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H3F3A G34 mutation DNA sequencing and G34W immunohistochemistry analysis in 366 cases of giant cell tumors of bone and other bone tumors

Lihua Gong[1], Marilyn M. Bui[2], Wen Zhang[1], Xiaoqi Sun[1], Ming Zhang[1] and Ding Yi[1]

[1]Department of Pathology, Beijing Jishuitan Hospital, The Fourth Medical College of Peking University, Beijing, China and [2]Department of Pathology, H Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA

Summary. H3F3A mutations and the expression of glycine 34 to tryptophan (G34W) mutants in giant cell tumors of bone (GCTBs) and other bone tumors were detected to compare H3F3A mutation types and the expression of G34W-mutant protein in order to provide a theoretical basis for using H3F3A mutations as a diagnostic and differential-diagnostic tool for GCTBs. A total of 366 bone tumor cases were investigated. The cases involved 215 men and 151 women, whose median age was 29 years (3-84). The cases included GCTB (n=180), recurrent GCTB (n=19), GCTB with lung metastasis (n=5), pediatric GCTB (n=15), primary malignant GCTB (n=5), chondroblastoma (CB, n=61), chondrosarcoma grade II (n=15), dedifferentiated chondrosarcoma (n=17), chondromyxoid fibroma (n=9), aneurysmal bone cyst (n=9), nonossifying fibroma (n=9), osteosarcoma (n=16), and undifferentiated sarcoma (n=6). Sanger DNA sequencing analysis was used to detect H3F3A mutations. Immunohistochemistry was used to assess the expression of the G34W-mutated protein in these bone tumors. DNA sequencing results revealed H3F3A mutations in 95.00% of GCTBs (171/180), including glycine 34 to tryptophan (G34W, 163/180, 90.56%), glycine 34 to leucine (G34L, 3/180, 1.67%), glycine 34 to valine (G34V, 3/180, 1.67%), and glycine 34 to arginine (G34R, 2/180, 1.11%). Recurrent GCTBs mostly had the H3F3A G34W mutation (18/19, 94.74%), and GCTBs with lung metastasis all had the H3F3A G34W mutation (5/5, 100%). Pediatric GCTBs had a mutation rate of 93.33% (14/15), including one case with G34L. Four cases of primary malignant GCTB showed the H3F3A G34W mutation (4/5, 80.00%), and the classical GCTB component and malignant component showed consistent mutation types.

Immunohistochemistry showed that GCTBs harboring G34W also expressed the mutant protein in tumor cell nuclei. Furthermore, one case of GCTB and one case of recurrent GCTB showed positive G34W immunostaining results despite being negative for the genetic mutation. Other bone tumors all showed wild-type expression in both DNA sequencing and immunohistochemistry. Our large-sample DNA sequencing analysis detected four different forms of mutations in GCTBs, including three rare mutation forms. The most common mutation of H3F3A was G34W, which was in accordance with the expression of G34W in GCTBs detected by immunohistochemistry. Although DNA sequencing analysis detected rare mutation types of H3F3A, false-negative results were also present due to the small number of cells in the samples. Detection of the most common (G34W) mutant protein by immunohistochemistry was more convenient. Given the high prevalence of these driver mutations, the detection of H3F3A mutant proteins can assist in the diagnosis of GCTB and its differential diagnosis from other bone tumors.

Key words: Giant cell tumors of bone, H3F3A, G34W, Mutation, Immunohistochemistry, Differential diagnosis, Ancillary testing

Introduction

The diagnosis of primary bone tumors relies on the combination of clinical, pathological, and imaging characteristics. However, the clinical, pathological, and imaging results for many cases might not all be typical, or different tumors might present similar clinical, pathological, and imaging characteristics. Additionally, there are very few markers with diagnostic value. All the above make differential diagnosis difficult. With the advancement of molecular genetic technology, the discovery of and research related to bone tumor-associated molecular events has also gradually expanded and become more detailed. Some molecular methods are gradually being applied to the ancillary diagnosis of bone tumors. The histone family of chromosomal proteins is subdivided into five classes, H1, H2A, H2B, H3, and H4, that occur in nearly all eukaryotic cell types. These proteins, except H4, consist of several subtypes, including H1°, H2A.X, H2A.Z, and H3.3. H3.3 is a subtype variant of the histone H3 protein. The H3.3 protein is encoded by two different genes: H3F3A and H3F3B. Both encode the same amino acid sequence but differ in nucleotide sequence and gene organization (Bramlage et al., 1997). Specific mutations of H3.3 vary by tumor type, such as pediatric glioblastoma, chondroblastoma (CB), and giant cell tumors of bone (GCTBs), each tumor containing a single mutant H3 allele. The somatic mutation of H3 lysine 27 to methionine (K27M) occurs in approximately 30% of pediatric high-grade glioblastomas. This mutation is most often found in H3F3A (>70%) (Gielen et al., 2013). Nearly 95% of CBs contain a lysine 36 to methionine (K36M) mutation. Unlike the K27M mutation in glioblastomas, approximately 90% of K36M mutations are found in H3F3B and this mutation is occasionally found in H3F3A (Cleven et al., 2015). In GCTBs, only H3F3A has these mutations, and it is mutated in 90% of cases. There are several forms of H3F3A mutation, including glycine 34 to tryptophan (G34W), which is the most common mutation, and less common are glycine 34 to leucine (G34L) and glycine 34 to arginine (G34R), the glycine 34 to valine (G34V) mutation being even less common (Kervarrec et al., 2017). This mutation is restricted to the neoplastic stromal cells (Behjati et al., 2014).

Recently, a monoclonal antibody targeting the G34W mutation of H3F3A has been developed. In this study, we comprehensively detected H3F3A DNA mutations and G34W mutant proteins in GCTBs and other bone tumors to investigate the mutation types and tumor distribution of H3F3A in GCTBs and other bone tumors. In addition, the DNA sequencing results and immunohistochemistry results for mutated proteins were compared to provide a theoretical basis for using H3F3A mutations as a tool for the diagnosis of GCTB and its differential diagnosis from other bone tumors.

Materials and methods

Patients and surgical specimens

With approval from the institutional ethics committee and following the research protocol, 366 cases of bone tumors were retrieved from surgical pathology records between 2016/12 and 2019/10 in the Department of Pathology in Beijing Jishuitan Hospital. None of the GCTB patients had ever received chemotherapy or radiotherapy. The clinical and radiological information was obtained from medical records and surgeons, while histopathological assessments were carried out according to the WHO Classification of Tumors of Soft Tissue and Bone and reviewed by three pathologists.

DNA mutation analysis

Total DNA was extracted from paraffin-embedded tumor tissue samples with an OMEGA DNA extraction kit according to the manufacturer's instructions. Hematoxylin-eosin sections were prepared to evaluate if the area of the tissue selected was representative of the tumor. Formic acid-decalcified specimens were not selected, to ensure consistent results. DNA was considered suitable for molecular analysis only if the A260/A280 ratio was greater than 1.8. The H3F3A primer sequences were as follows: H3F3A-F (ATCG TGGC AGGA AAAG TTGT) and H3F3A-R (ATAC AAGA GAGA CTTT GTCC CA). For PCR, 2 µl of 10× polymerase buffer, 1 µl of primer, 10 µl of Taq DNA Polymerase, and 7 µl of ddH2O were mixed in a final volume of 20 µl. The PCR conditions were as follows: 95°C for 5 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and 72°C for 7 min. Mutation analysis was conducted with the Basic Local Alignment Search Tool (BLAST) in the National Center of Biotechnology Information Database (http://www.ncbi. nlm.nih.gov/BLAST). Electropherograms were exported in FASTA format and were aligned to the NCBI BLAST sequence NM_002107.4 H3F3A mRNA.

Tissue samples and immunohistochemistry

Formalin-fixed, paraffin-embedded specimens from 366 cases of bone tumors were available for immuno-histochemical analysis. Immunohistochemical samples were prepared with an automated immunostainer (Autostainer 720, Lab Vision) according to standard heat-induced epitope retrieval and the avidin-biotin-peroxidase complex method. An anti-histone H3.3 G34W rabbit monoclonal antibody (clone RM263; RevMAb Biosciences USA) (diluted 1: 500) was used. The positive controls were GCTBs with the G34W mutation that had been verified by DNA sequencing and exhibited strong nuclear expression by immunohistochemical staining. CBs were the negative control. Blank controls were prepared by

substituting the primary antibody with non-immune mouse serum. To evaluate G34W immunoreactivity, tumor cells were considered immunopositive when they displayed a brownish nuclear immunoreactivity and negative when expression was not detected. All immunohistochemistry slides were evaluated independently by two pathologists who were blinded to the clinical information. Agreement was reached by careful discussion when the opinions of the two pathologists differed.

Results

Clinical information

Among 366 cases of bone tumors, 215 involved men and 151 involved women, whose median age was 29 years (3-84). There were 180 cases of GCTB, involving the femur (61 cases), tibia (52 cases), radius (17 cases), humerus (15 cases), sacrum (10 cases), fibula (nine cases), vertebra (seven cases), ulna (five cases), and ilium (four cases). The tumors were recurrent GCTB (n=19), GCTB with lung metastasis (n=5), pediatric GCTB (n=15), primary malignant GCTB (n=5), CB (n=61), chondrosarcoma grade II (CS, n=15), dedifferentiated chondrosarcoma (DDCS, n=17), chondromyxoid fibroma (CMF, n=9), aneurysmal bone cyst (ABC, n=9), nonossifying fibroma (NOF, n=9), osteosarcoma (OS, n=16), and undifferentiated sarcoma (UPS, n=6). Detailed information is shown in Table 1.

The H3F3A mutations investigated via molecular genetics

H3F3A mutations were found in 95.00% of GCTBs

(171/180) and included G34W (163/180, 90.56%) (Fig. 1A) and G34L (3/180, 1.67%) (Fig. 1B,C) and two different forms of base mutations (Table 2), G34V (3/180, 1.67%) (Fig. 1D) and G34R (2/180, 1.11%) (Fig. 1E). The other nine cases were determined to be wild-

Table 1. Clinical information.

Tumor type	Number	M	F	Median Age
GCTB*	180	98	82	33 (19-65)
GCTB(R)	19	12	7	31 (18-62)
GCTB (LM)	5	5	0	27 (13-29)
GCTB(P)	15	6	9	16 (11-18)
MGCTB	5	1	4	38 (29-57)
CB	61	45	16	15 (8-29)
CMF	9	7	2	13 (4-24)
CS GII	15	7	8	41 (7-61)
DDCS	17	9	8	51 (37-74)
ABC	9	5	4	8 (4-20)
NOF	9	5	4	12 (3-19)
OS	16	10	6	15.5 (5-47)
UPS	6	5	1	62 (43-84)
total	366	215	151	29 (3-84)

GCTB*, GCTB without recurrence and metastasis; GCTB(R), recurrent GCTB; GCTB (LM), GCTB with lung metastasis; GCTB(P), adolescent GCTB.

Table 2. H3F3A mutation types.

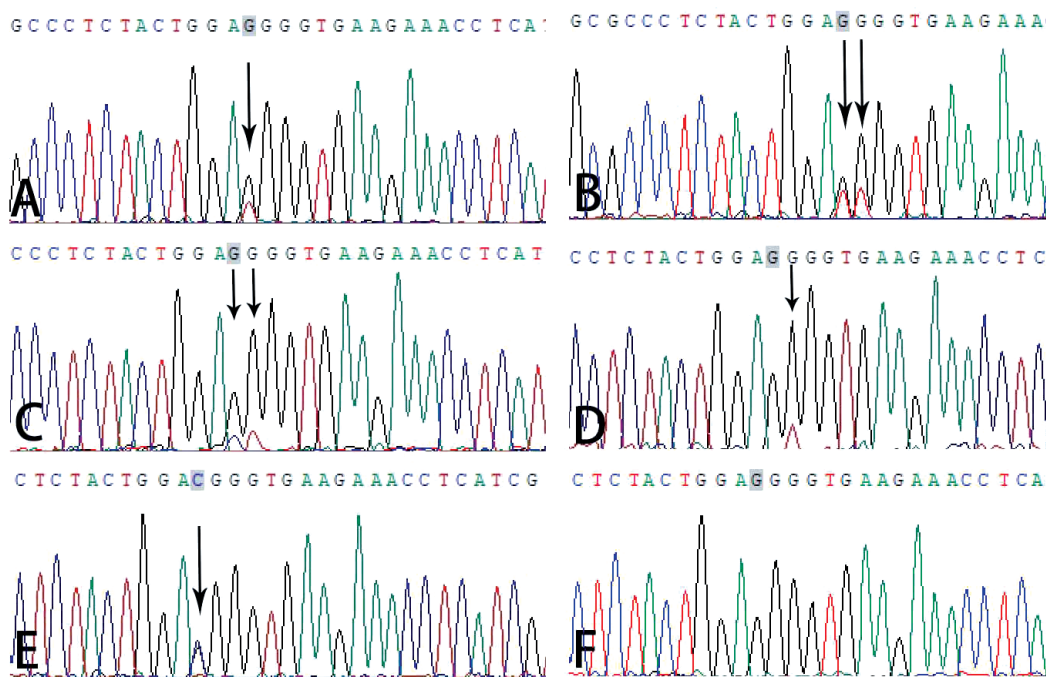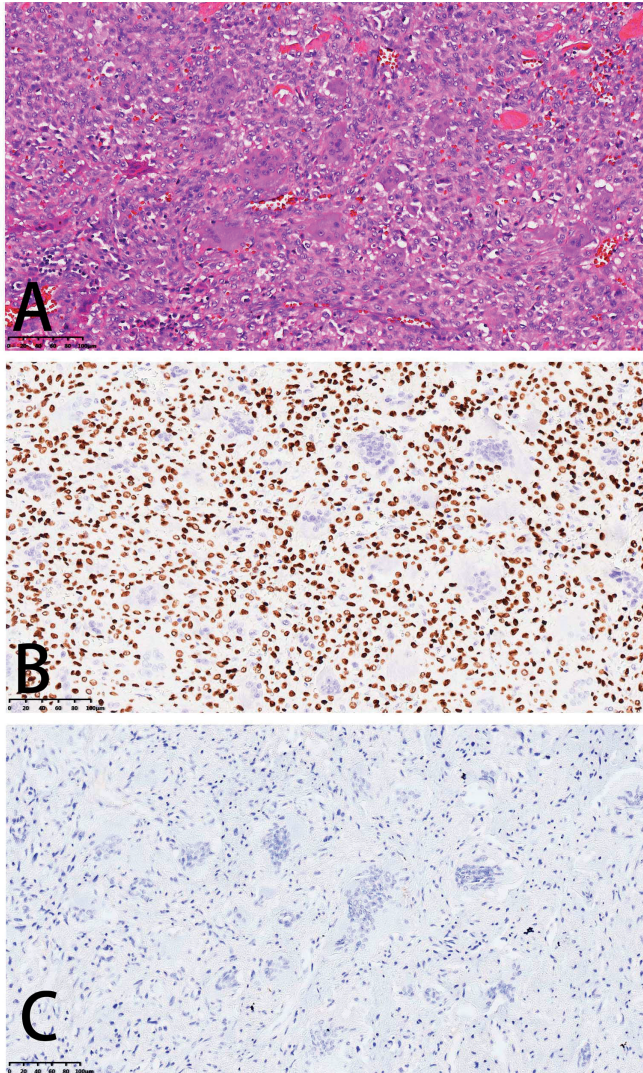| H3F3A WT	G34W	G34R	G34V	G34L
GGG	GGG>TGG	GGG>CGG	GGG>GTG	GGG>TTG GGG>CTG

Fig. 1. Detection of H3F3A mutations in GCTB. **A.** H3F3A G34W (GGG>TGG) mutation. **B.** H3F3A G34L (GGG>TTG) mutation. **C.** H3F3A G34L (GGG>CTG) mutation. **D.** H3F3A G34V (GGG>GTG) mutation. **E.** H3F3A G34R (GGG>CGG) mutation. **F.** H3F3A wild-type.

type (Fig. 1F) by Sanger DNA sequencing (Table 2). Among the nine wild-type cases, there were six females and three males, aged 19-46 years with an average age of 33 years. The lesion involved femur in five cases, tibia in two cases, radius in one case, and vertebrae in one case. Microscopic histomorphology examination showed classic GCTB morphology and did not reveal any recurrence. In addition, there were no differences in histomorphology and distributions between wild-type cases and various mutated cases Recurrent GCTBs mostly had the H3F3A G34W mutation (18/19, 94.74%), and GCTBs with lung metastasis all had G34W (5/5, 100%). Pediatric GCTBs had a mutation rate of 93.33% (14/15), including one case of the G34L mutation. Four cases of primary malignant GCTB had G34W (4/5, 80.00%). Sequencing analysis was performed on the classical GCTB region and the malignant region, and the results showed a consistent G34W mutation in these two components in four cases and the wild-type sequence in these two components in one case. The above mutations were not detected in other bone tumors (Table 3).

H3.3 G34W immunohistochemistry results

The immunostaining results showed H3.3-positive expression in the nucleus of neoplastic cells of GCTBs and negative expression in osteoclastic giant cells (Fig. 2A,B). All cells showed negative expression in negative cases (Fig. 2C). For GCTBs with a secondary ABC-like

Fig. 2. The histological features and detection of H3F3A G34W mutations by immunohistochemistry in GCTB. **A.** GCTB exhibits multinucleated giant cells and mononuclear cells with similar nuclear features (hematoxylin & eosin). **B.** Expression of the H3F3A G34W mutation protein in GCTB shown by strong and diffuse nuclear staining. Only the neoplastic mononuclear cells are highlighted, not the nonneoplastic multinucleated giant cells. **C.** H3F3A G34W negative in GCTB. x 20.

Table 3. H3F3A somatic alterations in bone tumors.

Tumor type	Number	Mutation (%)	G34W (%)	G34R (%)	G34V (%)	G34L (%)	H3F3A G34W positive (%)
GCTB*	180	171 (95.00)	163 (90.56)	2 (1.11)	3 (1.67)	3 (1.67)	164 (91.11)
GCTB(R)	19	18 (94.74)	18 (94.74)	0	0	0	19 (100)
GCTB (LM)	5	5 (100)	5 (100)	0	0	0	5 (100)
GCTB(P)	15	14 (93.33)	13 (86.67)	0	0	1 (6.67)	13 (86.67)
MGCTB	5	4 (80.00)	4 (80.00)	0	0	0	4 (80.00)
CB	61	0	0	0	0	0	0
CMF	9	0	0	0	0	0	0
CS GII	15	0	0	0	0	0	0
DDCS	17	0	0	0	0	0	0
ABC	9	0	0	0	0	0	0
NOF	9	0	0	0	0	0	0
OS	16	0	0	0	0	0	0
UPS	6	0	0	0	0	0	0

GCTB*, GCTB without recurrence and metastasis; GCTB(R), recurrent GCTB; GCTB (LM), GCTB with lung metastasis; GCTB(P), adolescent GCTB.

structure, the ABC structure showed H3.3-positive tumor cells (Fig. 3A,B), whereas all cells with a cystic structure of primary ABC were all H3.3-negative (Fig. 3C,D). GCTBs with lung metastasis were H3.3-positive (Fig. 3E,F). Five cases of primary malignant GCTB had the classical GCTB component and the malignant component. The malignant component showed high-grade sarcoma-like morphology. These two components were both consistently H3.3-positive in four cases (Fig. 4A-D) and negative in one case, and for giant cell-rich OS, the components were H3.3-negative (Fig. 4E,F). The other tumors all had negative expression. The positive cases were found to harbor the H3F3A G34W mutation by DNA sequencing, except for two cases

(Table 3). These two cases showed positive G34W antibody immunostaining with negative genetic mutation. Formic acid decalcification did not affect the immunostaining results. Immunostaining signals were absent in cases with wild-type H3F3A as well as with the G34R, G34V, and G34L mutations.

Discussion

GCTB is a benign and locally aggressive neoplasm that makes up approximately 5% of primary bone tumors. It mostly affects the epiphysis of the long bones in skeletally mature adults between the ages of 20 and 40 years (Amelio et al., 2016). It commonly involves the
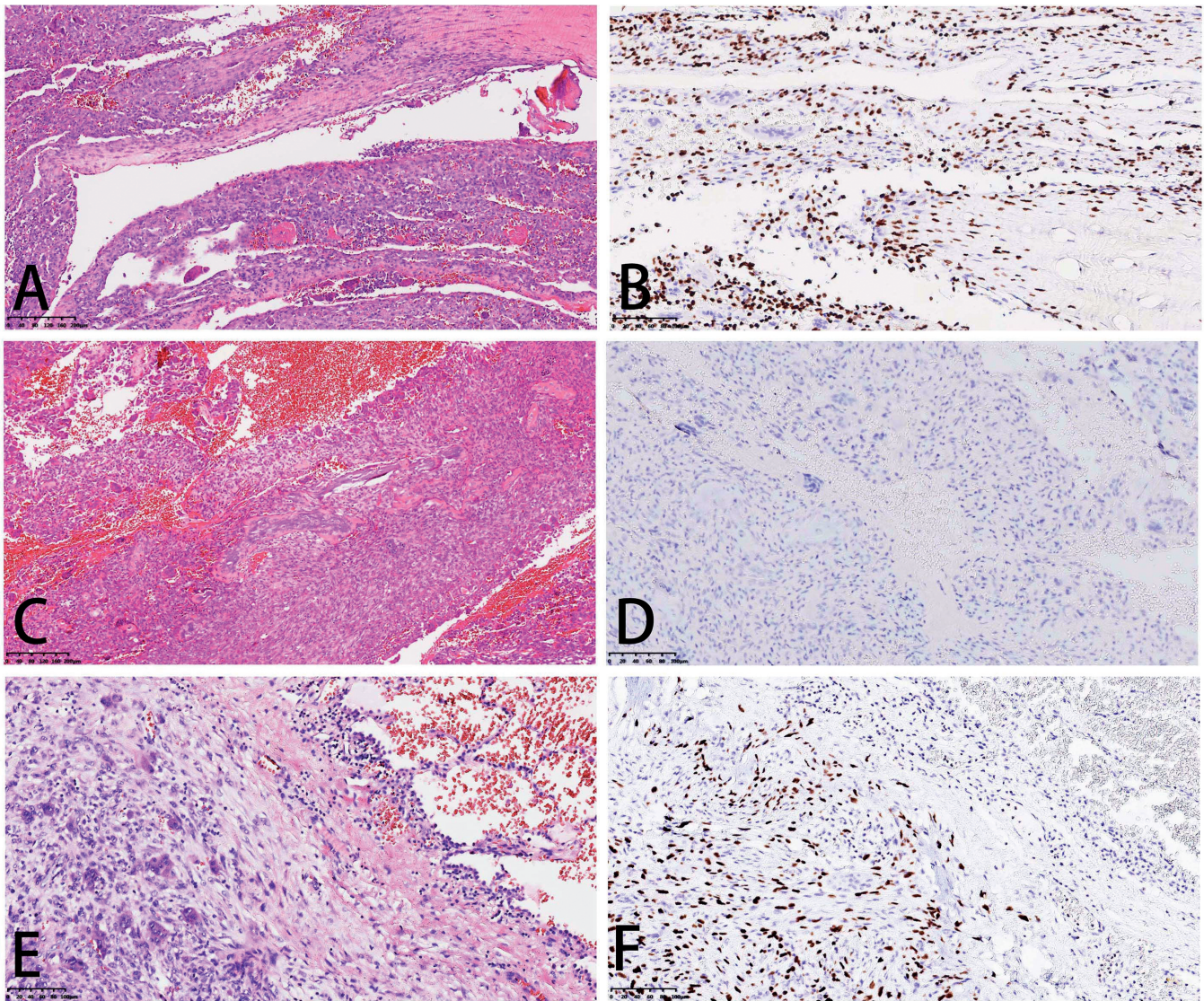

Fig. 3. The histological features and detection of the H3F3A G34W mutation by immunohistochemistry in GCTB and ABC. **A.** GCTB with secondary ABC (hematoxylin & eosin). **B.** Positive H3F3A G34W in the secondary ABC structure of GCTB. **C.** Primary ABC (hematoxylin & eosin). **D.** Negative H3F3A G34W in primary ABC. **E.** GCTB with lung metastasis (hematoxylin & eosin). **F.** Positive H3F3A G34W in GCTB with lung metastasis. A-D, x 10; E, F, x 20.

distal femur, proximal tibia, and distal radius, and the proximal humerus is not uncommonly affected (Miettinen et al., 2013). Interestingly, GCTB has a significantly (P<0.001) higher incidence in the authors' hospital than in the largest US series from the Mayo Clinic and accounts for 16.7% of primary bone tumors (Niu et al., 2015). Conventional GCTB may be locally aggressive, recurring in approximately 15% to 50% of cases (Broehm et al., 2018). The incidence of lung metastasis of GCTB ranges from 1% to 9%; the risk factors for lung metastasis include local recurrence, tumor location, and radiographic stage (Rosario et al., 2017). Although the nomenclature for GCTB is based on the morphological presence of multinucleated osteoclast-like giant cells, mesenchymal fibroblast-like stromal cells are considered the true tumor cells (Schwartz et al., 2002). There are variable features in the histological pattern; for example, the number of multinucleated osteoclast-like giant cells may be very different, the reactive component varies, and the secondary ABC structure is also very common. GCTB can also undergo malignant transformation (Gong et al., 2012); it can be easily diagnosed as other primary malignant sarcomas of bone, such as giant cell-rich OS, when the classical GCTB structure is not observed in the biopsy specimen, thus making malignant GCTB the most in need of
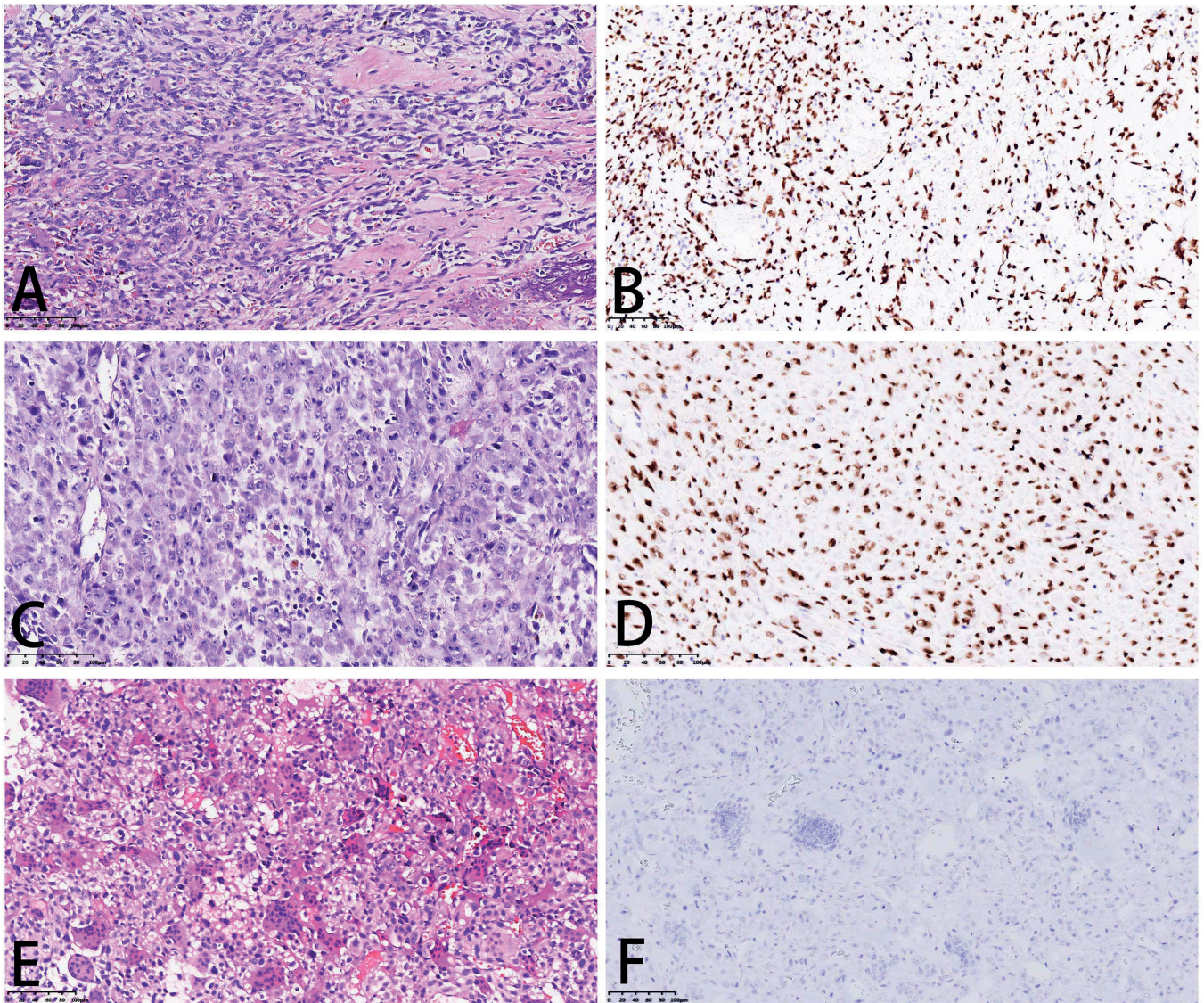

Fig. 4. The histological features and detection of the H3F3A G34W mutation by immunohistochemistry in MGCTB and giant cell-rich OS. **A.** Irregular osteogenesis in the classical GCTB region of primary malignant GCTB (hematoxylin & eosin). **B.** Positive H3F3A G34W in classical GCTB. **C.** Epithelioid morphology in the high-grade malignant component (hematoxylin & eosin). **D.** Positive H3F3A G34W in the high-grade malignant component. **E.** The histological features of giant cell-rich OS (hematoxylin & eosin). **F.** Negative H3F3A G34W in giant cell-rich OS. x 20.

identification.

Regarding the immunophenotype of GCTB, osteoclastic giant cells express RANK, CD51, and CD33 but do not express CD14. Mononuclear cells express RANK, CD51, CD33, and CD14 (Forsyth et al., 2009; Maggiani et al., 2011). These cells are believed to be derived from the fusion of blood monocyte-macrophage lineages and infiltrate tumors by chemotactic factors secreted by spindle-shaped stromal cells, which express RANKL (Zheng et al., 2001; Lau et al., 2005). However, these markers are not specifically expressed in GCTB and do not have significant implications for diagnosis. With the advancement of molecular detection techniques, the emergence of H3.3 mutation detection provides a powerful basis for tumor diagnosis. In one report (Behjati et al., 2013), mutations were found in 49 of 53 cases of GCTB (92%), 48 of which encoded G34W and one G34L. In accordance with these previous results, we detected 180 cases of GCTB, of which 171 cases were found to have a mutation in H3F3A, including G34W, G34R, G34V, or G34L. G34W was the major mutation form, and G34L contained two base mutation types.

In this study, 10 cases of recurrent GCTB and three cases of GCTB with lung metastasis all exhibited the H3F3A G34W mutation. Therefore, it was speculated that the H3F3A mutation might be related to the aggressive characteristics of GCTB. Whether it has implications in prognosis awaits further substantiation in a larger study. Although GCTB involving the immature skeleton is very rare, there are some cases of GCTB in individuals younger than 18 years. Our study included 15 cases of adolescent GCTB. Because the incidence was low and clinical and imaging features were all atypical, clinical diagnosis was difficult. Similar to Al-Ibraheemi et al. (2016), we also found H3F3A mutations among these 15 cases, including 13 cases with G34W and one case with G34R. This detection result provides a basis for the diagnosis of adolescent GCBT with atypical clinical and imaging presentations. Primary malignant GCTB has unique histological characteristics; in other words, it has both the classical GCTB component and the highly malignant component. Histological detection of five cases of primary malignant GCTB in this study showed both the classical giant cell tumor component and the high-grade sarcoma component. Mutation detection was performed on these two components. The results showed that the genetic presentations of these two were consistent; four cases had the H3F3A G34W mutation, and one case had wild-type H3F3A. Although 3.37% of primary malignant bone tumors (including OS and malignant GCTB) have the G34W mutation (Amary et al., 2017), no H3F3A mutation was detected in our 16 cases of OS, and it is questionable whether cases of OS with the H3F3A mutation are actually malignant GCTB. Therefore, if the classical GCTB structure is not observed, but an H3F3A mutation is detected in the malignant sarcoma component, the possibility of malignant GCTB should be considered. Samples should

be collected (as complete as possible) to fully confirm or rule out the GCTB component.

Unlike in GCTB, in CB approximately 90% of K36M mutations are found in H3F3B, and the rest are in H3F3A (Behjati et al., 2014). The H3F3A G34W mutation was not detected. Therefore, for tumors with similar histomorphology and those that occur in atypical locations, such as hands and feet, the H3F3A G34W mutation has differentiation value. Furthermore, H3F3A mutation has not yet been detected in other giant cell lesions, such as primary ABC, NOF, and cartilaginous lesions such as chondrosarcoma and CMF. Additionally, H3.3 mutations are mutually exclusive: two mutation types will not be present together (Gomes et al., 2014). Consequently, mutation detection could be a useful diagnostic tool to distinguish GCTB from other giant cell-rich mimics.

In this study, we performed both DNA sequencing analysis and G34W mutant protein detection. The results showed that except for the wild-type and some rare mutation types, both results were highly consistent. The application of antibodies against rare mutation types (G34R/V) using immunohistochemistry has been included in pathological diagnosis (Yamamoto et al., 2018) to make up for this shortcoming. In addition, compared to DNA sequencing, immunohistochemistry also has other advantages. On one hand, DNA sequencing analysis is influenced by the number of tumor cells. Lüke et al. (2017) found that H3F3A mutation-negative cases were immunoreactive for G34W (positive cells only 10-40%), while in mutation-positive cases, the G34W-positive cell proportion was above 40%. Therefore, the low number of neoplastic cells combined with bone formation, fibrosis, and hemorrhage could interfere with the results of DNA mutation analysis, especially in biopsy tissue. Similar to the results reported by Lüke et al. (2017) two of our cases were negative for genetic mutations but positive for G34W antibody by immunostaining in biopsy tissue. We think that the presence of a few neoplastic cells in the tested tissue explains this. If only a small portion of neoplastic stromal cells that harbor the mutation exist in the tested specimen, then the mutation testing results may be negative. Notably, compared with immunohistochemistry, DNA sequencing is more expensive and time consuming. As an alternative, G34W detection via immunohistochemistry is a useful surrogate marker for diagnosis, especially in biopsies.

Conclusions

H3F3A G34W/L/R/V mutations were highly specific for GCTB, and the expression of H3.3 G34W protein was consistent with the DNA mutation results. Compared to DNA sequencing, immunohistochemistry detection is more convenient and is not limited by the number of cells in the samples. Therefore, diagnosis and differential diagnosis of the majority of patients can be achieved. However, H3F3A mutations could be used to

diagnosis GCTB, but wild-type H3F3A should not rule out GCTB.

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