

Down-regulation of CD44 inhibits Wnt/ β -catenin mediated cancer cell migration and invasion in gastric cancer

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SUMMARY

Recent studies tried to find the genes that are associated with gastric cancer patient's survival. Many studies focused on CD44, which is associated with gastric cancer tumorigenesis and metastasis. However, the mechanisms by which molecules downstream of CD44 contribute to gastric cancer cell migration and invasion remain poorly studied. The lack of information on CD44 downstream mechanism limits the development of effective therapies for patients with gastric cancer. In this study, we aimed to characterize CD44-mediated regulation of the Wnt/ β -catenin signaling pathway, which promotes cancer invasion and metastasis. We hypothesized that CD44 down-regulation will inhibit gastric cancer cell migration and invasion by leading to down-regulation of Wnt/ β -catenin signaling. We found that CD44 up-regulation was significantly related to poor prognosis in gastric cancer patients. We demonstrated the CD44 down-regulation decreased β -catenin protein expression level. Our results suggest that CD44 down-regulation inhibits cell migration and invasion by down-regulating β -catenin expression level. We determined not only that CD44 regulates the expression level of β -catenin, but also discovered a novel CD44/ β -catenin pathway that regulates cell migration and invasion in gastric cancer. Our findings suggest that targeting the CD44/ β -catenin pathway may be an effective therapeutic strategy for gastric cancer patients.

INTRODUCTION

Gastric cancer, sixth most common type of cancer, causes second most human cancer death (1). Lymph node and liver metastases are main reason to decrease the survival rates of gastric cancer patients (2). As surgery is the only therapeutic treatment approach and common chemotherapy is limited by low efficacy and side effects, it is critical to develop novel molecular therapies to overcome these limitations (3). Understanding the molecular basis of metastasis in gastric cancer will be crucial to develop effective molecular therapies to improve the survival rate of gastric cancer patients.

A potential target for molecular therapies is CD44, which is involved in cancer proliferation and metastasis in gastric cancer (4, 5). CD44 is a common marker of cancer initiating

cells (CIC), a minor population of cells within the tumor that is necessary for tumor maintenance and progression (6). CD44 has also been identified as a major hyaluronic acid (HA) receptor that participates in uptake and intracellular degradation of HA. HA is known to cause high cellular motility and promote cancer aggressiveness (7, 8). Notably, CD44 is one of the most consistently reported markers related to gastric cancer invasion, lymph node metastasis, and poor outcomes (9, 10).

Another key signaling pathway in gastric cancer is Wnt/ β -catenin. This pathway is involved in cell adhesion and cancer development (11). When Wnt binds to receptor of cancer cell, it activates the Wnt/ β -catenin and β -catenin-independent pathways (12). Therefore, activation of the Wnt pathway depends on the expression of the Wnt ligand and the expression of receptors on target cells (12). β -catenin is a protein present in many types of tissue and cells, mainly found at adherent junctions that connect neighboring cells (11). β -catenin contributes to cell adhesion and communication between cells and plays an important role in the Wnt signaling pathway (13). However, over-activation of Wnt/ β -catenin signaling pathway is significantly connected to the development and progression of gastric cancer resulting in increased inflammatory cytokine production, abnormal apoptosis, and uncontrolled epithelial cell proliferation (14). In this study, we analyzed the effects of CD44 down-regulation on both Wnt/ β -catenin signaling and gastric cancer cell migration and invasion. Previous study indicates that CD44 is a positive regulator that activates Wnt/ β -catenin signaling (15). In addition, N-myc downstream-regulated gene 1 (NDRG1), an upstream regulator of CD44, inhibits proliferation of colorectal cancer and characteristic of stem cells by preventing β -catenin nuclear translocation (16). Thus, CD44 down-regulation may inhibit Wnt/ β -catenin signaling, resulting in prevention of gastric cancer cell migration and invasion.

Metastasis is a complex process that includes migration and invasion of cancer cells (17). The cancer cells often attempt to escape the primary tumor site (17). Through in vitro cell migration and invasion assays, we analyzed the live cell kinetics. We performed both cell migration assay and cell invasion assay to analyze the metastatic potential of the cancer cells (18).

Considering the close relationship between CD44 and β -catenin, we hypothesized that CD44 down-regulation will

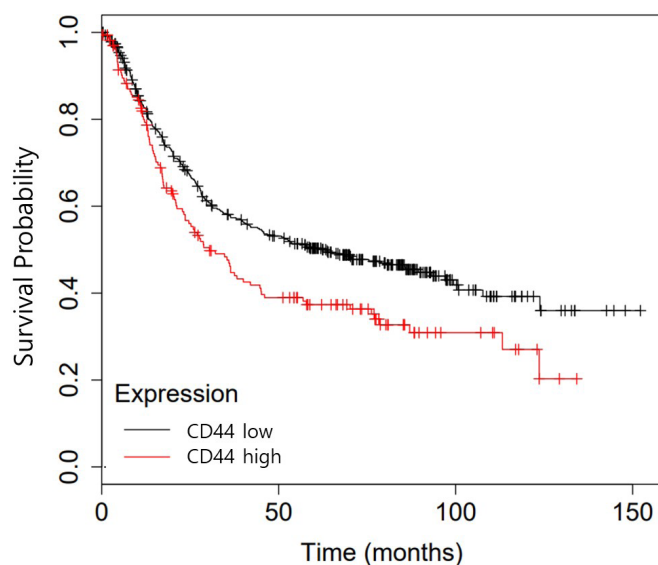


Figure 1: Kaplan-Meier survival plot of patients with gastric cancers, assessing overall survival probability in regard to CD44 expression. The publicly available datasets of three major cancer research centers (Berlin, Bethesda and Melbourne datasets) were used. The patient samples are split into two groups according to various quantile expressions of CD44: CD44 high (n = 463), CD44 low (n = 168). The overall survival curve of gastric cancer patients between two groups were analyzed by Kaplan–Meier survival plot (p=0.0034).

inhibit gastric cancer cell migration and invasion by leading to down-regulation of Wnt/ β -catenin signaling. We examined the effect of siRNA-dependent CD44 down-regulation on cell migration and invasion in MKN45 gastric cancer cells, which expresses a high level of CD44. Since CD44 down-regulation decreased β -catenin expression level and inhibited cell migration and invasion, we examined whether the down-regulation of migration and invasion phenotype can be reversed by overexpressing β -catenin expression level.

RESULTS

Higher CD44 expression is significantly associated with decreased overall survival of gastric patients

CD44 is associated with tumor proliferation and metastasis in gastric cancer (19). To determine if the expression levels of CD44 can be predictive for gastric cancer patient survival, 632 patient samples were analyzed using the public patient database (Berlin, Bethesda, and Melbourne datasets) to validate the gastric cancer survival biomarker candidates (20). The patient samples were divided into two groups according to the expressions of CD44. After we performed the patient survival analysis using Kaplan-Meier Plotter (<http://kmpplot.com/>), we compared two patient cohorts (low vs. high expression of CD44) according to the data provided by CD44 Affymetrix probe ID 1557905_s_at (20). As expected, patients with high expression of CD44 showed a statistically significant decrease in overall survival ($p = 0.0034$) (Figure 1). Overall, higher CD44 expression

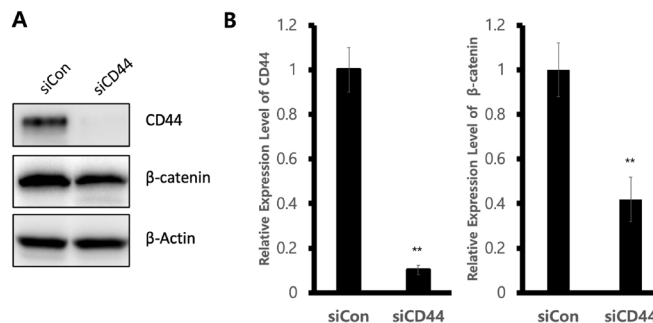


Figure 2: Western blot assay for detecting CD44 and β -catenin expression level on siRNA transfected (siCon and siCD44) MKN45 cells. (A) Western blots of CD44, β -catenin, and β -actin in MKN45 cells transfected with siCD44 or siCon (negative control). The blot of β -actin was used to normalize the expression of target proteins. (B) Quantitative, densitometric analysis of CD44 and β -catenin. Densitometric values were quantified using image analysis by Image Studio Lite of target protein bands to β -actin levels for normalization. Then, the relative expression level of CD44 and β -catenin was calculated based on siCon sample. Two technical replicates were measured (n = 2). Mean \pm S.D. was plotted with statistical significance (Student's t-test): **, $p \leq 0.01$.

is significantly associated with low survival rate of gastric patients.

CD44 down-regulation inhibits migration and invasion of MKN45 cells

According to patient survival analysis, we found that the level of CD44 expression may be associated with aggressive tumor cell behaviors (21). Therefore, to determine the effect of CD44 down-regulation on gastric cancer cell migration and invasion, CD44 siRNA (siCD44) was created and transfected into MKN45 cells, which show the highest level of CD44 mRNA among 20 gastric cancer cell lines provided in CellExpress microarray database (22). After transfection, CD44 protein expression level in MKN45 significantly decreased compared with control ($p = 0.008$) (Figure 2).

β -catenin is the central molecule in the Wnt signaling pathway, which is de-regulated in various malignancies (11). Since many studies indicated that CD44 is involved in Wnt signaling, we examined β -catenin protein expression in this study. β -catenin protein expression level was decreased to 40% relative to the control, siCon (Figure 2). Previously, CD44 was shown to regulate cell migration and invasion in various cancer cells, we also investigated the effect of CD44 down-regulation on cell migration and invasion (4). The siRNA-transfected MKN45 cells stained with green fluorescence CMFDA dye were placed on the top of the FluoroBlok insert, which is designed with a membrane that efficiently blocks the transmission of light between 400 and 700 nm (23). This insert allows to block fluorescently-stained cells present in the top chamber of the insert, which are shielded from bottom-reading fluorescence plate readers and microscopes by FluoroBlok membrane (23). Therefore, after fluorescently-labeled cells migrate through the membrane, they are easily

detected by an inverted fluorescence microscope (23). When migratory cells were analyzed from siCon- and siCD44-transfected samples, we found that CD44 down-regulation significantly decreased the migration of MKN45 cells ($p = 0.007$) (Figure 3). We also performed the invasion assay using Matrigel. Matrigel was pre-coated on the FluoroBlok insert to analyze both cell chemotaxis and the invasion of cells through extracellular matrix, a common phenomenon in cancer metastasis. When invaded cells were counted and analyzed from siCon- and siCD44-transfected samples, CD44 down-regulation significantly decreased invasion by MKN45 cells ($p = 0.009$) (Figure 3).

Rescue Experiments in MKN45 indicate that CD44-mediated migration and invasion depend on β -catenin

The over activation of the Wnt/ β -catenin signaling pathway is involved in the metastasis of gastric cancer (14). Nuclear β -catenin is correlated with an invasive phenotype, which is mediated by up-regulation of Wnt transcriptional

targets such as erythropoietin-producing hepatocellular and membrane type 3 matrix metalloproteinase genes (24, 25). To further test whether the CD44-mediated cell migration and invasion of gastric cancer are dependent on β -catenin, siRNA for CD44 and a vector overexpressing β -catenin were co-transfected to rescue β -catenin expression level in MKN45 cells (Figure 3). After the siCD44 was transfected for 24 hours (h), empty control vector (pcDNA3) or β -catenin overexpression vector (pcDNA3- β -catenin) was transfected.

To confirm the β -catenin overexpression, we performed western blot to check the β -catenin protein expression level (Figure 4). When siCD44 and pcDNA3 were co-transfected, β -catenin expression level remained similar compared to siCD44 transfected cells. Also, the expression level of β -catenin was increased in siCD44 + pcDNA3- β -catenin transfected cells (Figure 4). siCD44 cells overexpressing β -catenin increased both numbers of migrated and invaded cells relative to siCD44 and pcDNA3 co-transfected cells (Figure 3). Taken together, these results demonstrate that CD44 regulates cell migration and invasion by regulating β -catenin expression in gastric cancer cells.

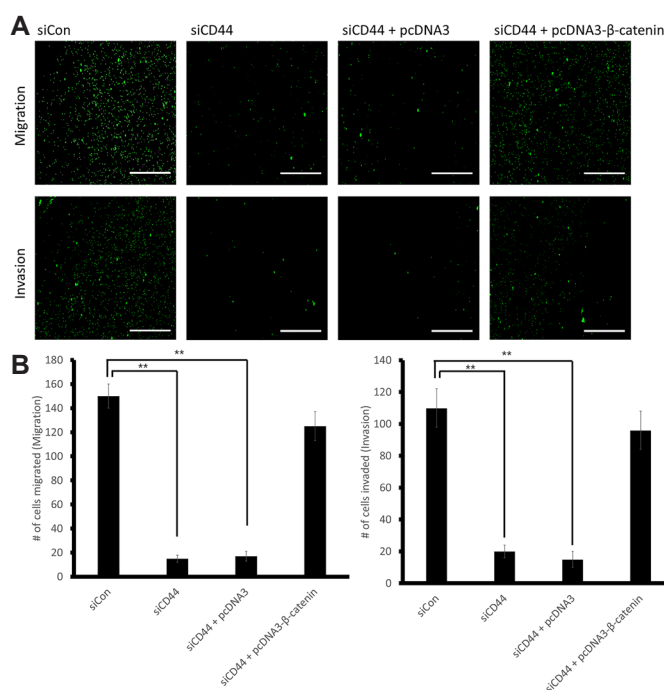


Figure 3. Migration and invasion assay on siRNA transfected (siCon a:d siCD44) and co-transfected (siCD44 + pcDNA3 and siCD44 + pcDNA3- β -catenin) MKN45 cells. (A) Representative images of migrated and invaded cells on the bottom of transwell inserts from siRNA transfected cells (siCon and siCD44), siRNA and empty vector co-transfected cells (siCD44 + pcDNA3), siRNA and β -catenin overexpressing vector co-transfected cells (siCD44 + pcDNA3- β -catenin). After 72 hour transfection, the cells were stained with CFDA to produce green fluorescence. The image was taken with a fluorescence microscope (Nikon). Scale bar: 500 μ m (B) Quantification of migrated or invaded MKN45 cells. The number of green fluorescence cells were counted by Image J. In this test, two technical replicates from the two biological replicates were measured for the same treatment. Mean \pm S.D. was plotted with statistical significance (two-way ANOVA and Tukey test): **, $p \leq 0.01$.

DISCUSSION

Many studies indicate that CD44 directly regulates Wnt/ β -catenin signaling, which is involved in tumor metastasis and progression (26, 27). Down-regulation of CD44 decreases Wnt activity in a concentration-dependent manner (15). On the other hand, when CD44 is overexpressed, Wnt activity is increased (15). Thus, we predicted that CD44 down-regulation in gastric cancer cells would inhibit cancer cell migration and invasion through impairment of Wnt/ β -catenin signaling.

Previous studies supported that CD44 activates post-translational modification of β -catenin. CD44 activates acetylation of β -catenin by HA-mediated CD44 interactions (28). As HA binds to CD44, it promotes p300 acetyltransferase activity by upregulating the expression level of p300 (28). Then, p300 mediates acetylation of both β -catenin and NF κ B-p65 (28). When acetylated β -catenin binds to the transcription factor T-cell factor/lymphocyte enhancer factor (TCF/LEF) in the nucleus, it mediates the transcriptional activation of c-myc, E-cadherin, and cyclin D1 (29). Therefore, HA/CD44-p300 pathway leads to β -catenin and NF κ B signaling and contributes to cell survival and chemoresistance in breast cancer (28). These results may indicate that CD44-dependent down-regulation of acetylated β -catenin decreases cell survival and chemoresistance in gastric cancer. Another study showed that HA-mediated CD44 interacts with the neuronal Wiskott-Aldrich syndrome protein (N-WASP) that leads to increased phosphorylated β -catenin (30). CD44 interaction with N-WASP and ErbB2 activates ovarian tumor progression by β -catenin signaling and actin polymerization (30). Our study showed that CD44 down-regulation in gastric cancer cells decreased the total β -catenin protein expression level. When CD44 is downregulated, CD44 interaction with N-WASP and ErbB2

may also decrease in gastric cancer cells. Therefore, this interaction may down-regulate phosphorylated β -catenin and deactivates actin polymerization to decrease cancer cell migration. Since CD44-dependent inhibition of cell migration and invasion may be mediated by decreased expression of both acetylated and phosphorylated β -catenin, it must be investigated in the future.

A previous study indicated that CD44 is required for Wnt-dependent LRP6 phosphorylation (31). Another study demonstrated that phosphorylation of LRP6 and the subsequent recruitment of axin leads to inhibition of the β -catenin degradation complex (15). These previous findings support that CD44 may increase the stability of β -catenin protein through phosphorylated LRP6. Therefore, further study is required to investigate the role of CD44 and phosphorylated LRP6 on protein stability of β -catenin.

This is the first report to demonstrate CD44/ β -catenin pathway regulates cell migration and invasion in gastric cancer. Our study shows that CD44-dependent β -catenin expression is crucial for cancer cell migration and invasion. We identified not only that β -catenin expression was regulated by CD44 but also revealed that inhibition of CD44 and β -catenin pathway could be a potential therapeutic target to treat gastric cancer patients. In conclusion, the effect of CD44 down-regulation on cell migration and invasion suggests that the inhibition of β -catenin signaling may result in beneficial to patient survival, especially in CD44-positive gastric cancer.

There are many follow-up experiments that could further confirm CD44's role in Wnt/ β -catenin signaling. First, we must analyze CD44 and β -catenin levels and validate our results in two different pathological types of gastric cancer: intestinal type and diffuse type. The intestinal tumors, which exhibit cancer cell adhesion, are arranged in tubular formations with less potential to invade surrounding tissues. By contrast, diffuse tumors are consisted of non-adhesive cells, leading to a population of scattered tumor cells. Therefore, it is important to check the role of CD44 and β -catenin in both types of tumors. Since tumor metastasis is a multi-step process that requires adaptation of cancer cells to various microenvironment conditions, the mouse xenograft model implanted with CD44-downregulated MKN45 cells should also be investigated in the future. Since inhibition of β -catenin signaling can be mediated by multiple treatments such as C2 (chemical β -catenin inhibitor) and siRNA targeting β -catenin, the beneficial method for cancer treatment should be further investigated.

MATERIALS AND METHODS

Overall patient survival analysis

Gene expression data from 632 gastric cancer patients was downloaded from GEO, EGA, and TCGA (32). The database was analyzed by PostgreSQL server, which integrates gene expression and clinical data (32). The prognostic values of specific genes were analyzed by splitting the patient samples into two groups by the quantile expression

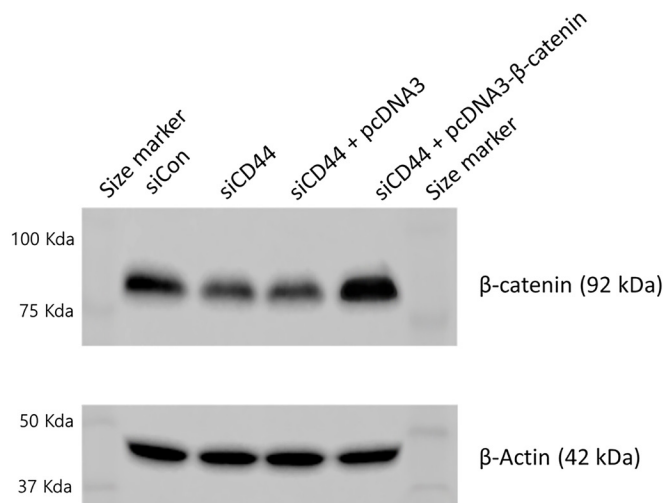


Figure 4: Western blot assay for detecting β -catenin expression level on siRNA transfected (siCon and siCD44) and co-transfected (siCD44 + pcDNA3 and siCD44 + pcDNA3- β -catenin) MKN45 cells. After transferring proteins from SDS-PAGE gel to PVDF membrane, the membrane was cut into small pieces by the size of the targeted proteins. The Bio-Rad protein marker (Precision Plus Protein 1610374) was used to check the size of targeted proteins.

of the CD44 (32). Two gastric patient groups were compared by Kaplan-Meier survival plot with 95% confidence intervals and log rank P-value was calculated (32). The databases and clinical data are updated regularly in <http://kmplot.com>.

Cell culture

The human gastric cancer cell line MKN45 was purchased from the Korean Cell Line Bank (Seoul, Korea). MKN45 cells were grown in RPMI-1640 medium (Gibco) with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin and streptomycin (Gibco) in a 5% CO₂ atmosphere at 37°C.

siRNA and vector transfection

Negative control scrambled siRNA med GC, which contains medium GC content (12935–300) and CD44 siRNA (HSS101596) were purchased from Thermo Fisher Scientific. For each siRNA and vector transfected cell line, transfection was performed when the cells were 60% confluent in 24-well culture plates. 20 μ M siRNA with lipofectamine RNAimax reagent (Thermo Fisher Scientific) with 1:3 ratio of siRNA (μ g) to lipofectamine (μ L) was used for siRNA transfection in MKN45. pcDNA3 (N/A) and pcDNA3- β -catenin vector (#19286) were purchased from Addgene. 1 μ g of DNA plasmid to 3 μ L of lipofectamine 2000 reagent (Thermo Fisher Scientific) was used for the plasmid DNA transfection in MKN45.

When siRNAs with pcDNA3 or pcDNA3- β -catenin vector were co-transfected in MKN45 cells, 20 μ M siRNA was transfected with 1:3 ratio of siRNA to lipofectamine before the plasmid vector transfection. After 24 hours siRNA

transfection, pcDNA3 or pcDNA3- β -catenin vector was transfected with 1 μ g of DNA plasmid to 3 μ L of lipofectamine 2000 reagent (Thermo Fisher Scientific). After 72 hours plasmid DNA transfection, cells were harvested for western blot.

Western blot and antibodies

To assess the protein expression level in MKN45, Passive Lysis Buffer (Promega) was used to prepare cell lysate. After the lysate was incubated at 95°C for 5 minutes to ensure all proteins are unfolded, it was loaded on a 15% sodium dodecyl sulfate-polyacrylamide (SDS) gel. After the electrophoresis, the proteins were transferred for 16 hours from SDS gel to polyvinylidene fluoride (PVDF) membranes with a Mini Trans-Blot tank (Bio-Rad) for 20 V at 4°C cold room. The transferred proteins on PVDF membrane were probed with antibodies against CD44 (Cell signaling #37259T), β -catenin (Cell signaling # 8480S), and β -actin (Santa Cruz, sc-47778) for 4 hours at 4°C. Then, PBS was used to wash away the un-probed antibodies. All secondary antibodies (Santa Cruz, sc-358914, sc-2005) were incubated for 1 hour at room temperature. After PBS washing on a shaking machine, Amersham ECL GST Western Blotting Detection kit (GE Healthcare) was used to detect the chemiluminescence signal on antibody-probed proteins. The densitometry of chemiluminescence signal was quantified with Image Studio ver. Light 5.2 (Licor). The expression value of protein bands was normalized against β -actin. All antibodies were validated by the manufacturer using cell lysates.

Cell migration and invasion

2.0 x 10⁵ MKN45 cells in RPMI1640 with 1% FBS were placed in the top chamber of 8.0 μ m pore size Fluoro-block inserts (Corning). Then, RPMI1640 with 10 % FBS were added to the lower wells on 24 well culture plate to initiate the cell migration. After 48 hours of incubation in CO₂ cell culture chamber, 2 μ M Carboxyfluorescein diacetate (CFDA) was placed in the chamber for 15 minutes to stain the cells with green fluorescence (ThermoFisher Scientific). The un-migrated cells remaining on the top membrane of the transwell were removed with a cotton swab. Then, 4% formaldehyde was used to fix the migrated cells on the lower membrane. After the image of migrated cells on the lower membrane was captured by 488/510 nm (Ex/Em) on an inverted fluorescent microscope (Nikon), the migrated cells were counted by Image J software.

To assess cell invasion, 50 μ L of Matrigel (200 μ g/mL) (BD Bioscience) was pre-coated on the top membrane of the transwell. Then, the transwell were incubated at 37°C for 2 hours before the cells were placed in the upper membrane of the chamber. The invasion assays were performed under the same conditions as the migration assays. After the images were taken by fluorescent microscope (Nikon), the number of invaded cells were counted by Image J software.

Statistical Analysis

For patient survival analysis, quality control for gene chips and control for duplicate samples were performed as described previously (20). Kaplan-Meier survival plot with 95% confidence intervals and log-rank p-values were calculated and plotted using PostgreSQL server as described previously (20). Data are expressed as the mean values \pm standard error and analyzed by Student's t-test, two-way ANOVA, or Tukey test using Excel and SPSS 11.5. All statistical results were considered significant if p-values were 0.05 or less.

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