Original

Immunohistochemical Analysis of the Distribution of RANKL-Expressing Cells and the Expression of Osteoclast-Related Markers in Giant Cell Tumor of Bone

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Abstract: To clarify the mechanism underlying the regulation of osteoclast differentiation and activation in giant cell tumor of bone, we investigated the expression of osteoclast-related markers in osteoclasts, neoplastic cells, and osteoclast precursors comprising giant cell tumor of bone using immunohistochemical analysis, and analyzed the distribution of receptor activator of nuclear factor κ-B ligand (RANKL)-expressing cells. We performed serial staining of sections using antibodies against osteoclast-related markers including RANKL, CD68, CD11b, c-Fms, RANK, and cathepsin K and analyzed the presence of the G34W mutation of H3F3A in giant cell tumor of bone. Mononuclear cells were CD11b+, CD68+, RANK+, and c-Fms+. In contrast, most multinucleate cells were CD11b+, CD68+, RANK+, and c-Fms+. RANKL was expressed in mononuclear cells and some multinucleate cells. Conversely, G34W was detected in mononuclear cells. RANKL+ mononuclear cells were also G34W+. RANKL expression was unevenly distributed in giant cell tumor of bone. RANKL+ cells were frequently localized along blood-containing cavity-like spaces. In areas with a high percentage of RANKL+ mononuclear cells, large numbers of osteoclasts were observed. In addition, the distribution of multinucleate cells (>100 μm in diameter) correlated with the distribution of RANKL+ cells. The distribution of RANKL+ mononuclear cells is uneven in giant cell tumor of bone and may have some effect on the localization of multinucleate cells. The distribution of mononuclear cells harboring the G34W mutation was identical to that of RANKL+ mononuclear cells.

Key words: Giant cell tumour of bone, RANKL, G34W, Immunohistochemistry, Osteoclast

Introduction

Giant cell tumor of bone (GCTB) is a primary bone tumor that typically develops in the epiphysis of long bones and in the bones of the pelvis in young adults. GCTB is characterized on plain radiographs by extensive osteolytic changes accompanied by significant cortical thickness, indicating aggressive bone destruction. While complete curettage of the pathological lesion is the mainstay of treatment for GCTB, it is still classified as an intermediate-grade tumor because of its aggressive behavior and frequent postoperative recurrence. The need for repeated surgery due to local recurrence is a significant clinical challenge as this can lead to serious functional morbidity. Also, curative resection can be difficult to perform in cases that involve the pelvic bone or spinal column1.

GCTB consists of multinucleate giant cells and mononuclear cells. The multinucleate cells have the functional and morphological characteristics of osteoclasts2-3, and the mononuclear cells include neoplastic cells (tumor cells) and osteoclast precursors, among others6-9. These multinucleate cells aggressively destroy bone, the features of which can be recognized clinically.

Osteoclasts are responsible for bone remodeling and regulation of blood calcium levels through bone resorption. These cells arise from monocytes/macrophages that are in turn differentiated from hematopoietic stem cells. Monocytes and macrophages express CD11b, CD14, and CD686-8. Among these markers, CD68 is expressed throughout the differentiation of monocytes/macrophages to osteoclasts6,9.

Receptor activator of nuclear factor κ-B ligand (RANKL) is a member of the tumor necrosis factor superfamily and is expressed by osteoblast/stromal cells and T lymphocytes. RANKL binds to its receptor RANK, which is expressed by osteoclast precursors, and induces osteoclast differentiation from the precursors and activates bone resorption. Osteoclast precursors express c-Fms, a receptor of macrophage colony-stimulating factor10,11.

Previous reports suggested that neoplastic cells express RANKL12,13, which plays a role in osteoclast differentiation in GCTB14,15. The immunohistochemical characteristics of the cells constituting GCTB, including the expression of RANKL, have been analyzed13,15. However, these observations do not indicate the distribution of RANKL+ neoplastic cells and multinucleate cells in GCTB, such as the relationship between the number of RANKL+ cells and the number of multinucleate cells16,17.

Recently, distinct driver mutations in H3F3A, encoded in histone

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In this study, to clarify the distributions of osteoclast-related marker expressing cells in GCTB tissue, we performed immunohistochemical analysis using osteoclast-related markers including RANKL, CD68, CD11b, c-Fms, RANK, cathepsin K, and G34W. Our results suggest that RANKL is unevenly distributed in tumor tissue and may contribute to osteoclast formation. These results offer new insights into how the differentiation and activation of osteoclasts is regulated in GCTB tissue.

Materials and Methods

Patients

Tissue samples were obtained from 5 patients (Table 1) at the time of surgery for therapeutic purposes. The diagnosis of GCTB was established by open biopsy prior to surgery at a university hospital in Japan.

Ethics approval

This study was carried out with approval from the ethics committees of our institution and in accordance with the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all participants included in the study.

Immunohistochemistry

We performed immunohistochemical staining to evaluate RANKL, CD68, CD11b, RANK, c-Fms, cathepsin K, and G34W expression in cells constituting GCTB and the expression of vascular endothelial markers including CD31, Factor VIII-related antigen, and D2-40. Tumor tissue was stored as paraffin-embedded formalin-fixed tissue and sectioned onto glass slides. After deparaffinization and inactivation of endogenous peroxidase at pH 9, the tissue sections were labeled with each antibody for 30 min. After the addition of a peroxidase-labeled antibody (MULTI™; Nichirei Biosciences Co. Ltd., Tokyo, Japan), sections were washed with phosphate buffer solution (Histofine®; Nichirei Biosciences Co. Ltd., Tokyo, Japan) and treated with a color development reagent (3,3′-diaminobenzidine tetrahydrochloride; Nichirei Biosciences Co. Ltd., Tokyo, Japan). Antibodies and concentrations used were as follows: RANKL (polyclonal, ab9957, ×500; Abcam Co. Ltd., Cambridge, UK), CD68 (monoclonal, M0876, ×100; DakoCytomation, Glostrup, Denmark), RANK (monoclonal, ab12008, ×100; Abcam Co. Ltd., Cambridge, United Kingdom), c-Fms (monoclonal, ab183316, ×100; Abcam Co. Ltd., Cambridge, United Kingdom), CD11b (monoclonal, ab52478, ×100; Abcam Co. Ltd., Cambridge, United Kingdom), cathepsin K (monoclonal, ab66237, ×50; Abcam Co. Ltd., Cambridge, United Kingdom), G34W (monoclonal, 31-1145-00, ×200; RevMAb Biosciences, San Francisco, USA), CD31 (monoclonal, NCL-CD31-1A10, ×100; Leica Biosystems, Wetzlar, Germany), Factor VIII-related antigen (monoclonal, M0616, ×100; DakoCytomation, Glostrup, Denmark), and D2-40 (monoclonal, 713451, prediluted; Nichirei Biosciences Co. Ltd., Tokyo, Japan). Analysis involving more than one antibody was performed on serial sections. We also performed immunofluorescent antibody staining to evaluate RANKL expression (monoclonal, MAB626, ×100; R&D Systems, Minneapolis, USA) and the G34W mutation. Neoplastic cells, which did not contain osteoclast precursors, were cultured for 24 h on 48-well plates and fixed in 99.5% acetone and 99% ethanol. After blocking with 1% bovine serum albumin in phosphate-buffered saline, the cells were labeled with each antibody for 1 h. After washing with phosphate-buffered saline, the cells were labeled with secondary antibodies (RANKL: Alexa Fluor 546 anti-mouse IgG, ×100; Life Technologies, Carlsbad, USA / G34W: Alexa Fluor 488 anti-rabbit IgG, ×100; Invitrogen, Carlsbad, USA).

Quantitative determination of RANKL+ and CD68+ cell distribution

We analyzed 5 square fields (1.0 × 1.0 mm) labeled with an anti-RANKL antibody and further segmented them into 4 fields (500 × 500 µm). The number of RANKL+ and RANKL+ mononuclear cells and multinucleate cells (i.e., those with >3 nuclei) per 500 × 500 µm field (total 20 fields) was counted. We then calculated the ratio of RANKL+ in all mononuclear cells (% of RANKL+). Next, we set a square field (2.0 × 2.5 mm) labeled with either anti-RANKL antibody or anti-CD68 antibody on serial sections and an area (2.0 × 2.5 mm) was then segmented into 20 medium-sized fields of 500 × 500 µm (Fig. 1a). These medium-sized fields were further segmented into 100 small fields of 50 × 50 µm (Fig. 1b). CD68+ multinucleate cells (i.e., those with >3 nuclei) with a longest diameter >100 µm were termed large osteoclasts, and the number of large osteoclasts in each medium-sized field was counted. The number of RANKL+ cells per small field was also counted. Small fields that contained more than 10 RANKL+ cells were termed RANKL+ high-density fields. The cells in each field were counted by the same person using printed photomicrographs at 200× magnification. Counts were obtained using 3 noncontiguous areas (2.0 × 2.5 mm) in GCTB tissues.

Statistics

Correlation was evaluated using Spearman’s coefficient of correlation. A coefficient of correlation >0.4 was considered correlation and p<0.05 was defined as significant. All statistical analysis was performed using JMP version, 12.0.1 (SAS Institute Inc., Cary, USA).

Results

Detection of CD11b+, CD68+, and cathepsin K+ cells in GCTB

To determine the expression of CD11b and CD68, which are markers of monocytes/macrophages, we labeled GCTB sections with anti-CD11b and anti-CD68 antibodies. All of the multinucleate cells and a few mononuclear cells were CD68+ (Fig. 2a, d). Conversely, multinucleate cells were CD11b+, but several mononuclear cells were CD11b+ (Fig. 2b, e). Multinucleate cells were cathepsin K+, which serves as a marker of osteoclasts, whereas mononuclear cells were cathepsin K+ (Fig. 2c, f). These results suggest that multinucleate cells in GCTB are CD11b+ and CD68+, and a minority of mononuclear cells are CD68+

Expression of RANK and c-Fms in GCTB

To examine the expression of markers related to osteoclast precursors, we labeled histological sections of GCTB with anti-RANK and anti-c-Fms antibodies. Several mononuclear cells and all of the multinucleate cells were RANK+ (Fig. 3a, c). c-Fms was expressed predominantly on the surface of multinucleate cells and a portion of mononuclear cells (Fig. 3b, d). These results suggest that multinucleate cells in GCTB are RANK+ and c-Fms+.

Correlation between the distribution of RANKL+ mononuclear cells and the number of osteoclasts

To clarify the relationship between the distribution of RANKL+ cells

Table 1. Patient characteristics

| Case number | Age (years) | Sex | Location | Remarks |
|-------------|-------------|-----|----------|---------|
| GCT1        | 28          | Male | Proximal tibia | Primary |
| GCT2        | 20          | Male | Distal femur | Primary |
| GCT3        | 25          | Male | Proximal tibia | Primary |
| GCT4        | 48          | Male | Proximal tibia | Recurrence |
| GCT5        | 30          | Female | Proximal fibula | Primary |
Figure 1. Osteoclast formation and receptor activator of nuclear factor κ-B ligand (RANKL)-expressing cell localization. A 2.0 × 2.5-mm area (green square) subdivided into 20, 500 × 500-µm medium-sized fields (pink square). Osteoclasts (diameter > 100 µm) were counted in these fields. RANKL+ cells were counted in 50 × 50-µm fields (yellow square); those containing >10 RANKL+ cells were counted and termed RANKL+ high-density fields.

Figure 2. Expression of CD68, CD11b, and cathepsin K in giant cell tumor of bone. Serial sections were labeled with anti-CD68 (a, d), anti-CD11b (b, e), and anti-cathepsin K (c, f) antibodies. Upper panels are magnified images of the lower panels.
and the number of osteoclasts, we labeled histological sections of GCTB with an anti-RANKL antibody. There were fields in which most mononuclear cells were strongly positive for RANKL and fields in which mononuclear cells were either weakly positive or negative for RANKL (Fig. 4a–c). Mononuclear cells were RANKL+, while multinucleate cells were either negative or weakly positive. Multinucleate cells were present in areas where most mononuclear cells were RANKL− cells and in areas with few RANKL+ cells (Fig. 4b, c). These findings suggest that in GCTB, RANKL+ mononuclear cells are variably located. In addition, we analyzed the relationship between the percentage of RANKL+ mononuclear cells and the number of multinucleate cells (i.e., those with >3 nuclei) by counting these cells in 500 × 500-µm fields. Five cases were included in this study (GCT1–5). We identified positive correlations between the percentage of RANKL+ mononuclear cells and the number of multinucleate cells in GCT2, GCT4, and GCT5. (Fig. 5A, Table 2). In contrast, the number of RANKL+ mononuclear cells was correlated with the number of multinucleate cells in GCT1 and GCT5 (Fig. 5B, Table 3).

### Table 2. Statistical analysis of the correlation between the percentage of receptor activator of nuclear factor κ-B ligand (RANKL)+ mononuclear cells and the number of multinucleate cells

|       | Rank-correlation coefficient (Spearman) | p-value  |
|-------|----------------------------------------|----------|
| GCT1  | 0.3881                                  | 0.0909   |
| GCT2  | 0.4955                                  | 0.0263   |
| GCT3  | 0.2863                                  | 0.2211   |
| GCT4  | 0.6997                                  | 0.0006   |
| GCT5  | 0.7935                                  | < 0.001  |

### Table 3. Statistical analysis of the correlation between the number of receptor activator of nuclear factor κ-B ligand (RANKL)+ mononuclear cells and multinucleate cells

|       | Rank-correlation coefficient (Spearman) | p-value  |
|-------|----------------------------------------|----------|
| GCT1  | 0.4804                                  | 0.0321   |
| GCT2  | 0.2493                                  | 0.2892   |
| GCT3  | 0.2373                                  | 0.3138   |
| GCT4  | 0.2929                                  | 0.2101   |
| GCT5  | 0.6429                                  | 0.0022   |

**Correlation between the density of RANKL+ mononuclear cells and the number of large osteoclasts**

We detected some high-density areas of RANKL+ mononuclear cells with large osteoclasts in GCT1 and GCT2. To determine whether there was a relationship between the density of RANKL+ mononuclear cells and the number of large-sized osteoclasts, we counted the number of RANKL+ mononuclear cells in small fields. Next, we counted the number of large osteoclasts with a longest diameter > 100 µm in medi-
Figure 5. Correlations between the number of osteoclasts and the proportion or number of receptor activator of nuclear factor κ-B ligand (RANKL) mononuclear cells. Plots showing the correlation between the number of osteoclasts and the proportion of RANKL⁺ mononuclear cells in all mononuclear cells (A). Plots showing the correlation between the number of osteoclasts and the number of RANKL⁺ mononuclear cells (B).
um-sized fields. We also counted the number of RANKL high-density fields in each medium-sized field. We identified a correlation between the number of large osteoclasts (>100 µm) and the number of RANKL high-density fields in the medium-sized fields in GCT1 and GCT2 (Fig. 6a, b, Table 4). Conversely, no large osteoclasts were identified in GCT3, GCT4, and GCT5; therefore, we did not analyze these 3 cases. These results suggest that the size of newly developed multinucleate cells is associated with the number of RANKL⁺ mononuclear cells around the multinucleate cells.

**Distribution of RANKL⁺ mononuclear cells and G34W⁺ mononuclear cells**

To determine the distribution of RANKL⁺ cells and G34W⁺ cells, we labeled histological sections of GCTB with anti-RANKL and anti-G34W antibodies. G34W was detected in mononuclear cells, which were interspersed among multinucleate cells, similar to RANKL⁺ cells, and was not detected in multinucleate cells (Fig. 7a–d). Conversely, most mononuclear cells were RANKL⁻, but were also G34W⁻, in another GCTB tissue (Fig. 7e, f).

To then clarify the correlation between RANKL⁺ cells and G34W⁺ cells, we performed immunofluorescence double staining. Cytoplasmic immunostaining for RANKL was observed in all cells; furthermore, G34W mutation was detected in the nucleus of each cell (Fig. 8). These results suggest that RANKL⁺ mononuclear cells also harbored the G34W mutation.

**Distribution of RANKL⁺ mononuclear cells and G34W⁺ multinucleate cells around cavity-like spaces**

There were some cavity-like spaces in the tissue, which were neither artefactual cracks in the sections nor blood vessels, with cell aggregates

Table 4. Statistical analysis of the correlation between the number of large osteoclasts and the number of receptor activator of nuclear factor κ-B ligand (RANKL)⁺ high-density fields.

|              | Rank-correlation coefficient (Spearman) | p-value |
|--------------|----------------------------------------|---------|
| GCT1         | 0.4811                                 | < 0.0001|
| GCT2         | 0.463                                  | 0.0002  |

Figure 6. Correlation between the number of large osteoclasts and the number of receptor activator of nuclear factor κ-B ligand (RANKL)⁺ high-density fields. Plots showing the correlation between the number of osteoclasts > 100 µm vs. the number of RANKL⁺ high-density fields in GCT1 (a) and GCT2 (b). Counts were obtained using 3 noncontiguous areas (2.5 × 2.0 mm) in giant cell tumor of bone tissues.

Figure 7. Receptor activator of nuclear factor κ-B ligand (RANKL) and G34W expression. Left panels (a, c) show RANKL-labeled sections; right panels (b, d) show G34W-labeled sections in GCT3. Upper panels are magnifications of the middle panels. Lower panels show RANKL (e) and G34W (f) expression in GCT2.
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Figure 8. Detection of the G34W mutation and receptor activator of nuclear factor κ-B ligand (RANKL) expression in giant cell tumor of bone-derived neoplastic cells in vitro. The G34W mutation (green) was labeled with anti-G34W antibody followed by a secondary antibody (Alexa Fluor 488 anti-rabbit IgG). RANKL (red) was labeled with an anti-RANKL antibody followed by a secondary antibody (Alexa Fluor 546 anti-mouse IgG). In merged images double positive areas appear as yellow.

Figure 9. Distribution of receptor activator of nuclear factor κ-B ligand (RANKL) + mononuclear cells around a cavity-like space. (a) Overall image of a cavity-like space. (b) Magnified view of rectangular shape in (a). (c) Magnified view of rectangular shape in (b). Note RANKL+ mononuclear cells uniformly lining the space with multinucleate cells.

Figure 10. Distribution of receptor activator of nuclear factor κ-B ligand (RANKL) + mononuclear cells (a) and G34W+ mononuclear cells (b) around a cavity-like space.

Figure 11. Detection of vascular endothelial related markers, CD31 (a), Factor VIII-related antigen (b), and D2-40 (c) around a cavity-like space.
that appeared to be red blood cells. RANKL+ mononuclear cells were present around the entire cavity-like space and appeared to be uniformly arranged lining the cavity-like space at high magnification (Fig. 9 a–c). Multinucleate cells were also present around the RANKL+ mononuclear cells. Furthermore, G34W+ mononuclear cells were also arranged lining the cavity-like space, similar to RANKL+ mononuclear cells (Fig. 10 a, b). There was no expression of endothelial markers (CD31, Factor VI-II-related antigen, and D2-40) in the cavity-like spaces (Fig. 11 a–c). These findings suggest that RANKL-expressing cells around the cavity-like spaces induce the formation of multinucleate cells.

**Discussion**

Many studies have reported that GCTB is composed of neoplastic cells, osteoclasts, and osteoclast precursor cells22,25. Our study revealed the distribution of each cell type within GCTB tissue.

Human osteoclast precursor cells have been reported to express CD11b21. Our immunostaining results indicated the presence of CD11b+ mononuclear cells interspersed among multinucleate giant cells in GCTB tissue. Fujikawa et al.20 and Quinn et al.8 reported that peripheral blood mononuclear cells differentiating into osteoclasts by co-culture with stromal cells express CD11b. Brunetti et al.21 reported that CD11b is expressed in osteoclast precursor cells that differentiate into multinucleate osteoclasts following treatment with macrophage colony-stimulating factor and RANKL. We detected CD11b expression in GCTB tissue. Together, the findings of these previous reports and ours suggest that some mononuclear cells are CD11b+ osteoclast precursors that differentiate into osteoclasts in GCTB tissue.

Furthermore, we found that multinucleate cells expressed cathepsin K, a specific marker of differentiated osteoclasts23. Multinucleate cells also expressed CD68, but not CD11b. CD11b and CD68 are markers of monocyte/macrophage lineage cells; however, while CD11b expression becomes negative during the process of differentiation into osteoclasts, CD68 expression is maintained even after differentiation22. Our results are consistent with the findings from these previous reports and suggest that multinucleate cells in GCTB tissue are osteoclasts that express cathepsin K and CD68, but not CD11b.

We found that c-Fms was expressed not only in mononuclear cells but also in multinucleate giant cells. c-Fms and RANK expression in human monocyte lineage cells and osteoclast precursor cells has been reported previously20. Atkins et al. described the expression of RANK in the OC-enriched fraction in GCTB24 and in CD14+ mononuclear cells21. Yamagishi et al. reported the detection of RANK in GCTB by real-time PCR26. In contrast, c-Fms expression in GCTB has not been examined previously. In the present study, we identified c-Fms and RANK expression in multinucleate cells in GCTB by using serial sections. These results suggest that macrophage colony-stimulating factor and RANKL are important factors for the survival of multinucleate giant cells in GCTB and also play an important role in continual bone resorption.

RANKL+ cells were mostly mononuclear cells, and the distribution of RANKL+ cells in GCTB tissue was uneven. Although previous studies have shown images of RANKL expression in GCTB tissues, the authors did not specifically analyze the localized distribution of RANKL+ cells or the relationship between the number of RANKL+ cells and the number of multinucleate cells4,17. The number of RANKL+ mononuclear cells was correlated with the number of osteoclasts only in parts of GCTB tissue. However, in areas with a high percentage of RANKL+ mononuclear cells, larger numbers of osteoclasts were observed. This may be due to the osteoclast differentiation of RANKL+ mononuclear cells. Areas with a high density of RANKL+ cells contained large osteoclasts. Given that multinucleate cells are formed by the fusion of multiple osteoclasts under the influence of dendritic cell-specific transmembrane protein and osteoclast-stimulatory transmembrane protein27, many osteoclasts likely formed in regions with a high density of RANKL+ cells. However, it remains unclear what kind of factor controls the expression of RANKL in mononuclear cells in GCTB. We speculate that some “special” microenvironments, such as the blood stream, stimulate the expression of RANKL in cells. Our findings suggest that the number of osteoclasts in GCTB tissue depends on the number of surrounding RANKL+ cells.

Mononuclear cells harboring the G34W mutation were found to be identical to RANKL+ mononuclear cells. The relationship between the expression of G34W and RANKL has not been analyzed previously. Bejhati et al.18 reported G34W expression in neoplastic stromal cells, but not in osteoclasts. Our results are consistent with those of this previous study. One of the GCTB cases analyzed in this study did not harbor G34W mutation. Additional studies also reported the presence of other mutations, such as G34L, G34R, G34M, and UPS6, but not G34W, in several cases23. In our case, which did not harbor G34W, most mononuclear cells were RANKL+. We found that RANKL+ mononuclear cells were identical to G34W+ mononuclear cells, but the detailed relationship between RANKL expression and the presence of the G34W mutation needs to be confirmed in further studies.

Furthermore, RANKL+ cells were observed around cavity-like spaces containing red blood cells. These cavities are histologically different from blood vessels because they lack endothelial cells. We speculate that factors that stimulate RANKL expression are obtained from these cavities. Bone marrow-derived stem cells, the progenitors of osteoclasts, migrate from the vascular system to the extravascular space and then move into bone tissue during differentiation27. Liao et al.29 reported that in multinucleate giant cell formation in GCTB, SDF-1, which is expressed in neoplastic cells, interacts with CXCR4 to recruit intravascular osteoclast precursor cells into tumor tissue. Also, Matsumoto et al.15 reported that vascular endothelial growth factor expressed in GCTB tissue leads to the recruitment of osteoclast precursor cells into tumor tissue. These reports suggest that blood flow is related to multinucleate giant cell formation in GCTB. Our results suggest that some osteoclasts are formed around these cavity-like spaces, which are not blood vessels, in GCTB.

In conclusion, we identified the expression of osteoclast-related factors (e.g., RANKL, RANK, c-Fms, CD11b, and CD68) in different types of cells (e.g., multinucleate cells, neoplastic cells, and osteoclast precursors) present in GCTB tissue. We also demonstrated the localized distribution of RANKL+ cells in GCTB; mononuclear cells harboring the G34W mutation were identical to RANKL+ mononuclear cells. These findings will contribute to the biological characterization and potential treatment of GCTB.

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**Conflict of Interest**

The authors have declared that no COI exists.

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