

Downregulation of B3GNT6 Predicts Poor Outcome in Colorectal Cancer Patients

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Abstract

Background: B3GNT6 encodes the core 3 synthase in O-glycan biosynthesis. It is commonly expressed in the GI tract, while its clinical significance in colorectal cancer remains largely unknown.

Methods: We gathered mRNA transcriptomic sequencing data from 3 Gene Expression Omnibus (GEO) datasets (GSE37182, GSE39582, GSE103512) and The Cancer Genome Atlas (TCGA) to compare the B3GNT6 mRNA level between colorectal cancer tissues and normal tissues and to evaluate its value as a prognostic marker. We further validated this in protein level using online database Human Protein Atlas and with immunohistochemical staining of B3GNT6 with our own cohort.

Results: B3GNT6 expression was downregulated in colorectal cancer tissue compared with that in normal tissue in both mRNA and in protein level. Downregulation of B3GNT6 was associated with poor overall survival of colorectal cancer in GSE39582 and in TCGA database. Low B3GNT6 mRNA level was significantly associated with chromosome stable (CIN negative) and KRAS mutated group colorectal cancer patient. GSEA revealed that low B3GNT6 level in colorectal cancer is associated with upregulated proteasome activity.

Conclusions: Downregulated B3GNT6 was correlated with poor overall survival of colorectal cancer patients. B3GNT6 could be used as a good prognostic marker in colorectal cancer.

Introduction

Colorectal cancer accounts for almost 900 000 death annually and ranks the world's 4th deadly cancer nowadays [1]. Though recent advance in researches in treatment options have doubled the overall survival for advanced disease to 3 years, prognosis is still best for those with non-metastasized disease [2]. Finding novel biomarkers that carries predictive values for colorectal cancer patients is of great importance.

Colorectal cancer has been known for its various types of genomic mutations which ultimately result in various prognostic endings. The chromosome instability (CIN), microsatellite instability (MIN), and CpG island methylator phenotype (CIMP) pathways are the three main known molecular pathways to colorectal cancer, with each containing different histology, risk factors, prognosis, and response to therapy [3]. The gene mutations related with these molecular pathways largely affect patients response to therapies. Finding prognostic and predictive biomarkers could provide more information in proficient management of colorectal cancer patients.

The B3GNT6 (UDP-GlcNAc:BetaGal Beta-1,3-N-Acetylglucosaminyltransferase 6) protein is a member of the beta-1,3-N-acetylglucosaminyl transferase family that adds an N-acetylglucosamine to N-acetylgalactosamine-modified serine or threonine. B3GNT6 is responsible for creating the core 3 structure of O-glycans that are important components of mucin-type glycoproteins [4]. O-glycans in various types of cancers are often unusual or dysregulated in structure, and greatly contribute to the abnormal biological activities of cancer cells [5]. The B3GNT protein family are commonly dysregulated in various types of cancers, including gastrointestinal tumors, cervical cancer and prostate cancer, etc.[6, 7]. B3GNT6 was largely downregulated in gastric and colorectal cancer [7]. The underlying mechanism remains largely unknown.

The ubiquitin-proteasome system (UPS) plays a pivotal role in cancer cells for their growth and survival. It is a central component of the cellular protein-degradation machinery that allows cellular misfolded and regulatory protein undergo degradation. As an important regulator of a variety of protein substrates, the proteasome participates in virtually every cellular function including proliferation, apoptosis, angiogenesis and metastasis [8]. The proper function of cellular proteasome is crucial for the survival of both normal and neoplastic cells, especially when apoptosis proteasome is inhibited. Understanding the function and regulation of the ubiquitin-proteasome system will help further illustrate cellular activity and tumor progression.

We conducted an in-depth bioinformatics analysis using online databases to investigate the mRNA and protein expression pattern and its clinical significance in colorectal cancer patients. Immunohistochemical analysis of B3GNT6 protein expression was also conducted in 23 paired tissues from our own cohort of colorectal cancer patients. Our results demonstrated that B3GNT6 was downregulated in colorectal cancer tissues compared with normal tissue in both mRNA and protein level. B3GNT6 mRNA expression

level is negatively correlated with overall survival. B3GNT6 level in colorectal cancer patients is associated with CIN status and KRAS mutation. Bioinformatics analysis revealed the B3GNT6 downregulation is enriched in proteasome pathway.

Materials And Methods

Data collection and processing.

To investigate B3GNT6 expression and its clinical significance in colorectal cancer, three datasets including gene expression data of colorectal cancer patients were downloaded from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). Datasets GSE39582 [9], GSE103582 [10] and GSE37182 [11] involve transcriptomic mRNA expression profile of 566, 50 and 88 colorectal cancer cases with 19, 84 and 10 normal cases, respectively. Level 3 HTSeq-FPKM files, comprising 612 transcriptome profiling RNA-Seqs of 544 cases, were collected from a TCGA dataset (<https://www.portal.gdc.cancer.gov/>) that included information on 452 and 96 patients with colon and rectal cancer, respectively. The clinicopathological characteristics, including age, gender, clinical TNM stage, T stage, M stage and lymph node status, CIN, MMR, KRAS and braf mutation status were included. The expression level of the B3GNT6 gene in other cell lines, organs and cancers was identified in the MediSapiens IST Online database (<http://ist.medisapiens.com/>). The protein expression level of B3GNT6 was reviewed by using immunohistochemical-staining data provided in the Human Protein Atlas (<http://www.proteinatlas.org/>)

Gene set enrichment analysis (GSEA)

In order to determine the function of B3GNT6, we conducted GSEA in patients with the top 25% and bottom 25% of B3GNT6 expression in GSE39582 dataset using GSEA 4.1.0 software (<https://www.gsea-msigdb.org/gsea>). The annotated gene sets c2.cp.kegg.v6.0.symbols.gmt and c2.cp.biocarta.v6.0.symbols.gmt from the pathway database were selected as the reference gene set. $p < 0.05$, $|\text{enrichment score (ES)}| > 0.3$ and $\text{gene size} \geq 30$ were set as the cutoff criteria.

Immunohistochemistry staining of B3GNT6 protein expression in colorectal cancer patients

To investigate B3GNT6 protein expression level in colorectal cancer patients, we conducted an immunohistochemical analysis on formalin-fixed and paraffin-embedded, surgically removed colorectal cancer specimen. From February 2016 to July 2018, we collected 23 colorectal cancer tissues and paired adjacent non-tumor tissues and related clinical information from patients who underwent a radical colorectal surgery at the Department of General Surgery, Xiangya Hospital of Central South University. All tissues collected were clinically and pathologically diagnosed as colorectal cancer. Recurrent cases or adjuvant chemo- or radiotherapy recipient were excluded from our study. The differential protein expression levels of B3GNT6 in 23 colorectal cancer and paired normal tissues were measured using IHC staining as has been stated elsewhere [12]. Rabbit polyclonal anti-B3GNT6 (21291-1-AP, ThermoFisher, US) was used at a working concentration of 1:100. The scores were evaluated based on staining intensity and the percentage of positive cells for each of the sections. The staining intensity was scored as follows: 0, no staining; 1, light yellow staining; 2, yellow-brown staining; and 3, deep brown staining. The percentage of positive cells was scored as follows: 0, 0~5%; 1, 6~25%; 2, 26~50%; 3, 51~75%; and 4, > 75%. The final score was calculated as follows: positive cell score \times staining intensity score. The total scores were condensed into four categories: 0 for negative (-); 1–3 for weakly positive (+); 4–7 for positive (++); and 8–12 for strongly positive (+++). All patients were sorted into two groups according to the total score. High expression of B3GNT6 was defined as a detectable immunoreaction with a total score of $\geq 1+$.

Statistical analysis.

Statistical analyses were performed using R studio (version 1.3.1056) and Graphpad Prism (Version 8.0.2). The comparison between B3GNT6 mRNA expression in colorectal cancer and normal tissues from the TCGA and GEO databases was performed using unpaired student's t-tests. The diagnostic value of B3GNT6 mRNA expression was evaluated by the receiver operating characteristic (ROC) curve. Survival analysis was conducted using log-rank (Mantel-Cox) test. The association between clinicopathological characteristics and B3GNT6 mRNA expression levels was determined using χ^2 test. All $p < 0.05$ was considered to indicate a statistically significant difference.

Results

B3GNT6 mRNA is downregulated in colorectal cancer tissues

To investigate B3GNT6 mRNA level in colorectal cancer tissues, we first analyzed B3GNT6 mRNA level by comparing mRNA level in tumor tissue compared with normal or para-tumor tissue in GSE37182, GSE39582 and GSE103512. B3GNT6 level was significantly lower in tumor tissue than that in normal tissue in all three of the microarrays. This was further validated by TCGA online data base (Fig. 1, A-D). The prognostic value of B3GNT6 was measured by ROC curve. TCGA, as well as GSE37182 and GSE39582 database showed B3GNT6 could be used as good prognostic marker for colorectal cancer with statistical significance (Fig. 1, E-H). To analyze B3GNT6 mRNA levels in other organs, we used the IST Online database (<http://ist.medisapiens.com/>). Results indicated that in the GI tract, the stomach, esophagus and normal colorectal tissue saw B3GNT6 mRNA level was largely elevated, with the exception of small intestine showed low expression. B3GNT6 level was also elevated in the bronchus (Fig. 1I).

Correlation between B3GNT6 mRNA level and clinicopathological characteristics in GSE39582

We then focused our study on GSE39582, which had the largest sample number and most detailed information (Table 1). The dataset was comprised of two independent cohort, testing cohort (n = 443) and validating cohort (n = 123). Subgroup analysis showed that chromosome stable (CIN negative) group and KRAS mutated group saw patients with lower B3GNT6 mRNA level.

Table 1
Association between B3GNT6 level and clinicopathological factors from dataset GSE39582.

Variables	Testing Cohort (n = 443)			Validating Cohort (n = 123)		
	High (n = 170)	Low (n = 273)	p-value	High (n = 49)	Low (n = 74)	p-value
Age						
>=65	101	166	0.7351	35	52	0.8901
<65	69	106		14	22	
Unavailable	0	1		0	0	
Gender						
Male	90	147	0.8527	32	41	0.2738
Female	80	126		17	33	
Stage						
I-II	84	137	0.9321	28	48	0.3882
III-IV	82	136		21	26	
T stage						
T1-T2	21	26	0.3574	2	11	0.0625
T3-T4	145	239		43	59	
Unavailable	4	8		4	4	
Lymph node metastasis						
Absent	88	142	0.8803	26	46	0.3906
Present	76	119		19	24	
Unavailable	6	12		4	4	
Distant metastasis						
Absent	150	226	0.1474	40	66	0.3078
Present	16	38		4	3	
Unavailable	4	9		5	5	
Tumor Location						
Distal	96	171	0.1971	27	48	0.2772
Proximal	74	102		22	26	
MMR						
dMMR	18	43	0.1222	5	9	0.6202
pMMR	139	209		41	55	
Unavailable	13	21		3	10	
CIMP status						
-	111	195	0.1855	38	61	0.4994
+	33	41		8	9	

Abbreviations: MMR: mismatch repair; dMMR: mismatch repair-deficient; pMMR: mismatch repair-proficient; CIMP: CpG island methylator phenotype; CIN: chromosome instability

Variables	Testing Cohort (n = 443)			Validating Cohort (n = 123)		
	High (n = 170)	Low (n = 273)	p-value	High (n = 49)	Low (n = 74)	p-value
Unavailable	26	37		3	4	
CIN status						
-	41	41	0.0205	17	11	0.0059
+	98	176		25	55	
Unavailable	31	56		7	8	
tp53 mutation						
mutated	51	84	0.9483	20	35	0.2642
wild type	42	68		24	27	
Unavailable	77	121		5	12	
kras mutation						
mutated	84	88	0.0002	20	25	0.496
wild type	78	174		29	47	
Unavailable	8	11		0	2	
braf mutation						
mutated	19	25	0.4532	4	3	0.3401
wild type	130	218		44	69	
Unavailable	21	30		1	2	

Abbreviations: MMR: mismatch repair; dMMR: mismatch repair-deficient; pMMR: mismatch repair-proficient; CIMP: CpG island methylator phenotype; CIN: chromosome instability

B3gnt6 Protein Expression Is Downregulated In Colorectal Cancer Tissues

To further address the change in B3GNT6 expression and its clinical significance in colorectal cancer patients, we turned to online database of protein expression together with our own clinical cohorts. We downloaded and analyzed B3GNT6 immunohistochemical micrographs in colorectal cancer tissue and normal colon tissue from the Human Protein Atlas (<https://www.proteinatlas.org/>). Negative (11/12) or low (1/12) staining of B3GNT6 was observed in colorectal cancer tissue compared with medium staining in normal colon (3/3) and rectal tissue (3/3) (Fig. 2). Staining of B3GNT6 protein were largely located in cytoplasmic area, supposedly in the golgi apparatus area [7]. We also conducted immunohistochemistry analysis in 23 colorectal cancer tissue with paired paratumor tissue that also showed downregulation of B3GNT6 in colorectal cancer (Fig. 2).

B3GNT6 upregulation is associated with better overall survival in colorectal cancer patients

Next, we examined B3GNT6 expression with regard to its prognostic significance in the cohort. In GSE39582, patients with high B3GNT6 level showed better overall survival than those with low B3GNT6 level. This was further validated by TCGA dataset (Fig. 3, A-C). Together, these findings indicate that high B3GNT6 expression has important clinical significance and could potentially serve as an important biomarker for predicting clinical outcome in colorectal cancer patients.

B3gnt6 Downregulation Is Correlated With Upregulated Proteasome Activity

Taken from the above-mentioned analysis, it is likely that B3GNT6 might act as a tumor suppressor in colorectal cancer microenvironment. To better understand the mechanism under which B3GNT6 facilitates its role as a tumor suppressor, gene set enrichment analysis (GSEA) was conducted in GSE39582. The top 25% and bottom 25% in B3GNT6 mRNA level of the patients enrolled in the study was taken to GSEA analysis. We adopted both KEGG and Biocarta pathway analysis in GSEA analysis and both pathway analysis indicated B3GNT6 mRNA level is negatively correlated with ubiquitin-proteasome system (Table 2 and Fig. 4).

Table 2
GSEA analysis of B3GNT6 mRNA expression in GSE39582

Geneset Name	NES	NOM p-val	FDR q-val
Biocarta pathway			
Upregulated			
Biocarta_RAB_PATHWAY	1.6703635	0.026	0.33836955
Biocarta_CERAMIDE_PATHWAY	1.6385397	0.03807615	0.34184185
Downregulated			
Biocarta_PROTEASOME_PATHWAY	-1.865893	0.011235955	0.15191999
KEGG pathway			
Upregulated			
KEGG_OLFACTORY_TRANSDUCTION	2.0274692	0	0.027611418
KEGG_TASTE_TRANSDUCTION	1.7740936	0	0.19784024
KEGG_GLYCOSPHINGOLIPID_BIOSYNTHESIS_LACTO_AND_NEOLACTO_SERIES	1.770417	0.001926782	0.13674085
KEGG_O_GLYCAN_BIOSYNTHESIS	1.7251008	0.007736944	0.16378903
KEGG_GNRH_SIGNALING_PATHWAY	1.7178557	0	0.14160849
KEGG_ALZHEIMERS_DISEASE	1.6913323	0.026923077	0.15125382
KEGG_NITROGEN_METABOLISM	1.6708751	0.001992032	0.13392551
KEGG_LONG_TERM_POTENTIATION	1.64782	0.024528302	0.14832915
KEGG_HUNTINGTONS_DISEASE	1.638019	0.04660194	0.14545798
KEGG_VASOPRESSIN_REGULATED_WATER_REABSORPTION	1.63744	0.018072288	0.1325422
KEGG_BUTANOATE_METABOLISM	1.6251645	0.01778656	0.13560429
KEGG_DRUG_METABOLISM_OTHER_ENZYMES	1.6193944	0.01178782	0.13172121
KEGG_PORPHYRIN_AND_CHLOROPHYLL_METABOLISM	1.5756354	0.025742574	0.17885368
KEGG_MATURITY_ONSET_DIABETES_OF_THE_YOUNG	1.5706768	0.027613413	0.17375107
KEGG_TERPENOID_BACKBONE_BIOSYNTHESIS	1.5477382	0.046092186	0.19446045
KEGG_AMYOTROPHIC_LATERAL_SCLEROSIS_ALS	1.5473746	0.023391813	0.18385722
KEGG_GLYCEROPHOSPHOLIPID_METABOLISM	1.5310374	0.015873017	0.1866673
KEGG_RETINOL_METABOLISM	1.526893	0.02970297	0.18341947
KEGG_GLYCOLYSIS_GLUONEOGENESIS	1.5237751	0.038	0.17888556
KEGG_VIBRIO_CHOLERAE_INFECTION	1.5188705	0.020876827	0.17640822
KEGG_PROXIMAL_TUBULE_BICARBONATE_RECLAMATION	1.4940959	0.048543688	0.20143263
KEGG_STARCH_AND_SUCROSE_METABOLISM	1.4879901	0.037848607	0.20171466
KEGG_ASCORBATE_AND_ALDARATE_METABOLISM	1.4647686	0.03976143	0.21592942
Downregulated			
NES: normalized enrichment score, that is, the enrichment score for the gene set after it has been normalized across analyzed gene sets. FDR q-val: false discovery rate, that is, the estimated probability that the normalized enrichment score represents a false positive finding. NOM p-val: normalized p-value, that is, the statistical significance of the enrichment score. The nominal p value is not adjusted for gene set size or multiple hypothesis testing; therefore, it is of limited use in comparing gene sets.			

Geneset Name	NES	NOM p-val	FDR q-val
KEGG_BASAL_TRANSCRIPTION_FACTORS	-1.7745181	0.009920635	0.3991878
KEGG_UBIQUITIN_MEDIATED_PROTEOLYSIS	-1.773844	0.013861386	0.20233367
KEGG_RNA_DEGRADATION	-1.6950728	0.028688524	0.21618877
KEGG_GLYCOSAMINOGLYCAN_BIOSYNTHESIS_HEPARAN_SULFATE	-1.5696611	0.020715632	0.3765442
KEGG_GLYCOSAMINOGLYCAN_BIOSYNTHESIS_CHONDROITIN_SULFATE	-1.5518181	0.04183267	0.3753312

NES: normalized enrichment score, that is, the enrichment score for the gene set after it has been normalized across analyzed gene sets. FDR q-val: false discovery rate, that is, the estimated probability that the normalized enrichment score represents a false positive finding. NOM p-val: normalized p-value, that is, the statistical significance of the enrichment score. The nominal p value is not adjusted for gene set size or multiple hypothesis testing; therefore, it is of limited use in comparing gene sets.

Discussion

We conducted a bioinformatics analysis of GEO and TCGA dataset and our own cohort and found B3GNT6 is significantly downregulated in colorectal cancer compared with normal tissue in both mRNA and protein level. Survival analysis showed that B3GNT6 downregulation is associated with poor overall survival in colorectal cancer patients. The upregulation of B3GNT6 is correlated with CIN status, KRAS mutation and proteasome pathway in colorectal cancer.

We found from our study that colorectal cancer patients with low level of B3GNT6 level are more likely to have KRAS mutation and chromosomal instability (CIN). This is interesting since it might shed light on the role of gene expression in modulating the development of colorectal malignancy. The chromosomal instability (CIN) pathway is generally driven by sequential mutational events most commonly by KRAS and leads to colorectal cancer that are typically aneuploid, microsatellite stable (MSS), and may have KRAS but not BRAF mutations [13]. Based on our findings, it is possible that B3GNT6 negatively modulates the gene mutation status of KRAS which could probably lead to the development of colorectal cancer. Also, different types of gene mutation might lead various response to chemo or biologic therapy. How B3GNT6 could regulate KRAS mutation and chromatin instability of colorectal cancer still need further study.

Our findings also suggest that high B3GNT6 level in colorectal cancer patients could lead to decreased proteasomal activity, which we tend to believe to eventually result in the poor clinical outcome of colorectal cancer patients. Under normal physiological conditions, the ubiquitin-proteasome system (UPS) is responsible for eliminating dysfunctional/misfolded proteins via the proteasome, and these specific functions enable the UPS to regulate protein quality in cells, thus maintaining cellular homeostasis and cell survival [14]. Dysregulation of UPS is generally found in various types of cancers [15]. Under tumorous environment, B3GNT6 downregulation lead to upregulation of proteasome activity, which in turn suppresses the accumulation of misfolded or toxic proteins in tumor cells. This disrupts the apoptosis of these tumor cells that eventually results in tumor formation. However, the mechanism under which B3GNT6 regulates proteasome activity still needs further illustration.

Previous studies on the tumor repressing role of B3GNT6 in colorectal cancer showed that B3GNT6 was commonly downregulated in colorectal malignancy [7]. In prostate cancer, B3GNT6 repressed tumor formation and metastasis through down-regulation of $\alpha 2\beta 1$ integrin complex [6]. We discovered that B3GNT6 upregulation is positively correlated with KRAS mutation and CIN status of colorectal cancer in genomic modification level. Down-regulation of B3GNT6 was associated with increased proteasomal activity. This is interesting since proteasome inhibitor use in colorectal cancer, as well as various other kind of solid tumors, was largely limited due to toxicity of the drug and limited efficacy. Further well designed study that focused on the role of B3GNT6 in proteasome regulation are warranted to illustrate this.

Abbreviations

chromosome instability (CIN), microsatellite instability (MIN), CpG island methylator phenotype (CIMP), microsatellite stable (MSS), B3GNT6 (UDP-GlcNAc:BetaGal Beta-1,3-N-Acetylglucosaminyltransferase 6), Gene Expression Omnibus (GEO), The Cancer Genome Atlas (TCGA), ubiquitin-proteasome system (UPS), receiver operating characteristic (ROC)

Declarations

This study was verified and ethically approved by the Medical Ethics Committee of Xiangya Hospital of Central South University. All patients provided written informed consent for the use of surgical specimens for pathological examination. All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>), TCGA dataset (<https://www.portal.gdc.cancer.gov/>), MediSapiens IST Online database (<http://ist.medisapiens.com/>) and the Human Protein Atlas (<http://www.proteinatlas.org/>).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CY, design of the work, the acquisition, analysis, interpretation of data, software, draft manuscript writing. CHH, the acquisition, analysis, interpretation of data, software. PWZ, the acquisition, analysis,

HYH, the acquisition, analysis. ZKC, conception, design of the work, interpretation of data, manuscript revision. CL, conception, design of the work, analysis, interpretation of data, manuscript revision

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Figures

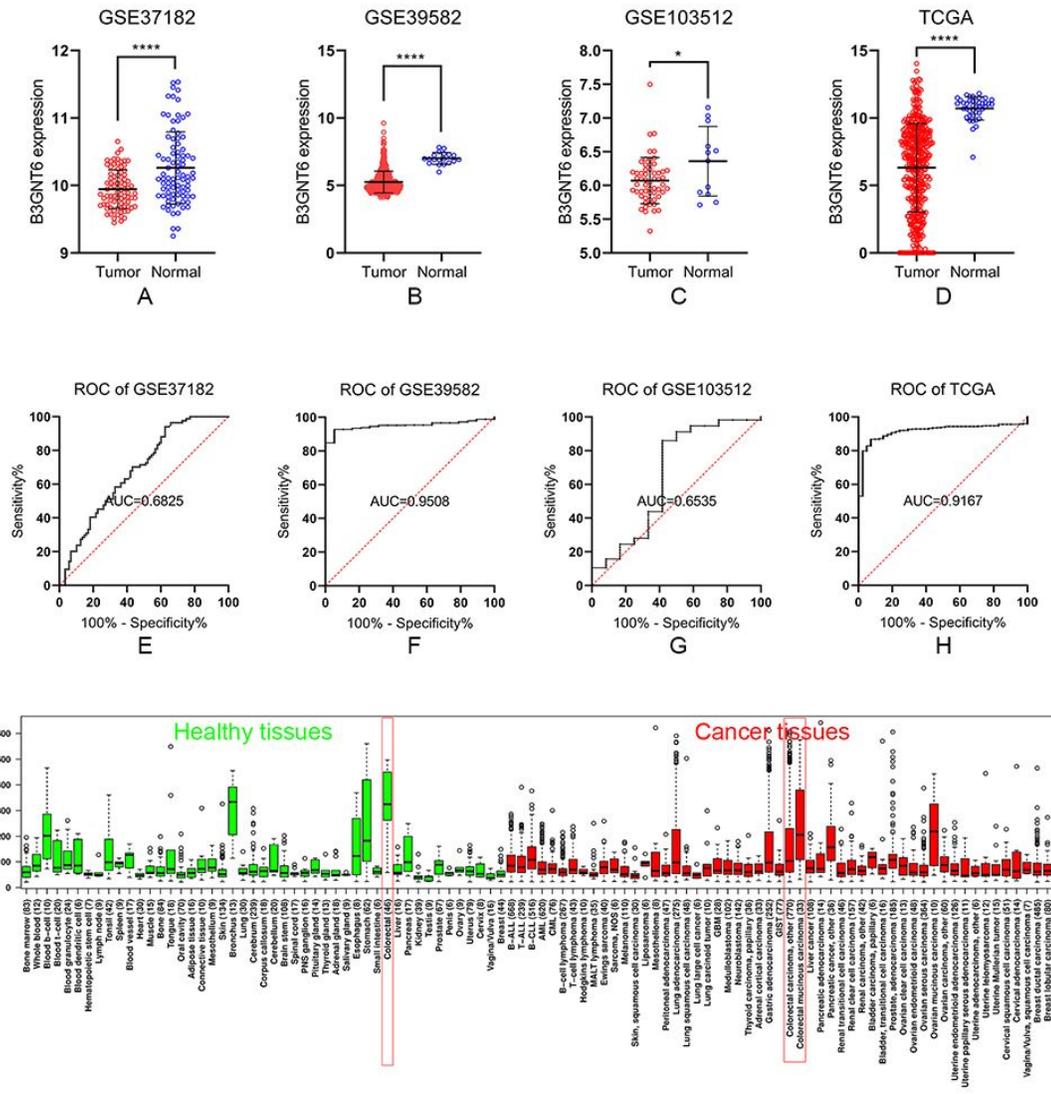


Figure 1

Bioinformatics analysis of B3GNT6 mRNA level online database. A-D. B3GNT6 mRNA level in colorectal cancer tissue compared with normal tissue from GEO datasets (GSE37182, GSE39582 and GSE103512) and TCGA database. E-H. Diagnostic value of B3GNT6 mRNA level in online datasets. I. B3GNT6 mRNA level in healthy tissues compared with cancer tissues.

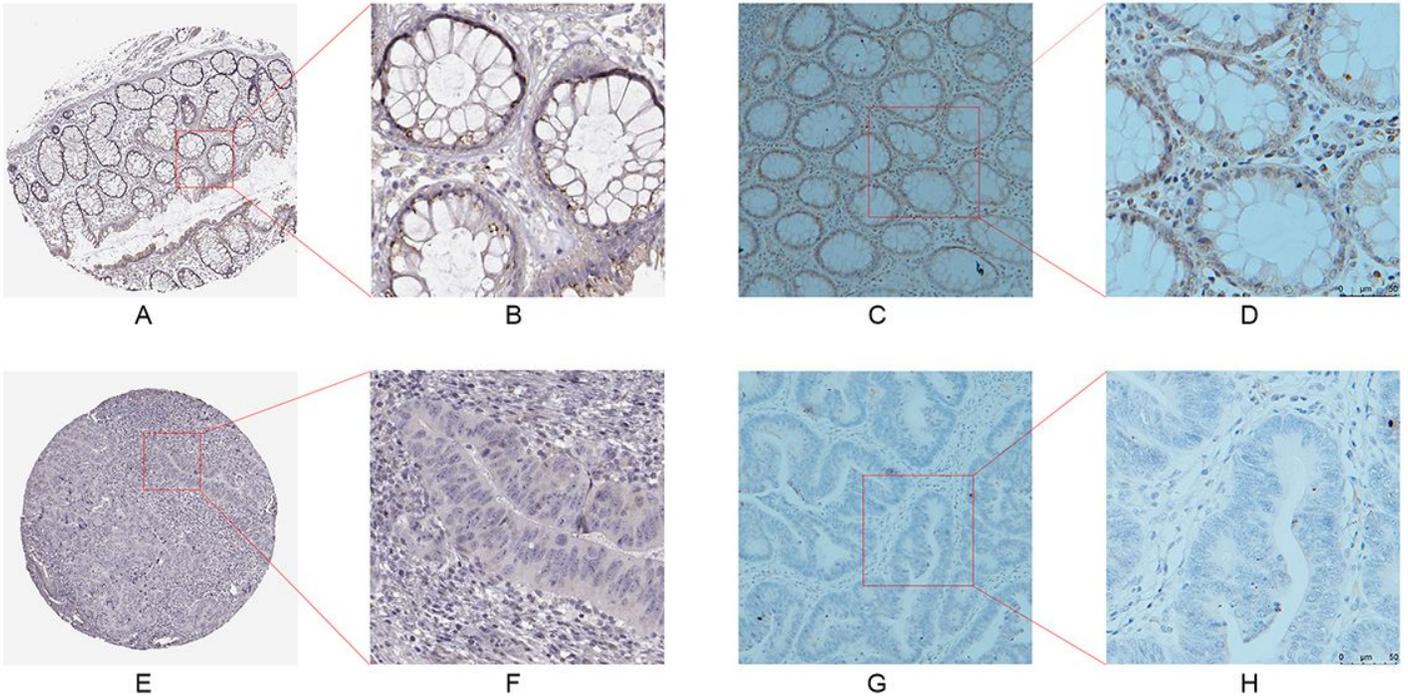


Figure 2

B3GNT6 protein expression level is lower in colorectal cancer tissue compared with normal tissue. A-B, E-F. Immunohistochemical staining of B3GNT6 protein in normal colon tissue and colorectal cancer tissue from online database Human Protein Atlas. C-D, G-H. Immunohistochemical staining of B3GNT6 protein in normal colon tissue and colorectal cancer tissue from our own clinical cohort.

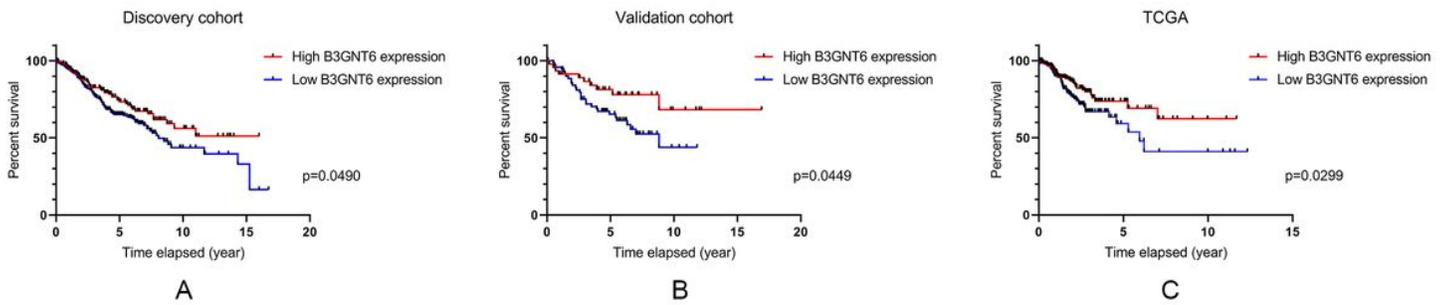


Figure 3

High B3GNT6 expression predicts better overall survival in colorectal cancer patients. A-B. Both discovery cohort and validation cohort in GSE39582 show patients with high B3GNT6 mRNA level have better overall survival. C. This is further validated with clinical data from TCGA online database.

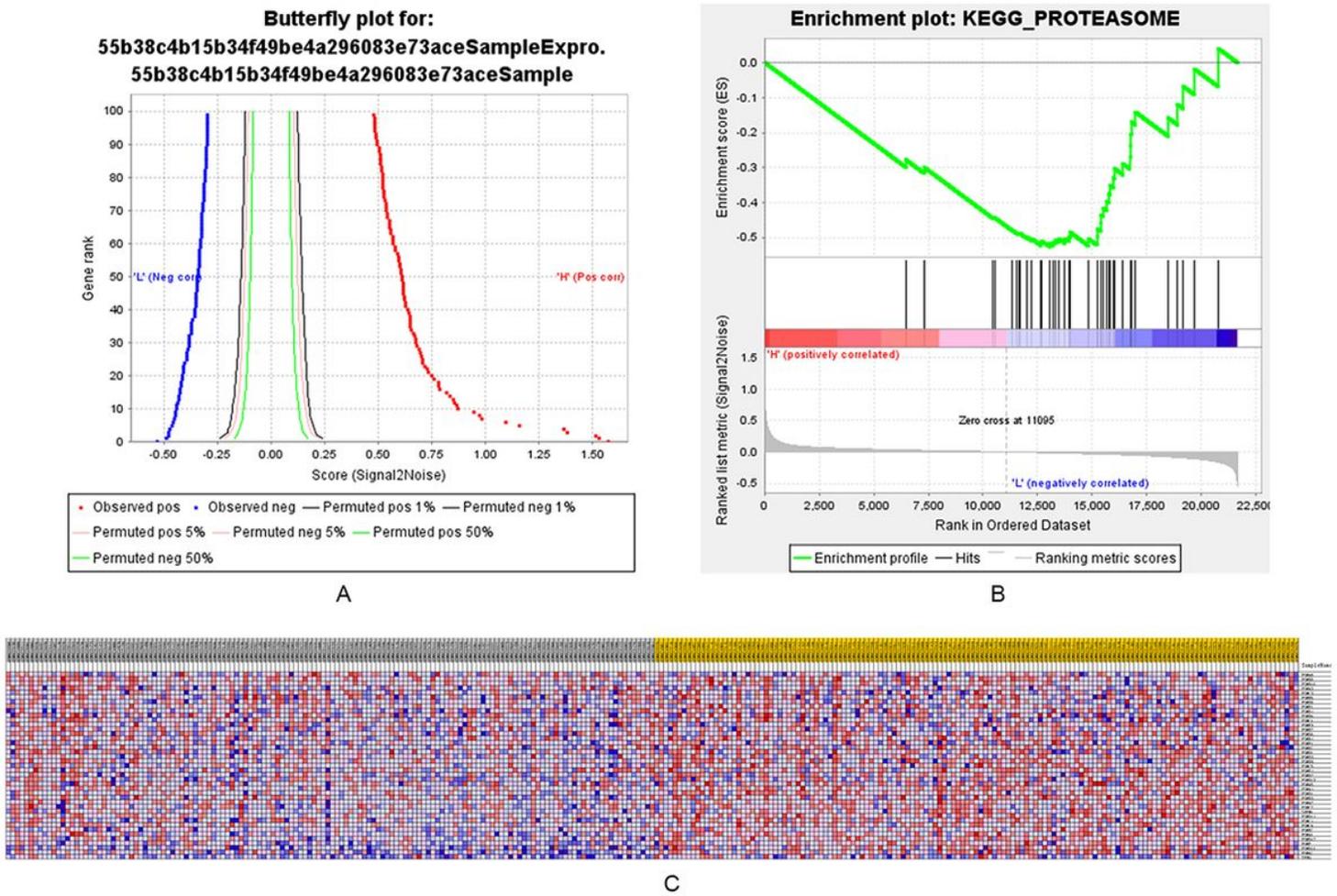


Figure 4

GSEA analysis indicates low B3GNT6 analysis is associated with increased proteasome activity. A. Butterfly plot shows the positive and negative correlation between gene rank and the ranking metric score. B. Enrichment plot indicates B3GNT6 level is negatively correlated with proteasome activity in KEGG pathway. C. Heat map shows patients with high B3GNT6 level (left) and low B3GNT6 level (right) and their gene expression in proteasome pathway.