Identification of differentially expressed genes and their subpathways in recurrent versus primary bone giant cell tumors

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Abstract. Giant cell tumor (GCT) of the bone is a benign but locally aggressive bone neoplasm with a strong tendency to develop local recurrent and metastatic disease. Thus, it provides a useful model system for the identification of biological mechanisms involved in bone tumor progression and metastasis. This study profiled 24 cases of recurrent versus primary bone GCT tissues using QuantiGene 2.0 Multiplex Arrays that included Human p53 80-Plex Panels and Human Stem Cell 80-Plex Panels. A total of 32 differentially expressed genes were identified, including the 20 most upregulated genes and the 12 most downregulated genes in recurrent GCT. The genes identified are related to cell growth, adhesion, apoptosis, signal transduction and bone formation. Furthermore, iSubpathwayMiner analyses were performed to identify significant biological pathway regions (subpathway) associated with this disease. The pathway analysis identified 11 statistically significant enriched subpathways, including pathways in cancer, p53 signaling pathway, osteoclast differentiation pathway and Wnt signaling pathway. Among these subpathways, four genes (IGF1, MDM2, STAT1 and RAC1) were presumed to play an important role in bone GCT recurrence. The differentially expressed MDM2 protein was immunohistochemically confirmed in the recurrent versus primary bone GCT tissues. This study identified differentially expressed genes and their subpathways in recurrent GCT, which may serve as potential biomarkers for the prediction of GCT recurrence.

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Introduction

Giant cell tumor of the bone is a relatively uncommon neoplasm, which is a benign but locally aggressive bone neoplasm characterized by massive bone destruction at the epiphysis of the long bone and has a strong tendency to develop local recurrence and metastasis (1). GCT accounts for 4-5% of primary bone tumors and up to 20% of benign bone tumors (2). Statistically, 80% of GCTs have a benign clinical course with a local recurrent rate of 20-50%. Approximately 10% will undergo malignant transformation and 1-4% will have pulmonary metastases even in cases with a benign histology (3). In China, GCT incidence is significantly higher and observed in roughly 20% of all primary bone tumors (4). To date, surgery is the primary treatment for GCT with unresectable tumors being treated with radiotherapy (5), and these treatment regimens have remained unchanged for much of the past three decades, which is partially due to the lack of randomized clinical trials (4) and lack of chemotherapy options. Since the tissue origin of GCT remains to be determined, and its clinical behavior is unpredictable, the accurate prediction of its recurrence and metastasis is still not available using clinical diagnosis, radiology and histology (6). Thus, novel approaches are urgently required to better understand the molecular mechanisms of GCT carcinogenesis and to therefore provide meaningful strategies for the effective control of GCT in the clinic.

Currently, profiling of altered genes and pathways using gene chips is a useful method and an efficient alternative strategy to establish disease-pathway relationships (7,8).

Information on disease-related genes, such as from the Genetic Association Database (GAD) (9), is increasingly available for constructing high quality disease-metabolic pathway relationships. Different expression profiles of the p53 pathway and stem cell pathway genes was considered to be a major cause of the occurrence of GCT and promoters of malignant transformation and metastasis (10). However, little is known about the role that the p53 pathway plays underlying tumorigenesis and development of recurrent GCT. Moreover, there is strong evidence showing that the neoplastic cells of GCT are developed from mesenchymal stem cells (11). In recent years, more attention has been paid to subpathway (local area of the entire biological pathway), which can provide more detailed information of complex diseases in high-throughput data analysis, because critical genes may not be significantly enriched in the whole pathway, but nevertheless play key roles (12,13). Therefore, in this study, we profiled differentially expressed genes in recurrent versus primary GCT tissues and identified significant subpathways to further explore the biological mechanisms involved in the recurrence of GCT. Thus, the aim of this study was to improve the understanding of these genes and pathways in the regulation of GCT invasion, recurrence and metastasis, and therefore to evaluate them as potential biomarkers for the early detection and prediction of tumor recurrence.

Materials and methods

Study population. A total of 24 cases of bone GCT, including 12 primary and 12 recurrent tumors, were obtained from 17 GCT patients who were surgically treated in the Department of Orthopedics Surgery, Shantou Hospital of Zhongshan University, between March 2001 and April 2010. All cases were diagnosed by the experienced subspecialty bone and soft-tissue pathologists, and confirmed by another study pathologist. The clinicopathological data of each patient were retrieved from their medical records and are summarized in Table I. The Institutional Review Board of Shantou Hospital of Zhongshan University approved the study protocol and each patient signed an informed consent form before recruitment into this study.

Whole genome cDNA QuantiGene 2.0 microarray analysis. Formalin-fixed and paraffin-embedded (FFPE) non-cancer and cancer tissues were isolated, separately by scraping, and placed into 1.5-ml microcentrifuge tubes for processing of tissue homogenates according to the procedure as described in the QuantiGene sample processing kit for FFPE tissues (Affymetrix, Inc., Santa Clara, CA, USA). Briefly, 300 μl of homogenizing tissue mixture, containing 10 deparaffinized 10-μm sections, were supplemented with 3 μl of proteinase K (50 μg/μl) and incubated overnight at 65˚C. The following day, the tissue homogenates were separated from debris by brief centrifugation and transferred to a new tube. The resulting tissue homogenates were frozen at -80˚C and stored until further use.

A QuantiGene 2.0 Multiplex assay system, containing Human p53 80-Plex Panels and Human Stem Cell 80-Plex Panels, was purchased from Affymetrix, Inc. The QuantiGene 80-Plex assay was performed according to the recommended protocol of QuantiGene 2.0 reagent systems (Affymetrix, Inc.). Briefly, 40 μl of tissue homogenate was mixed with 33.3 μl of lysis mixture, l μl of blocking reagent, 0.3 μl of 2.0 probe set, and 25.4 μl of nuclease-free water. The reactions were placed in a 96-well capture plate covalently coated with capture probes and incubated for 16 h at 54˚C. Wells were washed three times with wash buffer to remove unbound material. For signal amplification, with 100 μl of 2.0 Pre-Amplifier working reagent, 100 μl of Amplifier working reagent was added to each sample and incubated for 1 h each at 50˚C, respectively. To detect the signal, to each sample was added 100 μl of 2.0 substrate, the samples were sealed and incubated for 5 min. Luminescence levels were then measured using a luminometer (Victor Light; Perkin-Elmer, Waltham, MA, USA). Duplicate assays were performed for all samples, and homogenizing buffer was used as background control. To verify that the resulting assay signals were linearly proportional to the sample input, a 2-fold dilution series of each sample was performed. The RNA level of PGK1, TBP, HPRT1, GUSB and TFRC (reference genes) were measured to normalize the data.

Function enrichment analysis. We used the iSubpathwayMiner package that was developed by our laboratory (12) to identify the pathways of the differentially expressed genes in recurrent vs. primary bone GCT tissues. The tool was an R package for flexible biological pathway identification from the KEGG database (14), which covered not only the entire pathway level but also the subpathway level. During enrichment analyses, we performed entire pathway and subpathway identification for the differentially expressed genes based on the hypergeometric test. The corresponding GCT data were integrated with p53 gene and stem cell data and then into the corresponding gene product nodes (referred to as signature nodes) within the pathway. The lenient distance similar to the signature nodes within the pathway structure were analyzed to locate key cascade subpathway regions. Finally, a hypergeometric test was used to evaluate the enrichment significance of these subpathway regions.

Immunohistochemistry. We also performed immunohistochemistry using the PV-9000 2-step plus Poly-HRP anti-mouse/rabbit IgG detection system (ZSGB-BIO) and the liquid DAB substrate kit (ZSGB-BIO) to assess expression of MDM2 in GCT tissues using an MDM2 antibody (cat no. ZA-0519; ZSGB-BIO, Beijing, China). Briefly, 24 cases of GCT tissues were built to form a tissue microarray (TMA) and prepared for 4 μm sections. For immunohistochemistry, the TMA sections were subjected to dewaxing in xylene and rehydration in a series of graded alcohols, and then subjected to antigen retrieval with a pressure cooker for 10 min in 0.01 M sodium citrate buffer (pH 6.0). After that, the sections were submerged in a peroxidase quenching solution, containing one part of 30% hydrogen peroxide to nine parts of distilled water, for 10 min and then washed with phosphate-buffered saline (PBS) three times for 2 min each. The sections were incubated in a moist chamber with 0.1 ml of blocking serum solution for 10 min and then further incubated with 0.1 ml primary antibody for 30 min. After rinsing with PBS three times for 2 min each, 0.1 ml of HRP polymer conjugate was added to each section and incubated for 10 min, followed by a

Table I. Clinical characteristics of recurrent and primary bone GCT patients.

Case	Primary/recurrent	Sex	Age (years)	Site	Campanacci's grading	Surgical treatment
1	Primary	Female	25	Femur	I	Curettage
	Recurrences	Female	27	Femur	III	Wide resection
2	Primary	Male	44	Radius	I	Curettage
	Recurrences	Male	46	Radius	II	Curettage
	Recurrences	Male	48	Radius	III	Wide resection
3	Primary	Male	23	Tibia	I	Curettage
	Recurrences	Male	24	Tibia	II	Curettage
4	Primary	Female	20	Vertebra	I	Curettage
	Recurrences	Female	21	Vertebra	II	Curettage
5	Primary	Female	26	Tibia	I	Curettage
	Recurrences	Female	26	Tibia	I	Curettage
	Recurrences	Female	27	Tibia	II	Curettage
6	Primary	Male	18	Fibula	I	Wide resection
7	Recurrences	Male	40	Humerus	III	Wide resection
8	Recurrences	Male	49	Femur	III	Wide resection
9	Recurrences	Male	28	Tibia	III	Curettage
10	Recurrences	Male	48	Femur	III	Wide resection
11	Primary	Female	45	Radius	II	Curettage
12	Recurrences	Male	28	Femur	III	Wide resection
13	Primary	Female	19	Femur	II	Curettage
14	Primary	Male	50	Humerus	II	Curettage
15	Primary	Female	31	Femur	II	Curettage
16	Primary	Female	20	Metacarpus	I	Curettage
17	Primary	Male	23	Tibia	I	Curettage

rinse with PBS. Next, the sections were incubated with DAB chromogen solution for 3-10 min and subsequently counter-stained with Mayer's hematoxylin, dehydrated and mounted. The negative controls were incubated with 10% normal goat serum to substitute the primary antibody. Immunostained TMA sections were then reviewed and scored in a blinded manner by at least two independent investigators. The positive signal was observed in tumor cell cytoplasm, and scored as the estimated percentage of staining. MDM2 immunoreactivity was classified into three categories as negative (<20% tumor cells displaying cytoplasmic staining); heterogeneous (20-79% tumor cells with cytoplasmic reactivity); and homogeneous (>80% tumor cells with intense cytoplasmic staining).

Statistical analysis. All statistical analyses were performed by using SPSS 11.0 software (SPSS, Chicago, IL, USA). Statistical analyses between primary and recurrent groups were determined by using the Kruskal Wallis test. A P-value <0.05 was considered statistically significant.

Results

Identification of differentially expressed genes in recurrent vs. primary bone GCT. In this study, we analyzed differentially expressed genes in recurrent vs. primary bone GCT tissues using QuantiGene 2.0 Multiplex assay. We identified a total of 32 differentially expressed genes using fold-change (FD >2 or FD <0.5), including 20 most upregulated genes and 12 most downregulated genes in recurrent bone giant cell tumor tissues versus the primary tumors (Table II). These genes are related to cell growth, adhesion, apoptosis, signal transduction, and bone formation, indicating that they may play roles in bone GCT progression, such as recurrence or metastasis.

Functional analysis of the differentially expressed genes. We performed pathway enrichment analysis using the differentially expressed genes in recurrent GCT tissues and identified six gene pathways (Table III). We then used the gene pathway data to locate the important pathway regions, and tested the regions by entering the differentially expressed genes into the p53 and stem data set of 150 genes for the pathway enrichment. Thus, we found a total of 11 subpathways (Table IV). It needs to be pointed out that 6 of these subpathways were not identifiable by the entire pathway identification method. Only focal adhesion pathway is significant in both entire and subpathway identification methods. If we only adopted the entire pathway identification method, these pathways could be ignored due to their high

Table II. Differentially expressed genes between the primary and recurrent bone giant cell tumor tissues.

Name	Full name	Fold change
NANOG	Nanog homeobox	62.01
CD4	CD4 molecule	23.97
TIMP3	Tissue inhibitor of metalloproteinases 3	13.31
ADAR	Adenosine deaminase, RNA-specific	10.63
MDM2	Murine double minute 2	6.17
NUMB	Numb homolog (*Drosophila*)	4.98
STAT1	Signal transducer and activator of transcription 1	4.89
BAX	BCL2-associated X protein	4.16
PAFAH1B1	Platelet-activating factor acetylhydrolase 1b, regulatory subunit 1 (45 kDa)	3.96
RAC1	Ras-related C3 botulinum toxin substrate 1	3.62
CDH2	Cadherin 2, type 1, N-cadherin (neuronal)	3.47
NFKB1	Nuclear factor of κ light polypeptide gene enhancer in B-cells 1	3.32
NCSTN	Nicastrin	3.00
CD8A	CD8a molecule	2.27
PGK1	Phosphoglycerate kinase 1	1.71
IGF1	Insulin-like growth factor 1 (somatomedin C)	1.47
BTG2	BTG family, member 2	1.34
MME	Membrane metallo-endopeptidase	1.17
CXCL12	Chemokine (C-X-C motif) ligand 12	1.10
JUN	Jun proto-oncogene	0.21
FZD1	Frizzled family receptor 1	-1.81
HK2	Hexokinase 2	-1.86
TFRC	Transferrin receptor (p90, CD71)	-2.22
E2F1	E2F transcription factor 1	-3.38
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	-7.67
DVL3	Dishevelled, dsh homolog 3 (*Drosophila*)	-8.62
BMP1	Bone morphogenetic protein 1	-10.28
EGR1	Early growth response 1	-14.41
FGFR1	Fibroblast growth factor receptor 1	-20.25
BMP2	Bone morphogenetic protein 2	-36.26
COL1A1	Collagen, type I, α 1	-469.8
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	-37.99

Table III. The newly identified gene pathways after the pathway enrichment analysis of the differentially expressed genes.

Pathway ID	Pathway name	P-value	Ann molecule list	AnnBg molecule list
path:04640	Hematopoietic cell lineage	0.007708	CD8A; TFRC; MME; CD4	CD8A; TFRC; MME; CD44; IL6; TNF; CD4
path:04145	Phagosome	0.018792	TFRC; RAC1	TFRC; RAC1
path:04974	Protein digestion and absorption	0.018792	MME; COL1A1	MME; COL1A1
path:05340	Primary immunodeficiency	0.018792	CD8A; CD4	CD8A; CD4
path:04514	Cell adhesion molecules (CAMs)	0.036157	CD8A; CDH2; CD4	CD8A; VCAN; NCAM1; CDH2; CDH1; CD4
path:04510	Focal adhesion	0.049058	IGF1; COL1A1; JUN; RAC1	MAPK8; RHOA; IGF1; CCND1; COL1A1; PTEN; JUN; BCL2; PRKCA; IGF1R; RAC1

Table IV. The statistically significant subpathways identified by iSubpathwayMiner.

Subpathway ID	Pathway name	P-value	Differential genes within the subpathway	Genes within the subpathway
path:05200_1	Pathways in cancer	0.002258	DVL3; FZD1; IGF1; STAT1; FGFR1; NFKB1; MDM2; MYC; RAC1	DVL3; DVL1; FZD1; RHOA; FZD8; FZD3; IGF1; STAT1; TCF7; FGFR1; NFKB1; MDM2; MYC; KRAS; RELA; IGF1R; RAC1
path:04062_1	Chemokine signaling pathway	0.007513	STAT1; NFKB1; CXCL12; RAC1	STAT1; NFKB1; CXCL12; RELA; RAC1
path:04115_1	p53 signaling pathway	0.007513	IGF1; SERPINE1; MDM2; BAX	IGF1; TP53; SERPINE1; MDM2; BAX
path:04380_1	Osteoclast differentiation	0.007513	STAT1; NFKB1; JUN; RAC1	STAT1; NFKB1; JUN; RELA; RAC1
path:04010_1	MAPK signaling pathway	0.019088	NFKB1; MYC; JUN; RAC1	MAPK8; NFKB1; MYC; JUN; RELA; RAC1
path:04510_1	Focal adhesion	0.019088	IGF1; COL1A1; JUN; RAC1	MAPK8; IGF1; COL1A1; JUN; IGF1R; RAC1
path:05215_1	Prostate cancer	0.019088	IGF1; FGFR1; NFKB1; MDM2	IGF1; FGFR1; NFKB1; MDM2; RELA; IGF1R
path:05220_1	Chronic myeloid leukemia	0.030663	NFKB1; MDM2; MYC	NFKB1; MDM2; MYC; RELA
path:04310_2	Wnt signaling pathway	0.036904	DVL3; FZD1; MYC; JUN; RAC1	DVL3; DVL1; FZD1; FZD8; FZD3; TCF7; AXIN1; MYC; JUN; RAC1
path:04722_1	Neurotrophin signaling pathway	0.037737	NFKB1; JUN; BAX; RAC1	MAPK8; TP53; NFKB1; JUN; BAX; RELA; RAC1
path:00010_1	Glycolysis/ gluconeogenesis	0.044385	HK2; PGK1	HK2; PGK1

P-values threshold. However, these pathways were found statistically significant using the subpathway identification method. The result indicated that these subpathways may be associated with GCT recurrence.

The most significant subpathway was the 'pathways in cancer'. GCT, as tumor disease was obviously associated with the dysregulation of the pathway. We found that the differential gene IGF1, a growth factor, was located in the starting regions in the pathway (Fig. 1).

The third subpathway (path:04115_1) in Table I belonged to the p53 signaling pathway. The pathway was reported to be highly associated with giant cell tumor of bone (GCTB) (15-17). Gene p53 was located in the center of the pathway and identified within the subpathway path:04115_1 (Fig. 2). Moreover, MDM2 was localized in the central region of this subpathway.

The fourth subpathway path:04380_1, which belonged to the osteoclast differentiation pathway, was identified as significant in iSubpathwayMiner, which yielded a P-value of 0.0075. This pathway was associated with the localized bone destruction of GCTB (18). Mononuclear stromal cells in GCTs of bone have shown characteristics of the osteoblast lineage by expressing many osteoblast-associated differentiation markers (19). The bone resorption activity of osteoclasts can cause destructive osteolysis and consequent morbidity in GCT (18). The subpathway path:04380_1 contained four differential genes: JUN, NFKB1, STAT1 and RAC1 (Fig. 3).

The ninth subpathway (path:04310_2) belonged to the Wnt signaling pathway. The subpathway path:04310_2 contained MYC, JUN, and RAC1, most of which were localized in the endpoint of this pathway (Fig. 4).

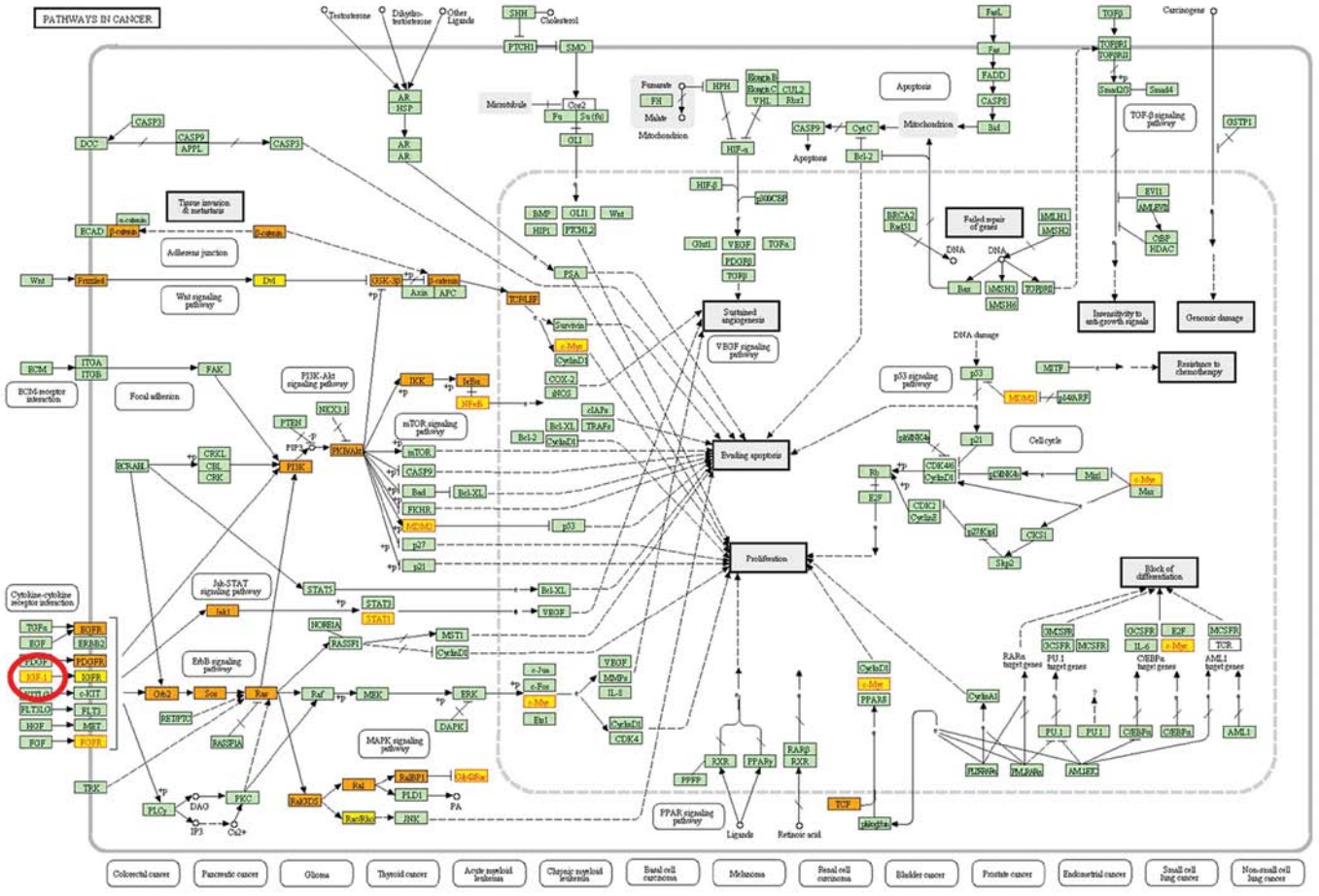

Figure 1. The differentially expressed genes in recurrent vs. primary bone GCT tissues belongs to the 'pathways in cancer'. The dark shade nodes indicate the key subpathway region (path:05200_1) identified by iSubpathwayMiner. The proteins mapped by differential genes are shown with red node labels and borders.

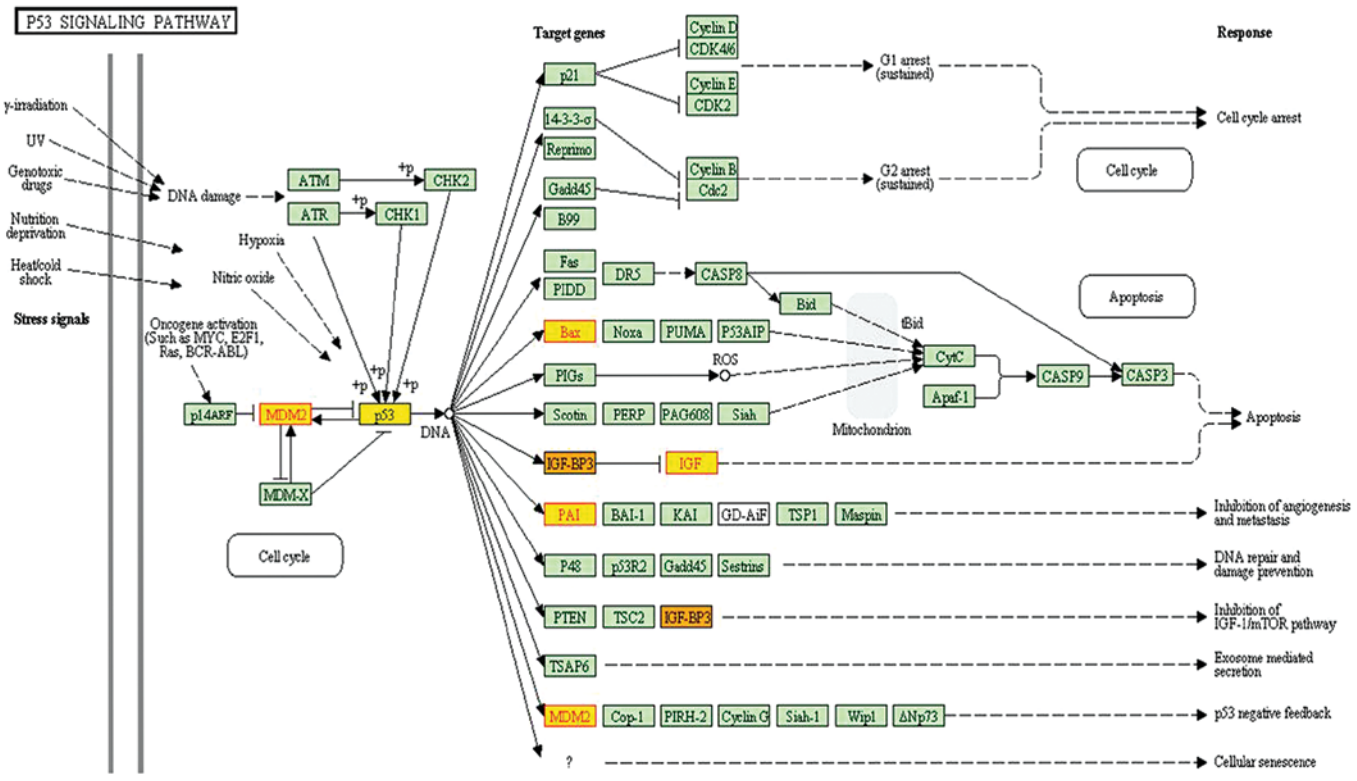

Figure 2. The *p53*-signaling pathway was annotated from the differentially expressed genes. The dark shade nodes indicate the key subpathway region (path:04115_1) identified by iSubpathwayMiner. The proteins mapped by differential genes are shown with red node labels and borders.

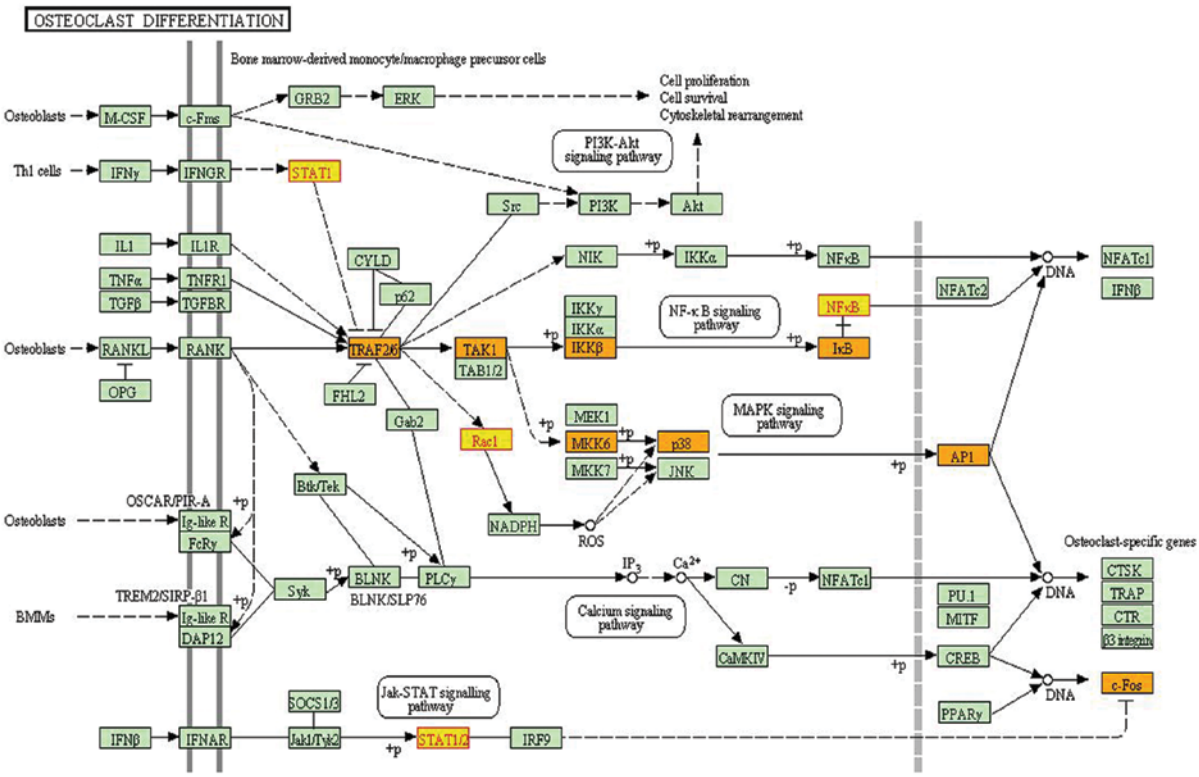

Figure 3. The osteoclast differentiation pathway was annotated from the differentially expressed genes. The dark shade nodes indicate the key subpathway region (path:04380_1) identified by iSubpathwayMiner. The proteins mapped by differential genes are shown with red node labels and borders.

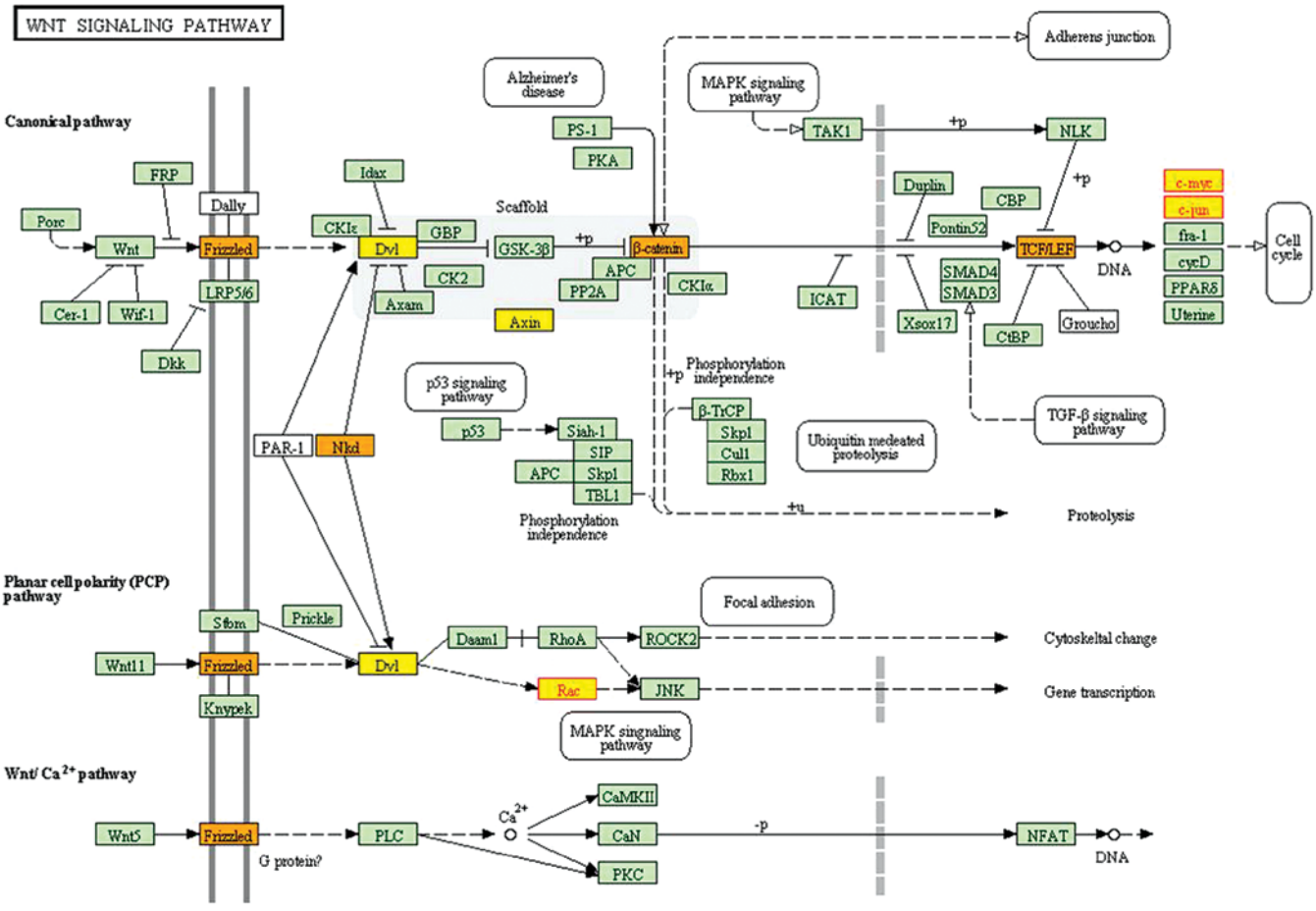

Figure 4. The Wnt signaling pathway was annotated from the differentially expressed genes. The dark shade nodes indicate the key subpathway region (path:04310_2) identified by iSubpathwayMiner. The proteins mapped by differential genes are shown with red node labels and borders.

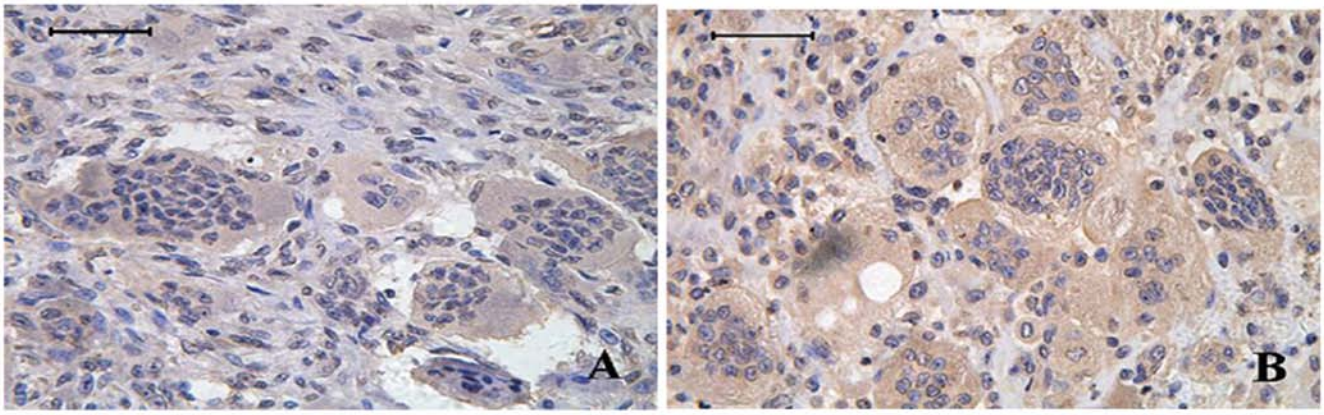

Figure 5. Immunohistochemical analysis of MDM2 expression in primary (A) and recurrent (B) bone GCT tissues. Bar, 50 μm.

Immunohistochemical validation of MDM2 expression in bone GCT tissues. After the above pathway analysis, we identified many risk genes in recurrent GCT. Among these risk genes, we chose MDM2 for immunohistochemical analysis in bone GCT tissues. MDM2 protein was observed in both multinucleated giant cells and mononuclear stromal cells in GCT tissues (Fig. 5). Comparing immunohistochemical results between the primary and recurrent bone GCT tissues, MDM2 was statistically significantly higher in recurrent tumors than primary tumors (P=0.015, χ^2=5.86). MDM2 is involved in the occurrence and development of GCT.

Discussion

Bone GCT is a benign but locally aggressive bone neoplasm with a strong tendency to develop a local recurrent and metastatic disease. Recently, two studies attempted to identify differentially expressed genes associated with GCT development and progression (20,21). Altered expression of Ephrin A receptor, Claudin 7, CD52, FGFR3, and AMFR was found by Guenther *et al* (20), whereas Skubitz *et al* (21) reported that genes found to be overexpressed in GCTs included tartrate-resistant acid phosphatase, the lysosomal H^+-transporting ATPase, and osteoprotegrin ligand (OPGL). In our present study, we identified 32 differentially expressed genes in the primary vs. recurrent bone GCT tissues and we then used iSubpathwayMiner to annotate them into gene subpathways resulting 11 statistically significantly enriched subpathways. This study is just the first step to identify genes that are associated with bone GCT recurrence and further study is warranted to investigate them mechanistically in bone GCT to provide biomarkers or therapeutic targets.

The present study identified several critical genes and gene pathways that are associated with bone GCT recurrence. The first gene was IGF1, which regulates cell proliferation, differentiation and survival (22). IGFI plays an important role in normal bone growth, bone cell turnover and metabolism, and is a key factor in osteoblast proliferation and bone formation (23). IGF1 may be a possible prognostic marker useful in the identification of GCT patients at a higher risk of relapse with potential for development into a therapeutic agent against GCT. Moreover, the third subpathway (path:04115_1) belonged to the p53 signaling pathway and p53 is localized in the center of this pathway and identified within the subpathway path:04115_1. MDM2 was localized in the central region of this subpathway. Indeed, p53 is frequently mutated in GCT and could be useful in predicting tumor progression and local recurrence (15). p53 mutations were detected in the cases of secondary malignant giant-cell tumor without irradiation therapy (10). Masui *et al* showed that p53 expression levels correlated with the rates of lung metastasis and recurrence of GCT (16). These results suggest that p53 mutations may play an important role in malignant transformation of conventional GCT (17). MDM2 is a negative regulator of p53 and plays an important role in the p53-signaling pathway, suggesting a potentially high association with bone GCT development. Indeed, MDM2 has been found widely expressed in GCT (24). Thus, we further evaluated MDM2 expression in bone GCT tissues and the immune-reactivity of anti-MDM2 antibody was observed in osteoclast-like giant cells and mononuclear stromal cells. Statistical analyses showed that MDM2 expression was significantly higher in recurrent tumors than in primary tumors, suggesting that MDM2 might be associated with bone GCT recurrence.

Furthermore, the fourth subpathway path:04380_1 was the osteoclast differentiation pathway. This subpathway path:04380_1 contained four differentially expressed genes, including JUN, NFKB1, STAT1 and RAC1. c-Jun is a component of the heterodimeric AP-1 transcription factor and was highly expressed in GCT stromal cells (25). JUN may also be involved in upregulation of matrix-metalloproteinases in GCT. MMP-2, MMP-13, and MMP-9 have been shown to be highly expressed in GCT tissues (26-28). Both MMP-2 and MMP-9 display several AP-1 consensus sequences within their promoter regions and may be directly upregulated by JUN (29). MMP-13 is responsible for optimizing the bone resorption capability of the giant cells, which is likely to be due to recruiting them to the bone surface (27,30). JUN could influence normal ECM physiology, thereby promoting growth and destructiveness of GCT (27). NF-κB has been shown to play an important role in many types of cancer and may also regulate tumor angiogenesis and invasiveness. NF-κB provides a mechanistic link between inflammation and cancer and is a major transcriptional factor controlling the ability of both pre-neoplastic and malignant cells to resist apoptosis-based tumor-surveillance mechanisms (30). RANKL, as a

negative regulator of NF-κB, was identified as essential for osteoclast physiology (31,32). RANKL is highly expressed in stromal cells of GCT (33,34). As a potential therapeutic target of bone disease, Amgen developed a monoclonal antibody to RANKL (denosumab). Denosumab was studied in a recent proof-of-principle phase II study of 35 patients with recurrent or unresectable GCT (35). Twenty-six of the 31 patients with data reported reduced pain or improvement in the functional status. Radiologic evidence of bone repair was reported in nine patients. The treatment was generally well tolerated without treatment-related serious adverse events. Thus, blockade of RANKL signaling in patients with advanced or unresectable GCT could provide objective changes in tumor composition, reduced bony destruction, and clinical benefit. Signal transducer and activator of transcription 1 (STAT1), localized in the starting region of the subpathway path:04380_1 (Fig. 3), was reported to be associated with human breast cancer, melanoma, leukemia, lymphoma, and other cancers (36,37). STAT1 has a critical role in regulation of bone growth and bone formation (38).

In addition, the ninth subpathway (path:04310_2) belongs to the Wnt signaling pathway. Several studies have shown that this pathway plays an important role in regulation of skeletal function, and the activation of Wnt signaling may induce osteoblast differentiation and osteoclastogenesis during the bone resorption process (39-41). Bone GCT was found associated with activation of the Wnt signaling pathway (42). This subpathway contained MYC, JUN, and RAC1, most of which were localized at the endpoint of this pathway. Gamberi *et al* (43) showed a strong correlation between c-Myc overexpression and GCT occurrence and its metastases. c-Myc protein was overexpressed in both giant cells and mononuclear cells, suggesting that both cell types are involved in progression of this tumor type (43). Previous studies found that Rac1 regulated a diverse array of cellular events, including formation of lamellipodia and membrane ruffles, cell cycle, cell adhesion and mobility (44-46). Rac1 is thought to play a significant role in the development of various cancers, including melanoma (45) and non-small cell lung cancer (46). As a result, it is now considered as a therapeutic target for these diseases (30). Rac1 can regulate survival signaling of osteoclasts and their bone resorption activity (47). A transgenic mouse model was used to confirm that Rac1 was the primary Rac isoform in regulating ROS production and the cytoskeleton organization during the multiple stages of osteoclast differentiation (48).

In conclusion, in the present study, we identified 32 genes that were differentially expressed in recurrent vs. primary bone GCT tissues and found them in multiple subpathways. Among them, four genes (IGF1, MDM2, STAT1 and RAC1) were located in key positions in these pathways. Further studies will confirm our current data and investigate their roles and functions in bone GCT progression.

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