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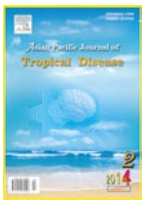

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Identification of specific proteins in colorectal cancer patients with *Schistosoma mansoni* infection as a possible biomarker for the treatment of this infection

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ABSTRACT

Objective: To identify the associated proteins or antigens in serum of colorectal cancer patients with and without the chronic *Schistosoma mansoni* (*S. mansoni*) infection in Sudan.
Methods: The study included 93 patients with colorectal cancer and/or *S. mansoni* infection. A clinical database of colorectal cancer patients was documented by pathologist and information of *S. mansoni* infection was collected through stool analysis. Blood samples were collected from the identified patients. Geographical distribution of patients and etiological factors of colorectal cancer and *S. mansoni* infection were also discussed in this study. The proteins extracted from serum samples of patients using Tri Reagent were electrophoretically separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis. Specific protein band was then subjected to matrix laser desorption ionisation-time assistance-of-flight mass spectrometry analysis.
Results: Stool analysis documented that 40 patients were colorectal cancer patients infected with *S. mansoni*, 26 patients were diagnosed with colorectal cancer only, and 27 patients were infected with *S. mansoni* only. Approximately 27% of participating patients with colorectal cancer and *S. mansoni* infection in this study came from Gezira State. A specific protein band was identified from the serum samples of colorectal cancer patients with *S. mansoni* infection on the separated protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The specific protein band was then analyzed by matrix laser desorption ionisation-time assistance-of-flight mass spectrometry and identified as a clusterin protein.
Conclusions: The study showed that the colorectal cancer patients infected with *S. mansoni* expressed clusterin protein, indicating that clusterin protein may be a potential biomarker to aid in the treatment of colorectal cancer patients infected with *S. mansoni* in the future.

1. Introduction

Human cancers are believed to caused by infectious diseases. It was estimated that 18% of cancer cases worldwide are related to infectious diseases[1]. This proportion varies by region, ranging from 25% in some areas of Africa to approximately 10% in developed countries. Infections with parasites, including *Schistosoma haematobium* (*S. haematobium*) that usually cause squamous cell carcinoma of the bladder and liver flukes[2], are often associated with human cancers. Schistosomiasis is a fairly prevalent communicable disease in tropical and subtropical regions, and is caused by a trematode of the genus *Schistosoma*. In general, human schistosomiasis is caused by three major species: *Schistosoma mansoni* (*S. mansoni*) endemic in Africa, the Middle East, and South America; *Schistosoma japonicum* (*S. japonicum*) common in Southeast Asia; and *S. haematobium*, which prevails in Africa and the Middle East[3]. Schistosomiasis affects more than 210 million people worldwide, and more than 700 million people live in areas that favour the transmission[4,5].

Schistosomiasis is a long-term infection with a progressive development of pathological changes throughout the course of the disease. *S. haematobium* can cause bladder cancer, whereas *S. mansoni* and *S. japonicum*

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result in schistosomal colitis. By a sequence of infection, treatment and re-infection, the disease causes a transient and recurrent inflammatory reaction in patients[6,7]. *S. mansoni* and *S. japonicum* remain sufficiently viable in the large intestine and can produce an inflammatory reaction, granuloma formation, papilloma, ulceration, bleeding, and subsequently fibrosis that gives rise to the long-term sequelae of the disease[8,9]. An association between schistosomiasis and colorectal malignancy has long been proposed in the literature. However, this link has not been uniformly accepted. In the Far East, the evidence supporting the etiological link between *S. japonicum* and colorectal cancer has frequently been discussed. However, available data on the role of *S. mansoni* and colorectal carcinogenesis is inconsistent and often does not show causality. There have been some studies on schistosomal colonic disease, but literature reports on the value of schistosomal colonoscopic biopsy in the diagnosis of colon disease remain scarce.

Scientists and clinicians have increasingly applied various techniques, including molecular approaches, to discover and validate new biomarkers or ensembles of biomarkers that display better specificity and sensitivity characteristics than existing methods. Indeed, an improved understanding of African schistosomal-related colorectal cancer initiation and progression is warranted[10]. Therefore, the identification of specific protein(s) or antigen(s) that indicate the proliferation of viable colorectal cancer cells could be useful for cancer staging and surveillance. This study aims to identify specific protein(s) or antigen(s) in the serum samples of colorectal cancer patients, some of whom were infected with *S. mansoni*, and also to assess the identity of the identified protein(s) or antigen(s) that to be used as a possible biomarker to aid in the treatment of colorectal cancer patients infected with *S. mansoni* in future.

2. Materials and methods

2.1. S. mansoni-infected population in Sudan

A total of 93 patients with colorectal cancer and/or *S. mansoni* infection from Khartoum Hospital in Sudan were involved in the study. The study was approved by the Tropical Medicine Research Institute in Sudan. Stool specimens were collected from all patients for analysis and were processed using a modified Kato-Katz technique as described by Teesdale and Amin[11]. Blood samples were also collected from the patients for protein extraction and specific protein identification.

2.2. Blood sample collection

Blood samples (2 mL) were collected in non-heparinised tubes from all patients involved in this study. The blood samples were allowed to clot at room temperature, and then serum of each sample was separated by centrifugation at 2000 r/min for 15 min and stored at −70 °C until use.

2.3. Protein extraction and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Tri Reagent was used to extract proteins from the blood samples. The concentration of each extracted protein was adjusted to 20 μg/μL using the DC protein assay reagent before SDS-PAGE analysis. The proteins were then separated by 10% reducing and discontinuous SDS-PAGE, as described by Laemmli[12], at 100 V for 2 h using a slab mini-gel system (Mini-V 8-10 Vertical Gel Electrophoresis Apparatus). The separation of the protein samples was performed according to their molecular weights.

2.4. Matrix laser desorption ionisation-time assistance-of-flight (MALDI-TOF) mass spectrometry

Bands of proteins (peptides) observed on the gel were excised and analysed by MALDI-TOF mass spectrometry. Subsequently, the identified peptides were analysed by Mascot Search Results software for the identification of specific peptides. Protein scores greater than 70 was regarded as statistically significant ($P<0.05$).

3. Results

Stool examination of all patients showed that 40 patients diagnosed with malignant colorectal cancer by colonoscopy and histopathology were infected with *S. mansoni*, 26 patients who were diagnosed with colorectal cancer were *S. mansoni* negative, and the remaining 27 patients were only infected with *S. mansoni*. Among the 93 patients who were examined and diagnosed, 75 patients (80.6%) were male, and 18 patients (19.4%) were female. Age of all patients involved in this study ranged from 15 to 66 years and above. The distribution of ages for this study is shown in Table 1.

Table 1
Age groups, number and percentage (%) of patients involved in this study.

Age group	Number	Percentage (%)
15-25	31	33.3
26-35	21	22.6
36-45	15	16.1
46-55	13	14.0
56-65	10	10.8
>66	3	3.2
Total	93	100.0

The geographic distribution analysis showed that high percentage of Sudanese patients with colorectal cancer and *S. mansoni* infection were from the Gazira State compared to other states (Figure 1).

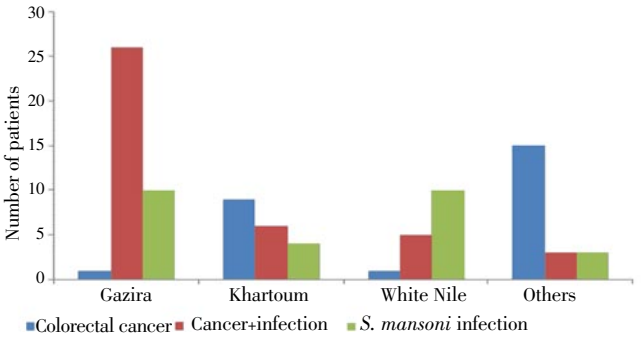


Figure 1. The geographical distribution of Sudanese patients with colorectal cancer and/or S. mansoni infection.

The electrophoretic separation of proteins extracted from serum samples of colorectal cancer patients, patients with *S. mansoni* infection only and colorectal cancer patients with *S. mansoni* infection were stained with Coomassie brilliant blue stain. A specific protein band (A) was identified from the serum samples of colorectal cancer patients with *S. mansoni* infection that served to distinguish the serum samples of the colorectal cancer patients who were infected with *S. mansoni* and those who were not infected with the parasite. Another protein band (B1) was identified in the serum samples of colorectal cancer patients with *S. mansoni* infection and B2 was identified in the serum samples of the patients with *S. mansoni* infection only (Figure 2).

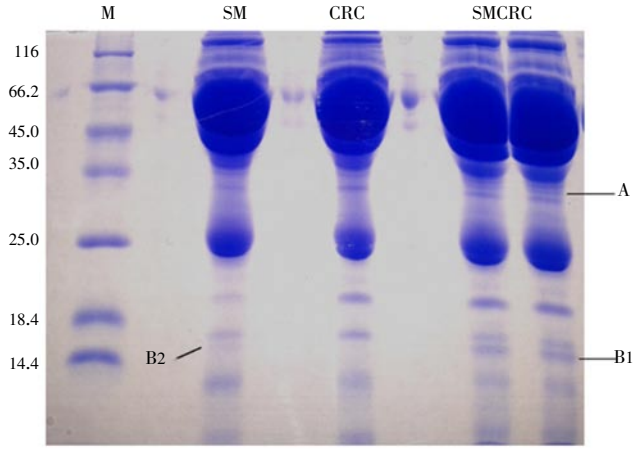


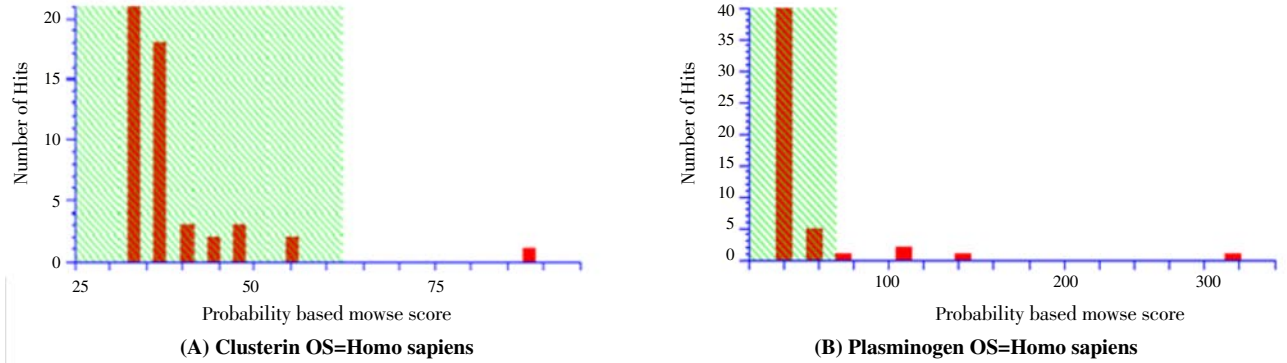
Figure 2. Analysis of proteins in serum samples collected from patients by SDS-PAGE.
M: Molecular weight marker; SM: Patients infected with *S. mansoni*; CRC: patients diagnosed with colorectal cancer; SMCRC: patients diagnosed with colorectal cancer and *S. mansoni* infection.

MALDI-TOF mass spectrometry analysis showed that the unknown protein band (A) detected in the serum samples analysed by Mascot Search, was a clusterin protein fragment. This peptide was specifically identified in the serum samples of colorectal cancer patients who were *S. mansoni* positive, but not in the colorectal cancer patients who were *S. mansoni* negative. Protein band (B1) revealed in colorectal cancer patients with *S. mansoni* infection and a faint protein band (B2) appeared in patients with *S. mansoni* infection only were plasminogen protein fragments (P<0.05) (Figure 3).

4. Discussion

Schistosomiasis is a major public health problem that has long existed in Sudan, particularly in area with irrigation schemes. The disease traces back to 2600 BC, and its introduction is thought to be a sign of political and economic ties to Egypt. The flow of thousands of pilgrims headed to Mecca from West Africa through Sudan may also have contributed to the establishment of the disease in Sudan[13]. Additionally, this disease has also demonstrated a close relation to colorectal cancer. The precursors of colorectal carcinomas are adenomatous polyps and chronic inflammatory bowel diseases, such as ulcerative colitis and Crohn's diseases. Additionally, infectious diseases that are common in the tropics may be related to colorectal malignancies[14]. *S. mansoni* and *S. japonicum* infections in the intestine begin with sequestered eggs in the mucosa and submucosa, inciting a severe inflammatory reaction with cellular infiltration and consequent granuloma formation. This reaction, in turn, leads to mucosal ulceration, microabscess formation, polyposis and neoplastic transformation[15], and may lead to colorectal cancer development.

In Sudan, approximately 51% of patients with colorectal cancer and *S. mansoni* infection are under the age of 35, as reported by Abou-Zeid and Mansour[16,17]. This phenomenon is in contrast to colorectal cancer prevalence in developed countries whereby approximately 90% of colorectal cancers are caused by environmental factors, and 10% are caused by genetics[18,19]. Similar to the aforementioned region, exposure to *Schistosoma* is responsible for 21% of the cases of childhood *S. mansoni* infection in Sudan[20]. The male predominance in *S. mansoni* infection observed in this work was in line with Hayne's study[21], but contrasts with McDermott's and a recent study[22,23], which found that infections were equally distributed between the sexes. This difference is not hormone dependent[24], but it is natural that the schistosomal infection is common in men and men are more prone to getting and suffering from this infection.



Protein name	Protein Score	Unique Peptides
(A) Clusterin	88 (P<0.05)	TSDSDVPSGVTEVVVK
(B) Plasminogen	296 (P<0.05)	WELCDIPR.C+Carbamidomethyl (C)

Figure 3. Analysis of MALDI-TOF mass spectrometry for protein bands identified from SDS-PAGE.

Approximately 27% of participating patients with colorectal cancer and *S. mansoni* infection in this study came from Gezira State, where the Gazira Agriculture Irrigation Scheme is known to be an area of high endemicity for schistosomiasis in Sudan[25,26].

In this study, we found that patients diagnosed with colorectal cancer and *S. mansoni* infection secreted clusterin protein that was not found in the serum samples of colorectal cancer patients who were *S. mansoni* negative. Clusterin protein has been reported to be involved in numerously physiological processes important for carcinogenesis and tumour growth, including apoptotic cell death, cell cycle regulation, DNA repair, cell adhesion, tissue remodelling, lipid transportation, membrane recycling and immune system regulation[27,28]. A previous study indicated that the clusterin protein expression is involved in tumorigenesis and the progression of 25% of colorectal cancer. Hence, in 75% of the tumor specimens, there is not clusterin expressing cancer cells[29]. Clusterin protein appears to be a sensitive and stable histological marker for murine and human intestinal tumours. Therefore, clusterin protein can be used as a potential biomarker for human colorectal cancer. Furthermore, the high secretion of clusterin protein from tumour cells can be easily detected in body fluids, such as serum. The correlation pattern of clusterin protein expression to the events of the other cells indicated that clusterin expression is involved in tumorigenesis and progression of 25% of colorectal cancer cells[30]. Perhaps, clusterin protein only presents in the cancer patients infected with certain infectious diasease, highlighting the potential of clusterin protein to be used as a biomarker in an effective treatment solution for colorectal cancer patients infected with *S. mansoni*.

This study also showed that patients with *S. mansoni* infection and those have colorectal cancer with *S. mansoni* infection released plasminogen protein. Plasminogen is a single chain glycoprotein, representing the monomeric proenzyme of the serine protease plasmin, which degrades fibrin and extracellular matrices[31]. Interactions of plasminogen with pathogens have been observed in bacteria, fungi, protozoa and helminths. The interactions show to increase invasiveness within the host[32-34]. The protozoa *Leishmania mexicana* and *Trypanosoma cruzi* have been reported to bind to the plasminogen, enhancing the activation of plasminogen by tissue-type plasminogen activator (t-PA)[35,36]. In helminths, two plasminogen-binding proteins have been identified: enolase in *Fasciola hepatica* and *Onchocerca volvulus* and glyceraldehyde-3-phosphate dehydrogenase in *Onchocerca volvulus*[37-39].

The identification of useful markers for the treatment of colorectal cancer patients infected with *S. mansoni* is the primary goal of our research. The lack of specific and sensitive biomarkers for colorectal cancer and schistosomal infections has resulted in delayed treatment for these patients. Therefore, the search for specific and sensitive markers for the treatment is warranted. High clusterin protein expression in the serum of colorectal cancer patients infected with *S. mansoni* indicate that clusterin may be a potential biomarker to aid in the treatment of colorectal cancer patients infected with *S. mansoni*, and plasminogen distinguih colorectal cancer patients with *S. mansoni* infection and patients infected with *S. mansoni* only in the future.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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