The H3F3 K36M mutant antibody is a sensitive and specific marker for the
diagnosis of chondroblastoma

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Running Title
Immunohistochemical biomarker for chondroblastoma

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Abstract

Aims

We recently reported that 95% of chondroblastomas harbour a p.Lys36Met (p.K36M) mutation in either the H3F3A (chromosome 1) or H3F3B genes (chromosome 17), with the majority involving H3F3B. In this study we sought to assess the expression of the K36M-mutated protein by immunohistochemistry in a large group of tumours.

Methods and Results

1894 tumours, including 85 chondroblastomas and 10 clear cell chondrosarcomas were studied, of these 82 chondroblastomas and 1 clear cell chondrosarcoma, known to harbour the H3F3 K36M mutation, expressed the mutated protein. 3 chondroblastomas and 9 clear cell chondrosarcomas wild-type for H3F3A/B were negative for K36M immunohistochemistry. The remaining 1799 cases tested, 545 of which were known to be wild-type for the H3F3A and H3F3B K36M substitutions, included 1047 primary bone tumours, 507 soft tissue and joint tumours. 245 other tumour types not expected to harbour the mutation were negative for K36M immunohistochemistry.

Conclusions

Our data demonstrate the specificity and sensitivity of this immunomarker and will be a useful adjunct for reaching a diagnosis of chondroblastoma.
Introduction

Chondroblastoma is a rare benign central cartilaginous bone tumour representing less than 1% of all primary bone tumours. Histologically, chondroblastoma has a characteristic heterogeneous appearance comprising sheets of discohesive neoplastic mononuclear cells with small grooved nuclei mixed and variable numbers of osteoclast-like giant cells: the latter can dominate or can be scant. Islands of eosinophilic chondroid (chondroid-osseous) matrix are also present whereas hyaline cartilage is uncommon. Pericellular ‘chicken wire’ calcification, when present, is a useful diagnostic feature. Mitoses can be frequent but atypical forms are not seen. Secondary aneurysmal cyst formation occurs commonly. Although the tumour is usually recognised to the ‘trained eye’, the heterogeneous composition of the tumour can be challenging particularly on biopsy material when the tumour occurs at unusual sites and outside the classic subarticular location.

The differential diagnosis of a chondroblastoma is extensive, including benign and malignant neoplasms. These comprise primary bone tumours including conventional cartilaginous tumours, and others such as clear cell chondrosarcoma which like chondroblastoma occurs in a subarticular site, and chordoma, in addition to osteoclast-rich tumours, bone-forming tumours, and fibrous dysplasia with areas of cartilage. A second group includes metastatic neoplasms to bone, although this is rarely a diagnostic challenge.

By combining our recent 2 studies we have found that 95% of chondroblastomas harbours a p.Lys36Met (p.K36M) mutation in either the H3F3A (chromosome 1) or H3F3B genes (chromosome 17), with the majority involving the latter. We also reported that H3F3A alterations were found in 96% (132/139) of giant cell tumours of bone, the majority of which harboured a p.Gly34Trp (p.G34W) mutation. In addition, 3 cases revealed a p.Gly34Leucine (p.G34L) alteration, and 2 tumours revealed a p.G34R mutation, one in H3F3A, one in H3F3B: a G34M mutation in H3F3A was detected in a single tumour. The H3F3 G34W and the K36M substitutions were mutually exclusive. These H3F3 mutations were highly specific for chondroblastoma and giant cell tumour of bone and the same
alterations were detected in only 2/103 osteosarcomas (one p.G34R in \(H3F3A\), one p.G34R mutation in \(H3F3B\)), 1/15 clear cell chondrosarcoma and 1/75 central chondrosarcomas with a \(H3F3B\) p.K36M alteration\(^1\). Since then, others have reported another \(H3F3A\) mutation in one other osteosarcoma (1/10, p.G34W)\(^5\).

Detecting a tumour type-specific nucleic acid substitution for diagnostic purposes is generally achieved by analysis of a variety of sequencing or PCR-based methodologies such as targeted DNA sequencing, exome and whole genome, droplet digital PCR and pyrosequencing amongst others. Each of these methods has benefits but they are not always available to diagnostic laboratories. Many of these approaches involve extensive analyses of panels of 10s to 1000s of genes, which is excessive when the aim is to confirm a suspected diagnosis made on a haematoxylin and eosin-stained tissue section. Furthermore, molecular genetic testing on extracted DNA remains expensive (from hundreds to thousands of dollars/pounds) and generally takes a minimum of a week with more extensive panels taking longer (http://www.foundationmedicine.com). In contrast immunohistochemistry is delivered at a significantly lower cost and within 24 hours. We therefore undertook this study to determine the utility of a K36M mutant antibody for helping to reach a diagnosis of chondroblastoma, and exclude other neoplasms. \(H3F3\) alterations have also been reported in paediatric brain tumours\(^6\)–\(^9\), specifically in up to 60% of diffuse intrinsic pontine gliomas\(^6\)–\(^9\). However, as these do not enter the differential diagnosis of bone and soft tissue tumours, we have excluded them from this study.

**Materials and Methods**

An electronic search of the pathology archives at the Royal National Orthopaedic Hospital, UK was undertaken to identify the histopathological diagnoses of interest. The tumours were classified using the criteria of the most recent WHO classification of soft tissue and bone\(^1\). Many of the samples selected for the study had already been used to construct tissue microarrays and in some cases it was known that the tumour harbourered a \(H3F3\) mutation as the DNA had been analysed for the mutation using a variety of technologies including whole genome sequencing, exome sequencing, capillary sequencing, targeted next generation
sequencing (Ion Torrent, targeted capture, and sequencing, and droplet digital PCR). In the majority of such cases the result of the p.K36M mutation was validated using at least 2 of these techniques\textsuperscript{2,3}.

We performed immunohistochemistry on 2 chondroblastomas which had been decalcified in nitric acid: immunoreactivity in these samples was patchy and weak (data not shown). Greater numbers were not available as we normally decalcify curettage specimens in EDTA. On the basis of this, samples decalcified in nitric were excluded from this study.

Either full tissue sections or sections of tissue microarrays were cut for immunohistochemistry as previously described\textsuperscript{10}. In brief, tissue microarrays in formalin-fixed paraffin-embedded blocks were constructed using a manual arrayer (Beecher Instrument, Sun Prairie, WI, USA). Duplicate 1.0 mm diameter cores were taken from each case.

The rabbit monoclonal histone H3 K36M mutant antibody (Clone RM193, RevMAb Biosciences USA Inc., CA, USA) was kindly provided as a gift by RevMAb Biosciences USA Inc. Immunohistochemistry was performed using the Leica Bond 3 fully automated immunohistochemistry stainer (Leica Microsystems Ltd., Milton Keynes, UK). Leica epitope retrieval 2 solution was used for 20 minutes. The antibody was diluted in 1/400.

Ethical approval was obtained from the Cambridgeshire 2 Research Ethics Service for the UCL biobank for health and disease (reference 09/H0308/165, renewed 2015, 15/YH/0311) project EC17.1. The biobank is covered by the Human Tissue Authority licence 12055.

Results
Immunohistochemistry undertaken using the rabbit monoclonal \textit{H3F3} K36M mutant antibody (Clone RM193) revealed that all 82 chondroblastomas harbouring the \textit{H3F3} p.K36M mutation (7= \textit{H3F3A}; 75=\textit{H3F3B}) expressed the mutated protein (Figure 1, Table 1). Three cases which failed to reveal immunoreactivity for the \textit{H3F3} p.K36M substitution were wild-type for the \textit{H3F3}
p.K36 as assessed by genotyping. Immuno-labelling on multiple tissue blocks from these cases failed to reveal a positive reaction.

The 1/10 clear cell chondrosarcomas reported previously to harbour the K36M alteration using next generation sequencing\(^2\) was found to be immunoreactive with the K36M antibody.

The K36M mutant antibody is directed against a nuclear protein and the immunoreactivity revealed crisp nuclear expression with virtually no cytoplasmic or matrix reactivity. Immunoreactivity was seen throughout the tumour. The antibody proved valuable in identifying immunoreactive cells in small clusters of cells in biopsy specimens (Figure 1).

Table 1 lists 1047 primary bone tumour cases (excluding chondroblastoma and clear cell chondrosarcoma) that are not immunoreactive for the \(H3F3\) p.K36M mutation: 545 of these samples are known not to harbour the mutation (see references in Table). Notably none of the giant cell tumours of bone with the \(HF3F\) p.G34 mutation showed immunoreactivity, and the 2 osteosarcomas (one p.G34R mutation in \(H3F3A\), one p.G34R mutation in \(H3F3B\)), and the single chondrosarcoma with the p.G34W alterations revealed no immunoreactivity for the mutant protein\(^2,3\).

Finally, immunohistochemistry was performed on 752 tumours comprising a wide variety of types the DNA of which had not been analysed for the presence of a \(H3F3\) K36M mutation (except for 42 tenosynovial giant cell tumours which were shown previously to be wild-type for the p.K36M alteration) (Table 1). Some of these lesions, such as soft tissue chondroma, plasma cell tumours and clear cell carcinomas, were chosen specifically as they can mimic chondroblastoma. However, to provide additional evidence for the specificity of the antibody we extended the immunohistochemistry testing to other varieties of bone and soft tissue tumours, carcinomas and other neoplasms, none of which has ever been shown to harbour a \(H3F3\) p.K36M mutation (http://cancer.sanger.ac.uk/cosmic). All these tumours failed to reveal immunoreactivity to this antibody \(^1\) (Table 1).
Discussion

We have demonstrated that the \( H3F3 \) K36M mutant monoclonal antibody (Clone RM193) used in this study is highly specific for tumours which harbour this mutation. To our knowledge this antibody has not been reported in previous studies. 82/82 chondroblastomas and 1/1 clear cell chondrosarcoma which were shown to possess this precise genetic alteration through analysis of extracted DNA revealed unequivocal immunoreactivity with this antibody. Furthermore, the 3 chondroblastomas and the 10 clear cell chondrosarcomas which did not harbour the mutation as assessed by DNA analysis failed to reveal immunoreactivity. As expected, the immunohistochemistry revealed positivity in the tumours with the mutation in either \( H3F3A \) or \( H3F3B \), as these represent paralogous genes, with the same amino acid sequence, on different chromosomes. The specificity of this antibody for the mutant protein is further supported by the absence of immunopositivity in at least 545 primary bone tumours comprising different types, which we have shown not to harbour the \( H3F3 \) p.K36M alteration\(^2,3\).

The analysis of another 1254 other tumour types, including a variety of primary bone tumours, carcinomas and other neoplasms in which the mutation would not be expected to be expressed \( \text{http://cancer.sanger.ac.uk/cosmic} \) supports the findings that this \( H3F3 \) K36M antibody is specific for the mutant protein.

The antibody was simple to optimise for use and we observed that it decorates crisply the nucleus of the mononuclear neoplastic population, on formalin-fixed paraffin-embedded tissue sections. We detected virtually no background immunoreactivity in the cytoplasm of the immuno-positive cells. Furthermore, neither the osteoclasts, representing a ‘passenger’ population in the tumour\(^2\) nor the extracellular cartilaginous matrix revealed any immunoreactivity. A number of the paraffin tissue blocks were at least 10 years old and where relevant the samples were strongly immunoreactive.

Our results demonstrate that this \( H3F3 \) K36M antibody is highly sensitive and specific for the p.K36M mutation, and as 96% of chondroblastomas harbour a
p.K36M mutation this antibody represents a valuable tool that can be used to support the histological diagnosis of chondroblastoma\(^1\). The antibody can be employed to distinguish chondroblastoma from other primary bone tumours that represent histological mimics of which there are several on account of the morphological heterogeneity of this neoplasm. Furthermore, as only \(~4\%\) of chondroblastoma do not harbour a \(H3F3\) K36M mutation, failing to detect it, should make the pathologist consider other diagnoses, and chondroblastoma should only be diagnosed with caution.

Apart from chondroblastoma, the only other bone or soft tissue tumour in which a p.K36M mutation was detected was the clear cell chondrosarcoma, a malignant bone tumour only accounting for \(2\%\) of all chondrosarcoma\(^1\). However, as reported previously the mutation was detected in a minority of cases analysed (1/15) (with only 10 cases being available for immunohistochemistry)\(^2\). Clear cell chondrosarcoma has many overlapping clinical and radiological features with chondroblastoma including the anatomical site (subarticular and apophyseal) but clear cell chondrosarcoma requires an \textit{en bloc} wide excision whereas chondroblastoma is generally curetted. Clear cell chondrosarcoma presents most commonly in the proximal femur and humerus in the 3\(^{\text{rd}}\) - 4\(^{\text{th}}\) decades, whereas chondroblastoma presents most commonly in the distal femur and proximal tibia in the 2\(^{\text{nd}}\) and 3\(^{\text{rd}}\) decades. However, both can present over the age of 40, and in any bone in the body. On imaging, clear cell chondrosarcoma can be challenging to distinguish from chondroblastoma as signal intensity of both tumour types on magnetic resonance imaging is very heterogeneous. Although clear cell chondrosarcoma is generally larger, and has less pronounced peritumoural oedema and joint synovitis than chondroblastoma these criteria are not sufficiently reliable to distinguish the 2 entities\(^1\). Histologically, clear cell chondrosarcoma is characterised by sheets of well-defined cells with clear – slightly eosinophilic cytoplasm: mitotic figures are rare and osteoclast-like cells can be present in large numbers. Zones of conventional chondrosarcoma with hyaline cartilage can be present, and there are foci of woven bone and ossification. A greater degree of cellular atypia, the presence of hyaline cartilage and entrapment of host bone are helpful in reaching the diagnosis. Nevertheless, this
is can be challenging on a needle core biopsy.

There are reports that TP53 mutations occur in 95% of clear cell chondrosarcoma\textsuperscript{12} whereas they have not been detected in 25 chondroblastoma\textsuperscript{2,3}. Therefore, testing for the presence of such pathogenic variants would be valuable for distinguishing these 2 neoplasms, although analysis of greater numbers would be necessary to establish this firmly.

Chondroblastoma can share occasionally morphological appearances with conventional central/surface cartilaginous tumours, mesenchymal chondrosarcoma which are characterised by \textit{HEY1-NCOA2} fusion genes\textsuperscript{13}, and chondromyxoid fibroma. The failure to show immunoreactivity for the K36M antibody in these tumours demonstrates the value of this marker.

Distinguishing chondroblastoma from a chondroblastic osteosarcoma can be challenging, not only because of the morphological overlap but also because on rare occasions chondroblastomas metastasise to the lungs\textsuperscript{1}. Such lesions can be managed with excision from the lung without progression of the disease. Distinguishing these 2 neoplasms is vital for delivery of appropriate clinical treatment, which involves neoadjuvant chemotherapy for patients with osteosarcoma. There is an exceptionally rare variant of osteosarcoma known as chondroblastoma-like osteosarcoma: this tumour resembles the histological phenotype of its benign counterpart but exhibits nuclear atypia and has a permeative growth pattern\textsuperscript{14}. Unfortunately we have not had the opportunity to test such cases. In our experience, chondroblastoma-like areas are seen occasionally alongside conventional areas of chondroblastic osteosarcoma, and therefore we consider that the term rarely needs to be used. Other rare tumours that on small biopsies may be considered as mimics of chondroblastoma include fibrous dysplasia with cartilaginous differentiation\textsuperscript{1,4}, and extra-axial chordomas\textsuperscript{15-17} but we have shown that these tumour types do not express K36M mutant protein.
Finally, the osteoclast-rich component of chondroblastoma overlaps with other osteoclast-rich lesions including giant cell tumour of bone, aneurysmal bone cyst non-ossifying fibroma, giant cell reparative granuloma of jaw, and osteoclast-rich and telangiectatic osteosarcoma. We have previously shown that these do not harbour a H3F3 p.K36M mutation³. Although 96% of giant cell tumours of bone, and 70% of aneurysmal bone cysts harbour a H3F3A p.G34 substitution and a USP6 rearrangement respectively, there are no diagnostic biomarkers reported for the other osteoclast-rich mimics¹⁸-²⁰. Hence, the K36M antibody is useful for excluding these diagnoses.

In summary, our data show that this immuno-marker is highly specific for the p.K36M mutant protein. Hence, this H3F3 K36M antibody is a valuable diagnostic tool and can be used by pathologists to provide more accurate diagnoses quickly and at low cost.

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Disclosure/conflict of interest
The Rabbit Monoclonal Histone H3 K36M Mutant Antibody (Clone RM193) was kindly provided as a gift by RevMAb Biosciences USA, Inc., CA, USA.

Author contributions
The work was conceived by AMF and MFA. Review of slides and immunohistochemistry scoring were undertaken by AMF, DB, MFA, RT and RM. FB, RM, MFA and RG selected the cases and constructed the tissue micro-array.
POD reviewed the radiology images. Molecular testing performed by AG. Analysis of the data was performed by FB, MFA and AMF. The MS was written by MFA and AMF.

Figure 1. Photomicrographs of a needle biopsy showing small fragments of a chondroblastoma (A). Tumour cells are crisply decorated by the antibody K36M (B).

References


