# Extracorporeal shock wave therapy ameliorates secondary lymphedema by promoting lymphangiogenesis

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***Objective:*** **Although secondary lymphedema is a common complication after surgical and radiation therapy for cancer, the treatment options for lymphedema remain limited and largely ineffective. We thus studied the effect of extracorporeal shock wave therapy on promoting lymphangiogenesis and improving secondary lymphedema.**

***Methods:*** **A rabbit ear model of lymphedema was created by disruption of lymphatic vessels. Two weeks after surgery, the lymphedematous ear was treated with or without low-energy shock waves (0.09 mJ/mm$^2$, 200 shots), three times per week for 4 weeks.**

***Results:*** **Western blot analysis showed that the expression of vascular endothelial growth factor (VEGF)-C (1.23-fold, $P < .05$) and VEGF receptor 3 (VEGFR3; 1.53-fold, $P < .05$) was significantly increased in the ears treated with shock wave than in the untreated lymphedematous ears. Compared with the control group, shock wave treatment led to a significant decrease in the thickness of lymphedematous ears (3.80 ± 0.25 mm vs 4.54 ± 0.18 mm, $P < .05$). Immunohistochemistry for VEGFR3 showed the density of lymphatic vessels was significantly increased by shock wave treatment ($P < .05$).**

***Conclusion:*** **Extracorporeal shock wave therapy promotes lymphangiogenesis and ameliorates secondary lymphedema, suggesting that extracorporeal shock wave therapy may be a novel, feasible, effective, and noninvasive treatment for lymphedema. (J Vasc Surg 2010;52:429-34.)**

***Clinical Relevance:*** **Therapeutic options for lymphedema are currently limited to supportive treatment. Thus, it is desirable to develop a curative treatment for lymphedema. The findings of the present study suggest that extracorporeal shock wave therapy is effective in treating lymphedema. Further clinical trials are required to confirm the efficiency of this therapy.**

Lymphedema is a pathogenic condition characterized by the excessive, regional interstitial accumulation of protein-rich fluid. Secondary lymphedema develops after disruption or obstruction of the lymphatic system as a consequence of surgery and radiotherapy for cancer.[1-4] In addition to chronic changes in the size and structure of the subcutaneous and integumentary structure, the presence of lymphedema markedly affects the quality of life and the self-perception of patients.[5,6] Despite recent advances in surgical and radiotherapeutic technical enhancements, therapeutic options for management of lymphedema are limited and largely ineffective.

Recent studies have elucidated the mechanism regulating the growth and formation of new lymphatic vessels (lymphangiogenesis) by means of the discovery of lymphangiogenic factors, the identification of lymphatic-specific markers, and the development of animal models to study lymphangiogenesis.[7-10] The accumulating evidence indicates that the signaling pathway of vascular endothelial growth factor-C (VEGF-C) and its receptor, VEGF receptor 3 (VEGFR3), are critically important in the regulation of the lymphatic vessel growth.[11-13] Importantly, transfer of the gene expressing VEGF-C effectively promoted the formation of lymphatic vessels and ameliorated lymphedema in an animal model.[14-16] Therefore, the induction of lymphangiogenesis might be a promising and effective approach for management of lymphedema.

Interestingly, extracorporeal low-energy shock wave (SW) therapy for myocardial ischemia up-regulated the expression of VEGF (or VEGF-A), increased vascular density, and improved myocardial ischemia and dysfunction in a pig model.[17] Moreover, a clinical study has shown that extracorporeal low-energy SW therapy ameliorates myocardial ischemia in patients with severe coronary artery disease.[18] Considering the similarity in the vascular morphogenesis between lymphatic and blood endothelium and the involvement of members of the VEGF and VEGFR family in the regulation of lymphangiogenesis as well as angiogenesis,[9,13] we hypothesized that extracorporeal low-energy SW treatment could induce the growth of lymphatic vessels, thereby ameliorating lymphedema. Therefore, the aim of the present study was to investigate the effect of extracorporeal low-energy SW therapy on promoting lym-

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This work was partly supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a competition of interest.

0741-5214/$36.00



doi:10.1016/j.jvs.2010.03.017

phangiogenesis and improving lymphedema in a rabbit ear model of secondary lymphedema.

## MATERIALS AND METHODS

All experiments were approved by the Institutional Animal Care and Use Committee of Yamaguchi University.

**Rabbit ear model of lymphedema.** Male Japanese white rabbits were purchased from the Oriental Yeast Company (Tokyo, Japan) and bred in the Animal Center of Yamaguchi University. The rabbit ear model of lymphedema was created as described previously[14,15,19] with minor modification. The rabbits were given general anesthesia before surgery. A 3-cm-wide circumferential strip of skin, subcutaneous tissues, and perichondrium was removed from the base of the ear, except for the central portion (1 cm in width) of the dorsal skin (ie, a "skin bridge") including the neurovascular bundle. The lymphatic vessels were identified by intradermal injection of 0.2 mL of 1% Evans blue dye into the acral aspect of the ear.

After the distal edge of the skin bridge was incised, the lymphatic channels were dissected and the lymphatic stumps were ligated and resected under an operating microscope. Skin edges were inverted and sutured to the perichondrium to prevent skin reapproximation and recanalization of the lymphatic vessels. A strip of bare cartilage was created in this way, leaving only the skin bridge for lymphatic growth. The surgery was performed on both ears in seven rabbits. All animals survived without any local complications on the ear until euthanasia.

**SW treatment.** Two weeks after surgery, we applied a low-energy SW (0.09 mJ/mm$^2$, about 10% of the energy for lithotripsy, 200 shots) to the distal edge of the skin bridge of the ear (SW group) using the Shock Wave Generator (Medispec Ltd, Germantown, Md). The SW treatment was performed three times within a week for 4 weeks (SW group). The conditions for the SW treatment have been reported previously.[17,18] The SW treatment was not performed in the contralateral ear (control group).

**Measurement of ear thickness.** Every week after surgery, the thickness of the ears was measured 1 cm medial and distal to the medial border of the skin bridge with a vernier caliper, as described previously.[14] Each ear was measured three times to derive an average value.

**Preparation of skin samples.** Rabbits were euthanized 4 weeks after treatment, and skin tissue from the bridge area of the ear was harvested. Each collected sample was used for histologic and Western blot analysis.

**Histologic analysis.** The harvested skin tissue from the bridge of the ear was fixed in 10% buffered neutral formalin (Wako, Tokyo, Japan), embedded in paraffin, and cut into sections 3-μm thick. Two independent sections of each ear deparaffinized, stained with hematoxylin and eosin, and examined under a light microscope (Nikon ECLIPSE, E1000M, Nikon, Tokyo, Japan) at original magnification ×20 or ×200. Observations were made in a blinded manner.

**Western blot analysis.** To examine the expression of VEGF-C and VEGFR3 in the skin tissue of the ear, we performed Western blot analysis as described previously[20,21] with slight modifications. The skin samples were homogenized in 5 volumes of a buffer (20 mmol/L Tris-HCl [pH 7.5]. 150 mmol/L NaCl, 1% Triton X-100, and complete protease inhibitor mixture tablets [Roche, Mannheim, Germany]), and centrifuged at 1000*g* for 10 minutes at 4°C. The supernatant was used for subsequent analysis.

Protein concentrations were determined using a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, Ill) with bovine serum albumin as a standard. The supernatant (80 μg/well) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene diflouride membrane (Millipore, Bedford, Mass). After blocking, the membranes were probed at 4°C overnight with goat anti-VEGF-C polyclonal antibody (1:1000, C-20, Santa Cruz Biotechnology, Santa Cruz, Calif) or mouse anti-VEGFR3 monoclonal antibody (1:1000, 9D9F9, Chemicon Intl Inc, Temecula, Calif). Horseradish peroxidase-conjugated donkey anti-goat immunoglobulin (Ig) G (1:3000, Santa Cruz Biotechnology) or goat antimouse IgG (1:3000, DakoCytomation Inc, Carpinteria, Calif) were used as a secondary antibody for detecting VEGF-C or VEGFR3, respectively.

Signals were visualized using an enhanced chemiluminescence Western blot detection system (GE Healthcare UK Ltd, Buckinghamshire, UK). The membranes were stripped and reprobed with antibody against β-actin (1:4000, Alpha Diagnostic Intl, San Antonio, Tex). Quantification of each band was carried out using National Institutes of Health image software, and levels of VEGF-C or VEGFR3 were normalized to that of β-actin.

**Immunohistochemical analysis.** To examine the formation of lymphatic vessels in skin tissue of the ear, we performed immunohistochemical analysis for VEGFR3, as described previously[20,21] with minor modifications. Deparaffinized sections were heated in a buffer (10 mmol/L Tris-HCl [pH 9.0], 1 mmol/L ethylenediaminetetraacetic acid) at 90°C for 10 minutes, followed by cooling in the buffer for 40 minutes at room temperature. The sections were incubated in methanol containing 30% hydrogen peroxide for 30 minutes and permeabilized with 0.2 % Triton X-100 in phosphate-buffered saline for 20 minutes.

After blocking with Protein Block Serum-free (DakoCytomation Inc), the sections were stained at 4°C overnight with mouse anti-VEGFR3 monoclonal antibody (1:4000, CHEMICON). The sections were then incubated with goat antimouse Ig conjugated with peroxidase-labelled polymer (DakoCytomation), and developed in 3,3′-diaminobenzidine tetrahydrochloride (DakoCytomation).

The sections were counterstained with hematoxylin. The number of VEGFR3-positive vessels was counted in 12 randomly selected microscopic fields (original magnification ×400) from two independent sections of each ear. Data are expressed as the number of VEGFR3-positive vessels/field. All measurements were made in a blinded manner.

**Statistical analysis.** All data are expressed as means ± standard error of the mean. Comparisons between two

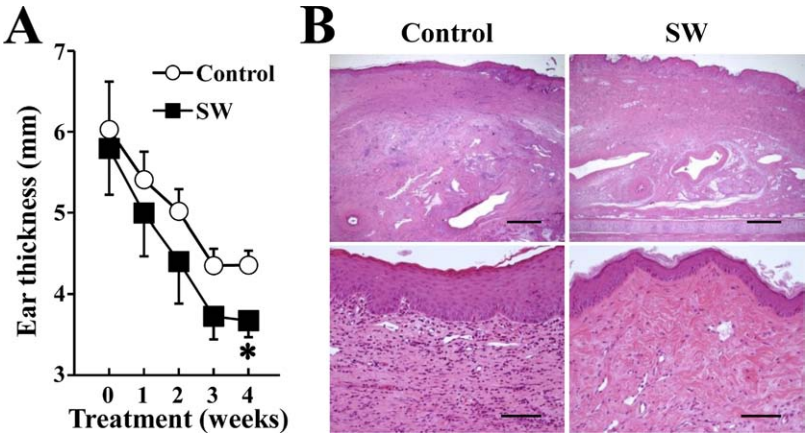


**Fig 1.** The effect of shock wave *(SW)* treatment on lymphedema. **A,** Measurement of ear thickness showed that the SW group had significantly thinner skin than the control group 4 weeks after treatment. $*P < .05$ vs control group (n = 7/group). Mean data are presented with the standard error of the mean. **B,** Representative histologic images for the SW-treated and untreated (control) lymphedematous ear 4 weeks after treatment. Bars indicate 1000 μm (*upper panels,* original magnification ×20) and 100 μm (*lower panels,* original magnification ×200; hematoxylin and eosin stain).

groups were made using the unpaired *t* test. A value of $P < .05$ was considered significant. All analyses were performed with StatView 5.0 software (SAS Institute, Cary, NC).

## RESULTS

**SW treatment induced the attenuation of lymphedema.** To investigate the effects of SW treatment on lymphedema, we measured the ear thickness of lymphedematous ears. Although there was no significant difference in the thickness of lymphedematous ear between the control and SW groups before treatment (2 weeks after surgery), skin in the SW group gradually became thinner than that of the control group after treatment onward. Four weeks after treatment, ears were significantly thinner in the SW group than in the control group ($3.80 \pm 0.25$ mm vs $4.54 \pm 0.18$ mm, $P < .05$; Fig 1, *A*). Histologic assessment of the lymphedematous ear showed a thinned epidermis, reduced cellularity in the dermis, and an overall decrease in thickness of the tissues in the SW-treated lymphedematous ear compared with the untreated (control) lymphedematous ear (Fig 1, *B*).

**SW treatment increased the expression of VEGF-C and VEGFR3 in the lymphedematous ear.** Western blot analysis showed that the expression of VEGF-C in the ear skin was significantly higher in the SW group than in the control group (1.23-fold, $P < .05$) (Fig 2, *A* and *B*), 4 weeks after SW treatment. We investigated changes in the expression of VEGFR3 in the skin tissue of lymphedematous ears in response to SW treatment. There was significantly increased expression of VEGFR3 in the skin tissue of the ear in the SW group compared with the control group (1.53-fold, $P < .05$), 4 weeks after SW treatment (Fig 3, *A* and B).

**SW treatment promoted the formation of lymphatic vessels in the lymphedematous ears.** To assess the formation of lymphatic vessels in response to SW treatment, we performed immunostaining for VEGFR3, a marker of the lymphatic vessel, in lymphedematous ears. Immunohistochemistry revealed that the density of VEGFR3-positive lymphatic vessels was significantly higher in the SW group than in the control group ($4.64 \pm 0.72$/field vs $1.81 \pm 0.31$/field, $P < .05$) 4 weeks after SW treatment (Fig 4, *A* and *B*).

## DISCUSSION

The present study documents the efficacy of extracorporeal SW therapy for lymphedema. Using a rabbit ear model of secondary lymphedema, we found that extracorporeal low-energy SW therapy enhanced the expression of VEGF-C and VEGFR3, increased the formation of lymphatic vessels, and improved lymphedema.

The main purpose of this study was to test the hypothesis that extracorporeal low-energy SW therapy reduces lymphedema by promoting lymphangiogenesis. We found significantly enhanced expression of VEGF-C protein in the SW-treated lymphedematous ear. VEGF-C is known to be a potent lymphangiogenic factor.[22] It has been demonstrated that VEGF-C gene therapy ameliorates lymphedema by inducing therapeutic lymphangiogenesis.[14-16] Furthermore, SW treatment significantly increased the expression of VEGFR-3 protein in the lymphedematous ear. We also observed a significant increase in VEGFR3-positive lymphatic vessels in the SW-treated lymphedematous ear. These results indicate that there is an augmentation of lymphangiogenesis in response to SW treatment because the expression of VEGFR3 becomes largely limited to the lymphatic endothelium.[23]

Given the importance of VEGF-C/VEGFR3 signaling in the processes of lymphangiogenesis,[11-13] we think that the SW-induced expression of VEGF-C and VEGFR3 proteins contributes to the growth of lymphatic vessels. More

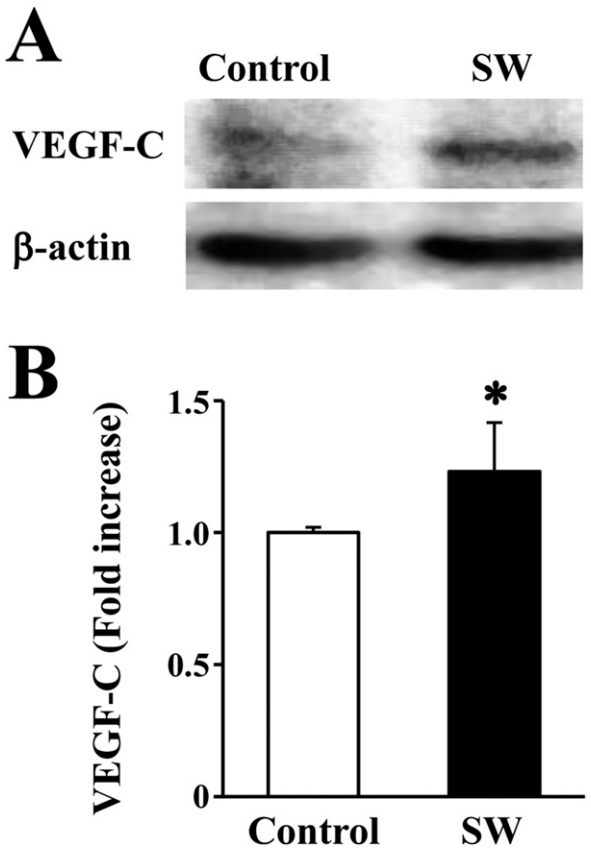


**Fig 2.** Analysis of vascular endothelial growth factor (*VEGF*)-C expression in skin tissue 4 weeks after shock wave (*SW*) treatment. **A,** Representative images of Western blot analysis for VEGF-C. **B,** Quantification after normalization to β-actin showed that the expression of VEGF-C was significantly higher in the SW group than in the control group. $*P < .05$ vs control group (n = 4/group). Mean data are presented with the standard error of the mean.

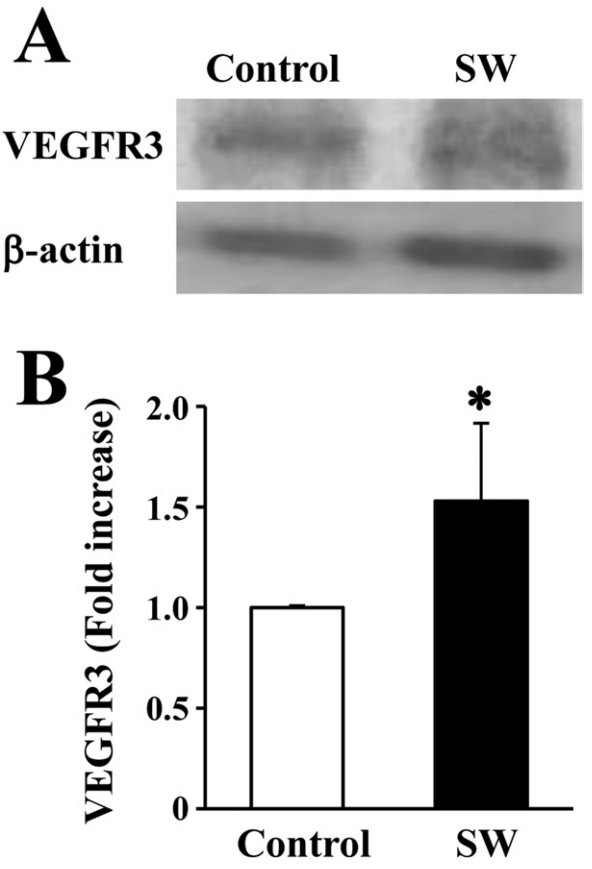


**Fig 3.** Analysis of vascular endothelial growth factor receptor 3 *(VEGFR3)* expression in skin tissue 4 weeks after shock wave (*SW)* treatment. **A,** Representative images of Western blot analysis for VEGFR3. **B,** Quantification after normalization to β-actin revealed significantly higher expression of VEGFR3 in the SW group than in the control group. $*P < .05$ vs control group (n = 4/group). Mean data are presented with the standard error of the mean.

importantly, we found that extracorporeal low-energy SW treatment results in the attenuation of lymphedema, as shown by a significant decrease in the thickness of lymphedematous ears. These results suggest that the amelioration of lymphedema by extracorporeal low-energy SW therapy can be attributed to the promotion of lymphangiogenesis.

The exact mechanisms for the SW-induced effects are incompletely understood and are being elucidated. It has been demonstrated that the biologic effects of SWs, amplitudes of acoustic pulse waves, are mediated by mechanical forces such as cavitation (a micrometer-sized violent collapse of bubbles) and shear stress.[24,25] These mechanical forces are known to increase the permeability of the cell membrane and lead to the induction of gene expression.[26-29] We therefore believe that these effects of SW may, at least in part, be responsible for the increased expression of VEGF-C and VEGFR3 and the subsequent growth of lymphatic vessels. Further studies are needed to elucidate the precise mechanisms for SW-induced lymphangiogenesis and amelioration of lymphedema.

It is crucial to develop a curative treatment for lymphedema because therapeutic options are currently limited to supportive treatment such as manual lymph drainage and compression bandaging. Extracorporeal SW therapy has been clinically used as an effective and safe treatment for lithotripsy to disintegrate kidney and ureteral stones for >25 years.[30,31] Extracorporeal SW therapy has subsequently been used in orthopedics and traumatology to heal tendons, surrounding tissue, and bones.[32,33] More recently, it has been reported that extracorporeal SW therapy ameliorates myocardial ischemia in patients with severe coronary artery disease.[18]

Our study, using a rabbit ear model of secondary lymphedema, showed that extracorporeal SW therapy ameliorated lymphedema by inducing therapeutic lymphangiogenesis. Considering that the advantages of SW therapy for clinical application include an avoidance of invasive surgical procedures and anesthesia, no procedural complications or adverse effects, and, if necessary, repeatable treatment for

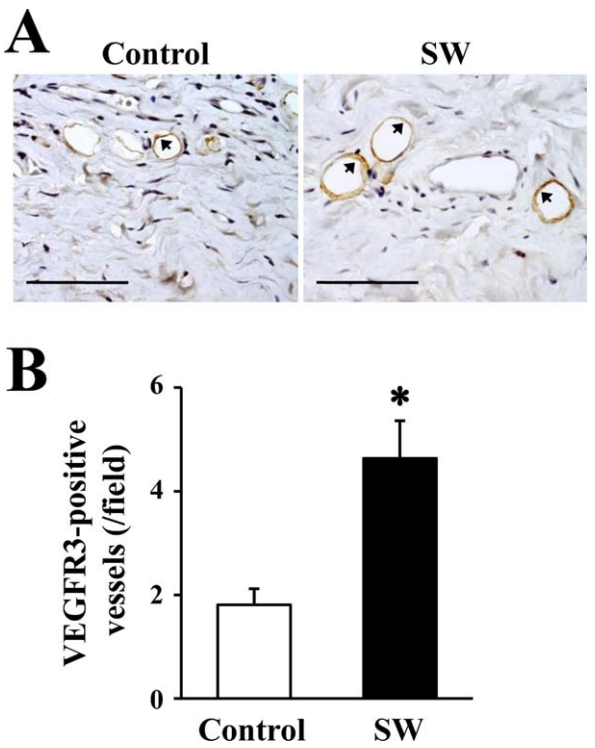


**Fig 4.** Histologic analysis of the lymphatic vessels in the lymphedematous ear 4 weeks after shock wave *(SW)* treatment. **A,** Representative images of immunostaining for vascular endothelial growth factor receptor 3 *(VEGFR3; arrows*). Bars indicate 50 μm (original magnification ×400). **B,** Quantification analysis revealed significantly increased density of the lymphatic vessels in the SW group compared with the control group. $*P < .05$ vs. control group (n = 3/group). Mean data are presented with the standard error of the mean.

patients (even outpatients), we propose that extracorporeal SW therapy could be a beneficial strategy for treatment of lymphedema.

Some limitations should be noted for the present study. First, the conditions for the SW therapy in this study were based on reports about SW-induced angiogenesis for myocardial ischemia.[17,18] The best regimen (dose, duration, frequency, etc) of SW therapy for lymphedema may differ from the purpose of inducing angiogenesis for the treatment of myocardial ischemia.

Second, although the expressions of VEGF-C and VEGFR3 were up- regulated by SW therapy, further studies with the neutralizing antibody of VEGF-C and/or VEGFR3 are required to confirm the precise mechanism for the SW-induced amelioration of lymphedema. Otherwise, the surgical ligation of lymphatic stumps will be complicated with inflammatory edema that it is known to decline spontaneously with time. Given the difference from the clinical entity of typical patients who present with secondary lymphedema months to years after surgery and radiotherapy, there may be limitations of the animal model to some extent. However, the rabbit ear model is well established and generally used for studies on secondary lymphedema, and we started SW therapy 2 weeks after surgery.

## CONCLUSIONS

The present study demonstrates for the first time, to our knowledge, that SW therapy is a novel and promising noninvasive strategy for the treatment of lymphedema. Further clinical trials are required to confirm the efficiency of this therapy.

## AUTHOR CONTRIBUTIONS

Conception and design: MK, TL, BS, KH
Analysis and interpretation: MK, TL, KH
Data collection: MK, TL, TK, MO
Writing the articles: MK, TL, KH
Critical revision of the article: MK, TL, KH
Final approval of the article: MK, TL, TK, MO, BS, KH
Statistical analysis: MK, TL
Obtained funding: MK, BS, KH
Overall responsibility: KH

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Submitted Dec 7, 2009; accepted Mar 7, 2010.