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Proteomic approaches reveal the deregulation of Apoptotic proteins in MCF7 breast cancer cell line treated byred seaweed, *Gracilaria corticota*

Ananthi Sivagnanam[1], Balasankar Thangasamy[2], Ambika Pushparaj[3], Pushparaj Arunachalam[*4]

[1]Dept of Preventive Oncology (Research), Cancer Institute (WIA), Adyar, Chennai – 600020, India

[2]Dept of Proteomics, Clinbiocare Technology, Chennai – 600042, India

[3]Dept of Proteomics, Clinbiocare Technology, Chennai – 600042, India

[4]Department of Zoology, T.D.M.N.S College, T Kallikulam – 627113, Affiliated to Manonmaniam Sundaranar University, Abishekapatti, Tirunelveli – 627012, Tamil Nadu, India

***Corresponding Author**

Pushparaj Arunachalam, Assistant Professor, Department of Zoology, T.D.M.N.S College, T Kallikulam – 627113, Tirunelveli, India. E-mail:tdmnspushparaj@gmail.com, clinbiocare@gmail.com

Contact no.: +91-9994097004, +91 9600075192

Abstract

Background: Breast cancer is always known to be a major health problem globally. Though many advancements are achieved in treatment strategy for breast cancer, still the patient survival rate is not promising for all the affected patients. Hence there is an emerging need for an alternative medicine for treating cancer patients.

Objective: Thus, we aimed to understand the possibility of using commonly available red seaweed, Gracilaria corticota in treating breast cancer. To analyse this, we aimed to check the seaweed extract's efficiency in breast cancer cell lines, MCF 7 using proteome wide analysis.

Method: The MCF7 cells were treated with red seaweed extract and incubated for 48 hrs. Further the treated cells were scraped and lysed to extract the proteins. The extracted proteins were estimated and further subjected to proteomic profiling. To analyse the differential protein expression and identification of the proteins, two-dimensional electrophoresis coupled with mass spectrometry was adapted.

Results: We found upon seaweed extract treatment in MCF7 breast cancer cell line, important apoptotic proteins were deregulated. These proteins include the down regulation of BCL2,BCL2L1 and MCL1 and upregulation of BAX protein upon treatment when compared to untreated cells.

Conclusion:This is a preliminary study to suggest that seaweed extract have very good potential against the breast cancer cell line by initiating the apoptotic proteins and target the cancercells. As well proteome-based approaches help us to identify the differentially expressed proteins and provide us the basic understanding about the tumorigenesis process which will eventually help us for better treatment strategy.

Introduction

Breast cancer is one of the major health problems globally. Reports suggest that approximately twenty lakh new cases were found and it led to about seven lakh deaths in 2018 (Bray *et al.*,2018).

As well reports describe that breast cancer incidence is getting increased with a higher rate in the developing countries.When breast cancer incidence known to increase about 0.5% worldwide, in Asia-Pacific countries the incidence was about 3-4%(Green M & Raina V, 2008).Researchers analysed that the rapid increase in incidence in the developing countries may be due to different socioeconomic changes such as delayed marriage and child birth, fewer number of children, increasing obesity and awareness. Irrespective of advancements in treatment modalities for breast cancer, still the patient survival is poor. To overcome this condition, researchers started looking for alternative medicine strategies. Recent studies suggest that Gracilaria red seaweed extracts induces apoptosis and inhibit tumor growth.Thus, this paper aims to understand the efficacy of Gracilaria red seaweed extracts in MCF7 breast cancer cells using proteomic approaches.

Materials and methods

Cell line and treatment

The MCF-7 cell lines were procured from the cell repository of National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco`s Modified Eagle Media (DMEM) was used for maintaining the cell line, which was supplemented with 10% Fetal Bovine Serum (FBS). Penicillin (100 U/ml), and streptomycin (100 µg/ml) were added to the medium to prevent bacterial contamination. The medium with cell lines was maintained in a humidified environment with 5% CO2 at 37°C. The cell lines were treated with 50 ug/ml of seaweed extract and incubated for 48 hrs, further cells were harvested and protein extraction was performed. Further the proteins were estimated and taken further for 2D analysis as described in the further sections.

Isoelectric focusing

The protein concentration applied for 2D gels were 100 µg. The gels were stained with colloidal coomassie blue G-250. The protein was added with 250 µl of rehydration buffer andthe passive rehydration method was employed for a minimum of 12 - 15 h[12]. After rehydration, the strips were focused at a temperature of 20 °C using the IPGphor IEF apparatus (GE Healthcare, Hong Kong, China) using the following voltage settings: 500 V for 1.30 h (step and hold), 1000 V for 1.30 h (gradient), 8000 V for 3.30 h (gradient), and 8000 V for 7 h (step and hold). Until the two-dimensional analysis, the focused strips were stored at −70 °C.

SDS–PAGE

Before the SDS PAGE run, the strips were equilibrated for 20 min each to reduce and alkylate the proteins. The equilibration buffer with DTT(2%) was used for the first step, and iodoacetamide (2.5%) replaced DTT for the second step(Ananthi *et al.,*2013). After these steps, the strips weretransferred to 12.5% polyacrylamide gel and sealed with 0.5% agarose dissolved in electrophoresis buffer. The run was performed in the DALT six apparatus (GE Healthcare, Hong Kong, China) at a constant temperature of 22 °C (18 W for 30 min and 50 W for 4.30 h). Gastric cancer andhealthycontrol samples were examined simultaneously to minimize experimental variations.

Staining

After completion of the SDS PAGE run, the gels were fixed in methonal (40%) and acetic acid (10%) in water for 1 h. After that, the gels were washed for 10min with water and this step was repeated thrice. Further the gels were stained with coomassie blue G-250 (Li *et al.,*2004) staining

solution made of 10% phosphoric acid, 10% ammonium sulphate, 0.12% coomassie blue G-250, and 20% methanol for minimum 6 – 10 hrs. Further the gels were then de-stained in water.

MALDI-TOF spectrometry of tryptic digests

In-gel trypsin digestion was performed as detailed earlier (Ananthi *et al.,* 2011). Mass spectrometric analysis of extracted tryptic peptides was performed as detailed earlier (Ananthi *et al.,*2008). The α–cyano-4- hydroxy cinnamic acid (CHCA) matrix, (prepared in 70% acetonitrile and 0.03% TFA at a concentration of 2 mg/ ml) was used in this study. Sandwich method was used for peptide samples which were applied on a stainless steel MALDI target plate. Whileusing the CHCA matrix, matrix(0.5 µl) was first placed on the plate and allowed to dry. A sample (0.5 µl) was placed and then immediately layered on top with the 0.5 µl of the matrix. In mass spectrometry analysis, reflector mode was adapted to acquire the peptide mass spectrum.The parameters used in the instrument was fixed to pulsed extraction and the acceleration voltage is 20,000 V. Bradykinin (757.39 Da), P14R (1533.85 Da), angiotensin II (1046.54 Da), and ACTH fragment 18– 39 (2465.19 Da) were used as calibration standards.Databases used for the search was Uniprot. The MASCOT score was considered significant if $p<0.05$.

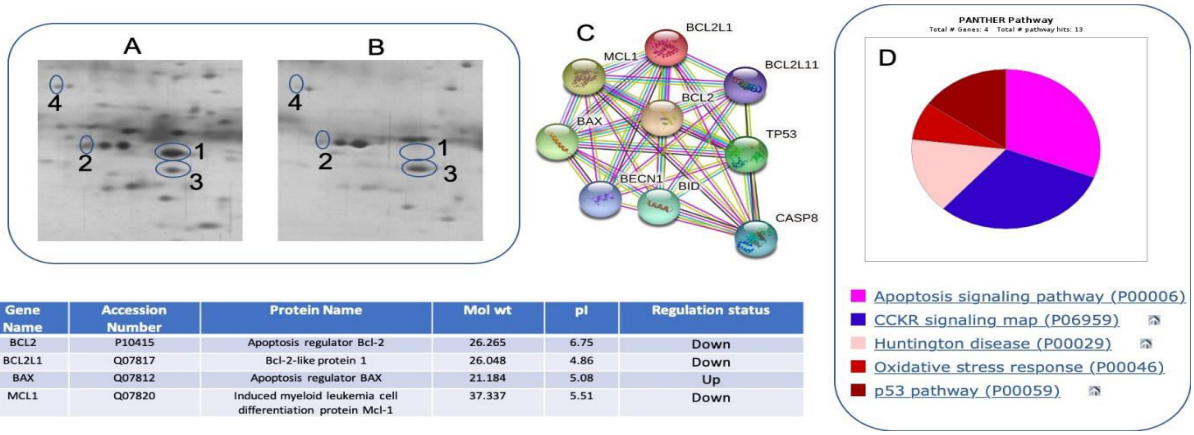
Results

Differential expression of apoptotic proteins in the2D gel electrophoresis

The seaweed treated cells were compared with the untreated MCF7 cells. Figure 1A(Untreated) and 1B(Treated) details the spots and their corresponding spot region which were highly differentially regulated and these were further selected for mass spectrometric identification which was detailed in 1E.Four apoptotic proteins were identified to be differentially regulated in the seaweed treated samples and they were BCL2,BCL2L1,BAX and MCL1. Among these four, anti-apoptotic proteins such as BCL2,BCL2L1 and MCL1 were downregulated and proapoptotic protein BAX was upregulated in the seaweed treated cells. This provides us the strong suggestion that Gracilaria seaweed extract induces the apoptosis and inhibit tumor cell proliferation.

Pathway and Interaction analysis for the identified proteins

All the identified proteins were subjected to pathway analysis by PANTHER database and protein - protein interaction analysis by STRING database. With respect to the protein interaction analysis as expected all the four proteins were interacting with all the other members of apoptosis pathway (Figure 1C) It was interesting to note that all these proteins were involved in very important tumorigenesis related pathways such as apoptosis signalling pathway, CCKR signalling map, Oxidative stress response and p53 pathway (Figure 1D).



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Spot ID	Gene Name	Accession Number	Protein Name	Mol wt	pI	Regulation status
1	BCL2	P10415	Apoptosis regulator Bcl-2	26.265	6.75	Down
2	BCL2L1	Q07817	Bcl-2-like protein 1	26.048	4.86	Down
3	BAX	Q07812	Apoptosis regulator BAX	21.184	5.08	Up
4	MCL1	Q07820	Induced myeloid leukemia cell differentiation protein Mcl-1	37.337	5.51	Down

Figure 1: Identification of differentially expressed proteins. A – Closer view of 2D gel of untreated MCF 7 cells; B - Closer view of 2D gel of treated MCF 7 cells with seaweed extract; C – Protein interaction analysis by STRING; D – Pathway analysis by PANTHER database.

Discussion

In this study, the 2D proteomic profiles of breast cancer cells were analysedupon treatment with red seaweed *Gracilaria corticota*using 2DE and differentially expressed proteins were identified through mass spectrometry. Four differentially expressed proteinswere identified with significant variations in expression levels, as compared to untreated control samples. Bcl-2, Bcl2-L1 and MCL1 were down regulated and Bax was upregulated upon treatment with the red seaweed.

Functional Implication of differentially expressed proteins

Bcl-2 is the important member of the Bcl-2 group of apoptotic regulatory proteins which is known to assist oncogenesis, not defined by cell growth or proliferation but it is due to enabling byapoptotic resistance.Reports states that Bcl-2 was known to be increased in non-Hodgkin's lymphomas, small cell lung cancers, follicular lymphoma, chronic lymphocytic leukemia, and macroglobulinemia (Yip and Reed 2008). Other mechanism including loss of endogenous miRNAs which repress Bcl-2 gene and gene hypomethylation were resulted in malignancies in most ofthe cancers (Hanada *et al.*,, 1993).

Bcl-2 like-1 (BCL2-L1) is an isoform of Bcl-xL and part of the Bcl-2 family of anti-apoptotic proteins. It is known to regulate apoptosis by binding to and inhibiting voltage-dependent anion channel, further it restricts the mitochondrial release of the caspase activator CYC1. Bcl2-L1 known to share the properties with Bcl-xL and are known to be highly expressed in glioblastoma, colorectal cancer, and gastric cancer (Lizarraga-Verdugo *et al.,* 2020). Reports states that Bcl2-L1 targeted drugs were shown to be highly effective in reducing the size of gastric cancer tumors (Park *et al.,* 2015). Hence, Bcl2-L1 could act as targets for different cancer therapies.

Mcl-1, in combination with other anti-apoptotic Bcl-2 family members, were shown to be highly increased in various cancers. Reports reveal that the somatically acquired amplification of the regions containing Mcl-1 was found in 10% of all human cancers (Beroukhim *et al.,,* 2010). Enhanced expression of Mcl-1 resulted in poor overall survival in triple-negative breast cancers. Expression of mutant forms of BH3-mimicking proteins might bind to Mcl-1 and arrest the metastasis and invasion of triple negative breast tumor cell lines in xenograft models (Campbell *et al.,*, 2018). It could be used as therapeutic targets for treating breast cancer.

Bax expression and its mutation hold an important role in the resistance of cancer cells to apoptosis. It is found to be downregulated in several cancers, including colorectal, lung, breast,

ovarian, and pancreatic cancer. Most of the chemoresistance in several cancers such as chronic lymphocytic leukemia, prostate, liver, colorectal, and lung cancers are known to have reduced levels of Bax (Liu *et al.,,* 2016; Naseri *et al.,,* 2015). Due to their role in enhancing apoptosis and its downregulation in most of the cancer cell lines, Bax was widely known to be targeted in different cancer therapies.

Conclusion

As there is an emerging need for evaluating the natural compounds which will be efficient in treating the cancer cells, in this study we aimed to analyse the efficiency of the red seaweed extract Gracilaria. By applying 2D gel electrophoresis coupled with mass spectrometry we could identify that key proteins involved in apoptosis pathway were altered upon Gracilaria treatment in MCF7 cells. Thus, this study paves the way for more validation studies using Gracilaria seaweed in treating the breast cancer cells.

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