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REVIEW

Unravelling undifferentiated soft tissue sarcomas: insights from genomics

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Unravelling undifferentiated soft tissue sarcomas: insights from genomics

Undifferentiated pleomorphic sarcoma now falls under the broader rubric of undifferentiated soft tissue sarcoma (USTS) in the 2020 World Health Organization classification of bone and soft tissue tumours. These rare cancers remain a diagnosis of exclusion, and show genomic complexity manifesting as extreme forms of aneuploidy and genetic rearrangement. This review covers some of the recent advances in the diagnosis and treatment of USTS based on genomic sequencing, cancer evolution and heterogeneity studies, and immunotherapy. We highlight the critical role that pathologists have to play in the diagnosis and treatment of patients with USTS, viewed through the lens of the hallmarks of cancer.

Keywords: bone and soft tissue, genomics, pathology, sarcoma

Introduction

Molecular profiling for sarcoma diagnostics is commonplace, but the complexity of interpreting newer tests such as clinical whole genome sequencing (WGS) for therapeutic purposes represents a new and significant challenge. Understanding how to place the genomic results in the context of the clinical history and pathology is important, as the use of high-resolution WGS is transforming our understanding of how these rare cancers develop and is illuminating new ways in which to classify and treat patients. This is especially important as molecular pathologists are increasingly integrators of diverse sets of information as part of molecular tumour boards.

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The changes in the histological classification and nomenclature of undifferentiated soft tissue sarcoma (USTS) over the last few decades have been reviewed elsewhere.[1,2] The conventional description of some sarcoma subtypes, such as USTS, high-grade myxofibrosarcoma, and osteosarcoma, is that they have 'complex genomes', a term emanating from karyotyping studies and the use of copy number arrays.[3] With the increased resolution now afforded through next-generation sequencing, this genomic complexity is now being unravelled, thereby revealing both novel mutational processes and potential therapeutic targets.

The 'hallmarks of cancer' (genomic instability, immortality, resisting cell death, avoiding immune destruction, sustained proliferation, angiogenesis, deregulation of cellular energetics, invasion and metastasis, evading growth suppressors, and tumour-promoting inflammation) introduced in seminal works by Hanahan and Weinberg[4] describe a set of biological characteristics that are considered to be fundamental requirements for the development of malignancy. By

using this conceptual blueprint, this review aims to describe the complex genomic patterns and highlight key pathological aspects seen in USTS through the lenses of a selection of these hallmarks (Figure 1A).

Hallmark 1: genomic instability and mutations in USTS

Cancer is considered to be a disease of the genome that arises because of an accumulation of mutations in cells throughout an individual's lifetime that confer a growth or 'fitness' advantage, often referred to as driver mutations.[5] The repertoire of somatic mutations in cancer is broad, and includes single base substitutions, small insertions or deletions (indels), copy number alterations, and genomic rearrangements.[6] For example, in USTS, this full spectrum of mutation classes is commonly seen in *TP53*, which is responsible for safeguarding genomic stability (Figure 1B).

As compared with many common cancers, which are predominantly epithelium-derived, sarcomas are enriched in macroevolutionary events such as whole genome doubling (WGD) and chromothripsis (described later), resulting in aneuploidy, loss of heterozygosity (LOH), and numerous genomic rearrangements. These events manifest as both structural and numerical chromosomal instability (CIN), and are particularly prevalent in USTS.[7,8] WGD is a key macroevolutionary event associated with a poor prognosis in multiple cancers.[9] The prevalence of WGD varies across different types of cancer, and reports from cancer sequencing datasets indicate ranges from 5% in non-Hodgkin lymphoma to 58% in germ cell tumours. Ninety percent of USTSs show WGD, which is a driving force in their evolution, and some of these cancers contain subclones that have undergone multiple WGD events.[7] The mutational timing of WGD events shows that WGD occurred just prior to diagnosis in some patients, suggesting that this is

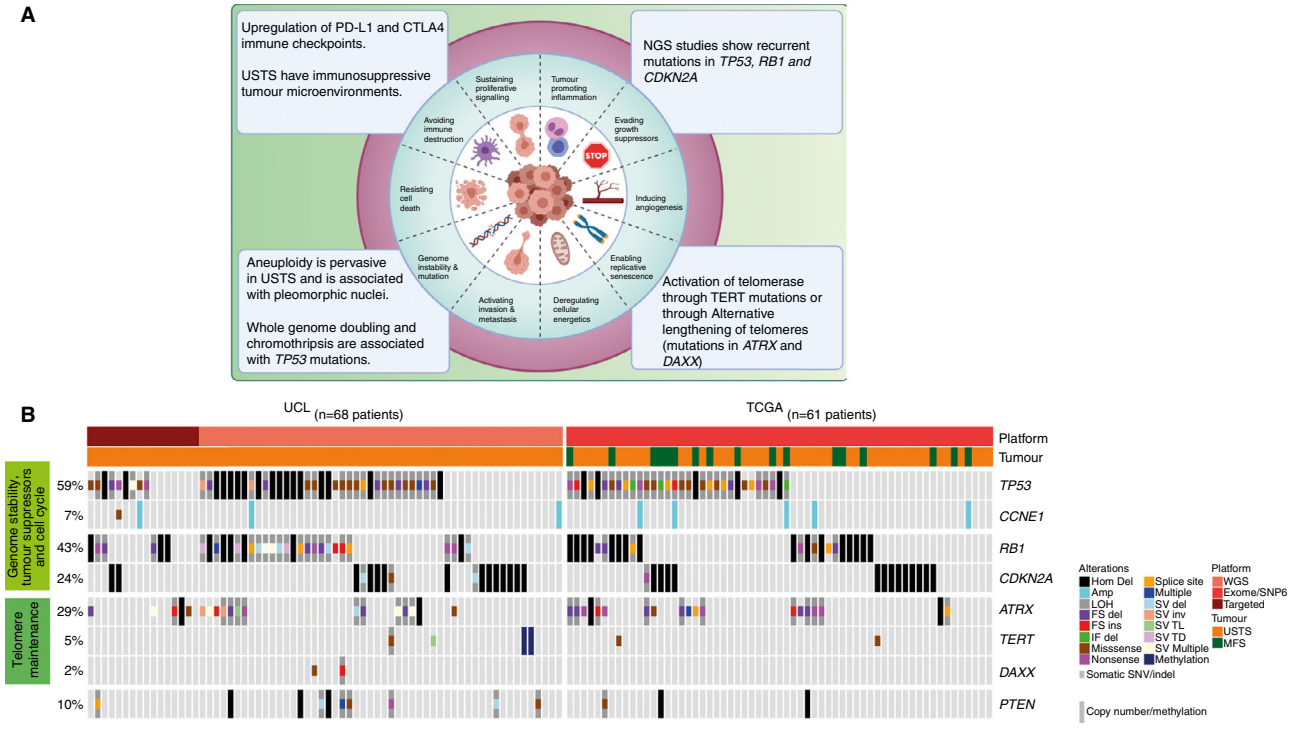

Figure 1. Selected hallmarks of undifferentiated soft tissue sarcoma (USTS). **A**, A selection of the hallmarks of cancer as they relate to recently described genomic aberrations seen in USTS. Created with BioRender.com. **B**, An OncoPrint of USTS driver genes and hallmarks identified in two large next-generation sequencing studies.[7,8] Driver genes are arranged for two common hallmarks of cancer. The broad spectrum of mutations in USTS includes single-nucleotide variants (SNVs) and indels, structural variants (SVs), and copy number alterations. Copy number alterations: Hom Del, homozygous deletion; Amp, amplification; LOH, loss of heterozygosity; FS del, frameshift deletion; FS ins, frameshift insertion; IF del, in-frame deletion. Structural variants are shown as small rectangles: SV del, deletion; SV inv, inversion; SV TL, translocation; SV TD, tandem duplication. SNVs, indels and structural variants are shown as small rectangles. Copy number and methylation alterations are shown as full blocks. Pale grey blocks indicate that no driver mutations were identified.

responsible for accelerating the growth of these tumours. In other patients, WGD appears at an early stage but a second WGD event is necessary before clinical presentation.[7]

Aberrations in nuclear morphology form the basis of multiple histopathological grading systems in cancer. One of the key morphological features of USTS is the presence of large cells with cytologically atypical nuclei and, in many cases, extensive pleomorphism (Figure 2A). Both intrinsic factors (genetic and epigenetic) and extrinsic factors (the microenvironment) can impact on the appearance of a neoplastic nucleus, including the amount of DNA present within the nucleus.[10,11] Aneuploidy, i.e. the presence of abnormal numbers of chromosomes and consequently DNA content, is a poor prognostic factor and is strongly associated with *TP53* mutations.[9,12]

Traditional morphological assessment of the nucleus is prone to subjectivity. With the development of computational analysis of digital pathological images, it is now possible to statistically quantify key morphometric aspects of nuclei, such as size, shape, area, convexity, texture, and staining intensity.[13] Such methods have shown that the extent of the pleomorphism and the large size of cells seen in USTS are associated with the number of WGDs, by utilising a combination of copy number profiling and digital pathology image analysis algorithms.[8] A more established way to assess the state of WGD of a sample is to perform DNA content analysis directly by using a DNA-binding dye on nuclear suspensions and performing ploidy analysis with flow or image cytometry, which can also provide a quantitative readout of the subclonal structure of a tumour[7] (Figure 2B). The limitation of this method is the loss of spatial information on the tumour cells.

Chromothripsis is a catastrophic genomic event leading to the generation of tens to thousands of clustered rearrangements across a few chromosomes.[14] Recent analyses using WGS have shown that chromothripsis events are widespread, being seen in 734 of 2543 (29%) tumours, with the highest frequency being seen in sarcomas, including 100% of dedifferentiated liposarcomas ($n = 18$) and 77% of

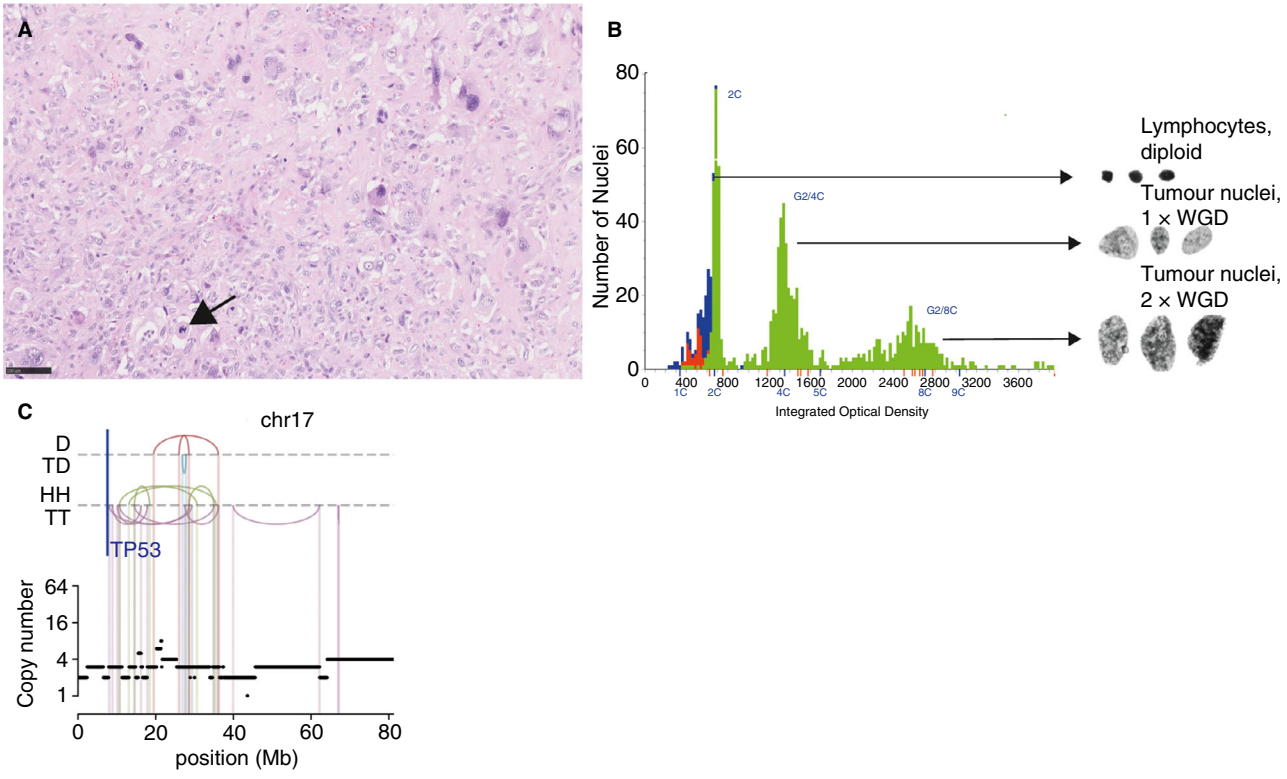

Figure 2. Macroevolutionary events seen in undifferentiated soft tissue sarcoma (USTS). **A**, Representative photomicrograph of USTS showing variation in nuclear size and pleomorphism. An abnormal mitotic figure is indicated with an arrow. Scale bar: 100 μm. **B**, An image cytometry histogram plot of DNA content, and representative images of large atypical nuclei associated with the different ploidy subpopulations. **C**, The characteristic pattern of chromothripsis, showing clustering of rearrangements, oscillating copy number states, and randomness of fragment joins seen across chromosome 17 involving *TP53*.

osteosarcomas ($n = 34$).[15] In one cohort of USTS cases,[7] 58% showed enrichment of disruptive rearrangements in the tumour suppressor genes *TP53*, *ATRX*, and *RB1* (Figure 2C). Furthermore, mutational timing has demonstrated that *TP53* mutations occur early in USTS evolution. This suggests that abrogation of the 'guardian' role of *TP53* is a prerequisite for the ability of these cells to tolerate the scale of DNA damage that occurs because of WGD and/or chromothripsis.[7]

Besides WGD and chromothripsis, a distinctive genomic phenotype seen in USTS is the presence of 'genome-wide' LOH (Figure 3A). A pan-cancer analysis of >10 000 cancers from the The Cancer Genome Atlas (TCGA) showed that 0.2% of cancers show almost genome-wide LOH, i.e. >80% of the genome with LOH (Figure 3B). This copy number pattern suggests a near-haploid precursor to the tumour. Such a near-haploid precursor will be under strong negative selection for subsequent copy number losses, as any such loss will lead to homozygous deletion and probably cell death, a particularly strong form of Muller's ratchet.[16] Such tumours are invariably found to be genome-doubled (Figure 3B), leading to genome-wide copy-neutral LOH, allowing them to escape their extreme selective constraints at a copy number level. Such tumours are rare among cancers (0.2% prevalence), and their occurrence is not evenly distributed across cancer types. Indeed, their prevalence is as high as 3% in TCGA sarcomas, and they are enriched in USTS (Figure 3C). Such tumours have previously been reported from karyotyping studies of sarcomas, particularly in USTS and inflammatory leiomyosarcoma.[3,17] Intriguingly, in our study of USTS, the genome-wide LOH events unmasked recessive mutations in DNA mismatch repair genes in some patients, resulting in a high tumour mutational load associated with immune cell infiltration and better overall survival.[7]

The mechanisms by which USTS is able to sustain such extreme levels of genomic instability remain poorly understood and cannot be explained by *TP53* mutation alone, as this is a prevalent driver in many

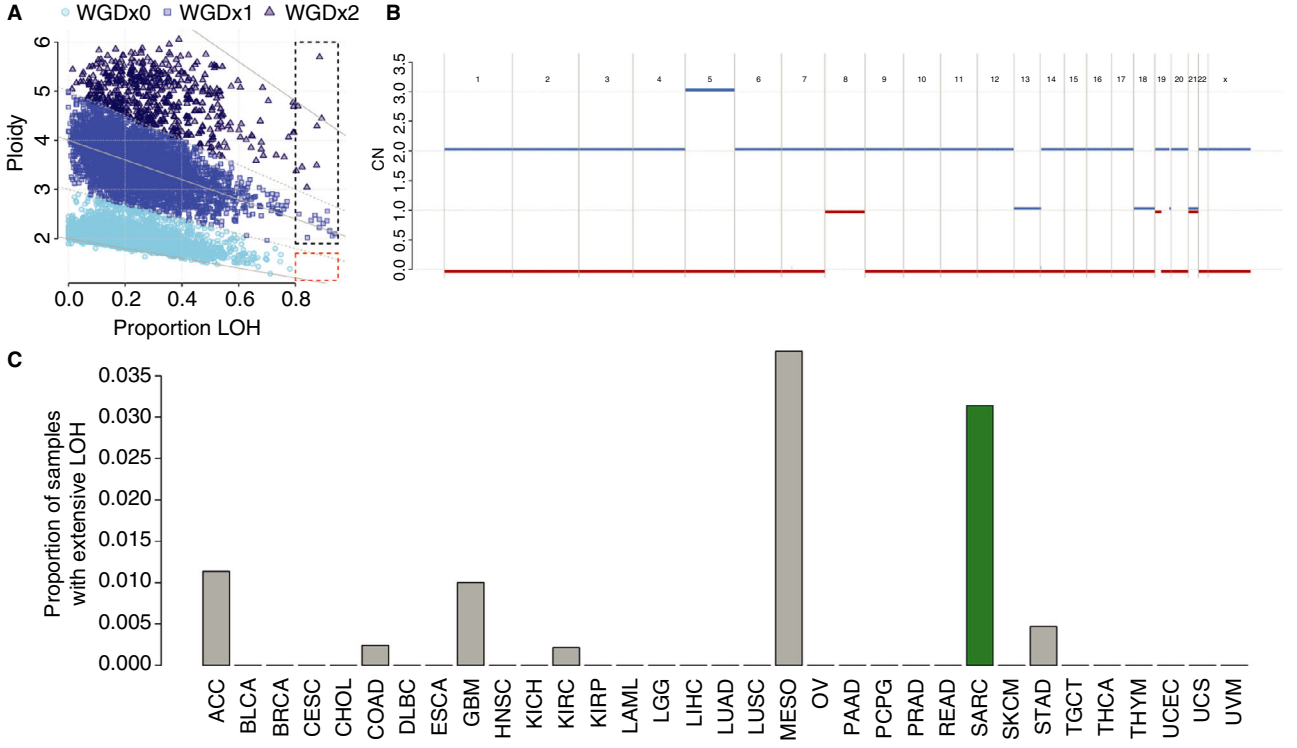

Figure 3. Undifferentiated soft tissue sarcomas (USTSs) show extensive genome-wide loss of heterozygosity. **A**, Allele-specific copy number profile of genome-wide loss of heterozygosity (LOH) in USTS. Blue: major allele. Red: minor allele. The *x*-axis shows the chromosome, and the *y*-axis shows the copy number (CN). **B**, Pan-cancer TCGA samples (~10 000 cases) classified into 0×, 1× or 2× whole genome doublings (WGDs) visualised as a function of ploidy and the proportion of the genome showing LOH. A subset of samples show extensive LOH (black box), but a 1*n* (haploid) population is not seen (red box). **C**, Distribution of extensive LOH events (>80% of the genome showing LOH) across tumour types represented in TCGA (cancer code abbreviations can be found at https://gdc.cancer.gov/resources-tcga-users/tcga-code-tables/tcga-study-abbreviations). TGGA, The Cancer Genome Atlas.

cancer types without elevated levels of copy number changes or rearrangements. Nevertheless, measures of genomic instability prior to therapy may prove to be informative in determining responses to chemotherapies. They could serve as biomarkers for newer classes of agent targeting replication stress and genomic instability, such as ATR, ATM and DNA protein kinase inhibitors. The development of a 67-gene expression signature related to mitosis and CIN has been shown to be superior to histological grading in determining metastasis-free survival, but it does not predict which patients could benefit from neoadjuvant chemotherapy[18,19] Newer methods for deconvolving this genomic complexity, such as the use of copy number signatures or rearrangement signatures derived from WGS of sarcomas, could also prove to be valuable tools providing prognostic or therapeutic benefit, as has been shown in other cancers.[7,20,21]

Hallmarks 2 and 3: evading growth suppressors and resisting cell death

Analyses of USTS using array comparative genomic hybridisation (CGH) or single-nucleotide polymorphism arrays, have shown recurrent copy number mutations in the form of deletions of tumour suppressor genes such as *TP53* on chromosome 17p, *RB1* on chromosome 13q, *CDKN2A* and *CDKN2B* on chromosome 9p, and *PTEN* on chromosome 10q.[12,22–24] More recently, next-generation sequencing data from two studies showed a broader repertoire of mutational types in USTS, demonstrated by frequent mutations in *TP53* (59%), *RB1* (43%), *ATRX* (29%), and *CDNK2A* (24%) (Figure 1B).[7,8] The benefit of using WGS is that structural rearrangement data can be integrated with the single-nucleotide variant (SNV) data, with data from our group showing that, without this integration, up to 50% of driver mutations in *TP53*, *RB1* and *ATRX* would have been missed if analysis had been restricted to only coding regions of the genome.[7]

TP53 is a classic tumour suppressor gene that orchestrates cell cycle arrest and can initiate apoptosis in response to cellular injury.[25] In addition, mutant forms of *TP53* have been shown to have a transdominant repressive effect over the wild-type form, and can promote invasion and metastasis.[26] USTSs show a diverse range of mutations in *TP53*, including homozygous deletions, and missense, frameshift, indel or splice site mutations,[7,8] but, to date, these have no diagnostic utility and are not used in routine sarcoma practice.

The G_1 to S phase cell cycle progression point is controlled by two canonical tumour suppressor genes: *RB1* and its upstream regulator *CDKN2A*. It has been shown in multiple studies that these two genes are mutated mainly through deep deletions in a mutually exclusive manner,[8,27] with USTS being more enriched for mutations in *RB1* (43%) than for mutations in *CDNK2A* (24%).[1,27]

By exploiting the knowledge of recurrent *RB1* and *TP53* mutations, it has been shown that inhibition of the downstream cell cycle progression molecule Skp2 leads to reduced proliferation and increased apoptosis in USTS cell lines and patient-derived mouse xenografts.[28]

Hallmark 4: sustained proliferative signalling in USTS

Up to 10% of USTSs show loss-of-function mutations or deletions of *PTEN* (Figure 1B). Loss of *PTEN* is associated with activation of phosphoinositide 3-kinase and subsequent downstream signalling to AKT and mammalian target of rapamycin, which is a serine/threonine kinase that regulates cell growth, survival, and proliferation. Targeting this pathway in preclinical models of USTS has shown some promise, and bears further investigation in a randomised control trial setting.[29]

Recurrent amplifications of chromosome 3p and 11q, in which regions *VGLL3* and *YAP1* are found, respectively, were identified in $\sim$10% of USTS cases by the use of array CGH.[30] This finding was corroborated by TCGA, which found amplification of *VGLL3* in 11% of cases and amplification of *YAP1* in 3% of cases.[8] These two genes are cofactors for TEA domain-containing transcription factors, and are responsible for activating the Hippo pathway, which is known to be involved in cellular survival, proliferation, and metastasis.[31,32] Knockdown of YAP has been shown to decrease the proliferation of murine autochthonous models of USTS both *in vitro* and *in vivo*. It may therefore be an attractive therapeutic candidate for a subset of patients.[33]

Hallmark 5: enabling replicative senescence

Sarcomas of multiple histological subtypes harbour long telomeres relative to other cancers and normal tissues.[34] Telomeres are simple tandem DNA repeat sequences (TTAGGG) at the ends of chromosomes that protect against the end replication problem, whereby chromosomes progressively shorten through

ongoing cell divisions.[35] The unprotected chromosome ends can trigger DNA damage response pathways, which can lead to recombination and end-to-end chromosomal fusions (dicentric chromosomes).[36] In 'healthy' somatic cells, when the Hayflick limit is reached, the cells undergo replicative senescence mediated by tumour suppressors such as p53 and retinoblastoma protein (RB1), and through the shortening of telomeres. Cells that can bypass this bottleneck undergo breakage–fusion bridge cycles of the dicentric chromosomes, resulting in complex derivative chromosomes and genomic instability (telomere crisis). This can lead to cell death or to continued growth that establishes a malignant phenotype.[34,37] This 'replicative immortality' is achieved either by up-regulation of the enzyme telomerase through mutations in *TERT*, or through the alternative lengthening of telomeres (ALT) pathway.

The increased expression of telomerase is predominantly caused by mutations of the *TERT* promoter[38] (Figure 4A) and, less frequently, by gene amplification or rearrangements.[34,39] Several studies have identified *TERT* promoter SNV mutations in different sarcomas.[40–45] *TERT* promoter mutations have been commonly identified in multiple subtypes, including atypical fibrous xanthoma (25 of 27 cases, 93%), pleomorphic dermal sarcoma (26 of 34 cases, 76%), myxoid liposarcoma (57 of 94 cases, 61%), and solitary fibrous tumour (38 of 176 cases, 22%). USTSs, on the other hand, rarely harbour *TERT* promoter SNV mutations (<2% of cases),[7,41,43,44] but a subset of cases show recurrent complex structural rearrangements of chromosome 5p15.33, which lies proximal to *TERT* (Figure 4B).[7] These 5p15.33 rearrangements juxtapose the *TERT* coding sequence to strong enhancer elements that transcriptionally up-regulate telomerase in a similar manner to that seen in some neuroblastomas.[7,46] In rare instances, recurrent translocations of *TRIO* are seen in USTS, often resulting in fusions with *TERT*.[47]

The more common way by which telomeres are maintained in USTS is through the ALT pathway, which is a telomerase-independent recombination mechanism.[48] Telomere lengths are generally much greater and more variable when the increases in length are generated via the ALT mechanism than when they are generated by telomerase, and this property can be exploited for the identification of USTS.[34] ALT is strongly correlated with inactivating mutations in α-thalassaemia/mental retardation syndrome X-linked protein (ATRX) or death domain-associated protein (DAXX).[34,49,50] *ATRX* and *DAXX* mutations were seen in 29% and 2% of cases of USTS, respectively, and ALT is thought to be the primary mechanism by which these cancers avoid telomere crisis.[7,8] The repetitive nature and structural complexities of telomeric DNA induce frequent replication fork stalling and chromosome breakage, and homologous recombination is required not only to maintain the length of the telomeres but also to utilise recombination to prevent and repair the stalled replication forks at the telomeres.[51] From a therapeutic perspective, the replication stress phenotype seen in preclinical ALT sarcoma models is targetable with drugs such as ATR kinase inhibitors, and this warrants investigation in a clinical trial setting with an appropriate biomarker.[52] A meta-analysis showed that ALT was present in 41% of 551 patients with soft tissue sarcoma and that ALT was associated with higher grade, a higher mitotic count, and a statistically higher risk of death.[53] Both ATRX and DAXX are nuclear proteins that have a role in telomeric chromatin remodelling, in which process they incorporate the histone variant H3.3. Mutations in these genes lead to loss of protein expression, which can be identified by immunohistochemistry (Figure 4C), but this may identify only a fraction of patients with the ALT phenotype, thereby limiting the potential of these mutations as biomarkers for stratification. Other methods for detecting abnormal telomeres and the ALT phenotype include the following:

1. Ultrabright telomeric DNA foci [fluorescence *in-situ* hybridisation (FISH)]. Telomere lengths can be quantified with FISH of peptide nucleic acid oligonucleotide probes that target the hexametric sequence.[54] The probes are assumed to bind in a proportional way such that the longer the telomeres, the greater the fluorescent signal, which has a resolution of 200 bp. This assay can be performed on cells either in interphase, when it can be used to quantify telomere lengths between cell groups, or on metaphase spreads, when it can be used to identify telomere lengths on specific chromosomes.[55] In ALT-positive cells, FISH demonstrates large, variably sized ultrabright telomere signals (Figure 4D). In comparison, normal or ALT-negative cells show only small, uniform FISH signals.
2. Promyelocytic leukaemia (PML) body assay. The ALT phenotype is characterised by ALT-associated PML protein nuclear bodies (APBs), which also can be detected by the use of anti-PML antibody staining together with a telomeric FISH probe (immune-FISH). APBs are structures that contain PML protein, large amounts of telomeric DNA,and

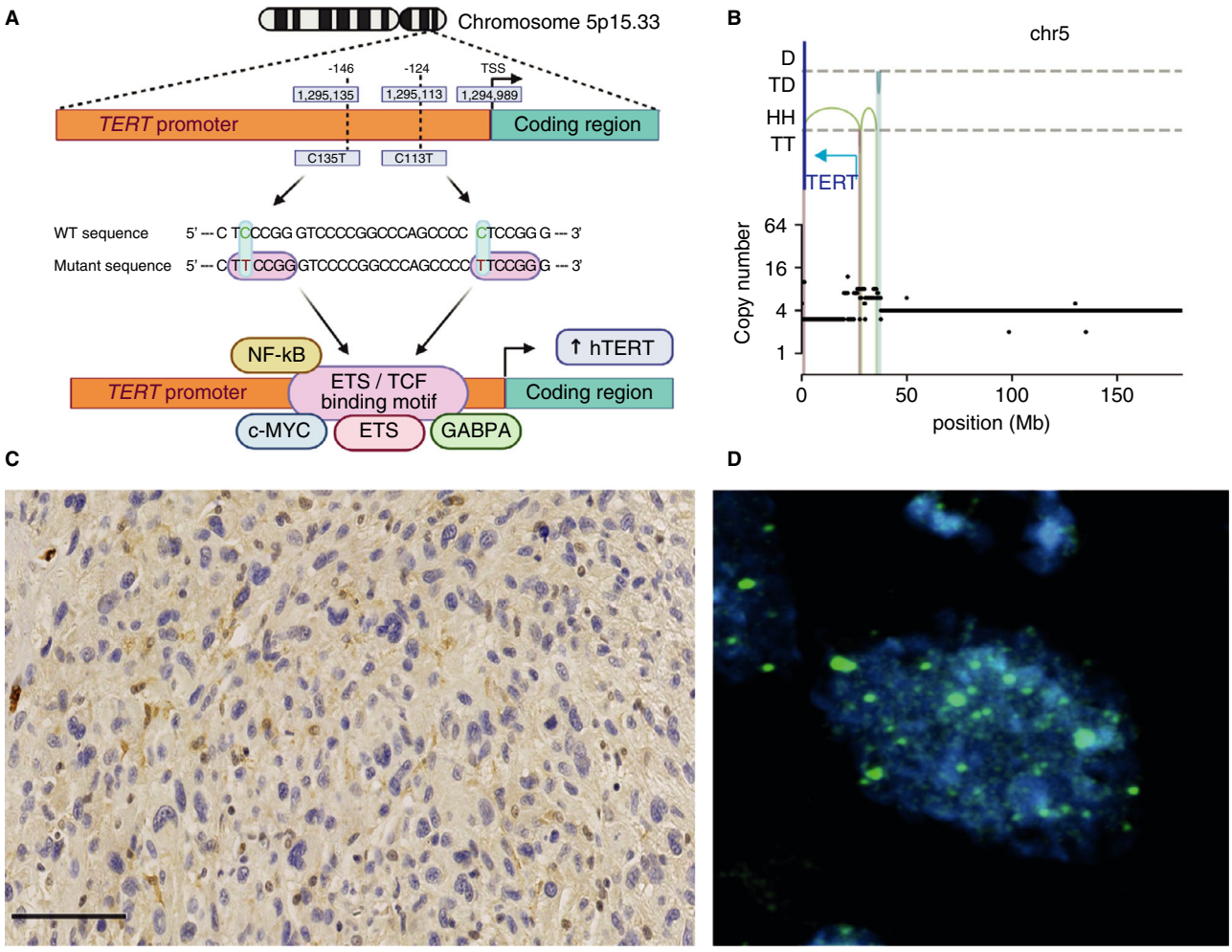

Figure 4. Replicative immortality. **A**, A schematic overview of the genomic location of hotspot *TERT* promoter region mutations seen in undifferentiated soft tissue sarcomas (USTSs). Transcription factors such as ETS, c-MYC, GA-binding protein-α (GABPA) and nuclear factor-κB (NF-κB) bind to the ETS/TCF binding motifs created by *hTERT* promoter mutations to increase transcription. WT, wild type. Created with BioRender.com. **B**, Genomic translocations occurring at chromosome 5p15.33 resulting in disruption of enhancer elements. Rearrangements are annotated as follows: D, deletion; TD, tandem duplication; HH, head-to-head inversion; TT, tail-to-tail inversion. **C**, α-Thalassaemia/mental retardation syndrome X-linked protein (ATRX) immunohistochemistry as a surrogate for loss-of-function *ATRX* mutations. A representative image of USTS shows loss of protein expression in tumour cells. Scale bar: 100 μm. **D**, A representative fluorescence *in-situ* hybridisation image of telomere fluorescence demonstrating large, ultrabright signals in USTS cells (taken with a confocal microscope).

proteins involved in telomere binding and DNA recombination, such as RAD51.[56] The APBs and the ultrabright telomere signals are often found to colocalise, and were detected in 33 of 52 (63%) of USTS cases.[57]

3. C-circle assays. Present within PML bodies are circular single-stranded telomeric DNA molecules called C-circles. C-circles are unique to ALT-positive cells, and provide the basis for the C-circle assay, which has shown utility as a non-invasive biomarker by detecting C-circles in blood samples from patients with ALT-positive osteosarcoma.[58] A protocol has recently been published to perform the C-circle assay on formalin-fixed paraffin-embedded tissue,[59] and this holds promise as a sensitive method for identifying ALT-positive tumours in both biopsy tissue and archival tissue.
4. Analysis of next-generation sequencing data. Several tools can provide estimates of telomere content from whole genome or whole exome sequencing data. Tools such as Telseq[60] can estimate telomere length, and TelomereHunter[61] can determine telomere content and detect ALT-positive cancer genomes by using matched tumour and normal pairs. Both

methods show a high degree of correlation with experimentally determined telomere lengths.

Therapeutically targeting telomerase maintenance mechanisms is an active area of research, and specifically targeting the ALT pathway may be a promising avenue for USTS. Trabectedin is an alkylating agent that binds to guanine in the minor groove, and disrupts the interaction of transcription factors with DNA by bending the DNA towards the major groove.[62] ALT-positive osteosarcoma and liposarcoma cell lines were shown to be highly sensitive to trabectedin, suggesting that selecting tumours with the ALT phenotype could be a useful strategy.[63] Although there is evidence of a response to trabectedin in some patients with USTS,[64] it remains to be seen whether response rates could be improved through the stratification of patients by their ALT status.

Hallmark 6: avoiding immune destruction and tumour-promoting inflammation

Cancer development and progression are regarded as evolutionary processes, and the strategies that tumours use to evolve are dynamic.[65] The tumour microenvironment plays a vital role in cancer evolution by creating selective pressures on cancer cells. Conversely, cancer cells can adapt to produce more permissive microenvironments, leading to distinctive tumour immune landscapes.[66] In large part, this interplay between the microenvironment and tumour cells is underpinned by the acquisition of immunogenic mutations and the loss of key genes involved in antigen presentation, either through genetic forces or epigenetic forces.[66] This immunity plays a dual role in the complex interactions between tumours and host, and has resulted in what are termed the three E's of the cancer immunoediting hypothesis, encompassing: (i) elimination of early-stage tumours (previously termed the immune surveillance hypothesis); (ii) equilibrium when the immune system controls the tumour; and (iii) escape when tumour cells are fully immunoedited and grow without immune control.[67] Although high levels of tumour-infiltrating lymphocytes are associated with an improved prognosis, it has been demonstrated in multiple cancer types that T cells can be dysfunctional. Seminal work involving the roles of cytotoxic T-lymphocyte antigen-4 (CTLA-4; CD152) and programmed cell death-1 (PD-1; CD279) in T-cell dysfunction, from Allison and Honjo, respectively, has revolutionised the understanding of the immune response to cancer.[68,69] Together with T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) and lymphocyte-activation gene 3 (LAG-3), these biomarkers are now in clinical use as a means of stratifying patients for immune checkpoint blockade therapy.[70] In multiple clinical trials of anti-PD-1 therapy in different cancer types, it was found that programmed death-ligand 1 (PD-L1) expression, as detected with immunohistochemistry, correlated with response to therapy, thereby leading to the approval of PD-L1 companion diagnostic tests for anti-PD-1/PD-L1 therapies.[71]

A subset of USTSs express high levels of the PD-1-binding ligand PD-L1, and results from recent clinical trials indicate that USTS and other sarcomas with complex genomes, such as dedifferentiated liposarcoma, are more responsive to immune checkpoint blockade than many other sarcoma subtypes.[72,73] The mechanisms for these responses are not entirely understood yet, but there are interesting lines of investigation to pursue, such as the role of tumour mutational burden, rearrangement-associated neoantigens, and copy number changes in the immune checkpoint and antigen presentation machinery.

THE USTS TUMOUR MICROENVIRONMENT

Investigating the USTS tumour microenvironment poses numerous biological and technical challenges. USTSs are inherently heterogeneous, and the distribution of immune cells can vary considerably within and adjacent to the tumour. Therefore, we support the idea that multiregional tissue sampling may be necessary to capture a global pattern of immune cell infiltration in order to better stratify patients for immunotherapy. With the increasing use of digital pathology-enabled image analysis, and a plethora of new multiplexing immunohistochemical technologies, there is now a convergence of tissue-based multiplexed immunohistochemistry and automated machine and deep learning imaging technologies. Such studies on USTS are awaited. This transformation has the potential to make complex information more accessible in clinical workflows, improving both predictive and prognostic power.

With other analysis methods, progress has started to be made in understanding the USTS immune microenvironment. The relationship between clinicopathological features such as grade, size and depth in USTS and immune cell infiltration has been shown by the use of multiplexed high-dimensional flow cytometry data on single cells.[74] Wustrack *et al.* found a positive correlation between the abundance of CD8+ T cells and overall survival, and, interestingly, an inverse correlation between the abundance

of CD8+ T cells and tumour size. Moreover, tumours that were located deep to the fascia showed a decrease in the amount of immunosuppressive CD4+/PD-1 + T cells as compared with superficial tumours, suggesting that deep and superficial USTSs could respond differently to immunotherapies. It will be important for prospective studies to evaluate how and why such anatomical features shape the USTS tumour microenvironment.

Using bulk RNA sequencing data, both the sarcoma landmark and pan-cancer TCGA studies[66] showed that there was considerable variation in the tumour microenvironment across different soft tissue sarcoma subtypes, including malignant peripheral nerve sheath tumour, synovial sarcoma, leiomyosarcoma, myxofibrosarcoma, and dedifferentiated liposarcoma, with USTS being the most immunologically active.[8,66] Using gene expression profiling of frozen tissue samples, Thorsson *et al*. found that cancers have one of six different global immune categories, i.e. wound healing, interferon-γ-dominant, inflammatory, lymphocyte-depleted, immunologically quiet, and transforming growth factor-β-dominant. Interestingly, across soft tissue sarcomas, samples were represented in every immune phenotype except for the immunologically quiet category.

Petitprez *et al.*[73] integrated multiple gene expression datasets and, through consensus clustering, found five distinct and varying immune profile categories across three different soft tissue sarcoma subtypes, including USTS, with every subtype being represented in every profile, highlighting the possible value of a transcriptomic-driven immune profile classification for therapeutic stratification. One of these profiles was characterised by B cells and tertiary lymphoid structures (TLSs). By evaluating molecular and clinical outcome data from the SARC028 immunotherapy clinical trial, they found that tumours with TLSs showed significant rates of response to PD-1 blockade therapy, and these patients showed improved progression-free survival irrespective of histological subtype. Among multiple sarcoma subtypes, USTSs have also been shown to have higher expression levels of antigen processing-related genes, activated and antigen-experienced T-cell-related genes, and PD-1/PD-L1 mRNA. USTSs also had the highest T-cell fraction and the highest T-cell clonality score.[75] These findings all suggest that immunotherapy may play a more prominent role in the future treatment of USTS, and that appropriate stratification strategies are needed to ensure the best therapeutic benefit.

Although the analyses of bulk RNA sequencing-based studies have provided a convenient starting point from which to begin to understand patterns in the sarcoma tumour microenvironment, the data produced are inherently biased towards tumour regions that have low levels of inflammation, as high-purity tumour samples are required for cancer sequencing studies. Moreover, these techniques result in a loss of spatial information, thereby hindering the assessment of the diverse nature of interacting cells in the tumour ecosystem. Spatial transcriptomic and proteomic approaches aim to bridge this gap by combining the power of global molecular profiling with near single-cell spatial representation.

IMMUNE EVASION AND USTS

The balance between T-cell infiltration and cancer cell death can be counteracted by immune-suppressive microenvironments containing large numbers of tumour-associated M2 macrophages; in USTS, this is mediated through increased production of retinoic acid by cancer cells.[76] In other cancers, such as melanoma and lung adenocarcinoma, the inability of T cells to recognise tumour neoantigens is achieved through diverse mutations within the antigen presentation machinery, chiefly in class I human leukocyte antigen genes,[77] but, to date, this has not been demonstrated in sarcomas.

Besides PD-1 and PD-L1, other coinhibitory receptors that suppress T-cell activation and cytokine secretion, such as CTLA-4 and LAG-3, are expressed in USTS (Figure 5A–D), and could prove to be useful therapeutic targets, as they are also positively correlated with tumour mutational load (Figure 5B). Immunohistochemical expression of PD-L1 is reported to be present in up to 34% of USTS cases, which approximately mirrors the response rates seen in clinical trials.[73] The genetic events associated with immune suppression in cancer include *PD-L1* copy number gains, which result in an increase in expression[78–80] (Figure 5B). The prevalence of *PD-L1* copy number gains in USTS samples has been shown in two different studies.[81,82] Budczies *et al.* found that *PD-L1* copy number gains correlated with higher PD-L1 expression[82] in up to one-third of USTSs, which was prognostically significant and impacted on the levels of the T-cell infiltrate.

Discussion

A catalogue of somatic alterations in USTS constructed by the use of WGS, exome sequencing, RNA sequencing and DNA methylation is now available,[7,8] and we have presented some of the key findings by providing a selection of cancer hallmarks. Despite the enigmatic

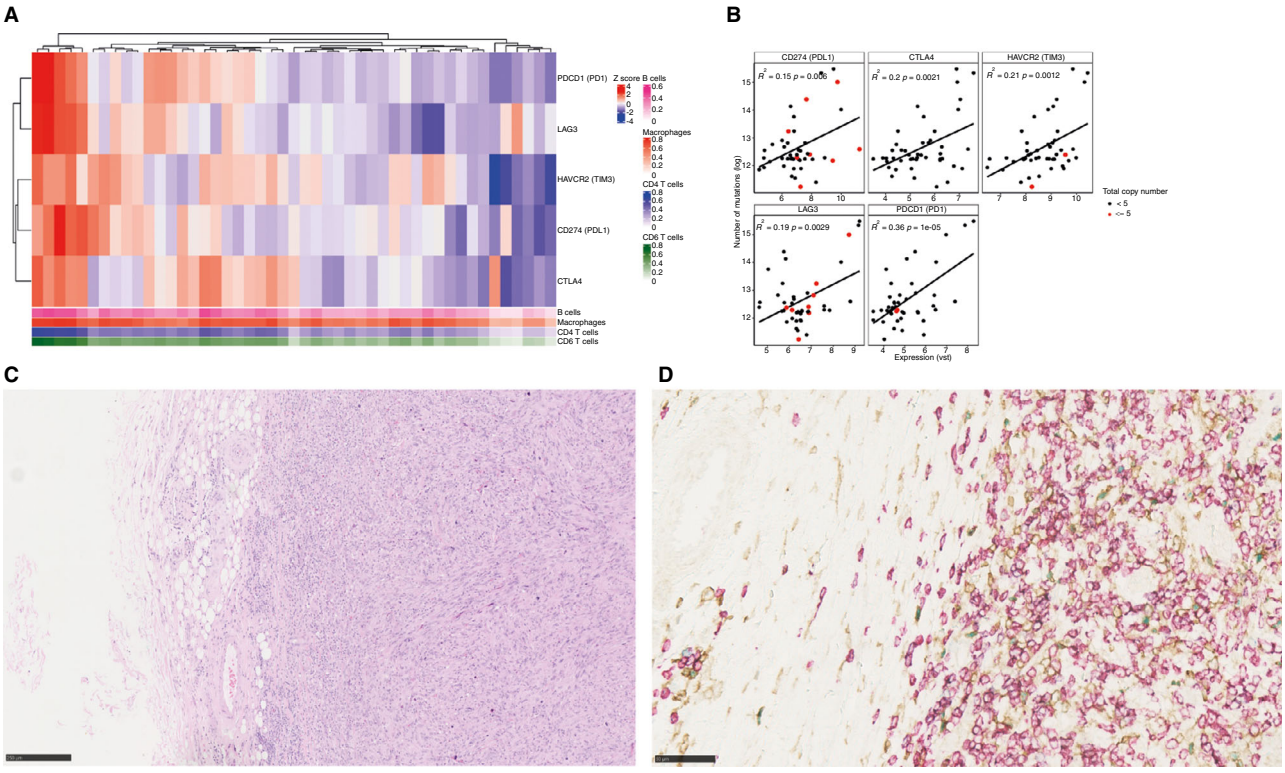

Figure 5. Immunogenomic features of undifferentiated soft tissue sarcoma. **A**, A gene expression heatmap of immune checkpoints that are targeted by antibodies currently being assessed in clinical trials. Tumours with high levels of immune cell infiltration also show concomitant up-regulation of immune checkpoint genes, suggestive of an immunosuppressive tumour microenvironment. The proportions of B cells, macrophages (M1 and M2), CD4+ T cells and CD8+ T cells were determined by the use of RNA immune cell deconvolution. **B**, The tumour mutational burden is significantly correlated with the expression of multiple immune checkpoint genes. A subset of cases show amplification (copy number of ≥5) of *PD-L1*, *TIM3*, *LAG3*, and *PD1*. Each immune checkpoint gene except for *CTLA4* shows a small subset of samples with copy number gains (copy number of ≥5), which may be related to expression of the gene. VST, variance-stabilising transformation of gene expression counts. **C**, A photomicrograph showing immune cell infiltration at the advancing edge of the tumour. Scale bar: 250 μm. **D**, A higher-magnification view of multiplexed brightfield immunohistochemistry of the T cells at the advancing edge of the tumour (CD4, brown; PD-1, blue; CD8, red; FOXP3, green). There is a complex melange of CD4+ (T-regulatory cells, CD4-positive and FOXP3-positive; conventional T-helper cells, CD4-positive only) and exhausted CD8+ cells (CD8-positive and PD-1-positive). Scale bar: 50 μm.

nature of these cancers, the future holds much promise if we can fully unlock and interpret the genetic code for patient benefit. The last decade of genomics research has enabled a more grounded understanding of the complexities of the USTS genome, and this has coincided with the development of targeted therapies, including immunotherapies. Despite this progress, only just over 100 samples have been profiled by the use of WGS or exome sequencing, and this is inadequate to capture the full repertoire of somatic variations that these tumours may harbour.

Genomic sequencing in some form is likely to become routine for all cancers, and for USTSs the most benefit is likely to be gained from global metrics of mutational change, such as tumour mutational burden, coupled with the judicious use of other biomarkers for immunotherapy and targeting the replication stress response that is pervasive in these tumours rather than single gene mutations. There will be much more to learn about the complexities of USTSs and, as these are rare cancers, it will be important for the accrual of data from research and personalised medicine programmes to be harmonised and accessible for research. Across Europe, there are efforts pointing in this direction for federated access and clinical data sharing for patient benefit.[83] By engaging with such efforts, pathologists with an interest in genomic medicine will continue to play a pivotal role in the diagnostic workup of USTS, as well as a meaningful role in the stratification of patients for newer therapies.

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Conflicts of interest

The authors state that they have no potential conflicts of interest.

Author contributions

N. Pillay, S. Hames and S. Nottley jointly conceived the layout of the review. S. Hames and S. Nottley prepared and wrote the initial draft with final review and revision by N. Pillay. S. Hames, S. Nottley and N. Pillay were responsible for data analysis and figure generation.

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