Glycoprotein screening in colorectal cancer based on differentially expressed Tn antigen

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Abstract. Colorectal cancer (CRC) is one of the most common cancers worldwide, and the identification of new biomarkers for CRC is valuable for its diagnosis and treatment. We aimed to screen differentially expressed glycoproteins (especially *O*-glycoproteins) and to identify diagnostic or therapeutic candidates for colorectal cancer (CRC) based on different Tn antigen expression levels. Fresh cancer tissues and adjacent healthy tissues were obtained from CRC patients and classified into three groups based on their Tn antigen expression: CRC with negative Tn expression (CRC Tn^-), CRC with positive Tn expression (CRC Tn^+) and normal control without Tn expression (NC). Protein extractions were separated and identified by iTRAQ technology. Glycoproteins and *O*-glycoproteins were selected using UniProt and DAVID. Deep bioinformatic analysis, including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KO), was used to annotate this *O*-glycoprotein interaction network. Subsequently, two *O*-glycoproteins were verified by western blotting and immunohistochemistry in either LS174T cells or CRC tissues. We found that 330 differentially expressed proteins were identified by iTRAQ between CRC Tn^- and NC tissues, 317 between CRC Tn^+ and NC tissues, and 316 between CRC Tn^- and Tn^+ tissues. Of the 316 proteins, 55 glycoproteins and 19 *O*-glycoproteins were identified and analyzed via deep informatics. Namely, different Tn antigen expression levels in CRC led to differential protein expression patterns, especially for glycoproteins and *O*-glycoproteins. Decorin and SORBS1, two representative functional *O*-glycoproteins, were significantly downregulated in the CRC Tn^+ tissues compared with the level in the CRC Tn^- or NC tissues. Based on this deep bioinformatic analysis, Decorin and SORBS1 are hypothesized to be involved in the TGF-β and PPAR-γ signaling pathways, respectively.

Introduction

Colorectal carcinoma (CRC) is one of the most common cancers worldwide. However, the early diagnosis rate of CRC is low, and surgery is less effective in patients with more advanced CRC (stage III), for whom the mortality rate is high and the prognosis is poor. Early diagnosis depends on a combination of early screening of non-specific biomarkers such as CEA and CA199 and pathological endoscopic biopsies (1,2). Consequently, most patients are diagnosed at the intermediate or late stages and deprived of the opportunity for surgery. Therefore, the lack of specific biomarkers for earlier diagnosis and therapeutic targets leads to high CRC mortality worldwide (2-4), and CRC is one of the primary causes of cancer-related death in the world. Intervention in the early stage could cure colorectal cancer, and the identification of CRC biomarkers may be a convenient way to achieve this objective.

Recent studies show that the pathogenesis of cancer is highly related to glycosylation (5,6). The increased expression of *N*-acetylgalactosamine (GalNAc) on the surface of cancer cells is one of the characteristics of the altered glycosylation pathway (7). Mucins are highly *O*-glycosylated proteins that play pivotal roles in cell adhesion, inflammation, cell proliferation, apoptosis and tumorigenesis (8-11). The mucin-associated carbohydrate Tn antigen is composed of a GalNAc residue covalently *O*-linked to a serine/threonine (12,13), which is shielded in healthy tissues and benign tumors but occurs in ~90% of all human primary carcinomas (14). The synthesis of sialyl-Tn antigen (sTn) is regulated by the sialyltransferase ST6GalNAc, which competes against *O*-glycan elongating glycosyltransferases and prevents tumors from exhibiting longer *O*-glycans (12,15). Abnormal Tn antigen expression

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Abbreviations: CRC, colorectal cancer; SORBS1, SH3 domain protein 1; iTRAQ, isobaric tags for relative and absolute quantitation

Key words: colorectal cancer, *O*-glycoprotein, proteomic, Tn antigen

has been observed in lung, breast, ovarian and CRC (16-20). Tn antigen is also associated with malignancy and represents a potential marker of malignant transformation (17,21,22). Recent studies have shown that higher Tn antigen expression is observed in CRC patients (14,23,24). Tn antigen may promote cancer metastasis by secretion of transforming growth factor-β (TGF-β) in the tumor microenvironment (25). In addition, Tn was found to be expressed in moderately differentiated tumors but not in poorly differentiated carcinomas (26), and a deficiency of core-1-derived *O*-glycans could delay the onset and progression of breast cancer (18). Furthermore, Tn antigen is related to poor prognosis in patients with breast cancer (27). Above all, we hypothesized that the Tn antigen plays a central role in the early diagnosis and the development of CRC. However, the Tn-related proteins participating in its pathogenic progression remain unclear. It is therefore worthwhile to analyze the expression of Tn-related proteins. In the present study, we used iTRAQ to screen differentially expressed proteins between Tn-positive and Tn-negative CRC tissues and to identify accessible proteins as useful diagnostic biomarkers or new therapeutic candidates for CRC.

Materials and methods

Patient information and sample preparation. A series of primary colon resections were prospectively collected between November 2011 and March 2012 from the Department of Minimally Invasive Surgery of the Second Xiangya Hospital in Changsha. Each resection contained both colon tumor samples and adjacent healthy tissues, and lymph nodes were also collected. Some of the samples were kept at -80˚C until analysis, and the remaining samples were fixed in 4% paraformaldehyde and sent to the Pathology Department for further analysis. CRC samples were screened for Tn staining via immunochemical examination by Dr Lijun Xia from the Oklahoma Medical Research Foundation (OMRF; Oklahoma City, OK, USA). Based on the Tn antigen expression, the samples were divided into three groups as follows: CRC with negative Tn expression (CRC Tn^-), CRC with positive Tn expression (CRC Tn^+) and normal control without Tn expression (NC). No patient was treated with any kind of chemotherapy. The patient information is shown in Table I.

This study was approved by the Institutional Ethics Committee of the Second Xiangya Hospital of Central South University. All participants provided written informed consent.

Protein extraction and iTRAQ labeling. One gram of fresh tissues was ground in liquid nitrogen and suspended in 5 ml of lysis buffer with supplements (7 mM urea, 2 mM sulfourea, 0.1% PMSF, 65 mM DTT) on ice for 30 min, followed by centrifugation for 15 min at 24,1488 x g. The supernatant was collected, and the protein concentration was determined using the Bradford Protein Assay kit (Bio-Rad, USA).

For peptide labeling, a peptide mixture from each group was labeled with iTRAQ tags according to the kit's protocol (Applied Biosystems, Foster City, CA, USA). Protein samples from adjacent healthy tissues were labeled with reagent 114. Tn^- CRC and Tn^+ CRC samples were labeled with reagent 117 and 118, respectively.

2D LC-MS/MS. The peptide mixture was reconstituted and acidified with buffer A (10 mM KH_2PO_4, pH 2.6, and 25% acetonitrile) (ACN; Thermo Fisher Scientific, Inc., Fair Lawn, NJ, USA) and loaded onto a strong cation exchange (SCX) chromatography column (The Nest Group, Inc. , Southborough, MA, USA) on a 20AD high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan). The peptides were eluted at a flow rate of 200 μl/min with a gradient of 0-80% buffer B for 60 min. The elution was monitored by absorbance at 214/280 nm, and fractions were collected every minute. The collected fractions were combined and desalted on a C18 cartridge (Waters Corp., Milford, MA, USA).

All samples were resuspended in 50 μl HPLC buffer A (5% ACN and 0.1% formic acid), loaded in the ZORBAX 300SB-C18 reverse-phase column (5 μm, 300 Å, 0.1x150 mm; Michrom BioResources, Auburn, CA, USA). The peptides were separated for >90 min using a linear gradient of 5-35% HPLC buffer B (95% ACN and 0.1% formic acid) at a flow rate of 300 nl/min. The MS analysis was performed using a QSTAR XL analyzer (Applied Biosystems) coupled with a 20AD HPLC system (Shimadzu). Precursor ions were selected across the mass range of 400-1,800 *m/z*. Four precursors were selected for MS/MS analyses across the mass range of 100-2,000 *m/z*.

Data analysis and bioinformatics. Protein identification and relative iTRAQ quantification were performed with the Paragon algorithm in the ProteinPilot Software 3.0 (revision 114732; Applied Biosystems). The results were further processed using the Pro Group algorithm (Applied Biosystems), where isoform-specific quantification was adopted to trace the differences between the expression levels of various isoforms. Quantitative ratios of reporter ions, calculated by comparing the peak areas of each of these reporter ions in the mass spectrum, were used to evaluate the expression change of the protein in different samples. Normalization was performed during the quantification to correct the experimental bias. To accept proteins as showing differential expression between different groups, we strictly followed the following criteria. It was mandatory for proteins to be identified with >95% confidence, and the protein confidence threshold cutoff was 1.3 (unused ProtScore). Proteins with significant P-values ($P<0.05$) were considered differentially expressed. The false discovery rate (FDR) for protein detection was calculated as FDR = (2 x reverse)/(forward + reverse). UniProt was used to annotate the proteins in the biological process and molecular function categories in the human UniProtKB/Swiss-prot database (version 3.52, November 2008).

Gene Ontology and pathway analysis. Gene Ontology (GO) was used to annotate the proteins by biological process and molecular function. We translated the genes into putative amino acid sequences and aligned these genes against a set of protein sequences from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Each protein was assigned to KEGG orthology (KO), and differentially enriched KO pathways were identified.

Cell culture and immunofluorescence staining of the Tn antigen. LS174T cells (a gift from Dr Lijun Xia, OMRF), were

Table I. Patient characteristics.

Pt. no.	Mean age (years)	Gender	Histological assessment	TNM	Tn	Category
1	64	Male	Rectal tubulovillous adenocarcinoma	Tis0	-	Tn^-
2	50	Female	Moderately-poorly differentiated rectal adenocarcinoma	T3N1bM0 (IIIB)	-	Tn^-/normal
3	81	Male	Moderately differentiated colon adenocarcinoma	T3N0M0 (IIA)	-	Tn^-
4	56	Female	Moderately differentiated rectal adenocarcinoma	T4aN1aM0 (IIIB)	+	Tn^+/normal
5	70	Male	Moderately and poorly differentiated rectal adenocarcinoma	T3N0M0 (IIA)	+	Tn^+/normal
6	52	Male	Moderately differentiated colon adenocarcinoma	T3N0M0 (IIA)	+	Tn^+

Table II. Differentially expressed proteins in Tn^+ compared with Tn^- tissues.

Pt. no.	Accession	Name	Tn^+:Tn^-	PValTn^+:Tn^-	EFTn^+:Tn^-
1	P24821	Tenascin	0.61944109201	0.041761569679	1.1803209782
2	P27797	Calreticulin	1.2941960096	0.10105329752	1.1912419796
3	O43852	Calumenin	1.5275659561	0.13953730464	1.2022639513
4	P02768	Serum albumin	2.0137240887	0.13020549715	1.213389039
5	P05787	Keratin, type II cytoskeletal 8	0.81658238173	0.38406300545	1.1168630123
6	P05783	Keratin, type I cytoskeletal 18	0.47424200177	0.00049924501218	1.213389039
7	P11047	Laminin subunit γ-1	0.65463608503	0.18409490585	1.2473829985
8	P12110	Collagen α-2(VI) chain	1.2589249611	0.007290690206	1.2705739737
9	Q01082	Spectrin β chain, brain 1	1.3677289486	0.18495669961	1.2941960096
10	P01023	α-2-macroglobulin	0.66680681705	0.34203439951	1.3061709404
11	P02766	Transthyretin	0.69823241234	0.21042379737	1.3182569742
12	P02763	α-1-acid glycoprotein 1	1.6749429703	0.49577480555	1.3304539919
13	Q9Y4L1	Hypoxia upregulated protein 1	1.5135610104	0.065106026828	1.3304539919
14	P55268	Laminin subunit β-2	0.71121352911	0.17134909332	1.3304539919
15	P02452	Collagen α-1(I) chain	0.55462568998	0.019404709339	1.3427649736
16	Q12864	Cadherin-17	0.74473202229	0.21211579442	1.3551889658
17	P05164	Myeloperoxidase	0.64863437414	0.12027399987	1.3677289486
18	P02787	Serotransferrin	1.8365379572	0.19530679286	1.4190570116
19	Q9Y6R7	IgG Fc-binding protein	1.3061709404	0.29926979542	1.4190570116
20	P08294	Extracellular superoxide dismutase [Cu-Zn]	0.64268767834	0.30398610234	1.4321880341
21	P10153	Non-secretory ribonuclease	1.3551889658	0.13421760499	1.4454400539
22	P69905	Hemoglobin subunit α	0.42461958528	0.0031579970382	1.4454400539
23	P01833	Polymeric immunoglobulin receptor	3.3419499397	2.79E-06	1.4859360456
24	P02675	Fibrinogen β chain	0.51050502062	0.46312698722	1.4859360456
25	P08123	Collagen α-2(I) chain	0.46131759882	0.20906309783	1.4859360456
26	P98160	Basement membrane-specific	1.3803839684	0.35451129079	1.4996850491
27	P01857	Ig γ-1 chain C region	1.3551889658	0.50612038374	1.4996850491
28	P00738	Haptoglobin	0.45289760828	0.10523310304	1.4996850491
29	P08238	Heat shock protein HSP90-β	1.7378009558	0.32500821352	1.5135610104
30	P01876	Ig α-1 chain C region	1.6443719864	0.16751100123	1.5135610104
31	P05155	Plasma protease C1 inhibitor	0.63679552078	0.38150951266	1.5135610104
32	P01009	α-1-antitrypsin	0.57543987036	0.51836198568	1.5135610104
33	P02788	Lactotransferrin	1.3803839684	0.40739420056	1.5275659561
34	P10645	Chromogranin A	2.6791679859	0.020165350288	1.5703630447
35	P10909	Clusterin	0.67920362949	0.50286757946	1.5995579958
36	P01871	IgM chain C region	0.66069352627	0.67946302891	1.5995579958
37	Q9BX66	Sorbin and SH3 domain-containing protein 1	0.47424200177	0.098883867264	1.6292959452
38	P01877	Ig α-2 chain C region	0.66680681705	0.44289419055	1.6443719864
39	P22105	Tenascin-X	0.79432821274	0.49807879329	1.6595870256

Table II. Continued.

Pt. no.	Accession	Name	Tn^+:Tn^-	$PValTn^+$:Tn^-	$EFTn^+$:Tn^-
40	P02750	Leucine-rich α-2-glycoprotein	0.50118720531	0.26861310005	1.6904410124
41	P51888	Prolargin	1.3182569742	0.95629411936	1.7060819864
42	P62807	Histone H2B type 1-C/E/F/G/I	0.48752850294	0.098780490458	1.7060819864
43	P02671	Fibrinogen α chain	0.082413807511	1.61E-06	1.7060819864
44	P04217	α-1B-glycoprotein	0.40550848842	0.16042810678	1.7060819864
45	P16070	CD44 antigen	0.67920362949	0.49087619781	1.7218689919
46	P02790	Hemopexin	1.7378009558	0.30744469166	1.7378009558
47	Q02817	Mucin-2	5.3951058388	0.0025611699093	1.9054609537
48	O00748	Cocainee sterase	1.9230920076	0.1603616029	1.7538809776
49	P04196	Histidine-rich glycoprotein	0.46558609605	0.19744589925	1.8197009563
50	P62937	Peptidyl-prolyl *cis*-trans isomerase A	3.0478949547	0.00011998559785	1.9230920076
51	P07585	Decorin	0.731139123	0.635718882	1.99843504
52	Q16363	Laminin subunit α-4	0.71121352911	0.70577037334	1.9230920076
53	P02751	Fibronectin	0.81658238173	0.24489469826	1.213389039
54	P12109	Collagen α-1(VI) chain	0.80167812109	0.50682651997	1.2359470129
55	P23229	Integrin α-6	0.80909591913	0.58197510242	1.4190570116

iTRAQ ratio indicates the relative quantification of the differentially expressed proteins. Statistical analysis of iTRAQ ratio was performed.

cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Zhejiang Tianhang Biological Technology Stock Co., Ltd., Zhejiang, China) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Gibco, USA).

For immunofluorescence staining of the Tn antigen, LS174T cells on cover slips were fixed in 4% paraformaldehyde (Sigma), permeabilized with 0.1% Triton X-100 (Promega) at room temperature for 15 min, and blocked with 1% BSA (Gibco) for 30 min at room temperature. The slides were incubated with a mouse monoclonal antibody against Tn (made by OMRF) at 1:50 overnight at 4˚C, followed by a 1-h incubation at room temperature with the secondary antibody (anti-mouse antibody, 1:2,000; Jackson ImmunoResearch, West Grove, PA, USA). The sections were counterstained with DAPI for 2 min and then visualized and photographed under a confocal laser scanning microscope (LaVision BioTec GmbH, Bielefeld, Germany). To confirm the expression of Decorin or SORBS1, $3x10^6$ LS174T cells were harvested, and the total proteins were prepared for subsequent western blotting.

Western blotting. The extracted proteins from tissues or cells were resolved by SDS-PAGE gradient gel (5% spacer gel and 8% separation gel). After electrophoresis, samples were transferred to a polyvinylidene fluoride membrane, blocked at room temperature for 1 h with 5% milk, and incubated with a mouse antibody against Decorin (1:500; Abcam, USA), a rabbit antibody against SORBS1 (1:1,000; Abgent, USA) or a mouse antibody against β-actin (1:1,000; Abcam) at 4˚C overnight. The membranes were washed with buffer three times, incubated with an HRP-conjugated anti-rabbit secondary antibody (1:5,000) or anti-mouse secondary antibody (1:20,000) (both from Abcam) at room temperature for 1 h, and visualized with an ECL kit (GE Healthcare). Specific signals were quantified from exposed X-ray film using a scanner with BandScan 4.30 densitometry software and are expressed as integrated intensity units relative to the β-actin signals.

Immunohistochemistry assay in tissues. To detect the immunohistochemical expression of Decorin or SORBS1, the embedded tissues were cut into 5-μm thin sections. These sections were incubated with an antibody against Decorin (1:50) or SORBS1 at 1:100 overnight at 4˚C after blocking with 2% BSA. Subsequently, the sections were treated with an HRP-conjugated anti-rabbit (1:5,000) or anti-mouse secondary antibody (1:20,000) at room temperature for 1 h. The DAB Detection kit (EliVision Super DAB; Maixin-Bio, Fuzhou China) for blocking non-specific binding and antibody detection was used. The sections were then counterstained with hematoxylin. The immunostaining results were detected under a light microscope.

Statistics. All observations were confirmed by at least three independent experiments. Image-Pro Plus 6.0 was used to analyze the results from IHC, and Quantity One v4.6.2 was used for western blotting. For quantitative data, t-test and ANOVA were used for statistical analysis. The analyses were performed using SPSS Statistics 20 (SPSS Inc. Chicago, IL, USA). $P<0.05$ was considered statistically significant.

Results

Tn antigen expression and clinicopathological factors in CRC patients. Sixteen patients with a confirmed diagnosis of CRC were included. The enrolled patients included three males and three females, with ages ranging from 50 to 81 years (mean age, 62). Most were diagnosed with moderately differentiated

Table III. Differentially expressed glycosylated proteins in Tn^+ compared with Tn^- tissues

Pt. no.	Unused	Accession	Name	Peptides (95%)	Differential expression		
					Tn^+:Tn^-	Tn^-:normal	Tn^+:normal
1	89.82	P02768	Seruma lbumin	84	2.013724089	0.194088593	0.440554887
2	101.57	P05787	Keratin, type II cytoskeletal 8	108	0.816582382	1.721868992	1.445440054
3	45.92	P05783	Keratin, type I cytoskeletal 18	42	0.474242002	4.055085182	1.958845019
4	37.56	Q01082	Spectrin β chain, brain1	26	1.367728949	0.691830993	0.963828981
5	40.35	P02452	Collage α-1(I) chain	34	0.55462569	1.066596031	0.586138189
6	23.09	P02787	Serotransferrin	14	1.836537957	0.487528503	0.862978518
7	8.5	P98160	Basement membrane-specific heparan sulfate proteoglycan core protein	4	1.380383968	0.505824685	0.704693079
8	10.12	P08238	Heat shock protein HSP90-β	22	1.737800956	1.096477985	1.923092008
9	24.54	P01876	Ig α-1chain C region	22	1.644371986	0.4487454	0.724435985
10	6	P05155	Plasma protease C1 inhibitor	3	0.636795521	1.853531957	1.224616051
11	22.08	P10645	Chromogranin-A	14	2.679167986	0.1599558	0.420726597
12	10.04	Q9BX66	Sorbin and SH3 domain-containing protein 1	7	0.474242002	0.505824685	0.248885706
13	6	P02750	Leucine-rich α-2-glycoprotein	3	0.501187205	0.772680581	0.390840888
14	21.35	P62807	Histone H2B type 1-C/E/F/G/I	32	0.487528503	0.90364939	0.432513803
15	6	P16070	CD44 antigen	3	0.679203629	1.923092008	1.342764974
16	5.43	P02790	Hemopexin	3	1.737800956	0.524807513	0.990831971
17	14.3	Q02817	Mucin-2	8	5.395105839	0.313328594	1.614359021
18	37.01	P02751	Fibronectin	21	0.816582382	0.963828981	0.787045777
19	2.99	P07585	Decorin	2	0.731139123	0.325087309	0.260615289

cancer. The Tn antigen expression was detected in 3 patients, as shown in Table I.

Identification of Decroin and SORBS1 as differentially expressed glycoproteins in CRC by iTRAQ. A total of 1,051 non-redundant proteins in the genome (UniProt) were significantly identified from the number of MS/MS spectra and number of peptides using a 2% false discovery rate (FDR) as the cutoff in the triplicate independent experiments. Of these, 750 had quantitative information, and more than two peptides were matched to these proteins. There were 330 differentially expressed proteins between CRC Tn^- and NC tissues, 317 between CRC Tn^+ and NC tissues, and 316 between CRC Tn^- and CRC Tn^+ tissues. Among the 316 proteins, there were 55 glycoproteins and 19 *O*-glycoproteins. Twelve of the 19 *O*-glycoproteins were also differentially expressed between the CRC Tn^-/CRC Tn^+ tissues and normal mucosal tissues (Tables II and III).

Sixty-five proteins were differentially expressed in CRC Tn^- tissue compared with NC, while 74 proteins were significantly altered in CRC Tn^+ compared with NC. Forty proteins displayed differential expression between CRC Tn^- and CRC Tn^+, and 25 of these proteins were simultaneously significantly differentially expressed in CRC Tn^- and Tn^+ tissues compared with NC (Fig. 1).

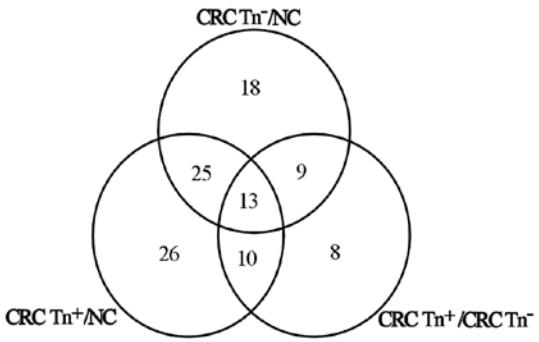

Figure 1. Numbers of unique differentially expressed proteins in different tissues. The total CRC Tn^-/normal (65) and CRC Tn^+/normal (74) numbers indicate the number of unique significantly differentially regulated proteins in CRC Tn^- and CRC Tn^+ tissues compared with NC tissues, respectively. The circle for CRC Tn^-/CRC Tn^+ indicates that 40 proteins were differentially expressed in CRC Tn^- compared with CRC Tn^+ tissues. The overlaps indicate the number of proteins simultaneously differentially expressed under two conditions compared with another. Namely, 25 represents the number of differentially expressed proteins in CRC Tn^- and Tn^+ tissues compared with NC tissues, 10 represents the number in CRC Tn^- and NC tissues compared with CRC Tn^+ tissues, and 9 indicates the number of CRC Tn^+ and NC tissues compared with CRC Tn^- tissues. The number 13 indicates the number of the proteins simultaneously differentially expressed in CRC Tn^+, CRC Tn^- and NC tissues.

Among the significantly differentially expressed proteins, there were a few glycosylated proteins, especially *O*-glycosylated proteins. Fifty-five glycosylated proteins exhibited differential expression between CRC Tn^+ and CRC

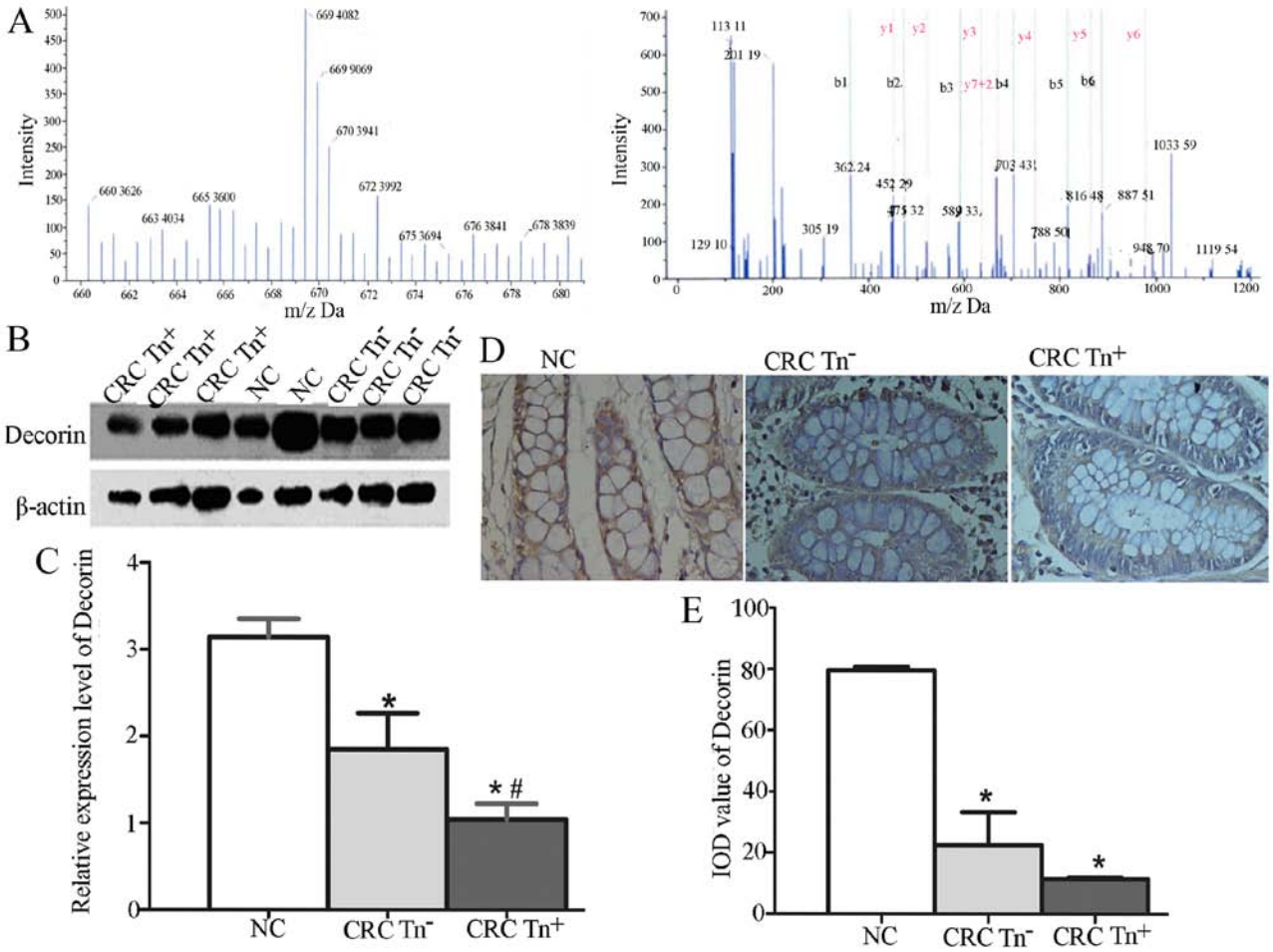

Figure 2. The identification of Decorin, a differently expressed protein in colorectal carcinoma (CRC), by iTRAQ analysis. The proteins were repeatedly identified by triplicate iTRAQ labeling and 2-dimensional (2D) LC-MS/MS analyses. (A) The released iTRAQ reporter ions provide the relative quantification of GLNNLAK (a peptide of Decorin) from each of the groups. (B) Western blotting shows changes in the Decorin expression levels in CRC Tn^+, Tn^- and NC tissues. (C) Protein expression analysis from tissues using one-way ANOVA. (D) Decorin was reduced in CRC Tn^+ tissues via IHC (magnification, x400). Decorin immunoreactivity is denoted by brown color, which is located mainly in the stromal tissue areas. (E) Using one-way ANOVA, Decorin expression decreased in CRC Tn^+ tissues compared with CRC Tn- (P=0.530) and NC tissues (P=0.003). CRC Tn^+, Tn-positive cancer tissues; CRC Tn^-, Tn-negative cancer tissues; NC, normal healthy tissues; *significantly different compared with NC ($P<0.05$); #significantly different compared with CRC Tn^- ($P<0.05$).

Tn^-, including 19 *O*-glycosylated proteins (Tables II and III). Among the differentially expressed *O*-glycosylated proteins, 12 were significantly differentially regulated in groups, including Keratin 8 (gene: KRT8), Keratin 18 (gene: KRT18), Decorin (gene: DCN), Sorbin and SH3 domain-containing protein 1 (SORBS1), and CD44 antigen (gene: CD44) (Tables II and III).

Differential expression of Decorin and SORBS1 in CRC tissues or cell lines with different expression levels of the Tn antigen. We investigated whether the expression of Decorin and SORBS1 is related to the Tn antigen expression level in CRC tissues. The relative quantification of Decorin using iTRAQ showed that Decorin was more highly expressed in normal tissues than in CRC tissues, especially Tn^+ tissues (Fig. 2A), as in the results for SORBS1 using iTRAQ. To verify the function of Decorin and SORBS1 in carcinogenesis, we used western blotting and IHC to quantify their expression in NC, CRC Tn^- and CRC Tn^+ tissues. As shown in Fig. 2B-E, Decorin expression was significantly decreased in the CRC Tn^+ tissues compared with the CRC Tn^- and NC tissues ($P<0.05$). A significant difference was also found in the expression of SORBS1 among Tn^+, Tn^- and NC tissues, as shown in Fig. 3.

To verify the Tn antigen expression levels in Tn^+ cells, we demonstrated that Tn antigen was significantly increased in Tn^+ cells compared with Tn^- cells via immunofluorescence assay (Fig. 4A). We also detected the expression levels of Decorin and SORBS1 in the LS174T cells with different Tn expression levels and found that the expression of Decorin was significantly decreased in the Tn^+ LS174T cells compared with that noted in the Tn^- cells ($P<0.05$) (Fig. 4B and C). Surprisingly, SORBS1 was not detected in the LS174T cells.

Functional analysis of 12 differentially expressed O-glycosylated proteins related to CRC. Functional analysis of the *O*-glycosylated proteins showed that the proteins participated in metabolic processes, in the regulation of biological processes, in the formation of membrane proteins of cells and organelles, and in the binding to other proteins (Table IV). In the present study, we paid particular attention to the analysis of metabolic processes, especially for SORBS1 and Decorin. SORBS1 was correlated with cell-cell adhesion via paxillin (PXN), the

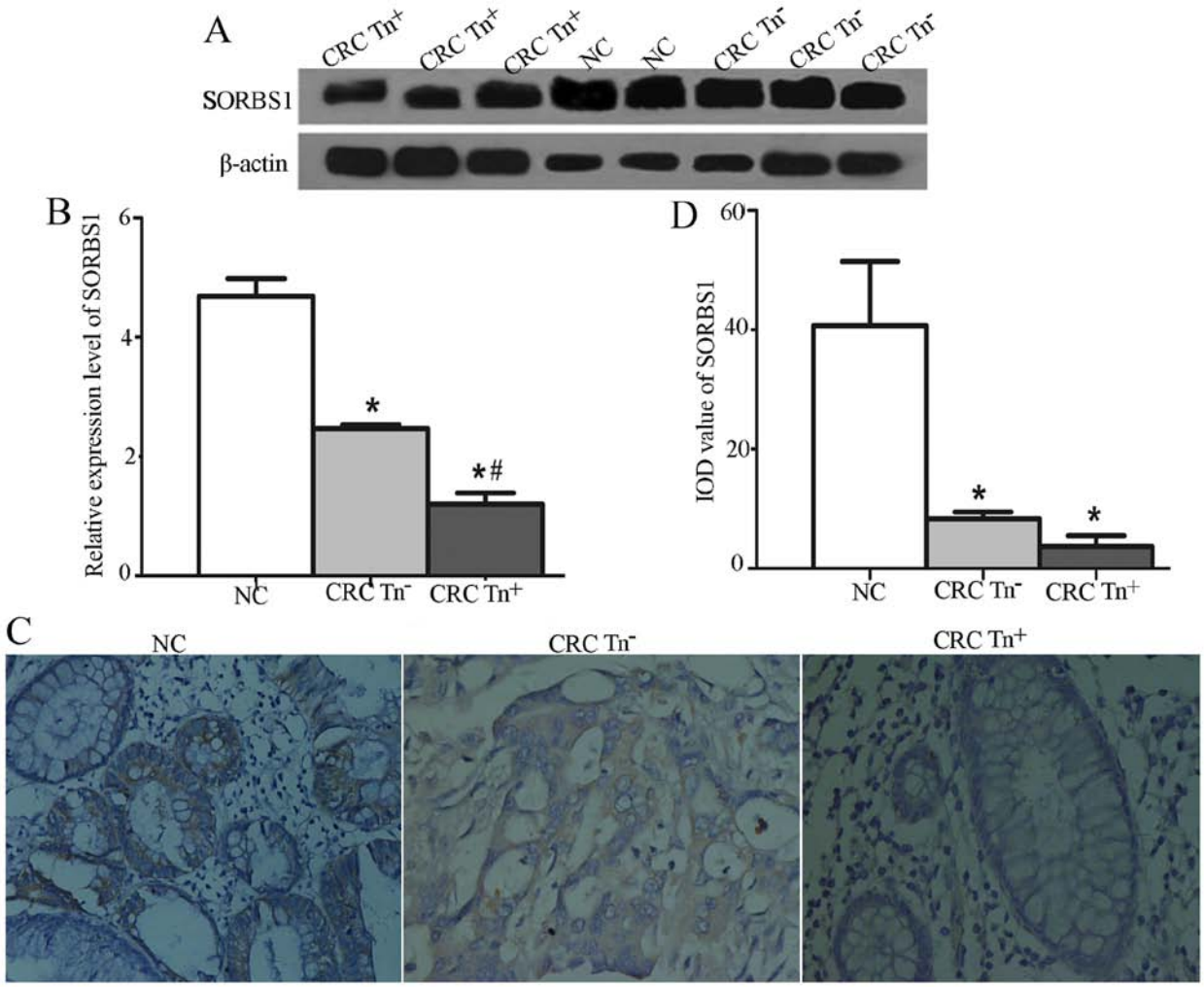

Figure 3. Reduced expression of SORBS1 in Tn+ tissues. (A) Western blotting shows changes in the SORBS1 expression levels in CRC Tn^+, CRC Tn^- and NC tissues. (B) Protein expression analysis from tissues using one-way ANOVA. (C) SORBS1 was reduced in CRC Tn^+ tissue via IHC (magnification, x400). SORBS1 immunoreactivity is visible as brown color, which is located mainly in the stromal tissue areas. (D) Using one-way ANOVA, SORBS1 expression decreased in CRC Tn^+ tissues compared with CRC Tn^- (P=0.732) and NC tissues (P=0.005). CRC Tn^+, Tn-positive cancer tissues; CRC Tn^-, Tn-negative cancer tissues; NC, normal healthy tissues; *significantly different compared with control, #significantly different compared with CRC Tn^- (P<0.05).

Table IV. Functional analysis of differentially expressed *O*-glycosylated proteins.

Functions	No. of genes	No. of proteins	Proteins (%)
Metabolic processes	27	12	63.16
Regulation of biological processes	41	10	52.63
Cell parts	63	11	57.89
Organelles	18	10	52.63
Organelle parts	26	14	73.68
Binding	34	13	68.42

PPAR signaling pathway and the insulin signaling pathway via INS/IRS1. It could also interact with the Cbl proto-oncogene (CBL). Decorin participated in the TGF-β pathway via TGFB1/3 and connected with JUN, neurocan (NCAN), syndecan (SDC) and FOS-like protein-1 (FOSL1), as shown in the network in Fig. 5 and Table V.

Correlation of Decorin and SORBS1 expression in CRC with clinicopathological factors. To ascertain whether the expression of Decorin and SORBS1 was related to neoplasm staging, we compared the expression of Decorin and SORBS1 with TNM stages. Decorin and SORBS1 were not significantly increased in TNM stage 0-II compared with stage III (Table VI, P>0.05). However, a significant correlation was found between the expression of the two proteins and the tumor differentiation stages. Decorin and SORBS1 were significantly increased in moderately differentiated tissues compared with poorly differentiated tissues (Table VI, P<0.05).

Discussion

Since the Tn antigen participates in the development of CRC (24,28,29), we investigated specific Tn antigen-related biomarkers as novel indicators for diagnostic or therapeutic targets. We analyzed the differentially expressed proteins

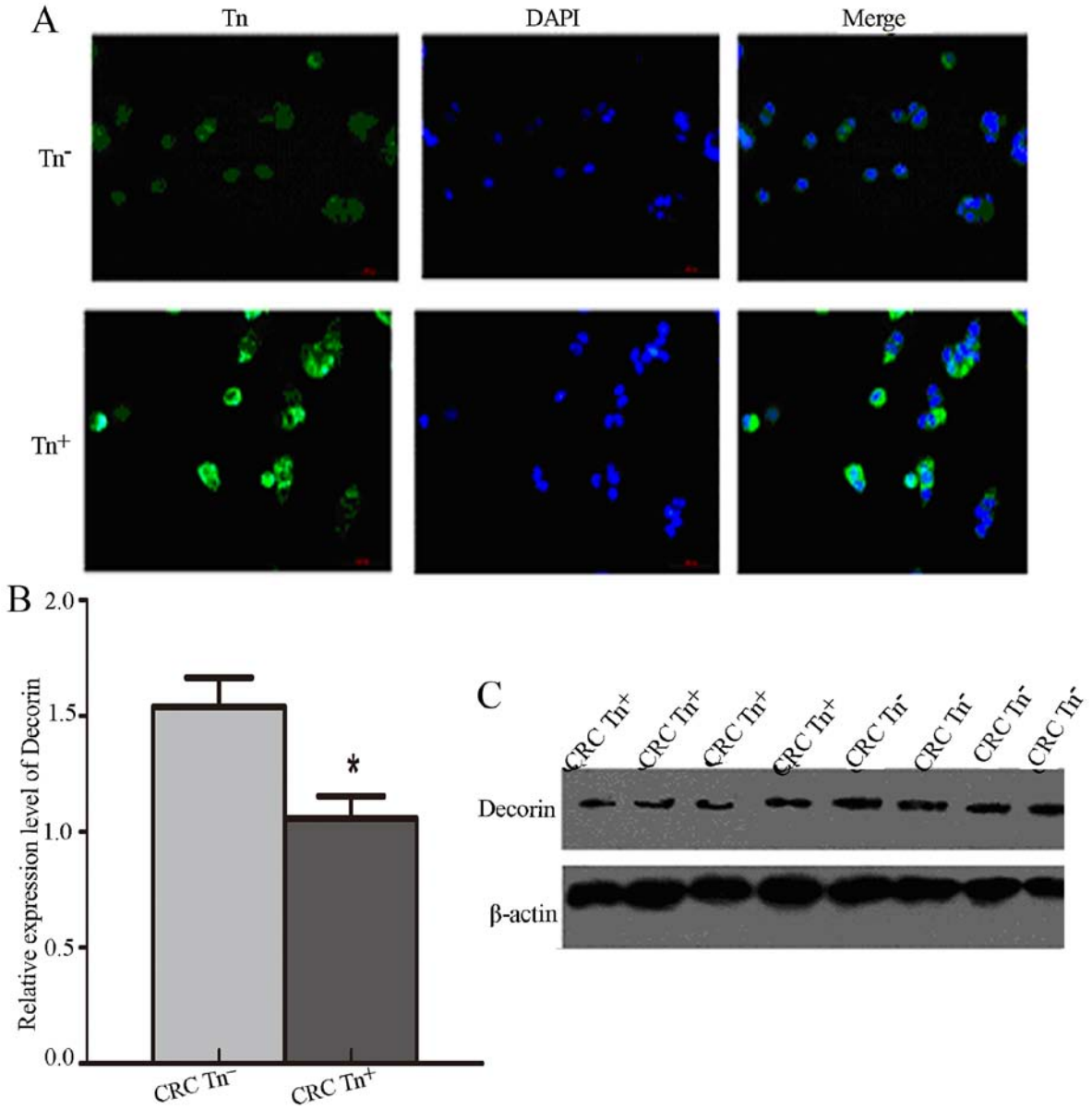

Figure 4. The expression level of the Tn antigen in the LS174T cells and the decreased expression of Decorin in the CRC Tn$^+$ cells. (A) The expression levels of the Tn antigen in the CRC Tn$^+$ and the Tn$^-$ cells (x200). The nuclei were stained blue, while the Tn antigen is green. The Tn antigen was not only detected in the nucleus but also in the cytoplasm. The Tn antigen (green) expression level was higher in the CRC Tn$^+$ than in the CRC Tn$^-$ cells, while the nuclear expression level (green) was the same in both cell types. (B) Decreased expression of Decorin in the CRC Tn$^+$ cells compared with the CRC Tn$^-$ cells. Protein expression analysis from CRC cells using a t-test. *Significantly different ($P<0.05$). (C) Western blotting shows changes in the Decorin expression levels in the CRC Tn$^+$ and Tn$^-$ cells. CRC Tn$^+$, Tn$^+$ CRC cells; CRC Tn$^-$, Tn$^-$ CRC cells.

between CRC Tn$^+$ and CRC Tn$^-$ tissues with the iTRAQ-based proteome technique. In the present study, 25 proteins were significantly expressed only in the CRC groups compared with the NC tissues, indicating that these proteins may participate in the development of CRC. The expression of 10 proteins was differently regulated in CRC Tn$^-$ tissues compared with CRC Tn$^+$ tissues but not in NC tissues. Considering that the Tn antigen was expressed mainly in moderately differentiated and not poorly differentiated tumors, we investigated the potential effects of these 10 proteins on the evaluation of the prognosis of carcinoma. Thirteen proteins were significantly differentially regulated among three groups, demonstrating the potential role of these 13 proteins in the initiation and progression of CRC.

Given that the Tn antigen is related to the disarrangement of *O*-glycosylation, we chose 12 differentially expressed *O*-glycosylated proteins for further study. Among these *O*-glycoproteins, Keratin 8, Keratin 18, Decorin, Sorbin, SORBS1 and CD44 antigen were reported to correlate with the initiation and progression of CRC. As yet, few studies have investigated the relationship between SORBS1, Decorin, the Tn antigen and the development of CRC (30,31).

SORBS1 belongs to a growing family of proteins containing a Sorbin homology (SoHo) domain and three SH3 domains in the C-terminal region. It plays an important role in adhesion between cells and the matrix via the SH3 domain (32,33). A study showed that c-Abl kinase, the product of the c-abl proto-oncogene, mediated the effects of cell adhesion on

Table V. KEGG pathway analysis of *O*-glycosylated proteins.

KEGG-controlled vocabularies	KEGG no.	Gene	Protein
Focal adhesion	Hsa04510	FN1	Fibronectin
Regulation of actin cytoskeleton	Hsa04810	FN1	Fibronectin
ECG-receptor interaction	Hsa04512	FN1	Fibronectin
Small cell lung cancer	Hsa05222	FN1	Fibronectin
Bacteria invasion of epithelial cells	Hsa05100	FN1	Fibronectin
Amoebiasis	Hsa05146	FN1	Fibronectin
Pathways in cancer	Hsa05200	FN1	Fibronectin
		HSP90AB1	Heat shock protein HSP90-β
Prostate cancer	Hsa05215	HSP90AB1	Heat shock protein HSP90-β
Antigen processing and presentation	Hsa04612	HSP90AB1	Heat shock protein HSP90-β
NOD-like receptor signaling pathway	Hsa04621	HSP90AB1	Heat shock protein HSP90-β
Protein processing in endoplasmic reticulum	Hsa04141	HSP90AB1	Heat shock protein HSP90-β
Progesterone-mediated oocyte maturation	Hsa04914	HSP90AB1	Heat shock protein HSP90-β
Adherens junction	Hsa04520	SORBS1	SORBS1
Insulin signaling pathway	Hsa04910	SORBS1	SORBS1
PPAR signaling pathway	Hsa03320	SORBS1	SORBS1
TGF-β signaling pathway	Hsa04350	DCN	Decorin

Table VI. Expression of Decorin/SORBS1 in different TNM stages and differentiation statuses (mean ± SD).

Tumor features	Samples	Decorin	SORBS1
TNM 0-II[a]	5	1.58±0.05	2.02±0.77
TNM III	4	1.05±0.45	1.38±0.53
Moderately differentiated[b]	6	1.69±0.29	2.23±0.41
Poorly differentiated	3	0.87±0.20	1.07±0.09

[a]Compared with TNM III, the expression of the two proteins was not significantly increased ($P>0.05$); [b]expression of the two proteins was significantly increased in the moderately differentiated group ($P<0.05$).

cell cycle progression or gene expression (34). Another study found that c-Abl could bind the SH3 domain of SORBS1 (30). Considering the abnormal adhesion between cancer cells, we hypothesized that SORBS1 may play a role in preventing CRC metastasis. Furthermore, SORBS1 together with vinculin was found to inhibit cell migration (35). p53 promoted the expression of SORBS1 in EB-1 cells (36,37). As SORBS1 is dependent on p53, mutations which correlate with cancer metastasis via the downregulation of adhesion proteins, we deduced that SORBS1 reduction may participate in the development and progression of carcinoma. In the present study, we found that SORBS1 expression was significantly downregulated in the CRC Tn^+ tissues compared with levels in the CRC Tn^- and the healthy tissues, especially in poorly differentiated tissues. From the GO network, it was observed that SORBS1 also is connected with the proto-oncogene Cbl (38). According to the functional analysis, SORBS1 participates in the PPAR-γ pathway, which has been identified as a 'driver gene' in CRC initiation and progression (39). Thus, we demonstrated that downregulation of SORBS1 plays a potential role in the development of CRC via the PPAR-γ pathway (Fig. 6).

Moreover, Decorin controls the expression of p21, affects the activation of EGFR, promotes the apoptosis of cancer cells, and plays a key role in the immune response (40). Downregulation of Decorin is related to poor prognosis in breast cancer, and a similar role was found in lung cancer and lymphoma (27,41-43). However, highly-expressed Decorin suppressed the development and metastasis of breast cancer (43). Few studies on the relationship between Decorin and CRC have been established. Bi *et al* found that the Decorin expression level was significantly reduced which may lead to tumorigenesis in mice (40). Our results showed that Decorin was decreased in the CRC Tn^+ tissue compared with this level in the CRC Tn^- and the control tissues, supporting the aforementioned conclusion. Furthermore, functional analysis revealed that Decorin is involved in the TGF-β pathway, which has been found to be associated with the onset and progression of intestinal cancer (44-46). In addition, Decorin is linked with JUN, NCAN, SDC1 and FOSL1. NCAN is involved in the modulation of cell adhesion and migration (47), and SDC1 functions as an integral membrane protein and participates in cell proliferation, cell migration and cell-matrix interactions via its receptor for extracellular matrix proteins. Altered SDC1 expression has been detected in several different tumor types (48-50). The FOS gene family consists of four members: FOS, FOSB, FOSL1, and FOSL2. These genes encode leucine zipper proteins that can

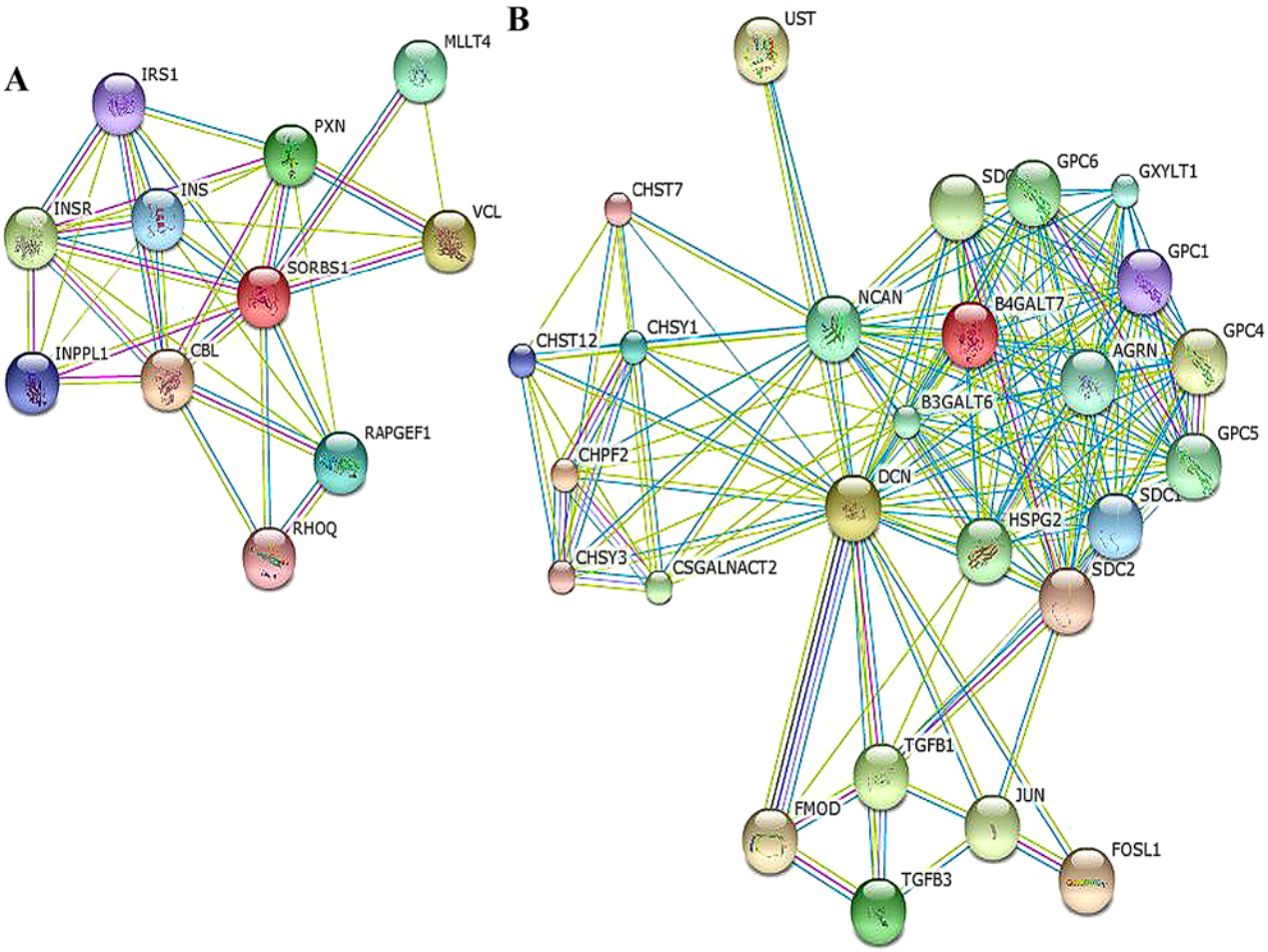

Figure 5. Gene Ontology and pathway analysis of Decorin and SORBS1. (A) The network indicates that SORBS1 is correlated with many molecules that are associated with cell adhesion or migration, such as CBL and PXN. (B) The network shows that Decorin (gene: DCN) participates in the TGF-β pathway via interaction with TGF, and is also linked with JUN, NCAN and other molecules.

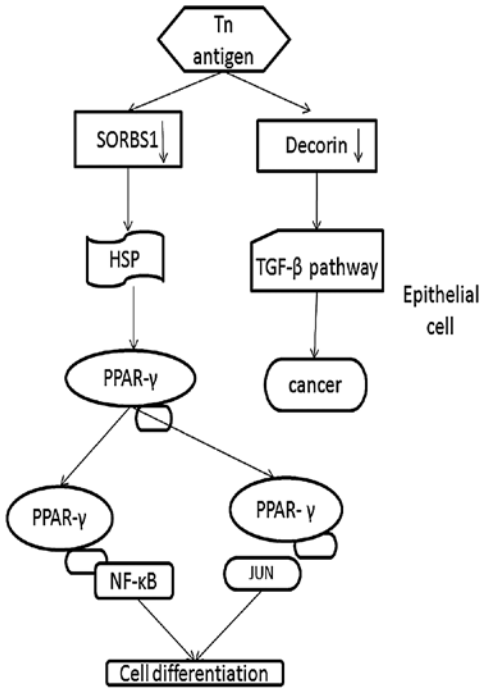

Figure 6. Schematic representation of the functions of Decorin and SORBS1 that are related to their carcinogenic properties via the PPAR-γ and TGF-β pathways.

dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1 (51). As such, the FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation (52,53). Therefore, we conclude that downregulated Decorin could lead to the development of cancer via the TGF-β pathway as our results confirmed (Fig. 6).

To verify our hypothesis that abnormal *O*-glycosylation may control the development of carcinoma via influencing the expression of SORBS1 and Decorin, we also tested their expression *in vitro*. The expression of Decorin was in accordance with the results obtained from western blotting. Notably, SORBS1 was not detected in the Tn^+ colorectal cells. As it is not a secretory protein, it is not possible that it was excreted outside the cell. We speculated that the SORBS1 protein level might be too low to be detected *in vitro*. Further studies with a larger number of samples are needed to clarify the relationship between Decorin, SORBS1 and neoplasm staging.

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