

Cytotoxicity and Subcellular Distribution of Sea Anemone Equinatoxin II S105-P142 Fragment in Cancer Cells

Yi-Lian Li, Xue-Mei Duan, Li-Qin Wu, Hu Wu, Xin-Yan Yang, Gong-Chu Li

Yi-Lian Li, Xue-Mei Duan, Li-Qin Wu, Hu Wu, Xin-Yan Yang, Gong-Chu Li, College of life sciences, Zhejiang Sci-Tech University, Hangzhou, Zhejiang, China

Yi-Lian Li and Xue-Mei Duan contributed equally to this paper

Correspondence to: Gong-Chu Li, College of life sciences, Zhejiang Sci-Tech University, Hangzhou, Zhejiang, China

Email: lgc@zstu.edu.cn

Telephone: +86-571-86843186 Fax: +86-571-86843185

Received: April 28, 2014 Revised: May 16, 2014

Accepted: May 22, 2014

Published online: July 18, 2014

ABSTRACT

Actinoporin Equinatoxin II (EqII), a sea anemone cytolytic, has been well known to lyse cells through forming pores on the cell membrane. We show here that an exogenous expression of the EqII (S105-P142) fragment through gene transferring significantly induced apoptosis in human hepatocellular carcinoma cell line BEL-7404. The EqII (S105-P142) expression did not alter the cleavage of PARP, as well as levels of Bcl-2 and Bax. Furthermore, the observation under a confocal laser scanning microscope showed that EqII (S105-P142) colocalized with mitochondria, but not with endoplasmic reticulum and Golgi apparatus. Therefore, the EqII (S105-P142) fragment was dramatically different from previously reported full length EqII, which specifically colocalized with Golgi apparatus. The cytotoxicity of EqII (S105-P142) reported here may provide insights into the utilization of EqII (S105-P142) as an anticancer agent in future cancer gene therapy.

© 2014 ACT. All rights reserved.

Key words: Equinatoxin II; EqII (S105-P142); Cancer gene therapy

Li YL, Duan XM, Wu LQ, Wu H, Yang XY, Li GC. Cytotoxicity and Subcellular Distribution of Sea Anemone Equinatoxin II S105-P142 Fragment in Cancer Cells. *Journal of Tumor* 2014; 2(7): 216-219 Available from: URL: <http://www.ghrnet.org/index.php/JT/article/view/>

INTRODUCTION

Diversified bioactive compounds have been identified from marine bioresources such as plants, mollusk animals, fishes, and fungi, and were found exhibiting potent anti-fungi and antitumor activity, as well as the capability to cure cardiovascular and cerebrovascular disease^[1-4]. Sea anemones produce a variety of toxins which are classified into three types: sea anemone neomimins, sea anemone cytolytic, and sea anemone potassium channel inhibitors^[5]. Sea anemone pore-forming toxins are typical cytolytic, which elicit cytotoxicity through forming pores on target cell membrane^[5,6].

Equinatoxin II (EqII), an actinoporin isolated from the sea anemone *Actinia equina*, is an archetypal example of α -helical pore forming toxins^[6,7]. EqII specifically binds sphingomyelin^[8-10], an important component concentrated in cell membrane lipid rafts, which provide binding targets for a variety of pathogens and toxins to penetrate the plasma membrane. EqII has become a useful marker to detect subcellular distribution of sphingomyelin^[11]. Previously, an intracellular expression of EqII-GFP fusion proteins was developed to detect the colocalization of EqII with Golgi apparatus, but not with other organelles including mitochondria, endoplasmic reticulum (ER), and nucleus, revealing the cytoplasmic exposure of Golgi sphingomyelin^[12].

Previous studies have shown that the EqII N-terminal α -helix (amino acid 11-31) is responsible for membrane penetrating, whereas the internal tryptophan-rich region is involved in initial membrane attachment^[13,14]. In this work, a sequence encoding the S105-P142 internal region including the tryptophan-rich motif was inserted into plasmid pEGFP-C1 to generate pEGFP-EqII (S105-P142)-C1. Human cancer cells were transfected with pEGFP-C1 or pEGFP-EqII (S105-P142)-C1. The exogenous expression, cytotoxicity, and subcellular distribution of EqII (S105-P142) were analyzed.

MATERIALS AND METHODS

Cell culture, transfection, and flow cytometry

BEL-7404 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). L02 cell line was purchased

from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin solution, and 1% L-Glutamine. Appropriate amounts of plasmids were transfected into cells by Thermo Scientific TurboFect Transfection Reagent (Thermo Fisher Scientific Inc, Canada) following the manufacturer's instruction.

For flow cytometry analysis, BEL-7404 cells were transfected with Plasmids pEGFP-C1 or pEGFP-EqtII (S105-P142)-C1. After 48 h, cells were harvested and stained with Annexin V-PE (BD Biosciences, San Jose, CA, USA) followed by analyzing under a BD FACSAria flow cytometry (BD Biosciences) to detect apoptotic cells. Cells without staining served as the negative control.

Plasmid construction

The plasmid pGH-genes carrying DNA sequences encoding EqtII (S105-P142) were purchased from Shanghai Generay Biotech Co., Ltd, China. The sequences were cut with XhoI, and then inserted into the corresponding site of pEGFP-C1 to form plasmids pEGFP-EqtII (S105-P142)-C1.

Western blotting analysis

The cell extracts were subjected to SDS-PAGE and electroblotted onto the nitrocellulose membrane. The membrane was then blocked with Tris-buffered saline and Tween 20 containing 5% of bovine serum albumin at room temperature for 2 h and incubated with primary antibodies overnight at 4°C. The membrane was washed and incubated with appropriate dilution of IRDye 800 donkey anti-mouse IgG or IRDye 700 donkey anti-rabbit IgG (LI-COR, Inc., Lincoln, NA, USA) for 1 h at room temperature. After washing with Tris-buffered saline, the membrane was then analyzed by an Odyssey Infrared Imaging System (LI-COR, Inc.)

Mouse anti-GFP antibody was purchased from Abmart Inc. (Shanghai, China). Rabbit anti-caspase3 antibody, anti-PARP antibody, anti-Bcl-2 antibody, and rabbit anti-GAPDH antibody were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Rabbit anti-Bax antibody was purchased from Epitomics (Burlingame, CA, USA).

Subcellular staining and colocalization study

ER-Tracker Red and Golgi-Tracker Red were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Mito Tracker Red Mitochondrion-Selective Probe was purchased from Invitrogen (Grand Island, NY, USA). Cells were plated on cover glass bottom dish (35 mm) at 1×10^5 per dish one day before transfection with plasmids. Cells were then transfected with plasmids as previously stated for 24 h or 48h. The cells were then stained with ER-Tracker Red, Golgi-Tracker Red or Mito Tracker Mitochondrion Selective Probes followed by observation under a confocal laser scanning microscope (Nikon, Inc., Japan).

RESULTS

EqtII (S105-P142) fragment was capable of being exogenously expressed in human cancer cells

A sequence encoding the S105-P142 region of EqtII was inserted into pEGFP-C1 to generate the pEGFP-EqtII (S105-P142)-C1 plasmid. To determine the exogenous expression of EqtII (S105-P142) in human cells, pEGFP-C1 or pEGFP-EqtII (S105-P142)-C1 plasmids were transfected into L02 and BEL-7404 human cancer cells and

the expression of EqtII (S105-P142) was examined by western blotting analysis with an anti-GFP antibody. Results showed that both enhanced green fluorescence protein (EGFP) and EGFP-EqtII (S105-P142) with molecular weights as expected were detected in these cells (Figure 1A), indicating that EqtII (S105-P142) is capable of being expressed in human cancer cells.

EqtII (S105-P142) expression induced apoptosis in BEL-7404 cells

We then examined the effect of EqtII (S105-P142) expression on hepatocellular carcinoma BEL-7404 cells. Plasmids pEGFP-C1 or pEGFP-EqtII (S105-P142)-C1 were transfected into BEL-7404. After 48 h, cells were harvested and stained with Annexin V-PE to detect apoptosis. As compared to pEGFP-C1 transfected cells which showed 0.494 ± 0.0339 of Annexin V-positive cells, upon the EGFP-EqtII (S105-P142)-C1 transfection, the percent of Annexin V-positive cells were significantly promoted to 7.22 ± 1.6140 (Figure 1B). Results indicated that the exogenous expression of EGFP-EqtII (S105-P142) induced apoptosis in BEL-7404 cells. Furthermore, pEGFP-EqtII (S105-P142)-C1 transfection also induced cell death in normal liver cell line L02 (data not shown), suggesting that the cytotoxicity of EqtII (S105-P142) fragment is not cancer specific.

To analyze the mechanism of EqtII (S105-P142)-induced apoptosis, apoptotic signal elements including poly (ADP-ribose) polymerase (PARP), Bax, and Bcl-2 were examined by Western blot. As shown in Fig. 1c, EqtII (S105-P142) did not significantly alter the cleavage of PARP, as well as the levels of proapoptotic factors Bcl-2 and Bax. Our densitometry analysis further confirmed that levels of these proteins were not significantly altered. Results suggested that EqtII (S105-P142) may induce apoptosis in Bel-7404 cells through other apoptotic signaling pathways pending further investigations.

EqtII (S105-P142) colocalized with mitochondria

Full length EqtII has been determined to specifically colocalize with Golgi apparatus^[12]. To further illustrated the subcellular distribution of EqtII (S105-P142). Plasmid pEGFP-EqtII (S105-P142)-C1 was transfected into BEL-7404. Cells were further stained with ER-Tracker Red, MitoTracker Mitochondrion-Selective Probes, and Golgi-Tracker Red. Results showed that EGFP-EqtII (S105-P142) did not colocalized with endoplasmic reticulum stained with ER-Tracker Red (Figure 2A) and Golgi apparatus stained with Golgi-Tracker Red (Figure 2B). Interestingly, EGFP-EqtII (S105-P142) was shown colocalizing with mitochondria stained with the MitoTracker Mitochondrion-Selective Probe (Figure 2C). Taken together, our data determined that EqtII (S105-P142) located in mitochondria, but not in Golgi apparatus and ER.

DISCUSSION

Previous studies revealed that full length EqtII colocalized with Golgi apparatus, but not with mitochondria and ER^[12]. However, our data showed that the EqtII (S105-P142) fragment colocalized with mitochondria, but not with Golgi apparatus and ER. Importantly, the expression of EqtII (S105-P142) significantly induced apoptosis. The dramatic locating and functional diversity between full length EqtII and the EqtII (S105-P142) fragment may be due to that the deletion of both N-terminal and C-terminal domains from EqtII resulted in a significant conformational change and subsequently exposed cryptic active sites, which showed binding specificity to molecules other than sphingomyelin, a molecule exposed on the Golgi apparatus as detected by full length EqtII^[10,12]. In fact, our

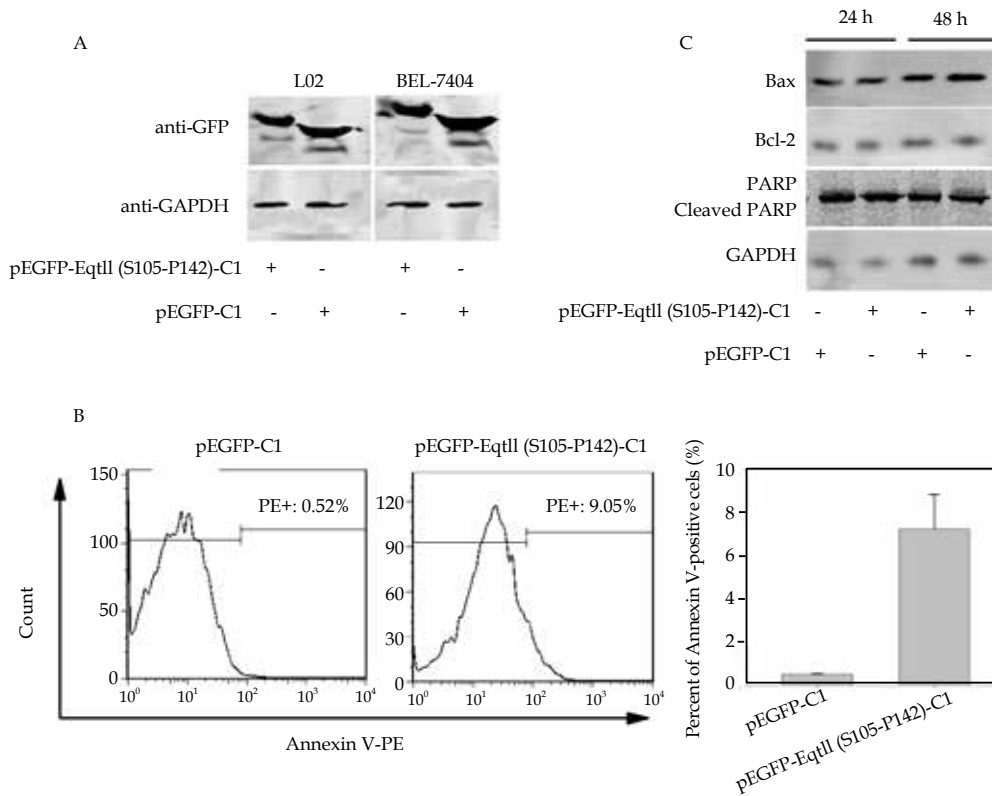


Figure 1 The expression and cytotoxicity of EqtlII (S105-P142). A: The expression of EGFP-EqtlII (S105-P142) in human cells. Transformed hepatic cell line L02 and hepatocellular carcinoma cell line BEL-7404 were transfected with pEGFP-C1 or pEGFP-EqtlII (S105-P142)-C1 plasmids. Cell lysates were collected after 48h and analyzed by western blot with antibodies against EGFP or GAPDH. B: Hepatocellular carcinoma cell line BEL-7404 was transfected with pEGFP-C1 or pEGFP-EqtlII (S105-P142)-C1 plasmids. After 48h, cells were harvested and stained with Annexin V-PE followed by flow cytometry analysis. Shown are representatives from 4 repeats and the statistics ($p < 0.05$). C: Hepatocellular carcinoma cell lines BEL-7404 were transfected with pEGFP-C1 or pEGFP-EqtlII (S105-P142)-C1 plasmids. Cell lysates were collected after 24h or 48h and analyzed by western blot with antibodies against Bax, Bcl-2, PARP, and GAPDH.

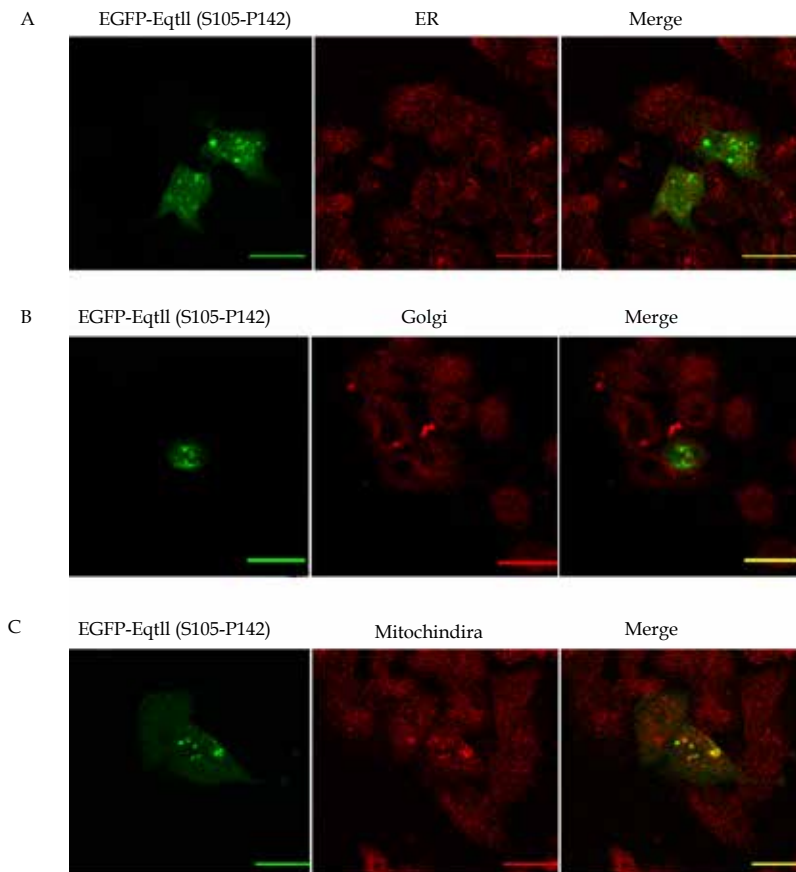


Figure 2 Localization of EGFP-EqtlII (S105-P142). A: BEL-7404 were transfected with pEGFP-EqtlII (S105-P142)-C1, and then stained with ER-Tracker Red at 24 h after transfection. The merge image was overlay of EGFP-EqtlII (S105-P142) image and ER-Tracker Red stained image. B: BEL-7404 cells were transfected with pEGFP-EqtlII (S105-P142)-C1 and stained with Golgi-Tracker Red. C: BEL-7404 cells were transfected with pEGFP-EqtlII (S105-P142)-C1 and stained with MitoTracker Mitochondrion-Selective Probes. All images were taken under 40 \times oil immersion objective of a confocal laser scanning microscope. Shown are representatives from at least 4 repeats. Bars show 20 μ m.

recent finding showing that EqtII (S105-P142) interacted with protein arginine methyltransferase 5 may have provided a further support for this hypothesis^[15].

As a cytotoxin, EqtII is capable of lysing cells through inserting the N-terminal α -helix into cell membrane and forming pores^[14]. Therefore, systemic administration of EqtII may result in deleterious effects. However, without the N-terminal α -helix, the EqtII (S105-P142) fragment may lose the pore-forming function, resulting in a safer EqtII isotype for medical use. Further tumor-specific controlling of EqtII (S105-P142) expression may provide insights into a novel role of EqtII (S105-P142) as an anticancer agent in future cancer gene therapy.

ACKNOWLEDGMENTS

We thank Shijing Wang for technical assistance.

CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

REFERENCES

- Ogawa T, Watanabe M, Naganuma T, Muramoto K. Diversified carbohydrate-binding lectins from marine resources. *J Amino Acids* 2011; **2011**: 838914
- Kim JA, Kim SK. Bioactive peptides from marine sources as potential anti-inflammatory therapeutics. *Curr Protein Pept Sci* 2013; **14**: 177-182
- Suarez-Jimenez GM, Burgos-Hernandez A, Ezquerro-Brauer JM. Bioactive peptides and decapeptides with anticancer potential: sources from marine animals. *Mar Drugs* 2012; **10**: 963-986
- Bhatnagar I, Kim SK. Marine antitumor drugs: status, shortfalls and strategies. *Mar Drugs* 2010; **8**: 2702-2720
- Aneiros A and Garateix A: Bioactive peptides from marine sources: pharmacological properties and isolation procedures. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004; **803**: 41-53
- Anderluh G, Pungercar J, Strukelj B, Macek P, Gubensek F. Cloning, sequencing, and expression of equinatoxin II. *Biochem Biophys Res Commun* 1996; **220**: 437-442
- Athanasiadis A, Anderluh G, Macek P, Turk D. Crystal structure of the soluble form of equinatoxin II, a pore-forming toxin from the sea anemone *Actinia equina*. *Structure* 2001; **9**: 341-346
- Bonev BB, Lam YH, Anderluh G, Watts A, Norton RS, Separovic F. Effects of the eukaryotic pore-forming cytotoxin Equinatoxin II on lipid membranes and the role of sphingomyelin. *Biophys J* 2003; **84**: 2382-2392
- Anderluh G, Razpotnik A, Podlesek Z, Macek P, Separovic F, Norton RS. Interaction of the eukaryotic pore-forming cytotoxin equinatoxin II with model membranes: 19F NMR studies. *J Mol Biol* 2005; **347**: 27-39
- Bakrac B, Gutierrez-Aguirre I, Podlesek Z, et al. Molecular determinants of sphingomyelin specificity of a eukaryotic pore-forming toxin. *J Biol Chem* 2008; **283**: 18665-18677
- Yachi R, Uchida Y, Balakrishna BH, et al.: Subcellular localization of sphingomyelin revealed by two toxin-based probes in mammalian cells. *Genes Cells* 2012; **17**: 720-727
- Bakrac B, Kladnik A, Macek P, et al. A toxin-based probe reveals cytoplasmic exposure of Golgi sphingomyelin. *J Biol Chem* 2010; **285**: 22186-22195
- Rojko N, Kristan KC, Viero G, et al. Membrane Damage by an alpha-Helical Pore Forming Protein Equinatoxin II Proceeds Through Succession of Ordered Steps. *J Biol Chem* 2013;
- Hong Q, Gutierrez-Aguirre I, Barlic A, et al.: Two-step membrane binding by Equinatoxin II, a pore-forming toxin from the sea anemone, involves an exposed aromatic cluster and a flexible helix. *J Biol Chem* 2002; **277**: 41916-41924
- Wu L, Yang X, Duan X, Cui L, Li G. Exogenous expression of marine lectins DIFBL and SpRBL induces cancer cell apoptosis possibly through PRMT5-E2F-1 pathway. *Sci Rep* 2004; **4**: 4505

Peer reviewers: Yoshinori Marunaka, MD, PhD, Professor and Chariman, Departments of Molecular Cell Physiology and Bioionomics, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 605-8566, Japan; Minghua Wu, Cancer Research Institute, Central South University, Xiangya Road 110#, Changsha, 410078, Hunan, China.