Histone 3.3 Mutations in Giant Cell Tumor and Giant Cell-Rich Sarcomas of Bone

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Abstract

Mutually exclusive histone 3.3 gene mutations have been recognized in chondroblastoma and giant cell tumor of bone (GCTB), which may be useful for differential diagnostic purposes in morphologically ambiguous cases. While over 90% of GCTB presents histone 3.3 variants exclusively in the H3F3A gene, chondroblastoma is mutated mainly in H3F3B. In this study we examined a series of giant cell rich primary bone tumors, aiming to evaluate the possible diagnostic role of histone 3.3 mutations in the differential diagnosis between GCTB and giant cell rich sarcomas. Sixteen cases of non-metastatic GCTB, 9 GCTB with lung metastases, and 35 giant cell-rich sarcomas were selected from our institutional archives. Eight chondroblastomas were used as controls. Direct sequencing for the presence of *H3F3A* and *H3F3B* variants in coding region between codons 1 and 42, including the hot spot codons (28, 35 and 37) was performed on DNA extracted from formalin-fixed paraffin-embedded tissue using conventional PCR and fast COLD-PCR. Overall, 24 GCTB (96%) presented a mutation in the *H3F3A* gene (15 of 16 non metastatic and 9 of 9 metastatic). Five sarcomas harbored a *H3F3A* mutation (3 p.G35W, 1 p.G35L and 1 p.G35E), and these were all secondary malignant GCTB. In conclusion, we confirm that *H3F3A* mutational testing may be a useful adjunct to differentiate GCTB from giant cell rich sarcomas. Although the presence of *H3F3A* mutations does not exclude with certainty a diagnosis of sarcoma, the possibility of a malignant evolution of GCTB should also be considered.

Key words: giant cell tumor of bone; giant cell rich sarcomas; malignant giant cell tumor; histone 3.3 gene mutations; COLD-PCR

Introduction

Giant cell tumor of bone (GCTB) is a locally aggressive tumor, which consists of primitive mesenchymal cells of putative osteoblastic lineage that represent the neoplastic population, plus osteoclast precursors and multinucleated osteoclast-like giant cells (1). It recurs in approximately 15-50% of patients following surgical treatment with curettage, and 2% develop lung metastases, usually 3-4 years after diagnosis (1). The risk of metastasis is increased in recurrent cases and in radiologically aggressive tumors, while no clear correlation has been demonstrated with histopathological features, including vascular invasion and grading, as well as with the expression of tumor markers (2-4).

Malignant transformation of GCTB is a rare event. It may be either present at the onset of disease (synchronous, primary malignant GCTB), or it may develop following repeated recurrences and radiation treatment (metachronous, secondary malignant GCTB) (1, 5-8). Histologically, these are always high-grade sarcomas with features of osteosarcoma, fibrosarcoma or pleomorphic sarcoma (malignant fibrous histiocytoma of the older literature), and carry a poor prognosis (7, 8). Their distinction from conventional GCTB may be difficult, especially for primary malignant GCTB, since areas of conventional GCTB are present in the lesion and the biopsy may not include the malignant component of the tumor (7). The differential diagnosis between GCTB and giant cell rich sarcomas is further complicated by the fact that several primary sarcomas of bone, including osteosarcoma, leiomyosarcoma, undifferentiated pleomorphic sarcoma, fibrosarcoma and dedifferentiated chondrosarcoma, may present giant cell rich areas. The distinction between GCTB and these giant cell rich sarcomas may be challenging, especially in small biopsy specimens, and the morphologic criteria for the differential diagnosis are not well defined.

Recently, mutually exclusive histone 3.3 gene mutations have been recognized in chondroblastoma and GCTB, which may be useful for differential diagnostic purposes in

morphologically ambiguous cases. While over 90% of GCTB presents histone 3.3 variants exclusively in the *H3F3A* gene, chondroblastoma is mutated mainly in *H3F3B* (9-14).

In this study we examined a series of giant cell rich primary bone tumors, aiming to evaluate the possible diagnostic role of histone 3.3 mutations in the differential diagnosis between GCTB and giant cell rich sarcomas. In addition, we studied a group of GCTB with lung metastases in order to assess whether the pattern of *H3F3A* mutations differs from non-metastasizing lesions.

Materials and Methods

Patient samples

Sixteen cases of GCTB and 35 giant cell-rich sarcomas were selected from our Institutional archives. All hematoxylin and eosin stained slides and formalin-fixed paraffin-embedded blocks of samples not submitted to decalcification, were retrieved for each case. All slides were reviewed and diagnosed according to the criteria of the current WHO classification (1). Individual cases were correlated with clinical and radiological information where available.

The group of sarcomas included 18 osteosarcomas (OS), 14 undifferentiated pleomorphic sarcomas (UPS), and 2 spindle cell sarcomas. Among the OS, 2 tumors developed in Paget's disease and one was radiation induced. In addition, we analyzed a giant cell rich osteosarcoma the represented the non-chondrogenic component of a dedifferentiated chondrosarcoma. Three OS and 4 UPS developed after multiple recurrences of a GCTB (secondary malignant GCTB). One UPS included in our analysis developed in the L1 vertebral body of a patient who had been treated for GCTB of the distal femur 21 years before, and who had developed lung metastases of the GCTB. In addition, we analyzed the primary tumor and the lung lesions in 9 patients affected by metastatic GCTB. Samples from 8 chondroblastomas were used as controls.

Histone 3.3 mutation analysis

The thick tissue sections (7-10µm) of FFPE blocks of tumor samples not submitted t o decalcification, underwent an overnight digestion with proteinase K at 56°C. DNA was ex tracted with the Qiamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) accordingly to the manufacturer's protocol in a final volume of 60µL. Nucleic acid samples were checked for concentration and quality by using the NanoDrop ND-1000 Spectrophotometer (Thermo S cientific, Inc., NYSE:TMO).

The primer sets were selected to cover the hot-spot sites of genes and were as follo wing: H3F3A (NC_000001.11) forward primer 5'-tgtttggtagttgcatatggtga-3' and reverse pri mer 5'-acaagagagactttgtcccatt-3' [240bp]; H3F3B (NC_000017.11) forward primer 5'-ttatct tcggggcgtctttc-3' and reverse primer 5'-gagcaggggaggagtgag-3' [238bp].

Two additional primer sets were designed to increase the amplification of the gene portions described above of fragmented DNA samples: H3F3A forward primer 5'- CAAATC GACCGGTGGTAAAG-3' and reverse primer 5'-acaagagagactttgtcccatt-3' [144bp]; H3F3B forward primer 5'-AAACAGCTGGCCACGAAA-3' and reverse primer 5'-gagcaggggaggagtg ag-3' [150bp]. The reduced amplicons length allows the amplification of the interested frag ments in degraded DNA samples.

The targeted sequences were tested to exclude nonspecific amplification of homolo gous regions by UCSC Genome Browser - BLAT Search (http://www.genome.ucsc.edu/cgi -bin/hgBlat?command=start) and Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer- blast/)].

Amplification of both genes was performed in an ABI 2720 thermal cycler (Applied B iosystems-Life Technologies, Foster City, CA 94404, USA) by starting from two different q uantity of DNA (20 ng and 100 ng) in a final volume of 20 µl and using HotStarTaq DNA Po lymerase (Qiagen, Milan, Italy) with 500nM of each primers. The thermal protocol entailed an initial denaturation at 95°C for 5 minutes, followed by 40 cycles at 95°C for 20 seconds,

58°C for 30 seconds, and 72°C for 30 seconds, with a final elongation step at 72°C for 10 minutes.

PCR products were purified by using FastGene Gel/PCR Extraction kit (Nippon Genetics Europe GmbH, Düren, Germany). DNA fragments were submitted to the cycle sequencing reaction with BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems-Life Technologies). The purified sequences, obtained by ZR DNA Sequencing Clean-up Kit (Zymo Research Corporation, Irvine, CA 92614, U.S.A.), were run on an ABI PRISM 310 Genetic Analyzer instrument (Applied Biosystems, Darmstadt, Germany) and manually analyzed.

The position of mutated codons was annotated following the reference sequences for H3F3 genes from Consensus CDS Database (https://www.ncbi.nlm.nih.gov/projects/CCDS/CcdsBrowse.cgi) [CCDS 1550.1 (NM_002107.4; NP_002098.1) for H3F3A and CCDS11729.1 (NM_005324.4; NP_005315) for H3F3B gene]. The first methionine and the related triplet ATG were counted in the coding regions.

H3F3A analysis by fast COLD-PCR (COamplification at Lower Denaturation temperature-PCR)

COLD-PCR is a designed protocol to enrich minor-variants amplification throughout the course of PCR (14). In this study, COLD-PCR was applied to improve the detection sensitivity of low-abundant mutations in *H3F3A* gene. In particular, the fast COLD-PCR platform was selected to validate results from samples with wild-type and/or uncertain genotype, because the most frequent mutations reported in the codon 35 of the H3F3A gene are Tm (melting)-reducing mutations (i.e. p.Gly35Trp, c.103G>T; p.Gly35Arg, c.103G>A; p.Gly35Val, c.104G>T).

The primers for shorter amplicon (144bp) of the *H3F3A* gene were used in COLD-PCR experiments. Briefly, to define the range of the critical denaturation temperature (Tc) a

nd the number of cycles appropriated for fast COLD-PCR of the given amplicon, a wild-typ e and a mutated samples were amplified in triplicate via conventional PCR in presence of Syto 9 ® dye (Thermo Fisher SCIENTIFIC, Carlsbad, USA) on a StepOnePlus™ Real-Tim e PCR System (Thermo Fisher SCIENTIFIC) and were analyzed for the temperature of me lting (Tm) and the Ct (cycle threshold). The Tc was experimentally determined, by other pa rallel amplifications performed at decreasing denaturation temperatures with a step of 0,5° C starting from the Tm. Finally the temperature was set at 85.5 °C and the number of cycl es, used in the first stage of COLD-PCR, was set equal to 25, the mean of the Ct obtained starting from 20ng of DNA/reaction. COLD-PCR products were sequenced as previously d escribed. The amplicon sequences obtained with different Tc were analyzed by Sanger se quencing, to determine the best PCR conditions for the enrichment of the mutant allele.

The fast COLD-PCR protocol included an initial step at 95°C for 5 minutes and a firs t stage of 25 PCR cycles at 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 secon ds, followed by a second stage of 30 cycles at specific Tc: dissociation at 85.5°C for 5 sec onds, 58°C for 30 seconds, 72° for 30 seconds, and a final extension at 72°C for 10 minut es.

Results

H3F3 gene analysis by Conventional PCR and fast COLD-PCR

Overall, in the present study we analyzed 75 specimens from 60 patients. According to conventional PCR analysis, there were 12 out of 16 GCT with *H3F3A* mutations. Among metastatic GCT the rate was lower, only 2 out of nine cases showing a *H3F3A* variant. Fin ally, among giant cell rich sarcomas, two samples presented a *H3F3A* variant.

Twenty-one samples resulting either wild type (n=11) or of uncertain interpretation (10) at conventional PCR analysis, were reanalyzed by fast COLD-PCR. Of these, 14 samples were reclassified as mutated (Figure 1), including all the samples with uncertain interpretation and 4 wild type samples, whereas 7 remained wild type.

H3F3 gene mutation in Giant Cell Tumor of Bone

At the end of our analysis, 15 GCTs presented a mutation in the *H3F3A* gene (12 pG35W, 1 pG35V, 1 pG35M and 1 pG35E). Considering the group of metastatic GCTB, all the nine lesions were mutated, with 7 tumors showing the p.G35W variant, one presenting a p.G35M and one showing a p.V36_K37insW. Thus, 24 of 25 (96%) GCTB were mutated in the present series. The lung lesions showed the same mutation of the primary bone tumor in 7 cases, while in the remaining 2 no mutation was identified.

H3F3 gene mutation in Giant Cell Rich Sarcomas of Bone

In the group of sarcomas, five tumors harbored a *H3F3A* mutation, three of which were p.G35W, one was a p.G35L and one was p.G35E. These were all secondary malignant giant cell tumors of bone, in which the sarcomatous component had features of high grade osteosarcoma in two cases and of undifferentiated pleomorphic sarcoma in three (Table 1, Figure 2). Two of these cases had been initially diagnosed as giant cell rich sarcomas, but upon review of all available histologic slides, they were reinterpreted as secondary malignant GCTB. In one of these cases, samples from multiple consecutive recurrences were analyzed, and all showed the same alteration (p.G35L). One tumor, that upon review was classified as high grade spindle cell sarcoma arising in GCTB showed no alterations in *H3F3A* in the primary tumor as well as in two further local recurrences.

Finally, we did not identify any *H3F3A* alteration in all the 28 giant cell rich sarcomas tested (Table 2, Figure 3). Notably, the UPS that developed in the L1 vertebral body of a patient who had been treated for GCTB of the distal femur 21 years before, showed no mutations in *H3F3A* gene, whereas a p.G35M mutation was identified in the GCTB of the distal femur. Thus the vertebral lesion was interpreted as a second primary sarcoma, rather than a metastasis from the femoral lesion.

No mutation was identified in the *H3F3B* gene in the group of GCTB, metastatic GCTB and giant cell-rich sarcomas, whereas all the chondroblastoma tested presented a p.K37M variant.

Discussion

In the present study we confirm the high frequency of *H3F3A* mutations in GCTB and we examine the presence of these mutations in secondary malignant GCTB and metastatic GCTB. Similarly to previous studies, p.G35W was the most frequently detected variant in GCTB (80% of mutations in GCTB and metastatic GCTB, 60% in secondary malignant GCTB), while the less frequently identified mutations were p.G35M in three cases, and p.G35L, p.G35V, p.V36_K37insW, and p.G35E in the remaining four. Thus, the distribution of the *H3F3A* alterations was similar in the clinical variants (primary, metastatic and secondary malignant) of GCTB analyzed.

The frequency of mutations in *H3F3A* reported in the literature varies greatly among different series, from 69% to 93% (9-13, 15). In general, some intrinsic factors can limit the detection of somatic mutations in human cancer samples. Indeed, most of the mutations affecting cancer cells are heterozygous and solid tumors have a complex cellular architecture. In particular, in GCTB a numerically relevant non-neoplastic population of multinucleated osteoclast like giant cells and mononuclear osteoclast precursors coexist with neoplastic cells. Finally, the clonal heterogeneity can account for the presence of cellular clones with different mutational status.

Thus, the main reasons for these discrepancies may be recognized in i) the type of samples analyzed (e.g., fresh frozen versus formalin-fixed, paraffin-embedded tissues, decalcified versus undecalcified specimens); ii) the analytical method used for the genotyping. Therefore, the difference in mutation frequencies observed in *H3F3A* is most probably due to the different sensitivity of protocols used.

To overcome these limitations, we have combined the specificity of direct sequencing with a COLD-PCR amplification protocol able to guarantee an enhancement of mutant alleles with G>T or G>A substitutions (16), which are the most frequent mutations at codon 35. With this method we were able to identify *H3F3A* mutations in 10 samples with ambiguous results and in 4 wild type samples. Thus, the mutation frequency of *H3F3A* in GCTB samples, despite the use of direct sequencing on DNA from FFPE specimens, reached 96% in our study. Therefore, COLD-PCR can be considered a suitable strategy for the correct identification of less represented mutations in GCTB samples, without requiring expensive and time-consuming procedures, and it appears also to be useful to define cases with ambiguous results, which occur even in analysis conducted with prescreening methods [3/28 cases in the study by Kervarrec et al (13)].

Distinguishing GCTB from a giant cell rich sarcoma may be a difficult task, especially in small biopsies. The presence of focal cellular atypia is not sufficient to classify a giant cell rich lesion as malignant, since rarely GCTB may show marked nuclear atypia, which is referred to as symplastic/pseudoanaplastic change (15). Similar changes, including presence of atypical cells, have also recently been reported in GCTB treated with Denosumab (17). Thus, the histologic diagnosis of malignant GCTB should rely on the identification of overt pleomorphism accompanied by abnormal mitotic figures (15).

Interestingly, mutation in *H3F3A* (p.Gly35Trp [G35W]) was found in 6 of the 7 cases of GCTB with symplastic/pseudoanaplastic changes studied by Sarungbam et al. (15). On the other hand, the occurrence of *H3F3A* mutations in giant cell rich sarcomas is extremely unusual. In their study, Presneau et al. examined 10 cases of giant cell rich sarcoma for the presence of *H3F3A* mutations and found two lesions harboring the p.G35W mutation (10). Joseph et al. reported another example of osteosarcoma with *H3F3A* mutation (18). Recently, Koelsche et al. reported six cases of *H3F3A* mutant osteosarcoma with distinctive clinical and molecular features, and concluded, among other hypothesis, that

these cases could represent malignant GCTB (19). Using a anti-histone H3.3 G35W rabbit monoclonal antibody, Amary et al. found a group of 11 positive osteosarcomas in a large series of primary bone tumors (20). Most of these osteosarcomas presented a giant cell rich component and presented in subarticular location, suggesting that they could represent true malignant GCTB (20). In the present study we found no *H3F3A* mutation in a series of 28 primary giant cell rich sarcomas of bone, including 16 osteosarcomas, 10 undifferentiated pleomorphic sarcomas, and 2 high-grade spindle cell sarcomas. This may depend on the relatively small number of cases analyzed, but in comparison with previous studies we focused our attention only on giant cell rich sarcomas. Interestingly, one undifferentiated pleomorphic sarcoma developed in the vertebral body of a patient who had been treated 21 years before for a GCTB of the distal femur, who developed lung metastases with the typical appearance of giant cell tumor of bone. A p.G35M mutation was present in the GCTB, but not in the vertebral lesion, indicating that the two lesions were genetically unrelated and the vertebral tumor most likely represented a second primary, as also suggested by the long time interval between the onset of the two lesions.

On the other hand, 5 of 7 sarcomas arising in GCTB after local recurrence (secondary malignant GCTB) were mutated in *H3F3A*. Malignant transformation of GCTB is a very rare event (1-6% of cases), which often follows radiation treatment of the primary lesion. The molecular mechanisms associated with this phenomenon have only partially been elucidated. Inactivation of *TP53* gene due to loss of heterozygosity or mutation has been observed only in the sarcomatous component of secondary malignant GCTB (21-23) and it is thought to play a role in tumor progression. *H-ras* mutation has also been detected in the sarcomatous component of a secondary malignant GCTB (24).

In the present study we observed that sarcomas arising in GCTB show the same pattern of *H3F3A* mutations of conventional GCTB, and in one instance we could demonstrate the presence of the same alteration in specimens from repeated recurrences

of the conventional GCTB and the sarcomatous evolution, providing the genetic evidence of a biologic link between these tumors. Similarly, two cases of malignant dedifferentiated giant cell tumor of bone included in a recent study, showed the p.G35W *H3F3A* mutation (13). Thus, the identification of a *H3F3A* mutation in a high-grade sarcoma of bone raises the possibility that the tumor may have originated from a GCTB, and a thorough review of the histopathological slides from previous surgical treatment(s) in case of a recurrent tumor, or extensive sampling in case of a primary lesion, is warranted.

In conclusion, we confirm that *H3F3A* mutational testing may be a useful adjunct to differentiate GCTB from giant cell rich sarcomas. A diagnosis of GCTB in absence of *H3F3A* mutation should be made with caution in view of the high prevalence of *H3F3A* mutations in GCTB. Although the presence of *H3F3A* mutations does not exclude with certainty a diagnosis of sarcoma, the possibility of a malignant evolution of GCTB should also be considered.

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Figure Legends

Figure 1. Representative electropherograms obtained from the same samples of giant cell tumor of bone analyzed with conventional and fast COLD PCR analyses. A: In this GCTB, a p.Gly35Trp (p.G35W) variant in *H3F3A* is identified only after fast COLD PCR (red arrow), while the result of conventional PCR could be interpreted as wild type.
B and C: samples from two recurrences of secondary malignant giant cell tumor of bone. The results of the conventional PCR are ambiguous (yellow arrows), while the fast COLD PCR shows a p.Gly35Leu (p.G35L) variant in both specimens.

Figure 2. A 17 year-old woman presented with a lytic lesion of the sacrum (A) which was diagnosed as giant cell tumor of bone on incisional biopsy (C). After curettage, she experienced three recurrences within a four year span that were surgically treated. After five years from the initial diagnosis, she presented a large recurrence, that was diagnosed as high grade sarcoma (C and D). A *H3F3A* variant was identified (E).

Figure 3. A 36 year-old woman presented with recurrent “giant cell tumor” of the proximal femur (A and B). Incisional biopsy showed a giant cell rich tumor (C), with focal areas of osteoid matrix formation, associated with atypical cells (D). Both *H3F3A* and *H3F3B* were wild type (E and F). These findings were considered more consistent with the diagnosis of giant cell rich osteosarcoma of bone.