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The investigation of serum protein profiles in anal fistula for potential biomarkers

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Abstract

Objectives: An anal fistula is an external abnormal anatomical connection between the rectum and the outer skin of the anus. Symptoms include anorectal pain, abscesses, perianal cellulitis, smelly or bloody drainage of pus, and, in some cases, difficulty controlling bowel movements. Diagnosis and evaluation of anal fistulas is crucial for prognosis and for the choice of the treatment method. In this study, we aimed to discover potential biomarkers from serum proteins for the prediction of anal fistulas.

Methods: Using antibody array technology, the expression of 40 proteins was simultaneously detected in serum samples from 13 patients with anal fistulas with chronic diarrhea, 14 patients with chronic diarrhea and six healthy volunteers. Differentially expressed proteins were subsequently validated by ELISA, with a sample population expanded to 30 patients with anal fistulas and chronic diarrhea, 30 patients with chronic diarrheas only and 20 healthy controls.

Results: Quantification analysis identified MIP-1 α , MIP-1 β and TNF-R1 with significant differential expression between the anal fistula with chronic diarrhea, chronic diarrhea only and healthy control groups. Bioinformatics analyses, including PCA and heat map creation, showed a clear separation between the three groups using the expression of MIP-1 α , MIP-1 β and TNF-R1. Validation by ELISA with the expanded sample population fistulas showed significant

differential expression levels of MIP-1 α , MIP-1 β and TNF-R1, displaying accuracy rates of 0.898, 0.987 and 1.0 between the anal fistula with chronic diarrhea and healthy control groups. Accuracy rates between the anal fistula with chronic diarrhea and the chronic diarrhea only groups were 0.9768, 0.909 and 0.964, respectively.

Conclusions: These results suggest the feasibility of employing serum proteins MIP-1 α , MIP-1 β and TNF-R1 as potential biomarkers for rapid and convenient diagnosis of anal fistula in chronic diarrhea patients.

Keywords: anal fistula; antibody array; biomarkers; ELISA.

Introduction

A fistula is an abnormal opening from inside to outside the body or abnormal tunnel between two or more organs. While fistulas can happen in various parts of the body, it is more common in the pelvic region [1]. An anal fistula is an external abnormal anatomical connection between the rectum and the outer skin of the anus. Symptoms include anorectal pain, abscesses, perianal cellulitis, smelly or bloody drainage of pus, and, in some cases, difficulty controlling bowel movements [2, 3]. Most anal fistulas are idiopathic (approximately 90% of cases) and arise from an injury to the tissue lining the anal canal or an infection and pus formation in that area. Epidemiological research shows that men are more commonly affected than women [4], with first presentation at a mean age of 40 years [5]. Anal fistulas are also associated with Crohn's disease, lymphogranuloma venereum, hidradenitis suppurativa, surgery, radiotherapy [6], and sexually transmitted diseases [7], as these can result in deep rectal/anal mucosal damage, which facilitates the development of fistulas. The treatment of perianal fistulas requires both surgical and medical approaches and very few heal without intervention [8]. Accurate identification of anal fistulas is crucial for prognosis and the choice of treatment method. Currently, exploration of the anal canal and distal rectum under anesthesia (EUA), performed by experienced surgeons, is the gold standard for assessing an anal fistula [9–11], with mandatory rectosigmoidoscopy when assessing perianal disease [9]. This procedure is an unpleasant and invasive

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procedure that is associated with patient trauma. Additional diagnostic methods used for assessing perianal disease include magnetic resonance imaging (MRI) or transanal endoscopic ultrasound (EUS). MRI is accurate and non-invasive, but the sensitivity and specificity are lower than EUA and an experienced radiologist or gastroenterologist is required to make reliable assessments [9, 12, 13]. The accuracy of EUS is comparable to pelvic MRI for assessing anal fistula and makes the assessment and histological examination of the rectal mucosa possible [12–15]. However, transanal EUS cannot be performed in the presence of rectal stenosis. Taken together, these limitations indicate a need for new, noninvasive diagnostic methods that are rapid, simple to perform and are efficient for the evaluation of anal fistula patients.

Proteins in serum have been associated with certain diseases and identified as ideal biomarkers for diagnostic and prognostic applications because they are easily collected in a non-invasive manner and are convenient for rapid detection, [16–20]. Cytokines in serum have been reported in many studies to be associated with chronic inflammatory bowel disease [21–23]. The aim of this study was to discover potential serum biomarkers for anal fistulas. With appropriate biomarkers, analysis of expression levels in patient serum could allow the accessing of anal fistulas, which could provide a simpler and more comfortable approach for patients. To accomplish this aim, we investigated the serum protein profiles in a total of 30 patients with anal fistula and chronic diarrhea, 30 patients with chronic diarrhea only and 20 healthy volunteers. Among the 40 target serum proteins assessed using an antibody array detection platform, three proteins were finally identified as being significantly differentially expressed between the three groups, and capable of distinguishing between anal fistula patients and chronic diarrhea patients with high specificity and sensitivity in subsequent enzyme-linked immunosorbent assay (ELISA) validation experiments. These results suggest the possibility of employing serum proteins as biomarkers for rapid and convenient diagnosis of anal fistula patients and are a good example of using a high-throughput antibody array approach to screen serum biomarkers for clinical applications.

Materials and methods

Patients: A total of 30 randomly selected chronic diarrhea patients, including 15 males and 15 females, with a mean age of 44.13 years (ranging from 20 to 68) and 30 anal fistulae with chronic diarrhea patients, including 29 males and one female, with a mean age of 39.42 years (ranging from 17 to 62) were recruited for this study. Blood

tests, X-rays and/or colonoscopy have been performed for all chronic diarrhea patients. Anal fistula with chronic diarrhea patients were diagnosed by rectosigmoidoscopy supplemented with MRI and/or ultrasound, according to the standard diagnosis criteria at the Department of Anorectal Diseases, Shuguang Hospital, within the last five years. A healthy control population of 20 persons without any diseases, including 10 males and 10 females, with a mean age of 40.75 years (ranging from 30 to 63) were also included in this study. Serum samples from individual patients were collected at the time of diagnosis and serum samples from healthy volunteers were obtained during routine physical examinations. The study was approved by the IRB of Shuguang Hospital Affiliated with Shanghai University of TCM (No. 2020-906-115-01).

Serum collection: All serum samples were processed following standard operating procedures. Peripheral blood was collected in BD vacutainer serum tubes (Becton Dickinson Company, Franklin Lakes, NJ, USA) and immediately transported to the laboratory. Serum separation was conducted by incubating samples at room temperature for 45 min and subsequently centrifuging them at 3,000 rpm for 15 min at 4 °C. All sera were stored immediately at –80 °C in small aliquots and repeat freeze–thaw cycles were avoided as recommended by the antibody array manufacturer. Sera from patients were collected at the time of diagnosis and before any treatment or therapy.

Antibody microarray analysis: Protein levels in serum were measured with human cytokine array QAH-INF (RayBiotech, Inc., Norcross, GA, USA), which measures the expression levels of a total of 40 cytokines (Table 1), according to the manufacturer's instructions. Briefly, glass slides containing different antibodies against corresponding protein targets were blocked for 1 h with blocking buffer and incubated with serum samples overnight at 4 °C. The slides were washed and incubated with a mix of biotin-conjugated detection antibodies. Finally, a streptavidin-conjugated Hylite Plus 555 fluor was incubated with the cytokine–antibody complexes on the slides for 1 h at room temperature. The fluorescent signals (532 nm excitation, 635 nm emission) were scanned and extracted using an InnoScan 300 scanner (Innopsys, Carbonne, France). Raw data from the array scanner were provided as images (tif files) and spot intensities (tab-delimited.txt file) via Mapix 7.3.1 Software. Individual array spots were background subtracted locally and normalized through two positive controls and internal controls on each array. All protein values were transformed with log2 transformation to facilitate data analysis, including principal components analysis, correlation heatmap and weighted correlation network analysis.

All data processing and statistical tests were performed in open-source R (<https://www.r-project.org/>) and Rstudio (RStudio, Inc., Boston, MA, USA; URL <http://www.rstudio.com/>). Figures were generated directly in Rstudio and then arranged for publishing using Photoshop CS5 (Adobe, San Diego, CA, USA).

Differential protein expression: To identify proteins with significantly different expression levels (based on log2 transformed values), false-discovery rates (FDR) for each protein were calculated with a non-parametric p-value using the *p.adjust* function in Rstudio. Proteins with an FDR <0.05 and absolute log2 fold-change >1 were identified as differentially expressed proteins (DEPs). Correlations between DEPs were evaluated using the *find correlation* function in R. Those with an average correlation higher than 0.7 were considered as highly collinear and were excluded from further statistical analyses. ROCs of the identified DEPs were analyzed using the “pROC” package in R.

Table 1: 40 cytokines detected for anal fistula with chronic diarrhea, chronic diarrhea only and healthy control groups.

BLC	I-309	IL-1ra	IL-6R	IL-12p40	IL-17	MIP-1 β	TIMP-2
Eotaxin	ICAM-1	IL-2	IL-7	IL-12p70	MCP-1	MIP-1 β	TNF α
Eotaxin-2	IFN γ	IL-4	IL-8	IL-13	MCSF	PDGF-BB	TNF β
G-CSF	IL-1 α	IL-5	IL-10	IL-15	MIG	RANTES	TNF-RI
GM-CSF	IL-1 β	IL-6	IL-11	IL-16	MIP-1 α	TIMP-1	TNF-RII

Classification analysis between patients and healthy controls: Classification analyses were conducted with principal component analysis (PCA), correlation heatmap analysis and an unsupervised linear discriminant analysis (LDA) model between AML patients and healthy controls with DEPs using the ggplot function (R package “ggfortify”). Correlation coefficients and complete clustering were used to analyze dissimilarities between the two groups using the heatmap.2 function (R package “gplots”).

Enzyme-linked immunosorbent assay: Single target detection sandwich-based ELISA kits ELH-MIP-1 α , ELH-MIP-1 β and ELH-TNFR1 were purchased from RayBiotech (Norcross, GA, USA) and used to detect corresponding protein expression levels in serum samples of the different groups, following the manufacturer’s instructions. Proper diluted serum samples were added into 96 well-plates precoated with corresponding capture antibodies. After incubation, washing and re-incubation with detection antibody, substrate reagents were added, and the signals were read at 450 nm using a BioTek800TS microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Results

Demographic parameters: To investigate potential biomarkers of anal fistula with chronic diarrhea, 30 patients with anal fistula and chronic diarrhea and 20 healthy volunteers were included in this study. A group of 30 chronic diarrhea only patients were recruited into the panel to distinguish the anal fistula from chronic diarrhea patients. All patients with other diseases or critical illness history were excluded from the study and no illness was reported in the healthy group (data not shown). The demographic parameters of the three groups are shown in Table 2. In the chronic diarrhea and healthy groups, the numbers of females and males were the same to avoid gender biases. In the anal fistula with chronic diarrhea group, however, far more males than females (29:1) were included because of the different incidence rate between male and female patients and the limited collection in our institution. The age of patients and controls were distributed evenly, and no significant difference was found between the groups with respect to age ($p=0.6359$). For all 60 patients, the history of chronic diarrhea had been investigated with the results ranging from less than one year to longer than 20 years in both patient groups. In anal fistula

Table 2: The demographic parameters of anal fistula, chronic diarrhea and healthy control groups.

Group	Patient number		Age, years	Disease history	
	Male	Female		Anal fistula	Chronic diarrhea
Chronic diarrhea	15	15	20–68	–	1–20 years
Anal fistula with chronic diarrhea	29	1	17–62	<1 month to 5 years	1 to >20 years
Healthy control	10	10	30–63	–	–

patients, the anal fistula history ranged from less than one month to five years.

Differential protein expression levels between patients and healthy controls: To investigate potential biomarkers of anal fistula disease, differentially expressed proteins in sera from 13 patients with anal fistula and chronic diarrhea, 14 patients with chronic diarrhea only (7 male and 7 female) and 6 healthy volunteers were screened individually using cytokine antibody arrays containing 40 target proteins. The experiments were performed successfully as described in the Methods section. According to the principal of the antibody array assay, a fluorescent signal was obtained for each of the quadruple spots corresponding to a different targeted protein after interaction with an equal volume sample. A higher concentration of targeted protein resulted in a stronger signal. Figure 1 shows an example image from arrays assayed with an anal fistula with chronic diarrhea patient sample, a chronic diarrhea patient sample, and a healthy control sample, including internal standard (positive control) and a dilution buffer (negative control) for each sample. The images confirmed differential expression patterns of serum proteins in both anal fistula with chronic diarrhea and patients with chronic diarrhea only when compared with the healthy controls. More importantly, a differential expression pattern was observed between the anal fistula with chronic diarrhea and chronic diarrhea groups, which suggests the biomarkers for anal fistula are capable of distinguishing chronic diarrhea patients.

Figure 1: Samples detected by antibody array assay. Example of laser scanned results from antibody array assay with samples of healthy control (left), chronic diarrhea patient, and anal fistula patient groups. Framed spots show significantly differential expression of protein targets MIP-1 α (red frame), MIP-1 β (orange frame) and TNF-R1 (blue frame). AF.CD, anal fistula with chronic diarrhea; CD, chronic diarrhea; HC, healthy control.

Biomarker identification of anal fistula with chronic diarrhea: Using the extracted fluorescent signals and standard curves, the concentration of each target was quantified from each sample and transformed into a log₂ value for convenience for subsequent data analysis. Our results indicate that more than 90% of the 40 target proteins were detected in most samples, and more than 75% of targets had concentrations within the confidence range (data not shown). To identify proteins with significantly differential expression between patients and healthy controls, false-discovery rates (FDR) and fold-change differences for each protein were calculated with a non-parametric p-value using the p.adjust function in Rstudio. Proteins with an FDR < 0.05 and an absolute log₂ fold-change > 1 were classified as differentially expressed proteins. Of the 37 targets (round spots) detected (Figure 2A), 33 DEPs were identified (right panel blue spots), including one up-regulated and 32 down-regulated proteins (left panel) between anal fistula with chronic diarrhea and the control samples. Table 3 shows the results of 33 DEPs in this serum biomarker screening. Similarly, 34 DEPs with one up-regulated (MIP-1 α) and 33 down-regulated proteins were identified among 38 detectable targets between the chronic diarrhea only group and healthy controls (Figure 2B left and right panel, Table 4). Between the anal fistula with chronic diarrhea and chronic diarrhea only groups, 16 targets presented different expression levels with five down-regulated and nine up-regulated proteins (Figure 2C left panel). Eliminating those proteins with an FDR > 0.05 and an absolute log₂ fold-change < 1, three DEPs MIP-1 α , MIP-1 β and TNF-R1 (Table 5) were identified between the two groups (Figure 2C right panel), showing the potential of biomarkers for anal fistula.

Principal component analysis showed that we could successfully distinguish anal fistula with chronic diarrhea from healthy controls using 33 proteins (Figure 3A), distinguish chronic diarrhea only patients from healthy controls using 34 proteins (Figure 3B), and distinguish anal

fistula with chronic diarrhea from chronic diarrhea only patients using three proteins (Figure 3C). Based on these detected proteins, each group belongs to a different area and can be completely separated from each other without overlap between corresponding groups. Correspondingly, correlation heatmap analysis based on the expression levels of the 33 DEPs and 34 DEPs showed clear differences between the two patient groups and healthy control group with one main cluster that consisted of anal fistula with chronic diarrhea patients or chronic diarrhea only patients and another cluster of healthy controls (Figure 4A, B). More importantly, correlation heatmap analysis between anal fistula with chronic diarrhea patients and chronic diarrhea only patients with the three DEPs clearly separated most samples into corresponding groups while one chronic diarrhea only patient misplaced into the anal fistula clusters. Collectively, these data indicate the potential of the three DEP panel expression profile as for use in diagnosis of anal fistula with chronic diarrhea.

To verify the use of MIP-1 α , MIP-1 β and TNF-R1 obtained from the first screen by antibody array, the samples were expanded to 30 samples anal fistula with chronic diarrhea, 30 of chronic diarrhea only and 20 of healthy controls. Respectively to detect the expression levels of the three DEPs were measured in the individual groups by ELISA. As shown in Table 6, the three proteins displayed differential expression levels between anal fistula with chronic diarrhea patients, chronic diarrhea only patients and healthy controls. While there were some differences, the expression trends of all three proteins were similar to the differences shown in the first screen (Figure 5 and Tables 3–5). With the detection results of MIP-1 α , MIP-1 β and TNF-R1, LDA analysis had accuracy rates of 0.898, 0.987 and 1.0, respectively, between anal fistula with chronic diarrhea and the healthy control, and accuracy rates of 0.9768, 0.909 and 0.964 between anal fistula with chronic diarrhea and chronic diarrhea only groups (Figure 6).

Figure 2: Analysis of differentially expressed proteins between the anal fistula with chronic diarrhea and healthy control groups (A), chronic diarrhea only and healthy control groups (B), and anal fistula with chronic diarrhea and chronic diarrhea patient groups (C). Based on the results of antibody array assay, proteins down-regulated, up-regulated or with no difference between two groups are shown as blue, red or gray spots, respectively, in left panels. The differentially expressed proteins were analyzed by fold changes between two groups shown as blue spots in the right panels. AF.CD, anal fistula with chronic diarrhea; CD, chronic diarrhea; HC, healthy control.

Table 3: Differential expressed serum proteins between 13 anal fistulas with CD and six healthy samples detected by antibody arrays.

Protein name	Mean AF.CD	Mean HC	log2 FC	p-Value
BLC	20.39	3.61	2.50	<0.0001
Eotaxin	66.89	14.61	2.19	<0.0001
G-CSF	16.15	1.36	3.57	0.01
GM-CSF	182.68	4.82	5.25	<0.0001
I-309	21.01	3.94	2.41	<0.0001
IFN γ	195.75	0.84	7.87	<0.0001
IL-1 α	322.58	14.73	4.45	<0.0001
IL-1 β	45.85	1.14	5.33	<0.0001
IL-1 α	537.33	14.64	5.20	<0.0001
IL-2	398.76	3.24	6.94	<0.0001
IL-4	20.01	1.88	3.41	<0.0001
IL-5	142.11	5.94	4.58	<0.0001
IL-6	196.44	12.81	3.94	<0.0001
IL-7	81.01	5.80	3.80	<0.0001
IL-8	84.28	2.02	5.38	<0.0001
IL-10	16.20	0.46	5.15	<0.0001
IL-11	132.18	11.44	3.53	<0.0001
IL-12p40	54.21	5.70	3.25	<0.0001
IL-12p70	0.54	0.22	1.29	0.01
IL-13	16.52	0.38	5.45	<0.0001
IL-15	78.99	9.53	3.05	<0.0001
IL-17	4.82	0.59	3.04	<0.0001
MCP-1	361.04	94.46	1.93	<0.0001
MCSF	2.50	0.62	2.01	0.02
MIG	481.35	1.04	8.85	<0.0001
MIP-1 α	40.53	17.56	1.21	<0.0001
MIP-1 β	79.65	12.29	2.70	<0.0001
MIP-1 δ	1861.53	484.42	1.94	<0.0001
PDGF-BB	1,010.95	174.43	2.54	<0.0001
TNF α	427.79	15.51	4.79	<0.0001
TNF β	1,230.52	15.04	6.35	<0.0001
TNF-R1	6,379.03	1,518.30	2.07	<0.0001
TNF-R2	7,344.53	857.15	3.10	<0.0001

AF.CD, anal fistula with chronic diarrhea; HC, healthy control.

Between the chronic diarrhea and healthy groups, the LDA analysis resulted in accuracy rates of 0.853, 0.876 and 0.879, respectively. Taken together, these data demonstrate that the differential expression levels of MIP-1 α , MIP-1 β and TNF-R1 could distinguish the 30 anal fistulae with chronic diarrhea patients from chronic diarrhea only patients and healthy controls, which successfully confirms the discriminating role of the three proteins identified in the initial screening. Therefore, our results suggest that proteins MIP-1 α , MIP-1 β and TNF-R1 are potential diagnostic biomarkers of anal fistula in chronic diarrhea patients. In addition, our results support the protein profiling investigation in the anal fistula clinical samples using antibody array technology, which has a powerful capacity as a high-throughput screening tool.

Table 4: Differential expressed serum proteins between 14 CD only and six healthy samples detected by antibody arrays.

Protein name	Mean CD	Mean HC	log2 FC	p-Value
BLC	24.27	3.61	2.75	<0.0001
Eotaxin	56.73	14.61	1.96	<0.0001
G-CSF	10.07	1.36	2.89	0.01
GM-CSF	182.61	4.82	5.24	<0.0001
I-309	18.01	3.94	2.19	<0.0001
IFN γ	187.02	0.84	7.80	<0.0001
IL-1 α	284.40	14.73	4.27	<0.0001
IL-1 β	48.12	1.14	5.40	<0.0001
IL-1 α	505.15	14.64	5.11	<0.0001
IL-2	411.30	3.24	6.99	<0.0001
IL-4	26.64	1.88	3.83	0.01
IL-5	133.29	5.94	4.49	<0.0001
IL-6	196.73	12.81	3.94	<0.0001
IL-7	77.67	5.80	3.74	<0.0001
IL-8	89.58	2.02	5.47	<0.0001
IL-10	17.46	0.46	5.26	<0.0001
IL-11	144.11	11.44	3.66	<0.0001
IL-12p40	75.52	5.70	3.73	<0.0001
IL-12p70	0.74	0.22	1.74	<0.0001
IL-13	17.44	0.38	5.53	<0.0001
IL-15	80.15	9.53	3.07	<0.0001
IL-16	58.53	24.85	1.15	<0.0001
IL-17	7.47	0.59	3.67	<0.0001
MCP-1	314.07	94.46	1.73	<0.0001
MCSF	1.89	0.62	1.60	<0.0001
MIG	299.23	1.04	8.16	<0.0001
MIP-1 α	8.76	17.56	-1.00	<0.0001
MIP-1 β	39.01	12.29	1.67	0.01
MIP-1 δ	2,156.80	484.42	2.15	<0.0001
PDGF-BB	1,088.77	174.43	2.64	<0.0001
TNF α	422.07	15.51	4.77	<0.0001
TNF β	1,262.89	15.04	6.39	<0.0001
TNF-R1	3,075.55	1,518.30	1.02	<0.0001
TNF-R2	6,255.84	857.15	2.87	<0.0001

CD, chronic diarrhea; HC, healthy control.

Table 5: Differential expressed serum proteins between 13 anal fistulas with CD and 14 CD only samples detected by antibody arrays.

Protein name	Mean AF.CD	Mean CD	log2 FC	p-Value
MIP-1 α	40.53	8.76	2.21	<0.0001
MIP-1 β	79.65	39.01	1.03	<0.0001
TNF-R1	6,379.03	3,075.55	1.05	<0.0001

Discussion

Worldwide, the prevalence of anal fistula is reported to be approximately 1–2 per 10,000 patients and the majority of anal fistulas are idiopathic and associated with

Figure 3: Principal component analysis (PCA) analysis between anal fistula with chronic diarrhea patients and healthy control group (A) based on 34 DEPs, chronic diarrhea patients and healthy control groups based on 33 DEPs (B), and anal fistula with chronic diarrhea patients and chronic diarrhea patient groups based on three DEPs (C). Each spot represents a single sample and different color means different sample group. AF.CD, anal fistula with chronic diarrhea; CD, chronic diarrhea; HC, healthy control.

Figure 4: Correlation heatmap analysis. Anal fistula with chronic diarrhea patients and healthy control groups (A) based on 34 DEPs, chronic diarrhea patients and healthy control groups based on 33 DEPs (B), and anal fistula with chronic diarrhea patients and chronic diarrhea patient groups based on three DEPs (C).

Table 6: Differential expressed serum proteins of MIP-1α, MIP-1β and TNF-R1 between anal fistula wit CD patients (n=30), CD patients (n=30) and healthy control (n=20) detected by ELISA.

	AF.CD		CD		HC		AF.CD vs. HC		AF.CD vs. CD only		CD only vs. HC	
	Average	STD	Average	STD	Average	STD	Fold change	p-Value	Fold change	p-Value	Fold change	p-Value
MIP-1α	3.95	1.71	1.01	0.58	1.96	0.59	2.01	<0.0001	3.89	<0.0001	0.52	<0.0001
MIP-1β	72.91	26.84	27.06	11.19	13.39	6.55	5.44	<0.0001	2.69	<0.0001	2.02	<0.0001
TNF-R1	5.21	1.96	1.56	0.79	0.65	0.28	7.97	<0.0001	3.35	<0.0001	2.38	<0.0001

CD, chronic diarrhea; AF.CD, anal fistula with chronic diarrhea; HC, healthy control.

Figure 5: Validation of DEPs by ELISA. Three DEPs, MIP-1α, MIP-1β and TNF-R1, were validated by ELISA with samples of anal fistula with chronic diarrhea patients (n=30), chronic diarrhea patients (n=30) and healthy controls (n=20). AF.CD, anal fistula with chronic diarrhea; CD, chronic diarrhea; HC, healthy control.

Figure 6: LDA analysis of MIP-1α, MIP-1β and TNF-R1 detected by ELISA.

MIP-1α, MIP-1β and TNF-R1 in samples of anal fistula with chronic diarrhea patients (n=30), chronic diarrhea only patients (n=30) and healthy controls (n=20) were detected individually by ELISA and analyzed by LDA between each pair of groups to obtain the accuracy rate. AF.CD, anal fistula with chronic diarrhea; CD, chronic diarrhea; HC, healthy control.

inflammatory conditions [24, 25]. Diagnosis and evaluation of perianal chronic diarrhea are important for treatment and therapy. Traditional diagnostic tools, such as EUA and MRI, however, are complicated and must be performed by professionals and are painful for the patients. Serum proteins including cytokines, chemokines, growth factors and secreted receptors have been proven to be associated with a wide spectrum of diseases and could be ideal biomarkers with the advantages of rapid, simple and noninvasive detection.

To discover the potential biomarkers related to anal fistulas with chronic inflammatory conditions, in this study we simultaneously analyzed 40 proteins in the serum

samples of anal fistula with chronic diarrhea patients, chronic diarrhea only patients and healthy controls. By analyzing the expression levels of 40 proteins between the three groups, we identified three proteins, MIP-1α, MIP-1β and TNF-R1, which can distinguish the anal fistula with chronic diarrhea patients from healthy controls and patients suffering from chronic diarrhea alone. These proteins were confirmed by ELISA with expanded sample numbers. To our knowledge, this is the first study to discover potential biomarkers from serum proteins for anal fistula in a certain amount of patient samples. There was no evidence of sample degradation, despite some samples

having been stored for up to five years, because no significant variance of most of the targets was noted when compared with freshly collected samples in the same group (data not shown). As shown in Table 2, a total of 30 anal fistula with chronic diarrhea patients, 30 chronic diarrheas only patients and 20 healthy volunteers were recruited for the initial screen and follow-up validation in this study, and samples with any other significant diseases were excluded. There was no statistical difference with respect to distribution of age within the three groups, indicating that the observed differences in expression could not be attributed to other clinical features such as age or disease history. In the chronic diarrhea only and healthy control groups, equal numbers of male and female patients were selected there by avoiding gender bias. In the anal fistulae with chronic diarrhea group, however, there were 29 male patients and only one female patient. The gender difference in this group was caused by limits of the female patient source that we could obtain and consistent with the low incidence rate of anal fistula in females reported worldwide [4]. Therefore, the gender bias in the anal fistula with chronic diarrhea group should not be of concern in this investigation.

In the initial screening with 13 anal fistulas with chronic diarrhea samples, 14 chronic diarrheas only samples and six healthy control samples, the expression levels of 40 proteins were analyzed simultaneously using an antibody array for each sample. Antibody array is a new technical platform developed to facilitate the global analysis of expression patterns of proteins in cells, tissues and serum or plasma, in a high-throughput manner, to improve the single protein detection bottleneck that currently plagues clinical detection attempts. The application of antibody arrays in biomarker discovery has allowed considerable progress in improving the diagnosis and prognosis of several diseases, including cancer, neurodegeneration, and cardiovascular disease [19, 20, 26]. The application of antibody arrays has rarely been reported in anal fistula disease, and this is our first attempt to comprehensively evaluate serum protein profiles using this new platform.

The 40 proteins detected in this array consist mostly of inflammatory factors but include some growth factors and secreted receptors as well. The comparison of anal fistula with chronic diarrhea group to healthy control group identified 33 DEPs (Table 3). With the panel of 33 proteins, anal fistula with chronic diarrhea patients was clearly separated from healthy volunteers (Figures 2, 3A, 4A). Of the 33 proteins, most are inflammatory factors, such as IL-1, IL-2, 4, 5, 6, 7, 8, 10, 11, 12, 13, 15, 17, MIP-1 α , 1 β , and TNF- α , which is not surprising due to the chronic inflammatory characteristic of chronic diarrhea. In previous studies, several researchers have also reported the elevated inflammatory factors in anal fistulas. Using fistula tract tissue, Onkelen et al. [27]

accessed the level of IL-1 β , IL-8, IL-10, IL-12p40, IL-17A, IL-18, IL-36 and TNF- α using immuno-enzyme staining methods and found high expression of IL-1 β , IL-8, IL-12p40 and TNF- α in the large majority of cryptoglandular anal fistulas. Ruffolo et al. [28] quantified the expression of TNF- α , IL-6, IL-1, IL-12, and TGF-1 by ELISA in 17 anal fistula patients with Crohn's disease, seven Crohn's disease patients without perianal involvement, and 17 healthy controls using rectal mucosal samples and concluded that mucosal levels of IL-6 and IL-12 are predictors of recurrence and of the need for surgery in anal fistula patients. In the present study, we significantly expanded the number of targets, many of which have not been targeted in anal fistula samples before. Our results have not only further confirmed previous discoveries but revealed more DEPs in anal fistula with chronic diarrhea due to a wider range of proteins being investigated. In our expectation, 34 DEPs between the chronic diarrhea only and healthy control groups were similar to the 33 DEPs between the anal fistula with chronic diarrhea and healthy control groups, with the addition of IL-16 (Table 4 and Figures 2, 3B, 4B). This result is basically coincident with a recent report from Haddow et al. who systematically compared the cytokine and phosphoprotein profiles between idiopathic and CD-related perianal fistulae by investigating the concentrations of 30 cytokines and 39 phosphoproteins in 48 idiopathic and 13 CD perianal fistula patients using a chemiluminescent antibody array respectively, resulting in no major differences in cytokine and phosphoprotein profiles between two groups [29]. While Adegbola et al. have more recently revealed 41 differentiated metabolites including amino acid, carnitine and lipid metabolism from tissue samples of Crohn's vs. idiopathic anal fistula patients [30], we have fortunately in this study narrowed over 30 DEPs down to three proteins, MIP-1 α , MIP-1 β and TNF-R1, when comparing the anal fistula with chronic diarrhea group to the chronic diarrhea only group (Figure 2C). The PCA and heatmap analysis showed successful separation between the anal fistula with chronic diarrhea and chronic diarrhea only groups (Figures 3C and 4C). Validation of the three DEPs using ELISA with a sample population expanded to 30 anal fistulae with chronic diarrhea, 30 chronic diarrheas only and 20 healthy control samples showed the trend of expression levels similar to the initial screening (Figure 5 and Table 6), increasing our confidence in the proteins and the reliability of the methodology.

Limitations

Because of limited number of samples employed, conclusions based on this study should be drawn cautiously,

although the data resulting from the experiments is reliable. Further testing of these three proteins, separately or together, is required with a larger sample number to verify and validate their future use for diagnosis of anal fistula with chronic diarrhea.

Conclusions

Altogether, our investigation combined the screening of 40 protein expression levels by antibody array and validation by ELISA in three patient groups, including anal fistula with chronic diarrhea, chronic diarrhea only and healthy volunteers. We identified three proteins, MIP-1 α , MIP-1 β and TNF-R1, with significantly differential expression levels which can clearly distinguish the patients from corresponding groups and could be used as biomarkers for diagnosis of anal fistula with chronic diarrhea patients. It is worth noting that some samples were classified into the wrong group by correlation heatmap analysis, suggesting either that these patients and healthy samples need to be identified with more strict clinical parameters or that a wider spectrum of proteins should be evaluated and included into the current profile.

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