Molecular testing of sarcomas

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Abstract

Connective tissue tumours, particularly sarcomas, are rare and present as a variety of histological subtypes with diverse management protocols and have varied prognoses. Many of these tumours have specific cancer driver genetic alterations that can be leveraged for diagnostic purposes. For practical purposes, these tumours can be categorised as harbouring genetic alterations including chromosomal rearrangements, gene amplifications, single nucleotide substitutions, and complex genetic abnormalities. Herein we discuss different tumour subtypes with their associated genetic abnormalities that may be used in clinical practice, when interpreted in the context of the relevant clinical, histological and radiological information.

The benefits of large scale sequencing studies of sarcoma are leading to new insights into sarcoma development and are providing a biological rationale for personalised medicine. Genomic profiling and other "omic" studies will likely play a fundamental part in the development of new diagnostic and predictive biomarkers in the near future.

Keywords Amplification; connective tissue; FISH; RT-PCR; sarcoma; translocations

Benign connective tissue tumours represent a relatively common group of neoplasms that derive from mesenchymal cells. In contrast, their malignant counterpart, sarcomas, are classified as rare cancers with an overall incidence of 6/100,000 per year in the UK.[1–3] The rarity of sarcoma makes delivery of accurate diagnoses challenging: this is compounded not only by the existence of more than 50 histological subtypes but the overlapping histological features between benign, locally aggressive and malignant connective tissue tumours. Until about 10 years ago most soft tissue tumours were resected after which a more definitive diagnosis could be provided but it is now recognised that patients can benefit from a more diagnostic-specific treatment of their tumour. Furthermore, today we have a greater knowledge about the behaviour of the different subtypes which allows a more informed prognosis to be provided to patients. Hence it is now necessary to provide an accurate diagnosis on a needle core biopsy whenever possible. Fortunately, many of these tumours have specific, recurrent genetic alterations which can be employed as molecular tests to aid diagnosis.[2,4–6] Currently it is recommended that such tests are used when the clinical and/or histopathological features are not typical for a diagnosis and or when the differential diagnosis could warrant different treatment.[7,8]

The molecular classification of connective tissue tumours for practical and diagnostic purposes is divided into tumours that have a) recurrent chromosomal rearrangements – mainly translocations b) copy number variation – mainly amplifications, c) recurrent single nucleotide substitutions and small deletions (indels) and d) complex karyotypes suggestive of genomic instability. Over the years molecular techniques have improved and most of these abnormalities can be detected in formalin-fixed paraffin-embedded (FFPE) tissue.[9,10] The most commonly employed tests include fluorescence in situ hybridisation (FISH) and PCR-based techniques, and are currently available in a large number of histopathology diagnostic laboratories allowing those tests to be performed routinely with a fast turnaround time of approximately 48 hours. Access to next generation sequencing (NGS) tests is more restricted as the cost of these technology platforms and DNA input requirements are relatively high and turnaround times are longer compared with FISH and PCR. However, with improvements in NGS technology, particularly when combined with improved library preparation techniques and target capture methodologies, along with reduced turnaround time and cost, we expect that such technology will be used more commonly in clinical practice in the near future. Despite many advances, in some cases a standard immunohistochemistry test will provide a diagnosis as there is no specific DNA mutation: for example the expression of brachyury for the diagnosis of chordoma. Nevertheless, regardless of the method used, it is crucial that the molecular results are interpreted in the light of the appropriate clinical, radiological and morphological information as different tumour types share similar molecular signatures. Herein, a selection of tumour types will be discussed in the context of the four categories of genetic alteration described above.

Sarcomas associated with chromosomal translocations

Numerous sarcomas have a chromosomal translocation as their genetic hallmark. These cytogenetic events usually lead to the formation of novel "chimeric" genes that are transcribed and translated into chimeric proteins. Some of these chimeric proteins may act as transcription factors.

Cytogenetic techniques demonstrate that chromosomal translocations are reciprocal (balanced) in the majority of the cases, that is, there is reciprocal exchange of genetic material between two chromosomes without any loss or with minimal loss of genetic material. This type of rearrangement may be indirectly detected by interphase FISH on FFPE tissue with the use of break-apart DNA probes which represent DNA sequences that flank the known breakpoints where the rearrangement occurs. Each probe is tagged with a different colour fluorochrome: spectrum red/orange and spectrum green probes are commonly employed. The probes are designed to hybridise adjacent to the 5 and 3 prime ends of the gene of interest, telomeric and

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centromeric sites respectively. When there is no rearrangement or when normal cells are targeted, each allele is visible as one green signal virtually abutting one red/orange signal: when this occurs the combination of colours results in a yellow signal. When a gene rearrangement is present, the pattern is that of a combined signal in one allele and a break-apart signal (red/orange distant from green) representing the rearrangement in the second allele (Figure 1).

In a few sarcoma subtypes the translocation is unbalanced resulting in loss of chromosomal material (deletion). In the unbalanced translocations, the use of break-apart probes is not ideal, as the signal from one of the probes may be "lost" resulting in a potential inconclusive interpretation.[9,11] A fusion-type probe is preferable in such cases, however its use requires having evidence *a priori* that such a complex rearrangement is present. The fusion probes are designed to target each gene involved in the formation of the fusion transcript. In normal or tumour cells in which the translocation is not present, the expected pattern is shown as two separate signals of each probe (generally two spectrum orange/red and two spectrum green). If a translocation is present, a fused signal is detected in the affected cells.

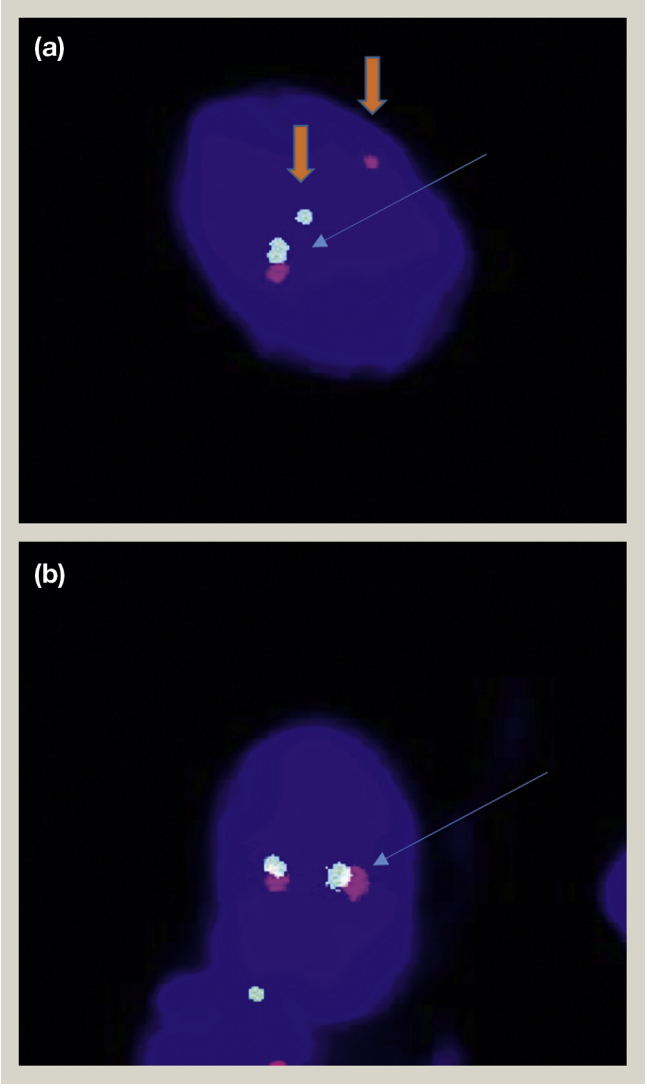

Figure 1 Break-apart dual colour FISH probes to detect gene rearrangement. (**a**) Nucleus with rearrangement: one (unaffected) allele is visible as one green signal virtually abutting one red/orange signal (long thin blue arrow). The second allele (thick orange arrows) shows a break-apart signal (red/orange distant from green) representing the rearrangement.

The alternative to FISH where the probes are tagged with a fluorescent label is the use of a chromogenic signal detection (CISH) method similar to that used in immunohistochemistry: this is more convenient for diagnostic laboratories because it uses bright-field microscopy rather than the fluorescence microscopes used in FISH. The other benefit of CISH is that the slides can be held indefinitely whereas the fluorescent signal fades with time, though the availability of digital scanners which can capture fluorescence images should overcome this problem. CISH is currently less sensitive than FISH, particularly for break-apart assays, although improvements are being made in this area. CISH is more frequently used in amplification/copy number variation assays than for detection of translocations.

Reverse transcriptase PCR (RT-PCR)-based assays are frequently used to detect chimeric fusion transcripts. These assays are more specific than FISH as they target both gene partners in a rearrangement through the use of specific oligonucleotide sequences (primers) designed to anneal to both parts of the fusion gene. The amplification is performed using cDNA (complementary DNA generated from single stranded RNA via reverse transcriptase). Due to multiple possible breakpoints and transcript variations, the use of multiple primers sets may be required, making this approach time consuming and labour intensive. Nevertheless, it is a popular, well established technique used by many molecular pathology laboratories.

In most scenarios, clinical, histological and immunohistochemical features, associated with a gene rearrangement detected by FISH are sufficient to give the pathologist sufficient information to provide a specific diagnosis. FISH is therefore currently the method of choice for chromosomal translocation-associated sarcomas in the majority of specialist laboratories.

Below we will discuss some specific sarcoma subtypes associated with chromosomal translocations:

Ewing sarcoma/PNET

Ewing's sarcomas are tumours of primitive cells composed of a monomorphic population of round cells with scant cytoplasm and little intervening stroma. The tumour may occur in bone, where it is more prevalent in children and young adults, and in soft tissue, more frequently in adults. This was the first sarcoma recognised to be associated with a recurrent chromosomal translocation. The t(11;22)(q24;q12) was characterised through karyotyping. Later, a specific fusion transcript was detected involving the *EWSR1* gene in chromosome 22 and the *FLI1* gene on chromosome 11. *EWSR1-FLI1* fusion is detected in approximately 85% of the cases.[6,12–16] Other translocations generating different fusion transcripts were subsequently described.

Immunohistochemistry is used as a triage approach to exclude other round cell tumours in the differential diagnosis. Molecular genetics tests are required in all cases suspicious for Ewing's sarcoma. As *EWSR1* is rearranged in a series of different sarcoma subtypes (Table 1), this rearrangement is not specific for Ewing's sarcoma although when interpreted in the context of clinical, histological and immunohistochemical features, the test supports

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