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ORIGINAL ARTICLE

Observational Study

Comprehensive serum proteomics profiles and potential protein biomarkers for the early detection of advanced adenoma and colorectal cancer

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Abstract

BACKGROUND

The majority of colorectal cancer (CRC) cases develop from precursor advanced adenoma (AA). With the development of proteomics technologies, blood protein biomarkers have potential applications in the early screening of AA and CRC in the general population.

AIM

To identify serum protein biomarkers for the early screening of AA and CRC.

METHODS

We collected 43 serum samples from 8 normal controls (NCs), 19 AA patients and 16 CRC patients at China-Japan Friendship Hospital. Quantitative proteomic analysis was performed using liquid chromatography–mass spectrometry/mass spectrometry and data independent acquisition, and differentially expressed proteins (DEPs) with *P*-values < 0.05 and absolute fold changes > 1.5 were screened out, followed by bioinformatics analysis. Prognosis was further analyzed based on public databases, and proteins expression in tissues were validated by immunohistochemistry.

RESULTS

A total of 2132 proteins and 17365 peptides were identified in the serum samples. There were 459 upregulated proteins and 118 downregulated proteins in the NC

vs AA group, 289 and 180 in the NC *vs* CRC group, and 52 and 248 in the AA *vs* CRC group, respectively. Bioinformatic analysis revealed that these DEPs had different functions and participated in extensive signaling pathways. We also identified DIAPH1, VASP, RAB11B, LBP, SAR1A, TUBGCP5, and DOK3 as important proteins for the progression of AA and CRC. Furthermore, VASP ($P < 0.01$), LBP ($P = 0.01$), TUBGCP5 ($P < 0.01$), and DOK3 ($P < 0.01$) were associated with a poor prognosis. In addition, we propose that LBP and VASP may be more promising protein biomarkers for the early screening of colorectal tumors.

CONCLUSION

Our study elucidated the serum proteomic profiles of AA and CRC patients, and the identified proteins, such as LBP and VASP, may contribute to the early detection of AA and CRC.

Key Words: Serum proteomics; Advanced adenoma; Colorectal cancer; Protein biomarker; Early screening

Core Tip: In this study, serum proteomics analysis of advanced adenoma (AA) and colorectal cancer (CRC) was comprehensively performed using liquid chromatography-mass spectrometry/mass spectrometry and data independent acquisition methods, screening for a variety of differentially expressed proteins. Among them, we identified DIAPH1, VASP, RAB11B, LBP, SAR1A, TUBGCP5, and DOK3 as important proteins for the progression of AA and CRC. Furthermore, LBP, VASP, TUBGCP5, and DOK3 were associated with a poor prognosis. In addition, we proposed that LBP and VASP may be more promising protein biomarkers for the early screening of colorectal tumors.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the world and the second leading cause of cancer-related death[1]. Over the past few decades, the incidence of early-onset CRC has been increasing in many countries[2,3], and some factors that likely contribute to this phenomenon include dietary changes, obesity, physical inactivity, and environmental factors[4]. The majority of CRCs develop from colorectal adenomas[5], especially advanced adenomas (AAs), which are defined as having any of the following features: a size equal to or greater than 10 mm, high-grade dysplasia, or a ≥ 25% villous component[6]. The accumulation of genetic mutations and epigenetic changes is expected to take 5-10 years[7], providing an ample opportunities for early diagnosis and clinical intervention. According to previous studies, the five-year survival rate of patients with localized CRC after curative surgery is approximately 90%, but it decreases to approximately 65% in patients with regional lymph node metastasis[8]; therefore, early diagnosis and treatment of CRC are key measures for improving patient prognosis.

Currently, there are a range of methods for the early screening of CRC, including fecal occult blood test (FOBT), multitarget stool DNA (mt-sDNA) testing, colonoscopy and sigmoidoscopy[9]; however, each method has advantages and limitations. FOBT and mt-sDNA testing are noninvasive and do not require bowel preparation, but the sensitivities of the FOBT for detecting CRC and precancerous lesions are only 33-75% and 11-25%, respectively[10], whereas the sensitivity of mt-sDNA testing for screening CRC rather than precancerous lesions has greatly increased[11]. Colonoscopy has high sensitivity for detecting both cancerous and precancerous lesions; lesions can be removed at the time of discovery with this approach[12], but the invasive nature of the examination and bowel preparation and manipulation procedures are uncomfortable for patients[13], resulting in low follow-up compliance[14]. Therefore, the optimal screening method should be sensitive, minimally invasive, inexpensive, and able to accurately identify patients who require further colonoscopy.

Blood protein biomarkers have potential for large-scale cancer screening in the general population[15]; one such marker is carcinoembryonic antigen (CEA), but this marker has relatively low sensitivity and specificity for detecting AA and early-stage CRC[16]. With the development of proteomics technologies, some studies have reported potential diagnostic or prognostic biomarkers for CRC in serum or plasma[17,18], but research on AA, an important precursor lesion of cancer, has been limited.

In this study, we aimed to elucidate the serum proteomic profiles of patients with AA and CRC by performing proteomics analysis and identified characteristic proteins from differentially expressed proteins (DEPs) as biomarkers for AA and CRC screening.

MATERIALS AND METHODS

Study population

Between June 2023 and August 2023, we recruited 43 participants at China-Japan Friendship Hospital, including 19 AA patients, 16 CRC patients, and 8 normal controls (NCs) without colorectal neoplasms. The final clinical diagnoses of AA and CRC were confirmed by pathology reports. Patients with familial adenomatous polyposis, Lynch syndrome, or inflammatory bowel disease; patients who had received treatments, including chemoradiotherapy, targeted therapy, and immunosuppressive therapy; and patients with missing data were excluded from the study. We collected clinicopathological information, such as sex, age, neoplasia size and location, pathology, and TNM stage, from medical records. This study was approved by the Ethics Committee of China-Japan Friendship Hospital.

Sample collection and preparation

Fresh whole blood samples were collected from NCs, AA patients and CRC patients before surgery and centrifuged at 2-8 °C and 3000 r/min for 15 min at room temperature. The supernatant serum was collected into 2 mL freezing tubes and transferred to a -80 °C refrigerator for storage.

The sample preparation steps included low-abundance protein enrichment, protein denaturation, reduction, and alkylation as well as the digestion and peptide cleanup. Briefly, 1 mg of PuriMag magnetic beads (PuriMag Biotech, Xiamen, China) was diluted with 100 μL of wash buffer (10 mmol/L Tris (pH = 7.4), 150 mmol/L KCl, 0.05% CHAPS), and then 100 μL of serum was added. Then, the mixture was incubated at 37 °C for 1 h at 1000 rpm. After incubation, the beads were collected by the magnetic separation device and further washed with 300 μL of wash buffer three times with the magnetic separation device. The precipitate was resuspended in 40 μL of Lyse buffer (0.1 M urea, 5 mmol/L TCEP, 10 mmol/L CAA) and heated at 95 °C for 5 min at 1000 rpm with agitation. After cooling to room temperature, Lys-C and trypsin solution were added, and the sample was incubated at 37 °C for 2 h at 500 rpm with shaking. The digestion process was stopped with 10% TFA. Sample clean-up and desalting were carried out by a C18 peptide cleaning column. Peptides were eluted twice with 30 μL of elution buffer (90% ACN, 0.2% TFA) and then dried in a speed vacuum concentrator.

Liquid chromatography-mass spectrometry analysis

The UltiMate 3000 (Thermo Fisher Scientific, MA, United States) liquid chromatography system was connected to a timsTOF Pro 2 ion-mobility spectrometry quadrupole time-of-flight mass spectrometer (Bruker Daltonics). The samples were reconstituted in 0.1% FA, and 200 ng of peptide was separated by an AUR3-15075C18 column (15 cm length, 75 μm inner diameter, 1.7 μm particle size, 120 Å pore size, IonOpticks) with a 60 min gradient starting with 4% buffer B (80% ACN with 0.1% FA) followed by a stepwise increase to 28% in 25 min, 44% in 10 min, 90% in 10 min, a static step for 7 min, and then equilibration at 4% for 8 minutes. The column flow rate was maintained at 400 nL/min with a column temperature of 50 °C.

Data independent acquisition (DIA) data were acquired in the diaPASEF mode. We defined 22 × 40 Th precursor isolation windows from m/z 349 to 1229. During PASEF MS/MS scanning, the collision energy increased linearly as a function of the mobility from 59 eV at 1/K0 = 1.6 Vs/cm^2 to 20 eV at 1/K0 = 0.6 Vs/cm^2.

Protein identification

The DIA data were processed and analyzed by Spectronaut 18 (Biognosys AG, Switzerland) with the default settings. The database was Homo_sapiens (version 2022, 20610 entries), which was downloaded from UniPort, and specific trypsin sequences were used as the digestion type and digestion enzyme. Carbamidomethyl on cysteine was specified as the fixed modification. Oxidation of methionine was specified as the variable modification. The retention time prediction type was set to dynamic iRT. Data extraction was determined by Spectronaut based on extensive mass calibration. Spectronaut can dynamically determine the ideal extraction window depending on iRT calibration and gradient stability. The Q-value (FDR) cutoff at the precursor level was 1%, and that at the protein level was 1%. Decoy generation was set to mutated, which is similar to scrambled generation but will only apply a random number of AA position swamps (min=2, max=length/2). The normalization strategy was set to local normalization. Peptides that passed the 1% q-value cutoff were used to calculate the major group quantities *via* the MaxLFQ method.

Immunohistochemistry

Paraffin blocks were cut into 4-μm-thick sections, and the sections were sequentially placed in dewaxing solution I for 10 min, dewaxing solution II for 10 min, dewaxing solution III for 10 min, and anhydrous ethanol I for 5 min, anhydrous ethanol II for 5 min, anhydrous ethanol III for 5 min and then washed with distilled water. The slides were placed in a repair box filled with EDTA antigen repair buffer (pH 9.0) in a microwave oven for antigen repair, and after natural cooling, the slides were placed in phosphate-buffered saline (PBS) (pH = 7.4) on a decolorization shaker for 3 washes, each lasting 5 min. The sections were placed in 3% hydrogen peroxide solution, incubated for 25 min at room temperature and protected from light, and the slides were placed in PBS (pH = 7.4) on a decolorizing shaker for 3 washes, each lasting 5 min. The tissue slides were covered uniformly with 3% BSA in a dropwise manner and then blocked at room temperature for 30 min. The blocking solution was removed, and an LBP antibody (Immunoway, 1:50) or VASP antibody (Immunoway, 1:100) was added to the sections. The sections were incubated flat in a wet box overnight at 4 °C. After the slides were washed and shaken, we added HRP-conjugated goat anti-rabbit IgG and incubated them at room temperature for 50 minutes. After washing and shaking the slides dry, freshly prepared DAB color development solution was added dropwise, the color development time was controlled under the microscope, and the positive color was brownish yellow.

Table 1 Basic characteristics of the study population, *n* (%)

	NC (*n* = 8)	AA (*n* = 19)	CRC (*n* = 16)
Sex			
Male	4 (50.0)	12 (63.2)	11 (68.7)
Female	4 (50.0)	7 (36.8)	5 (31.3)
Age (yr), mean ± SD	54.5 ± 10.5	57.1 ± 11.6	59.6 ± 10.1
Diameter size (cm), mean ± SD	-	2.4 ± 1.0	4.7 ± 1.5
Location			
Proximal colon	-	8 (42.1)	4 (25.0)
Distal colon	-	7 (36.8)	4 (25.0)
Rectum	-	4 (21.1)	8 (50.0)
Stage distribution			
Early stage (I/II)	-	-	8 (50.0)
Late stage (III/IV)	-	-	8 (50.0)

AA: Advanced adenoma; CRC: Colorectal cancer; NC: Normal control.

Hematoxylin was used to restrain the nuclei for approximately 3 min, and the sections were subsequently washed with tap water. Finally, microscopic examination was performed, and images were acquired for analysis.

Statistical analysis

All the statistical analyses were conducted using R version 4.2.2 and SPSS version 26.0, with the use of relevant software packages such as mixOmics, ggplot2, and pheatmap. Quality control showed good stability and excellent accuracy of the proteomics results (Supplementary Figure 1). To screen for DEPs, the average of the relative quantitative values of each group of samples for each protein in the pairwise comparisons was calculated, and the ratio of the mean values of each group of samples in the pairwise comparisons was the fold change (FC). Student's *t* test was used to calculate the p value to judge the significance of the difference. In this study, a *P*-value < 0.05 and absolute FC > 1.5 were selected as the screening criteria for DEPs. All DEPs identified were subjected to enrichment analyses, such as Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses, to test whether the proteins were significantly enriched in particular functional categories. Genes with |log2FC| values > 1 and *P*-values < 0.01 were considered differentially expressed.

The Kaplan-Meier curves and the log rank test were generated by Kaplan-Meier Plotter (https://kmplot.com/analysis/index.php?p=background), and this tool was applied to assess the correlation between the expression of genes and overall survival (OS).

Quantitative analysis of the immunohistochemistry images was performed using ImageJ (2.14.0) software. Five fields of view were randomly selected after magnification of each section at 200 × to assess the integrated optical density (IOD). The average optical density (AOD) was calculated by dividing the IOD by the area.

RESULTS

From June 2023 to August 2023, we collected 43 serum samples from 19 AA patients, 16 CRC patients, and 8 healthy subjects at China-Japan Friendship Hospital. There were no statistically significant differences in sex or age distribution among the three groups. The basic characteristics of the study population are summarized in Table 1.

Proteomic signatures

We identified a total of 2132 proteins and 17365 peptides in the serum samples. In the comparison of the NC and AA groups, partial least squares discriminant analysis (PLS-DA) indicated a separation of clusters for samples from the two groups (Figure 1A), and we identified 577 DEPs (*P*-value < 0.05 and |FC| > 1.5), 459 of which were upregulated and 118 of which were downregulated in the AA group, as shown in the volcano plot and heatmap (Figure 1B and C). Further functional annotation and enrichment analysis of the DEPs were carried out. GO analysis revealed that the DEPs in this group were enriched mainly in biological processes (BPs), such as fatty acid metabolic process, lipid catabolic process, and alpha-amino acid metabolic process; molecular functions (MFs), such as cell adhesion molecule binding, cadherin binding, and phospholipid binding; and cellular components (CCs), such as vesicle lumen, cytoplasmic vesicle lumen, and extracellular matrix (Figure 2A). KEGG enrichment analysis indicated that DEPs were mainly enriched in pathways related to metabolic pathways, carbon metabolism, and ribosomes (Figure 2B).

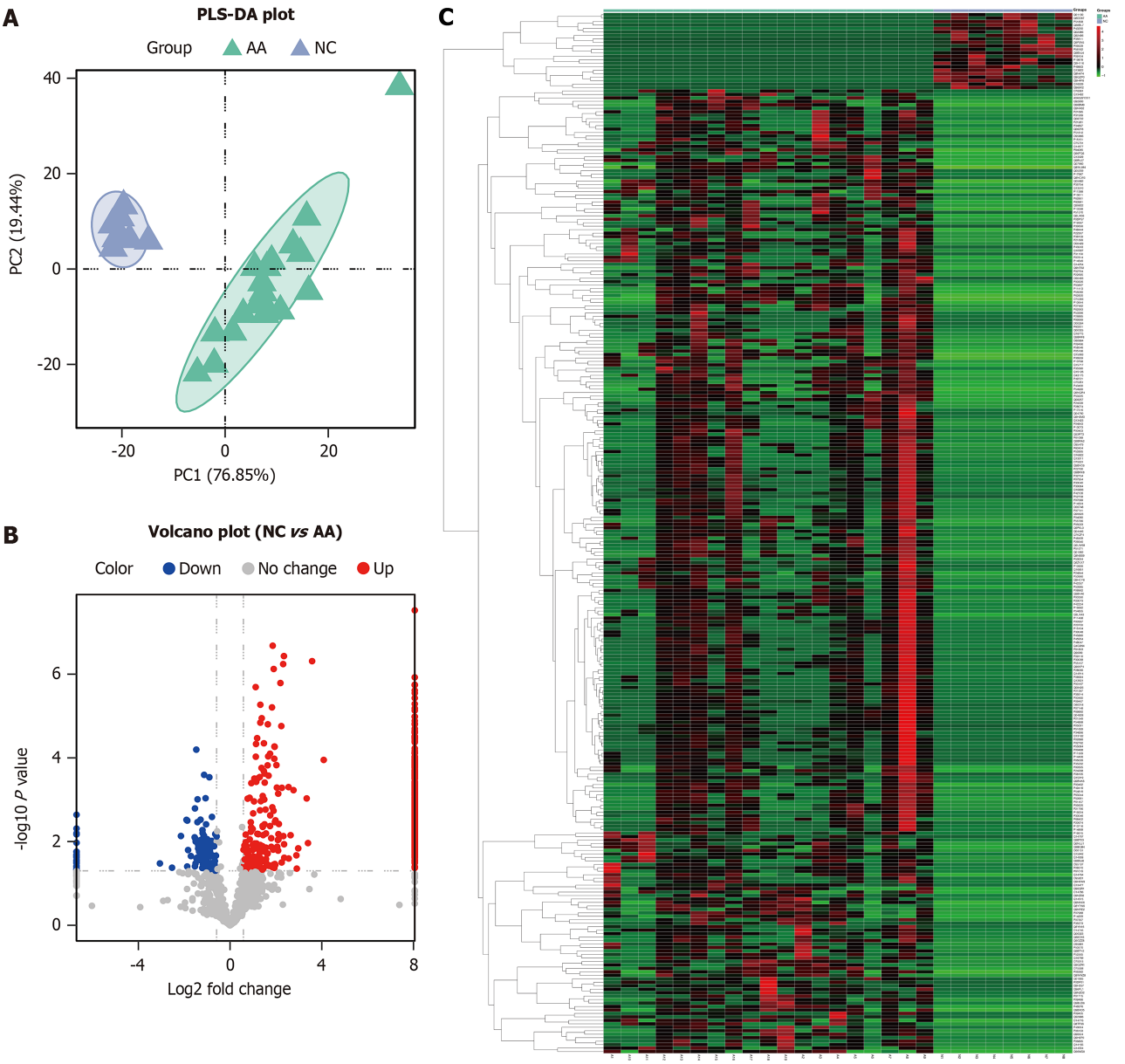

Figure 1 Partial least squares discrimination analysis plot, volcano plot, and heatmap between the normal control group and advanced adenoma group. A: Partial least squares discrimination analysis shows the protein expression in the normal control group (blue) and advanced adenoma group (green); B: Volcano plot shows the fold change of the identified proteins between the two groups; C: Hierarchical clustering analysis shows the upregulated (red) and downregulated (green) differentially expressed proteins between the two groups. NC: Normal control; AA: Advanced adenoma; PLS-DA: Partial least squares discrimination analysis.

PLS-DA clearly distinguished the NC group from the CRC group (Figure 3A). The volcano plot showed 469 DEPs between the two groups, with 289 upregulated and 180 downregulated in the CRC group (Figure 3B). Hierarchical clustering and heatmaps were generated to visualize the expression of the top 300 DEPs (Figure 3C). Furthermore, GO and KEGG enrichment analyses were performed on the DEPs. GO analysis indicated that the DEPs were enriched in BPs such as amide biosynthetic process, cellular macromolecule biosynthetic process and translation; MFs such as protein binding, organic cyclic compound binding, and RNA binding; and CCs such as secretory granule lumen, ribosome, and cytosolic ribosome (Figure 4A). The metabolic pathways, ribosome, endocytosis, spliceosome, and proteoglycans in cancer were significantly enriched according to the KEGG analysis (Figure 4B).

PLS-DA also revealed distinct clusters for the AA and CRC samples, with a total of 300 DEPs between the two groups; 52 proteins were upregulated, and 248 proteins were downregulated in the CRC group (Figure 5A and B). We performed hierarchical clustering analysis, and the results are shown in a heatmap (Figure 5C). Bioinformatics analysis, including GO and KEGG enrichment, was used for further analysis of the DEPs. According to GO analysis, the DEPs were enriched in BPs such as amide biosynthetic process, fatty acid metabolic process, and nucleobase-containing small molecule metabolic process; MFs such as protein binding, small molecule binding, and anion binding; and CCs such as extracellular exosome, membrane-enclosed lumen, and organelle lumen (Figure 6A). According to KEGG analysis, the DEPs were mainly enriched in metabolic pathways, carbon metabolism, ribosomes, and some disease pathways (Figure 6B).

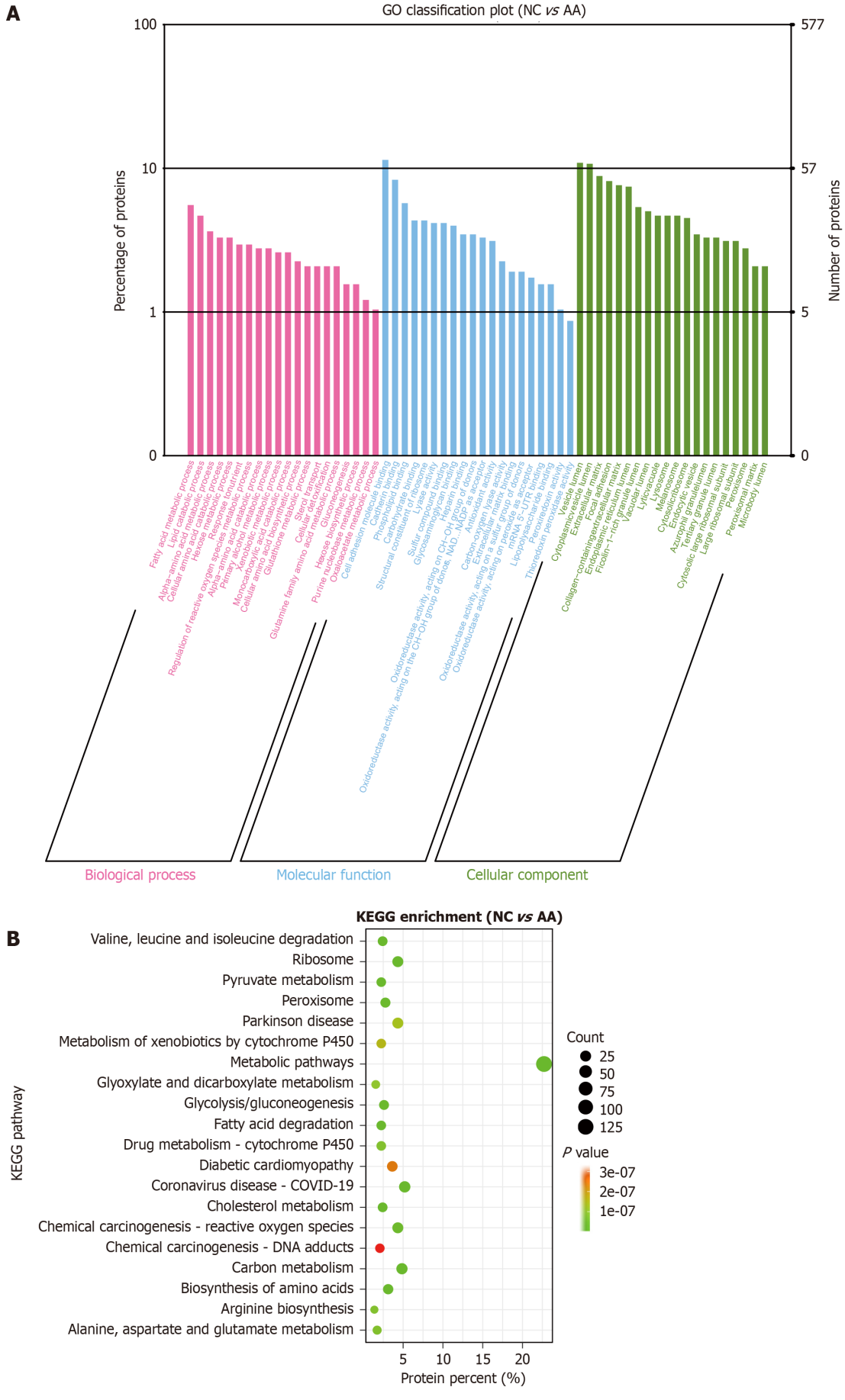

Figure 2 Gene Ontology enrichment and Kyoto Encyclopedia of Genes and Genomes enrichment analysis of differentially expressed

protein between the normal control group and advanced adenoma group. A: Gene Ontology enrichment analysis: differentially expressed proteins (DEPs) are mainly enriched in fatty acid metabolism process, cell adhesion molecule binding, and vesicle lumen; B: Kyoto Encyclopedia of Genes and Genomes enrichment analysis: DEPs are mainly enriched in metabolic pathways and ribosomes. NC: Normal control; AA: Advanced adenoma; DEPs: Differentially expressed proteins; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

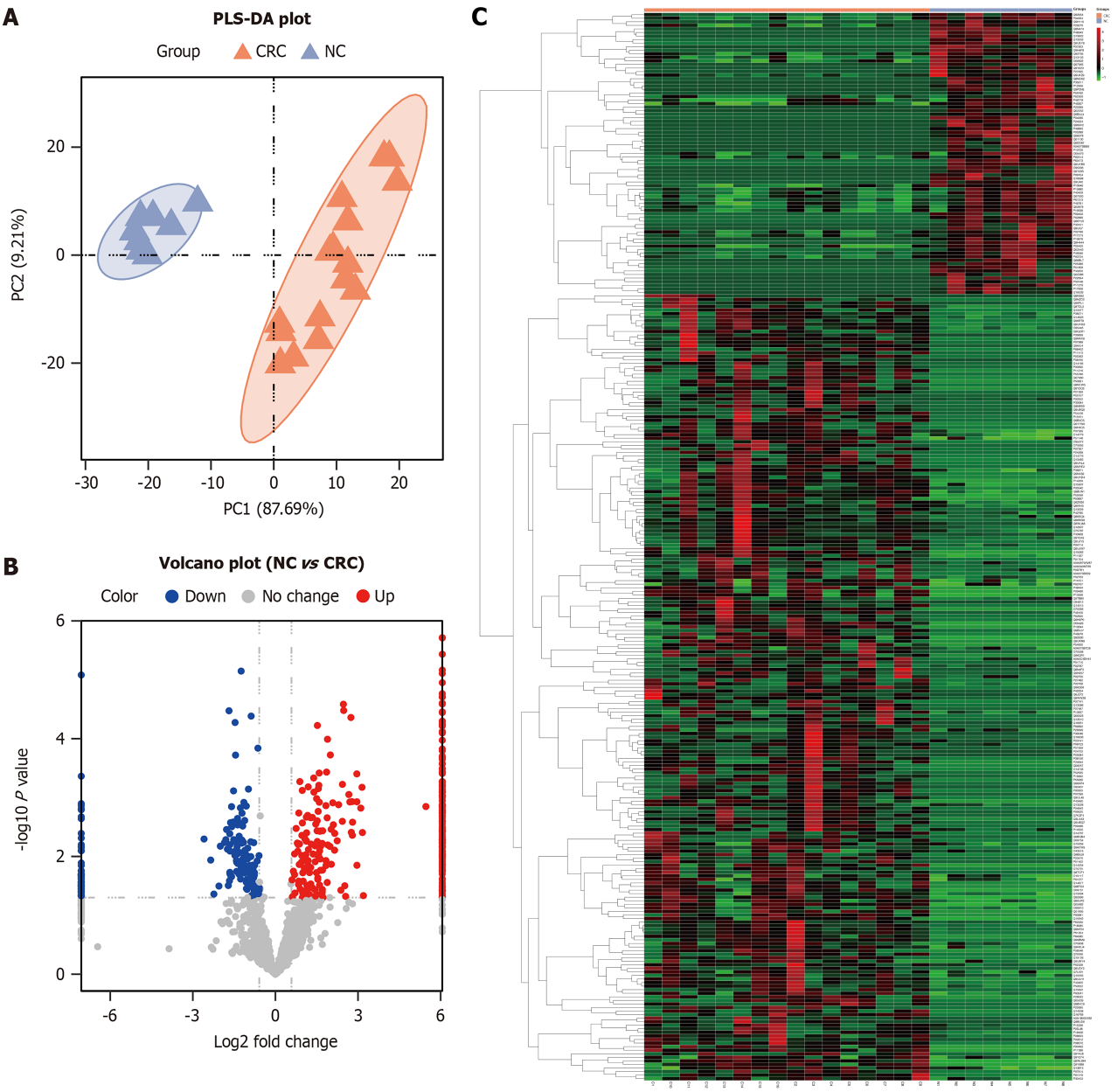

Figure 3 Partial least squares discrimination analysis plot, volcano plot, and heatmap between the normal control group and colorectal cancer group. A: Partial least squares discrimination analysis shows the protein expression in the normal control group (blue) and colorectal cancer group (orange); B: Volcano plot shows the fold change of the identified proteins between the two groups; C: Hierarchical clustering analysis shows the upregulated (red) and downregulated (green) partial least squares discrimination analysis between the two groups. NC: Normal control; CRC: Colorectal cancer; PLS-DA: Partial least squares discrimination analysis.

Potential protein biomarkers for AA and CRC

After elucidating the serum proteomic profiles of AA and CRC patients, we identified potentially characteristic DEPs as biomarkers by generating a Venn diagram (Figure 7A). Ideally, the characteristic proteins should exhibit low expression or no expression in the NC group and high expression in the AA and CRC groups, and show a consistent trend of change during the disease progression of "normal-adenoma-carcinoma". As shown in Figure 7A, there were 21 proteins at the intersection of the three comparison groups. Comparing their relative levels in the NC, AA, and CRC groups, we found that only 7 proteins gradually increased along the disease progression axis of “normal-adenoma-carcinoma”, including DIAPH1, VASP, RAB11B, LBP, SAR1A, TUBGCP5, and DOK3; these findings indicate that these proteins could be used

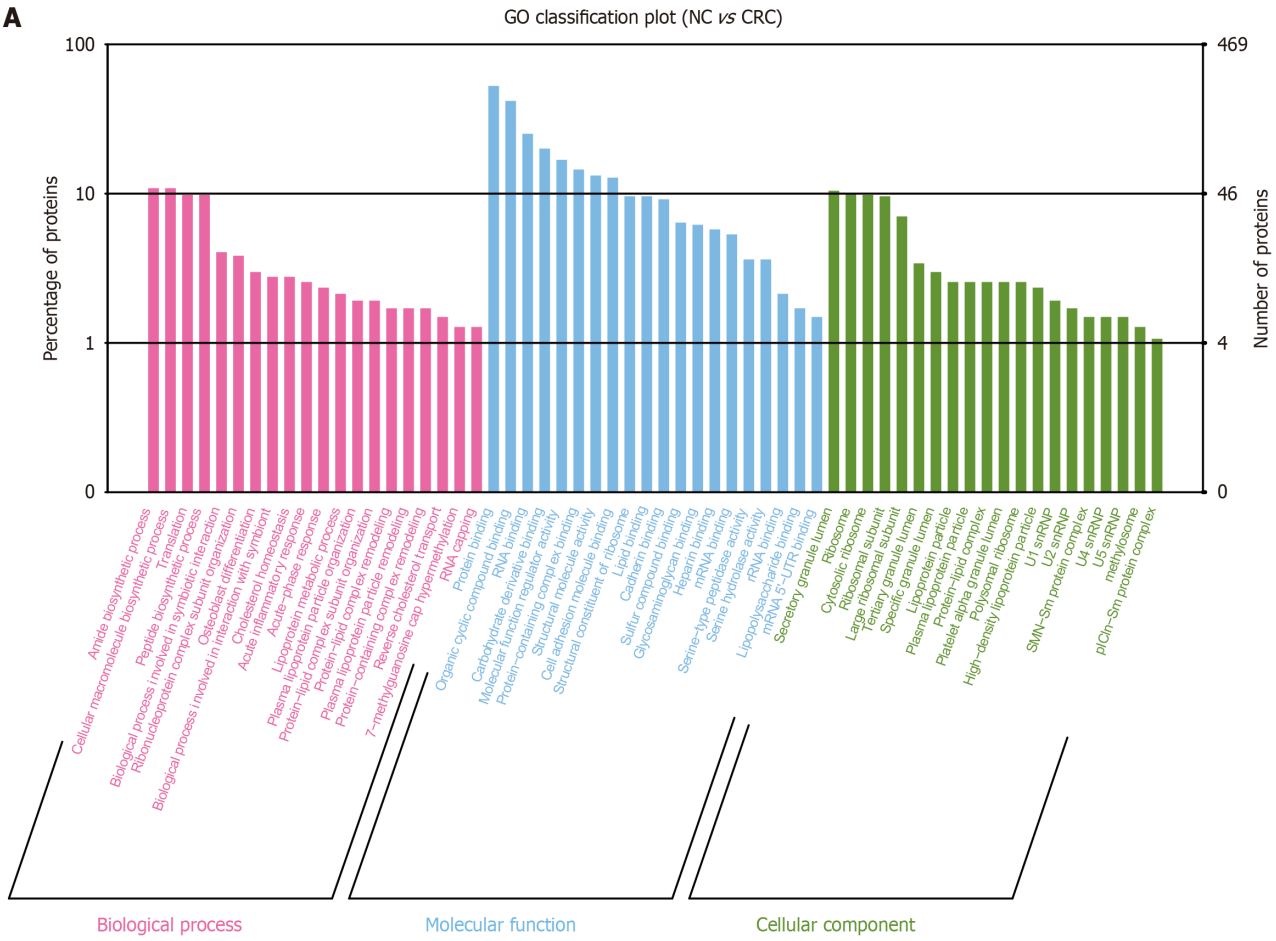

Figure 4 Gene Ontology enrichment and Kyoto Encyclopedia of Genes and Genomes enrichment analysis of differentially expressed proteins between the normal control group and colorectal cancer group. A: Gene Ontology enrichment analysis: Differentially expressed proteins (DEPs) are mainly enriched in amide biosynthetic process, protein binding, and secretory granule lumen; B: Kyoto Encyclopedia of Genes and Genomes enrichment analysis: DEPs are mainly enriched in metabolic pathways, ribosomes, and endocytosis. NC: Normal control; CRC: Colorectal cancer; DEPs: Differentially expressed proteins; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

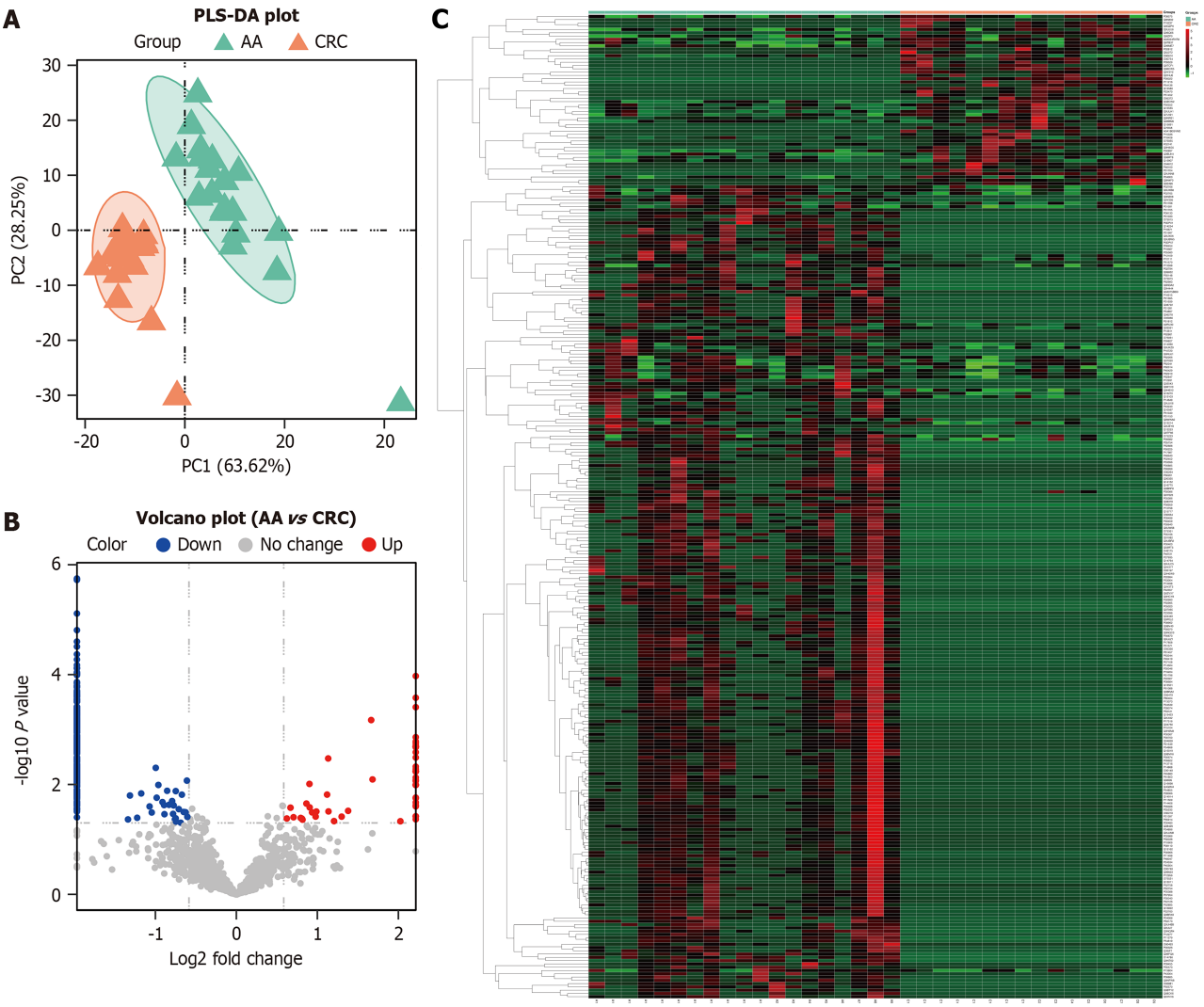

Figure 5 Partial least squares discrimination analysis plot, volcano plot, and heatmap between the advanced adenoma group and colorectal cancer group. A: Partial least squares discrimination analysis shows the protein expression in the advanced adenoma group (green) and colorectal cancer group (orange); B: Volcano plot shows the fold change of the identified proteins between the two groups; C: Hierarchical clustering analysis shows the upregulated (red) and downregulated (green) differentially expressed proteins between the two groups. AA: Advanced adenoma; CRC: Colorectal cancer; PLS-DA: Partial least squares discrimination analysis.

for early serum screening in AA and CRC patients. The relative levels of these seven proteins in AA and CRC samples compared with healthy subject samples are presented in Figure 7B-H, and there were significant differences between all three groups.

Based on the UniProt database, we summarized the biological processes and molecular functions of the above seven protein biomarkers. As shown in Table 2, these genes were enriched in a wide variety of biological processes, including actin cytoskeleton and microtubule organization, acute-phase response, secretion, signal transduction, *etc.* In addition, the biomarkers appeared to be primarily associated with protein binding molecular functions.

Survival analysis

For survival analysis, we applied the online tool Kaplan-Meier Plotter to plot the 5-year OS of patients grouped based on the expression levels of seven proteins. Patients with high expression of the four proteins [VASP ($P < 0.01$), LBP ($P = 0.01$), TUBGCP5 ($P < 0.01$), and DOK3 ($P < 0.01$)] had significantly lower OS rates, indicating that these proteins may serve as prognostic biomarkers and potential therapeutic targets, whereas the expression of DIAPH1, RAB11B, and SAR1A did not appear to be associated with prognosis (Figure 8). Based on the protein function, impact on prognosis, and availability of antibodies, we selected LBP and VASP from the seven potential characteristic proteins for validation in AA and CRC tissues.

Immunohistochemistry

A total of 9 tissue samples from healthy controls, 26 tissue samples from AA patients, and 27 tissue samples from CRC patients were collected.

Table 2 Functions of potential protein biomarkers

Biomarkers	Protein names	Uniprot ID	Biological process	Molecular function
DIAPH1	Protein diaphanous homolog 1	O60610	Actin cytoskeleton organization, cytoskeleton organization	Actin binding, RNA binding, signaling receptor binding
VASP	Vasodilator-stimulated phosphoprotein	P50552	Actin cytoskeleton organization	Actin binding, Cadherin binding
RAB11B	Ras-related protein Rab-11B	Q15907	Constitutive secretory pathway, endocytic recycling	Cadherin binding, GTP binding
LBP	Lipopolysaccharide-binding protein	P18428	Acute-phase response	Lipopeptide binding
SAR1A	GTP-binding protein SAR1a	Q9NR31	COPII-coated vesicle cargo loading	GTP binding, GTPase activity
TUBGCP5	Gamma-tubulin complex component 5	Q96RT8	Cytoplasmic microtubule organization	Gamma-tubulin binding, microtubule binding
DOK3	Docking protein 3	Q7L591	Ras protein signal transduction	-

Immunohistochemical staining revealed that the positive expression of the LBP protein was mainly located in the cytoplasm. In the AA and CRC tissues, LBP protein exhibited predominantly dark brown high expression (Figure 9B and C), while in normal colon mucosa, it showed no expression or low expression (Figure 9A). Moreover, the protein expression of LBP gradually increased among the three groups, and there were statistically significant differences among the three groups ($P < 0.001$; Figure 9D). Similarly, the positive expression of VASP protein was also mainly located in the cytoplasm. In normal colon mucosa tissue, VASP protein was not expressed or was expressed at low levels (Figure 9E), while in AA and CRC tissues, it was highly expressed (Figure 9F and G). By calculating the AOD values from the images, we found that the expression of VASP protein gradually increased in normal tissue, AA tissue, and CRC tissue, with statistically significant differences among the three groups ($P < 0.001$; Figure 9H).

DISCUSSION

Although previous multiomics studies of CRC have been carried out, there have been limited studies on the serum proteomes of patients with AA and CRC. In this study, 43 serum samples from 19 patients with AA, 16 patients with CRC, and 8 healthy subjects were collected for proteomic analysis, and a total of 2132 proteins were identified. Through pair-by-pair comparisons of the NC, AA, and CRC groups, we characterized their DEPs and performed bioinformatics analysis. Upon further analysis, seven proteins, DIAPH1, VASP, RAB11B, LBP, SAR1A, TUBGCP5, and DOK3, were found to be progressively elevated from normal samples to adenoma samples to adenocarcinoma samples. These findings indicate that these proteins might be serum screening biomarkers for AA and CRC. In addition, we found that LBP, VASP, TUBGCP5, and DOK3 were associated with a poor prognosis. The protein expression levels of LBP and VASP were significantly greater in the tissues of patients with AA and CRC than in those of healthy controls. Therefore, we propose that LBP and VASP could serve as potential novel biomarkers for noninvasive screening of early colorectal tumors.

The three groups of proteomic characteristics share similarities; for example, KEGG enrichment analyses revealed that all three groups were enriched in metabolic pathways related to cellular behaviors and ribosomes[19]. Apart from their canonical functions, many metabolic enzymes are also involved in gene expression, cell cycle progression, and DNA repair[20]. Ribosomes play important roles in sustaining tumor cell growth and proliferation to facilitate tumorigenesis [21], and ribosome-targeted therapies are currently a promising approach for cancer treatment[22]. Moreover, the DEPs were mainly enriched in fatty acid metabolic processes and amide biosynthetic processes, which participate in the modulation of CRC cell proliferation[23]. Furthermore, the differences among the three groups were also significant. As reported in the Results section, the greatest number of DEPs was found in the NC group *vs* the AA group, while the lowest number of DEPs was found in the AA group *vs* the CRC group, suggesting that a variety of proteins are regulated in the development of adenomas.

As previously mentioned, LBP and VASP might serve as promising screening biomarkers. LBP is an acute phase protein produced by hepatocytes that contributes to an inflammatory response cascade[24]. Previous studies have shown that serum levels of LBP are elevated in patients with metabolic-related diseases such as obesity, diabetes, and atherosclerosis[25,26], which may be related to chronic inflammation caused by changes in the gut microbiota and endotoxins [27]. Additionally, the protein expression of LBP is significantly increased in various malignant tumors, such as ovarian cancer, renal cancer, and CRC[28-30], but the mechanisms underlying its involvement in tumor initiation and progression remain unclear, and it may be involving the NF-κB signaling pathway[31].

VASP belongs to the Ena/VASP family and is involved in actin dynamics, cell motility and epithelial cell adhesion[32, 33]. It was found to be aberrantly expressed in many malignant diseases, such as gastrointestinal cancer, breast cancer, and hepatocellular carcinoma[34-37], and was linked to a poor prognosis of CRC patients in this study. The mechanism

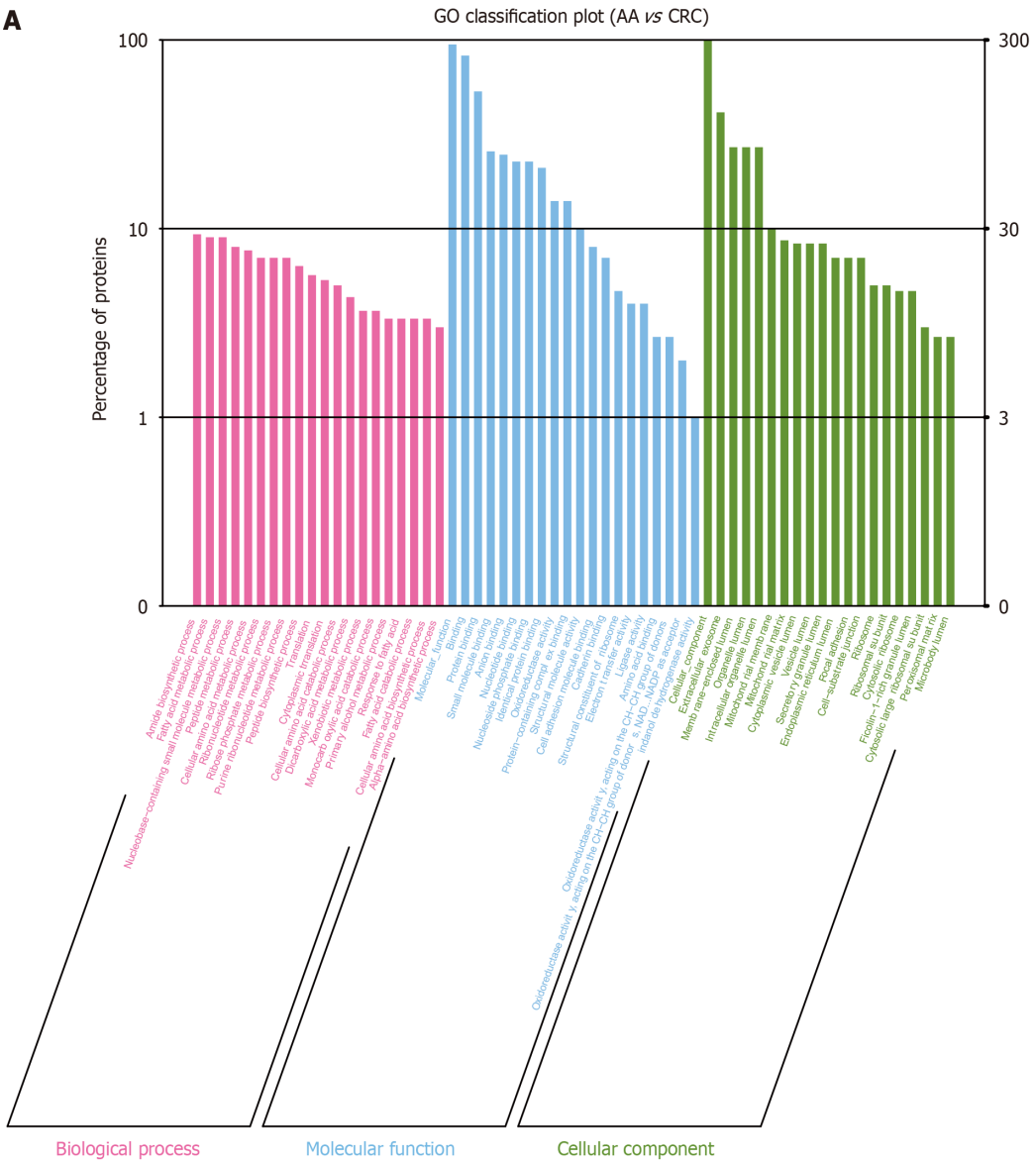

Figure 6 Gene Ontology enrichment and Kyoto Encyclopedia of Genes and Genomes enrichment analysis of differentially expressed

proteins between the advanced adenoma group and colorectal cancer group. A: Gene Ontology enrichment analysis: Differentially expressed proteins (DEPs) are mainly enriched in amide biosynthetic process, protein binding, and extracellular exosome; B: Kyoto Encyclopedia of Genes and Genomes enrichment analysis: DEPs are mainly enriched in metabolic pathways, ribosomes, and PPAR signaling pathway. AA: Advanced adenoma; CRC: Colorectal cancer; DEPs: Differentially expressed proteins; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

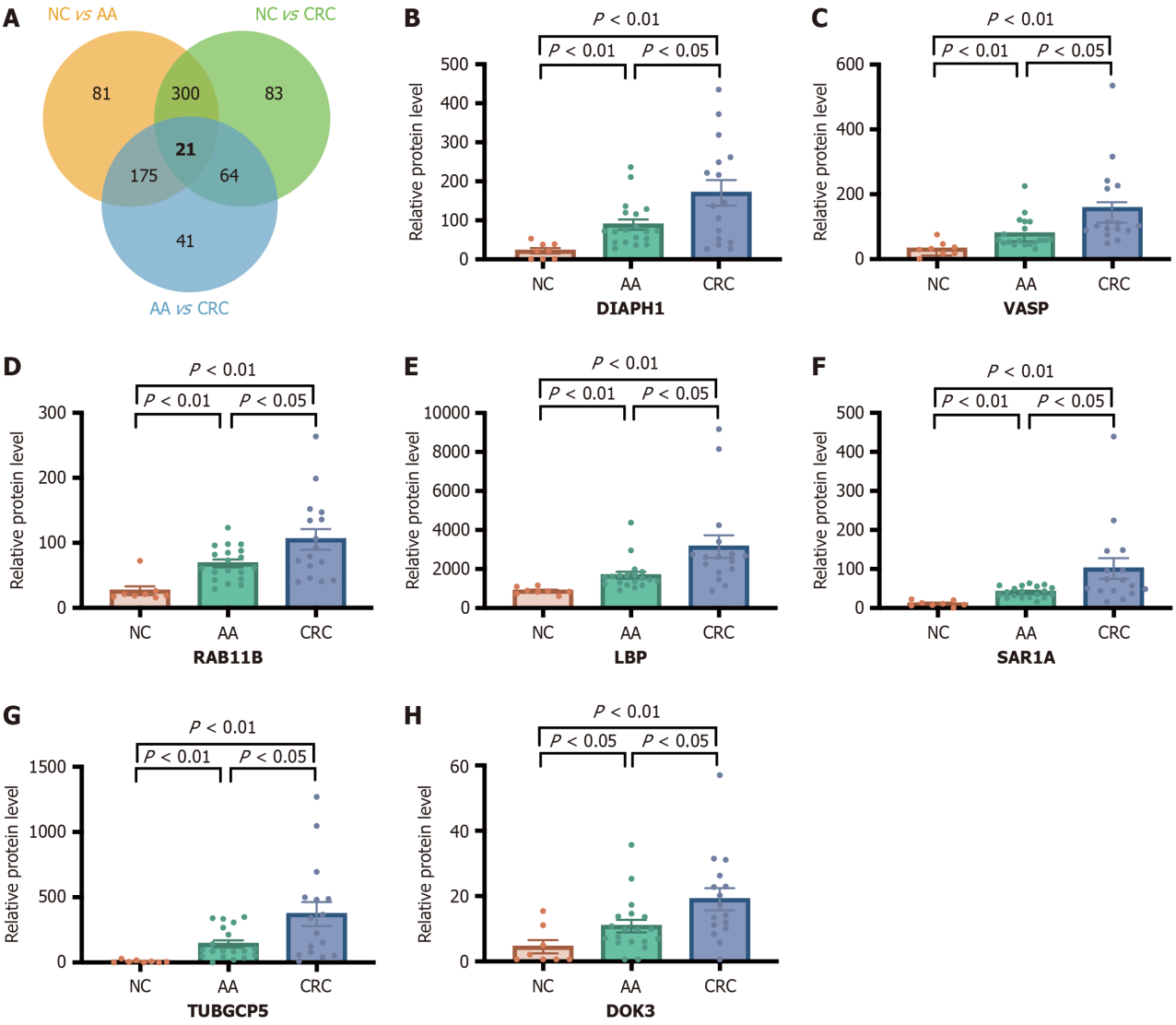

Figure 7 Intersecting differentially expressed proteins of the normal control, advanced adenoma, and colorectal cancer groups and serum relative protein level of seven biomarkers. A: Venn diagram indicates the intersecting differentially expressed proteins of the normal control (NC), advanced adenoma (AA), and colorectal cancer (CRC) groups; B-H: Serum relative protein level of DIAPH1, VASP, RAB11B, LBP, SAR1A, TUBGCP5, and DOK3 in NC, AA, CRC groups. DEPs: Differentially expressed proteins; NC: Normal control; AA: Advanced adenoma; CRC: Colorectal cancer.

may be related to the activation of the PI3K/AKT signaling pathway[35-37]. Since the PI3K/AKT signaling pathway is involved in various processes of tumor cell proliferation, differentiation, migration, and epithelial mesenchymal transition[38], we speculate that the mechanism of occurrence and development of VASP in AA and CRC may be related to this pathway.

DIAPH1 is a member of the diaphanous-related formin subfamily. In recent studies, DIAPH1 was also shown to mediate the formation of focal adhesions and cell spreading by stabilizing microtubules and inducing epithelial-to-mesenchymal transition, migration and invasion through its roles in the actin cytoskeleton[39,40]. It has been identified as a participant in CRC and ovarian cancer[41,42]. Additionally, RAB11B, a component of the RAB11 family, is responsible for regulating the recycling of internalized proteins and transporting them back to the plasma membrane[43]. Moreover, SAR1A is a GTPase and plays a key role in regulating the trafficking of proteins from the endoplasmic reticulum to the Golgi apparatus[44]. TUBGCP5 is a member of the γ-tubulin complex, which is necessary for microtubule nucleation at the centrosome[45]. DOK3, a member of the regulatory protein DOK family, plays an important role in the receptor tyrosine kinase signaling pathway[46], and its expression may predict the response of CRC to chemoradiotherapy[47]; furthermore, TUBGCP5 and DOK3 were found to be associated with poor CRC prognosis in our study.

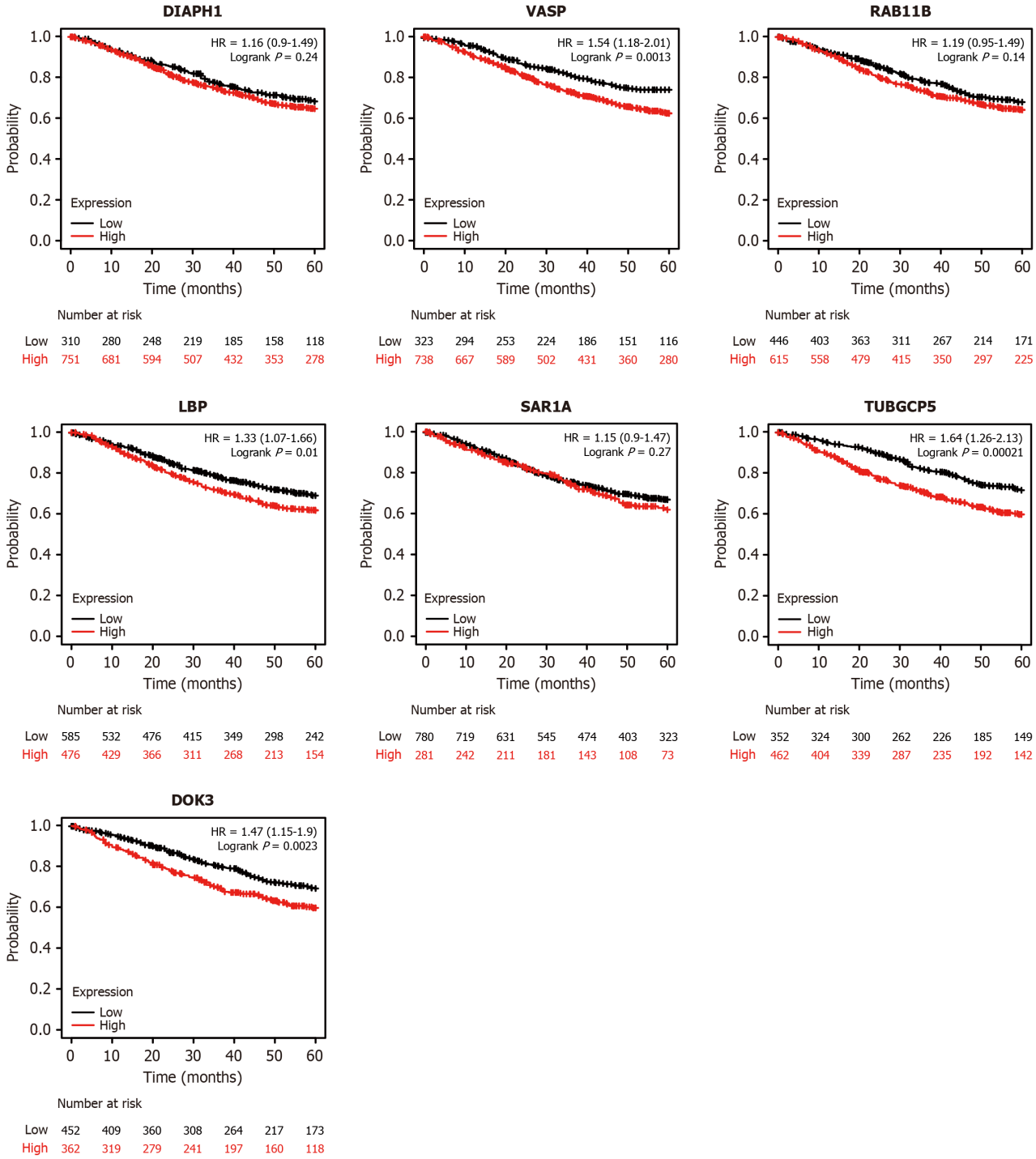

Figure 8 The relationship between seven protein biomarkers and 5-year overall survival in colorectal cancer patients. Patients with high expression of VASP, LBP, TUBGCP5, and DOK3 had significantly lower overall survival rates. OS: Overall survival; CRC: Colorectal cancer.

There are several strengths in our study. Comprehensive serum proteomic profiles of NC, AA, and CRC patients were quantified and analyzed using liquid chromatography-mass spectrometry and 4D DIA methods, and more than 2000 proteins were identified, which is much greater than in previous studies. Although there has been research on blood-based protein biomarkers of CRC, little attention has been given to its precursors, AA. Moreover, after in-depth screening, we identified seven DEPs that may play important roles in the progression of "normal-adenoma-carcinoma". We propose that LBP and VASP may be more promising protein biomarkers for early screening in AA and CRC patients. There are some limitations to our research as well. First, as a single center study, we included only a limited number of patients; thus, we cannot rule out the possibility of selection bias, so we need to validate the findings in a larger sample size in the future. In future studies, it will be possible to further increase the nonadvanced adenoma subgroup and follow up on their recurrence and prognosis outcomes to observe the impact of characteristic proteins on disease progression. Second, the lack of complete genomic and transcriptomic data hinders the integrated study of gene-mRNA-protein interactions. In addition, in the future, we will further analyze the core genes through the PPI network. Third, the protein mechanisms

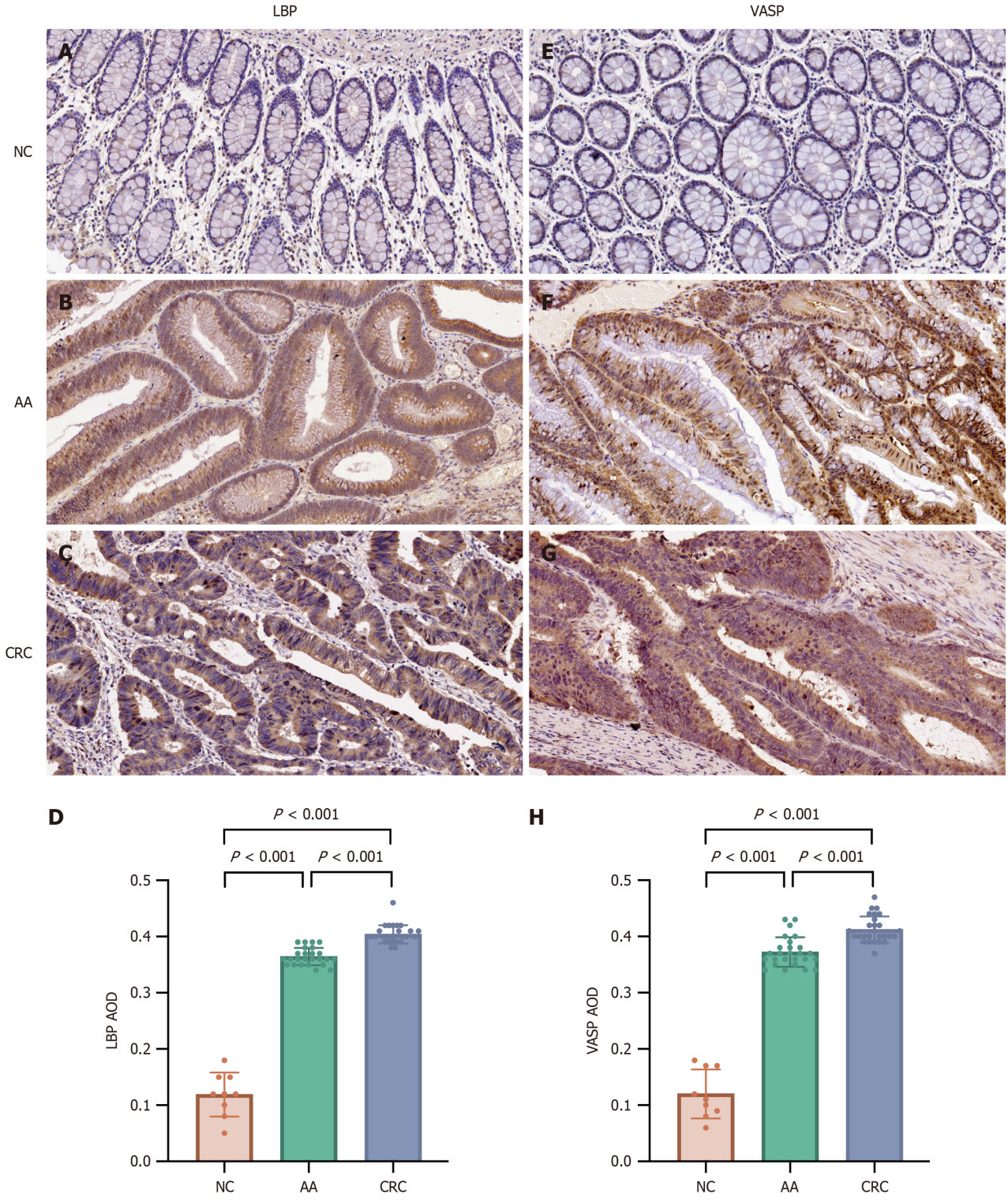

Figure 9 Immunohistochemical staining of LBP protein and VASP protein (200 ×). A-C: LBP is not expressed in normal colon tissues, while highly expressed in advanced adenoma (AA) tissues and colorectal cancer (CRC) tissues; D: Expression levels of LBP protein among the three groups; E-G: VASP is not expressed in normal colon tissues, while highly expressed in AA tissues and CRC tissues; H: Expression levels of VASP protein among the three groups. AA: Advanced adenoma; CRC: Colorectal cancer; AOD: Average optical density; NC: Normal control.

need to be further investigated and validated in CRC tissues. Omics technologies have greatly advanced cancer biomarker research, but much research is still needed before they can be clinically applied.

CONCLUSION

In summary, we comprehensively elucidated the serum proteomic profiles of AA and CRC patients and clarified the

functions of DEPs. We also identified seven proteins, DIAPH1, VASP, RAB11B, LBP, SAR1A, TUBGCP5, and DOK3, which may play important roles in the progression of "normal-adenoma-carcinoma". In addition, we propose that LBP and VASP may be more promising protein biomarkers for the early screening of colorectal tumors.

FOOTNOTES

Author contributions: Tan C conceived the study, collected the samples, prepared the original draft, and reviewed this manuscript; Wang QQ, Li KM and Zhou YC critically participated in the data analysis and manuscript revision. Qin G provided guidance on the study design; Yao SK supervised the study, revised the manuscript, and obtained funding; all authors read and approved the final manuscript.

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