Discovery and verification of gelsolin as a potential biomarker of colorectal adenocarcinoma in a Chinese population: Examining differential protein expression using an iTRAQ labelling-based proteomics approach

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OBJECTIVE: To identify and validate potential biomarkers of colorectal adenocarcinoma using a proteomic approach.
METHODS: Multidimensional liquid chromatography/mass spectrometry was used to analyze biological samples labelled with isobaric mass tags for relative and absolute quantitation to identify differentially expressed proteins in human colorectal adenocarcinoma and paired normal mucosa for the discovery of cancerous biomarkers. Cancerous and noncancerous samples were compared using online and offline separation. Protein identification was performed using mass spectrometry. The downregulation of gelsolin protein in colorectal adenocarcinoma samples was confirmed by Western blot analysis and validated using immunohistochemistry.
RESULTS: A total of 802 nonredundant proteins were identified in colorectal adenocarcinoma samples, 82 of which fell outside the expression range of 0.8 to 1.2, and were considered to be potential cancer-specific proteins. Immunohistochemistry revealed a complete absence of gelsolin expression in 86.89% of samples and a reduction of expression in 13.11% of samples, yielding a sensitivity of 86.89% and a specificity of 100% for distinguishing colorectal adenocarcinoma from normal tissue.
CONCLUSIONS: These findings suggest that decreased expression of gelsolin is a potential biomarker of colorectal adenocarcinoma.

Key Words: *Biomarker; Colorectal cancer; Gelsolin; Proteomic*

La découverte et la vérification de la gelsoline comme biomarqueur potentiel de l'adénocarcinome colorectal au sein d'une population chinoise : l'examen de l'expression protéique différentielle au moyen de la protéomique par marquage iTRAQ

OBJECTIF : Identifier et valider les biomarqueurs potentiels de l'adénocarcinome colorectal au moyen de la protéomique.
MÉTHODOLOGIE : Les chercheurs ont utilisé la chromatographie liquide multidimensionnelle et la spectrométrie de masse pour analyser des échantillons biologiques marqués par masse isobare afin d'obtenir une quantification relative et absolue pour parvenir à la détermination différentielle des protéines exprimées dans les adénocarcinomes colorectaux humains et la muqueuse normale appariée, afin de déceler les biomarqueurs cancéreux. Ils ont comparé des échantillons cancéreux et non cancéreux au moyen d'une séparation virtuelle et réelle. Ils ont identifié les protéines par spectrométrie de masse. Ils ont confirmé la régulation à la baisse de la protéine de gelsoline dans les échantillons d'adénocarcinome colorectal par transfert Western et l'ont validée par immunohistochimie.
RÉSULTATS : Au total, les chercheurs ont repéré 802 protéines non redondantes dans des échantillons d'adénocarcinome colorectal, dont 82 se situaient hors de la plage d'expression de 0,8 à 1,2 et étaient considérées comme des protéines potentiellement spécifiques au cancer. L'immunohistochimie a révélé une absence complète de l'expression de gelsoline dans 86,89 % des échantillons et une réduction de l'expression dans 13,11 % des échantillons, pour une sensibilité de 86,89 % et une spécificité de 100 % à distinguer l'adénocarcinome colorectal des tissus normaux.
CONCLUSIONS : Selon ces observations, une diminution de l'expression de gelsoline serait un biomarqueur potentiel de l'adénocarcinome colorectal.

Approximately 1,529,560 cancer-related deaths are estimated to occur annually in the United States; of these, 142,570 are attributed to colorectal cancer (CRC) (1). Due to early detection, clinical prognoses have improved significantly; however, robust screening tests are needed for all types of gastrointestinal cancer. Although a wide variety of screeening modalities for CRC exist, only 40% of cases are detected at early stages (2). The identification of novel protein biomarkers or therapeutic targets may ultimately improve patient care and survival.

Proteomics combined with mass spectrometry (MS) has become a powerful method for the global examination of proteins in the post-genomics era, enabling the discovery of cancer biomarkers and drug targets. Although transcriptomics provides the tools for unravelling gene expression networks, proteomics links these networks to protein products and provides additional insight into post-translational modifications that regulate cellular functions, thereby complementing genomic analyses. The identification of differentially expressed proteins in CRC using proteomics suggests that expression patterns of proteins may have some utility in the discovery of cancer biomarkers.

Differential tagging with isotopic reagents, such as isotope-coded affinity tags (3), or the more recent variation that uses isobaric tags for relative and absolute quantitation (iTRAQ) reagents followed by multidimensional liquid chromatography (LC) and tandem MS/MS analysis, is emerging as a powerful methodology in the search for disease biomarkers. Many recent studies (4-11) using iTRAQ reagents resulted in the identification and relative quantification of proteins that led to the discovery of potential cancer markers for prostate, radiotherapy-resistant, renal cell, lung, oral, head and neck, breast and

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TABLE 1
Gelsolin expression in colorectal cancer patients (n=61)

Characteristics	Cases (n=61)	Gelsolin expression: Absent (n=53)	Gelsolin expression: Reduced (n=8)	P
Sex				0.975*
Male	34	29	5	
Female	27	24	3	
Age, years, mean ± SD	61.00±9.33	61.79±7.58	55.75±16.82	0.348†
Histological differentiation				0.409*
Poorly differentiated	19	15	4	
Moderately differentiated	42	38	4	
Well-differentiated	0	0	0	
TNM stage				0.164*‡
I	13	12	1	
II	20	19	1	
III	18	14	4	
IV	10	8	2	
Histological classification				<0.0001*§
Adenocarcinoma	49	47	2	
Mucinous adenocarcinoma	10	6	4	
Signet-ring cell carcinoma	2	0	2	
Undifferentiated carcinoma	0	0	0	
Tumour localization				0.474*
Colon	18	17	1	
Rectum	43	36	7	

*Data presented as n unless otherwise indicated. *Determined using χ2 test (continuity correction); †Determined using t test; ‡Given the limited sample size, patients were combined: stage I and II as the early stage group, stage III and IV as the advanced stage group; §Mucinous adenocarcinoma, signet-ring cell carcinoma and undifferentiated carcinoma were combined into one group because of the limited sample size. TNM Tumour, node, metastasis*

hepatocellular cancers. To the best of our knowledge, iTRAQ reagents have seldom been used in biomarker discovery studies in CRC.

In the present study, we used iTRAQ labelling in combination with two-dimensional (2D) liquid chromatography (LC) and MS to identify potential biomarkers of CRC by comparing protein profiles of tumour tissue with paired normal mucosa, which in turn may facilitate the early diagnosis of CRC. Some of the proteins identified were confirmed by Western blot analysis and immunohistochemistry (IHC). These approaches ensure that the selected proteins demonstrated a consistent pattern of differential expression in CRC and significantly increased the confidence of the observations obtained from the iTRAQ analysis. In addition to their potential utility as biomarkers for CRC, these proteins also provide valuable insight into the still unknown molecular networks and mechanisms that govern the normal-to-malignant conversion of the epithelium.

METHODS

Patients

Data from all CRC patients who underwent surgical resection at the 150th Hospital, PLA, Luoyang, China, between January and June 2010, were retrieved from the files of the department of pathology. The criteria for inclusion in the study were as follows: presence of sporadic tumours without evidence of polyposis or familial predisposition syndrome; availability of clinical information and tumour stage; availability of tissue material; and a diagnosis of colorectal adenocarcinoma. The criteria for exclusion from the study were the presence of palindromic or metastatic tumour(s), and patients receiving antitumour therapy including chemotherapy, radiotherapy or biotherapy. Of the patients fulfilling these criteria, 66 cases were randomly selected. The patient consent forms and tissue-banking procedures were approved by the Research Ethics Board of the 150th Hospital. Histological diagnosis of each sample was confirmed using microscopic examination of hematoxylin and eosin-stained sections obtained during radical correction surgery.

Tissue material

Paired tissue samples of primary tumours (obtained from surgical resections) and their normal mucosa (not less than 5 cm from the cancerous margin) were flash frozen in liquid nitrogen within 20 min of devitalization after radical correction surgery (n=5, used for iTRAQ and Western blot analysis) or fixed in buffered formalin and embedded in paraffin (n=61, used for IHC).

The frozen samples were sectioned and stored at −80°C. The tissue from the opposite face of the histological section was then washed three times in approximately 1 mL of phosphate buffered saline (PBS) containing a mixture of protease inhibitors as described previously (12) (1 mM 4-[2-aminoethyl] benzenesulfonyl fluoride, 10 μM leupeptin, 1 μg/mL aprotinin and 1 μM pepstatin). The washed tissues were then homogenized in 0.5 mL of PBS containing protease inhibitors using a handheld homogenizer. These homogenates were then flash frozen in liquid nitrogen and stored at −80°C until use.

Neoplastic lesions were classified histologically according to the Vienna classification of digestive epithelial neoplasia (13). The tumour, node, metastsis (TNM) stage of the adenocarcinomas was determined according to the American Joint Committee on Cancer classification (14). The following data were recorded: localization, histological classification and TNM stage for adenocarcinomas (Table 1).

Protein digest, iTRAQ labelling and strong cation-exchange fractionation

Cancerous and paired normal flash-frozen samples from five patients were thawed and clarified by centrifugation, and protein concentrations were determined with a Bradford-type assay using a protein quantification reagent (Bio-Rad, USA). Cancerous samples and paired normal samples were pooled in equal parts and precipitated.

The samples (100 mg each) were reduced, alkylated and digested with trypsin overnight at a protein:enzyme ratio of 20:1 at 37°C. Each digest was concentrated to a volume of 15 μL in a speed vacuum followed by the addition of 15 μL of 1 M TEAB. The iTRAQ reagent was dissolved in 70 μL of ethanol and added to the digest, and the mixture was incubated at room temperature for 1 h. Cancer and normal samples labelled with different iTRAQ reagents were mixed and dried to a volume of 50 μL. The combined peptide mixture was fractionated by strong cation-exchange (SCX) chromatography on an UltiMate high-performance liquid chromatography (HPLC) system (LC Packings, The Netherlands) using a polysulfoethyl A column (2.1100 mm, 5 m, 300 Å [PolyLC, USA]). The sample was subsequently dissolved in 1 mL of SCX loading buffer (25% v/v acetonitrile, 10 mM KH_2PO_4 [pH 2.8]), and pH was adjusted to 2.8 by adding 1 M phosphoric acid. The entire sample was loaded onto the column and washed isocratically for 30 min at a flow rate of 200 μL/min. Peptides were eluted with a linear gradient of 0 mM to 500 mM KCl (25% v/v acetonitrile, 10 mM KH_2PO4, pH 2.8) over 30 min at a flow rate of 200 μL/min. The absorbance at 214 nm was monitored, and 15 fractions were collected along the gradient.

LC/MS analysis

Each SCX fraction was dried down, dissolved in 0.1% formic acid and analyzed by MS (Qstar Pulsar [Applied Biosystems-MDS Sciex, USA) interfaced with an Agilent 1100 HPLC system. Peptides were separated on a reverse-phase column packed with 10 cm of C18 beads (36,075 m, 5 m, 120 Å, YMC ODS-AQ [Waters Associates, USA]) with an emitter tip (New Objective, USA) attached. The peptides retained were washed using a gradient ranging from 5% to 40% solvent B (98%

acetonitrile, 0.1% formic acid) in solvent A (0.1% formic acid) for 60 min at a flow rate of 300 nL/min. Survey scans were acquired from a spectra of 400 m/z to 1200 m/z, with up to three precursors selected for MS/MS using a dynamic exclusion of 45 s. Rolling collision energy was used to promote fragmentation, with the collision energy range 20% higher than that used for unlabelled peptides because of the presence of iTRAQ tags.

Data analysis

The MS/MS spectra data were extracted and searched for in the Uniprot-Sprot database (version 4, total number of entries: 230,093, entries for *Homo sapiens*: 14,515) using ProteinPilot version 3.0 software (revision 114732 [Applied Biosystems, USA]) with the Paragon method using the following search parameters: *H sapiens* as species, trypsin as enzyme (one missed cleavage allowed), cysteine static modification with methylmethanethiosulfate and iTRAQ (peptide labelled at the N terminus and lysine) as sample type. Mass tolerance was set to 0.15 atomic mass units for the precursor, and 0.1 atomic mass units for the fragment ions. The raw peptide identification results from the Paragon algorithm (Applied Biosystems, USA) searches were further processed by the Pro Group algorithm (Applied Biosystems, USA) within the ProteinPilot software before final display. The Pro Group algorithm uses the peptide identification results to determine the minimal set of proteins that can be reported for a given protein confidence threshold. For each protein, Pro Group algorithm reports two types of scores: unused ProtScore and total ProtScore. The total ProtScore is a measurement of all the peptide evidence for a protein, and is analogous to protein scores reported by other protein identification software packages. The unused ProtScore, however, is a measurement of all peptide evidence for a protein that is not better explained by a higher ranking protein. In other words, the unused ProtScore is calculated by using the unique peptides (peptides that are not used by the higher ranking protein) and is a true indicator of the presence of protein. This is how a single-protein member of a multiprotein family was isolated. The protein confidence threshold cut-off for the present study was ProtScore 2.0 (unused) with at least one peptide with 99% confidence. The mean, SD and P values to estimate statistical significance of the protein changes were calculated using Pro Group software. Differentially expressed proteins (ratio >1.2 or <0.8) that were consistent between two independent biological experiments were manually validated and quantified. Peak areas for each of the signature ions (113 and 114) were obtained and corrected according to the manufacturer's instructions to account for isotopic overlap. Only signature ions with intensities <1500 counts were used for quantitation. When intesities of the signature ions were >1500 counts, 1:1 ratios were generated due to detector saturation.

Western blot analysis

To verify the iTRAQ data, samples (1 µg) were separated by 12% sodium dodecyl polyacryamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking for 1 h at room temperature with blocking buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Tween 20 [TBS/Tween 20]) and 5% nonfat milk powder, membranes were incubated overnight at 4°C in a 1:25,000 dilution of primary antibody (rabbit monoclonal antibody [Abcam (Hong Kong) Ltd, Hong Kong, China) in blocking buffer. The membranes were washed with TBS/Tween 20, incubated for 1 h at room temperature in secondary antibody (donkey antirabbit immunoglobulin G, Santcruz Biotech, USA) at a 1:2000 dilution in blocking buffer and, finally, washed with TBS/Tween 20. Blots were developed using Immun-Star TMAP Substrate Pack (BioRad, USA) and scanned on an Epson Scan (Agilent Technologies, USA) scanner within the linear range of detection.

Evaluation of immunohistochemical staining

A standard two-step indirect streptavidin-biotin method was applied to sections (3 µm thick) of deparaffinized tissue (StreptABComplex/HRP Duet, Mouse/Rabbit Amplification kit, ZhongShan Company, China). After autoclave sterilizer antigen retrieval (5 min in sodium citrate buffer [pH 7.3]) and peroxidase quenching with 3% hydrogen peroxide for 15 min, a 1:150 dilution of antigelsolin antibody (Abcam (Hong Kong) Ltd, Hong Kong, China) was applied overnight at 4°C. Biotinylated goat immunoglobulin G and streptavidin-biotin complexes were then applied for 30 min each. Sections were counterstained with Mayer's hematoxylin. Primary antibody omission served as a negative control. Smooth muscle cells and endothelial cells served as an internal positive control.

To evaluate the percentage of gelsolin-positive cells, the entire section was first scanned to determine the overall distribution of gelsolin expression within the tumour and its degree of heterogeneity; then, the percentage of gelsolin-positive cells was determined in representative areas by examining 10 randomly selected microscopic fields (magnification ×400) of each tissue section. Observations were made by two different observers who were blinded to clinical outcome (ie, the slides were coded, and the pathologists did not have previous knowledge of the local tumour burden, lymphonodular spread and grading of the tissue samples while scoring the immunoreactivity). Sections were scored positive if epithelial cells showed immunopositivity in the cytoplasm, plasma membrane and/or nucleus when judged independently by two scorers. A grading system was used to express the proportion of positive cells in each case as follows: grade (G) 0, negative; G1, <25% positive cells; G2, 25% to 50% positive cells; G3, 50% to 75% positive cells; and G4, >75% positive cells per lesion. In each type of lesion, results were expressed as the percentage of cases of each grade.

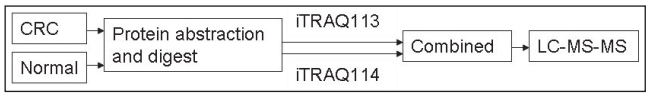

Figure 1) *Mass spectrometry (MS)-based approach (isobaric tags for relative and absolute quantitation [iTRAQ]) to quantitative analysis of colorectal cancer (CRC). Proteins were extracted from CRC tissue and paired normal mucosa, digested with trypsin, labelled with iTRAQ reagent and combined. The resulting peptides were fractioned using strong cation exchange chromatography and analyzed by liquid chromatography (LC)-tandem MS-MS*

Statistical analysis

ProteinPilot software using the Paragon method was used to analyze MS/MS spectra. Results of IHC were analyzed using the χ^2 test (continuity correction) or *t* test. Differences between two means with $P<0.05$ were considered to be statistically significant.

RESULTS

Quantitative proteomics analysis of CRC and normal tissue

In the present study, iTRAQ-based stable isotope labelling of colorectal tissues was performed to identify dysregulated proteins in CRC. The experimental strategy is shown in Figure 1.

After labelling with iTRAQ reagents (113 for cancer samples and 114 for normal samples), the two samples were mixed and analyzed by LC-MS/MS. The MS/MS fragmentation of the iTRAQ-labelled peptides resulted in signature peaks (at 113.1 and 114.1) for quantitation, while the fragmentation along the peptide backbone resulted in b- and y-type fragments, which were used to identify the peptide sequence. Only proteins with at least two peptides per protein and a high annotation confidence (≥95%) were considered, resulting in a total of 802 nonredundant proteins, which included structural proteins, signalling components, enzymes, receptors, transcription factors and chaperones. Eighty-two altered proteins outside the expression range of 0.8 to 1.2 were considered to be potential CRC-specific proteins (Appendix). Among these 82 proteins, gelsolin was specifically identified as a potential biomarker given the following: gelsolin, one of the major actin-binding proteins, is involved in the regulation of actin cytoskeleton organization via its severing and capping activity of actin filaments (15); and loss of gelsolin, a tumour suppressor,

TABLE 2
Peptide sequences, confidence, ratios, protein ratios and P values of gelsolin calculated by ProteinPilot* software

Peptide sequence	Peptide		Protein	
	Confidence, %	Ratio†	ratio†	P
AGALNSNDAFVLK	99	0.4295	0.5012	<0.0001‡
EPAHLMSLFGGKPMIIYK	99	0.2144		
EVQGFESATFLGYFK	99	0.4734		
HVVPNEVVVQR	99	0.1181		
QTQVSVLPEGGETPLFK	99	0.3839		
SEDCFILDHGKDGK	99	0.2984		
TGAQELLR	99	0.3147		
TPSAAYLWVGTGASEAEK TGAQELLR	99	0.9661		
YIETDPANR	99	0.5898		

**Applied Biosystems, USA; †Cancerous/normal; ‡Actual value 9.04×10^{-5}*

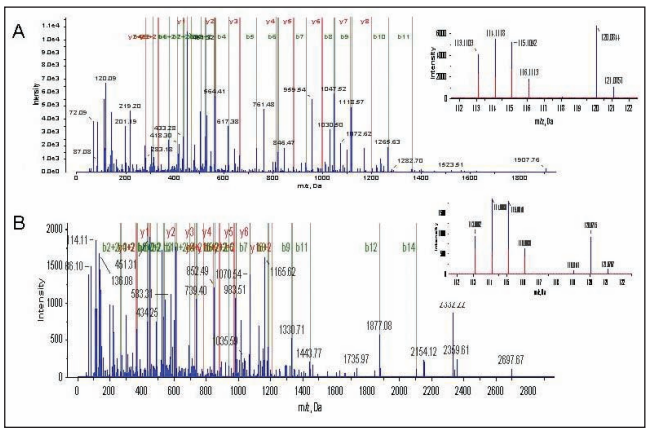

Figure 2) *Representative tandem mass spectrometry (MS/MS) spectra for peptides derived from gelsolin. For each MS/MS spectrum, b- and y-type fragment ions enabled peptide identification, whereas the peak areas of each of the iTRAQ signature ions (insets) enable quantification of the peptides and protein.* **A** *MS/MS spectrum of the peptide sequence AGALNSNDAFVLK from gelsolin.* **B** *MS/MS spectrum of the peptide sequence EPAHLMSLFGGKPMIIYK from gelsolin. Colorectal cancer and the paired normal sample were iTRAQ tagged by 113 and 114, respectively. The isobaric tag 115 and 116 were used in another experiment of esophageal carcinoma. iTRAQ Isobaric tags for relative and absolute quantitation*

is one of the most frequently occurring molecular defects in ovarian, breast, colon and pancreatic cancers (16-19). Gelsolin peptide sequences, peptide confidence, peptide ratios, protein ratios and P values calculated by ProteinPilot are presented in Table 2.

According to the protein sequence coverage map, protein sequences with matching peptides could be divided into groups with the highest confidence, moderate confidence, lowest confidence and the portions of the protein sequence for which no peptides were identified. The MS/MS spectra of two matching peptides (AGALNSNDAFVLK and EPAHLMSLFGGKPMIIYK) with the highest confidence are shown in Figure 2, with the peaks for the signature ions shown in the insets.

Western blot analysis

Western blot experiments were performed to validate the iTRAQ assay results. Based on a literature search, gelsolin was chosen. Gelsolin was detected by Western blot from the pooled sample used in a 2D LC-MS/MS experiment. Gelsolin is considered to be downregulated in CRC based on gray scale analysis of the protein strap, which was consistent with the discovery experiment (Figure 3).

Figure 3) *Validation of gelsolin by Western blot. Western blot results using frozen tissue samples used in the discovery experiment.* **A** *β-actin in normal sample.* **B** *β-actin in cancerous sample.* **C** *Negative control.* **D** *Gelsolin in normal sample.* **E** *Gelsolin in cancerous sample. The gray scale in the cancerous sample (E) is 0.48 of that of the normal sample (D)*

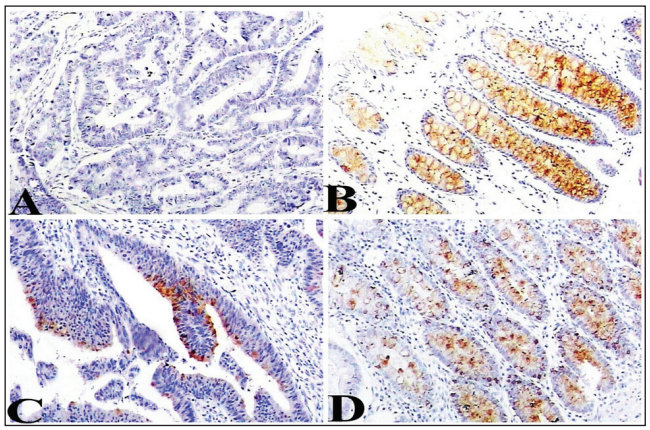

Figure 4) *Validation of gelsolin using immunohistochemical staining.* **A** *Cancerous sample showing a complete absence of detection.* **B** *Paired normal mucosa of sample A showing an expression of grade 4.* **C** *Cancerous sample showing decreased expression from grade 3 to grade 1 compared with sample* **D** *(paired normal mucosa of sample C). All images original magnification ×100*

Immunohistochemical staining

Gelsolin expression was tested using IHC in 61 primary human colorectal adenocarcinomas and their paired normal mucosa for further validation. Gelsolin expression was detected in all normal colon samples examined (61 of 61 [100%]) (Figure 4B and 4D). In epithelial cells, gelsolin was detected in both enterocytes and goblet cells. Gelsolin showed cytoplasmic expression and, in part of the cytoplasm surrounding the mucus vacuoles, no nuclear labelling was detected. Compared with normal mucosa, gelsolin expression was decreased in all tumours analyzed (61 of 61 [100%]) (Figure 4A and 4C), resulting either in a complete absence of detection in 86.89% (53 of 61) of samples (Figure 4A) or in focal staining of clusters of tumour cells in 13.11% (eight of 61) of samples (Figure 4C). Gelsolin achieved a sensitivity of 86.89% and specificity of 100% in distinguishing colorectal adenocarcinoma from normal tissue.

In cancer samples (reduced expression in eight cases) with clusters of gelsolin-positive cells, the mean (±SD) percentage of gelsolin-positive tumour cells was 21.18±7.73%. The percentage of gelsolin-positive cells was reduced in cancer compared with paired normal mucosa (P=0.034 [paired sample *t* test]) (Figure 4C and 4D). No adenocarcinoma was scored as G4 (>75% of positive cells) or G3 (50% to 75% of positive cells), whereas 37.5% of cases (three of eight) were classified as G2 (25% to 50% of positive cells) and 62.5% of the cases (five of eight) were classified as G1 (<25% of positive cells). Gelsolin-positive cells showed cytoplasmic expression in adenocarcinoma (three of eight samples [37.5%]); mucinous adenocarcinoma (three of eight samples [37.5%]); and signet-ring cell carcinoma (two of eight samples [25%]). Compared with tumours devoid of expression, gelsolin expression tended to be reduced in mucinous adenocarcinoma and signet-ring cell carcinoma (P<0.0001). This decrease in gelsolin expression in CRC was not correlated with age, sex, histological differentiation, TNM stage or tumour localization (Table 1).

DISCUSSION

Compared with traditional proteomic methods, such as 2D gel analysis followed by MS/MS identification, the iTRAQ-coupled 2D-LC-MS/MS

approach provides higher detection sensitivity and holds the promise of effectively depicting cellular protein profiles in tissue (9,11,20). In recent years, this approach has been used in prostate, radiotherapy-resistant, renal cell, lung, oral, head and neck, breast and hepatocellular cancers (4-11).

In the present study, we applied isobaric peptide tags (ie, iTRAQ) and multidimensional LC-MS/MS to identify proteins that are differentially expressed in CRC and paired normal mucosa samples. Differential gelsolin expression was validated by Western blot analysis and IHC. Because adenocarcinoma comprises more than 50% of CRCs, only colorectal adenocarcinoma was included in the present study.

Using a combination of subfractionation, iTRAQ-based labelling, 2D-LC peptide separation, MS and database searches for human proteins, 82 proteins altered outside the range of 0.8 to 1.2 were considered as potential CRC-specific proteins. Downregulation of gelsolin was confirmed by Western blot analysis and IHC. These serial studies suggest that combined proteomics and bioinformatics analysis can generate valid candidates that may be further evaluated for their role in the progression from normal tissue to adenocarcinoma. Although the validation of a protein cannot be extrapolated to the full protein list, it did suggest that the iTRAQ results were reliable.

Abnormal expression of gelsolin has been reported in many types of tumours. The expression of gelsolin has been reported to be frequently silenced in various cancers (16,18,21-27). Conversely, overexpression of gelsolin has been found to be a negative prognostic predictor in a subpopulation of patients with nonsmall cell lung cancer, urothelial cancer, and $EGFR^+$/$erB\text{-}2^+$ breast cancer (25,28,29). Gelsolin regulates the architecture and dynamics of cells by capping, severing and nucleating actin filaments. However, the precise molecular mechanism behind the reduction of gelsolin expression has not yet been clarified (22,30). Despite the high incidence of CRC, only a few contradictory studies regarding the expression of gelsolin in this type of malignancy and its precursor lesions are available. One IHC study (31) reported weak gelsolin staining in 30 normal human colon mucosa and 22 adenocarcinoma samples. In contrast, another report based on 69 primary human colon adenocarcinomas and their paired normal mucosa (18) showed decreased gelsolin expression in all tumours analyzed, resulting in either a complete absence of detection in 34.8% of cases, or in focal staining of clusters of tumour cells in 65.2% of cases. This latter result was supported by DNA microarray studies that reported reduced expression of gelsolin complementary DNA in colon cancer compared with normal colon tissue (32,33). Furthermore, gelsolin expression and its subcellular distribution seem to correlate with metastatic potential in human colon adenocarcinoma cells (15). Using 2D-LC-MS/MS, gelsolin expression was found to be decreased in CRC in the present study. This result was further validated by Western blot analysis and IHC, with gelsolin expression tested using IHC in 61 primary human CRC and paired normal mucosa samples. Gelsolin expression was detected in all normal colon and rectum samples examined. Compared with normal mucosa, gelsolin expression was decreased in all tumours analyzed (61 of 61 [100%]), resulting either in a complete absence of detection in 86.89% (53 of 61) of samples or in focal staining of clusters of tumour cells in 13.11% (eight of 61) of samples. The rate of complete absence of detection surpasses the rate reported by Gay et al (18). In cases of reduced gelsolin expression, 62.5% showed mucinous adenocarcinoma and signet-ring cell carcinoma, while 37.5% of cases showed adenocarcinoma. This difference was statistically significant ($P<0.0001$), which suggests that the role of gelsolin in CRC may correlate with histological classification.

CONCLUSION

The results presented reinforce the idea that iTRAQ and 2D-LC-MS/MS represent an effective and reliable proteomic process in cancer biomarker discovery; the downregulation of gelsolin expression is a general mechanism during the transition from normal tissue to cancer; and that gelsolin may be a potential biomarker for colorectal adenocarcinoma in the Chinese population.

COMPETING INTERESTS: The authors have no conflicts of interest to declare.

AUTHORS' CONTRIBUTIONS: Nai-jun Fan was responsible for the conception and design of this study, providing samples and clinical data, drafting and revising the article and performing the experiments. Chun-fang Gao contributed to the design of this study. Chang-song Wang, Jian-Yin and Qing-yin Liu contributed to the validation of gelsolin. Jing-Jing Lv, Guang Zhao, Xin-hua Sheng, Xiu-li Wang and Dong-hui Li contributed to samples and clinical data collection. All authors have read and approved the final manuscript.

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APPENDIX

Dysregulated proteins (n=82) in colorectal adenocarcinoma identified using an iTRAQ-based proteomic approach

Protein	Accession	Expression level in cancer
Histone H3.3	sp|P84243|H33_HUMAN	Increased
Neutrophil defensin 3	sp|P59666|DEF3_HUMAN	Increased
40S ribosomal protein S19	sp|P39019|RS19_HUMAN	Increased
Collagen alpha-1(XII) chain	sp|Q99715|COCA1_HUMAN	Increased
Histone H4	sp|P62805|H4_HUMAN	Increased
Elongation factor 2	sp|P13639|EF2_HUMAN	Increased
Histone H2B type 1-N	sp|Q99877|H2B1N_HUMAN	Increased
Annexin A2	sp|P07355|ANXA2_HUMAN	Increased
60 kDa heat shock protein, mitochondrial	sp|P10809|CH60_HUMAN	Increased
Fibrinogen beta chain	sp|P02675|FIBB_HUMAN	Increased
78 kDa glucose-regulated protein	sp|P11021|GRP78_HUMAN	Increased
Protein disulfide-isomerase	sp|P07237|PDIA1_HUMAN	Increased
Thymidine phosphorylase	sp|P19971|TYPH_HUMAN	Increased
Tenascin	sp|P24821|TENA_HUMAN	Increased
Filamin-B	sp|O75369|FLNB_HUMAN	Increased
Keratin, type I cytoskeletal 1	sp|P05783|K1C18_HUMAN	Increased
Stress-70 protein, mitochondrial	sp|P38646|GRP75_HUMAN	Increased
Fibrinogen alpha chain	sp|P02671|FIBA_HUMAN	Increased
Macrophage-capping protein	sp|P40121|CAPG_HUMAN	Increased
Periostin	sp|Q15063|POSTN_HUMAN	Increased
Cytoskeleton-associated protein 4	sp|Q07065|CKAP4_HUMAN	Increased
Protein disulfide-isomerase A3	sp|P30101|PDIA3_HUMAN	Increased
SH3 domain-binding glutamic acid-rich-like protein 3	sp|Q9H299|SH3L3_HUMAN	Increased
Annexin A1	sp|P04083|ANXA1_HUMAN	Increased
Fibrinogen gamma chain	sp|P02679|FIBG_HUMAN	Increased
Plastin-2	sp|P13796|PLSL_HUMAN	Increased
Myosin-9	sp|P35579|MYH9_HUMAN	Increased
Neutral alpha-glucosidase AB	sp|Q14697|GANAB_HUMAN	Increased
40S ribosomal protein S17	sp|P08708|RS17_HUMAN	Increased
Serum albumin	sp|P02768|ALBU_HUMAN	Increased
Plectin-1	sp|Q15149|PLEC1_HUMAN	Increased
Keratin, type II cytoskeletal 8	sp|P05787|K2C8_HUMAN	Increased

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APPENDIX – CONTINUED
Dysregulated proteins (n=82) in colorectal adenocarcinoma identified using an iTRAQ-based proteomic approach

Protein	Accession	Expression level in cancer
Heat shock protein HSP 90-alpha	sp|P07900|HS90A_HUMAN	Increased
Fibronectin	sp|P02751|FINC_HUMAN	Increased
Myosin-11	sp|P35749|MYH11_HUMAN	Decreased
Hemoglobin subunit alpha	sp|P69905|HBA_HUMAN	Decreased
PDZ and LIM domain protein 7	sp|Q9NR12|PDLI7_HUMAN	Decreased
Collagen alpha-1(VI) chain	sp|P12109|CO6A1_HUMAN	Decreased
Neuroblast differentiation-associated protein AHNAK	sp|Q09666|AHNK_HUMAN	Decreased
Synaptopodin-2	sp|Q9UMS6|SYNP2_HUMAN	Decreased
Alpha-actinin-1	sp|P12814|ACTN1_HUMAN	Decreased
ATP synthase subunit beta, mitochondrial	sp|P06576|ATPB_HUMAN	Decreased
Collagen alpha-1(XIV) chain	sp|Q05707|COEA1_HUMAN	Decreased
Tensin-1	sp|Q9HBL0|TENS1_HUMAN	Decreased
Decorin	sp|P07585|PGS2_HUMAN	Decreased
Collagen alpha-2(VI) chain	sp|P12110|CO6A2_HUMAN	Decreased
Talin-1	sp|Q9Y490|TLN1_HUMAN	Decreased
Gelsolin	sp|P06396|GELS_HUMAN	Decreased
Myosin light chain kinase, smooth muscle	sp|Q15746|MYLK_HUMAN	Decreased
Desmuslin	sp|O15061|DMN_HUMAN	Decreased
ATP synthase subunit alpha, mitochondrial	sp|P25705|ATPA_HUMAN	Decreased
Sorbin and SH3 domain-containing protein 1	sp|Q9BX66|SRBS1_HUMAN	Decreased
Ig gamma-1 chain C region	sp|P01857|IGHG1_HUMAN	Decreased
Desmoplakin	sp|P15924|DESP_HUMAN	Decreased
Membrane primary amine oxidase	sp|Q16853|AOC3_HUMAN	Decreased
Lamin-A/C	sp|P02545|LMNA_HUMAN	Decreased
Lumican	sp|P51884|LUM_HUMAN	Decreased
Polymerase I and transcript release factor	sp|Q6NZI2|PTRF_HUMAN	Decreased
Peroxiredoxin-2	sp|P32119|PRDX2_HUMAN	Decreased
Tropomyosin beta chain	sp|P07951|TPM2_HUMAN	Decreased
Tropomyosin alpha-1 chain	sp|P09493|TPM1_HUMAN	Decreased
Prolargin	sp|P51888|PRELP_HUMAN	Decreased
Myosin regulatory light polypeptide 9	sp|P24844|MYL9_HUMAN	Decreased
Profilin-1	sp|P07737|PROF1_HUMAN	Decreased
Vinculin	sp|P18206|VINC_HUMAN	Decreased
Creatine kinase B-type	sp|P12277|KCRB_HUMAN	Decreased
Filamin-C	sp|Q14315|FLNC_HUMAN	Decreased
Calponin-1	sp|P51911|CNN1_HUMAN	Decreased
Mimecan	sp|P20774|MIME_HUMAN	Decreased
Spectrin alpha chain, brain	sp|Q13813|SPTA2_HUMAN	Decreased
Ig gamma-2 chain C region	sp|P01859|IGHG2_HUMAN	Decreased
Glyceraldehyde-3-phosphate dehydrogenase	sp|P04406|G3P_HUMAN	Decreased
Ig kappa chain C region	sp|P01834|IGKC_HUMAN	Decreased
Transgelin	sp|Q01995|TAGL_HUMAN	Decreased
Phosphatidylethanolamine-binding protein 1	sp|P30086|PEBP1_HUMAN	Decreased
Peroxiredoxin-5, mitochondrial	sp|P30044|PRDX5_HUMAN	Decreased
EH domain-containing protein 2	sp|Q9NZN4|EHD2_HUMAN	Decreased
Tenascin-X OS=Homo sapiens	sp|P22105|TENX_HUMAN	Decreased
Cysteine and glycine-rich protein 1	sp|P21291|CSRP1_HUMAN	Decreased
Ig lambda chain C regions	sp|P01842|LAC_HUMAN	Decreased
Hemoglobin subunit beta	sp|P68871|HBB_HUMAN	Decreased
Heat shock 70 kDa protein 1	sp|P08107|HSP71_HUMAN	Decreased
Filamin-C	sp|Q14315|FLNC_HUMAN	Decreased
Calponin-1	sp|P51911|CNN1_HUMAN	Decreased
Spectrin alpha chain, brain	sp|Q13813|SPTA2_HUMAN	Decreased
Ig gamma-2 chain C region	sp|P01859|IGHG2_HUMAN	Decreased
Glyceraldehyde-3-phosphate dehydrogenase	sp|P04406|G3P_HUMAN	Decreased
Ig kappa chain C region	sp|P01834|IGKC_HUMAN	Decreased
Transgelin	sp|Q01995|TAGL_HUMAN	Decreased
Phosphatidylethanolamine-binding protein 1	sp|P30086|PEBP1_HUMAN	Decreased
Peroxiredoxin-5, mitochondrial	sp|P30044|PRDX5_HUMAN	Decreased
EH domain-containing protein 2	sp|Q9NZN4|EHD2_HUMAN	Decreased
Tenascin-X OS= Homo sapiens	sp|P22105|TENX_HUMAN	Decreased
Cysteine and glycine-rich protein 1	sp|P21291|CSRP1_HUMAN	Decreased
Ig lambda chain C regions	sp|P01842|LAC_HUMAN	Decreased
Hemoglobin subunit beta	sp|P68871|HBB_HUMAN	Decreased
Heat shock 70 kDa protein 1	sp|P08107|HSP71_HUMAN	Decreased
Filamin-C	sp|Q14315|FLNC_HUMAN	Decreased
Calponin-1	sp|P51911|CNN1_HUMAN	Decreased

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