

**Regulation of CXCR4-Mediated Invasion by DARPP-32 in Gastric Cancer Cells**

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**Abstract**

Although Dopamine and cAMP-regulated phosphoprotein, Mr 32000 (DARPP-32) is overexpressed in two-thirds of gastric cancers, its impact on molecular functions has not been fully characterized. In this study, we examined the role of DARPP-32 in gastric cancer cell invasion. Using matrigel-coated Boyden chamber invasion assay, DARPP-32-overexpressing AGS cells showed a three-fold increase in invasion relative to the vector control ($P < 0.01$). We also tested the transendothelial cell invasion as a measure of cell aggressiveness using the impedance-based human umbilical vein endothelial cells invasion assay and obtained similar results ($P < 0.001$). Western blot analysis indicated that overexpression of DARPP-32 mediated an increase in the membrane-type 1 matrix metalloproteinase (MT1-MMP) and CXCR4 protein levels. Consistent with the role of MT1-MMP in cleaving extracellular matrix proteins initiating the activation of soluble MMPs, we detected a robust increase in MMP-2 activity in DARPP-32-overexpressing cells. The knockdown of endogenous DARPP-32 in the MKN-45 cells reversed these signaling events and decreased cell invasive activity. We tested whether the invasive activity mediated by DARPP-32 might involve sustained signaling via CXCR4-dependent activation of the MT1-MMP/MMP-2 pathway. The small-molecule CXCR4 antagonist (AMD3100) and CXCR4-siRNA blocked DARPP-32-induced cell invasion. We further examined our hypothesis that DARPP-32 could interact with CXCR4 and stabilize its levels following stimulation with its ligand, CXCL12. Using reciprocal coimmunoprecipitation and immunofluorescence experiments, we found that DARPP-32 and CXCR4 coexist in the same protein complex. DARPP-32 prolonged the CXCR4 protein half-life and reduced ubiquitination of the CXCR4 protein, following treatment with its ligand, CXCL12. In conclusion, these findings show a novel mechanism by which DARPP-32 promotes cell invasion by regulating CXCR4-mediated activation of the MT1-MMP/MMP-2 pathway.

**Introduction**

Gastric cancer is one of the most common cancers worldwide (1). The prognosis for gastric cancer patients remains poor, especially in more advanced stages (2). Dopamine and cAMP-regulated phosphoprotein, Mr 32000 (DARPP-32), is abundantly expressed in striatal neurons and in striatoni-gral fibers (3). We have previously shown that DARPP-32 is overexpressed in approximately two-thirds of gastric cancers (4, 5). The expression of DARPP-32 occurs in the early stages of the gastric cancer progression cascade (4). Overexpression of DARPP-32 is associated with potent anti-apoptotic advantage and drug resistance in gastric cancer cells by promoting interaction between epidermal growth factor receptor and ERBB3 and activating phosphoinositide 3-kinase-AKT signaling (6, 7).

The invasion capacity of cancer cells determines their penetration power into surrounding tissues, a crucial early step in the metastatic cascade (8, 9). Metastasis, the major cause of morbidity and mortality in most cancers, is a complex pathophysiological process (10). Recently, it was suggested that chemokine stromal cell-derived factor-1α (SDF-1α, also known as CXC-chemokine ligand 12, CXCL-12) and its receptor, CXC-chemokine receptor 4 (CXCR4), are involved in gastric cancer invasion and metastasis (11–13). CXCR4 expression is associated with gastric cancer cell migration in vitro, and strong expression of CXCR4 by gastric cancer cells is significantly associated with lymphatic metastasis in patients with gastric cancer, suggesting that CXCR4 plays an important role during gastric cancer progression (14). Members of the matrix metalloproteinase (MMP) family are multifunctional zinc-dependent endopeptidases that can degrade a variety of ECM components. Almost all MMPs are secreted into the extracellular milieu, except 6 membrane-type (MT; MT1-MMP to MT6-MMP), also called MT1-MMP, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25, respectively; refs. 15, 16). MMPs share a conserved structure that consists of a signal peptide, a propeptide, and a catalytic domain.
Overexpression of MT1-MMP has been observed in gastric cancers, which is associated with the invasiveness of the cancer cells (17). It has been reported that the expression of MT1-MMP may influence prognosis via tumor invasion of the gastric wall and lymph node metastasis, and activation of MMP-2 correlates with local invasion and lymphatic permeation in gastric cancers (18).

Although several molecules have been implicated in cancer metastasis, the detailed mechanism of gastric cancer invasion is not completely understood. In the present study, we have uncovered a novel mechanism by which DARPP-32 regulates CXCR4 and gastric cancer cell invasion. We have shown that DARPP-32 interacts with CXCR4 promoting its stability and enhancing the MT1-MMP–regulated activation of MMP-2.

**Materials and Methods**

**Cell culture and reagents**

Human gastric cancer cell lines (AGS and MKN-45) and the immortalized human embryonic kidney epithelial cell line (HEK-293) were maintained in culture using either F12 medium or Dulbecco’s Modified Eagle’s Medium (DMEM, GIBCO). CXCL-12 and AMD3100 were purchased from BioVision and Tocris Bioscience, respectively. Anti-mouse and anti-rabbit DARPP-32 antibodies were obtained from Santa Cruz Biotechnology and Abcam, respectively. Anti-MT1-MMP and anti-CXCR4 antibodies were purchased from Abgent and Sigma-Aldrich, respectively. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit secondary antibodies; anti-MMP-2 and anti-β-actin antibodies were obtained from Cell Signaling Technology.

**DARPP-32 expression and shRNA vectors**

The flag-tagged coding sequence of DARPP-32 was cloned in pCDNA3.1 mammalian expression construct (Invitrogen). AGS cells were transfected with the expression construct and stable cell lines expressing DARPP-32 were generated (DP01 and DP02; ref. 5). Lentivirus particles expressing DARPP-32, short hairpin RNA (shRNA)-DARPP-32, or control shRNA were produced by GeneCopoeia.

**Invasion assay and HUVEC invasion assay**

The effect of DARPP-32 on the invasion ability of AGS cells or MKN-45 cells was determined using matrigel invasion chambers (BD Biosciences). AGS cells stably expressing DARPP-32 were seeded into inserts at 1 × 10⁴ cells per insert in serum-free medium and then transferred to wells filled with the culture medium containing 10% FBS. After 24 hours of incubation, non-invading cells on the top of the membrane were removed by cotton swabs. Invaded cells on the bottom of the membrane were fixed with 4% paraformaldehyde for 10 minutes, followed by staining with 0.05% crystal violet for 3 hours. Photographs were taken and all the cells on the entire membrane were counted. The relative invasion activity was calculated after normalization to cell migration.

We also used the transendothelial cell invasion assay as a measure for cell aggressiveness. The interaction of tumor cells with endothelial cells is a key event in tumor metastasis (19). The various components of tumor cell-endothelial cell interaction can be replicated in vitro by challenging a monolayer of human umbilical vein endothelial cells (HUVEC) with cancer cells. We modified an established in vitro invasion assay system in which the invasion of tumor cells after...

![Figure 1](https://example.com/figure1.png)
interaction with endothelial cells can be examined (20). The xCELLigence system (Roche Diagnostics) was used to monitor the change in the cell index. It measures the effect of any perturbations in a label-free real-time setting and measures as change in electrical impedance (change in resistance at cell-electrode interphase), which is recorded as cell index, and data point is collected every 5 minutes. The relative rate of invasion in transendothelial tumor cell invasion can be defined as: A reduction in the cell index of the HUVEC cells monolayer after the addition of the invasive cell line as a function of time, compared with the drop in the cell index with the addition of a non-invasive cell line, normalized with the cell index of HUVEC cell monolayer at a given time, as measured by the xCELLigence system. For transendothelial invasion assays, Roche E-plates (Cat# 05469813001) were treated with 100 µL of 0.1% sterile gelatin (Sigma) overnight at 4°C. Plates were washed once with sterile PBS before the addition of early passage HUVEC cells, obtained from Lonza Biosciences. HUVEC cells were grown in EBM-2 basal media (Cat# CC3156, Lonza Biosciences) supplemented with endothelial growth medium 2 (EGM-2) growth factors (Cat# CC4176, Lonza Biosciences). The E-plates were seeded with 25,000 HUVEC cells/100 µL and incubated for 18 hours at 37°C. The cell index was monitored on the xCELLigence system while the monolayer was formed. Following the formation of the HUVEC monolayer, which is indicated by the plateau in the cell index, the EGM-2 media was removed and 100 µL of RPMI + 5% serum containing media was added. The cell index was monitored for 4 hours and allowed to stabilize. After the cell index stabilized, the invading cells were added to each well at a density of 5,000 cells/100 µL. The cell index was normalized to the HUVEC monolayer and invasion was monitored over time. The experiments were conducted in 6 wells per cell line. Rate of invasion of the cell lines were calculated according to the RTCA software version 1.2, within particular time intervals.

Gelatin zymography

Gelatin zymography was conducted in 12% SDS-PAGE that had been cast in the presence of 0.1% gelatin. Samples were prepared in nonreducing loading buffer. After electrophoresis, SDS was removed by 2.5% Trion X-100 to renature gelatinases. Gels were then incubated at 37°C for 72 hours in an incubation buffer [50 mmol/L Tris-HCl (pH 8.0), 50 mmol/L NaCl, 10 mmol/L CaCl2]. Following incubation, the gels were stained with 0.05% Coomassie Brilliant Blue R-250 and destained with 10% acetic acid.

**Figure 2.** DARPP-32 enhances gastric cancer cells invasion. A and B, Five thousand cells each of AGS or MKN-45 were used to challenge the HUVEC monolayer. The cell index was measured every 5 minutes by the xCELLigence system. The rate of change of cell index as a function of time was calculated as a measure of invasive activity. MKN45 cells have a higher change in cell index than AGS cells. C and D, the same as in A and B, using AGS cells stably expressing DARPP-32 or pcDNA shows a significant increase in the rate of change of cell index in DARPP-32 expressing AGS cells (DP02) as compared with control (pcDNA; P = 0.0002). E and F, the same as in A and B, following knockdown of DARPP-32 (DP shRNA) or control (SC shRNA) in MKN45 cells, shows a significant decrease in the rate of change of cell index (P = 0.0004).
7.5), 200 mmol/L NaCl, 5 mmol/L CaCl₂, and 0.7 mg ZnCl₂, and were then stained with 0.125% Coomassie Brilliant Blue R-250. TPA (12-O-Tetradecanoylphorbol 13-acetate) was used as positive control.

Immunochemistry
Following cell fixation and permeabilization, immunochemistry was conducted with both anti-DARPP-32 (1:800) and anti-CXCR4 (1:200) antibodies. The cells were then washed with cold PBS 3 times for 3 minutes each, and incubated with both Alexa-Fluor 488 goat anti-rabbit secondary antibody (green, 1:400) and Alexa-Fluor 568 goat anti-mouse secondary antibody (red, 1:800; Invitrogen) at room temperature for 1 hour. The cells were examined by fluorescence microscopy (Olympus America Inc.).

Immunoprecipitation and Western blotting
Cells were lysed with lysis buffer containing 0.5% Triton X-100, 150 mmol/L NaCl, 5 mmol/L EDTA, and 50 mmol/L Tris supplemented with protease and phosphatase inhibitors. Immunoprecipitations of equivalent total protein amounts were conducted at room temperature for 1 hour by using a primary antibody previously bound to 50 μL of Protein A Dynabeads per sample. The beads were washed 4 times with wash buffer. The beads in each tube were heated to 100°C for 5 minutes in 20 μL of sample buffer, and then clarified by magnet. Proteins were separated on 12.5% SDS-PAGE and transferred to Immobilon polyvinylidene difluoride membrane (Millipore). Membranes were probed with specific antibodies. Proteins were then visualized by using HRP-conjugated secondary antibodies and Immobilon Western Chemiluminescent HRP Substrate detection reagent (Millipore). β-actin was used as loading control.

Quantitative Real-time RT-PCR analysis
Twenty pairs of de-identified gastric adenocarcinomas and their adjacent histological normal specimens were obtained from the NCI Human Cooperative Human Tissue Network in accordance with approved protocols. Total RNA was isolated from cell lines and human tissue samples by using the RNeasy Mini Kit (Qiagen). Total RNA (1 μg) was reverse transcribed by an iScript cDNA synthesis kit (Bio-Rad). The quantitative real-time RT-PCR analysis (qRT-PCR) was conducted using an iCycler and iCycler software version 3.0 (Bio-Rad). The primers for CXCR4 were forward: 5′-AATCTTCCTGCCCACCATCT-3′; reverse: 5′-GACGCCAAATACAGACCACCT-3′; the primers for CXCL-12 were forward: 5′-GGTGGTGCGTTGGTCCTC-3′; reverse: 5′-AGATGCTTGACGTTGGCTCT-3′; and primers for HPRT1 were forward: RT-F: 5′-TTGGAAAGGTTTTATTCCCTA-3′; and reverse: RT-R: 5′-CAGCAGGTCAGCAAGA-3′. The results of 3
independent experiments were subjected to statistical analysis. Fold change was calculated using the ΔΔC(t) method [21]. HPRT1 was used as normalization control.

Ubiquitination assays for CXCR4

HEK293 cells were transiently transfected with HA-CXCR4, His-ubiquitin and pcDNA or DARPP-32. The next day, cells were transferred onto 6 cm dishes and allowed to grow for an additional 24 hours. The cells were then washed by aspirating medium and then replacing it with 4 mL of warm DMEM supplemented with 20 mmol/L HEPES. We then incubated the cells in the same medium in the presence of 30 nmol/L CXCL-12 for 30 minutes, rapidly washed cells on ice with cold PBS, and collected samples in 1 mL of lysis buffer. Samples were transferred into microcentrifuge tubes and placed at 4°C for 20 minutes, followed by sonication and centrifugation to pellet cellular debris. Cell lysates were incubated with an anti-HA polyclonal antibody and the immunoprecipitates were analyzed by SDS-PAGE followed by Western blotting using an anti-His antibody conjugated to HRP.

Statistical analyses

Data were expressed as mean ± SD of 3 independent experiments. Statistical significance of the in vitro studies was analyzed by a Student’s t test. Differences with P values 0.05 or less are considered significant.

Results

DARPP-32 enhances gastric cancer cells invasive activity

We detected a 5-fold increase in the invasive activity in DARPP-32-expressing cells, as compared with control AGS cells (Fig. 1A and B). The knockdown of endogenous DARPP-32 using DARPP-32-specific shRNAs in MKN-45 cells (DARPP-32-shRNA01 and DARPP-32-shRNA02) inhibited the invasive activity (Fig. 1C and D). We also conducted invasion assay using CXCL-12/SDF-1 (100 ng/mL) with low serum (1% FBS medium), a minimum amount to maintain cell survival. The results showed that DARPP-32 enhances CXCL-12-mediated cells invasion as compared with ctrl cells (Supplementary Fig. S1).

To address the question of whether DARPP-32 is able to mediate invasion, we used an impedance based endothelial cell invasion assay. This technique involves challenging a confluent monolayer of HUVEC with a second layer of metastatic cells that attach to and invade the HUVEC monolayer. First, we measured the invasive activity of wild-type AGS and MKN-45 cells; MKN-45 cells have a higher expression level of DARPP-32 protein as well as higher invasive activity compared with AGS cells (Fig. 2A and B). The AGS cells stably overexpressing DARPP-32 resulted in significantly increased invasive activity (Fig. 2C and D, P < 0.001). In contrast, the knockdown of endogenous DARPP-32 in MKN-45 cells decreased cell invasion (Fig. 2E and F, P < 0.05). Taken together, these results have established an important, previously unknown, role for DARPP-32 in enhancing the invasive activity in gastric cancer cells.

DARPP-32 induces CXCR4 and MT1-MMP protein expression and enhances MMP-2 activity

The expression and activity levels of MMPs are known to play an important role in cell invasion. To detect possible effects of DARPP-32 on the expression of MMPs, we used a RayBio Human MMP Antibody Array, which comprises spotted antibodies specific for MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, MMP-13, TIMP-1, TIMP-2, and TIMP-4. We did not detect any significant changes in the MMPs protein levels (Supplementary Fig. S2). Therefore, we thought to examine the activity of MMP-9 and MMP-2, important mediators of cell invasion, using a zymography assay. Although we did not observe changes in the MMP-9
activity (data not shown), we detected a robust increase in MMP-2 activity in DARPP-32 overexpressing cells (Fig. 3A) that was reversed upon the knockdown of endogenous DARPP-32 in MKN-45 cells (Fig. 3B). Our next step was to examine the expression of CXCR4, a major protein that mediates regulation of MMPs and cell invasion in cancer. Using qRT-PCR, we could not detect significant changes in CXCR4 mRNA levels following overexpression of knockdown of DARPP-32 (Fig. 3C, D and E). On the other hand, we detected changes in the protein level of CXCR4 and MT1-MMP in these cells, whereas MMP2 levels were unchanged (similar to our array results; Fig. 3F and G). Conversely, knockdown of endogenous DARPP-32 resulted in decreased CXCR4 and MT1-MMP protein levels in MKN-45 cells (Fig. 3H). The MT1-MMP protein plays a major role in cleavage of extracellular matrix proteins initiating the activation of soluble MMPs (22). Therefore, our findings explain the noted changes in activity of MMP2. Taken together, these results suggest that the increased invasive activity of DARPP-32-expressing cells could be attributed to DARPP-32-mediated upregulation of CXCR4 and MT1-MMP with a subsequent increase in the activity of MMP-2.

DARPP-32 interacts with CXCR4 and blocks CXCR4 degradation

Because our data suggested that the DARPP-32-mediated upregulation of CXCR4 was not transcriptional, we tested the hypothesis that the DARPP-32 protein may bind to
CXCR4, thereby affecting CXCR4 protein stability. Indeed, we found that CXCR4 co-immunoprecipitated with the exogenous DARPP-32 protein in HEK-293 cells (Fig. 4A) and confirmed that endogenous DARPP-32 and CXCR4 proteins are also present in the same complex in MKN45 cells (Fig. 4B). In line with these results, the immunofluorescence assay showed the colocalization of DARPP-32 and CXCR4 on the cell membrane (Fig. 4C). We also measured the changes in the CXCR4 protein half-life, following treatment with the protein synthesis inhibitor cyclohexamide (CHX) and found that DARPP-32 prolonged the CXCR4 protein half-life, as compared with control (Fig. 5A). Next, we examined whether the stabilization of DARPP-32-induced CXCR4 was mediated through the ubiquitin-proteasome pathway. IP-Western blot analysis showed reduced ubiquitination of CXCR4 protein after overexpression of DARPP-32 in HEK-293 cells (Fig. 5B). To analyze the effects of DARPP-32 on CXCL12-induced CXCR4 degradation, DARPP-32-expressing AGS cell were treated with CXCL12 (30 nmol/L) for 1 hour. As shown in Fig. 5C and D, CXCR4 remained mainly localized to the plasma membrane after vehicle or CXCL12 treatment of DARPP-32-expressing AGS cells. On the other hand, CXCR4 was mainly localized to punctuate vesicles distributed throughout the cytoplasm in AGS control cells (Fig. 5C and D). These data indicated that DARPP-32 significantly delayed the CXCL12-induced degradation of CXCR4 protein.

Blocking CXCR4 attenuates DARPP-32-mediated invasive properties in gastric cancer cells

To determine if CXCR4 is indeed the major determinant of the DARPP-32-mediated increased invasion potential, we used the small-molecule CXCR4 antagonist (AMD3100) and CXCR4 siRNA approaches. AMD3100 is a symmetric bicyclam, prototype nonpeptide antagonist of the CXCR4 chemokine receptor. Using the migration and invasion assay as described above, an overnight treatment with AMD3100 (0.2 μg/mL) was sufficient to significantly diminish cell invasion in DARPP-32-expressing cells (Fig. 6A and B). We next investigated if the changes in MT1-MMP expression in DARPP-32 expressing cells are also mediated by CXCR4. Indeed, the inhibition of CXCR4 using an overnight treatment with AMD3100, led to a remarkable reduction in MT1-MMP (Fig. 6C). Similar results were obtained following the use of CXCR4 siRNA (Fig. 6D). Taken together, the results indicate that CXCR4 is a major player of DARPP-32-mediated invasion in gastric cancer cells.

CXCL-12 and CXCR4 mRNA levels positively correlate with DARPP-32 expression

We detected high levels of DARPP-32, CXCR4, and CXCL-12 in tumors, as compared with adjacent normal tissues (P<0.01; Supplementary Fig. S3). Statistical analysis using the Pearson’s method confirmed the positive correlation between CXCR4 and DARPP-32 or CXCL-12 and...
DARPP-32 in these tissue samples, with a correlation coefficient of 0.636 or 0.397, respectively (Fig. 7A and B). These results suggest that the expression of these components of cell invasion is intact in gastric tumors.

Discussion

Although overexpression of DARPP-32 has been identified in several malignancies such as gastric, esophageal, colon, and breast cancers (4, 23, 24), the full spectrum of its biologic functions in cancer remains uncharacterized. The ability of cancer cells to invade the nearby tissues and blood vessels is one of the crucial steps that determine the metastatic potential of a tumor (8, 9, 25, 26). In this study, we show a novel molecular mechanism by which DARPP-32 regulates CXCR4 and mediates cancer cell invasion.

The human chemokine superfamily includes at least 46 ligands, which bind to 18 functionally signaling G-protein-coupled receptors and 2 decoy or scavenger receptors (27). The chemokine CXCL-12/SDF-1 binds to the receptors CXCR4 and CXCR7 (28, 29). The chemokine receptor CXCR4 and its ligand CXCL-12/SDF-1 are known to play an important role in the development of invasion and metastases in several malignancies including gastric cancer (30, 31). The current study examined a hypothesis that cancer cell invasion mediated by DARPP-32 in gastric cancer cells might involve a CXCR4-dependent pathway. We have shown that DARPP-32 promotes invasion in gastric cancer cells by increasing CXCR4 protein levels. CXCR4 activated by CXCL-12 is rapidly internalized and targeted into the degradative pathway by an ubiquitin-dependent mechanism (32). Activation by CXCL-12 induces phosphorylation of CXCR4, then promotes binding to the E3 ubiquitin ligase, AIP4 (33). This is followed by internalization of CXCR4 onto early endosomes where the ubiquitin moiety serves as a sorting signal to direct the receptor to lysosomes for proteolysis (32). Because of our finding that exogenous and endogenous DARPP-32 interacted with CXCR4, we examined the protein stability levels of CXCR4. The results uncovered a novel mechanism whereby DARPP-32-CXCR4 interaction prolongs the CXCR4 protein half-life and blocks the CXCL-12-induced CXCR4 protein ubiquitination and degradation. Taken together, our findings suggest that DARPP-32 and CXCR4 binding could block CXCR4 internalization, thereby preventing its ubiquitination.

We did not identify a significant role for DARPP-32 in regulating the protein levels of several MMPs that included MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, MMP-13, TIMP-1, TIMP-2, and TIMP-4. However, we observed an increase in the activity of MMP2 and showed an increase in the MT1-MMP protein level in DARPP-32 expressing cells. High expression levels of MT1-MMP were reported in gastric cancer metastasis (18). Various cell types use MT1-MMP to alter their surrounding environment where MT1-MMP–mediated activation of MMP2 plays a key role during tumor invasion and metastasis (34, 35). It was also previously reported that CXCR4 and the MT1-MMP are mutually required during melanoma metastasis to lungs (36) and CXCL-12/SDF-1 is a key signaling event for MT1-MMP/MMP-2–dependent melanoma and breast cancer cell invasion (11, 35). These observations support our findings that DARPP-32 upregulated MT1-MMP expression and promoted MMP-2 activity. Using small molecule inhibitors and siRNA knockdown approaches, we have confirmed the requirement of CXCR4 in promoting DARPP-32-dependent signaling events and cell invasion. Therefore, the sustained signaling via CXCR4-dependent activation of the MT1-MMP/MMP-2 pathway is a critical step in the DARPP-32-mediated cancer cell invasion. We also detected a positive correlation between high expression levels of DARPP-32, CXCR4, and CXCL-12 in primary gastric cancers, supporting the integrity of this axis in tumors.

In conclusion, our findings suggest that DARPP-32 plays an important role in invasion of gastric cancer cells. The ability of DARPP-32 to bind to CXCR4 and activate MT1-MMP/MMP-2 signaling provides a novel mechanism in mediating CXCR4 overexpression and metastasis in cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: A. Belkhiri, V. Sehdev, W. El-Rifai
Development of methodology: J. Hong, W. El-Rifai

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