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Risk of osteomyelitis of the jaw induced by oral bisphosphonates in patients taking medications for osteoporosis: A hospital-based cohort study in Japan

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ABSTRACT

Oral bisphosphonates (BPs) represent the first line of prevention and treatment for osteoporosis. However, several studies have reported osteonecrosis of the jaw (ONJ), also known as osteomyelitis of the jaw (OMJ), as a side effect of these drugs. Although absolute risk is suggested to be low, information to date on the relative risk or attributable risk has in fact been limited. Here, we estimated risk of oral BPs for OMJ in osteoporosis patients taking oral BPs compared with other osteoporosis drugs. Using an electronic medical records retrieval system and manual confirmation of OMJ, we conducted a retrospective cohort study of patients taking medications for osteoporosis at Kyoto University Hospital between November 2000 and October 2010. To evaluate relative risks of oral BPs for OMJ, logistic regression analysis was performed and odds ratios (ORs) and 95% confidence interval (CIs) were estimated. In addition, sensitivity analyses were performed according to hierarchical diagnosis. A total of 4129 patients (59.6%) were prescribed BPs while 2794 (40.3%) received other osteoporosis drugs. Absolute risk for OMJ was estimated to range from 0.46% to 0.99% (95% CIs: 0.25–0.66 to 0.69–1.2) among patients receiving oral BPs and 0.071% to 0.17% (95% CIs: 0–0.17 to 0.022–0.33) among patients receiving other osteoporosis drugs. The attributable risks of oral BPs for OMJ were estimated to range from 0.38% to 0.81% (95% CIs: 0.38–0.39 to 0.80–0.81). ORs (95% CIs) adjusted for confounding factors were 5.0 (1.9–12.9) to 6.0 (1.3–26.1) for confirmed cases only. In terms of absolute and attributable risks, the risk of oral BPs for OMJ is considered to be less than 1% in patients with osteoporosis. However, oral BPs may increase the risk of OMJ compared with patients treated with other osteoporosis medications and the risk of side effects should be kept in mind.

Introduction

Bisphosphonates (BPs) are used for a range of conditions involving the bone, such as osteoporosis and bone metastases of malignant cancer, and their efficacy in increasing bone mineral density, preventing further bone fractures, and reducing bone pain has been confirmed [1,2]. Nevertheless, in 2003 Marx reported bisphosphonate-related osteonecrosis of the jaw or bisphosphonate-induced osteonecrosis of the jaw as a side effect of BP treatment [3]. Since this initial report, the association between BP exposure and the incidence of osteonecrosis of the jaw (ONJ) and osteomyelitis of the jaw (OMJ) has been clarified in several case series, reviews, epidemiologic studies and clinical trials [4–16]. Here, we group OMJ together with ONJ for case ascertainment, as in previous studies and a review [15–17].

Several studies have reported prevalence of OMJ on intravenous administration of BPs ranging from 0.7% to 18.6% [6–8]. In contrast, surveillance data reported that estimated prevalence or incidence in patients treated with oral BPs (alendronate) ranged from 0.01% to 0.04% [9], or approximately 0.7 cases per 100,000 person-years' exposure [10]; while several studies reported a prevalence or incidence of OMJ on oral BP administration ranging from 0.05% to 4.3% [11–13], or 3.0 to 6.3 cases per 100,000 person-years [14]. These data suggest that the risk of OMJ is much lower in patients receiving oral than intravenous BPs. However, the low incidence of OMJ among BP-naïve patients precludes any direct estimation of the risk ratio of OMJ among osteoporosis patients treated with oral BPs, and few reports have described the relative risk of oral BPs for OMJ [14,15].

Oral BPs are the drug treatment of first choice in osteoporosis [18], a condition which affects more than 75 million people in the United States, Europe and Japan [19]. Nevertheless, effective decision making on the risks of oral BPs is hampered by a lack of information for both patients and the physicians who prescribe them. It is therefore of

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interest to evaluate the particular risk of OMJ as a side effect of oral BPs and to offer clinically relevant information to patients, physicians and dentists.

Here, we conducted a historical cohort study of patients diagnosed with and treated for osteoporosis at Kyoto University Hospital using an electronic medical records (EMR) retrieval system. Our purpose was to estimate specific risks for OMJ in osteoporosis patients taking oral BPs compared with other osteoporosis medications, with comprehensive data extraction and manual confirmation of OMJ.

Material and methods

Study design and cohort

We conducted a retrospective cohort study of patients diagnosed with osteoporosis at Kyoto University Hospital between November 2000 and October 2010. Among these patients, analysis was limited to those aged 20 years or older who had been treated with osteoporosis medications. This criterion was based on previous findings that age at first onset of BP-induced OMJ was approximately 20 years [20–23]. Eligible patients were identified using their ID at Kyoto University Hospital, and dental and medical records were examined by two reviewers from September 2011 to December 2011.

Data extraction

We used an electronic medical records retrieval system to extract data from the EMR [24]. This system retrieves electronic data for both outpatients and inpatients at Kyoto University Hospital, including demographic data, diagnosis and 10th edition of the International Classification of Diseases (ICD-10) code [25], medications and injections, laboratory tests, radiological or pathological studies, etc. First, we searched for patients who were diagnosed with osteoporosis as specified by ICD-10 code (Appendix 1) and prescribed osteoporosis medications approved in Japan (Appendix 2). We then extracted the following data for these patients: sex; date of birth; diagnosis; date of diagnosis; names, doses and dates of osteoporosis medications, hypoglycemic agent and insulin, corticosteroids and chemotherapy; and diagnosis related to malignant tumors in the oral region and diabetes as specified by ICD-10 code.

Exclusion criteria

Patients with primary or metastatic tumors or a history of trauma in the maxillofacial region were excluded, because these often induce inflammation of the jaw. Patients with a history of radiation therapy were excluded from analysis of the risk of oral BPs for OMJ, because craniofacial radiation for malignant tumors in the maxillofacial region causes osteoradionecrosis of the jaw [26]. Patients treated with intravenous BPs were also excluded, because our purpose here was to evaluate the risk of OMJ in osteoporosis patients receiving oral BPs.

Definition of OMJ cases

The American Association of Oral and Maxillofacial Surgeons, a task force of the American Society for Bone and Mineral Society and the Canadian Consensus Practice Guideline for Bisphosphonate-associated Osteonecrosis of the Jaw have stated that the hallmark of BP-induced ONJ is exposed necrotic bone in the maxillofacial region that has persisted for more than 8 weeks [27–29]. However, radiographic findings in infected jawbone in patients treated with BPs have shown that it has similar characteristics to those in BP-induced ONJ even if necrotic bone could not be clinically visualized [30,31]. In addition, the presence of osteonecrosis is a common histopathologic finding in both BP-induced ONJ and OMJ [32]. We therefore considered it appropriate to group cases of OMJ together with ONJ, as was done in previous studies and a review [15–17].

Hierarchical diagnostic criteria of OMJ

We proposed interim diagnostic criteria for OMJ in this study, using four hierarchical diagnostic criteria defined as follows: possible cases were diagnosed by increased uptake on technetium bone scan with characteristic signs and symptoms of bone infection and/or findings on dental panoramic X-ray; probable cases were diagnosed by imaging findings on computed tomography (CT) scans which were consistent with findings of possible cases; confirmed cases were diagnosed by a histological picture consistent with OMJ and/or the isolation of a microorganism in samples obtained by extraoral open surgery, percutaneous biopsy of bone, removed bone or intramedullary tissue, or pus aspiration from adjacent tissues, with findings of probable cases; and non-cases were diagnosed if not applicable to the above criteria.

Reconfirmation of OMJ

OMJ was diagnosed independently by two oral and maxillofacial surgeons. To minimize diagnosis bias, the records were examined in the following order: 1. observation of findings on dental panoramic X-ray; 2. observation of findings on technetium bone scan; 3. observation of findings on CT scan; 4. observation of findings of histological study; 5. observation of findings of surgical treatment; and 6. observation of clinical symptoms by checking progress notes. Before reviews, reviewers were trained to diagnose OMJ using a standardized protocol. We then examined inter-examiner reliability of diagnosis using 20 patients with a diagnosis of OMJ who were not included from the study population using kappa statistics. Inter-observer agreement was moderate (kappa value = 0.64 to 0.81).

First, the reviewers examined radiographic imaging and clinical records of patients with diagnoses of an inflammatory condition of the jaws (Appendix 3) to confirm their diagnoses of OMJ. Examination of X-rays, technetium bone scans, and CT scans was done using the Centricity Enterprise web v.3.0 software (GE Health Care, Little Chalfont, Buckinghamshire, England). Next, they examined radiographic imaging and records of patients with diseases possibly related to OMJ (e.g. fracture or cellulitis in the oral and maxillofacial regions, periodontal disease, or osteomyelitis and osteonecrosis in other regions, etc.) as well as records of patients suspected to have OMJ (Appendix 3), in order to decrease the false-negative rate.

In their review, the reviewers examined when oral BPs were prescribed and when OMJ occurred: cases of OMJ which developed before or without prescription of an oral BP were regarded as non BP-induced cases. They also examined whether patients had malignant tumors or any history of craniofacial radiation therapy or trauma in the maxillofacial region. In addition, they collected data concerning BP prescriptions at other hospitals when available from referral letters from physicians, and patients with a confirmed prescription in another hospital were regarded in the same way as those with a prescription in our hospital. Cases with diagnostic disagreement were discussed until they could be classified by consensus into an appropriate case category.

Confounding factors

The following confounding factors were included into the statistical analysis: age, sex, diabetes, steroid use and chemotherapy. Diabetes was diagnosed if the patient had received a diagnosis of diabetes, and had either received any treatment with hypoglycemic medication (hypoglycemic agent and/or insulin) or had an HbA1c ratio $\geq$6.5% [33]. Steroid use was defined as the receipt of any

treatment with corticosteroids, and chemotherapy use as the receipt of any treatment with cancer chemotherapy.

Statistical analysis

Patient characteristics were summarized using descriptive statistics (median, range, interquartile range, 95% confidence intervals (CIs) and percentages). Medians for continuous variables were compared using the Wilcoxon rank-sum test. Proportions across levels of categorical variables were compared using the Fisher exact test. In the analysis of characteristics by case definition, we compared the differences between overall cases and non-cases. Duration of drug administration was calculated by the sum of the number of prescription days. Once-weekly medication was treated as equivalent to 7 days' prescription. The incidence of OMJ was calculated using the cumulative incidence method, which is defined as the number or proportion of a cohort of people who experience the onset of OMJ during a specified time interval [34]. Attributable risk was defined as the difference in the population risk of disease in exposed versus unexposed patients [34]. The relative risk of oral BPs for OMJ was evaluated by logistic regression analysis with OMJ as the dependent variable, and odds ratio (ORs) for OMJ cases and 95% CIs were estimated using three models: Model 1, crude; Model 2, adjusted for sex and age; and Model 3, adjusted for Model 2 and diabetes, steroid, and chemotherapy use. In addition, the following sensitivity analyses were performed: ORs for overall cases; ORs for both probable and confirmed cases; and ORs for only confirmed cases. Goodness-of-fit of the model was examined using the Hosmer–Lemeshow goodness of fit test. All *P* values were two-sided at a significance level of 5%. All statistical analyses were performed using Stata 11.2 software (Stata Corporation, College Station, TX, USA).

Sample size estimation

Almost all previous studies reported that the prevalence or incidence of ONJ or OMJ in patients treated with oral BPs was low, at up to 0.34% [9,11,12]. In contrast, the incidence of ONJ or OMJ among BP-naïve osteoporosis patients was unclear at the time this present study began. Black et al. identified only 1 patient with possible ONJ among 3852 postmenopausal women without BPs (0.025%) [35]. Hence, to compare the proportion of patients receiving oral BPs with those receiving other osteoporosis medications, at least 2842 patients in each group were estimated for inclusion with an alpha set at 0.05 and beta set at 0.10, assuming that the proportion of OMJ in patients receiving BPs was 0.50% and that in patients receiving other osteoporosis medications was 0.025%.

Ethical approval

This study protocol was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine and conducted according to the 'Ethical Guidelines for Epidemiological Research' [36].

Results

A total of 7062 patients treated with osteoporosis medications and aged 20 years or older were included. Among these, the reviewers examined records of 84 patients suspected of having OMJ and 1986 patients with diseases possibly related to OMJ. In their review, they confirmed that 7 patients had been treated with BPs (including 1 patient receiving intravenous BPs) in other hospitals and 6 had a history of craniofacial radiation therapy in the maxillofacial region. No patient was confirmed to have developed OMJ due to trauma in the maxillofacial region or before receiving a prescription of BP. After exclusion of 29 patients with primary or metastatic tumors in the oral region and/or a history of craniofacial radiation therapy and 110 patients receiving intravenous BPs, 6923 (98.0%) eligible patients were entered into the analysis.

Patient characteristics are summarized in Table 1. The total number of patients prescribed oral BPs was 4129 (59.6%), while 2794 (40.3%) received other osteoporosis drugs. Median age was lower ($P=0.022$) and the percentage of females, and steroid and chemotherapy users was higher among those prescribed BPs ($P<0.001$), whereas the prevalence of diabetes did not differ between the two groups ($P=0.15$).

Patient characteristics according to our four case definitions are shown in Table 2. Forty-six patients receiving osteoporosis medications developed OMJ (0.66%, 95% CIs: 0.47–0.85). Compared with non-cases, cases were older ($P=0.049$) but the percentage of females, diabetes patients, and steroid or chemotherapy users did not differ between the two groups ($P>0.05$). All patients who developed OMJ received one or more kinds of nitrogen-containing BPs (NBPs), namely alendronate, risedronate and minodronate. The median duration of BP administration among case patients prescribed oral BPs was longer than that in non-cases. Among the 4129 patients prescribed oral BPs, 19 cases were diagnosed as confirmed, 7 as probable, and 15 as possible, giving an estimated absolute risk (i.e. incidence) of oral BPs for OMJ ranging from 0.46% to 0.99% (95% CIs: 0.25–0.66 to 0.69–1.2). In contrast, among the 2794 patients prescribed other osteoporosis drugs, 2 cases were diagnosed as confirmed, 1 as probable, and 2 as possible, giving an estimated absolute risk ranging from 0.071% to 0.17% (95% CIs: 0–0.17 to 0.022–0.33). The attributable risks of oral BPs for OMJ were estimated to range from 0.38% to 0.81% (95% CIs: 0.38–0.39 to 0.80–0.81).

Table 3 shows sensitivity analyses and adjusted ORs for OMJ according to case definitions. Crude ORs (95% CIs) were 5.5 (2.2–14.1) for overall cases, 5.8 (1.7–19.4) for both probable and possible cases, and 6.4 (1.5–27.7) for confirmed cases only. After adjustment for potential confounding factors, ORs were 5.0 (1.9–12.9) for overall cases, 5.4 (1.6–18.3) for both probable and possible cases, and 6.0 (1.3–26.1) for confirmed cases only. The final multivariable adjusted model was reliable ($P=0.55$ to 0.61 by the Hosmer–Lemeshow test).

Table 1
Characteristics of patients taking medications for osteoporosis at Kyoto University.

	BP administration	Other osteoporosis drugs	*P* value
Number	4129	2794	
Median age (range)	65.0 (20–99)	65.5 (20–97)	0.022
Sex, n (%)			
Male	814 (19.7)	725 (25.9)	<0.001
Female	3315 (80.2)	2069 (74.0)	
Diabetes, n (%)			
Yes	707 (17.1)	442 (15.8)	0.15
No	3422 (82.8)	2352 (84.1)	
Steroid use, n (%)			
Yes	2934 (71.0)	1508 (53.9)	<0.001
No	1195 (28.9)	1286 (46.0)	
Chemotherapy use, n (%)			
Yes	551 (13.3)	256 (9.1)	<0.001
No	3578 (86.6)	2538 (90.8)	
Oral BP administration[a]			
Etidronate, n (%)	548 (13.2)	N.A.	N.A.
Alendronate, n (%)	2871 (69.5)	N.A.	
Risedronate, n (%)	1604 (38.8)	N.A.	
Minodronate, n (%)	38 (0.92)	N.A.	
Duration of administration (days)			
Median duration (IQR)	364.0 (90–966)	439.5 (98–1413)	<0.001

BPs = bisphosphonates; N.A. = not applicable; IQR = interquartile range.
Medians for continuous variables were compared using the Wilcoxon rank-sum test.
Proportions across levels of categorical variables were compared using the Fisher exact test.
[a] In some cases, several oral BPs were prescribed for one patient.

Table 2
Demographic and risk factor characteristics of patients by case definition.

	Hierarchical diagnostic criteria			Overall cases	Non-cases	*P* value
	Confirmed cases	Probable cases	Possible cases			
Number	21	8	17	46	6877	
Median age (range)	68.0 (35–84)	74.5 (50–83)	66.0 (23–79)	69.0 (23–84)	65.0 (20–99)	0.049
Sex, n (%)						
Male	3 (14.2)	1 (12.5)	3 (17.6)	7 (15.2)	1532 (22.2)	0.29
Female	18 (85.7)	7 (87.5)	14 (82.3)	39 (84.7)	5345 (77.7)	
Diabetes, n (%)						
Yes	2 (9.5)	1 (12.5)	4 (23.5)	7 (15.2)	1142 (16.6)	1.0
No	19 (90.4)	7 (87.5)	13 (76.4)	39 (84.7)	5735 (83.3)	
Steroid use, n (%)						
Yes	15 (71.4)	6 (75.0)	14 (82.3)	35 (76.0)	4407 (64.0)	0.12
No	6 (28.5)	2 (25.0)	3 (17.6)	11 (23.9)	2470 (35.9)	
Chemotherapy use, n (%)						
Yes	3 (14.2)	0	2 (11.7)	5 (10.8)	802 (11.6)	1.0
No	18 (85.7)	8 (100)	15 (88.2)	41 (89.1)	6075 (88.3)	
Route of administration, n (%)						
Oral BPs	19 (90.4)	7 (87.5)	15 (88.2)	41 (89.1)	4088 (59.4)	<0.001
Other osteoporosis drugs	2 (9.5)	1 (12.5)	2 (11.7)	5 (10.8)	2789 (40.5)	
Oral BP administration[a]						
Etidronate, n (%)	4 (19.0)	0	3 (17.6)	7 (15.2)	541 (7.8)	N.A.
Alendronate, n (%)	15 (71.4)	3 (37.5)	9 (52.9)	27 (58.7)	2844 (41.3)	
Risedronate, n (%)	7 (33.3)	4 (50.0)	9 (52.9)	20 (43.4)	1584 (23.0)	
Minodronate, n (%)	1 (4.7)	1 (12.5)	0	2 (4.3)	36 (0.52)	
Duration of BP administration (days)[b]						
Median days (IQR)	1267 (182–2009)	380 (84–1342)	588 (273–1630)	707 (210–1630)	358.5 (89.5–966)	0.001

BPs = bisphosphonates; N.A. = not applicable; IQR = interquartile range.
Wilcoxon rank-sum test or Fisher exact test were performed to compare the differences between overall cases and non-cases.
[a] In some cases, several oral BPs were prescribed for one patient.
[b] Duration was calculated for 4129 patients treated with oral BPs.

Discussion

We conducted a retrospective cohort study with comprehensive data extraction using an EMR retrieval system and manual confirmation of ONJ according to standardized procedures. We evaluated not just the absolute risk, but also the attributable risk of OMJ induced by the administration of oral BPs. In addition, we estimated the relative risks (ORs) for OMJ, which ranged from 5.0 (95% CIs: 1.9–12.9) to 6.0 (95% CIs: 1.3–26.1) after adjustment for confounding factors. This study provides a significant and comprehensive estimation of the specific risks of OMJ in osteoporosis patients taking oral BPs compared with other osteoporosis medications.

Few reports have examined the relative risk of oral BPs for OMJ, and the risk remains unclear. The Dental Practice-based Research Network (DPBRN) reported an unadjusted OR for ONJ of 15.5 (95% CIs: 6.0–38.7) in two health-care organizations [14], and a Danish group reported an adjusted hazard ratio of alendronate for inflammatory jaw disease of 3.1 (95% CIs: 1.4–6.8) and for etidronate of 2.2 (95% CIs: 1.1–4.3) in Danish population [15]. Our results are consistent with these findings and indicate an increased risk of OMJ in osteoporosis patients treated with oral BPs even after adjustment for confounding factors.

The estimated absolute risk of oral BPs for OMJ in this study was slightly higher than those of previous reports. Among findings to date, surveillance data from Australia estimated a prevalence of ONJ ranging from 0.01% to 0.04% or 0.09% to 0.34% after tooth extraction [9]; a Korean group reported a prevalence ranging from 0.05% to 0.07% among patients treated with oral BPs in a university hospital [11]; and the DPBRN in the US reported the occurrence of ONJ in 6 of 21,163 cohort members who had at least one oral BP dispensed, giving an estimated incidence of approximately 0.028% [14]. On the other hand, another US group reported a prevalence of ONJ of approximately 4% in patients treated with alendronate in a university hospital [13]. Characteristics of our study design include a hospital-based setting; inclusion of patients treated with other osteoporosis medications; classification of OMJ and ONJ cases together; and diagnosis of OMJ according to four hierarchical

Table 3
Adjusted odds ratios for osteomyelitis of the jaw by case definition.

	Osteomyelitis of the jaw		Odds ratio		
	Cases, n (%)	Non-cases, n (%)	Crude	Age- and sex-adjusted	Multivariable-adjusted
Possible cases ≥					
Oral BPs (+)	41 (89.1)	4088 (59.4)	5.5 (2.2–14.1)	5.5 (2.1–14.1)	5.0 (1.9–12.9)
Oral BPs (−)	5 (10.8)	2789 (40.5)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Probable cases ≥					
Oral BPs (+)	26 (89.6)	4103 (59.5)	5.8 (1.7–19.4)	5.9 (1.7–19.5)	5.4 (1.6–18.3)
Oral BPs (−)	3 (10.3)	2791 (40.4)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Confirmed cases only					
Oral BPs (+)	19 (90.4)	4110 (59.5)	6.4 (1.5–27.7)	6.4 (1.4–27.7)	6.0 (1.3–26.1)
Oral BPs (−)	2 (9.5)	2792 (40.4)	1.0 (ref)	1.0 (ref)	1.0 (ref)

BPs = bisphosphonates; ref = reference.
Odds ratios (95% confidence interval) are shown.
These multivariate odds ratios for osteomyelitis of the jaw were adjusted for age, sex, diabetes, and steroid and chemotherapy use.

diagnostic criteria. Estimated incidence among the studies may vary by setting, design, or population. Additionally, incidence might also be influenced by dental hygiene [37], albeit that our present and previous studies have not examined dental hygiene at the population level. Clinical decision making for osteoporosis patients should be done in consideration of the risk and benefit of oral BPs in the target population.

BPs can be classified into two groups, with different molecular modes of action, namely NBPs and non-nitrogen-containing BPs (NNBPs) [1]. In this study, we confirmed that all patients who developed OMJ received one or more kinds of NBPs. Several clinical studies have also shown that NBP use is associated with an increased risk of ONJ [6,8,11,16]. Further, NBPs were shown to exert a strong negative effect on human oral keratinocytes at different cellular levels in vitro compared to NNBPs [38]. These results indicate that patients taking NBPs have a higher risk of OMJ than those receiving NNBPs even among oral bisphosphonate users. However, although a few studies have shown the occurrence of ONJ or increased incidence of inflammation of the jaw in users of oral NNBPs (i.e. clodronate or etidronate) [9,15], the risk of oral NNBPs for OMJ remains unclear. Further investigation in a different population is required to examine the hypothesis that patients taking oral NNBPs have a lower risk of OMJ than those receiving oral NBPs.

An additional characteristic of this study was our proposal of four interim hierarchical diagnostic criteria for OMJ. In general, OMJ is diagnosed by the presence of a compatible clinical picture; consistent imaging findings on plain radiographs and/or computed tomography scans and/or increased uptake on technetium bone scan; and a histological picture consistent with OMJ and/or the isolation of microorganisms in samples obtained by extraoral open surgery, percutaneous biopsy of bone, removed bone or intramedullary tissue, or pus aspiration from adjacent tissues [39]. Diagnosis is often difficult, however, particularly in the early stage [26], and these criteria are not always consistently applied to different stages of OMJ. Osteomyelitis is caused by a certain inciting focus that enables the infection to propagate and has various clinical expressions, and the clinical and laboratory features of infections are not always present [26,40]. This background explains why diagnostic imaging has long played a major role in the investigation of suspected osteomyelitis [41]. To date, however, the accuracy of radiographic imaging for OMJ has not been clarified. Accordingly, we set priority on these radiographic findings for OMJ with reference to previous reviews of osteomyelitis in the other regions [40,41]. A review of osteomyelitis reports as follows: plain radiographs should always be the first step in the imaging assessment of osteomyelitis, but sensitivity or specificity is low; scintigraphic procedures are an essential part of the diagnostic procedure; and CT scans are a useful adjunct to conventional radiography when findings are normal in cases clinically suspected to have skeletal infection [40]. In addition, there is an agreement that the objective standard for diagnosing osteomyelitis is bone biopsy and culture [41]. On the other hand, a review of OMJ reports that routine radionuclide bone scans have low specificity and other problems that may be mitigated by the addition of CT scans, because increased uptake on blood flow phase images may be seen with soft tissue infection or at surgical sites in the jaw, etc. [26]. These reviews indicated that CT scans were of greater value in diagnosing OMJ than technetium bone scans or plain radiographs, but that the highest priority was given to a histological picture consistent with OMJ and/or the isolation of a microorganism in samples obtained at surgical treatment.

Data extraction from EMR in this study was conducted using an EMR retrieval system [24]. In two previous large cohort studies of the risk of oral BPs, data were extracted from a health maintenance organization database and an administrative claims database [14,15]. However, these databases have not been designed for medical research, and EMR data are richer than information in claims databases [42]. In addition, outcome was diagnosed using codes for osteomyelitis [15], but automated data extraction without human intervention has not reached a suitable level of accuracy [43]. In this study, two oral and maxillofacial surgeons diagnosed cases by chart review with observation of imaging findings. Furthermore, to guard against false-negative cases, they examined a total of 1986 patients (28.1%) among the included patients. This comprehensive data extraction process and manual confirmation of OMJ likely improve the reliability of our results.

Several limitations of this study warrant mention. First, selection bias is inherent to single-center studies, and the present study was additionally subject to inherent referral bias toward the selection of more severe cases, given that our department is a lead institution for oral and maxillofacial surgery in Kyoto City. A positive aspect of this limitation, however, is that almost all patients likely consult our department in the clinical problem of oral and maxillofacial regions. Additionally, OMJ is an uncommonly encountered clinical condition, and such patients are likely to be referred to our clinic to establish a diagnosis. The impact of selection bias is thus somewhat unclear. Second, although our estimation models were adjusted for confounding factors, including diabetes, and steroid and chemotherapy use [28,44], no adjustment was made for other possible confounding factors related to OMJ, such as smoking, immune disorders, or oral BP dose, etc. [14,27]. However, several studies reported that there was no association between dose of oral BP or other risk factors and inflammation of the jaw [15,45], and risk factors of BP-induced OMJ are controversial. Further investigation is required to clarify other risk factors for oral BP-induced OMJ. Third, we might have overestimated the risk of oral BPs. In the chart review, we confirmed 6 patients treated with oral BPs in other hospitals; however, other patients who were treated without oral BPs in our hospital may have received oral BPs in other hospitals. We therefore performed sensitivity analyses with exclusion of these patients, but the significant results did not change (data not shown). Fourth, due to the limited number of events, the 95% CIs of estimated ORs were wide. This prevents the drawing of reliable conclusions from the results, and indicates the need to assess relative risks in a larger number of patients with OMJ.

Conclusions

In terms of absolute and attributable risks, the risk of oral BPs for OMJ is considered to be less than 1% in osteoporosis patients. However, oral BPs may increase the risk of OMJ compared with patients treated with other osteoporosis medications, and the extent of side effects should be kept in mind. This study provides important information for patients, physicians and dentists involved in the treatment of osteoporosis using oral BPs.

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TY participated in the design of the study, performed the statistical analysis and drafted the manuscript. MY participated in the design of the study, checked the statistical analysis and helped to draft the manuscript. KY participated in the design of the study, and programmed and extracted data from the hospital database using the EMR retrieval system. KS and KA participated in the design of the study and performed the chart review. ES participated in the design of the study and helped to draft the manuscript. KG and KT participated in the design of the study. TN participated in the design and checked the statistical analysis. KB participated in the design of the study and was the principal investigator of the study. All authors have read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bone.2012.08.115.

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Increased incidence of osteonecrosis of the jaw after tooth extraction in patients treated with bisphosphonates: a cohort study

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Abstract. This study estimated the cumulative incidence and risk ratio for osteonecrosis of the jaw (ONJ) after tooth extraction in patients with and without administration of bisphosphonates (BP) and identified potential risk factors for bisphosphonate-induced osteonecrosis of the jaw (BIONJ). A cohort study was conducted in all patients undergoing tooth extraction at a university hospital in Japan from April 2006 to June 2009. Of 3216 patients, 126 had BP administration, of whom 5 (3.9%, 95% confidence interval (CI): 1.2–9.2) developed ONJ, *versus* 1 (0.032%, 95% CI: 0.00081–0.18) among 3090 patients without BP administration. BP administration was associated with the development of ONJ after tooth extraction, with an unadjusted risk ratio of 122.6 (95% CI: 14.4–1041.8). When stratified by age and route of BP administration, the risk ratio for ONJ patients aged 65 years or older with intravenous BP administration compared to those without was 200.2 (95% CI: 23.8–1679.4, $P < 0.001$). Patients receiving BP showed a significant association between the incidence of BIONJ and alveolar bone loss score. The risk of ONJ is higher in patients with than without BP administration, particularly intravenous administration. Severe periodontitis might be a risk factor for BIONJ.

Key words: alveolar bone loss; bisphosphonate; incidence; jaw; osteonecrosis; relative risk; tooth extraction.

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Bisphosphonates (BP) are used for the treatment of a range of bone problems, such as osteoporosis or bone metastases of malignant cancer, and their efficacy in preventing further bone damage, reducing bone pain, and increasing bone mineral density has been confirmed. In 2003, Marx reported bisphosphonate-related osteonecrosis of the jaw or bisphosphonate-induced osteonecrosis of the jaw (BIONJ), as a side effect of BP treatment.[1] The incidence and mechanism of BIONJ have not been determined accurately.

Since that initial report, the association between BP exposure and the incidence of osteonecrosis of the jaw (ONJ) has been clarified in several case series, reviews,

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epidemiologic studies and clinical trials,[2–11] which reported a prevalence of BIONJ ranging from 0.7% to 18.6% on intravenous[5–7] and 0.01% to 4.3% on oral administration.[8,9] The low incidence of ONJ among BP-naïve patients has prevented any direct estimation of the risk ratio of ONJ among patients receiving BP. Black et al. identified only 1 patient with possible ONJ among 3852 postmenopausal women during a 3-year period.[10] In their 6-year population-based cohort study using medical claims data, Wilkinson et al. found that 0.30% of naïve patients had been diagnosed with inflammatory conditions or osteomyelitis of the jaw but not ONJ.[11]

Tooth extraction has been reported to be the main initiating factor and one of the most common risk factors for BIONJ among patients receiving BP (approximately 86% of cases),[8,12,13] and relative risk of BIONJ in these patients is 5.3–53 times higher than in BP patients who do not experience tooth extraction.[5,14,15] A current guideline recommends non-surgical treatment rather than tooth extraction in dental patients at high risk of BIONJ,[16] but given that bacterial infection is reported as a critical risk factor for BIONJ,[16] avoiding extraction might be problematic in cases in which the bacterial infection remains. Information on the incidence or risk factors for BIONJ after tooth extraction among patients receiving BP is limited. A better understanding of this condition, particularly with regard to risk factors and incidence, will be helpful to dentists in the care of patients receiving treatment with BP.

The purpose of this study was to estimate the cumulative incidence and risk ratio for ONJ after tooth extraction in patients with and without administration of BP, and to identify potential risk factors for BIONJ, including oral status.

Materials and methods

The authors conducted a retrospective analysis of patients who had undergone tooth extraction between April 2006 and June 2009 at the Department of Oral and Maxillofacial Surgery, Kyoto University Hospital. Patients were identified using administrative data, and dental and medical records were reviewed from January 2010 to August 2010. This study protocol was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine.

Inclusion was restricted to patients aged 20 years or older. ONJ in this study was diagnosed by the presence of exposed bone in the maxillofacial region that had persisted for more than 8 weeks, in reference to diagnostic criteria formulated by the American Association of Oral and Maxillofacial Surgeons.[16] Diagnoses were determined independently by two oral and maxillofacial surgeons. Among patients undergoing tooth extraction at the authors' institution, the following were excluded: tooth extraction in patients with metastatic tumours to the oral region; signs of osteomyelitis or exposed bone in the maxillofacial region before extraction; any history of craniofacial radiation therapy; and patients for whom panoramic radiographs before extraction were not available. Cases with diagnostic disagreement were discussed. Finally, ONJ cases with diagnostic disagreement were regarded as non-ONJ cases and analysed with the non-ONJ patients. The kappa value for inter-observer agreement was 0.86 (95% confidence interval (CI); 0.82–0.89).

For each chart reviewed, the following information was collected: demographics; medical history; test results; and potential risk factors associated with ONJ, namely the use of steroids, chemotherapy (including anticancer agents, immunosuppressive agents and thalidomide), current smoking status, current alcohol intake, diabetes, and details of BP treatment (indication, type, dose, duration). Recommended regimens for oral BP administration in the treatment of osteoporosis in Japan are etidronate sodium, 200 mg per day (Didronel®); alendronate sodium, 5 mg per day or 35 mg per week (Fosamac® or Bonalon®); and risedronate sodium, 2.5 mg per day or 17.5 mg per week (Actonel® or Benet®), while those for intravenous administration in the treatment of osteolytic bone metastases of malignant cancer, multiple myeloma, or hypercalcemia of malignancy are incadronate, 10 mg per time (Bisphonal®); pamidronate, 30–45 mg for hypercalcemia of malignancy, or 90 mg every 4 weeks for osteolytic bone metastases of malignant cancer (Aredia®); and zoledronic acid, 4 mg every 4 weeks (Zometa®). Intravenous BP has not been approved for osteoporosis in Japan.

For patients treated with BP in the hospital, these medications were entered into the electronic medical record system to obtain the type and duration of BP administration, and the number of patients to whom they were administered, to estimate the incidence of BIONJ. For patients treated with BP in other hospitals, the authors reviewed the record of their first examination at the authors' hospital, at which the type and duration of BP administration as obtained from the referring physician by letter is recorded.

Using the patient's panoramic radiograph before tooth extraction, an experienced examiner calculated the DMF index and severity of alveolar bone loss. The DMF index, which comprises the number of decayed (D), missing (M), and filled (F) teeth, has been established as a key measurement of caries experience in dental epidemiology.[17] The severity of alveolar bone loss was measured to examine periodontal status as a percentage of missing bone at the mesial and distal surfaces of each tooth present.[18] Severity was estimated from $(a - b)/a \times 100$ (%) using the panoramic radiograph (where a is the distance from radiographic apex to cement–enamel junction (mm) and b is the distance from radiographic apex to interproximal alveolar bone crest (mm)). If the location of the cemento-enamel junction was obscured by interproximal fillings, the cervical margin of these fillings was chosen as standard. If the cervical margin of these fillings was obscured, bone loss height was characterized as unmeasurable. Each tooth surface was assigned a score corresponding to a bone loss of 0–24%, 25–49%, 50–74%, and 75–100%, respectively. These measurements were averaged to yield a single mean bone loss score for each patient, with a higher score indicating more severe periodontal disease.[19]

Statistical analysis

The incidence of ONJ was calculated using the cumulative incidence method, which is defined as the number or proportion of a cohort of people who experience the onset of ONJ during a specified time interval.[20] In the calculation of the CI for incidence, the Poisson distribution was used. Medians for continuous variables were compared using the Wilcoxon rank-sum test. Proportions across levels of categorical variables were compared using the Fisher exact test. Risk ratios were calculated for all dichotomous variables and Wald CIs were calculated.[20] All P values were two sided at a significance level of 5%. For missing data, available-case analysis was performed in addition to multiple imputation analysis. All statistical analysis was performed using Stata 11.2 software (Stata Corporation, College Station, TX, USA).

Results

3240 patients underwent tooth extraction at the authors' institute. After exclusion of 8 patients with a history of craniofacial radiation therapy and 16 without a panoramic radiograph, 3216 (99.2%) eligible patients were entered into the analysis. Patient

Table 1. Patient characteristics.

Characteristic	BP administration	
	Yes ($n = 126$)	No ($n = 3090$)
Age (years)		
Median	66.0	39.0
Range	26–88	20–94
Sex (%)		
Male	23 (18.3)	1490 (48.2)
Female	103 (81.7)	1600 (51.8)
Primary disease (%)		
Osteoporosis*	99 (78.6)	74 (2.4)
Breast cancer*	13 (10.3)	61 (2.0)
Multiple myeloma	11 (8.7)	5 (0.2)
Prostate cancer	1 (0.8)	41 (1.3)
Kidney cancer	3 (2.4)	1 (0.03)
Other cancer	6 (4.8)	184 (5.6)
Route of BP administration (%)		
Oral	99 (78.6)	N.A.
Intravenous	27 (21.4)	N.A.

BP: bisphosphonate; N.A.: not applicable.
* Seven patients with BP administration were diagnosed with both osteoporosis and breast cancer.

characteristics are summarized in Table 1. 126 patients (3.9%) had received BP; among the 103 women (81.7%), 86 (83.5%) were treated with oral BP and 17 (16.5%) with intravenous BP, while among the 23 men, 13 (56.5%) received oral and 10 (43.5%) received intravenous BP. Seven patients were diagnosed with both osteoporosis and breast cancer, all of whom had been treated with oral BP. The total number of patients without BP administration was 3090 (96.0%), of whom 51.8% were women. Median age was lower (39.0 years) than that of patients administered BP (66.0 years).

Cumulative incidence of ONJ after tooth extraction

Five of the 126 patients receiving BP developed ONJ (3.9%, 95% CI: 1.2–9.2), *versus* 1 (0.032%, 95% CI: 0.00081–0.18) of the 3090 without BP administration. Individual data from the 6 patients with ONJ are

Table 2. Demographic and clinical characteristics of ONJ patients.

Sex	Age	Primary disease	Type of BP	Dosage of BP (mg)	Duration of BP (months)	Other risk factors	Cure and prognosis
F	75	Osteoporosis	A	1990	13	None	Sequestrectomy, healing
F	75	Breast cancer	P	1455	45	Chemo therapy	Currettage, nonhealing
			Z	16	4		
F	68	Multiple myeloma	P	1305	19	Thalidmide Steroid	Sequestrectomy, healing
M	75	Prostate cancer	Z	32	8	Chemo therapy	Die of primary disease
M	67	Kidney cancer	Z	28	7	None	Sequestrectomy, healing
M	73	None	None	–	–	None	Sequestrectomy, healing

ONJ: osteonecrosis of the jaw; BP: bisphosphonate; A: alendronate; P: pamidronate; Z: zoledronic acid.

Table 3. Stratified analysis of osteonecrosis of the jaw after tooth extraction.

	ONJ (+) ($n = 6$)	ONJ (–) ($n = 3210$)	Total ($n = 3216$)	Risk ratio (95% CI)	*P* value
Total					
BP (+)	5 (83.3)	121 (3.8)	126	122.6 (14.4–1041.8)	<0.001
BP (–)	1 (16.7)	3089 (96.2)	3090	1.0 (ref)	
BP-stratified					
Oral BP (+)	1 (16.7)	98 (3.1)	99	31.2 (1.9–495.4)	0.061
IV BP (+)	4 (66.6)	23 (0.7)	27	457.7 (52.8–3962.7)	**<0.001**
BP (–)	1 (16.7)	3089 (96.2)	3090	1.0 (ref)	
Age-stratified (≥65 years)					
BP (+)	5 (16.7)	63 (8.3)	68	51.5 (6.1–434.8)	**<0.001**
BP (–)	1 (83.3)	700 (91.7)	701	1.0 (ref)	
Age-stratified (<65 years)					
BP (+)	0	58 (2.4)	58	N.A.	N.A.
BP (–)	0	2389 (97.6)	2389	1.0 (ref)	
BP- and age-stratified (≥65 years)					
Oral BP (+)	1 (16.7)	53 (7.0)	54	12.9 (0.82–204.6)	0.138
IV BP (+)	4 (66.6)	10 (1.3)	14	200.2 (23.8–1679.4)	**<0.001**
BP (–)	1 (16.7)	700 (91.7)	701	1.0 (ref)	
BP- and age-stratified (<65 years)					
Oral BP (+)	0	45 (1.8)	45	N.A.	N.A.
IV BP (+)	0	13 (0.53)	13	N.A.	N.A.
BP (–)	0	2389 (97.6)	2389	1.0 (ref)	

The Fisher exact test was used to compare differences in the incidence of osteonecrosis of the jaw between patients with and without BP administration.
Statistically significant *P* values appear in bold.
ONJ: osteonecrosis of the jaw; CI: confidence interval; BP: bisphosphonate; IV: intravenous; ref: reference; N.A.: not applicable.

shown in Table 2. The cumulative incidence of ONJ among patients with BP administration was significantly higher than that among those without ($P < 0.001$). The crude risk ratio for ONJ after tooth extraction for patients with BP administration compared to those without was 122.6 (95% CI: 14.4–1041.8). By route of administration, cumulative incidence was 1.0% (95% CI: 0.025–5.6) among patients treated with oral BP and 14.8% (95% CI: 4.0–37.9) in those treated with intravenous BP. The risk ratio for ONJ was 31.2 (95% CI: 1.9–495.4) among patients treated with oral BP and 457.7 (95% CI: 52.8–3962.7) in those treated with intravenous BP. Large differences were seen in age and prevalence of cancer or osteoporosis between BP and BP-naïve patients. The authors therefore performed stratified analysis by age and route of BP administration to estimate risk ratios for ONJ to control for these factors (Table 3). Among patients aged 65 years or older (median 73, range 65–94 years), the risk ratio for ONJ for patients with oral or intravenous BP administration compared to those without was 12.9 (95% CI: 0.82–204.6, $P = 0.138$) or 200.2 (95% CI: 23.8–1679.4, $P < 0.001$), respectively.

Association with possible risk factors for BIONJ after tooth extraction

The authors investigated potential risk factors associated with BIONJ among patients with BP administration. The incidence of BIONJ was significantly associated with the type of BP ($P = 0.007$) but not with the other potential risk factors (Table 4). The crude risk ratio for intravenous compared with oral BP administration was 14.6 (95% CI: 1.7–125.8). The type, dosage and duration of intravenous BP administration are shown in Table 5. Median duration in patients with BIONJ (13.5, range 7–49 months) was longer than that in patients without BIONJ (7.5, range 1–96 months), but this difference was not significant. Regarding oral BP, etidronate, alendronate and risedronate were given as single agents to 4 (4.0%), 54 (54.5%), and 31 patients (31.3%), respectively, while the rest (10.2%) received a series of double BP agents.

Regarding oral status among patients receiving BP, the authors found a significant association between the incidence of BIONJ and bone loss score but not DMF index. The median bone loss score among BIONJ patients was significantly higher than that among those without BIONJ ($P = 0.024$) (Table 4). While median bone loss score among those without BIONJ was 1.3 (interquartile range 1.1–1.6) and the prevalence of severe periodontal status was 7.4%, median score among BIONJ patients was 1.6 (interquartile range 1.5–1.9) and the prevalence of severe periodontal status was 20.0%. Similarly, this association was found significantly among patients aged 65 years or older (median, 1.4 *versus* 1.6, $P = 0.034$).

Table 4. Potential risk factors for osteonecrosis of the jaw after tooth extraction.

Variable	Bisphosphonate-induced osteonecrosis of the jaw			
	Yes ($n = 5$)	No ($n = 121$)	RR (95% CI)	P value
Sex—no. (%)				
Male	2 (40.0)	21 (17.4)	2.9 (0.52–16.8)	0.225
Female	3 (60.0)	100 (82.6)	1.0 (ref)	
Age				
Median	75.0	65.0	N.A.	0.133
Range	67–77	26–88		
Administration route—no. (%)				
Intravenous	4 (80.0)	23 (19.0)	14.6 (1.7–125.8)	0.007
Oral	1 (20.0)	98 (81.0)	1.0 (ref)	
Steroid use—no. (%)				
Yes	2 (40.0)	54 (44.6)	0.83 (0.14–4.8)	1.000
No	3 (60.0)	67 (55.4)	1.0 (ref)	
Chemotherapy use—no. (%)				
Yes	3 (60.0)	23 (19.0)	5.7 (1.0–32.7)	0.059
No	2 (40.0)	98 (81.0)	1.0 (ref)	
Diabetes—no. (%)				
Yes	0 (0)	9 (7.4)	N.A.	N.A.
No	5 (100)	112 (92.6)		
Current smoking—no. (%)*				
Yes	0 (0)	21 (18.4)	N.A.	N.A.
No	5 (100)	93 (81.6)		
Current alcohol intake—no. (%)*				
Yes	1 (20.0)	29 (25.4)	0.74 (0.086–6.3)	1.000
No	4 (80.0)	85 (74.6)	1.0 (ref)	
DMF count				
Median	21	19	N.A.	0.548
Range	13–28	0–28		
D count				
Median	2	1	N.A.	0.085
Range	1–4	0–15		
M count				
Median	7	5	N.A.	0.209
Range	6–14	0–27		
F count				
Median	10	10	N.A.	0.910
Range	4–14	0–25		
Bone loss score				
Median	1.6	1.3	N.A.	**0.022**
Range	1.5–2.2	1.0–3.5		

P values were calculated using the Fisher exact test or Wilcoxon rank-sum test.
The Fisher exact test was used to compare differences in the presence of potential risk factor between patients with and without BIONJ.
Statistically significant P values appear in bold.
RR: risk ratio; CI: confidence interval; ref: reference; N.A.: not applicable; D: decayed teeth; M: missing teeth; F: filling teeth.
* Data were not available for seven patients and the valid percentages are shown.

Missing data

Potential risk factors were not available for eight patients, namely the duration of BP administration for one patient and current alcoholic intake and current smoking for seven patients. The authors therefore performed available-case analysis (Tables 4 and 5). Additionally, they performed multiple imputation analysis, but the results did not change from those of available-case analysis (data not shown).

Discussion

The authors found that the cumulative incidence of ONJ among patients who had received BP administration was significantly higher than that among patients who had not received this treatment (crude risk ratio 122.6, 95% CI: 14.4–1041.8). To the authors' knowledge, this study is the first cohort study to evaluate the cumulative incidence and risk ratio for ONJ after tooth extraction among patients with or

Table 5. Type, dosage and duration of intravenous BP administration.

	Bisphosphonate-induced osteonecrosis of the jaw		
	Yes ($n = 4$)	No ($n = 22^*$)	*P* value
Dosage of IV BP administration (mg)			
Single-drug administration			
Incadronate			
No.	0	1	
Median (range)	0	40	
Pamidronate			
No.	1	2	
Median (range)	1305	30 (15–45)	
Zoledronic acid			
No.	2	11	
Median (range)	30 (28–32)	44 (4–104)	
Combined-drug administration			
Incadronate + pamidronate			
No.	0	2	
Median (range)	0	*I*: 1640 (210–3070) *P*: 1608 (470–2745)	
Pamidronate + zoledronic acid			
No.	1	6	
Median (range)	*P*: 1455 *Z*: 16	*P*: 75 (30–2160) *Z*: 50 (12–56)	
Duration of IV BP administration (month)			
Median (range)	13.5 (7–49)	7.5 (1–96)	0.333

P values were calculated using the Wilcoxon rank-sum test.
BP: bisphosphonate; I: incadronate; P: pamidronate; Z: zoledronic acid; IV: intravenous.
* Data were not available for one patient.

without BP administration. Additionally, risk ratio for ONJ was particularly elevated in the subpopulation of patients with intravenous BP administration.

Age-stratified analysis showed no significant association between the incidence of ONJ after tooth extraction and oral BP among elderly patients aged over 65 years, but a significant association with intravenous BP administration (risk ratio 200.2, 95% CI: 23.8–1679.4).

These results indicate that intravenous BP may be associated with an increased risk of ONJ after tooth extraction among elderly patients over 65 years. Dental practice-based research network (DPBRN) reported an unadjusted odds ratio for ONJ for patients with oral BP administration compared to those without of 15.5 (95% CI: 6.0–38.7) in two healthcare organizations, regardless of a history of tooth extraction.[21] The impact of oral BP on the risk of ONJ after tooth extraction requires further investigation.

In the DPBRN study, the investigators had so little hospital information that they could not estimate the risk ratio of intravenous BP.[21] Although the present study was a single-centre study, the authors were able to collect detailed BP administrative data from the hospital database and estimate the risk ratio stratified by the route of BP administration. In addition, oral and maxillofacial surgeons followed up all patients after tooth extraction, diagnosed ONJ, and excluded patients with non BP-induced ONJ. This extraction of detailed information and manual confirmation of ONJ likely improved the reliability of the results.

Previous studies have reported that preventative dental treatment decreased BIONJ risk among patients with intravenous BP administration.[22–24] All patients who consulted the outpatient clinic of the authors' department before BP administration underwent an oral examination, screening for periodontal disease, and oral cleaning. For patients receiving BP, particularly intravenous BP, an extensive oral examination was performed and preventive dental treatment was conducted if needed. When tooth extraction was required following ineffective conservative treatment, preventive dental treatment was performed before extraction in all patients receiving BP, and extraction was conducted with particular care and antibiotic use, with complete wound closure when possible.

The estimated incidence of BIONJ associated with tooth extraction in the literature ranges from 8.3% to 40%.[5,15,25] The present study found a cumulative incidence of BIONJ after extraction at 42 months among all patients receiving BP and those receiving intravenous BP of 3.9% and 14.8%, respectively. Saia et al. reported that 5 of 60 patients receiving BP by either route developed BIONJ after tooth extraction (8.3%) within 12 months.[25] In a large cohort of 3994 patients with intravenous BP administration, Hoff et al. reported that 16 (10.5%) of 152 patients with tooth extraction developed BIONJ within 90 months, while in 1621 patients receiving intravenous BP,[5] Vahtsevanos et al. reported that 46 of 115 patients with a history of tooth extraction developed BIONJ (40%) within 106 months.[15] The results of the present study are consistent with these other studies which also employed positive preventive dental treatment before tooth extraction, and they are better than that of the study which did not describe the use of preventive care.[15] In addition, Mawardi et al. reported that bacterial infection at tooth extraction sites caused diminished keratinocyte growth factor expression in gingival fibroblasts, leading to a delay in the epithelial wound-healing process in *in vitro* and *in vivo* experiments.[26] These results are consistent with the hypothesis that poor oral hygiene might be associated with an increased risk of BIONJ after tooth extraction. Preventive and therapeutic treatment of oral bacterial infection before extraction might be important in preventing BIONJ in patients with BP administration.

Periodontal disease, an infection caused by oral bacteria, is characterized by inflammation that leads to alveolar bone loss.[27] In the present study, the authors found that not only intravenous BP administration but also the loss of alveolar bone was associated with an increased risk of BIONJ after tooth extraction. Given that all patients with BP administration underwent preventive and therapeutic treatment of oral bacteria before tooth extraction and that any effect of infection at the time of extraction was accordingly minimum, these findings suggest that previous inflammation of periodontal tissue may predispose to BIONJ after tooth extraction.

Several limitations of this study warrant mention. First, large differences were seen in age and prevalence of cancer or osteoporosis between BP and BP-naïve patients. The authors therefore performed stratified analysis by age and route of BP administration to estimate risk ratios for ONJ to control for these factors. Owing to the limited number of events, the authors were unable to estimate relative risks adjusted for the other potential risk factors such as steroid use or smoking, and the 95% CIs were wide. This prevents the

drawing of any reliable conclusions from the results, and indicates the need to evaluate possible risk factors or relative risks in a larger number of patients with ONJ, or to conduct a case–control study. Second, selection bias is inherent in single-centre studies, and the present study was additionally subject to inherent referral bias towards the selection of more severe cases, given that the department is a leading institution for oral and maxillofacial surgery in Kyoto City. A positive aspect of this latter limitation, is that almost all patients consult the department again in the event of subsequent problems at the tooth extraction site. Additionally, ONJ is an uncommon clinical condition, and such patients are likely to be referred to the authors' clinic to establish a diagnosis. The impact of selection bias is thus unclear. Subsequent multicentre regional (or national) studies would be required to address this bias. Third, the authors were unable to eliminate the possibility that some of the patients who developed ONJ might have had unidentified ONJ in the submucosa at the time of tooth extraction, hampering assessment of the impact of tooth extraction on ONJ development. A comprehensive understanding of the mechanism of ONJ therefore awaits additional studies.

In conclusion, BP administration, particularly intravenous administration, is associated with an increased risk of ONJ after tooth extraction. Severe alveolar bone loss might be a risk factor for BIONJ after tooth extraction. This study provides important information for physicians and dentists concerned with the prevention of ONJ in patients receiving BP.

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None.

Competing interests

None declared.

Ethical approval

This study protocol was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine (E-838).

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What is This?

RESEARCH REPORTS
Biological

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The Effects of C-type Natriuretic Peptide on Craniofacial Skeletogenesis

ABSTRACT

C-type natriuretic peptide (CNP) is a potent stimulator of long bone and vertebral development *via* endochondral ossification. In the present study, we investigated the effects of CNP on craniofacial skeletogenesis, which consists of both endochondral and membranous ossification. Morphometric analyses of crania from CNP knockout and transgenic mice revealed that CNP stimulates longitudinal growth along the cranial length, but does not regulate cranial width. CNP markedly increased the length of spheno-occipital synchondrosis in fetal murine organ cultures, and the thickness of cultured murine chondrocytes from the spheno-occipital synchondrosis or nasal septum, resulting in the stimulation of longitudinal cranial growth. Mandibular growth includes endochondral and membranous ossification; although CNP stimulated endochondral bone growth of condylar cartilage in cultured fetal murine mandibles, differences in the lengths of the lower jaw between CNP knockout or transgenic mice and wild-type mice were smaller than those observed for the lengths of the upper jaw. These results indicate that CNP primarily stimulates endochondral ossification in the craniofacial region and is crucial for midfacial skeletogenesis.

KEY WORDS: chondrocyte(s), maxillofacial surgery, craniofacial anomalies, craniofacial biology/genetics, growth factor(s), growth/development.

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INTRODUCTION

Mammalian skeletons are formed through intramembranous or endochondral ossification. The former occurs when mesenchymal precursor cells directly differentiate into bone-forming osteoblasts, whereas in the latter process, mesenchymal cells differentiate into chondrocytes and form a cartilage anlage, which is eventually remodeled into calcified bone (Lenton *et al.*, 2011). Although most mammalian bones are formed through endochondral ossification, the craniofacial skeleton contains bones that develop through either intramembranous or endochondral ossification; bones of the face and cranial vault are thought to develop *via* intramembranous ossification, whereas the skull base, nasal septum, and condyle in the mandible are created *via* endochondral ossification (Takigawa *et al.*, 1984; Takano *et al.*, 1987).

C-type natriuretic peptide (CNP) is one of the members of the natriuretic peptide family (Nakao *et al.*, 1992). CNP exerts its biological actions through a subtype of membranous guanylyl cyclase receptor, guanylyl cyclase-B (GC-B), by elevating the intracellular cGMP production (Suga *et al.*, 1992). We previously disclosed that the CNP/GC-B system is a potent stimulator of endochondral bone growth; both CNP and GC-B are expressed in the growth plate of long bones and vertebrae (Chusho *et al.*, 2001), and mice lacking CNP or GC-B exhibit severely impaired growth of long bones and vertebrae (Chusho *et al.*, 2001; Tamura *et al.*, 2004). In contrast, transgenic mice with targeted overexpression of CNP in growth plate cartilage or transgenic mice with increased levels of circulating CNP exhibit prominent skeletal overgrowth (Yasoda *et al.*, 2004; Kake *et al.*, 2009).

Regarding the effects of CNP in the craniofacial region, previous studies in transgenic or knockout mice showed that CNP stimulates longitudinal growth of the skull (Chusho *et al.*, 2001; Yasoda *et al.*, 2004; Kake *et al.*, 2009). Nevertheless, the comprehensive effects of CNP on craniofacial skeletogenesis, including the growth of various components of the craniofacial skeleton, have not been fully elucidated. In the present study, we performed closer analyses of the effects of CNP on craniofacial skeletogenesis.

MATERIALS & METHODS

Animals

Transgenic mice expressing CNP under the control of the mouse type II collagen promoter (*Col2-Nppc-Tg* mice) and CNP knockout mice ($Nppc^{-/-}$ mice) were described previously (Chusho *et al.*, 2001; Yasoda *et al.*, 2004). Animal care and all experiments were conducted in accordance with the institutional guidelines of the Kyoto University Graduate School of Medicine.

Imaging of Skulls

Skulls from adult mice were fixed in 95% ethanol. Three-dimensional reconstructions were made with microcomputed tomography (μCT, SMX-100CT-SV3, Shimadzu Co., Kyoto, Japan). The details of the methods are included in the Appendix.

Soft X-ray Imaging

Soft x-ray analyses were performed with an SRO-M5 system (30 kVp, 5 mA for 1 min; Softron, Tokyo, Japan).

Organ Culture

Organ culture experiments of mandibles and cranial bases from WT or *Nppc*$^{-/-}$ mice at birth were performed as previously reported (Lei *et al.*, 2008). The details of the methods are included in the Appendix.

Micromass Cultures

The micromass culture of chondrocytes from mandibular condylar cartilage (MCC), spheno-occipital synchondrosis (SOS), or nasal septal cartilage (NSC) was performed as described in the Appendix.

Histological Analysis

For light microscopy, sections were cut from paraffin-embedded specimens. The details of the methods, including immunohistochemical staining, are described in the Appendix.

Real-time Reverse-transcription/Polymerase Chain-reaction (RT-PCR) Analyses

The details of the methods are included in the Appendix.

Statistical Analysis

Data are expressed as means ± SEM. Statistical analysis was performed by analysis of variance (ANOVA) with Fisher's least significant difference method when appropriate. *P* values less than 0.05 were considered statistically significant. We used a non-parametric statistical technique to evaluate the significance of differences observed with Euclidean distance matrix analysis (EDMA).

RESULTS

Morphologic Analyses of Skulls from CNP Mutant Mice

To investigate the effects of CNP on craniofacial skeletogenesis, we examined the skulls of CNP mutant mice. Gross morphologic analyses and skeletal preparations demonstrated that, compared with WT mice, the skulls of *Nppc*$^{-/-}$ mice were longitudinally shorter, whereas those of *Col2-Nppc-Tg* mice were longer (Figs. 1A, 1B). Further, skeletal preparations revealed shorter lower jaws in *Nppc*$^{-/-}$ mice than in WT mice. In contrast, lower jaws of *Col2-Nppc-Tg* mice did not differ from those obtained from WT mice (Fig. 1B).

To further analyze morphologic changes in the skulls of CNP mutant mice, we performed morphometric analyses using μCT images (Figs. 1C, 1D). Linear measurements—defined in Fig. 1C—confirmed significantly reduced and increased skull lengths for *Nppc*$^{-/-}$ crania and *Col2-Nppc-Tg* crania, respectively. Nose length, nasal bone length, and upper jaw length, which together comprise the skull length, were each significantly reduced in *Nppc*$^{-/-}$ crania and increased in *Col2-Nppc-Tg* crania, compared with results from WT crania. The lower jaws of *Nppc*$^{-/-}$ mice were also shorter than those of WT mice, although the phenotype was milder than those of bones that contribute to the growth of skull length. The lower jaw length of *Col2-Nppc-Tg* mice did not markedly differ from that of WT mice. Skull widths and inter-orbital distances were not affected by the genetic modifications.

Next, we investigated morphologic craniofacial changes in the mutant mice using Euclidean distance matrix analysis (EDMA) (Fig. 1E) (Richtsmeier *et al.*, 2000; Arron *et al.*, 2006). The nasal, premaxilla, maxilla, and frontal bones were more markedly affected than the neurocranium, resulting in hypoplasia in *Nppc*$^{-/-}$ crania or hyperplasia in *Col2-Nppc-Tg* crania along the sagittal plane. *Nppc*$^{-/-}$ mice showed mandibular hypoplasia at the center of the condylar process and angular process in the sagittal direction. No difference in EDMA measurements was noted among the mandibles of WT and *Col2-Nppc-Tg* mice.

Effects of CNP on the Growth of the Skull Base

The spheno-occipital synchondrosis (SOS) is an important growth center in the craniomaxillofacial skeleton; this structure regulates temporal and positional cues for the growth and development of the maxilla (Singh, 1999; Lei *et al.*, 2008). The longitudinal length of the skull base is defined as endochondral bone growth occurring at SOS. Because CNP is a potent stimulator of long bone and vertebral growth *via* endochondral ossification, we investigated the effects of CNP on endochondral bone growth in the skull base. μCT imaging showed that the skull base was shorter in *Nppc*$^{-/-}$ mice and longer in *Col2-Nppc-Tg* mice compared with WT mice (Fig. 2A). In fact, both the occipital and sphenoid bones that comprise the skull base were significantly shorter in *Nppc*$^{-/-}$ mice and longer in *Col2-Nppc-Tg* mice than in WT mice (Fig. 2B). Furthermore, histological examination revealed that, compared with WT skull bases, SOS was markedly narrower in the *Nppc*$^{-/-}$ skull base and wider in the *Col2-Nppc-Tg* skull base at the ages of 2 and 4 wks (Figs. 2C, 2D). Immunohistochemical analysis of type X collagen—a marker of hypertrophic chondrocyte differentiation—revealed a narrower and wider hypertrophic chondrocyte layer in *Nppc*$^{-/-}$ synchondroses and *Col2-Nppc-Tg* synchondroses, respectively (Figs. 2C, 2D). In *Col2-Nppc-Tg* synchondroses, chondrocytes in the hypertrophic and proliferative layers were enlarged compared with cells in the WT synchondroses, especially at the age of 4 wks (Figs. 2C, 2D). As for the expression pattern of GC-B, the receptor of CNP, in WT SOS, immunohistochemical analysis revealed that GC-B is expressed in prehypertrophic chondrocytes (Fig. 2E). Furthermore, CNP was also, but weakly, expressed in the prehypertrophic chondrocytes in WT SOS (Fig. 2E).

To further analyze the effects of CNP on endochondral bone growth at SOS, we performed organ culture experiments using

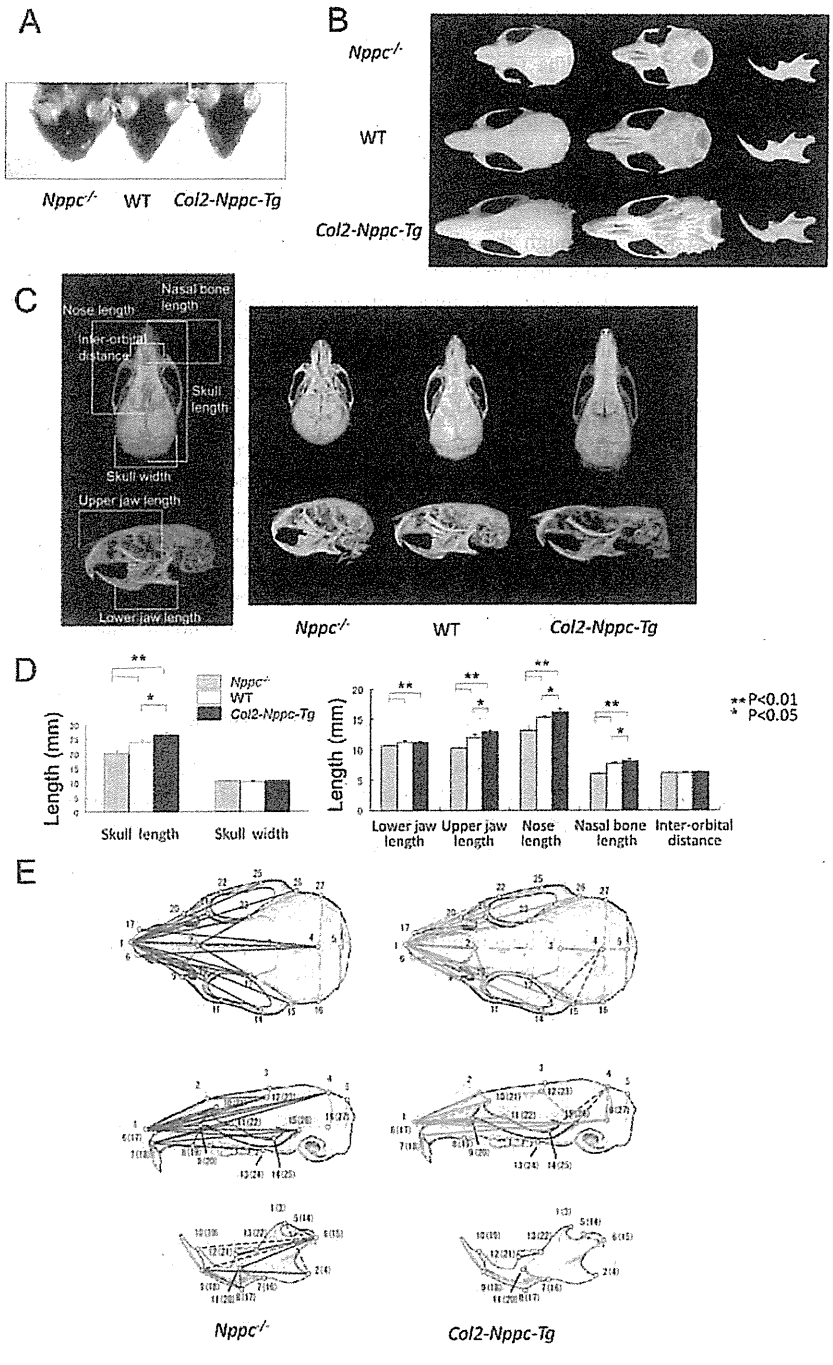

Figure 1. Craniofacial morphology of *Nppc*$^{-/-}$, WT, and *Col2-Nppc-Tg* mice. **(A)** Gross morphologies of 12-week-old *Nppc*$^{-/-}$, WT, and *Col2-Nppc-Tg* mice. **(B)** Skeletal preparations from 12-week-old *Nppc*$^{-/-}$, WT, and *Col2-Nppc-Tg* mice. **(C)** Left: Linear measurements for analysis of normal mouse skulls. Adapted from Richtsmeier *et al.* (2000) and the Craniofacial Resource from The Jackson Laboratory (www.jax.org/cranio/index.html). Right: Three-dimensional reconstructed images of the skulls of 12-week-old *Nppc*$^{-/-}$, WT, and *Col2-Nppc-Tg* mice. **(D)** Linear measurements from 12-week-old *Nppc*$^{-/-}$, WT, and *Col2-Nppc-Tg* mice. **(E)** Landmarks used for EDMA. Schematic images of the mouse cranium (upper, superior view; middle, lateral view) and mandible (lower, lateral view). Significantly smaller or larger values ($n = 8$, $P < 0.05$ or 0.01) compared with those for WT mice are denoted by different lines. Blue lines show significant hypoplasia, and red lines show significant hyperplasia compared with WT mice (solid lines, $p < 0.01$; dotted lines, $p < 0.05$).

skull base explants from fetal *Nppc*$^{-/-}$ and WT mice. At the end of 6-day cultures, synchondroses of skull base explants from *Nppc*$^{-/-}$ mice were approximately 15% shorter than those from WT mice (Figs. 2F, 2G). In contrast, treatment of WT skull base explants with CNP significantly increased the lengths of SOS (Figs. 2F, 2H). SOS in WT skull base explants treated with 10^{-8} or 10^{-7} M CNP for 6 days were approximately 30% longer than results observed in explants treated with vehicle (Fig. 2H). Moreover, chondrocytes in the CNP-treated SOS were enlarged compared with vehicle-treated chondrocytes (Fig. 2F). As for the effect of CNP on the proliferation of chondrocytes in SOS, we performed immunohistochemical staining for PCNA and found that CNP did not significantly, but tended to, increase the ratio of PCNA-positive cells (Fig. 2I). In contrast, immunohistochemical analysis of type X collagen revealed a narrower and wider hypertrophic chondrocyte layer in *Nppc*$^{-/-}$ synchondroses and WT synchondroses treated with 10^{-7} M CNP, respectively (Fig. 2J).

We also studied the effects of CNP on chondrocytes from SOS using micromass cultures. CNP dose-dependently increased the micromass thickness of chondrocytes from WT SOS (Fig. 2K). The chondrocytes dose-dependently increased in size in response to CNP (Fig. 2K). Further, we analyzed expressions of chondrogenic differentiation markers of micromass using real-time RT-PCR. The expression of aggrecan, a major sulfated proteoglycan of the cartilage matrix and a highly specific marker of differentiated chondrocytes, was significantly higher in the CNP-treated group than in the vehicle-treated group (Fig. 2L). The expression of type X collagen, a specific marker for hypertrophic chondrocytes, was also significantly higher in the CNP-treated group than in the control group (Fig. 2L).

Effects of CNP on Endochondral Bone Growth from Nasal Septal Cartilage (NSC)

Growth of NSC is essential for longitudinal facial growth (Wealthall and Herring, 2006). Because μCT analysis revealed that nasal bone length or nose

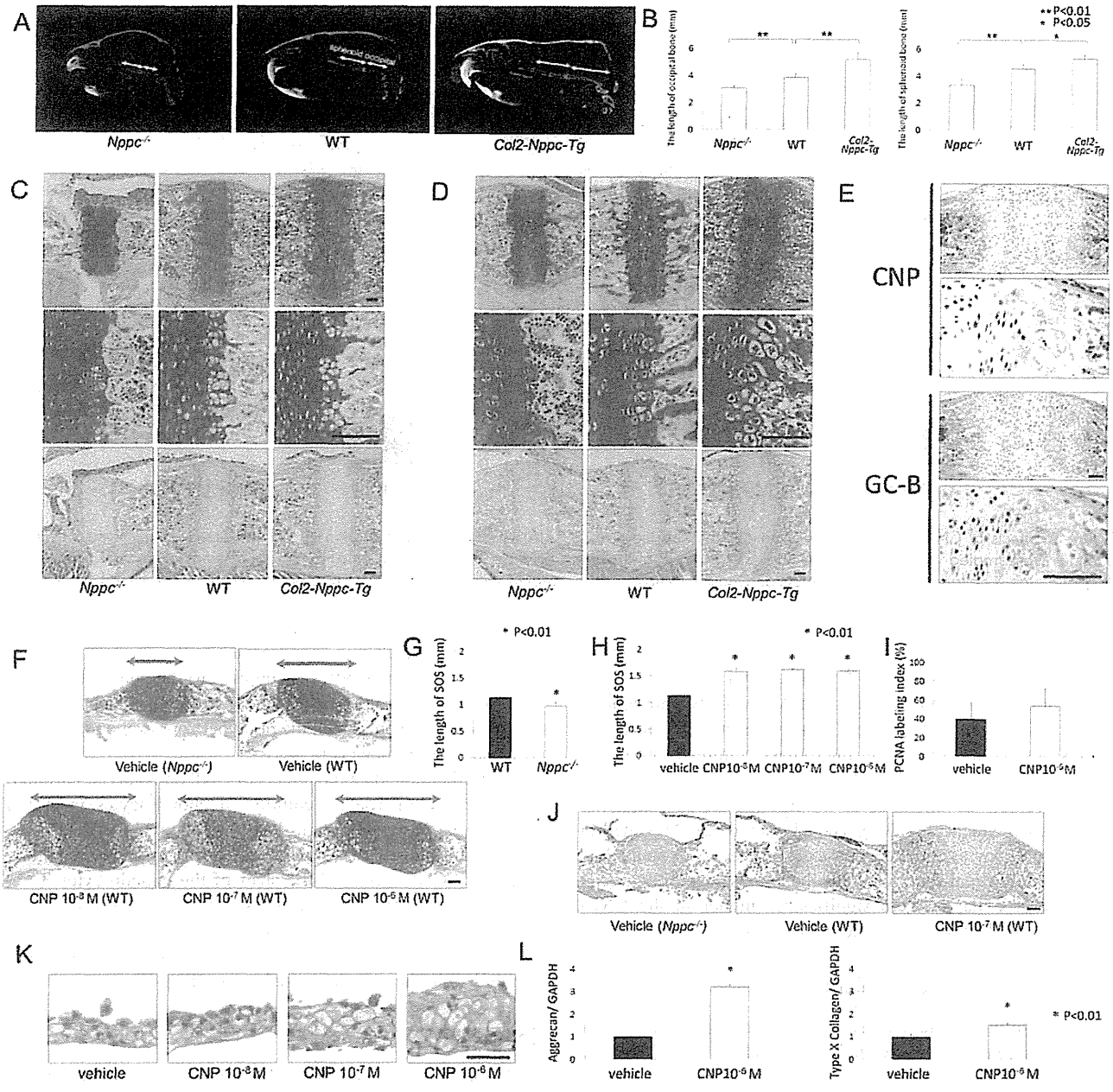

Figure 2. Effects of CNP on growth of the skull base. **(A)** Sagittal sections from μCT images of 12-week-old *Nppc*$^{-/-}$, WT, and *Col2-Nppc-Tg* mice. **(B)** Sagittal lengths of occipital and sphenoid bones from 12-week-old *Nppc*$^{-/-}$, WT, and *Col2-Nppc-Tg* mice. **(C, D)** Histological examination of spheno-occipital synchondrosis (SOS) from two-week-old (C) and four-week-old (D) Nppc$^{-/-}$, WT, and Col2-Nppc-Tg mice. Samples were stained with Alcian blue-HE (upper, and middle with higher magnification) and immunohistochemically labeled for type X collagen (lower). **(E)** Immunohistochemical staining for CNP (upper) and GC-B (lower) of SOS from 2-week-old WT mice. Lower panel in each set is shown with higher magnification, and the arrowhead indicates positive staining. **(F)** Histological examination of SOS after a six-day organ culture and staining with Alcian blue-HE in skull base explants from fetal *Nppc*$^{-/-}$ and WT mice treated with vehicle or 10^{-8} to 10^{-6} M CNP. **(G)** Lengths of SOS from fetal WT and *Nppc*$^{-/-}$ mice at the end of six-day organ cultures. **(H)** Lengths of SOS from WT mice treated with vehicle or 10^{-8}–10^{-6} M CNP at the end of six-day organ cultures. **(I)** PCNA labeling index of SOS in skull base explants from fetal WT mice treated with vehicle or 10^{-6} M CNP at the end of six-day organ culture. **(J)** Immunohistochemical staining for type X collagen of SOS in skull base explants from fetal *Nppc*$^{-/-}$, WT, and WT treated with 10^{-7} M CNP after six-day organ culture. **(K)** WT SOS-derived micromass cultures on day 21 were stained with Alcian blue-HE after treatment with vehicle or 10^{-8} to 10^{-6} M CNP. **(L)** Expressions of genes for aggrecan (left) and type X collagen (right) in the SOS-derived micromass cultures treated with vehicle or 10^{-6} M CNP for 10 days, analyzed by real-time RT-PCR. All scale bars: 100 μm.

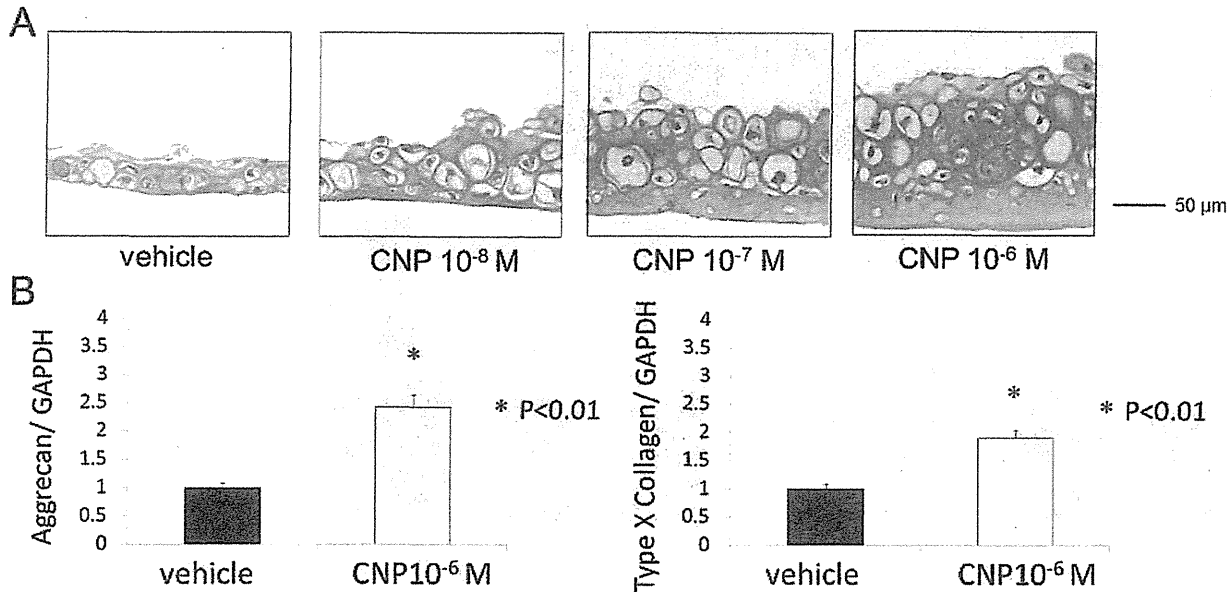

Figure 3. Effects of CNP on the growth of nasal septal cartilage (NSC). **(A)** Histological examination of WT NSC-derived micromass cultures. Samples were treated with vehicle or 10^{-8} to 10^{-6} M CNP and stained on day 21 with Alcian blue-HE. **(B)** Expressions of genes for aggrecan (left) and type X collagen (right) in the NSC-derived micromass cultures treated with vehicle or 10^{-6} M CNP for 10 days, analyzed by real-time RT-PCR.

length was significantly shorter in *Nppc*$^{-/-}$ mice and significantly longer in *Col2-Nppc-Tg* mice, after confirming the expression of both CNP and GC-B in WT NSC by immunohistochemical staining (Appendix Fig.), we examined the direct effects of CNP on NSC using micromass cultures of chondrocytes from WT nasal septum. Histological sections revealed that CNP increased the thickness of the micromass, an effect that was dose-dependent (Fig. 3A). The addition of CNP resulted in larger chondrocytes and increased extracellular space stained by Alcian blue. In real-time RT-PCR, the expressions of aggrecan and type X collagen were significantly higher in the CNP-treated group than in the vehicle-treated group (Fig. 3B).

Effects of CNP on Mandibular Growth

Because morphometric analysis revealed impaired mandibular growth in *Nppc*$^{-/-}$ mice, we analyzed the effect of CNP on the growth of condylar cartilage in organ cultures of fetal murine mandibular explants. The length of condylar cartilage of *Nppc*$^{-/-}$ mice, measured by means of a linear ocular scale mounted on a dissecting microscope, was significantly shorter than that of wild-type mice at the end of the 6-day culture period (Figs. 4A, 4B). Histological examination revealed that the chondrocytes in the *Nppc*$^{-/-}$ condylar cartilage were smaller than those in the WT condylar cartilage, resulting in hypoplasia in the *Nppc*$^{-/-}$ explants (Fig. 4C). Furthermore, the area of hypertrophic chondrocytes in the *Nppc*$^{-/-}$ condylar cartilage was smaller than that in the WT condylar cartilage, as is observed in SOS or growth plate of long bones (Fig. 4C). In contrast, treating WT mandibular explants with CNP dose-dependently increased the length of condylar cartilage (Figs. 4D, 4E); 10^{-7} or 10^{-6} M CNP significantly increased the condylar cartilage length ($p < 0.05$, n = 5 for each group) (Fig. 4E). Not only each chondrocyte but also the area of hypertrophic chondrocytes in the condylar cartilage was enlarged when CNP was supplied to the culture (Fig. 4F). In fact, immunohistochemical study revealed that both GC-B and CNP were expressed in the prehypertrophic chondrocytes in WT condylar cartilage (Fig. 4G).

To further elucidate the effects of CNP on condylar cartilage, we examined micromass cultures of chondrocytes from WT condylar cartilage. Histological sections showed that CNP dose-dependently increased the thickness of the micromass cultures (Fig. 4H). CNP increased the sizes of the chondrocytes in the micromass, and the extracellular space stained by Alcian blue (Fig. 4H). In real-time RT-PCR, the expressions of aggrecan and type X collagen were significantly higher in the CNP-treated group than in the vehicle-treated group (Fig. 4I).

DISCUSSION

In the present study, we investigated the effects of CNP on craniofacial skeletogenesis. Craniofacial skeletogenesis consists of both endochondral and membranous ossification (Nie *et al.*, 2006). Morphometric analyses of *Nppc*$^{-/-}$ and *Col2-Nppc-Tg* mice by μCT images clearly demonstrated that CNP affects longitudinal skull growth. Because longitudinal skull growth mainly depends on endochondral bone growth of the skull base at SOS and of the nasal septum, these results indicate that CNP stimulates endochondral bone growth during craniofacial skeletogenesis. Furthermore, using organ and micromass cultures, we demonstrated that CNP stimulates endochondral bone growth in the skull base and nasal septum. Of note, these results agreed with those from our previous study showing that CNP is a potent stimulator of the endochondral ossification-based growth of long bones and vertebrae (Yasoda *et al.*, 2004). Actually, histological analyses revealed that the morphological alterations of

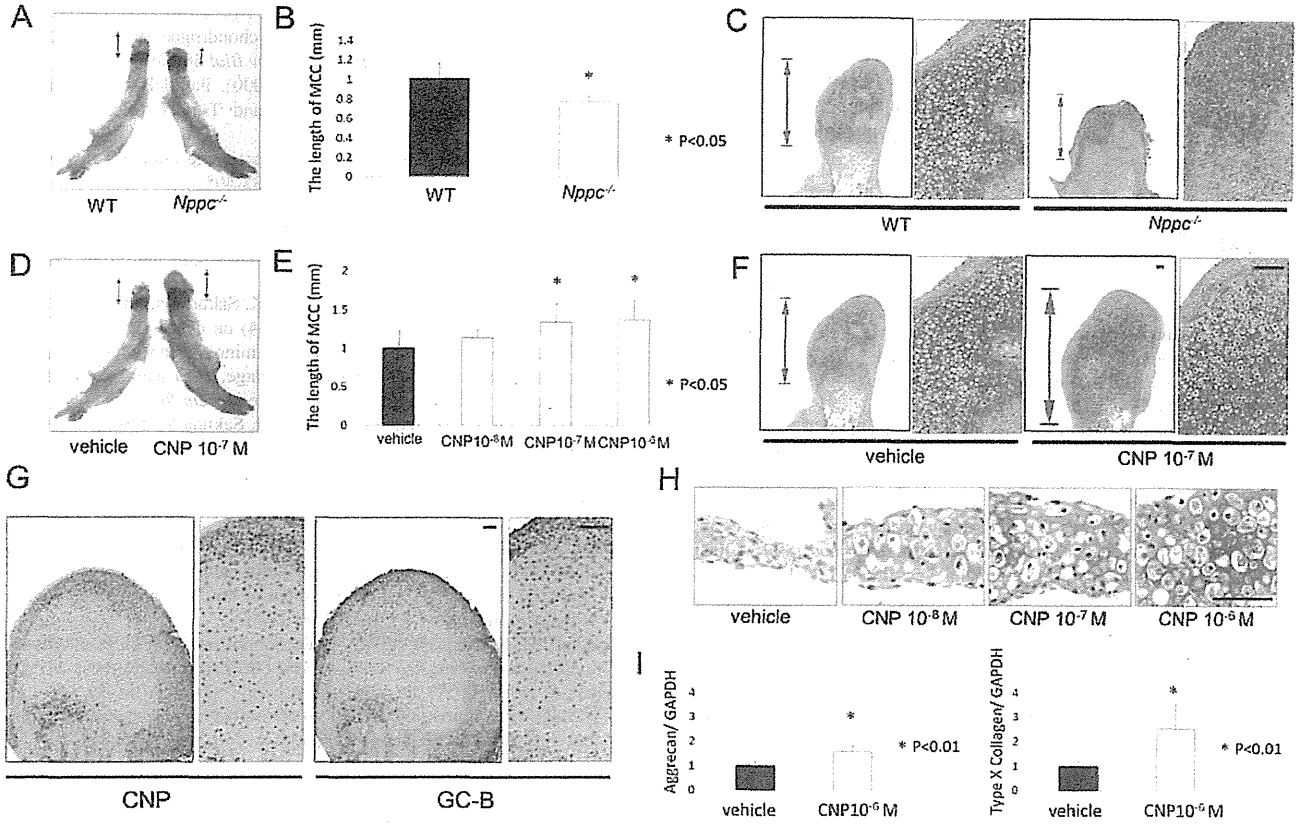

Figure 4. Effects of CNP on mandibular growth. **(A)** Mandibular explants from fetal WT and *Nppc*$^{-/-}$ mice were stained with Alcian blue and Alizarin red after six-day organ cultures. **(B)** Lengths of MCC from fetal WT and *Nppc*$^{-/-}$ mice after a six-day organ culture. **(C)** Histological analysis of mandibular condylar cartilage (MCC) stained with Alcian blue-HE after six-day organ cultures with mandibular explants from fetal WT and *Nppc*$^{-/-}$ mice. Right panel in each set of panels is exhibited with higher magnification. **(D)** Mandibular explants from fetal WT mice were treated with vehicle or CNP 10^{-7} M in six-day organ cultures and stained with Alcian blue and Alizarin red. **(E)** Lengths of MCC from WT mice treated with vehicle or 10^{-8} to10^{-6} M CNP in six-day organ cultures. **(F)** Histological examination of MCC six-day organ cultures and staining with Alcian blue-HE. Fetal WT murine mandibular explants were treated with vehicle or CNP 10^{-7} M. **(G)** Immunohistochemical staining for CNP (left set) and GC-B (right set) of MCC from two-week-old WT mice. Right panel in each set is exhibited with higher magnification. **(H)** Histological examination of WT MCC-derived micromass cultures treated with vehicle or 10^{-8} to 10^{-6} M CNP and stained with Alcian blue-HE on day 21. **(I)** Expressions of genes for aggrecan (left) and type X collagen (right) in the MCC-derived micromass cultures treated with vehicle or 10^{-6} M CNP for 10 days, analyzed by real-time RT-PCR. All scale bars: 100 μm.

SOS in *Nppc*$^{-/-}$ and *Col2-Nppc-Tg* mice compared with those in WT mice are the same as those observed in growth plates of long bones in *Nppc*$^{-/-}$ and *Col2-Nppc-Tg* mice, respectively.

As for the lower jaw, mandibular development depends on endochondral and membranous ossification. Specifically, endochondral ossification contributes to the formation of condylar cartilage, whereas the mandibular body forms *via* membranous ossification (Oka *et al.*, 2007). Although we confirmed the stimulatory effects of CNP on endochondral bone growth in condylar cartilage using organ and micromass culture experiments, morphometric analyses revealed that the impaired growth of the lower jaw was weaker than the upper jaw phenotype in *Nppc*$^{-/-}$ mice, and lower jaws of *Col2-Nppc-Tg* mice were not affected. These results indicated that the stimulatory effects of CNP on bone growth were weaker in the mandible compared with the skull base. The reason might be that the mandible does not grow by primarily endochondral ossification. During development, bones of the mandible undergo intramembranous ossification surrounding Meckel's cartilage. The condylar cartilage makes up the mandibular portion of the temporomandibular joint. Differential growth of the condylar cartilage may make the mandible seem larger, but that might be due to displacement in the joint, rather than mandibular growth. Conversely, mandibular hypoplasia of *Nppc*$^{-/-}$ mice might be due to disturbed growth caused by malocclusion. This may explain why the *Col2-Nppc-Tg* mandibles are the same length as WT mandibles.

In conclusion, we investigated the effects of CNP on craniofacial skeletogenesis. CNP is a pivotal stimulator of endochondral bone growth in the craniofacial region, and plays an important role in midfacial skeletogenesis.

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