Tubeimoside-1 Protects Against Renal Ischemia Reperfusion Injury In Vivo and In Vitro

Xiaoli Yuan¹, Jing Wang¹, and Yun Zhang¹

Abstract
Renal ischemia reperfusion injury (RIRI) is one of the main causes of acute kidney injury. This study aimed to explore whether tubeimoside-1 (TBMS1) could protect against RIRI. RIRI mice model and hypoxia/reoxygenation (H/R)-induced NRK-52E cells were used in this study. The renal pathology was observed by hematoxylin and eosin staining to calculate the tubular injury score. The levels of serum creatinine and blood urine nitrogen were analyzed by a Hitachi model 7180 automatic analyzer. The expressions of tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin 6 (IL-6), Bax, cleaved caspase-3, cleaved caspase-9, total caspase-3, and total caspase-9 in renal tissues and NRK-52E cells were detected by western blot analysis. The levels of TNF-α, IL-1β, and IL-6 in serum and NRK-52E cells were measured by a commercial enzyme-linked immunosorbent assay kit. The renal cell apoptosis in renal tissues was analyzed by TUNEL assay, and NRK-52E cell apoptosis was detected by flow cytometry analysis. CCK-8 assay was used to analyze the viability of NRK-52E cells after the indicated treatment. As a result, the renal tissues that were seriously damaged in mice with RIRI could be alleviated by TBMS1. Therefore, 50 mg/kg TBMS1 was chosen for the animal experiment. Renal cell apoptosis was increased in renal tissues of mice with RIRI. These changes could be partially reversed by TBMS1 treatment. TBMS1 improved the viability, and reduced the inflammation and apoptosis of H/R-induced NRK-52E cells. In conclusion, TBMS1 ameliorates RIRI by promoting viability and suppressing apoptosis and inflammation of renal cells.

Keywords
tubeimoside-1, renal ischemia reperfusion injury, apoptosis, inflammation

Received: September 29th, 2020; Accepted: November 6th, 2020.

Ischemia reperfusion injury (IRI) refers to the damage caused by the restoration of blood supply following a long time of ischemia in tissues or organs.¹ Renal ischemia reperfusion injury (RIRI) is a common cause of acute renal failure (ARF) and the main factor affecting the early recovery of renal function after kidney transplantation. It not only causes delayed graft function, but also affects the immune mechanism to promote the occurrence of acute rejection.² RIRI can lead to apoptosis of renal tubular epithelial cells, renal tubular-interstitial inflammatory response, and ARF.³⁻⁵ During the development of RIR, the occurrence of inflammatory cell infiltration and the production of inflammatory factors plays important roles in the early stage of RIRI.⁶ Therefore, inhibiting renal tubular epithelial cell apoptosis and inflammatory response, and promoting proliferation of renal tubular epithelial cells induced by RIR may be useful for RIRI treatment.

Rhizoma bolbostemmae (Bolbostemma paniculatum [Maxim.] Franquet, family Cucurbitaceae) is widely used in traditional Chinese medicine. With detoxifying and detumescent properties, it can be used for the treatment of breast gland hyperplasia and breast cancer.⁷⁻⁹ Tubeimoside-1 (TBMS1) is the main active ingredient of Rhizoma bolbostemmae, and studies have demonstrated that it suppresses proliferation and induces apoptosis of cells in cancers such as glioma,¹⁰ glioblastoma,¹¹ non-small-cell lung cancer,¹² and oral squamous cell carcinoma.¹³ TBMS1 is conventionally used to treat snake venoms and inflammation, and was included in Supplements to Compendium of Materia Medica published in 1765.¹⁴ Furthermore, in acute lung-injured mice, TBMS1 could alleviate inflammation in lipopolysaccharide-induced RAW 264.7 cells.
Nevertheless, information about the role of TBMS1 in RIRI is limited. Therefore, we examined whether TBMS1 could exert protective effects on RIRI mice and hypoxia/reoxygenation (H/R)-induced NRK-52E cells for the further understanding and future use of this kind of drug in RIRI.

Materials and Methods

Animals

All animal experiments were approved by the Animal Ethics Committee of Beijing Tongren Hospital, Capital Medical University. Eighty wild-type C57BL/6 male mice (10 weeks old, 25-30 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All mice were raised and had free access to food and water in a controlled room with moderate temperature (22 ± 2 °C) and humidity (55%-60%) with a 12:12 hours light–dark cycle.

RIRI Model

Mice adaptively fed for 7 days were randomly divided into 8 groups (n = 10), including control group (sham), ischemia/reperfusion (I/R) group, I/R + 1 mg/kg TBMS1 group, I/R + 25 mg/kg TBMS1 group, I/R + 50 mg/kg TBMS1 group, I/R + 75 mg/kg TBMS1 group, I/R + 100 mg/kg TBMS1 group, and I/R + resveratrol (RVT) group. Except for control group, mice in the other groups were weighed and anesthetized with 1.25% avertin (0.2 mL/10 g body weight) by intraperitoneal injection. Mice were fixed on the operating plate in a supine position, with their abdomens disinfected with alcohol after skin preparation. A median abdominal incision about 2 cm long was made, and the abdominal cavity was opened. The left and right kidney pedicles were bluntly dissected and clamped using an atraumatic vascular clamp. The color of the kidney was observed and the change from ruddiness to grayness indicated that the clamping was effective, and the timing was started. The vascular clamp was removed after 40 minutes of...
ischemia in both kidneys to observe the color of the kidney changing from purple black to ruddy indicating good reperfusion, and the abdominal incision was performed layer by layer. Mice in I/R + TBMS1 groups were intraperitoneally injected with TBMS1 at concentrations of 1, 25, 50, 75, and 100 mg/kg, and mice in the I/R + RVT group were intraperitoneally injected with 30 mg/kg RVT during reperfusion. For the control group, mice received the same procedures except clamping of renal pedicles. After reperfusion for 24 hours, all mice were sacrificed, and the blood and renal samples were harvested and stored at −80 °C for further analysis.

**Hematoxylin-Eosin Staining**

Renal tissues were fixed in 4% (v/v) paraformaldehyde for 48 hours at room temperature, embedded into paraffin, and sliced into 4-µm-thick sections. Then, the sections were stained with hematoxylin and eosin. The tubular injury score was calculated according to the percentage of damaged tubules in the outer medulla. The magnitude of cell loss and necrosis was scored based on 5 levels. Scores ranged from 0 to 4 based on the percentage of tubules affected (0: <10%; 1: 10%-25%; 2: 25%-50%; 3: 50%-75%; 4: >75%). A renal pathologist blinded to the mice group assignments graded the tubular injury under the high-power field of a light microscope.

**Cell Culture**

NRK-52E cells were provided from American Type Culture Collection (Rockville, MD, USA). All the cells were cultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin in an incubator with 5% CO2 at 37 °C. The medium was exchanged every 24 hours.
H/R Induction and TBMS1 Treatment

The H/R procedure was conducted as previously described with modifications. Briefly, confluent NRK-52E cells were incubated for 24 hours in an anaerobic chamber equilibrated with 5% CO₂, 5% H₂, and 90% N₂ to form hypoxia cells. After the hypoxia treatment, the cells were moved to a regular incubator with 21% oxygen and treated with TBMS1 in different concentrations (1, 5, 10, 15, and 20 µM) and 50 µM RVT for 4 hours. NRK-52E cells in dimethyl sulfoxide (DMSO) group were conventionally cultured in medium containing DMSO. After H/R induction and TBMS1 treatment, a hemocytometer was used to count cell numbers.

CCK-8 Assay

After the indicated treatment of NRK-52E cells, they were seeded in 96-well plates to which was added 10 µL of CCK-8 reagent per well. When the plates were incubated in the dark for 4 hours, the absorbance value of each well was measured at 450 nm with a PerkinElmer Microplate reader.

Serum Biochemistry and Enzyme-Linked Immunosorbent Assay

The levels of serum creatinine (Scr) and blood urine nitrogen (BUN) were analyzed by a Hitachi model 7180 automatic analyzer. Tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) in serum and NRK-52E cells were measured by a commercial ELISA kit referring to the manufacturer’s instructions.

TUNEL Assay

Briefly, renal tissue sections were washed with phosphate-buffered saline (PBS) and treated with protease K at room temperature. Next, sections were sealed with 3% H₂O₂ for 5 minutes and then incubated completely with TUNEL reaction solution at 37 °C for 1 hour. After PBS washing, sections were treated with 50 mL converter-POD at 37 °C for 30 minutes. Diaminobenzidine was added for full color development at room temperature for 10 minutes. Finally, fluorescence microscopy was used to analyze the stained sections.

Western Blot Analysis

RIPA lysis buffer was used to lyse renal tissues and NRK-52E cells. After lysis, total proteins were extracted from both and quantified by a protein assay kit. Then, proteins (40 µg/lane) were separated with 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes, which were blocked with 5% fat-free milk for 2 hours at room temperature. The membranes were incubated with primary antibodies against TNF-α, IL-1β, IL-6, Bax, cleaved caspase-3, cleaved caspase-9, total caspase-3, and total caspase-9 at 4 °C overnight, followed by buffer washing. Next, horseradish peroxidase-conjugated antibody was added to the membranes for incubation at room temperature for 1 hours. Finally, the protein bands were observed using an enhanced chemiluminescence system.
Flow Cytometry Analysis

Apoptosis of NRK-52E cells was detected by fluorescein isothiocyanate (FITC) Annexin V and propidium iodide (PI) dual staining in accordance with the manufacturer’s protocol. Annexin V-FITC/PI binding was analyzed by fluorescence-activated cell sorting, and FlowJo software was used to analyze the data.

Statistical Analysis

Data are presented as mean ± standard deviation. One-way analysis of variance was used coupled with Tukey’s post hoc test to evaluate intergroup differences. SPSS 23.0 statistical software was performed for statistical analysis. *P* < .05 was considered statistically significant.

Results

**TBMS1 Protects Against RIRI**

In mice with RIRI, renal tissues were seriously damaged. RIRI mice were, respectively, treated with different concentrations of TBMS1. After TBMS1 treatment with concentrations from 1 to 50 mg/kg, the renal tissue injury was gradually improved. After
50-100 mg/kg TBMS1 treatment, the renal tissue showed little, but not significant injury as TBMS1 concentration increased, which was consistent with the alleviative effect of RVT treatment on RIRI (Figure 1(A)). The tubular injury score was increased in mice with RIRI, while decreased when mice were treated with 1-50 mg/kg TBMS1. The tubular injury scores were not statistically changed after 50-100 mg/kg TBMS1 treatment on mice with RIRI. The tubular injury score after 50 mg/kg TBMS1 treatment was not statistically different from RVT treatment, either. Therefore, 50 mg/kg TBMS1 was determined for the next experiment (Figure 1(B)).
The levels of Scr (Figure 1(C)) and BUN (Figure 1(D)) in serum were upregulated in mice with RIRI, and TBMS1 treatment downregulated their levels.

**TBMS1 Alleviates Inflammation in Renal Tissues of Mice With RIRI**

The expression of TNF-α, IL-1β, and IL-6 in renal tissues of mice with RIRI was increased, but this was inhibited by TBMS1 treatment (Figure 2(A)). Also, the levels of TNF-α, IL-1β and IL-6 in serum of mice with RIRI were increased, and TBMS1 treatment decreased their levels (Figure 2(B)-(D)).

**TBMS1 Inhibits Apoptosis of Renal Cells in Mice With RIRI**

The apoptosis of renal cells was enhanced in mice with RIRI, but TBMS1 treatment suppressed this effect (Figure 3(A)), which was consistent with the changes in apoptosis index (Figure 3(B)).

**TBMS1 Affects the Expression of Apoptosis-Related Proteins in Renal Tissues of Mice With RIRI**

As shown in Figure 4, the expressions of Bax, cleaved caspase-3, and cleaved caspase-9 were increased in renal tissues of mice with RIRI, which could be reversed by TBMS1 treatment. The expression of total caspase-3 and total caspase-9 was not changed in these 3 groups.

**TBMS1 Promotes Proliferation of H/R-Induced NRK-52E Cells**

Observed by microscopy, the cell number in the H/R group was significantly decreased compared with the DMSO group. When H/R-induced NRK-52E cells were treated with TBMS1, the numbers of H/R-induced NRK-52E cells were increased after treatment with 1 and 5 µM TBMS1, while there was no significant difference in cell numbers after 1 and 5 µM TBMS1 treatment of the cells. The number of H/R-induced NRK-52E cells was further increased after 10 µM TBMS1 treatment, and there was no significant difference in cell numbers after...
TBMS1 treatment at doses of 10, 15, and 20 µM (Figure 5(A)). CCK-8 assay showed that NRK-52E cells induced by H/R showed sharp declines in their growth curve, while the growth curve of H/R-induced NRK-52E cells treated with 1, 5, 10, 15, and 20 µM TBMS1 rose. The improvement produced by 10, 15, and 20 µM TBMS1 on cell growth was better than that of 1 and 5 µM TBMS1. There was no significant difference in cell growth after 1 and 5 µM TBMS1 treatment, and there was no significant difference in cell growth after TBMS1 treatment at doses of 10, 15, and 20 µM (Figure 5(B)). Therefore, 10 µM TBMS1 was chosen for the cell experiments.

**TBMS1 Alleviates Inflammation in H/R-Induced NRK-52E Cells**

The protein expression of TNF-α, IL-1β, and IL-6 in H/R-induced NRK-52E cells was increased, but this effect was decreased by treatment with TBMS1 (Figure 6(A)). In the culture supernatant of NRK-52E cells, ELISA indicated that levels of TNF-α, IL-1β, and IL-6 were increased in the H/R group, but decreased in the H/R + TBMS1 group (Figure 6(B-D)).

**TBMS1 Inhibits Apoptosis of H/R-Induced NRK-52E Cells**

The apoptosis of NRK-52E cells after H/R induction was increased, but TBMS1 treatment suppressed this (Figure 7(A)). Likewise, the expressions of Bax, cleaved caspase-3, and cleaved caspase-9 were increased, but TBMS1 treatment decreased these. The expressions of total caspase-3 and total caspase-9 were not statistically different in these 3 groups (Figure 7(B)).

**Discussion**

After renal ischemia, reperfusion can aggravate the injury of tissues or organs, and even cause irreversible injury. The diagnosis of RIRI has been greatly improved, the incidence and mortality of the disease are still high. Therefore, reducing new RIRI cases is a hot topic in clinical research and discussion.

The use of traditional Chinese medicine in the treatment of diseases has a long history. Wang et al indicated that RSV alleviated oxidative stress and inflammation to relieve I/R-induced renal injury in rats. Han et al demonstrated that cordycepin suppressed the development of RIRI by inhibiting inflammation, apoptosis, and oxidative stress. Long et al reported that macelignan increased the expressions of Bax and caspase-3 while decreasing the expression of Bel-2, and suppressed inflammation in renal epithelial cells rats with RIRI. TBMS1 extracted from the tubers of *Rhizoma bolbostemmae* has been demonstrated to effectively treat gastric cancer, liver cancer, and cervical cancer. Previous studies indicated that TBMS1 and TBMS3 had anti-inflammatory and antitumor effects. In this study, we found that TBMS1 alleviated RIRI in vivo and in vitro by inhibiting apoptosis and inflammation in renal tissues of mice with RIRI and H/R-induced NRK-52E cells.

In conclusion, TBMS1 ameliorated RIRI by inhibiting apoptosis of renal cells and inflammation in mice with RIRI. Also, TBMS1 improved viability, and inhibited apoptosis and inflammation of H/R-induced NRK-52E cells. The above experimental results demonstrated that TBMS1 has a potential therapeutic effect on RIRI.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) received no financial support for the research, authorship, and/or publication of this article.

**ORCID ID**

Yun Zhang https://orcid.org/0000-0001-7618-8677

**References**

1. Iñiguez M, Dotor J, Feijoo E, et al. Novel pharmacologic strategies to protect the liver from ischemia-reperfusion injury. *Recent Pat Cardiovasc Drug Discov*. 2008;3(1):9-18. doi:10.2174/157489008783331643

2. Smith SF, Hosgood SA, Nicholson ML. Ischemia-reperfusion injury in renal transplantation: 3 key signaling pathways in tubular epithelial cells. *Kidney Int*. 2019;95(1):50-56. doi:10.1016/j.kint.2018.10.009

3. Cao Q, Wang Y, Niu Z, et al. Potentiating tissue-resident type 2 innate lymphoid cells by IL-33 to prevent renal ischemia-reperfusion injury. *J Am Soc Nephrol*. 2018;29(3):961-976. doi:10.1681/ASN.2017070774

4. Bonventre JV, Yang L. Cellular pathophysiology of ischemic acute kidney injury. *J Clin Invest*. 2011;121(11):4210-4221. doi:10.1172/JCI45161

5. Eltzschig HK, Eckle T. Ischemia and reperfusion—from mechanism to translation. *Nat Med*. 2011;17(11):1391-1401. doi:10.1038/nm.2507

6. Malek M, Nematbakhsh M. Renal ischemia/reperfusion injury; from pathophysiology to treatment. *J Renal Inj Prev*. 2015;4(2):20-27. doi:10.12861/jrip.2015.06

7. Xu Y, Wang G, Chen Q, et al. Intrinsic apoptotic pathway and G2/M cell cycle arrest involved in tubeimoside I-induced EC109 cell death. *Chin J Cancer Res*. 2013;25(3):312-321. doi:10.3978/j.issn.1000-9604.2013.06.03

8. Wang F, Ma R, Yu L. Role of mitochondria and mitochondrial cytochrome c in tubeimoside I-mediated apoptosis of human cervical carcinoma HeLa cell line. *Cancer Chemother Pharmacol*. 2006;57(3):389-399. doi:10.1007/s00280-005-0047-y
9. RD M, LJ Y, WM S. Induction of cell cycle arrest and apoptosis by tubeimoside I isolated from Bolbostemma paniculatum in HeLa cells. Chin J Clin Pharm Therap. 2004;9(3):261-269.

10. Cao L-J, Xie H-T, Chu Z-X, Ma Y, Wang M-M, Shi Z. Tubeimoside-1 induces apoptosis in human glioma U251 cells by suppressing PI3K/Akt-mediated signaling pathways. Mol Med Rep. 2020;22(2):1527-1535. doi:10.3892/mmr.2020.11224

11. Cao J, Zhao E, Zhu Q, et al. Tubeimoside-1 inhibits glioblastoma growth, migration, and invasion via inducing ubiquitilation of Met. Cells. 2019;8(8):774. doi:10.3390/cells8080774

12. Shi H, Bi H, Sun X, et al. Tubeimoside-1 inhibits the proliferation and metastasis by promoting miR-126-5p expression in non-small cell lung cancer cells. Onco Lett. 2018;16(3):3126-3134. doi:10.3892/ol.2018;9051

13. Wu T, Cui H, Xu Y, et al. The effect of tubeimoside-1 on the proliferation, metastasis and apoptosis of oral squamous cell carcinoma in vitro. Onco Targets Ther. 2018;11:3989-4000. doi:10.2147/OTTS.164503

14. Wang Y. A Study on the Inhibitory Effects of Tubeimoside I on Cultured Human Hepatoma (Hepg2) Cells and Associated Mechanisms. Chongqing University; 2011.

15. Wu Q, Sun G, Yuan X, et al. Tubeimoside-1 attenuates LPS-induced inflammation in RAW 264.7 macrophages and mouse models. Immunopharmacol Immunotoxicol. 2013;35(4):514-523. doi:10.3109/08923973.2013.810643

16. Savransky V, Molls RR, Burne-Taney M, Chien C-C, Racusen L, Rabb H. Role of the T-cell receptor in kidney ischemia-reperfusion injury. Kidney Int. 2006;69(2):233-238. doi:10.1038/ki.2006.50

17. Sauvant C, Schneider R, Holzinger H, Renker S, Wanner C, Gekle M. Implementation of an in vitro model system for investigating of reperfusion damage after renal ischemia. Cell Physiol Biochem. 2009;24(5-6):567-576. doi:10.1159/000257153

18. Zhou Y, Cai T, Xu J, et al. Ucp2 attenuates apoptosis of tubular epithelial cells in renal ischemia-reperfusion injury. Am J Physiol Renal Physiol. 2017;313(4):F926-F937. doi:10.1152/ajprenal.00118.2017

19. Zheng H, Lan J, Li J, Lv L. Therapeutic effect of berberine on renal ischemia-reperfusion injury in rats and its effect on Bax and Bcl-2. Exp Ther Med. 2018;16(3):2008-2012. doi:10.3892/etm.2018.6408

20. Fan W, Ankawi G, Zhang J, et al. Current understanding and future directions in the application of TIMP-2 and IGFBP7 in AKI clinical practice. Clin Chem Lab Med. 2018;57(5):567-576. doi:10.1515/cclm-2018-0776

21. Lafrance J-P, Miller DR. Acute kidney injury associates with increased long-term mortality. J Am Soc Nephrol. 2010;21(2):345-352. doi:10.1681/ASN.2009060636

22. Wang M, Weng X, Chen H, Chen Z, Liu X. Resveratrol inhibits TNF-α-induced inflammation to protect against renal ischemia/reperfusion injury in diabetic rats. Acta Cir Bras. 2020;35(5):e202000506. doi:10.1590/s1012-86502020005000006

23. Han F, Dou M, Wang Y, et al. Cordycepin protects renal ischemia/reperfusion injury through regulating inflammation, apoptosis, and oxidative stress. Acta Biochim Biophys Sin. 2020;52(2):125-132. doi:10.1093/abbs/gmz145

24. Long J, Qian K, Tan S, Liu J, Li J. Macelignan protects against renal ischemia-reperfusion injury via inhibition of inflammation and apoptosis of renal epithelial cells. Cell Mol Biol. 2020;66(1):55-59. doi:10.14715/cmb/2019.66.1.9

25. Jia G, Wang Q, Wang R, et al. Tubeimoside-1 induces gloma apoptosis through regulation of bax/bcl-2 and the ROS/Cytochrome C/Caspase-3 pathway. Onco Targets Ther. 2015;8:303-311.

26. Gu Y, Körbel C, Scheuer C, Nenciu A, Mengen MD, Laschke MW. Tubeimoside-1 suppresses tumor angiogenesis by stimulation of proapoptotic VEGFR2 and Tie2 degradation in a non-small cell lung cancer xenograft model. Oncotarget. 2016;7(5):5258-5272. doi:10.18632/oncotarget.6676

27. Peng Y, Zhong Y, Li G. Tubeimoside-1 suppresses breast cancer metastasis through downregulation of CXCR4 chemokine receptor expression. BMB Rep. 2016;49(9):502-507. doi:10.5483/BMBRep.2016.49.9.030

28. Bian Q, Liu P, Gu J, Song B. Tubeimoside-1 inhibits the growth and invasion of colorectal cancer cells through the Wnt/β-catenin signaling pathway. Int J Clin Exp Pathol. 2015;8(10):12517-12524.

29. Yang J-B, Khan M, He Y-Y, et al. Tubeimoside-1 induces oxidative stress-mediated apoptosis and G0/G1 phase arrest in human prostate carcinoma cells in vitro. Acta Pharmacol Sin. 2016;37(7):950-962. doi:10.1038/aps.2016.34

30. Cui Z, Shi X, Liu X, et al. Determination of tubeimoside I content in Bolbostemma paniculatum (Maxim.) Franquet and its anti-proliferative effect on human erythroleukemia K562 cells. Medicine. 2016;27:1171-1173.

31. Yu L, Ma R, Wang Y, Nishino H. Potent anti-tumor activity and low toxicity of tubeimoside 1 isolated from Bolbostemma paniculatum. Plants Med. 1994;60(3):204-208. doi:10.1055/s-2006-959459

32. Yu L, Ma R, Wang Y, et al. Potent anti-tumorogenic effect of tubeimoside 1 isolated from the bulb Bolbostemma paniculatum (Maxim.) franquet. Int J Cancer. 2010;50(4):635-638. doi:10.1002/ijc.2910500425

33. Yu L, Yu T, Ma R. Inhibition of the tumor promoting action of 12-O-tetradecanoylphorbol-13-acetate by tubeimoside III isolated from Bolbostemma paniculatum. Carcinogenesis. 1995;16(12):3045-3048. doi:10.1093/carcin/16.12.3045