly connected genes in the interaction network if they had an interaction degree (interD) > 20 . In the present study, NEBL and CIQL1 were identified as hub genes, whose association with CRC remains unclear.

Gene Ontology (GO) analysis of NEBL and CIQL1. In order to obtain additional insight into the functional enrichment of NEBL and CIQL1, GO analysis was performed. The Database for Annotation, Visualization, and Integrated Discovery (DAVID; david.ncifcrf.gov) version 6.8 was used to investigate the relevant biological meaning of the hub genes of interest.

Table I. Characteristics of patients with colorectal cancer in microarray and validation experiments.

Characteristics	Microarray (n)	Validation (n)	P-value
Age (years)			0.074
Mean \pm SD	63.30 \pm 13.98	59.33 \pm 13.27	
Range	19-87	22-87	
Gender			0.156
Female	86	10	
Male	96	20	
AJCC stage			0.002
I	28	3	
II	48	16	
III	49	11	
IV	57	0	
TNM stage			0.518
Tumor			
T1	4	1	
T2	34	3	
T3	131	25	
T4	13	1	
Node			0.245
N0	93	18	
N1	46	9	
N2	43	3	
Metastasis			<0.01
M0	125	30	
M1	57	0	

SD, standard deviation; AJCC, American Joint Committee on Cancer; TNM, tumor-node-metastasis.

A P-value < 0.05 indicated a statistically significant functional annotation.

Cell lines and cell culture. The HCT116, HT29, DLD1 human CRC cell lines and the NCM460 normal human colorectal epithelial cell line were used in this study. All the cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). These cell lines were cultured in Dulbecco's modified Eagle's medium (GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cells were cultured at 37°C in a humidified atmosphere containing 95% O₂ and 5% CO₂.

Clinical sample collection and ethical approval. Fresh CRC tissue samples and paired adjacent non-cancerous tissue samples were obtained after surgical resection prior to radiation or chemotherapy from 30 patients at the Department of General Surgery, Zhongnan Hospital of Wuhan University (Wuhan, China) from March 2017 to June 2017. The samples were frozen and stored in liquid nitrogen. The morphological classification of the tumor was performed according to the American Joint Committee on Cancer (AJCC) staging

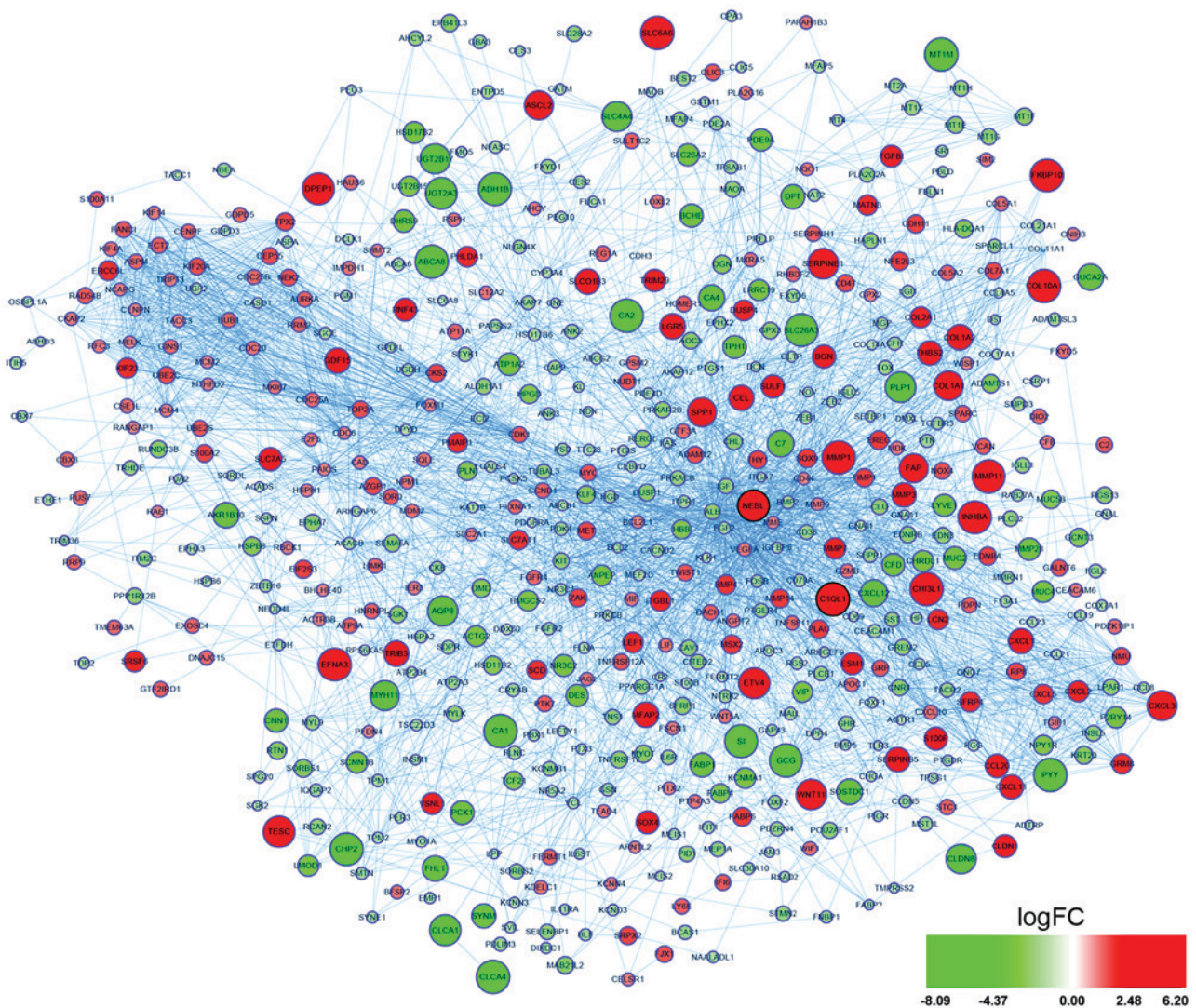


Figure 1. Protein-protein interaction network of total DEGs in colorectal cancer. Green nodes represent downregulated genes, red nodes represent upregulated genes. The size of the nodes is proportional to the expression level of DEGs. NEBL and CIQL1 (black circle outline) were the two hub genes based on highly connected degree and upregulated DEGs. DEGs, differentially expressed genes; NEBL, nebulin; CIQL1, complement C1q like 1.

system (19). Written informed consent was obtained from all patients. The study was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethical Committee of Zhongnan Hospital of Wuhan University, and with the principle of the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The sample characteristics for CRC patients are presented in Table I.

RNA extraction and RT-qPCR. Total RNA was extracted from the cells and tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA quantification was performed using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Inc.). Total RNA (1 μ g) was used with a first-strand cDNA using a synthesis kit (Thermo Fisher Scientific, Inc.) to perform the RT reaction (65°C for 5 min, 42°C for 60 min and 70°C for 5 min). The mRNA expression levels of the selected genes were subsequently evaluated by qPCR using a QuantStudio™

6 Flex Real-Time PCR instrument (Thermo Fisher Scientific, Inc.) with SYBR Premix Ex Taq™ II mix (Takara Bio, Inc., Otsu, Japan). The qPCR reaction was performed with an initial denaturation step of 95°C for 5 min followed by 40 cycles of 95°C for 3 sec and 61°C for 30 sec. The NEBL primers were forward (F) 5'-GGAATGCAAGCTGGCACTGACA-3' and reverse (R) 5'-GAGTGTCTGTGCTCACCTGCAT-3'; CIQL1 F 5'-AGTATGTGGGAGACCTCTGCA-3' and R 5'-CCA GCTTGATGAAGACCTCGTC-3'; and GAPDH F 5'-AGA AGGCTGGGGCTCATTG-3' and R 5'-GCAGGAGGCATT GCTGATGAT-3'. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method (20). GAPDH was used as the internal control.

Statistical analysis. All analyses of experimental validation were performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Student's t-test was used for comparisons between groups. Differences between the CRC cell lines and control cell line were analyzed using one-way analysis of variance with a Bonferroni correction. $P < 0.05$ was considered to indicate

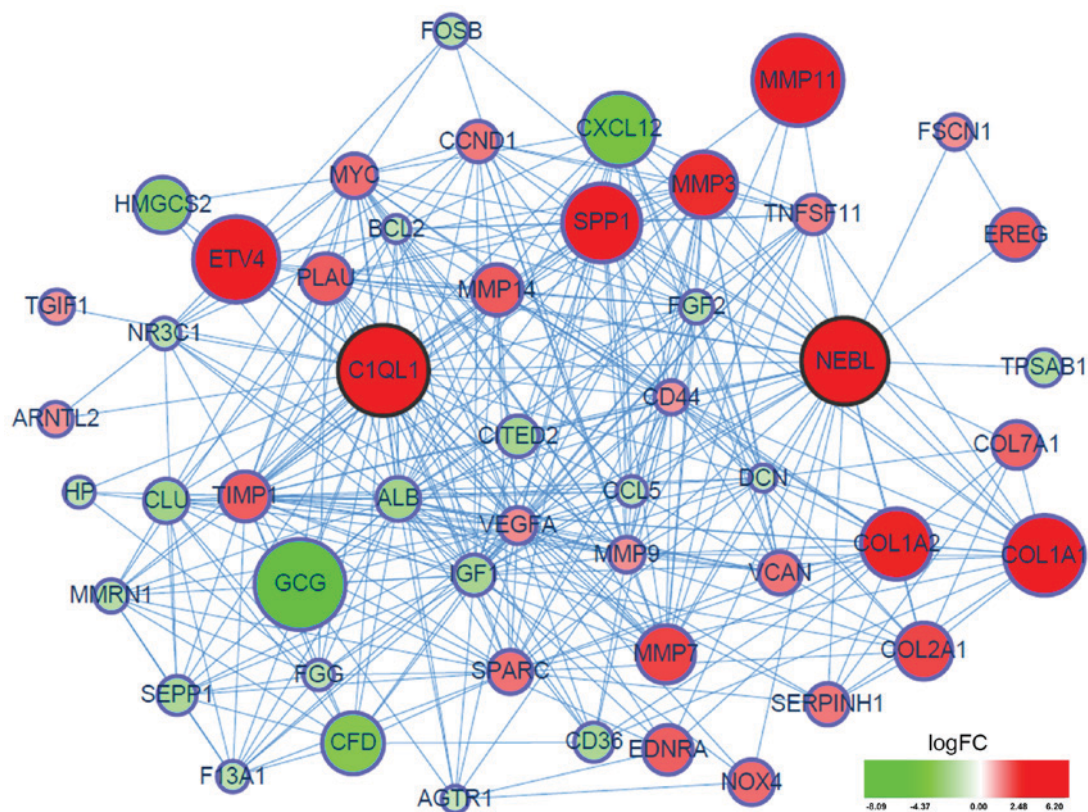


Figure 2. Protein-protein interaction network of the two hub genes (NEBL and C1QL1) and linker genes in colorectal cancer. Green nodes represent down-regulated genes, red nodes represent up-regulated genes. The size of the nodes is proportional to the expression level. NEBL, nebulin; C1QL1, complement C1q like 1.

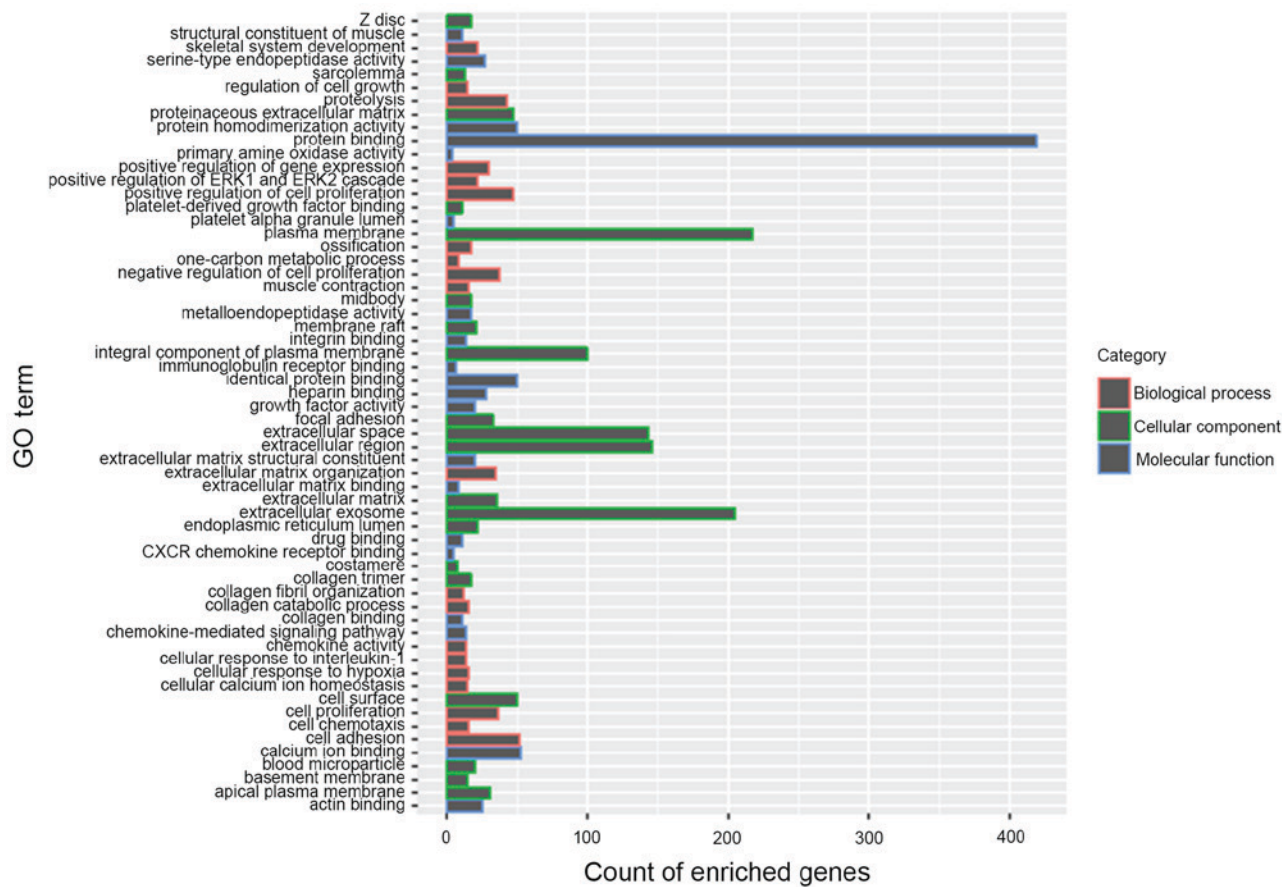


Figure 3. Gene Ontology analysis of differentially expressed genes.

Table II. Hub genes with their representative significantly enriched terms of GO biological processes.

A, NEBL		
GO ID	Description	P-value
65008	Regulation of biological quality	5.79×10^{-9}
51128	Regulation of cellular component organization	1.57×10^{-5}
65007	Biological regulation	1.37×10^{-4}
90066	Regulation of anatomical structure size	4.28×10^{-4}
33043	Regulation of organelle organization	4.98×10^{-4}
50789	Regulation of biological process	3.71×10^{-3}
B, C1QL1		
GO ID	Description	P-value
50896	Response to stimulus	2.74×10^{-7}
7626	Locomotory behavior	1.56×10^{-6}
7610	Behavior	1.92×10^{-6}

NEBL, nebullette; C1QL1, complement C1q like 1.

a statistically significant difference. Data are presented as mean \pm standard deviation. Experiments were repeated independently at least three times.

Survival analysis of NEBL and C1QL1. The median value of each hub gene in tumor samples was calculated. Samples with expression higher than the median value were placed in the high expression group, and the samples with expression lower than the median value were placed in the low expression group. Survival analysis was performed using PROGeneV2 tool for both groups (21), which allowed the investigation of prognostic implications of gene expression associated with CRC in the corresponding microarray datasets. The GSE41258 dataset was used to predict the overall survival of the hub genes of interest in the present study. In addition, survival models were adjusted for multiple covariates such as age, gender and cancer stages. The survival curves were visualized using Kaplan-Meier plots for the high and low expression groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

DEGs screening and identification of hub genes. A total of 759 DEGs were identified, including 279 upregulated and 480 downregulated DEGs in primary CRC samples compared with normal colon samples (Fig. 1). By constructing PPI network of DEGs, NEBL and C1QL1 were identified as the two hub genes based on highly connected degree (interD= 34 and 33, respectively) as well as being overexpressed DEGs in the GSE41258 dataset (Fig. 1). Additionally, a PPI network of the two hub genes and their linker genes in CRC was presented in Fig. 2.

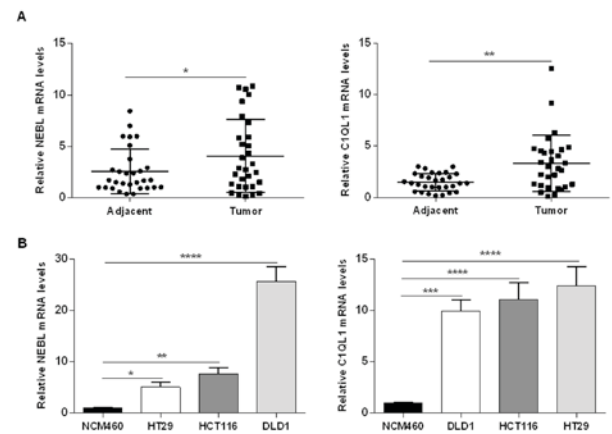


Figure 4. mRNA expression of NEBL and C1QL1 in CRC samples. (A) Relative mRNA levels of NEBL and C1QL1 in 30 CRC patient tissues. (B) Expression levels in the 3 CRC cell lines were detected by RT-qPCR. Each experiment was repeated independently at least three times. Data were presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. adjacent/NCM460 control group. CRC, colorectal cancer; NEBL, nebullette; C1QL1, complement C1q like 1.

GO analysis of NEBL and C1QL1. Functional enrichment map of DEGs, including NEBL and C1QL1 was presented in Fig. 3, which represents a holistic view of the functions of all screened DEGs. In addition, under the threshold of $P < 0.05$, the biological processes NEBL was significantly enriched in were 'regulation of biological quality', 'regulation of cellular component organization', 'biological regulation', 'regulation of anatomical structure size', 'regulation of organelle organization' and 'regulation of biological process'. The biological processes C1QL1 was enriched in were 'response to stimulus', 'locomotory behavior' and 'behavior' (Table II).

Validation of NEBL and C1QL1 mRNA expression. The mRNA expression levels of NEBL and C1QL1 in the present study were confirmed in the tissues of patients with CRC and CRC cell lines using RT-qPCR. The mRNA expression levels of NEBL and C1QL1 were higher in CRC tissues when compared with normal adjacent tissues (NEBL, $P = 0.049$; C1QL1, $P = 0.001$; Fig. 4A). NEBL and C1QL1 expression levels were upregulated in the 3 CRC cell lines when compared with NCM460 cell line (Fig. 4B).

Prognosis of NEBL and C1QL1. Survival analysis was performed for NEBL and C1QL1 using PROGeneV2 based on the GSE41258 dataset. In the overall dataset, 91 samples were demonstrated to exhibit a high expression of NEBL, and a further 91 samples were demonstrated to exhibit a low expression of NEBL. During >16 years of follow up, the incidence of death in high and low expression groups was 42 and 60 events, respectively. The median survival of high and low expression of NEBL were 3,600 and 2,580 days, respectively, which indicated that the overexpression of NEBL in patients with CRC was significantly associated with a positive prognosis [$P = 0.019$; hazard ratio (HR), 0.75; 95% confidence interval (CI), 0.59-0.95; Fig. 5A]. Additionally, the prognosis of NEBL was associated with age and gender (Table III). However, the expression of C1QL1 was not associated with the prognosis of CRC ($P = 0.504$; HR, 1.6; 95% CI, 0.41-6.29; Fig. 5B).

Table III. Overexpression of NEBL and C1QL1 associated with the prognosis of colorectal cancer using PROGgene version 2.0 and GSE41258 dataset.

Survival models ^a	NEBL	P-value	C1QL1	P-value
Overall	0.75 (0.59-0.95)	0.02	1.60 (0.41-6.29)	0.50
After adjustment for age	0.75 (0.59-0.95)	0.02	1.91 (0.47-7.69)	0.36
After adjustment for gender	0.76 (0.59-0.97)	0.03	1.32 (0.33-5.24)	0.70
After adjustment for clinical stage	1.08 (0.84-1.38)	0.54	0.65 (0.15-2.92)	0.58
After adjustment for TMN stage	0.87 (0.68-1.12)	0.29	0.96 (0.18-4.99)	0.96

^aSurvival models may be adjusted for corresponding covariates. NEBL, nebullette; C1QL1, complement C1q like 1.

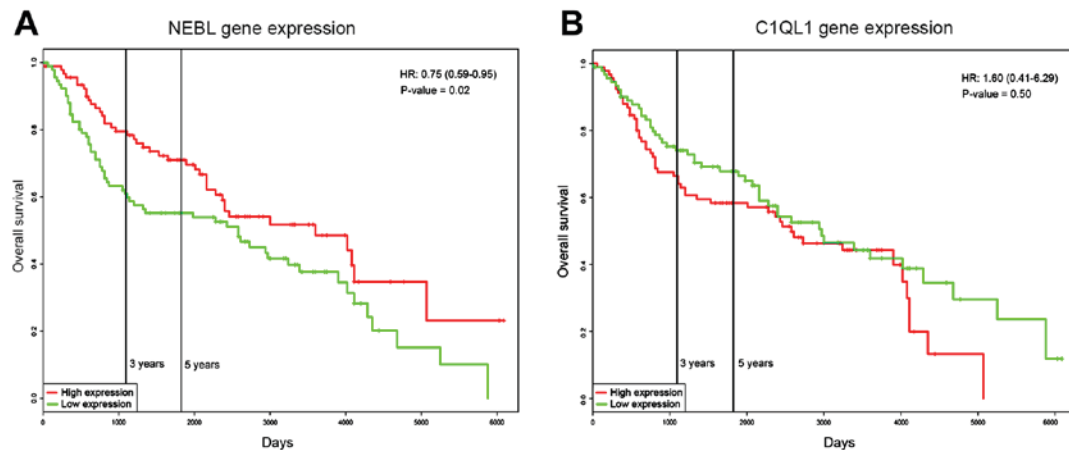


Figure 5. Kaplan-Meier survival plots for the expression of NEBL and C1QL1 in the overall survival of CRC patients. (A) Kaplan-Meier survival curve indicates the higher expression of NEBL is associated with longer the overall survival of patients with CRC. (B) Kaplan-Meier survival curve indicates that the expression of C1QL1 is not associated with the overall survival of patients with CRC patients. CRC, colorectal cancer; NEBL, nebullette; C1QL1, complement C1q like 1.

Discussion

CRC is one of the most frequent malignant tumors worldwide (1). The overall survival of patients with CRC remains poor, primarily due to the late diagnosis and metastasis (22). There are a few prognostic biomarkers identified at present that may be applied successfully in clinical practice; therefore, it is essential to identify novel potential biomarkers of prognosis in patients with CRC in order to facilitate early intervention.

The NEBL gene was previously reported to be highly expressed in cardiac muscle, regulating the attachment and migration of extracellular matrix (23). It was primarily identified as a novel mixed lineage leukemia fusion partner gene in an infant that suffered from acute myeloid leukemia (24). Additionally, NEBL has previously been demonstrated to have a significant effect on the regulation of cardiac function (25), concurrently its mutation may lead to various cardiomyopathies (26). NEBL was also identified to be associated with foot processes in podocyte injury and osteogenic abilities (27,28). The aforementioned validated biological processes of NEBL were in accordance with the findings of the GO analysis performed in the present study. However, information regarding the expression and role of NEBL in tumors, particularly in CRC is insufficient. The present study revealed that NEBL was highly expressed in CRC tissues and cell lines. It is of note that the present study observed that higher NEBL expression

was associated with improved patient prognosis. A prognostic biomarker refers to a measurable and quantifiable method that is closely associated with clinical outcomes in the certain context of patients. Therefore, the findings of the current study suggested that NEBL overexpression may be a prognostic biomarker for CRC. However, although NEBL was highly expressed in CRC, its effect on clinical phenotypes of CRC remains to be elucidated. Instead of acting as an oncogene, it may have an important role in combating oncogenes involved in CRC progression, and once a tumor develops it may be overexpressed in patients with CRC, thus contributing to an improved prognosis for CRC.

The C1QL1 gene was originally cloned as a senescence-associated gene that was highly expressed in the brain, which may also have a role in neuronal differentiation (29). It has been previously demonstrated that C1QL1 specifically binds to its receptor, the adhesion G protein-coupled receptor 3, controlling the stereotyped pattern of connectivity and regulating maturation of synapses between climbing fibers and Purkinje cells (30,31). However, its expression and role in CRC have remain to be investigated. To the best of our knowledge the present study was the first to demonstrate that C1QL1 was upregulated in CRC; however, it was unable to identify a significant correlation between C1QL1 expression and the prognosis of patients with CRC.

NEBL and C1QL1 have been investigated in various other diseases in the recent years (25,26,31). The present study

identified NEBL and C1QL1 to be involved in CRC using microarray analysis, where both DEGs were overexpressed in CRC tissues. Additionally, the experimental findings revealed were consistent among CRC cell lines and tissues and confirmed the outcomes of bioinformatics analysis. However, several limitations of the present study should be mentioned. Firstly, a larger sample size of CRC tissues with survival time are required in order to confirm the current conclusions. In addition, the biological function of the two hub genes remains unknown, and the relationship between them and the tumor malignant phenotype should be evaluated in future studies.

In conclusion, numerous DEGs including NEBL and C1QL1 were identified in CRC tissues, NEBL was overexpressed in CRC tissues and may be used as a prognostic biomarker for patients with CRC.

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