Identification of Potential Anticancer Protein Targets in Cytotoxicity Mediated by Tropical Medicinal Fern Extracts

Siok-Thing Tan¹, Hean-Chooi Ong¹, Tsun-Thai Chai³,⁴, Fai-Chu Wong¹,³

¹Centre for Biodiversity Research, Universiti Tunku Abdul Rahman, ²Biochemistry Program, Department of Chemical Science, Faculty of Science, Universiti Tunku Abdul Rahman, 31900 Kampar, Perak, ³Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

ABSTRACT

Background: Medicinal fern species represent a potentially important source for both food and medicinal applications. Previously, two underutilized tropical fern species (Blechnum orientale and Phymatopteris triloba) were reported with cytotoxic activities against selected cancer cell lines. However, the exact mechanism remains elusive. Objective: In this paper, we reported the identification of six differentially expressed proteins isolated from cancer cells, following exposure to the cytotoxic fern extracts. Materials and Methods: The identities of these cancer proteins were determined by matrix-assisted laser desorption ionization time-of-flight protein sequencing. Results: The cancer proteins were identified as follows: elongation factor 1-γ, glyceraldehydes-3-phosphate dehydrogenase, heat shock protein 90-β, heterogeneous nuclear ribonucleoprotein-A2/B1, truncated nucleolar phosphoprotein B23, and tubulin-β chain. To the best of our knowledge, this paper represents the first time these cancer proteins are being reported, following exposure to the aforementioned cytotoxic fern extracts. Conclusion: It is hoped that further efforts in this direction could lead to the identification and development of target-specific chemotherapeutic agents.

Key words: Anticancer, Blechnum orientale, medicinal fern, Phymatopteris triloba, protein expression

SUMMARY

• Cytotoxic fern extracts were tested in anti-cancer proteomic works.
• Six differentially-expressed cancer proteins were identified.
• Potential anti-cancer protein targets were reported.

INTRODUCTION

Since ancient age, plant species represent important sources of food, treasured for their nutritional and medicinal applications. In modern pharmaceutical science, plants with their rich secondary metabolites represent a reservoir of diverse phytochemicals waiting for medicinal discoveries. Notorious examples include vinblastine and vincristine, chemotherapeutic drugs which were originally derived from the alkaloid compounds in Catharanthus roseus (Madagascar periwinkle), a plant endemic to Madagascar. Likewise, paclitaxel (a commercial antitumor drug) was originated from the bark extract of Taxus brevifolia (Pacific yew tree).

Among the different plant species, fern species represent a group of underutilized food and medicinal sources. Examples include Selaginella willedenowi which is reportedly used to treat wounds and for tonic medicine, as well as consumed as vegetables in the tropical areas. Likewise, the young fronds of Stenochlaena palustris are harvested from the wild and consumed as vegetable in Southeast Asia. Although fern species have been consumed by different ethnic groups across the world for both culinary and medicinal purposes, the full extent of their potentials has never been thoroughly investigated.

Blechnum orientale and Phymatopteris triloba are two fern species found in the tropical regions. The leaves of B. orientale were reportedly used to treat blister, sores, stomach pain, and urinary bladder-related complications, while P. triloba was found in the tropical mountain forest. No much information is available on P. triloba’s medicinal bioactivity; however, two fern members in this same genus Phymatopteris (Phymatopteris hastata and Phymatopteris quasidivaricata) were reportedly used in traditional medicines. P. hastata was used to treat diarrhea and bronchitis-related diseases, while P. quasidivaricata was applied to treat musculoskeletal related problems.

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

Cite this article as: Tan ST, Ong HC, Chai TT, Wong FC. Identification of potential anticancer protein targets in cytotoxicity mediated by tropical medicinal fern extracts. Phcog Mag 2018;14:227-30.
Previously, both *B. orientale* and *P. triloba* had been reported with anticancer bioactivities,
however, their exact cytotoxic mechanism remains elusive. It is not clear that which cancer signaling pathways or cancer enzymes are being targeted by these cytotoxic fern extracts. For instance, it is curious to determine whether the observed fern-induced cytotoxicity is exerted through cell cycle arrest, through microtubule interference, or by inhibiting the DNA synthesis pathway in cancer cells. In this paper, selected cancer cell lines were exposed to the cytotoxic *B. orientale* and *P. triloba* fern extracts. Proteomic works were performed to identify the differentially expressed cancer proteins implicated in the fern-induced cytotoxic mechanism. The identities of these cancer proteins were then confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI TOF-TOF) protein sequencing. Through these efforts, we hope to contribute toward the understanding of fern-induced cytotoxic mechanism and potential anticancer protein targets.

**MATERIAL AND METHODS**

**Preparation of fern extracts**

Tropical fern species were collected from Perak and Pahang states (Malaysia) from July to November of 2013. The fern species were authenticated by Professor Dr. Hean-Chooi Ong at the Institute of Biological Sciences, University of Malaya, Malaysia. Following harvesting and rinsing with distilled water, the ferns were dried in oven at 40°C, until constant weights were achieved. The dried ferns were then pulverized using a warring blender, followed by solvent extraction. The extracted supernatants were filtered, concentrated, and stored in −20°C.

**Solation of proteins from cancer cells for SDS-PAGE analysis**

Human cervical cancer cells (HeLa) and human myelogenous leukemia cancer cells (K562) were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Both cancer cell lines were maintained in a humidified incubator with 5% CO₂ at 37°C. Subculturing was performed when the cultured cancer cells reached 75% confluency, and cultured cells in the exponential phase were used in experiments. Cancer cell cultures were treated with optimal concentrations of the filtered fern extracts (0.1–1 mg/ml), and sterile water was used as the negative control. The cytoplasmic and nuclear proteins of cancer cells were extracted using NE-PER nuclear and cytoplasmic protein extraction kit (Thermo Scientific).

**SDS-PAGE gel analysis and identification of differentially expressed cancer cellular proteins**

SDS-PAGE gel electrophoresis was performed as previously described. Gel electrophoresis was performed with constant electric current of 135 mV for approximately 90 min. The protein gels were then stained with Coomassie Brilliant Blue R-250 (Fisher Scientific). Stained protein bands were visualized under densitometer (Bio-Rad), and the protein band densities were determined with Image Lab software (Bio-Rad). Differentially expressed cancer proteins were excised with sterile razor blades and subjected to analysis using MALDI TOF-TOF mass spectrometry (4800 Proteomics Analyzer, AB Sciex) (Proteomics International, Perth, Australia). The spectra were analyzed using Mascot sequence matching software (Matrix Science) with Ludwig NR Database to identify the proteins of interest.

**Western blotting**

Extracted cancer proteins were resolved using 12% SDS-PAGE gel and then transferred onto a nitrocellulose membrane. After blocking the nitrocellulose membrane with Tris-buffered saline containing 1% Tween-20 (TBST) and 5% bovine serum albumin for an hour, the membrane was probed overnight with anti-heat shock protein 90 (HSP90) primary antibody and anti-β-actin antibody (as control) at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Thermo Scientific). After washing the nitrocellulose membrane with TBST, the protein bands were detected using enhanced chemiluminescence kit (Thermo Scientific) and visualized by exposing the membrane to X-ray film. Band intensities were determined using Image Lab software (Bio-Rad).

**RESULTS AND DISCUSSION**

In this paper, a total of six differentially expressed proteins were pinpointed and isolated from cancer cells, following exposure to cytotoxic fern extracts [Figure 1]. The identities of these differentially expressed proteins were then determined by MALDI TOF-TOF protein sequencing, and their characteristics were summarized in Table 1.
In our works with B. orientale fern extract, four differentially expressed cancer cellular proteins were identified (Protein bands a to d) [Figure 1]. Based on MALDI-TOF-TOF protein sequencing results, protein bands a to d were determined as elongation factor 1-γ (EF1-γ), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HSP90-β, and heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2/B1), respectively. EF1-γ was previously reported with functional role in transporting aminoacyl-tRNA to ribosome, during the elongation stage of protein synthesis. [13] Earlier studies had also reported on the increased expression of EF1-γ in assorted cancer cells, compared to normal cells. [14,15] Interestingly, a separate study reported on the downregulation of EF1-γ in cervical cancer cells, following treatment with antitumor drugs (paclitaxel and cisplatin). [16] In our study, the expression of EF1-γ was downregulated, following exposure to B. orientale [Figure 1a]. It is tempting to speculate that B. orientale extract may exert its cytotoxic effect, partly by interfering with the cancer cell’s protein synthesis pathway, through reduction of EF1-γ expression level. However, further analysis is needed to determine if this is indeed the case or not.

On the other hand, GAPDH was reported as an enzyme involved in glycolysis pathway, [17] the metabolic pathway that proliferating cancer cells rely on for energy generation. Previous studies had reported on increased glycolysis activities in rapidly growing tumor cells, together with the upregulated glycolysis-related genes, which include GAPDH. [17,18] On the other hand, methyl jasmonate (a plant-derived lipid derivative) was previously reported with anticancer and apoptosis-inducing effects, which correlating closely with its inhibition activities on components in the glycolytic pathway. [19,20] In our study, GAPDH was found to be downregulated, following exposure to B. orientale fern extract [Figure 1b]. The reduced expression of GAPDH could possibly be part of the reasons which contributed to the cytotoxic effect of B. orientale extract.

Meanwhile, HSP90-β is a molecular chaperone with a mass of 90 KD, reported with functional role to stabilize cellular proteins and enhance proper folding. [21] Previously, it was reported that HSPs were abundantly expressed in cancer cells, partly to enhance protein stabilization and to promote cellular proliferation. [16,22] In addition, other researchers have reported on antitumor agents which are targeting HSP90. [20,22] In our study, the expression of HSP90-β was found to be downregulated [Figure 1c], following exposure to the cytotoxic B. orientale extract. The downregulation of HSP90 was also verified by western blot study [Figure 2]. However, it remains to be determined whether B. orientale extract induced cytotoxicity by modulating the HSP90-β expression level, through similar mechanism observed in other HSP inhibitors.

The fourth identified differentially expressed cancer protein was hnRNPA2/B1, which was previously reported as a multifunctional RNA-binding protein, associated with cell proliferation and carcinogenesis. [23] In addition, an earlier study documented the overexpression of hnRNPA2/B1 in cancer cells. [24] Although the exact reason remains unclear, it was proposed that this may be linked to the functional roles of hnRNP proteins in telomere regulation. [24,25]

Furthermore, hnRNPA2/B1 may also be regulated by the presence of other related hnRNPA2/B1 family members. [24] In our study, hnRNPA2/B1 was found to be upregulated, following exposure to B. orientale extract [Figure 1d]. This phenomenon may be due to the accelerated metabolic activities of cancer cells, in response to cytotoxic stress. However, we could not rule out the possibility that the cytotoxic B. orientale extract may stimulate the cancer cells to produce more hnRNPA2/B1, to compensate for other downregulated hnRNPA2/B1 family members. Further detailed research is needed to distinguish which is the case.

In our works with P. triloba fern extract, two differentially expressed cancer proteins were isolated. The identities of these two proteins were determined through MALDI-TOF-TOF protein sequencing as truncated nucleolar phosphoprotein B23 (nucleophosmin [NPM]) and tubulin beta chain (tubulin-β) [Figure 1e and f], respectively. NPM was previously reported as a multifunctional protein linked to cell proliferation and cancer pathogenesis. [26,27] Meanwhile, PNM was also found to overexpress in many types of malignant cells. [28] Interestingly, a recent study reported on the downregulation of NPM protein, in cancer cells treated with antitumor triterpenoid compounds (derived from Thai medicinal plant Trichosanthes cucumerina). [29] Collectively, these studies highlighted the potential of NPM as a chemotherapeutic target. In our study, the observed downregulation of NPM expression, following exposure to P. triloba extract, hinted the possible presence of bioactive compounds which may induce NPM-mediated cytotoxic activities.

On the other hand, literature search revealed tubulin-β as an important component of microtubules. In the cells, microtubules are the major constituents of cytoskeletons, which are involving in cell division, chromosome separation, as well as maintaining cellular structures. [30,31] Meanwhile, paclitaxel (a chemotherapeutic drug) functions by stabilizing the microtubule polymers, which leads to suppression of microtubule dynamics and subsequently preventing the metaphase spindle configuration in chromosome. [32] Recently, efforts to study synthetic small molecules (benzenesulfonamide derivatives) which demonstrated anticancer and tubulin-targeting activities were also reported. [33] Similarly, a related study reported on phenethyl isothiocyanate (a phytochemical found in many edible cruciferous vegetables), which reduced tubulin expression.
and exerted cytotoxic effects in prostate cancer cells.\textsuperscript{[14]} In our study, the tubulin-β expression was found to be downregulated, following exposure to the cytotoxic \textit{P. triloba} extract [Figure 1f]. It is possible that the \textit{P. triloba} extract may inhibit tubulin-β expression and subsequently lead to cytotoxicity in cancer cells. However, the detailed relationship between the reduced tubulin-β expression and the anticancer activity of \textit{P. triloba} extract required further elucidation in the future study.

CONCLUSION

We reported in this paper the identification of six differentially expressed cancer proteins, following exposure to cytotoxic tropical fern extracts. These identified cancer proteins could further be classified into cancer cellular pathways pertaining to protein synthesis, glycolysis, chaperone-mediated protein stabilization, proliferative-related factors, as well as microtubule dynamics. It is hoped that further study in this direction could eventually lead to the identification of fern-derived, target-specific chemotherapeutic agents.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Fachini PJ, De Luca V. Opium poppy and madagascar periwinkle: Model non-model systems to investigate alkaloid biosynthesis in plants. Plant J 2008;54:763-84.
2. Kusan S, Singh S, Jayabaskaran C. Rethinking production of taxol® (paclitaxel) using endophyte biotechnology. Trends Biotechnol 2014;32:304-11.
3. Hanum IF, Hamzah N. The use of medicinal plant species by the Temuan tribe of Ayer Hitam Forest, Selangor, Peninsular Malaysia. Pertanika J Trop Agric Sci 1999;22:95-94.
4. Eswani AD. Traditionally utilization of \textit{Selaginella antiqua} and \textit{Phymatopteris hastata}. J Serbian Chem Soc 2011;76:1465-55.
5. Chai TT, Panichthumvum E, Ong HC, Wong FC. Phenolic contents and antioxidant properties of \textit{Stenochlaena paluensis}, an edible medicinal fern. Bot Stud 2012;53:439-46.
6. Lai HY, Lim YY, Kim KH. Blechnum orientale linn – A fern with potential as antioxidant, anticancer and antibacterial agent. BMC Complement Altern Med 2010;10:15.
7. Chai TT, Quach Q, Ong KF; Ismail NI, Ang YV, Elamparuthi S, et al. Analysis of the proliferative activity of a cell using new monoclonal antibodies to \textit{P. hastata}. J Agric Sci 2010;2:189-210.
8. Sambasivarao AD. Traditionally utilization of \textit{Selinigella}, Field research and literature review. Nus Biosci 2009;1:146-55.
9. Su WJ, Li P, Hsu W, Wu C, Guo N, Liu L. Phenolic content and antioxidant activity of \textit{Phymatopteris hastata}. J Serbian Chem Soc 2011;76:1486-95.
10. Upasri Y, ASSelin H, Boon EK, Yudav S, Shrestha KK. Indigenous use and bio-ecofy of medicinal plants in the Rasuwa District, Central Nepal. J Ethnobiol Ethnomed 2010;6:3.
11. Wong FC, Tan ST, Chai TT. Phytochemical-mediated protein expression profiling and the potential applications in therapeutic drug target identifications. Crit Rev Food Sci Nutr 2016;56 Suppl 1:1562-70.
12. Yong AL, Ong KF; Ong HC, Chai TT, Wong FC. Investigation of antibacterial mechanism and identification of bacterial protein targets mediated by antibacterial medicinal plant extracts. Food Chem 2015;186:32-6.
13. Ogawa K, Utsunomiya T, Mimori K, Tanaka Y, Tanaka F, Inoue H, et al. Clinical significance of elongation factor-1 delta mRNA expression in oesophageal carcinoma. Br J Cancer 2004;91:282-6.
14. Al-Maghrabi M, Anum JT, Olalu AA. Up-regulation of eukaryotic elongation factor-1 subunits in breast carcinoma. Anticancer Res 2005;25:2573-7.
15. Mimori K, Mori M, Tanaka S, Akiyoshi T, Sugimachi K. The overexpression of elongation factor 1 gamma mRNA in gastric carcinoma. Cancer 1995;76:1446-9.
16. Liu H, Han Y, Mi R, Zhang Y, Su G, Wang H, et al. Identification of cervical cancer proteins associated with treatment with paclitaxel and cisplatin in patients. Int J Gynecol Cancer 2011;21:1462-7.
17. Pelciano H, Martin DS, Xu RH, Huang P. Glycogenesis inhibition for anticancer treatment. Oncogene 2006;25:4653-46.
18. Revillion F, Pawlowski V, Homex L, Peyrat JP. Glyceroldehydrogenase gene expression in human breast cancer. Eur J Cancer 2000;36:1038-42.
19. Wang Z, Wang N, Chen J, Shen J. Emerging glycolysis targeting and drug discovery from chinese medicine in cancer therapy. Evid Based Complement Alternat Med 2012;2012:873175.
20. Goldin N, Arzzone L, Heyfets A, Israelson A, Zaslavsky Z, Brauman T, et al. Methyl jasmonate binds to and detaches mitochondria-bound hexokinase. Oncogene 2008;27:4366-43.
21. Hong DS, Banerji U, Tavaha B, George GC, Aaron J, Kurzrock R, et al. Targeting the molecular chaperone heat shock protein 90 (HSP90): Lessons learned and future directions. Cancer Treat Rev 2013;39:375-87.
22. Sarkar D, Mukherjee S, Roy M. Targeting heat shock proteins by phenethyl isothiocyanate results in cell-cycle arrest and apoptosis of human breast cancer cells. Nutr Cancer 2013;65:480-93.
23. Choi HS, Lee HW, Jang YJ, Kim CH, Ryu CJ. Heterogeneous nuclear ribonucleoprotein A2/B1 regulates the self-renewal and pluripotency of human embryonic stem cells via the control of the G1/S transition. Stem Cells 2013;31:2647-58.
24. Pino I, Pio R, Toledo G, Zabalegu N, Vicent S, Rey N, et al. Altered patterns of expression of members of the heterogeneous nuclear ribonucleoprotein (HnRNP) family in lung cancer. Lung Cancer 2003;41:131-43.
25. Ford LP, Wright WE, Shay JW. A model for heterogeneous nuclear ribonucleoproteins in telomere and telomerase regulation. Oncogene 2002;21:580-3.
26. Bulacheva TI, Dergunova NN, Artemenko EG, Dunik OA, Shpakova AP, Malashenko OS, et al. Analysis of the proliferative activity of a cell using new monoclonal antibodies to nucleolar protein B23/nucleophosmin. Titoologia 2000;42:944-54.
27. Lim MJ, Wang XW. Nucleophosmin and human cancer. Cancer Detect Prev 2006;30:481-90.
28. Grisendi S, Mecucci C, Falini B, Pandolfi PP. Nucleophosmin and human cancer. Cancer Detect Prev 2006;6:493-505.
29. Lu R, Lim PW, Saksena MK, Law CS, Lim WT, Wei SP, et al. The potential for using nucleolar protein B23 expression as a novel biomarker for lung cancer diagnosis. Lung Cancer 2003;41:131-43.
30. Mukhtar H, Adhami VM, Mukhtar H. Targeting microtubules by natural agents for cancer therapy. Mol Cancer Ther 2014;13:275-84.
31. Kavallaris M. Microtubules and resistance to tubulin-binding agents. Nat Rev Cancer 2010;10:194-204.
32. Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. Nat Rev Cancer 2004;4:253-65.
33. Yang J, Yang S, Zhou S, Lu D, Li L, Li Z, et al. Synthesis, anti-cancer evaluation of benzeneulfonamide derivatives as potent tubulin-targeting agents. Eur J Med Chem 2016;122:488-96.
34. Yin P, Kawamura T, He M, Vanaja DK, Young CY. Phenethyl isothiocyanate induces cell cycle arrest and reduction of alpha- and beta-tubulin isotypes in human prostate cancer cells. Cell Biol Int 2009;33:57-64.