Screening of Auto-antigens as Diagnostic Biomarkers in Sera of Patients with Cholangiocarcinoma by SERPA Technique

Urwa Tanveer^{1*}, Hafsa Tariq², Nargis Haider Kakar³, Tariq. M. Tahir⁴, Qurat-ul-ain Fatima⁵, Hina Sadaf⁶ and Taimoor Anwar⁷

¹Allied Burn and Reconstructive Surgery Center, Faisalabad, Pakistan.

²Services Institute of Medical Sciences, Lahore, Pakistan.

³Department of Physiology, Bolan University of Medical and Health Sciences, Quetta, Pakistan.

⁴Department of Pathology, Independent Medical College, Faisalabad, Pakistan.

⁵Department of Physiology, Avicenna Medical College, Lahore, Pakistan.

⁶Physiology Department, Pak Red Crescent Medical College, Dina Nath, Kasur, Pakistan.

⁷Department of Neurosurgery, Allied Hospital, Faisalabad, Pakistan.

* Corresponding author: dr.urwatanveer@gmail.com

Abstract

Cholangiocarcinoma (CC) is one of the main crucial hepatic malignancies; hence CC is the prime cause for cancer death occurred due to bile duct cancer. A wide range of studies showed that the expression of intracellular proteins associated with the progression of tumor process might be a response of autoantibody. Unlike intracellular components, autoantibodies can appear in cancer patients long before clinical appearance of the cancer. Apparently, CC autoantibodies can appear at any point in the transformation of chronic liver disease; those autoantibodies may not apparent in erstwhile non-transformation phases, lead to a significant increase in the quantity of patients with CC positive for the presence of autoantibodies. The aim of present study was to detect the cellular proteins involved in bile duct cancer process by the use of immunoblotting technique and to predict the future biomarkers of CC. SERPA technique was applied to detect the differentially expressed proteins from nine CC patients' sera adopting standard protocol. 2-DE maps revealed a number of protein spots after gels staining. Proteins of interest were observed between pH 5 and pH8 having molecular mass range between 20 and 90 kDa. Comparative analysis of blots indicated four common immunoreactive spots in CCSW1 cell lines. These cancer specific proteins might be used for CC diagnosis at early stage.

Keywords: Biomarkers, SERPA, Cholangiocarcinoma

ARTICLE INFORMATION

Received: 13.07.2018

Revised: 26.11.2018

Accepted: 03.12.2018

DOI: 10.31580/pjmls.v1i1.983

© Readers Insight Publication

INTRODUCTION

There cholangiocarcinoma (CC) is one of the main crucial hepatic malignancies, however CC is the prime cause for cancer death occurred due to bile duct cancer (1). Among all hepatic cancer reports, CC contributes almost 3% of gastrointestinal cancers worldwide and disease rate has been rising over the past 2-3 years (2).

A wide range of studies showed that intracellular proteins associated with the progression of tumor process, there might be a response of autoantibody (3) indicated that the prospect of use of auto-immunoglobulin of clinical cancer is not only evident, but actively involved in tumor conversion (4).

Unlike intracellular components, autoantibodies can occur in cancer patients (5). Apparently, CC autoantibodies can appear at any stage in the transformation of chronic liver disease; those autoantibodies may not apparent in erstwhile non-transformation phases, lead to a significant increase in the number of patients with CC positive for the presence of autoantibodies. The nuclear or cellular proteins associated with the cancer process, capable of inducing the generation of

antibodies. Antibodies reactive to atypical antigens of the cytoplasm and exact nucleus have been documented by patients having CC to have positive indirect immunofluorescence tests. A review of the literature indicates a higher frequency of suspicious nucleolar fluorescent-positive patients with CC, although some have noted fluorescent prototypes in various researches (6, 7). The subsistence rate of people that positively screened with cholangiocarcinoma was not satisfactory as a result the majority of the patients deceased during the first 6-12 months of identification of CC. It cannot be found on diagnostic methods in the early incidence of the cause of high mortality situations (8).

Changes that occurred due to the increase in previously detected autoantibody frequencies in patients with chronic liver disease in terms of Immunogenic tumorigenesis stimuli. The mapping of most patients to novel antibody responses, and others were seen with high titers of autoantibodies previously found. If the costs associated with CC related with an autoantigen tumor are the defining characteristic of the person concerned in these early studies (9, 10).

The aim of present study was to detect the cellular proteins involved in bile duct cancer process by SERPA technique.

METHODOLOGY

Sera Included in the Study

We examined 09 sera (5 men and 4 women, mean of age $\pm$ 67 year) patients suffering from CC of various etiologies and serum samples from non-diseased subjects (n = 05) were also investigated. Sera were processed and stained using immunoblots with two CC cell lines (CCLP1 and CCSW1) as antigenic extracts. The gels from SDS-PAGE were prepared from the same cell lines.

2D-Electrophoresis and Iso-Electric Focusing (IEF)

IPG (immobilized polyacrylamide gel) strips (GE Healthcare) were rehydrated prior to IEF in Ettan IPGphor (GE Healthcare) cups. They measure 13 cm long and contain a 3-10 pH gradient. The rehydration solution was composed of urea 7.5 M, thiourea 2.2 M, CHAPS 4%, DTT 0.28%, De Streak® reagent 1.3 % and IPG buffer 0.6 %. Orange G (tracking dye) was added to allow a better visualisation of the migration. The rehydration solution was mixed with cell protein samples (250 µg for 250 µL per strip). Urea and thiourea solubilized and denatured proteins, CHAPS is a zwitterionic detergent that solubilized hydrophobic proteins, DTT is a reductant, DeStreak® reagent improved reproducibility by preventing streaking. During the overnight rehydration, proteins penetrated passively into the gel.

IEF was performed with Ettan IPGphore system (GE Healthcare). Voltages were increased gradually from 100 V to 8000 V in order to deliver a total amount of 23,000 Vh. The amperage was limited to 50 µA per IPG strip. During IEF, the current decreased while the voltage increased as proteins and other charged components migrated to their equilibrium positions.

SDS-PAGE and Immunoblotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique was employed to separate the proteins according to their molecular weight using ionic buffer system(15). After separation, proteins were transferred onto PVDF (polyvinylidene difluoride) membranes according to Towbin's protocol(16) and then incubated with patient's sera (by diluting 1:2500) and then incubated with secondary antibody (horseradish peroxidase) conjugated with IgG antihuman antibody (Bio-Rad), 1:3500 dilution was used. Proteins were detected by signals generated by the reaction of chemiluminescence (ECL) with the instructions of manufacturer (ECL detection kit; GE Healthcare). Subsequent gels were stained by silver staining technique. All experiments were performed in triplicate. Sera of all patients were systematically compared with non-diseased subject's sera.

Silver Staining

Silver staining is a highly sensitive method that can detect protein amounts between 1 and 20 ng. Silver ions are reduced to insoluble silver metal granules in the vicinity of the protein molecules. Sufficient silver deposition remain visible as a dark brown band on the gel. Some gels were stained using this technique (Silver staining kit, GE Healthcare) following the manufacturer's procedure.

Immuno Blotting Detection

The nitrocellulose membranes were incubated for two hours in PBS-T (Phosphate Buffer Saline-TWEEN) Tris 1.5M 2.5 % v/v, NaCl 0.9 % w/v, pH 7.5) containing 5 % dry skimmed milk and 0.05 % TWEEN 20. The nitrocellulose membranes were incubated with the sera diluted to 1/2500 12 hours. After multiple washings, the detecting 2ndry H+L antibody (BioRad) conjugated to peroxidase and diluted in TBS- TWEEN® (1/3500) was applied. The membranes were washed and covered by ECL substrate (GE Healthcare). After elimination of ECL substrate, the membranes were shifted to the cassette for X-ray film blots.

RESULTS

Total proteins extracted from the human CC cell lines (CCSW1, CCLP1), were separated individually by isoelectric focusing according to their pI, then, by SDS-PAGE according to their molecular weight. Further they were transferred onto nitrocellulose membrane, solid support for antigen-antibody reaction with sera studied (CC, and controls).

Immunoreacting Antigens in Cholangiocarcinoma Cell Lines (CCSW1 and CCLP1)

2-D electrophoresis maps of CC cell lines showed different protein distributions. After silver staining, an average of 160-180 spots were detected from CCSW1, mainly between pH 4 and 8, with molecular weights ranging from 40 to 90 kDa (Figure 1). With CCLP1, 80-100 spots were observed in acidic parts of the map with MW ranging from 20 to 90 kDa.

Fig. 1. Two dimensional electrophoretic pattern of CCSW1. Silver staining coloration.

Fig. 2. Immunoreactive spots by control sera (black arrows) and by CC sera (green arrows) with CCSW1.

Fig. 3. Immunoreactive spots by CC sera with CCLP1.

By comparative blotting analysis, four common immunoreactive spots were detected in 77.77% of CCSW1 blots (7 out of 9 CC patient sera), whereas two common immunoreactive spots were found with control sera (Figure 2). With CCLP1, two common immunoreactive spots were stained in the immunoblots of 55.55% (5 out of 9) CC sera (Figure 3). Whereas, no spot was detected with control sera.

DISCUSSION

Cholangiocarcinoma (CC) has very bad prognosis due to its late detection (2). Many studies demonstrated that cancer sera contain antibodies which react with a unique group of autologue cellular antigens, generally known as tumour associated antigens TAA (11). During carcinogenesis of many cancers there are different protein modifications, such as over-expression, post-transduction and post-translational modifications. These modifications can convert some proteins to antigens recognized by antibodies. In the present study, we have applied a SERPA approach to immunoscreen 9 sera from patients with CC as well as 5 sera from normal individuals. We used 2DE separation for total proteins of 2 human CC cell lines (CCSW1 and CCLP1).

Concerning CC, some immunoreactive spots were detected on immunoblots of CCLP1 cell line with all CC sera studied and also all of control sera studied, two common spots were detected with sera of CC and not detected with sera of the control group. Their MW/pI values were 55/7 and 55/7, respectively. These spots may correspond to proteins, especially glucose regulated protein 75. By contrast, four immunoreactive protein spots were detected from CCSW1 with CC sera tested, with MW/pI values of 70/4.5, 45/7.5, 45/4.7 and 70/6.5. These spots indicate the proteins that might be potential TAA in CC detection, which can produce strong reacting autoantibodies in almost all of CC cases.

A number of published literature indicated the occurrence of cancer related immune-proteins in CC patients sera (13) which includes anti-p53 antibody (14), anti-telomerase antibody (15), alpha-fetoprotein and DCP etc (14). The detected immunoreactive protein spots in the present study should further be identified by mass spectrometry for candidate biomarkers.

REFERENCES

1. Hill-Baskin A, Markiewski M, Buchner D, Shao H, Desantis D, Hsiao G, Subramaniam S, Berger N, Croniger C, Lambris J, Nadeau J. Diet-induced hepatocellular carcinoma in genetically-predisposed mice. *Hum Mol Genet.* 2009;18(16):2975-88.

2. Sakamoto M. Early HCC: Diagnosis and molecular markers. *J Gastroenterol.* 2009;44Suppl 19:108-11.

3. Li C, Tan YX, Zhou H, Ding SJ, Ma DJ. Proteomic analysis of Hepatitis B virus associated Hepatocellular carcinoma: Identification of potential tumour markers. *Proteomics.* 2005;5:1125-39.

4. Lim S, Park S, Kim W, Park SG, Kim HA. Proteome Analysis of Hpatocellular Carcinoma. *Biochim, Biophys. Resear commun.* 2002;291:1031-7.

5. Troeles Z, Harcha HC, Mads G, Anirban M, Akhilesh P. Differential membrane proteomics using O-labeling to identify biomarkers for Cholangiocarcinoma. *J. Proteomics Res.* 2008;11:4670-77.

6. Khan S, Thomas H, Davidson B, Taylor-Robinson S. Cholangiocarcinoma. *Lancet.* 2005;366(9493):1303-14.

7. Reddy S, Patel T, Current approach and to the diagnosis and treatment of cholangiocarcinoma. *Curr Gastroenterol Rep.* 2006;8:30-37.

8. Yalcin S. Diagnosis and management of cholangiocarcinomas: a comprehensive review. *Hepatogastroenterology.* 2004;51:43-50.

9. Scarlett C, Saxby A, Nielsen A, Bell C, Samra J, Hugh T, Baxter R, Smith Rc. Proteomic profiling of cholangiocarcinoma: diagnostic potential of SELDI-TOF MS in malignant bile ductstricture. *Hepatology.* 2006;44:658-66.

10. Looi K, Nakayasu E, Diaz R, Tan E, Almeida I, Zhang J. Using proteomic approach to identify tumor-associated antigens as markers in hepatocellular carcinoma. *J Proteome Res.* 2008;7:4004-12.

11. Zhang JY, Zhu W, Imai H, Kiyosawa K, Chan EKL, Tan EM. De-novo humoral immune responses to cancer-associated autoantigens during transition from chronic liver disease to hepatocellular carcinoma. *Clin Exp Immunol.* 2001;125(1):3-9.

12. Scott DI. Immunoblotting and dot blotting. *J Immunol Methods* 1989; 119: 15387.

13. Mustafa MZ, Nguyen VH, Le Naour F, De Martin E, Beleoken E, Guettier C, Johanet C, Samuel D, Duclos-Vallee JC and Ballot E. Autoantibody signatures defined by serological proteome analysis in sera from patients with cholangiocarcinoma. *Journal of Translational Medicine.* 2016;14(1):17.

14. Jisnusun S, Chantragan S, Pantipa S, Siriporn K. Proteomics profiling of cholangiocarcinoma cell line treated with pomiferin from derris malaccensis. *PROTEOMICS.* 2005;5: 4504-4509.

15. Soussi T. P 53 Antibodies in the sera of patients various types of cancers: a review. *Cancer RES* 2000;60:1777-88.