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BONE BIOLOGY

Effect of GNAQ alteration on RANKL-induced osteoclastogenesis in human non-small-cell lung cancer

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Aims

Receptor activator of nuclear factor-κB ligand (RANKL) is a key molecule that is expressed in bone stromal cells and is associated with metastasis and poor prognosis in many cancers. However, cancer cells that directly express RANKL have yet to be unveiled. The current study sought to evaluate how a single subunit of G protein, guanine nucleotide-binding protein G(q) subunit alpha (GNAQ), transforms cancer cells into RANKL-expressing cancer cells.

Methods

We investigated the specific role of GNAQ using *GNAQ* wild-type cell lines (non-small-cell lung cancer cell lines; A549 cell lines), *GNAQ* knockdown cell lines, and patient-derived cancer cells. We evaluated GNAQ, RANKL, macrophage colony-stimulating factor (M-CSF), nuclear transcription factor-κB (NF-κB), inhibitor of NF-κB (IκB), and protein kinase B (Akt) signalling in the *GNAQ* wild-type and the *GNAQ*-knockdown cells. Osteoclastogenesis was also evaluated in both cell lines.

Results

In the *GNAQ*-knockdown cells, RANKL expression was significantly upregulated ($p < 0.001$). The expression levels of M-CSF were also significantly increased in the *GNAQ*-knockdown cells compared with control cells ($p < 0.001$). *GNAQ* knockdown cells were highly sensitive to tumour necrosis factor alpha (TNF-α) and showed significant activation of the NF-κB pathway. The expression levels of RANKL were markedly increased in *GNAQ* mutant compared with *GNAQ* wild-type in patient-derived tumour tissues.

Conclusion

The present study reveals that the alterations of *GNAQ* activate NF-κB pathway in cancers, which increase RANKL and M-CSF expression and induce osteoclastogenesis in cancers.

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Keywords: GNAQ, RANKL, Osteoclastogenesis, Bone, Lung cancer

Article focus

- The detailed mechanisms of guanine nucleotide-binding protein G(q) subunit alpha (GNAQ) in the signal transduction pathway responsible for receptor activator of nuclear factor-κB ligand (RANKL) are not yet understood.
- This study investigated the significant roles of *GNAQ* in osteoclastogenesis.

Key messages

- The study showed that the expression level of RANKL and the number of osteoclasts were significantly increased in *GNAQ* knockdown cells ($p < 0.001$).

Strengths and limitations

- To our knowledge, this is the first study to assess whether suppression of *GNAQ* can transform lung cancer cells into RANKL-expressing cells.
- The absence of an in vivo model is a principal limitation of this study.

Introduction

Refractory bone metastasis, for which all conventional treatments such as chemotherapy and radiation therapy have failed, often results in pathological fractures. Efforts have been made to prevent pathological fractures of metastatic bone. Bisphosphonates are

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commonly used to reduce the risk of pathological fractures. A recent study showed that denosumab, which targets receptor activator of nuclear factor-κB ligand (RANKL) and is also known as tumour necrosis factor (TNF) ligand superfamily member 11 (TNFSF11) or TNF-related activation-induced cytokine (TRANCE), prevents or delays skeletal-related events (SREs) and can improve a prognosis.[1–3]

Receptor activator of nuclear factor-κB ligand, a cell membrane-bound TNF superfamily member, binds to receptor activator of nuclear factor-κB (RANK) expressed on osteoclast precursors, which then leads to the fusion, differentiation, and maturation of osteoclast.[4] The RANK/RANKL/osteoprotegerin (OPG) system is a master regulator of the bone resorption process by activating the osteoclasts. OPG functions as a decoy receptor for RANKL, which inhibits RANKL-induced osteoclast differentiation.[5,6] RANKL is expressed at a high level in stromal cells. Therefore, cancer cells indirectly induce RANKL expression via stromal cells, which accelerates bone metastasis. Interestingly, some cancers have been observed to express RANKL by themselves.[7,8]

RANKL-expressing cancers are correlated with poor prognosis. Among gastric cancer patients, RANKL expression was observed in 33% of the patients with a poor prognosis.[9] Patients with renal cell carcinoma which expresses high levels of RANKL showed shorter bone metastasis-free survival and disease-free survival.[6] Breast cancer patients with RANKL-positive primary tumours exhibited poorer clinical outcomes than patients with RANKL-negative primary tumours.[2] In addition, inhibition of RANKL has been shown to improve the overall survival in patients with metastatic lung cancer.[2,7,10,11] However, the mechanisms by which cancer cells transform to RANKL-expressing cells have yet to be fully researched.

Guanine nucleotide-binding proteins (G proteins) and G protein-coupled receptors (GPCRs) transduce extracellular signals and involve multiple processes of mammary cells including hormonal signal transduction, metabolism, development, cell survival, and sensory functions.[12,13] The heterotrimeric G proteins of α, β, and γ subunits provide the specificity and functionality of GPCRs in a cell type- and tumour-specific way. Guanine nucleotide-binding proteins are classified into four subfamilies: $G\alpha_i$; $G\alpha_s$; $G\alpha_{12/13}$; and $G\alpha_{q/11}$. Gαq is encoded by the *GNAQ* gene.[14,15] *GNAQ* mutations have been associated with several carcinomas.[16,17] About 85% of melanoma patients presenting metastasis and higher rates of mortality exhibit mutations in *GNAQ*.[10,18] In the current study, we found that the alteration of *GNAQ* induced RANKL expression in lung cancer cells. This study aimed to determine how this *GNAQ* alteration is involved in the signal transduction pathway responsible for RANKL expression.

Methods

Patient samples. Primary tumour cells were obtained from patients who underwent surgery at the Samsung Medical Center, Sungkyunkwan University, Seoul, South Korea, and signed the informed consent form according to the relevant guidelines and the regulation for the cell storage. A total of six lung cancer tissues obtained from metastatic bone lesion were included in the present study (Supplementary Table i).

Cell culture. Non-small-cell lung cancer (NSCLC) A549 cell lines were purchased from the American Type Culture Collection (Manassas, Virginia, USA). A549 cells were cultured in RPMI1640 medium (HyClone Laboratories, Logan, Utah, USA) supplemented with 10% fetal bovine serum, and 1% antibiotic-antimycotic (Gibco, Waltham, Massachusetts, USA). Cell lines were incubated in a 5% CO_2-humidified atmosphere at 37°C. RAW 264.7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (HyClone Laboratories) with 10% fetal bovine serum and 1% antibiotic-antimycotic. Patient tissues were dissociated at 37°C with collagenase, and then patient-derived cells (PDCs) were grown in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic.

Evaluation of the GNAQ status. The *GNAQ* status of the samples was evaluated according to the same process as we described in the previous study.[19] Briefly, the genomic status of *GNAQ* was screened using CancerSCAN, a Next-Generation-Sequencing-based analysis which used Illumina HiSeq 2000 (Illumina, San Diego, California, USA). Sanger sequencing was used as a confirmative method to find a specific genetic alteration.

Reverse transcription polymerase chain reaction and quantitative real-time polymerase chain reaction analysis. Total RNA isolated from the cultured cells was reverse-transcribed using a SuperScriptIII cDNA Synthesis kit (Life Technologies, Carlsbad, California, USA). Polymerase chain reaction (PCR) was performed using AccuPower® Hotstart PCR PreMix (Bioneer, Daejeon, Korea), and quantitative real-time PCR (qRT-PCR) was carried out in the Applied Biosystems 7900HT (Applied Biosystems, Foster City, California, USA). The target genes were amplified using the following primers: *GNAQ* forward, 5′-GCACAATAAGGCTCATGCAC-3′ and reverse, 5′-TGGAACCAGGGGTATGTGAT-3′; *RANKL* forward, 5′-TATGCCAACATTTGCTTTCG-3′ and reverse, 5′-CTTGGGATTTTGATGCTGGT-3′; macrophage colony-stimulating factor (*M-CSF*) forward, 5′-AGCAGGAGTATCACCGAGGA-3′ and reverse, 5′-TAATTTGGCACGAGGTCTCC-3′; glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) forward, 5′-TGATGACATCAAGAAGGTGG-3′ and reverse, 5′-TCCTTGGAGGCCATGTGGGC-3′; β-*actin* forward, 5′-TCATGAAGTGTGACGTGGAC-3′ and reverse, 5′-GCAGTGATCTCCTTCTGCAT-3′.

Western blotting. The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors (Thermo Fisher Scientific, Piscataway, New Jersey, USA). Protein supernatants were obtained by centrifugation at 14,000 × *g* for 15 minutes at 4°C. Protein quantification was performed using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, California, USA) for western blotting. The membrane was blocked with 5% skim milk for one hour and then immunoblotted with a primary antibody, GNAQ (Abcam, Cambridge, UK), RANKL, β-actin (Santa Cruz Biotechnology, Dallas, Texas, USA), or β-tubulin (Cell Signaling Technology, Danvers, Massachusetts, USA), at a dilution of 1:1,000 overnight at 4°C.

Osteoclastogenesis assay. RAW 264.7 cells were seeded in 24-well plates at a density of 2×10^3 cells per well. Negative control lentiviral vector (A549_shNC) and GNAQ short hairpin RNA lentiviral vector (A549_shGNAQ) cells were subsequently seeded in minimum essential medium-alpha modification(α-MEM) (HyClone Laboratories) overlaying the RAW 264.7 cells at a density of 2×10^3 cells per well. Cells were treated with RANKL (50 ng/ml) and M-CSF (20 ng/ml) (R & D Systems, Minneapolis, Minnesota, USA) to stimulate osteoclast differentiation. After five days, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity using a TRACP Assay Kit (Takara Bio, Mountain View, California, USA), as per the manufacturer's instructions. For quantification, osteoclasts were defined as multinucleated (more than three nuclei) TRAP-positive cells.[9] Cells were visualized at 200 × magnification using a light microscope. Electronic images of five pre-determined areas per well were obtained, and TRAP-positive cells were counted in each image.

Statistical analysis. The results were presented as means and standard deviations (SDs). The p-values were evaluated using Mann-Whitney test and paired *t*-test in GraphPad Prism5 (GraphPad Software, La Jolla, California, USA). Statistical significance was set at $p < 0.05$.

Results

GNAQ knockdown induces osteoclastogenesis. To investigate the role of *GNAQ* in osteoclastogenesis, we performed knockdown experiments using short hairpin RNA (shRNA) specific to *GNAQ*. The human lung adenocarcinoma A549 cells were transfected with shGNAQ. We found that both the messenger RNA (mRNA) and protein levels of *GNAQ* were decreased in the shGNAQ compared with the shNC group (Figure 1). We have successfully established a stable A549_shGNAQ cell line for further studies, which is confirmed using qRT-PCR and western blotting.

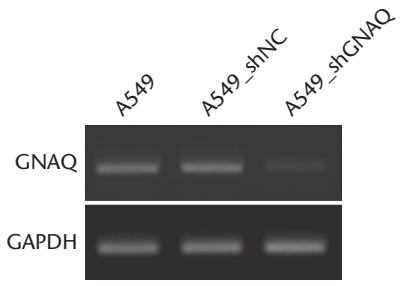

Fig. 1a

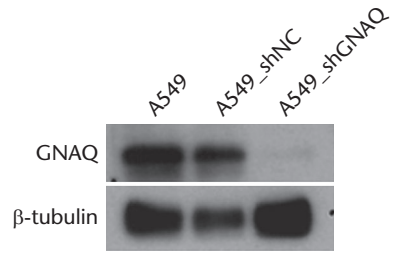

Fig. 1b

Stable knockdown of guanine nucleotide-binding protein G(q) subunit alpha (GNAQ) in A549 cells. GNAQ short hairpin RNA lentiviral vector (A549_shGNAQ) and short hairpin RNA negative control lentiviral vector (A549_shNC) were constructed. The vectors were transfected into A549 cells. a) Messenger RNA and b) protein levels of GNAQ were assessed using semi-quantitative polymerase chain reaction and western blot assay, respectively. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

RANKL activates the RANK receptor on the surface of osteoclast precursor cells and promotes their differentiation into multinucleated giant cells.[20] Our qRT-PCR revealed that mRNA expression of RANKL was significantly ($p < 0.001$) increased in *GNAQ* knockdown cells compared with that expressed in control cells (Figure 2a). These results were validated using semi-quantitative PCR (Figure 2b). Western blotting analysis showed that RANKL was markedly increased in *GNAQ* knockdown cells (Figure 2c). We investigated whether *GNAQ* knockdown cells can stimulate osteoclastogenesis in an osteoclast precursor cell line, RAW 264.7. A high number of TRAP-positive cells and the higher TRAP activity were observed in a co-culture of RAW 264.7 cells and *GNAQ* knockdown cancer cells. However, the wild-type *GNAQ* cells failed to induce differentiation of RAW 264.7 cells into TRAP-positive osteoclasts (Figures 2d and 2e). Taken together, these results indicated that the suppression of *GNAQ* influences osteoclast formation by increasing the expression of *RANKL*. Moreover, the expression of *M-CSF* was significantly ($p < 0.001$) elevated up to 5.3-fold in the *GNAQ* knockdown cells compared with that in the control cells (Figure 3). These results indicated that the suppression of *GNAQ* regulates osteoclastogenesis by inducing the expression of RANKL and M-CSF in cancer cells.

Effect of GNAQ knockdown on protein kinase B (Akt)/nuclear transcription factor-κB (NF-κB) signalling pathway. As noted in many studies,[21–23] TNF-α, a major

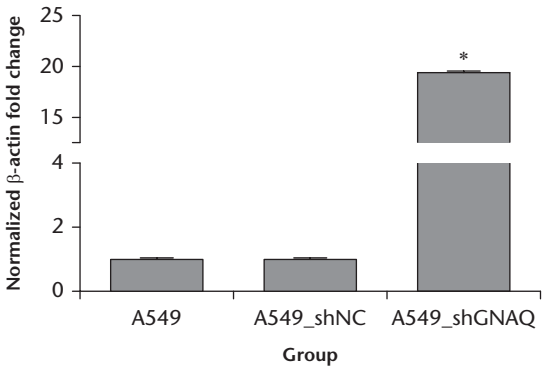

Fig. 2a

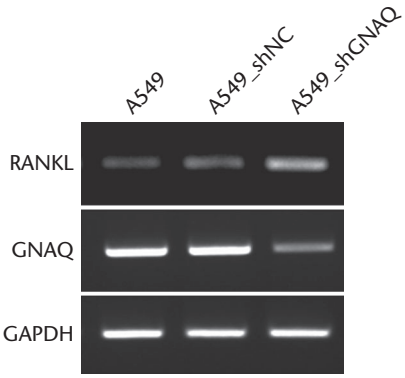

Fig. 2b

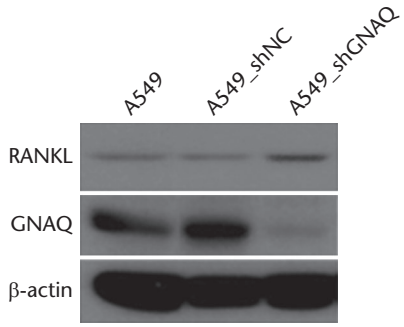

Fig. 2c

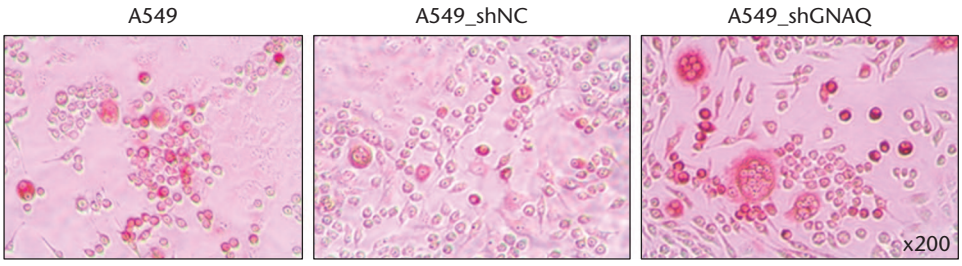

Fig. 2d

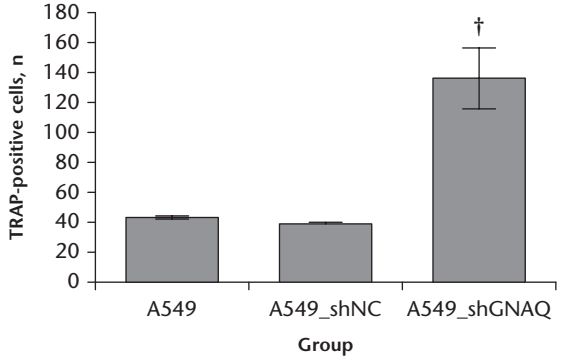

Fig. 2e

Induction of receptor activator of nuclear factor-κB ligand (RANKL) by knockdown in guanine nucleotide-binding protein G(q) subunit alpha expression (GNAQ). The expression level of RANKL mRNA was determined by a) real-time polymerase chain reaction and b) semi-quantitative polymerase chain reaction. c) Protein levels of RANKL were determined by western blotting. d) Representative images of tartrate-resistant acid phosphatase (TRAP) staining after induction of osteoclastogenesis by co-culture of RAW 264.7 cells with A549, short hairpin RNA negative control lentiviral vector (A549_shNC), or GNAQ short hairpin RNA lentiviral vector (A549_shGNAQ) cells. Cells were 1:1 co-cultured and treated with RANKL (50 ng/ml) and macrophage colony-stimulating factor (M-CSF, 20 ng/ml). The number of TRAP-positive cells was higher in the GNAQ knockdown group than in the control group. Images were captured using a light microscope (magnification × 40 or × 200). e) Evaluation of the number of TRAP-positive cells (n = 3). Error bars represent mean (SD). *Statistically significant ($p < 0.001$). †Statistically significant ($p < 0.05$). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

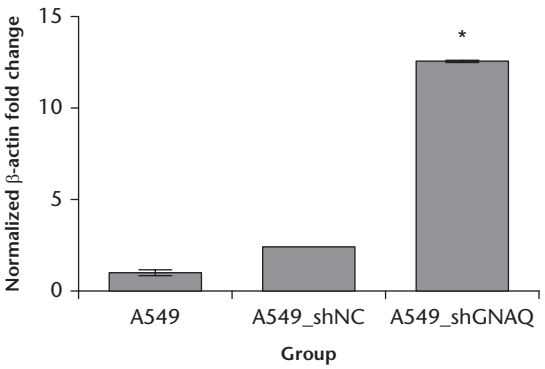

Fig. 3

Effect of suppression of guanine nucleotide-binding protein G(q) subunit alpha (GNAQ) on macrophage colony-stimulating factor (M-CSF) mRNA expression. Real-time polymerase chain reaction analysis was performed to detect M-CSF levels. The expression levels of M-CSF were normalized to the expression of β-actin. Error bars represent mean (SD). (*p < 0.001,Mann-Whitney U test).

pro-inflammatory cytokine, plays an important role in RANKL expression via the nuclear transcription factor-κB (NF-κB) signalling pathway. Therefore, to determine the effect of GNAQ on the NF-κB signalling pathway, NF-κB signalling was evaluated using western blot. We found that the expression levels of inhibitor of NF-κB (IκB) were reduced in *GNAQ* knockdown cells. In contrast, IκB was activated in the control cells two hours after the administration of TNF-α. We observed that the suppression of *GNAQ* activated NFκB signalling pathway by inducing phosphorylation of NFκB-p65 protein and protein kinase B (Akt) (Figure 4). Furthermore, the phosphorylation levels of Akt and NF-κB were increased upon the administration of TNF-α in *GNAQ* knockdown cells. Our results indicate that suppression of *GNAQ* facilitated Akt/NF-κB signalling pathway and elevated the sensitivity to TNF-α.

Expression of RANKL in PDTT extracted from metastatic bone lesion. We conducted genetic analysis for NSCLC tissues obtained from metastatic bone lesion using CancerSCAN and Sanger sequencing. Among six samples, the *GNAQ* mutation was found in three cases (Supplementary Table i). The *RANKL* mRNA level was increased in patient-derived tumour tissue (PDTT) with the *GNAQ* mutation (Figure 5). These results demonstrated that suppression of *GNAQ* affected the expression of *RANKL* in metastatic bone lesion.

Discussion

In this study, we found the silencing of *GNAQ* upregulated the NF-κB signalling pathway in lung cancer cells and increased the expression of RANKL and M-CSF. In vitro experiments showed that cancer cells with *GNAQ* knockdown effectively transformed macrophage (RAW 264.7) cells into TRAP-positive osteoclasts.

RANKL was initially identified on the surface of stromal cells, a major cell that mediates osteoclastogenesis. RANKL expression has been found in many tissues. It has recently been reported that human articular cartilage can express and produce RANKL.[24] High expression of RANKL is commonly detected in the lymph nodes, thymus, and lungs.[25] A weak level of RANKL expression can be found in bone marrow, the stomach, spleen, peripheral blood, placenta, leukocytes, heart, thyroid, or skeletal muscle.[26] In addition, RANKL expression was found in several malignant tumour cells and is localized on the surface of activated T-cell lymphocytes.[25–29] RANKL expression is upregulated during the progression of prostatic carcinoma in bone.[30,31] Breast cancer induces osteolytic metastases in bone by inducing RANKL expression.[32–33] In renal cell carcinoma, RANKL promotes cancer cells to metastasize to bone.[7]

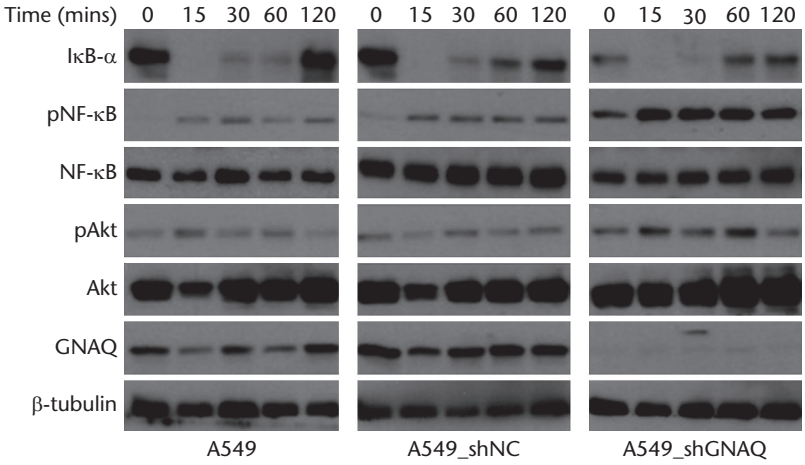

Fig. 4

Effect of suppression of guanine nucleotide-binding protein G(q) subunit alpha (GNAQ) on protein kinase B (Akt)/nuclear transcription factor-κB (NF-κB)/inhibitor of NF-κB (IκB) pathway after tumour necrosis factor α (TNF-α) treatment. Cells were incubated with 10 ng/ml TNF-α for zero, 15, 30, 60, or 120 minutes. Suppression of GNAQ induced several signalling pathways including protein kinase B (Akt) and inhibitor of NF-κB (IκB)/ nuclear transcription factor-κB (NF-κB).

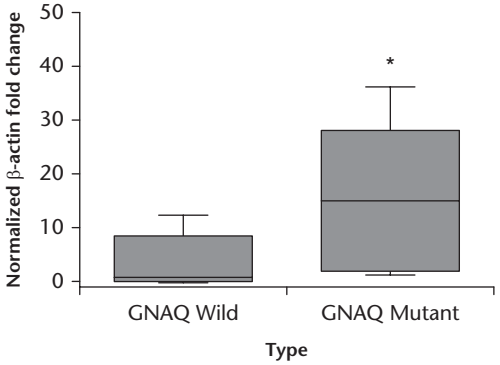

Fig. 5

Expression of receptor activator of nuclear factor-κB ligand (RANKL) in patient-derived tumour tissue extracted from metastatic bone cancer. *RANKL* expression was significantly different between samples from patients with guanine nucleotide-binding protein G(q) subunit alpha (*GNAQ*) wild-type and *GNAQ* mutant groups. Error bars represent mean (SD). *$p < 0.010$, paired *t*-test.

In addition, it has been reported that lung cancer cells that metastasize to bone showed increased levels of RANKL.[34] However, the mechanism by which RANKL expression is induced in cancer cells has not yet been fully examined. Our study first found that *GNAQ* mutation can transform cancer cells into RANKL-expressing cells. A previous study[35] demonstrated that M-CSF plays a critical role in the regulation of multiple processes including regulation of human osteoclasts. We detected the increased expression of M-CSF in *GNAQ* knockdown cells, which is also a crucial molecule for osteoclastogenesis. Therefore, *GNAQ* knockdown is sufficient for transforming macrophages into osteoclasts. Indeed, *GNAQ* knockdown cells alone were sufficient to induce RAW 264.7 cells into TRAP-positive osteoclasts.

NF-κB pathway activation has been shown as a vital contributing factor to GNAQ-mediated oncogenesis.[36] However, the alteration in *GNAQ* expression and the correlation of NF-κB pathway to osteoclastogenesis require further investigation. Our study confirmed that Akt/IκB/NF-κB pathway was constitutively active in *GNAQ* knockdown cells. In addition, we showed that *GNAQ* knockdown cells were highly sensitive to TNF-α.

Analysis of gene expression databases revealed that lung cancer cells harvested from metastatic bone lesions frequently harboured *GNAQ* mutations (M59L, T96S, or Y101X). We also found that the RANKL expression was increased in the metastatic bone lesion where the *GNAQ* mutations were found. This result indicated that the alteration in *GNAQ* expression in lung cancer contributes to osteoclastogenesis.

Willeumier et al[37] reported that the prognosis of NSCLC patients with bone metastasis was related to the growth transfer receptor (EGFR) mutations. However, the clinical significance of *GNAQ* mutations in metastatic bone cancers is still unknown. Further research should be carried out on clinically relevant signature genes that influence the prognosis and that have potential to be used in a targeted therapy. Nevertheless, our results improve our understanding of the function of GNAQ and provide a new potential target which can offer a novel therapeutic approach to osteolytic pathologies in bone metastasis.

In conclusion, our study demonstrated that RANKL expression in metastatic bone cancers is regulated by *GNAQ*. The alterations of *GNAQ* activate the NF-κB pathway in cancers, which transforms cancer cells to express RANKL and M-CSF and induces osteoclastogenesis.

Supplementary Material

Table showing GNAQ status of patient-derived cancer cells.

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Ethical review statement
- This study was approved by the Institutional Review Board of the Samsung Medical Center (approval number: 2019-01-098).