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Immunohistochemical analyses of β-catenin and cyclin D1 expression in giant cell tumor of bone (GCTB): a possible role of Wnt pathway in GCTB tumorigenesis

Short title: β-catenin and cyclin D1 in giant cell tumor of bone

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Abstract

Giant cell tumor of bone (GCTB) is a benign neoplasm but occasionally shows local recurrence, and histologically consists of osteoclast-like giant cells (GC) and stromal mononuclear cells (SC), which possess the ability of proliferation and osteoblastic differentiation. Activation of Wnt signaling can induce osteoblast differentiation and osteoclastgenesis during bone resorption process. This study analyzed the profiles of β-catenin and cyclin D1 expression in GCTB to elucidate an involvement of Wnt pathway in tumorigenesis. We performed immunohistochemistry for β-catenin, cyclin D1, and Ki-67 in 16 GCTB tumors, including 5 recurrent cases which were surgically resected. All 16 cases of GCTB displayed β-catenin, cyclin D1, and Ki-67 expression. Immunoreactivity for β-catenin was observed in nuclei of SC and GC. Cyclin D1 immunoreactivity was mainly found in nuclei of GC, while Ki-67 immunoreactivity was restricted to nuclei of SC. The nuclear β-catenin labeling index (LI) in both SC (60.6 vs. 41.8%, p=0.074) and GC (41.7 vs. 20.1%, p=0.095) was higher in recurred tumors than in primary tumors in all of 4 cases. However, Ki-67 LI in SC (18.8 vs. 19.9%, p=0.851) and cyclin D1 LI in GC (55.4 vs. 70.1%, p=0.225) were not higher in recurred tumors than in primary tumors. Our results suggested activation of Wnt/ β-catenin pathway in GCTB tumorigenesis. Since cyclin D1 in GC was never associated with the expression of the well-known proliferative marker Ki-67, cyclin D1 expression might exert a role in GC formation instead of promoting cell proliferation during GCTB tumorigenesis. Importantly, it was suggested that the level of nuclear β-catenin staining might be associated with tumor recurrence in GCTB.

Introduction

Giant cell tumor of bone (GCTB), also known as osteoclastoma, is a benign but locally aggressive neoplasm of bone characterized by massive bone destruction at the epiphysis of long bone that has a strong tendency for local recurrence. Histologically, GCTB consists of numerous scattered multinucleated osteoclast-like giant cells (GC) which are the characteristic hallmark of this tumor, and mononuclear stromal cells (SC) which represent the true neoplastic (proliferative) component [1]. Although their exact origin is as yet undefined, it is likely that SC might originate either from an osteoblastic lineage or from bone marrow mesenchymal cells, and regulate the formation of GC in the neoplasm.

Cyclin D1 is a critical cell cycle regulator that drives the cell cycle from the G1 to the S phase. Elevated nuclear cyclin D1 expression has been found in human tumors including GCTB [2, 3]. We have previously reported that cyclin D1 overexpression is significantly correlated with cytoplasmic β-catenin expression in thyroid tumors [4-6]. β-catenin has been shown to be a key downstream effector of the Wnt signaling pathway to regulate cell growth/survival [7]. This pathway is activated by genetic mutations that stabilize the β-catenin protein, which accumulates in the cytoplasm, and then translocates to the nucleus. It then binds to the T-cell factor/lymphoid enhancer factor (Tcf/Lef) [8-10] to activate genes such as cyclin D1 and contributes to the oncogenesis of various human cancers [11-14].

Recent studies have implicated an important role for Wnt signaling in the regulation of skeletal function and have suggested that activation of Wnt signaling can induce osteoblast differentiation and osteoclastgenesis during bone resorption process

[15-17]. This study analyzed the profiles of β-catenin and cyclin D1 expression in GCTB to elucidate an involvement of Wnt pathway in its tumorigenesis. In our results, we found an increased level of nuclear β-catenin in recurrent GCTB as compared with primary tumors.

Subjects and Methods

Materials

A total of 16 cases of GCTB including 11 primary and 5 recurrent tumors were selected from a file of surgically resected specimens performed from 1977 to 2006 in our department. These tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections 4μm in thickness were routinely stained with hematoxylin and eosin and prepared for immunohistochemistry. The clinical profile of each case including Campanacci's radiographic grading system [18] and treatment is summarized in Table 1.

Immunohistochemistry

After antigen retrieval, sections were immersed in 0.3% H_2O_2/methanol and, subsequently, preincubated with 10% normal goat serum. Then, tissues were incubated overnight at 4^0C with polyclonal anti-β-catenin antibody (GenWay Biotech, San Diego, CA) at a 1:50 dilution, monoclonal anti-cyclin D1 antibody (Zymed Labs, South San Francisco, CA) at a 1:50 dilution, or monoclonal anti-Ki-67 antibody (DAKO, Carpinteria, CA) at a 1:50 dilution. The slides were subsequently incubated with biotinylated goat anti-rabbit (for β-catenin) or anti-mouse IgG antibody (for cyclin D1 and Ki-67) for 1 hr, followed by incubation with avidin-peroxidase for 30 min, and

visualized with diaminobenzidine (DAB). A case of callus from a 17 yo-male patient

was also used as a non-neoplastic tissue control. Control experiments included

incubation with non-immunized rabbit serum (for β-catenin) or mouse serum (for cyclin

D1 and Ki-67) instead of the primary antibodies. They did not show any staining.

Evaluation of immunohistochemical results

Tumor cells with nuclear and/or cytoplasmic staining were considered as positive

in immunohistochemistry for β-catenin, while only tumor cells with nuclear staining

were considered as positive in immunohistochemistry for cyclin D1 and Ki-67. The

number of β-catenin, cyclin D1, and Ki-67 positive cells was counted in five different

tumor areas at 200-fold magnification, and the percentage of immunoreactive cells from

the total number of SC or GC was calculated as the labeling index (LI) in each case. For

statistical analysis, the paired Student's t-test was used to assess differences in the

β-catenin, cyclin D1, and Ki-67 LI between primary and recurrent tumors. Associations

between the β-catenin, cyclin D1, and Ki-67 LI and Campanacci's radiographic grading

were assessed by using the Jonckheere-Terpstra test. A p-value of less than 0.05 was

accepted as statistically significant.

Results

All 16 cases of GCTB displayed β-catenin, cyclin D1, and Ki-67 expression.

Immunoreactivity for β-catenin was observed in nuclei of SC and in nuclei and/or

cytoplasms of GC (Fig. 1A and D). Cyclin D1 immunoreactivity was mainly found in

nuclei of GC and, occasionally, in a much small number of SC (Fig. 1B and E), while

Ki-67 immunoreactivity was restricted to nuclei of SC (Fig. 1C and F). Nuclear

immunoreactivities of both β-catenin and cyclin D1 were observed throughout almost

all nuclei in a GC showing staining. The co-localization of β-catenin and cyclin D1

immunoreactivity were shown in nuclei of GC by using serial sections (Fig. 2).

In comparison of immunohistochemical results between GC in which the number

of nuclei was less than 15 (GC<15) and GC in which the number of nuclei was 15 or

more (GC≥15), statistical analyses revealed that both β-catenin and cyclin D1 LI were

significantly higher in GC<15 than GC≥15 (p<0.001, respectively). Furthermore,

although there was no significant difference, nuclear β-catenin LI in both SC (60.6 vs.

41.8%, p=0.074) and GC<15 (41.7 vs. 20.1%, p=0.095) were higher in recurred tumors

than in primary tumors in all of 5 cases. However, Ki-67 LI in SC (18.8 vs. 19.9%,

p=0.851) and cyclin D1 LI in GC<15 (55.4 vs. 70.1%, p=0.225) was not higher in

recurred tumors than in primary tumors. On the basis of Campanacci's radiographic

grading, although there was no significant difference between the mean value of

β-catenin/cyclin D1 LI in tumor and grading of case, Ki-67 LI in SC was significantly

increased with a higher level of grade (14.5, 23.1, and 24.0% in grade I, II, and III,

respectively, p=0.033). Immunohistochemical results for each case are presented in

Table 2, and these results are summarized in Tables 3 and 4.

In a case of callus, immunoreactivity for β-catenin was observed only in nuclei and

cytoplasms of osteoblasts lining the surface of bone trabecule but not in osteoclasts (Fig.

3A and B). However, cyclin D1 immunoreactivity was found in neither osteoblasts nor

osteoclasts (Fig. 3C and D).

Discussion

GCTB histologically consists of osteoclast-like GC and SC, which possess the ability of proliferation and osteoblastic differentiation. Wnt signaling has been shown to play a substantial role in the control of bone formation. This study analyzed the immunohistochemical profile of β-catenin and cyclin D1 expression in GCTB to elucidate an involvement of Wnt pathway in tumorigenesis. It is well established that the cytoplasmic stabilization of β-catenin via Wnt signaling leads to translocation of β-catenin into the nucleus and subsequent activation of cyclin D1 transcription [19]. Our results demonstrated both β-catenin and cyclin D1 expressions in all cases of GCTB. Furthermore, immunostainings with serial sections revealed the co-localization of β-catenin and cyclin D1expression in nuclei of GC, suggesting a role of activated Wnt/β-catenin pathway during GCTB tumorigenesis. However, activation of Wnt signaling can induce osteoblast differentiation and negatively regulate osteoclastgenesis through osteoblast during a physiological bone resorption process [20, 21]. Actually, nuclear β-catenin expression was revealed only in osteoblatsts but not in osteoclasts which appeared in callus tissue as an activated state of bone remodeling. These observations suggest Wnt/β-catenin might be abnormally activated in GC during GCTB tumorigenesis. Thus, molecular analyses regarding its activation machinery remain to be elucidated.

Similarly to a previous report [3], cyclin D1 staining in GC was never associated with the expression of Ki-67. Due to the fact that Ki-67 is a well characterized proliferation marker which is expressed by all cells undergoing cell cycle, we hypothesized that nuclear cyclin D1 protein might function mainly in a role instead of

promoting cell proliferation. However, the functional role of cyclin D1 expression in GC of GCTB tumorigenesis is not fully understood. Interestingly, the level of cyclin D1 immunoreactivity was significantly higher in GC<15 than GC≥15. Thus, cyclin D1 expression may play a role on the maturation and multinucleation of GC in GCTB. Indeed, cyclin D1 protein overexpression has been shown to be associated with giant cell formation, multinucleation, and increased ploidy in different cell models [22-25]. Further studies are required to elucidate the functional role of cyclin D1 overexpression on GC formation during GCTB tumorigenesis.

GCTB can be locally aggressive with a tendency for recurrence whose clinical behavior is difficult to predict based on its microscopic appearance alone [26]. Campanacci's radiographic grading system has been considered as a reliable predictive factor of local recurrence in GCTB [18]. Our statistical analysis revealed that a case of higher Campanacci's radiographic grade seems to exhibit a high proliferative activity of SC component. In our subjects, 5 of 16 cases were with recurrence. It is worthy to note that the level of nuclear β-catenin expression in both SC and GC was higher in recurred tumors than in primary tumors in all cases, although there was no significant difference. Similarly, although there was no significant difference, the level of nuclear β-catenin immunoreactivity in SC was going up with higher Campanacci's grading. Thus, the level of nuclear β-catenin immunoreactivity might be related with tumor recurrence in GCTB. However, because the level of Ki-67 staining in SC was not higher in recurred tumor than in primary, the level of nuclear β-catenin expression was not directly associated with the proliferative activity of SC, which is considered as the true neoplastic component, in tumor recurrence [1, 26-28]. Biological activity other than proliferative capability of tumor cells, which was regulated by Wnt/β-catenin pathway,

might be important in recurrence of GCTB.

In summary, the present study demonstrated a frequent expression of both β-catenin and cyclin D1 proteins in GCTB. Furthermore, immunoreactivity for β-catenin localized to nuclei of both SC and GC, suggesting an activation of Wnt/β-catenin pathway in GCTB tumorigenesis. Cyclin D1 staining in GC was never associated with the expression of the well-known proliferative marker, Ki-67, and the level of cyclin D1 immunoreactivity was mainly expressed in GC possessing fewer nuclei. Thus, cyclin D1 expression might exert a role in GC formation instead of promoting cell proliferation during GCTB tumorigenesis. Importantly, it is suggested that the level of nuclear β-catenin staining might be associated with tumor recurrence in GCTB. Thus, additional studies are needed to further clarify the functional role of Wnt/β-catenin pathway in GCTB.

Acknowledgements

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Figure Legends

Figure 1. Immunohistochemical analysis for β-catenin, cyclin D1, and Ki-67 expression in primary (A-C) and recurrent cases (D-F) of giant cell tumor of bone (GCTB). A primary case of GCTB showed cytoplasmic β-catenin expression in osteoclast-like giant cells (GC) (A), while recurrent GCTB widely showed nuclear β-catenin expression in both GC and stromal mononuclear cells (SC) (D). Insets in both A and D display representative cytoplasmic and nuclear β-catenin immunoreactivity in tumor cells, respectively. Cyclin D1 immunoreactivity was found in nuclei of GC in primary case (B) and in nuclei of both GC and SC in recurrent case (E). Arrows indicate cyclin D1-positive GC in which the number of nuclei was less than 15, while asterisks indicate cyclin D1-negative GC in which the number of nuclei was 15 or more. Ki-67 immunoreactivity was restricted to nuclei of SC but not in GC (C and F). No apparent difference is evident in the level of cyclin D1 expression between primary (C) and recurrent (F) cases[u1][u2].

Figure 2. Co-localization of β-catenin (A) and cyclin D1 (D) immunoreactivity in nuclei of osteoclast-like giant cells (GC) by using serial sections. Arrows indicate double-positive GC, while asterisks indicate double-negative GC.

Figure 3. Immunohistochemical analysis for β-catenin (A, B) and cyclin D1 (C, D) expression in a case of callus as a non-neoplastic tissue control. Nuclear staining for β-catenin was observed in osteoblasts but not in osteoclasts, while no cyclin D1 immunoreactivity was evident in any cells.
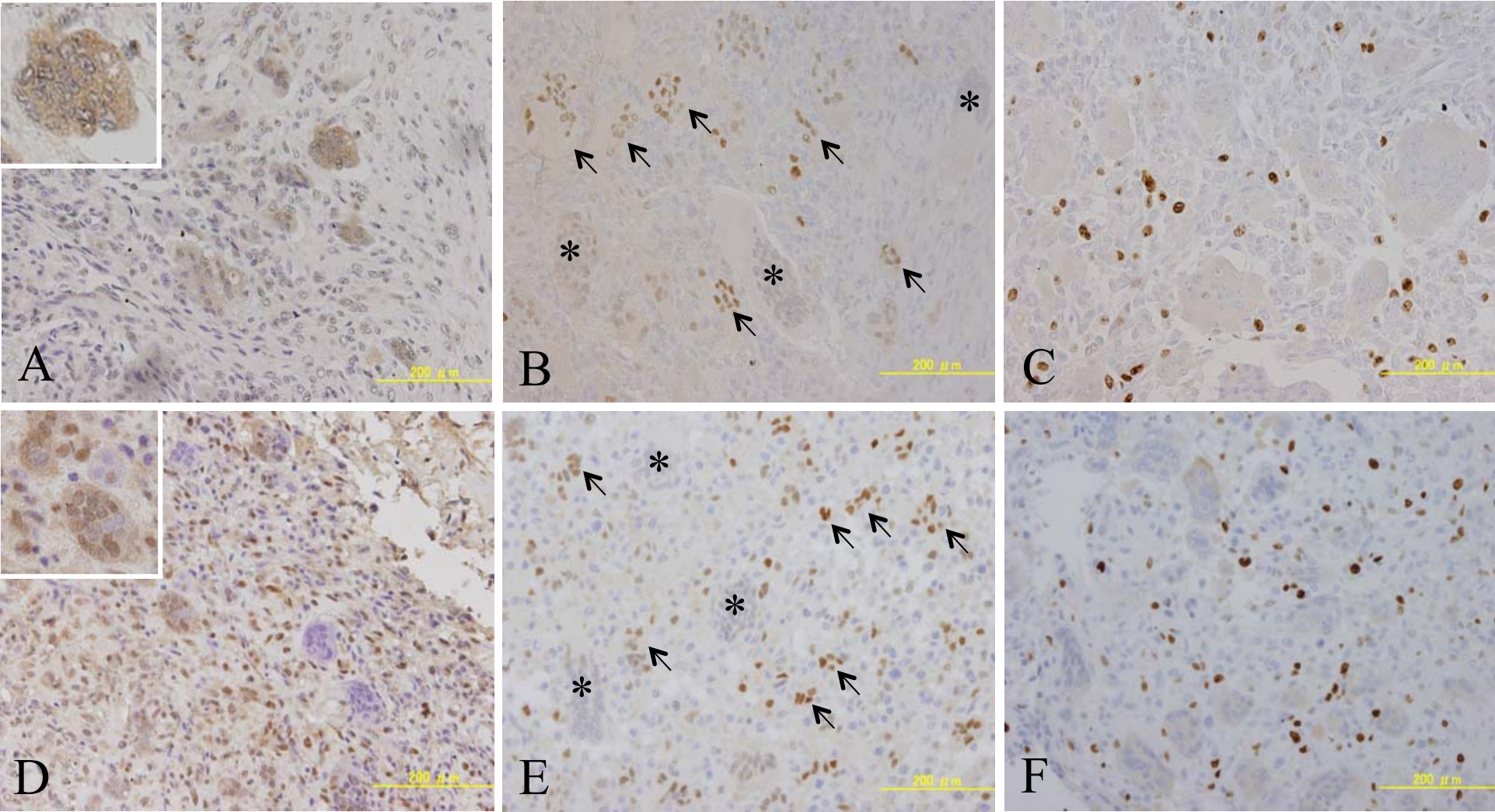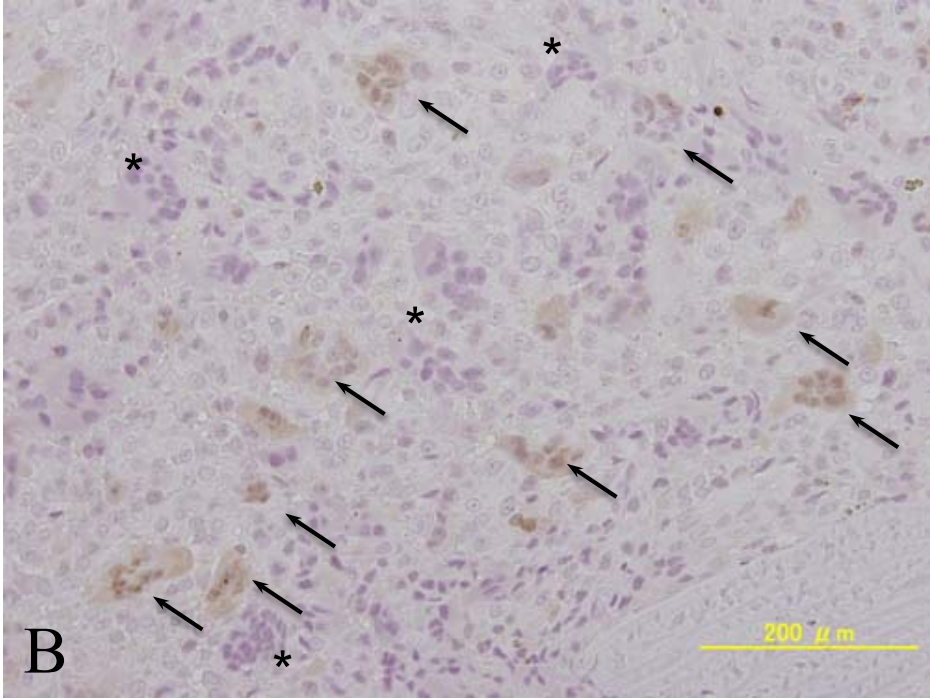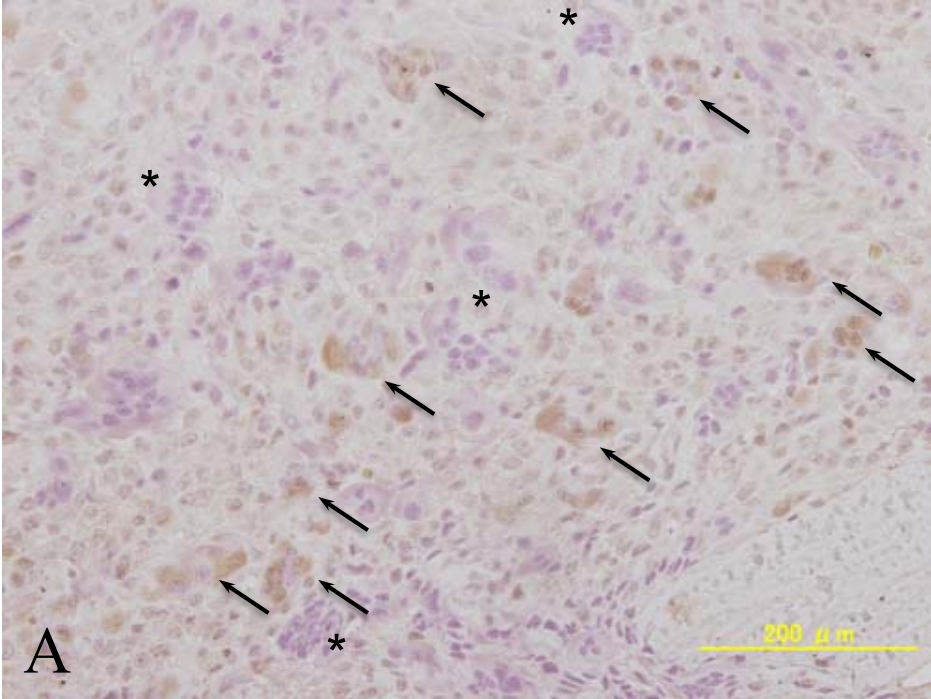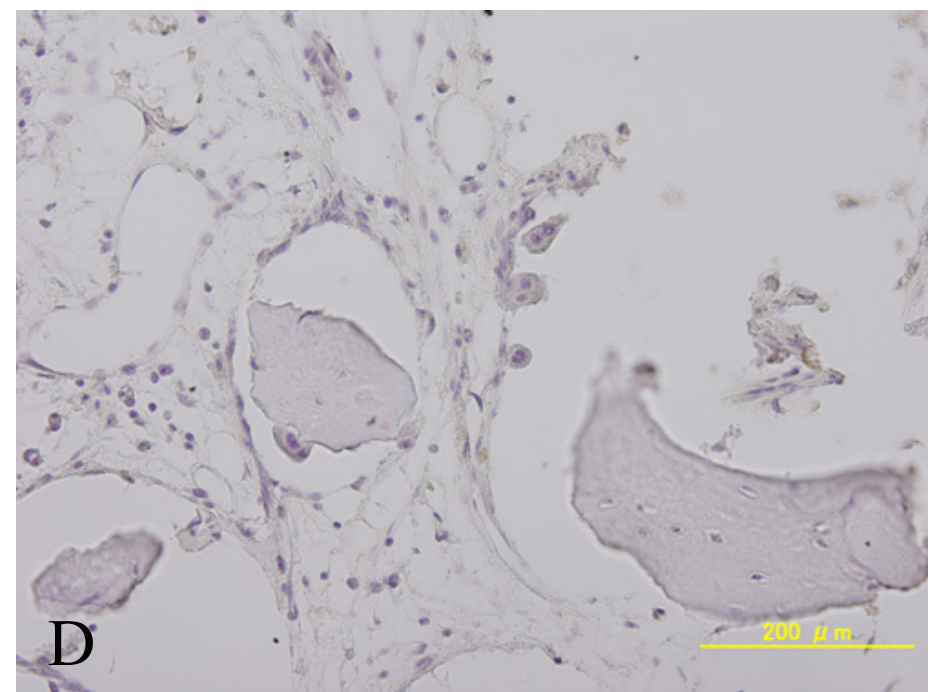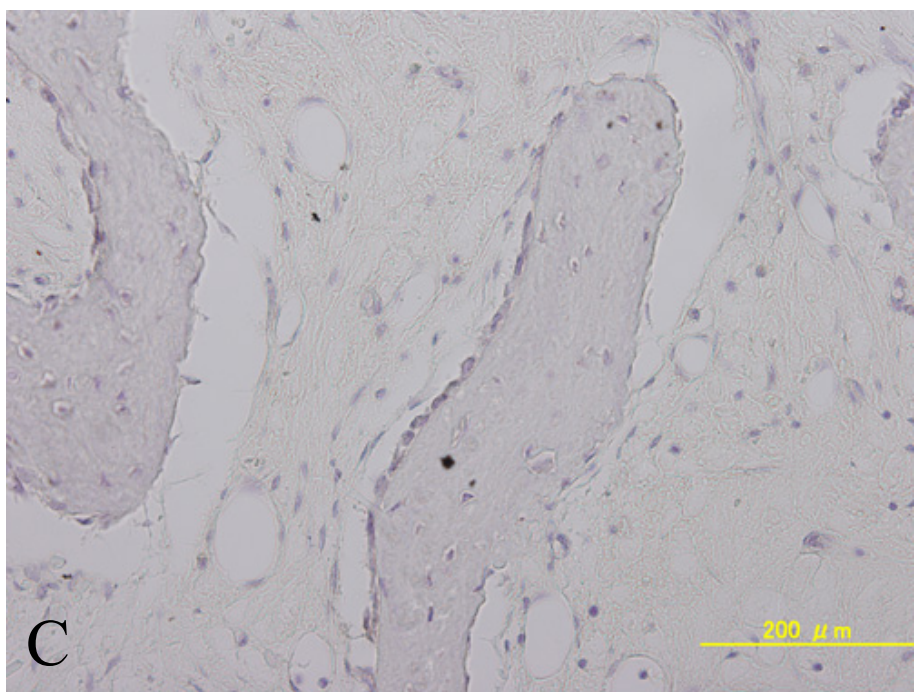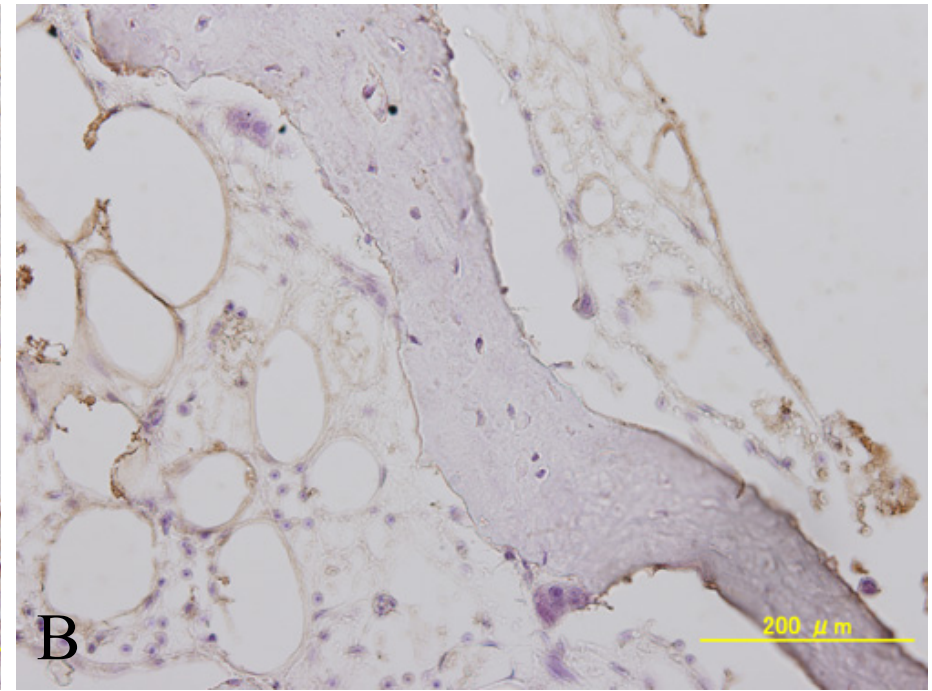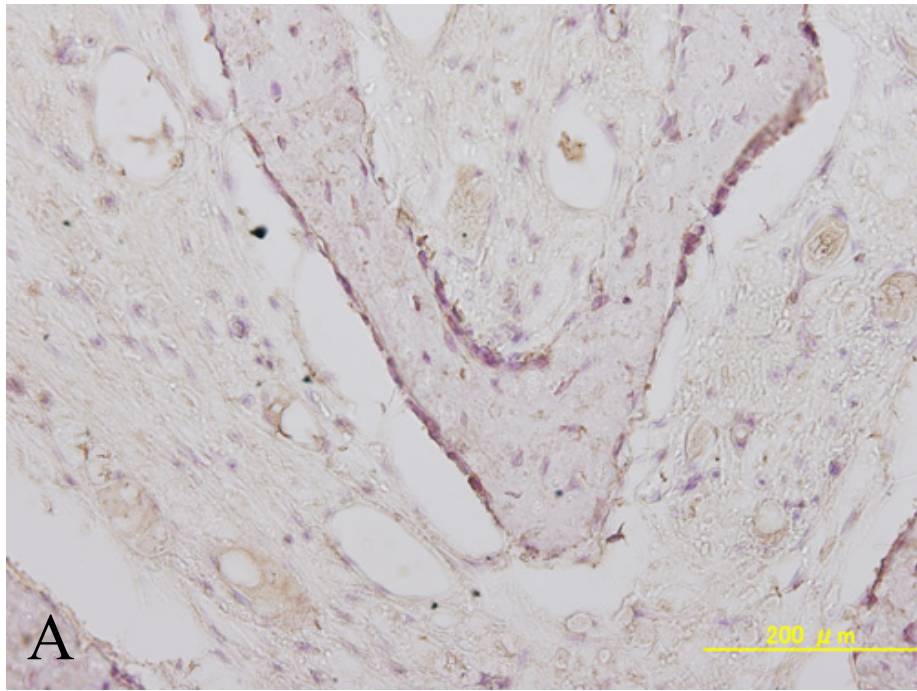
Figure 1

Figure 2

Figure 3

Table 1. Clinicopathological profiles of patients

Patient No	Sex	Primary/ Recurrent	Age	Size [mm]	Site	Campanacci's grading	Treatment Curettage	Treatment Burning	Treatment Spacer
1	M	Primary	33	53x35x30	tibia	I	+	ethanol	artificial bone
		Recurrent	34	30x30x25	tibia	II	+	airtome, ethanol	polymethylmetacrylate
2	F	Primary	17	30x30x25	tibia	I	+	airtome	artificial bone
		Recurrent	17	35x30x30	tibia	I	+	airtome, ethanol	polymethylmetacrylate
3	F	Primary	62	60x60x35	femur	I	+	airtome	polymethylmetacrylate
4	F	Primary	28	60x55x35	sacrum	I	-, radiation	-	-
5	M	Primary	59	60x50x40	tibia	I	+	-	-
6	M	Primary	55	100x45x40	humerus	III	+	-	artificial bone
		Recurrent	57	100x48x44	humerus	III	+	ethanol	polymethylmetarylate
7	M	Primary	23	40x20x20	radius	II	+	ethanol	artificial bone
		Recurrent	23	40x20x20	radius	II	+	ethanol	artificial bone
8	M	Primary	22	70x60x43	humerus	III	+, radiation	ethanol	artificial and autobone
9	F	Primary	58	43x40x25	fibula	III	wide resection	-	-
10	F	Primary	17	78x45x38	femur	II	+	-	polymethylmetacrylate
		Recurrent	35	60x50x40	femur	III	+	-	polymethylmetacrylate
11	M	Primary	44	33x23x22	radius	III	wide resection	-	-

Table 2. Immunohistochemical results

Patient No	Sex	Primary/ Recurrent	Campanacci's grading	Stromal cell		Giant cell			
						Nuclear No <15		Nuclear No ≥15	
				β-catenin [%]	Ki-67 [%]	β-catenin [%]	Cyclin D1 [%]	β-catenin [%]	Cyclin D1 [%]
1	M	Primary	I	21.9	11.6	31.3	84.6	3.1	7.3
		Recurrent	II	68.1	28.1	42.9	86.6	15.9	7.2
2	F	Primary	I	56.6	17.0	20.8	53.3	25.0	36.7
		Recurrent	I	61.6	7.0	26.1	33.3	8.7	6.7
3	F	Primary	I	52.0	13.3	65.0	80.2	13.3	17.6
4	F	Primary	I	63.2	10.2	50.0	72.5	6.0	8.2
5	M	Primary	I	17.9	28.1	8.3	78.9	0	6.1
6	M	Primary	III	44.5	22.9	0	67.1	0	26.8
		Recurrent	III	67.2	25.3	2.4	74.4	4.8	23.9
7	M	Primary	II	26.5	14.5	0	70.5	0	9.0
		Recurrent	II	44.3	16.4	54.2	58.5	0	0
8	M	Primary	III	58.7	35.1	49.1	69.4	3.9	4.2
9	F	Primary	III	48.3	18.4	51.4	69.2	16.8	5.3
10	F	Primary	II	59.6	33.5	48.4	75.2	22.6	1.2
		Recurrent	III	62.0	17.0	82.8	24.2	3.5	0
11	M	Primary	III	69.3	25.4	80.9	71.9	4.8	1.1

Table 3. Comparison of immunohistochemical results between primary and recurrent giant cell tumor of bone

Primary or recurrent	n	Age	Size [mm]	Stromal cell		Giant cell			
						Nuclear <15		Nuclear ≥15	
				β-catenin [%]	Ki-67 [%]	β-catenin [%]	Cyclin D1 [%]	β-catenin [%]	Cyclin D1 [%]
Primary without recurrence	6	45.5	54.4x48.0x32.8	51.6	21.8	50.8	73.7	7.5	7.1
Primary with recurrence	5	29.0	60.2x35.0x30.6	41.8	19.9	20.1	70.1	10.1	16.2
Recurrent	5	33.2	53.0x35.6x32.2	60.6	18.8	41.7	55.4	6.6	7.6
P-value*				0.074	0.851	0.095	0.225	0.593	0.195

*Primary with recurrence vs. Recurrent

Table 4. Comparison of immunohistochemical results on the basis of Campanacci's radiographic grading

| Campanacci's grading | n | Age | Primary/Recurrent | Stromal cell | | Giant cell | | | |
| | | | | | | Nuclear <15 | | Nuclear ≥15 | |
				β-catenin [%]	Ki-67 [%]	β-catenin [%]	Cyclin D1 [%]	β-catenin [%]	Cyclin D1 [%]
I	6	36.0	5/1	45.5	14.5	33.6	67.1	9.4	13.8
II	4	24.3	2/2	49.6	23.1	36.4	72.7	9.6	4.4
III	6	45.2	4/2	58.3	24.0	44.4	62.7	5.6	10.2
P-value*				0.209	0.033	0.468	0.288	0.592	0.227

*Jonckheere-Terpstra test among Campanacci's radiographic grading