Prognostic role of serum AZGP1, PEDF and PRDX2 in colorectal cancer patients

Dengbo Ji1,4, Ming Li1,4, Tiancheng Zhan1, Yunfeng Yao1, Jing Shen2, Huifang Tian2, Zhiqian Zhang1 and Jin Gu1,3,*

1Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), 2Central Laboratory, and 3Department of Cell Biology, Peking University Cancer Hospital and Institute, Beijing 100142, China

4To whom correspondence should be addressed. Department of Colorectal Surgery, Peking University Cancer Hospital & Institute, No. 52, Fucheng Rd., Haidian District, Beijing, China, 100142. Tel: +86-10-88196238; Fax: +86-10-88196238; Email: zlgui@bjmu.edu.cn

This study was designed to develop novel and better reliable serum prognostic biomarkers for colorectal cancer (CRC). A 50 sample set including CRC, adenoma and healthy control sera was used to identify the serum proteins involved in CRC carcinogenesis using serum proteomic approach. Alpha-2-glycoprotein 1, zinc-binding (AZGP1), pigment epithelium derived factor (PEDF) and peroxiredoxin 2 (PRDX2) were selected as good candidates. Two independent cohorts of 868 individuals were enrolled. The expression of selected proteins in serum from cohort 1 (n = 534) was quantified with enzyme-linked immunosorbent assays. CRC sera of this cohort (n = 405) were assigned to training and test sets, which were used to identify and verify the prognostic markers. The prognostic values of identified proteins were further validated in cohort 2 (n = 334) using quantitative reverse transcription PCR and immunohistochemical staining. Our data showed that the elevated AZGP1 and decreased PEDF and PRDX2 expressions in CRC serum and tissues were correlated with liver metastases. In the training set, higher AZGP1 and lower PEDF levels in sera were significantly associated with a poorer overall survival (OS), higher AZGP1 was also associated with a poorer disease-free survival (DFS). This association was verified in the testing set and further validated in patients in cohort 2. Patients with lower PEDF or PRDX2 levels in their CRC tissues had a significantly poorer DFS or OS than patients with high levels of these proteins in cohort 2. Univariate and multivariate analyses indicated that the prognostic performance of serum AZGP1 and PEDF was independent of other clinicopathological factors. We propose that they may serve as prognostic markers and potential therapeutic targets in CRC.

Introduction

Colorectal cancer (CRC) is the third predominant cancer in the world. Survival rate of patients with CRC has increased during the past few years, possibly due to early diagnosis and improved treatment. However, about 40–50% of patients are still succumbed to death from either local or distant recurrence after surgery (1). Although AJCC Tumor, Node, Metastases (TNM) classification has been a valuable tool in staging CRC and choosing for specific treatment, it is acknowledged that patients at similar clinical stages can have very different outcomes. Therefore, it is desirable to identify biomarkers that are capable of predicting high-risk patients and might benefit from adjuvant chemotherapy.

Abbreviations: 2-DE, two-dimensional gel electrophoresis; CEA, carcinoembryonic antigen; CRC, colorectal cancer; DFS, disease-free survival; ELISA, enzyme-linked immunosorbent assay; HR, hazard ratio; LC-MS/MS, liquid chromatography tandem mass spectrometry; OS, overall survival; Prxs, peroxiredoxins; qRT-PCR, quantitative reverse transcription PCR; ROC, receiver-operating characteristic; TMAs, tissue microarrays.

1These authors contributed equally to this study.

Carinoembryonic antigen (CEA) has been the only biomarker recommended by the American Society of Clinical Oncology and the European Group on Tumor Markers for use in the post-operative period for the early detection of recurrent or metastatic CRC (2,3). However, diagnostic sensitivity of CEA is always questionable and unsatisfactory. Therefore, an appropriate molecular marker is a necessity for evaluation of adequate treatment of aggressive CRC and prognosis.

Despite a number of limitations, a two-dimensional gel electrophoresis- (2-DE) and mass spectrometry (MS)-based proteomics strategy can provide high throughput simultaneous identification of hundreds of proteins and is still considered a valuable method to screen biomarkers for tumor. A number of studies using 2-DE-based separation techniques have identified differential protein expression in clinical CRC tissue samples (4–7). Such studies undoubtedly provide not only critical information regarding potential biomarkers but also insight into the pathophysiology of the disease. However, tissue markers do not represent the ideal for clinical use as tissue sampling procedures such as biopsy do have associated morbidity. Markers present in the periphery circulation would be more directly and easily applicable to the clinical setting. However, the existence of highly abundant heterogenous proteins in the serum usually makes the small size proteins with low concentration difficult to be detected. Those small proteins or peptides often serve as valuable biomarkers in certain diseases, particularly in cancers. In order to remove or reduce the interference from other proteins, macromolecular protein depletion prior to proteomic analysis has become a mainstay in clinical proteomic studies, particularly for searching biomarkers (8).

In this study, differentially expressed proteins were profiled from CRC, adenoma and healthy control sera. We noticed that AZGP1, PEDF and PRDX2 were significantly dysregulated in CRC compared with adenoma and normal controls. The candidate proteins have been found to be associated in some way with cancer progression. AZGP1 was found to be associated with tumor differentiation status of several cancers such as breast and prostate cancer (9,10). PED F exhibited potential activity as an inhibitor of angiogenesis (11). PRDX2 was found to play a role in cancer development or progression (12–16). In view of their potential functional relevance, their possible correlation with CRC progression, AZGP1, PEDF and PRDX2 were chosen as the potential biomarkers for further analysis.

Materials and methods

Subjects and samples

This research was approved by the ethical committee of Health Science Center of Peking University and the Oncology Center at Peking University. A written informed consent was obtained from all the participants prior to the enrollment.

Serum samples were collected from 584 subjects including 435 patients with CRC, 55 patients with adenoma and 94 healthy controls between 2003 and 2008. A 50 sample set was used to identify the serum proteins involved in CRC carcinogenesis by a 2-DE and MALDI-TOF/TOF-based proteomics approach. The 50 samples were divided into 5 groups with 10 samples in each group: healthy control, adenoma, early stage CRC (I and II), developing stage CRC (III) and CRC with live metastasis (IV). The rest 534 serum samples constitute cohort 1. A total of 405 CRC serum samples were randomly assigned to training and test sets, which were used to identify and verify the prognostic biomarkers. The CRC serum samples were collected prior to surgical operation, and no prescribed chemotherapy or radiotherapy was given before sample collection. All those subjects were cleared of having any other malignant disorders. Among CRC patients, post-operative sera were collected from 62 subjects. The post-operative serum samples were collected 7 days post the surgeries.

CRC tissues samples were directly collected after surgical resection. All samples were immediately frozen in liquid nitrogen and stored at −80°C or fixed in 10% formalin for paraffin embedding.

© The Author 2013. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com

1265

Downloaded from https://academic.oup.com/carcin/article-abstract/34/6/1265/2463164 by guest on 20 January 2019
Paraffin-embedded CRC specimens and surrounding non-tumor tissues from 294 independent patients and 40 adenoma specimens who underwent surgical resections from 1999 to 2006 were microdissected to perform TMAs (cohort 2). TMAs were analyzed by immunohistochemistry. Cases with familial adenomatous polyposis CRC were excluded from the study. CRC patients did not receive any preoperative chemotheraphy or radiotherapy, and the samples were pathologically verified under microscope to have 80% cancer tissues. Among cohort 2, 137 paired fresh CRC tissue samples and surrounding non-tumor tissues were obtained and analyzed by quantitative reverse transcription PCR (qRT–PCR). A summary of the clinical characteristics of these patients is presented in Supplementary Tables 1–3, available at Carcinogenesis Online.

Serum preparation and two-dimensional electrophoresis
A 50 sample set was used for 2-DE. The samples were divided into five groups: healthy control, adenoma, early stage CRC, developing stage CRC and CRC with liver metastasis, with ten cases in each group. Serum was separated by centrifugation at 2000g for 10 min at 4°C to remove the cellular components. After introducing a protease inhibitor cocktail (Roche diagnostics, Mannheim, Germany) to prevent protein enzymatic breakdown or modifications, samples were aliquoted and stored at −80°C until analysis.

Fourteen high-abundance serum proteins were depleted using the Agilent (Palo Alto, CA) Multiple Affinity Removal System Human 14 LC Column immunopurity-based depletion system according to the manufacturer’s instructions. The proteins removed were albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha2-macroglobulin, alpha1-acid glycoprotein, IgM, apolipoprotein A-I, apolipoprotein A-II, transthyretin and complement C3. The low-abundance fractions and the high-abundance proteins eluted were either analyzed immediately or aliquoted and stored at −80°C until use.

Four hundred microliters samples from each individual of same group were pooled together and concentrated using a 5Kda molecular weight cutoff spin concentrator (Agilent). The samples were subjected to three rounds of buffer addition, with centrifugation at 7500g for 20 min at 10°C every time. Protein concentration was determined with the Bradford assay using a bovine serum albumin standard. Protein quantities were normalized and 100 μg of total proteins were used for analyses. Two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis was applied to determine the target proteins as described previously (17). Subsequently, the signal images were acquired using a GS-800 Calibrated Densitometer (Bio-Rad) and quantitated with ImageMaster 2-D Platinum software, version 3.0 (GE Healthcare, Piscataway, NJ). The spots of interest were immediately cut and stored at −80°C until analysis. The 2-DE separations were performed in triplicate.

Identification of proteins by liquid chromatography tandem mass spectrometry
Proteins were identified following gel spot excision and trypsin digestion according to the protocols of Courchesne and Patterson (18). Peptide mixture was analyzed on the nano-liquid chromatography tandem mass spectrometry (LC-MS/MS) system, which consists of an Ultimate HPLC system ( Dionex) and quadrupole time-of-flight (Q-TOF) mass spectrometer (micrOTOF-Q II mass spectrometer, Bruker) equipped with a nano-ESI source, as described previously (19).

MS/MS data were processed using the Mascot search engine (www.matrixscience.com). The TOF mass analyzer of the instrument was calibrated using 10 mM Na Formate (pos). For the MS/MS ion search, a peptide charge state of +2 or +3 and peptide/fragment mass tolerance of ±0.1 Da was used. Probability-based MASCOT scores were estimated by comparison of search results against estimated random match population and reported as −10 x log(P), where P is the absolute probability. The significance threshold was set at P<0.05.

Enzyme-linked immunosorbent assays
Serum AZGP1, PEDF and PRDX2 concentrations were measured by sandwich enzyme-linked immunosorbent assay (ELISA) method using AZGP1 kit (Abnova, Heidelberg, Germany), PEDF kit (BioVendor, GmbH, Heidelberg, Germany) and PRDX2 kit (Uscn Inc, Wuhan, China) according to the manufacturer’s instructions.

RNA isolation and quantitative real-time RT–PCR
Total RNA was extracted from 137 CRC tissues and the surrounding non-tumor tissues using TRIZOL reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA was treated with DNase for removal of contaminating genomic DNA using DNase Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The yield of total RNA was determined by the absorbance at 260 nm using the Nanodrop. The quality of total RNA was analyzed using agarose gel electrophoresis and the OD A260/A280 ratio and the OD A260/A230 ratio by the Nanodrop. Total RNA (3 μg) treated with DNase was used in 20 μl of reverse transcriptase reaction to synthesize cDNA by using Moloney murine leukemia virus reverse transcriptase (M-MLV RT) for RT–PCR (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA) on an ABI7500 PCR machine. Assays were designed to be public available in order to be verified by using Roche UPL design software (ProbeFinder, v. 2.45) operated under National Center for Biotechnology Information. All assays were designed to span an intron–exon boundary to prevent amplification of DNA. All the primer sequences and probe names were provided in Supplementary Table 4, available at Carcinogenesis Online.

Cycling conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, 57°C for 30 s and 72°C for 30 s. Data analysis was performed using the ΔΔCt method using AZGP1 kit (Sigma, St Louis, MO), anti-human PRDX2 kit (Epitomics, Burlingame, CA) and mouse monoclonal anti-human PEDF (Millipore, Billerica, MA). All images were examined by two experienced pathologists independently. The immunoreactivity of the proteins detected was recorded through the intensity of staining and the percentage of immunoreactive cells are as follows: tissues with no staining were rated as 0, with a faint or moderate staining to strong staining in <25% of cells rated as 1, or strong staining in 25–50% of cells rated as 2 and strong staining in >50% of cells rated as 3.

Western blot analysis
Total protein lysates extracted from samples were quantitated using bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Twenty microgram of total protein lysates extracted from samples were separated with 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to a polyvinylidine fluoride membrane. The membrane was blocked with 3% bovine serum albumin, followed by incubation with mouse monoclonal anti-human PEDF (Millipore, Billerica, MA), rabbit monoclonal anti-human AZGP1 (Sigma, St Louis, MO), anti-human PRDX2 and anti-human β-actin antibody (Epitomics, Burlingame, CA). The membrane was then incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (Jackson Immune Research Laboratories Inc, West Grove, PA), and visualized using Immobilon Western Chemiluminescent HRP substrate (Millipore). β-Actin was used as the loading control.

Statistical analysis
All data are presented as the mean ± SE and were analyzed using the statistical package SPSS13.0. Data are expressed as median values (interquartile range) in tables or as means ± SE in the figures. Comparisons were performed using the non-parametric Mann–Whitney U-test or Kruskal–Wallis test for continuous variables. Spearman rho correlation test (two tailed) was used to estimate the correlation between the expression in CRC tissue of each marker and clinical–pathological variables. Survival curves were plotted using the Kaplan–Meier analysis, and the log–rank test was used to test for significant differences. Cox univariate and multivariate proportional hazard models were used to estimate the hazard ratio for each marker. P ≤ 0.05 was considered statistically significant.

Results
Proteomic analysis and identification of differentially expressed proteins
The differential proteins were identified using 2-DE gel from five group serum samples (Figure 1A). In each gel, about 1300 spots were clearly detected by silver staining, and the overlap rate for all the parallel gels derived from the same samples were >80%. To improve image analysis quality, the spots located at the similar 2-DE positions in different gels with 3-fold change were defined as the differential spots in the 2-DE gels. A total of 60 differential spots were obtained, including 26 upregulated and 34 downregulated in CRC and adenoma group. Figure 1(A and B) showed the typical differential
AZGP1, PEDF and PRDX2 as prognostic marker for CRC

spots. MALDI-TOF/TOF MS was employed and 27 unique proteins were identified.

AZGP1 was identified in the upregulated proteins spots. PEDF and PRDX2 were observed in the downregulated proteins group. The differential protein spots were annotated on CRC, adenoma and control 2D gels, respectively (Figure 1A). The mass spectrometry data were summarized in Supplementary Table 5, available at Carcinogenesis Online.

Validation of serum AZGP1, PEDF and PRDX2 by ELISA

For the direct validation and quantification of the identified AZGP1, PEDF and PRDX2 in all serum samples, ELISA cohort 1 (n = 534) was applied. The serum levels of AZGP1 and PRDX2 in patients with CRC (n = 405), in patients with benign bowel disease (n = 45) and in healthy controls (n = 84) showed significant difference (P < 0.0001, Kruskal-Wallis test). The ELISA results further confirmed the discoveries by MALDI-TOF MS as described above. AZGP1 was significantly elevated in sera from patients with CRC (P < 0.0001, Mann–Whitney test) and adenoma (P < 0.0001) compared with healthy controls. Interestingly, AZGP1 in CRC patients was significantly higher than in adenoma patients (P < 0.0001; Figure 2A). PRDX2 and PEDF were significantly decreased in serum of patients with CRC than those in healthy controls (P < 0.0001; Figure 2A), but no significant difference was found between adenoma patients and healthy controls.

To verify whether dysregulated AZGP1, PEDF and PRDX2 are associated with CRC, their serum levels were measured in 62 patients with CRC before and 7 days after surgical removal of the tumor. It was found that the levels of AZGP1 were significantly reduced in the post-operative

Fig. 1. Proteomics analysis of normal, adenoma and CRC tissues using 2-DE gels. (A) Comparison of protein profiles among normal, adenoma and CRC tissues by 2-DE gels. Three representative spots dysregulated among normal, adenoma and CRC tissue group are indicated. (B) Typical dysregulated spots in CRC. Spot 1 is AZGP1, spot 2 is PEDF and spot 3 is PRDX2.
samples when compared with the preoperative samples \((P = 0.0015,\) Wilcoxon test; Figure 2B), whereas the levels of PRDX2 were significantly elevated in the post-operative samples when compared with the preoperative samples \((P < 0.0001)\). No significant differences were found between the preoperative and post-operative serum PEDF levels.

Receiver-operating characteristic (ROC) curve analyses revealed that the serum level of AZGP1 was a useful biomarker for differentiating patients with CRC from controls within ROC curve areas of 0.9572 [95% confidence interval (CI) = 0.9173–0.9971]. At the cutoff value of 2060 ng/ml for AZGP1, the sensitivity was 100% and the specificity was 79.5%. The ROC curve areas for PEDF and PRDX2 were 0.5744 (95% CI = 0.52–0.63) and 0.7045 (95% CI = 0.63–0.77), respectively. At the cutoff value of 4323 ng/ml for PEDF and 22.4 ng/ml for PRDX2, the sensitivity and the specificity were 34% and 96%, 87% and 51%, respectively (Figure 2D).

**Clinical pathological data and correlation with serum AZGP1, PEDF and PRDX2**

To confirm the association among serum AZGP1, PEDF, PRDX2 and clinical–pathologic characteristics, their serum levels were analyzed in 405 CRC patients of cohort 1.

Serum AZGP1 levels increased in accordance with the advanced CRC TNM stages \((P < 0.0001;\) Figure 2C). There was no significant correlation between AZGP1 level and gender, age at surgery, extent of local tumor invasion (T stage), histological subtype and venous invasion. However, high serum AZGP1 levels were associated with the presence of liver metastases \((P < 0.0001)\) and CEA \((P = 0.0045)\). In addition, serum AZGP1 level was negatively correlated with the differentiation degree of CRC \((P = 0.0191)\) (Supplementary Table 6, available at Carcinogenesis Online).

The serum levels of PEDF and PRDX2 decreased remarkably with the advanced CRC TNM stages (Figure 2C). In addition, low serum PEDF levels were associated with liver metastases \((P < 0.0001)\) and CEA \((P = 0.0336)\). Serum PEDF level in mucinous adenocarcinoma was significantly higher than in adenocarcinoma \((P = 0.0127)\) (Supplementary Table 6, available at Carcinogenesis Online).

**Prognostic value of serum AZGP1, PEDF and PRDX2 and correlation with distant metastases and recurrence**

The training set \((n = 225)\) was analyzed for the correlation between the clinical outcome and the levels of these three proteins. Kaplan–Meier analyses for OS were performed using the median levels of proteins.
AZGP1, PEDF and PRDX2 as prognostic marker for CRC

as the cutoff for the definition of the subgroups. Higher serum AZGP1 or lower PEDF levels were associated with poor prognosis \( (P = 0.005, P = 0.003, \) respectively, log-rank test; Figure 3B and A). The OS probabilities at 1, 3 and 5 years of the high AZGP1 group (86.2%, 67% and 56%, respectively) were significantly lower than those of the low AZGP1 group (88.8%, 76.7% and 74.1%, respectively; \( P = 0.005; \) Figure 3B). The patients in the low PEDF group had a significantly shorter OS than those in the high PEDF group \( (P = 0.003; \) Figure 3A). For stages I–III CRC patients, higher serum AZGP1 levels were associated with a shorter DFS \( (P = 0.001, \) log-rank test; Figure 3C). The probabilities of DFS at 1 and 3 years in the low AZGP1 group were 96.3 and 90%, respectively, whereas in the high AZGP1 group were 82.7 and 57.7%, respectively. However, no significant association was found between the PEDF level and DFS. No significant association was found between the PRDX2 level and OS or DFS.

The prognostic ability of serum levels of AZGP1, PEDF or PRDX2 were further verified in the test set \( (n = 180) \). The median levels of proteins were used as the cutoff value to classify patients. As shown in Figure 3F, the probabilities of DFS at 1 and 3 years in the low AZGP1 group were 96.1 and 80.8%, respectively, which were significantly higher than in the high AZGP1 group (81.8 and 63.6%, respectively; \( P = 0.01 \)). The OS probabilities at 1, 3 and 5 years of the high AZGP1 group (78, 53 and 48%, respectively) were also significantly lower than those of the low AZGP1 group (98.8, 93.8 and 88.8%, respectively; \( P < 0.0001 \); Figure 3E). The patients in the low PEDF group had a significantly shorter OS than those in the high PEDF group \( (P = 0.038; \) Figure 3D). However, no significant association was found between the PEDF level and DFS in the testing set. Finally, serum PRDX2 was not correlated with DFS or OS by Kaplan–Meier analysis in the testing set.

On the basis of Cox univariate proportional hazards analysis, TNM staging, CEA, microvascular invasion, elevated serum AZGP1 and decreased PEDF levels were prognostically correlated with OS. Multivariate analysis identified CEA [hazard ratio (HR) for death = 2.067, 95% CI = 1.099–3.885, \( P = 0.024 \)], elevated serum AZGP1 (HR for death = 6.65, 95% CI = 1.077–41.07, \( P = 0.041 \)) and decreased PEDF levels (HR for death = 0.421, 95% CI = 0.221–0.801, \( P = 0.008 \)) as independent risk factors for the prognosis (Supplementary Table 7, available at Carcinogenesis Online).

Expression of AZGP1, PEDF and PRDX2 in colorectal cancer tissues

To determine the source of AZGP1, PEDF and PRDX2 other than CRC cells, their differential expression in CRC tissues were assayed by qRT–PCR, western blot and immunohistochemistry analysis at both mRNA and protein levels.

We examined the mRNA expression of AZGP1, PEDF and PRDX2 by qRT–PCR in a set of 137 primary CRC tissues, among CRC cohort 2. Analysis showed that there was a significant increase in AZGP1 and decrease in PEDF and PRDX2 expression in the tumor tissues \( (P < 0.001) \) compared with the matched adjacent normal tissues (Figure 4A).

We also detected the protein expression by western blot and found that AZGP1 abundance in tumor tissues was dramatically higher than in the adjacent normal tissues. Interestingly, the PEDF and PRDX2 expressions were lower in tumor tissues compared with normal tissues. These findings were consistent with the data obtained by qRT–PCR (Figure 4B).

To identify the cells within a tumor that expressed AZGP1, PEDF and PRDX2, we used immunohistochemistry to visualize the protein expressions in tumor, corresponding non-tumor tissue and adenoma tissue. AZGP1 was strongly expressed in colorectal epithelial cells compared with normal and adenoma tissue. In cancer specimens, AZGP1 expression was detected only in the cytoplasm and cytomembrane of tumor cells and not in the surrounding stroma. Although the expression of AZGP1 was also detected in some normal mucosa, the expression was remarkably weak (Figure 5A).

![Fig. 3. Kaplan–Meier analysis for OS and DFS in (A–C) training and (D–F) test set according to serum AZGP1 and PEDF levels. In each set, different subgroups were plotted according to the cutoff value of AZGP1 and PEDF levels defined as the median of the set.](https://academic.oup.com/carcin/article-abstract/34/6/1265/2463164)
To address the clinical implication of the upregulation of AZGP1 in CRC, the correlation of AZGP1 with clinical-pathological features from cohort 2 (n = 294) was investigated. The results showed that the upregulation of AZGP1 was significantly correlated with advanced clinical stage (r = 0.152, P = 0.014, Spearman test), lymph node metastasis (r = 0.124, P = 0.041, Spearman test) and distant metastasis (r = 0.145, P = 0.018, Spearman test), whereas no significant correlation was observed with other clinical-pathological parameters (Supplementary Table 8, available at Carcinogenesis Online). To further evaluate the prognostic significance of AZGP1 in CRC, Kaplan–Meier analysis was performed illustrating that high AZGP1 expression was associated with poorer OS and DFS in the CRC cohort 2 (P = 0.031, P = 0.008, respectively, Kaplan–Meier test; Figure 5B).

PEDF and PRDX2 were expressed in the cytoplasm and cell membrane in the normal mucosa, the primary cancer and adenoma cells. However, the expression of PEDF in cancer cells was remarkably weaker than in normal mucosa (Figure 5A).

The downregulation of PEDF was significantly correlated with advanced clinical stage (r = -0.13, P = 0.026, Spearman test), lymph node metastases (r = -0.138, P = 0.023, Spearman test) and distant metastases (r = -0.127, P = 0.03, Spearman test). The lower expression of PRDX2 was also significantly correlated with advanced clinical stage (r = -0.134, P = 0.022, Spearman test), poorer tumor differentiation (r = 0.234, P < 0.0001, Spearman test) and distant metastasis (r = -0.12, P = 0.04, Spearman test). The decreased PEDF and PRDX2 expressions in tumor tissue were significantly associated with a shorter survival time (P = 0.008, P < 0.0001, respectively, Kaplan–Meier test; Figure 5B). The decreased PEDF expression was also significantly associated with a shorter DFS time (P = 0.024, Kaplan–Meier test; Figure 5B).

**Discussion**

Clinical staging plays a significant role in predicting survival and directing treatment for patients with CRC. However, it is acknowledged that patients at similar clinical stages can have very different outcomes. It has agreed that biomarkers may provide assistant information to identify patients at high risk who might be benefited from adjuvant chemotherapy.

In this study, we evaluated the differential expression pattern of proteins among CRC, adenoma and healthy control sera using proteomic technology. Three proteins associated with CRC were identified and further confirmed by ELISA, real-time PCR and immunohistochemical assays. The elevated expression of AZGP1, decreased expression of PEDF in CRC sera and tissues were correlated with liver metastases and poorer OS. The elevated expression of AZGP1 and decreased PEDF were also correlated with DFS. The decreased expression of PRDX2 in CRC tissues was mainly seen in the patients with liver metastases and associated with poorer OS.

We compared the global protein profiles among CRC, adenoma and healthy control serum based on affinity depletion of high-abundance proteins. A total of 27 proteins were identified to be associated with carcinogenesis in CRC. In view of their potential functional relevance, their possible correlation with CRC progression and the fact that they were among the proteins identified as changing significantly by both image analysis packages, AZGP1, PEDF and PRDX2 were chosen as the potential biomarkers for further analysis.

AZGP1 is a 41 kDa soluble protein with a major histocompatibility complex-1-like fold in its structure (21). AZGP1 protein is ubiquitous in normal prostate, breast, skin, salivary gland, liver, kidneys, respiratory and the gastrointestinal tract. It is synthesized by epithelial cells of many tissues and is secreted in various body fluids (22,23). AZGP1 has also been associated with cancer cachexia due to its high level of

---

**Fig. 4.** (A) Relative mRNA expression levels of AZGP1, PEDF and PRDX2 in 137 paired tumor and non-tumor samples from patients in cohort 2. AZGP1 was significantly upregulated in primary CRC compared with their corresponding non-tumor tissues. PEDF and PRDX2 were downregulated in CRC tissues compared with their corresponding non-tumor tissues. Expression was shown in log10 scale and was normalized against an endogenous control glyceraldehyde 3-phosphate dehydrogenase. (B) Immunoblot analysis of AZGP1, PEDF and PRDX2 levels of human CRC tissues and normal mucosa tissues from the same patient. Expression levels of AZGP1, PEDF and PRDX2 were normalized by β-actin.
Amino acid sequence homology with tumor-derived lipid-mobilizing factor (24). In a mouse AZGP1-producing tumors model, AZGP1 stimulated lipolysis in adipocytes leading to cachexia (25). It has been reported that AZGP1 is also a potential serum marker for prostate cancer that may be elevated early in tumor growth (26). It was observed that AZGP1 production was associated with tumor differentiation status of breast and prostate cancer (9,10).

Our 2DE data showed the elevated expression of AZGP1 in tumor tissue was correlated with an increased concentration of the protein in serum. Our results demonstrated significant increase of serum AZGP1 in CRC patients as compared with adenoma patients and normal controls. The ROC curve analysis showed that the area under the curve of AZGP1 reached 0.95, which means that AZGP1 can be used as a potential serum biomarker for diagnosis, prognosis and treatment evaluation purposes. The elevated serum AZGP1 levels in CRC patients were correlated with advanced clinical stage and distant metastasis. Serum AZGP1 was significantly higher in patients with metachronous liver recurrence than in other patients in stages I–III CRC patients. Elevated serum AZGP1 levels were significantly associated with poor survival and were an independent risk factor for poor survival in stages I–IV CRC patients with curative intent surgery. These data suggested that elevated serum AZGP1 level may be a useful marker for predicting recurrence of liver metastases.

Using pre- and post-operative serum samples, we confirmed that elevation of plasma AZGP1 is likely derived from CRC as most patients have markedly reduced levels after surgical resection of tumor. To confirm that the enhanced AZGP1 levels detected in serum were derived from the primary malignancy, the mRNA and protein expression levels in CRC tissues were measured by real-time PCR and immunohistochemistry analysis. The results confirmed that expression of AZGP1 was remarkably higher in tumor tissue than in normal epithelium at the mRNA level. Immunohistochemistry analysis by TMA revealed that the high level of AZGP1 was significantly associated with liver metastasis, poorer DFS and OS in CRC. Our results suggested that AZGP1 may play an important role in the development and progression of CRC.

PEDF is a 50 kDa secreted glycoprotein that is a non-inhibitory member of the serpin (serine protease inhibitor) superfamily proteins and initially isolated from conditioned media of primary human fetal retinal pigment epithelial cells (27). Many reports suggested that it has biological relevance and exerts influences on many physiological processes, likely through diffusion from blood into the extracellular matrix, where it can be immobilized by binding with type I collagen. It exhibits potent activity as an inhibitor of angiogenesis. It has been reported that PEDF was twice as potent as angiostatin and seven times more potent than endostatin (11,28).

Our data showed that PEDF expression was lower in CRC serum than in healthy control, which was consistent with the results of
Wagsater (29). Low serum PEDF levels were associated with TNM staging, liver metastases and poorer OS. This result was confirmed at the tissue level. Low PEDF expression in CRC tissues was also associated with poorer DFS. These results suggested the potential value of the PEDF in the prognosis of CRC.

Peroxiredoxin II (Prx-II), a member of the peroxiredoxin of antioxidant enzyme family, which reduce hydrogen peroxide and alkyl hydroperoxides. Many studies indicated aberrant expression of Prx-II in a variety of cancers. Moreover, some members of Prxs were thought to be a biomarker of cancer cells. Decreased expression of Prx-II protein was found in bladder cancer (12) and malignant melanomas (13). In controversy, increased Prx-II was found in human breast cancer and hepatocellular carcinoma (15,16).

The assay of 2-DE analysis revealed that expression of PRDX2 was significantly lower in CRC sera than in normal control. The serum level of PRDX2 decreased remarkably with the advanced CRC TNM stages. Our results showed that the lower expression of PRDX2 in CRC tissues was significantly correlated with advanced clinical stage, poorer tumor differentiation, distant metastasis and poor survival. The serum PRDX2 levels may reflect the complex systemic antioxidative status of individuals, whereas PRDX2 in CRC tissue represents antioxidative activity within the tumor cells.

In summary, three potential biomarkers, AZGP1, PEDF and PRDX2 were identified and confirmed for diagnosis and prognosis of CRC based on proteomics screening and molecular biology assays. The elevated expression of AZGP1 and decreased expression of PEDF and PRDX2 were correlated with liver metastases and poorer OS. The elevated expression of AZGP1 and decreased expression of PEDF were also correlated with DFS. Our results might provide certain useful information for the development of novel biomarkers for clinical diagnosis and prognostic evaluation of CRC. This finding may potentially enable us to identify and select high-risk patients for effective and aggressive therapy after surgery.

Supplementary material
Supplementary Tables 1–8 can be found at http://carcin.oxfordjournals.org/

Funding
Supporting Program for Science and Technology Research of China (2008BAI52B03); the National High Technology Research and Development Program of China (863 Program) (2012AA02A506); the National Natural Science Foundation (81201965); Beijing Municipal Natural Science Foundation (7132052).

Acknowledgements
We thank Dr Zhihua Tian for technical assistance and Dr Aiguo Wu at George Mason University (USA) for critical reviewing and editing of the article.

Conflict of Interest Statement: None declared.

References

Received October 18, 2012; revised December 28, 2012; accepted February 3, 2013