Identification of Biomarkers to Improve Diagnostic Sensitivity of Sporadic Colorectal Cancer in Patients with Low Preoperative Serum Carcinoembryonic Antigen by Clinical Proteomic Analysis

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ABSTRACT

Purpose. Colorectal cancer (CRC) is the third most common type of cancer worldwide. Carcinoembryonic antigen (CEA) assays usually give false negative results. To improve the diagnosis of primary sporadic CRC, there is an urgent need to identify new biomarkers.

Methods. We used laser pressure catapulting and proteomics to analyze overexpressed cancer associated proteins from 48 sporadic CRC patients with low preoperative serum CEA (LPSC) (<5 ng/ml). Real-time Q-PCR was used to identify the target gene transcripts. Immunoblots were carried out to validate the biomarkers.

Results. Alpha-enolase, HSP27 and macrophage migration inhibitory factor (MIF) were overexpressed in all tumor tissues from 48 LPSC CRC patients, as assessed by 2DE image analysis. The genes were also overexpressed at the transcript level in all tumor tissues from the same patients. In the immunoblot assay, only serum levels of Alpha-enolase and MIF were significantly overexpressed in the LPSC group compared to the mean levels in the control group. Combined with the determinations of preoperative CEA levels, screening for serum Alpha-enolase and MIF were shown to improve the diagnosis of primary CRC.

Conclusions. Serum Alpha-enolase and MIF may be potential biomarkers that can be used to improve clinical predication of primary CRC with LPSC.

INTRODUCTION

Colorectal cancer (CRC) is one of the world's most common malignancies, representing 15% of all diagnosed cancers with an annual incidence of over 1 million new cases worldwide [1]. CRC is also the third leading cause of cancer-related death in Taiwan. Currently, the most widely used screening procedures are flexible sigmoidoscopy and colonoscopy. However, both tests are relatively time consuming, inconvenient and expensive in comparison to blood tests. In addition, fecal occult blood tests (FOBT) are somewhat inaccurate and highly user-dependent. A serum tumor marker with acceptable diagnostic sensitivity and specificity would be more suitable for the detection of sporadic CRC. Tumor-associated antigens, such as carcinoembryonic antigen (CEA), have been proposed as markers of CRC [2]. CEA is the most widely-used marker for CRC, but it lacks sensitivity and specificity in early-stage disease, thus precluding the general use of CEA alone [3].

Clinically and pathologically, CRC can be classified as either the sporadic (approximately 80% of cases) or the hereditary (approximately 20% of cases) form of disease [4]. To date, the vast majority of oligonucleotide and cDNA microarray studies have focused on delineating the genetic mechanisms of sporadic CRC have frequently been reported [5]. In the past decade, the proteome has emerged as a complementary component to the genome. The supposition is that it could drastically

aid in unraveling the biochemical and physiological mechanisms of complex, multivariate diseases. Although genetic mutation and/or errant gene expression may underlie a disease, the biochemical bases for most diseases are protein defects [6]. Therefore, the global analysis of protein abundance in human tumors, called cancer proteomics, could offer many opportunities and challenges in identifying new tumor markers and therapeutic targets, as well as in understanding tumor pathogenesis [7-9]. Many investigators have performed genomic and proteomic studies using cell lines or full cancer tissues, which contain both the tumor itself and surrounding normal tissues. However, the data presented were preliminary and several questions remain, which include defining the optimal time points and appropriate specimens to use in monitoring protein expression, determining the appropriate size of tumor tissues to be tested, discerning which cell line is the best to test, and, finally, determining whether the proteomic dada will actually be translatable to the clinic. Laser Pressure Catapulting (LPC) is a recent development that can be employed to procure highly representative subpopulations of cells from complex, heterogeneous tissues [10]. This technology has been used successfully in a diverse array of studies, involving downstream analysis at the DNA, RNA and protein levels [11].

There are three major aims of this study: (1) to develop systemic proteomic analysis coordinated with powerful sampling in order to improve the quality of

current clinical studies; (2) to verify new biomarkers for Taiwanese sporadic CRC based on low CEA level from tissue samples; and (3) to test the diagnostic sensitivity of the serum levels of these new candidate biomarkers in combination with serum CEA to determine the feasibility of future clinical applications. This study will provide an update on the clinical characteristics of CRC using a rare proteomic method to facilitate more accurate analyses.

PATIENTS AND METHODS

Patients and Donors

From January to December 2007, 125 patients with primary sporadic CRC, which had been treated by resection at the Department of Surgery, China Medical University Hospital, were evaluated in this study. HNPCC and FAP patients were identified by screening patient histories and immunohistochemical staining. Patients that could also be diagnosed based on Amsterdam criteria were excluded. CRC patients who had been treated with preop-chemoradiation were also excluded. Initial staging work-up included history and physical examination, routine biochemistry, determination of CEA levels and X-ray and abdominal computed tomographic (CT) scans. The CRC diagnosis was performed by histological evaluation. In the study, 48 of 125 patients had low preoperative serum levels of CEA (<5 ng/ml, LPSC) and were used in the experiments for determining new potential biomarkers. The main characteristics of the LPSC population are reported in Supplemental data 1**.** Overall, no tumor cells were detected in any of the corresponding surrounding tissues. Specimens were kept at −80 °C until used in the assays. In the immunoblot assay, samples from the control group (50 healthy volunteers) were collected from our Health Examination Center. Use of the tissues and serum for research purposes complied with the regulations set by the Institutional Review Board (DMR-IRB 96-0704). The study was approved by the ethical committee of the China Medical

University Hospital.

Tissue Sample Preparation

Sections for microdissection were prepared according to our previously developed protocol. Briefly, using a cryostat, 12 μm frozen tissue sections were mounted on uncharged glass slides without embedding media, and slides were placed immediately in 70% ethanol for 1 min. Slides were then dried in a laminar flow hood for 10 min prior to microdissection, and the first section was stained with hematoxylin and eosin (H&E) for histological examination.

Microdissection by Laser pressure catapulting (LPC)

LPC was performed according to Bazan *et al.*, with modifications, using a Zeiss inverted microscope PALM Laser Micro-Beam System UV laser at 337 nm, linked to a PC with the required software programs (LMPC; PALM MicroLaser Technologies AG, Bernried, Germany) [10]. A clear discrimination between the observed tumor and normal tissues was demonstrated, which facilitated the cutting and subsequent catapulting procedures on the remaining serial sections (Supplemental data 2 A and B). The entire catapulted target tissues from serial sections were collected on the caps of Eppendorf tubes (Supplemental data 2 C). After catapulting, the material was removed from the cap for analysis. Target tissue collection was microdissected from 30 serial sections from each sample. This collected tumor tissue

sufficiently concentrated protein solutions to be suitable for subsequent 2D gel electrophoresis.

Two-Dimensional Gel Electrophoresis (2DE) Analysis

We used 70 μg of tissue extract per sample for 2D gel separation. The extracted samples were separated using 7 cm immobilized pH 3–10 nonlinear gradient strips (ReadyStrip IPG strip, Bio-Rad) overnight. Electrophoresis in the first dimension was focused for a total of 60 kV h (Protean IEF cell, Bio-Rad) at 20°C, and the strips were stored at -20°C until SDS-PAGE. The strips were transferred to the top of 12% polyacrylamide gels and held in position with molten 0.5% agarose in running buffer. Gels were routinely stained with silver nitrate and scanned, and spots were quantified using a GS-800 imaging Densitometer with PDQuest software version 7.1.1 (Bio-Rad). To compare each tumor to its pair normal sample, we screened for proteins that constituted over-expressed spots (>3 fold) in tumor tissue samples for advanced analysis.

Enzyme Digestion and Nanoelectrospray Mass Spectrometry Analysis

Each protein spot of interest (1~2 mm diameter) was picked using a pipette tip. The gel pieces were digested with trypsin (Promega, Madison, WI) solution (20 ng/μL in 50 mM ammonium bicarbonate), according to the method described in our previous report [12].Protein identification was performed using an Ultimate capillary

LC system (LC Packings, Amsterdam, The Netherlands) coupled to a QSTARXL quadrupole-time of flight (Q-TOF) mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA, USA). The nanoscale capillary LC separation was performed on a RP C18 column. Data acquisition was performed using Automatic Information Dependent Acquisition (IDA; Applied Biosystem /MDS Sciex). The product ion spectra generated by nanoLC-MS/MS were searched against NCBI databases for exact matches using the ProID program (Applied Biosystem/MDS Sciex) and the MASCOT search program.

Real-Time Quantitative RT-PCR

To define relative gene expression, PCR products from tumor samples were compared with PCR products from matched surrounding normal tissues obtained from the same 48 patients. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN). All PCR reactions were performed using the real-time fluorescence detection method and LightCycler System (Roche Diagnostics, Mannheim, Germany), with a FirstStart DNA Master SYBR Green I kit (RocheMolecular Biochemicals, Indianapolis, IN). The primers used for real-time quantitative RT-PCR are listed in Supplemental data 3. The *GAPDH* gene was used to normalize the data.

Immunoblot Assays

For immunoblot analysis, 50 μg of each serum sample from the LPSC CRC

group (48 patients) and the control group (50 healthy donors) were individually separated by 12% SDS-PAGE and electro-transferred onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). After blocking, the membranes were probed with monoclonal antibodies against Alpha-enolase or MIF (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The chemiluminescent signals were detected using an Enhanced Chemiluminescence Plus developer (Amersham Biosciences, Piscataway, NJ.). Signals were captured with the CHEMI GENIUS Bio Imaging System (Syngene, Frederick, MD, USA), and the signal intensities were analyzed using the GeneTools software (Syngene).

Statistical Analysis

All results are expressed as means ± SD. Initially, all data (folds of relative expressions) that were identified as normal distributions by the Biostatistics Center, China Medical University were then analyzed in the current study. The Student's *t*-test was used to analyze statistical significance between tumor and matched normal tissues and between the LPSC CRC group and control group. A $P<0.05$ was regarded as significant.

RESULTS

In this study, we investigated the feasibility of the determined experimental conditions for analyzing the additional biomarkers obtained by LPC integrated proteomics analysis, using the Taiwanese sporadic CRC as the model system. A flow chart of the methods used in this work is depicted in Figure 1.

Screening for overexpressed proteins in tumor tissues

Proteins were quite evenly distributed within the gel, with P/S in the range of 3-10 and molecular masses of 10-70 kDa. Figure 2 shows representative 2DE images of the tumor tissue (Figure 2 A) and the matched normal tissue (Figure 2 B) from LPSC CRC. Some proteins were present in train spots, indicating that isoforms were well separated by 2DE. Averages of 270-483 spots were detected across all gels. The ratio values were compared, and abundance ratios between the CRC and the pair normal tissue samples from different gels were calculated. From the image analysis, we determined that three protein spots constituted overexpression (>3 fold) in each of the CRC samples from the 48 patients. The statistical data for the average of the 48 tissue pairs for Alpha-enolase, HSP27 and MIF proteins are summarized in Table 1, with the fold elevations in protein expression.

Based on the image analysis described above, the three overexpressed protein spots were excised and subjected to in-gel trypsin digestion, nano LC-MS/MS analysis

and database matching. A summary of the three over-expressed proteins is presented in Table 1, which includes the accession number, the Experimental and Theoretical Mr (kDa) / pI, and indicates that most of the matched proteins had high sequence coverage. Overall, the sequence coverage of the three proteins was more than the minimum of 30%, which indicates the data from the nanoLC-MS/MS analysis are dependable.

Quantization of *Alpha-enolase* and *MIF* gene expression

The dynamic results from real time quantitative RT-PCR analysis suggested that all three of the candidate genes were also overexpressed in the tumor tissues. Compared with the paair normal tissues, the *Alpha-enolase, Hsp27* and *MIF* genes were overexpressed by an average of 2.99-fold (95% CI, 2.58-3.22), 1.05-fold (95% CI, 0.85-1.36) and 4.93-fold (95% CI, 3.85-5.38) in the tumor tissues, respectively (Figure 3). This result indicated the overexpression phenomena were not only manifested in protein levels but also in mRNA levels and that only *Alpha-enolase* and *MIF* were demonstrated to be significantly overexpressed.

Validation of the serum protein expressions

In both the control and LPSC groups, the HSP27 signals appeared weak compared to the Alpha-enolase and MIF in the same serum samples (Figure 4A). Quantification of the data indicated that the relative protein levels of Alpha-enolase, HSP27 and MIF (averages of 3.06-, 1.18- and 3.51-fold) were higher in LPSC group

than the mean levels of image intensities from the control group, and only Alpha-enolase and MIF were significantly overexpressed (P<0.01) (Figure 4B).

Improvement in diagnosis of primary CRC

In the clinical guide of CRS, the positive likelihood ratio (diagnostic ratio) for patients with preoperative CEA levels of more than 5 ng/ml was 61.6 % (77/125 patients identified as high risk) in this study. Among the other 48 patients whose CEA levels were less than 5 ng/ml, 26 patients had serum Alpha-enolase, and 37 patients had serum MIF, levels that were 3-fold higher than the mean levels of the image intensities from the control group (n=50), determined by quantitative analysis as described above (data not show). Combining the determinations of serum CEA (cut-off of 5 ng/ml) with Alpha-enolase or MIF levels to diagnose primary CRC increased the number of diagnoses from 61.6 % (77 /125 patients) to 82.4 % (26+77 /125 patients) or 91.2 % (37+77/125 patients), respectively (Figure 5).

Discussion

In recent years, the search for new cancer biomarkers has received a strong impetus from high-throughput genomic and proteomic techniques [13, 14]. Biomarkers should enable scientists and medical staff to make more reliable diagnoses of certain human diseases, especially malignant tumors. Furthermore, biomarkers can facilitate the prediction of disease progression. Cellular heterogeneity has been a significant barrier to the molecular analysis of normal and diseased tissues [15]. An extremely important advantage of the LPC is that it removes all contact between the operator and the prepared sample, which means that this method can enormously reduce human error and eliminate the risk of damage caused by possible contamination or infection [16]. The technique has been applied successfully in several biomarker studies of CRC, but the sample size in previous studies has been too small [10, 17]. Based on the first purpose, in this study, LPC has been used as an optical strategy to improve our skillful proteomic approach.

To date, several groups have reported proteomic analyses of CRC. Dundas *et al.* found that mitochondrial HSP70, which is involved in cell cycle regulation and correlated with poor survival [18]. Alfonso *et al.* reported the up-regulation of annexin Ⅳ and MTA-1 in CRC tissues, along with the down-regulation of NCF2, PMM2 and other factors [19]. Friedman *et al.* identified adenosyl homocysteinase and leukocyte

elastase inhibitor as up-regulated proteins in CRC tissues [20]. Zhang *et al.* identified proliferating cellular nuclear antigen and heterogeneous nuclear ribonucleoprotein overexpression in both the nuclei and cytoplasm of tumor cells by tandem mass spectrometry and tissue microarray [21]. However, the sensitivity of these biomarkers have not yet been satisfactorily established, and there were no data regarding the screening for "sporadic CRC" with "low preoperative serum level of CEA" until now. The second purpose of this study was to identify low serum levels of CEA-related biomarkers present in the CRC tissues, which would lead to a better understanding of the mechanisms driving the 25-40% cases of Taiwanese sporadic CRC progression with low CEA. In the current study, we found that 3 proteins, Alpha-enolase, Hsp27 and MIF, were overexpressed in CRC tissues, which, by inference, might indicate that these proteins are functionally linked to the multi-step transformation processes in sporadic CRC patients with low levels of CEA. This study differs from other previous reports in that we simultaneously monitored differences in expression at both the protein and gene levels from the same samples (Table 1 and Figure 3). The integrated results indicated that significantly increased levels of the transcripts for Alpha-enolase and MIF, but not for HSP27, were the origins of the observed significant protein overexpression (Figure 3).

In cancer research, high levels of HSP27 protein have been commonly demonstrated in cancer tissues, including prostate [22], breast [23], ovarian [24] and gastric tumors [25], but have not previously been reported to be significantly overexpressed in the serum of cancer patients. In our previous report, the HSP27 protein was also shown to be overexpressed in OSCC tissues by clinical proteomic analysis, but there was no significant difference in gene transcription between OSCC and matched normal tissue [26]. In fact, HSP27 is known to be a constitutively expressed protein in many cell types. Therefore this protein was only significantly overexpressed at the protein level, but not at the gene levels in CRC tissues, which is a common phenomenon. Alpha-enolase has been the focus of recent research because of its multifunctional roles in diseases such as cancer and autoimmune disorders [27-31]. The Alpha-enolase enzyme system has been implicated in tissue remodeling, embryogenesis and cell spreading in tumor-cell invasion and metastasis because it can degrade fibrin and extracellular matrix [31]. MIF is a secretory cytokine that is known to contribute to the development and promotion of malignant tumors. Overexpression of serum MIF was reported to be related to poor outcome and early metastasis in cancer [32]. In a gastrointestinal study, MIF was generally increased in sporadic adenomas and carcinomas [33]. In colon cancer, Alpha-enolase and HSP27 have been reported to be overexpressed, but the sample size was too small [17]. Serum MIF

level has also been observed to be an early biomarker for CRC, but the pervious experiment was not gated to screen for sporadic or nonsporadic CRC [34]. Thus, Alpha-enolase and MIF may be potential biomarkers to aid in the diagnosis of LPSC patients in this study, which employed well-established clinical proteomic techniques.

Moreover, we carried out immunoblot analyses to identify serum levels of three candidate biomarkers. Based on the statistical analyses, only Alpha-enolase and MIF were shown to be significantly overexpressed in the LPSC patient group. Thus, we tested the ability of Alpha-enolase and MIF to improve the diagnostic sensitivity of primary LPSC CRC in a total of 125 patients. HSP27 did not appear to be a suitable candidate serum biomarker in this study. Generally, biomarkers were evaluated in body fluid, such as serum, to allow for quick detection. Our third purpose was to check the serum biomarkers, Alpha-enolase and MIF, to determine whether these markers would help in the diagnosis of CRC. We compared the diagnostic sensitivity between determinations of preoperative CEA alone and combined with serum candidate biomarkers by image analysis. These results suggest that serum Alpha-enolase and MIF might be potential biomarkers that would provide higher sensitivity, of over 80 and 90% (82.4 % and 91.2%), for identifying individuals with increased risk of CRC when CEA levels were under 5 ng/ml.

To date, many methods are available for CRC detection when the CEA level is

under 5 ng/ml. However, the costs and risk of complications and discomfort have prevented these tests from being widely used. Thus, developing noninvasive biological markers that can be isolated from body fluid would be of great benefit. Taken together, this study demonstrated that Alpha-enolase and MIF levels in LPSC CRC associated tissue and serum proteins can be used as additional biomarkers to improve diagnosis of primary CRC.

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Figure legends

Figure 1 Outline of the strategy used for screening the helper biomarkers in the sporadic CRC based on low levels of CEA (LPSC).

Figure 2 2DE protein patterns from the tumor and the pair normal tissue extracts from series sections of one patient. Seventy micrograms proteins were separated on 7cm gels followed by silver staining. (A) 2DE map of the tumor tissue that was isolated by LPC. (B) The pair normal tissue from the same patient.

Figure 3 Relative gene expressions of *Alpha-enolase, Hsp27* and *MIF* were identified between the tumor and the pair normal tissues from 48 sporadic CRC patients with LPSC by real time quantitative RT-PCR. *The significantly different from levels of the pair normal tissues based on student's *t*-test ($p< 0.01$).

Figure 4 Validations of serum Alpha-enolase, HSP27 and MIF from 48 LPSC CRC patients (LPSC group) and 50 healthy donors (Control group). (A) 50μg of each sample was separated on 12% SDS-PAGE and immunodetected by the respective primary antibodies. (B) The quantization of the average expression levels of serum Alpha-enolase, HSP27 and MIF relative to mean levels from the control group in

triplicate experiments. White column: control group; black column: LPSC group. ** The significantly different from the health donors based on student's *t*-test ($p< 0.01$).

Figure 5 Among 125 CRC patients, the serum CEA levels more than 5ng/ml were 61.6% (77/125 patients). There were 48 patients whose serum levels of CEA were less than 5ng/ml. Twenty-six of the 48 LPSC CRC patients also had serum Alpha-enolase were 3 fold higher than the mean levels of control group. Combining the determinations of serum CEA and Alpha-enolase levels improved the diagnostic sensitivity for primary CRC from 61.6% to 82.4% (26+77/125 patients) (left). Similarly, there were 37 patients among the same 48 LPSC CRC patients who had 3 fold higher serum MIF levels than the mean of control group, improving the sensitivity from 61.6 % to 91.2% (37 + 77/125) (right).