Title: The genomics of undifferentiated sarcoma of soft tissue: progress, challenges and opportunities.

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ABSTRACT

Undifferentiated sarcoma of soft tissue (USTS) are aggressive sarcomas that remain a diagnosis of exclusion and show extreme genomic complexity. Many advances in diagnostic criteria have resulted in several revisions in the definition of this rare cancer type. Recent sequencing efforts have illuminated the nature of the genome complexity and have revealed extensive copy number heterogeneity and multiple evolutionary patterns of development. This review places these recent advances into their historical and translational context and covers the changes in nomenclature, molecular classification, and the promise of personalised medicine.

KEYWORDS

sarcoma, cancer, genomics, copy number signatures, evolution

INTRODUCTION

Sarcomas are rare cancers of mesenchymal origin and can occur anywhere in the body but are predominantly located in soft tissues and bone. Soft tissue sarcomas have an age adjusted incidence rate of 45 per million per year and represent less than 1% of all malignant tumours[1]. They comprise roughly seventy subtypes with many displaying distinctive histologies, molecular genetic underpinnings, divergent natural histories and clinical outcomes[2]. The historical pathological classification of sarcomas is based on histological observations based on the shapes of cells (round, spindled, pleomorphic and epithelioid), patterns of growth (storiform, fascicular, plexiform, patternless) and the nature of the extracellular matrix (myxoid, fibrous,fatty). When this information was considered in conjunction with the patient's age and sex, the location of the tumour and clinical history a rudimentary classification was born. The concepts of differentiation and dedifferentiation were established by cell biologists in the 1800s and pathologists in the main were able to distinguish specific cell differentiation phenotypes by light microscopy alone e.g. smooth or skeletal muscle, nerve tissues or melanocytes but it was only when the ability to probe the differentiation patterns in cells became mainstream through the use of immunohistochemistry that pathologists began to use this information to

improve upon the classification. With these tools, pathologists had the ability to, for example, distinguish amongst the spindle cell sarcomas which were of smooth muscle, skeletal muscle or neural differentiation where cell phenotypes were atypical. Importantly they were now able to identify other cancers masquerading as sarcomas such as sarcomatoid carcinomas or melanomas. Moreover, as cytogenetics and other molecular biological techniques were being used to understand the genetic underpinnings of cancer, it was found that many sarcoma subtypes harboured gene translocations which were prototypical of particular entities and could thus be used as diagnostic tools[3–5].

Groups of sarcomas which did not demonstrate a particular line of differentiation and that were difficult to classify were all included under the rubric of malignant fibrous histiocytomas (MFH) which was regarded as a distinct sarcoma subtype by many pathologists (discussion below). MFH is no longer regarded as a distinct tumour entity and is obsolete as terminology, the more current diagnostic appellation being undifferentiated sarcoma of soft tissue (abbreviated here as USTS) which is an umbrella term that incorporates multiple undifferentiated morphological subtypes[2] including undifferentiated pleomorphic sarcoma (UPS). USTS is now considered a diagnosis of exclusion and accounts for 10% of adult soft tissue sarcomas and most commonly occurs in patients over the age of 50 but the age range is quite wide[2]. The lower extremities are the most commonly affected sites and USTS are frequently deep-seated lesions within muscle but can be located above the fascia (Figure 1A). USTS are a group of high-grade tumours frequently demonstrating necrosis and can have a pleomorphic morphology (Figure 1B and C) and with a local recurrence rate between 19-31% and a metastatic rate of 30-35%[6]. The prognosis for these patients is poor, with a median survival for those with advanced, metastatic disease of approximately 12 months[7]. Treatment of localized disease in the majority of cases in the United Kingdom comprises surgical excision with neo-adjuvant radiotherapy. The use of chemotherapy remains a matter of discussion but recent improvements in calculating risk of metastases strongly support perioperative chemotherapy in patients with high risk[8–10].

Until recently, the genetics of USTS has been poorly defined, limited to small series of cases and low resolution techniques such as karyotyping and arrayCGH[11,12]. In the current era of massive parallel sequencing and genome wide multi-omic profiling a clearer picture of the extent of genomic complexity (Figure 1D), heterogeneity and the variable evolutionary histories of USTS is beginning to be appreciated[13,14]. Whole exome (TCGA) and whole genome sequencing (Steele *et al.*) efforts have demonstrated differences in mutational frequencies, identification of new cancer driver gene mutations in USTS as well as new ways to classify genomic instability in these tumours[13,15]. It is hoped that this rapid expansion of biological information will have a clear impact in the way that future clinical trials and translational research are performed but there are still many bottlenecks that need to be addressed foremost amongst them are consistent criteria for the diagnosis of USTS and the relative dearth of appropriate preclinical models. This review will summarise the evolving nature of the diagnostic criteria for USTS, and provide an updated view of the compendium of molecular advances in this cancer and how this may relate to cancer evolution. A discussion on how future translational studies could be improved

by incorporating comprehensive genomic profiling to improve patient stratification and enable personalised medicine for USTS will also be addressed.

Histological classification and nomenclature

The terminology used for undifferentiated sarcomas has a long and interesting history (Figure 2). It was first known as MFH which was first introduced in the 1960s by Arthur Purdy Stout who thought it to be histiocytic in origin as it demonstrated histiocyte-like properties such as fibroblast transformation and phagocytic abilities in cell culture[16]. In 1971, Kempson described that MFH comprised multiple variants, some with benign features and others with more aggressive behaviour typified by high local recurrence rates and metastatic potential. One of these historical variants was known as fibroxanthosarcoma, an aggressive tumour with cells that contained foamy cytoplasm and multinucleated giant cells[17].

In early 1980s, MFH became the most commonly diagnosed adult soft tissue sarcoma and constituted multiple different subtypes with most diagnoses being made on morphological grounds on haematoxyln and eosin (H&E) stained slides. The MFH tumours showed a wide variety of histological appearances and were categorised into 5 types: storiform-pleomorphic, giant cell, myxoid (myxofibrosarcoma), inflammatory and angiomatoid. The prototypical pattern was the storiform-pleomorphic type showing admixed areas of pleomorphic cells with cartwheeled storiform areas containing a prominent vasculature. Characteristically, the pleomorphic areas contained numerous large, atypical cells including giant forms with hyperchromatic nuclei and abundant cytoplasm. During this period a revolution in diagnostic histopathology was taking place with the routine use of immunohistochemistry and electron microscopy becoming a staple part of the pathologists' armamentarium. It soon became apparent to some pathologists that MFH did not express immunophenotypical nor ultrastructural features reminiscent of histiocytic cell neoplasms. In an instructive study, the validity of MFH as a diagnostic entity was interrogated by Fletcher *et al.* using both immunohistochemistry and electron microscopy on a retrospective series of 159 cases[18]. Only twenty-one cases (13%) could be classified as MFH. The most common diagnoses were pleomorphic and dedifferentiated liposarcoma, which were discerned thorough identification of lipoblasts and areas of well-differentiated liposarcoma respectively. The other common diagnoses were leiomyosarcoma and non-sarcomatous neoplasms (carcinoma, lymphoma and melanoma). These were distinguished by expert pathology review and using a panel of antibodies that included desmin, smooth muscle actin, HHF-35, myoglobin, S100, CD45 and pancytokeratin[18]. Over time it also became clear that the angiomatoid variant was a distinctive tumour characterised by translocations involving *ATF1- EWSR1* or *CREB1-EWSR1*) [19,20]. There was therefore an increasing awareness that MFH may not be a distinct entity particularly in the late 1990s early 2000s. Many of these tumours were rather subtypes of other sarcomas or even other cancer types, and pleomorphic sarcoma or undifferentiated sarcoma were deemed more appropriate designations for those that were not[21,22]. However, probably reflecting the entrenched nature of the term MFH in the medical community it took at least two decades from the discovery that most MFHs were other tumour types before it was finally removed from the World Health Organisation (WHO) tumour classification[23,24]. In the 2013 WHO

classification, the term MFH was removed and UPS was introduced as a soft tissue sarcoma with no identifiable cell line or differentiation[23].

Undifferentiated soft tissue sarcoma subtypes

In the 2006 WHO classification[24], this group of tumours was labelled as UPS, however the 2013 WHO classification considered the variable morphological traits that USTS exhibit i.e. spindle, epithelioid, pleomorphic (UPS) , round cell and mixed and created an overarching grouping of which these subtypes form a part[23]. The undifferentiated sarcomas of round cell phenotype are morphologically and genetically a separate grouping more closely related to the small round blue cell tumours such as Ewing Sarcoma which are more prevalent in the paediatric setting and are not discussed further in this review.

The distinction of USTS subtypes from other tumours is largely dependent on the clinical scenario, availability of resources and the desire to pursue a specific line of differentiation by the attending pathologist. In routine practice a line has to be drawn between costs, time and the clinical benefits of assiduously distinguishing other sarcoma subtypes or even other types of cancer from USTS. In most instances many high-grade soft tissue sarcomas of adults are subjected to similar treatment regimens so making the distinction between histological subtypes is predominantly for prognostic information or academic interest. However, in the era of precision medicine and genomic medicine, the sarcoma treatment landscape is changing and increasingly patients are being recruited to biomarker-based clinical trials using small molecular inhibitors, antibodies to elicit an immune response, cellular-based studies as well as modified radiotherapy protocols which may require judicious subtyping for recruitment[25–27].

The ability to accurately workup a diagnosis of USTS in pathology depends on the extent of tissue sampling, use of a wide range of antibodies and molecular testing to distinguish these tumours from other more well-characterised sarcomas (Table 1). A common clinical scenario for instance is the distinction that needs to be made between a dedifferentiated liposarcoma (DDLPS) which occurs when a non-lipogenic appearing sarcoma arises on the background of a well differentiated liposarcoma. Here it is important to note that dedifferentiation is the process of a tumour losing its differentiation shared with the tissue it is presumed to arise from, which can be viewed as becoming more immature. Conversely, a tumour classed as undifferentiated lacks any evidence of a differentiated phenotype; morphologically an undifferentiated and dedifferentiated tumour can be indistinguishable. Hence, the non-lipogenic component often mimics USTS but is distinguished from USTS by the identified of *MDM2*/*CDK4* gene amplification[28]. Recent clinical trial data suggest the *CDK4* inhibitors may be beneficial for patients with advanced dedifferentiated liposarcomas and the prognosis for patients with DDLPS is superior than for USTS[28–30]. The clinical scenario, particularly many less distinctive (“soft criteria”) features e.g. location of tumour, previous history of cancer, sun exposed skin or age of patient may influence the decision to label a tumour as USTS. Moreover, the recent demonstration by the Cancer Genome Atlas (TCGA) that high grade myxofibrosarcoma shares many common transcriptomic features with UPS may blur

the lines even further and more research is clearly required in this regard[15]. It is also noteworthy that up to 25% of soft tissue sarcomas that are radiation induced fall into the category of USTS[2].

GENOMICS OF USTS

Historical context

During the 1990s using cytogenetic techniques it was evident that USTS showed high degrees of genomic complexity and intratumour heterogeneity[31], including the presence of double minute chromosomes, suggesting amplification of potent oncogenes in extrachromosomal material, and telomeric associations, suggesting possible telomeric dysfunction[32]. Despite this complexity, a hint of recurrent alterations in chromosomes 1, 3, 5, 7, 9, 11 and 12 emerged[2]. Using array based genetic profiling in the 2000s, targets of these alterations and more could be refined, revealing recurrent losses of *CDKN2A* on 9p, *RB1* on 13q, and *TP53* on 17p[33–35]. Other recurrent gains and losses were identified, some of which were associated with improved prognosis for patients, such as 1p33 gain[12]. However, recurrent genetic aberrations that could be used as diagnostic markers for USTS remained elusive. Similarly, genetic features that correlated with histological subtypes of USTS, or indeed defined novel subtypes of USTS, failed to emerge, other than the now recognised use of *MDM2* amplification as a diagnostic marker to exclude dedifferentiated liposarcoma[28].

Driver genes and pathways

Both technical breakthroughs and falling costs of sequencing have enabled efforts to investigate tumours at a base pair resolution on a genome wide scale, even in rare tumours such as USTS. Two large scale studies of USTS that provide a comprehensive understanding of its underlying biology have recently been published, one of which is based on whole genome sequencing (WGS) and a TCGA study which used exome sequencing[13,15]. Additional omic data from both studies included RNA sequencing and methylation array analysis. Here we provide an overview of the findings from both studies by combining the driver mutational events identified in each study, utilising single nucleotide variants (SNVs), insertions and deletions (indels), structural rearrangements, methylation alterations and ASCAT[36] called copy number variation, where each is available (Figure 3A).

Mutations in *TP53* are prevalent across all cancers[37], and USTS is no exception. TCGA study showed that 43% of USTS (UPS variant in TCGA study) and myxofibrosarcomas (MFS) have a mutation in this key tumour suppressor gene[15]. Using WGS a wider repertoire of *TP53* mutations was identified and increased the frequency of mutation in *TP53* to 65% of USTSs (Figure 3A), many of which included structural alterations in the gene highlighting the added benefit of using a technique with more breadth of coverage across genes[13].

Likewise, alterations in cell cycle regulators, chiefly through *CDKN2A* and *RB1* mutations, are common in USTSs, affecting 71% of WGS samples[13], 61% of samples from TCGA and 66% of samples overall. The biallelic alterations in these genes are mutually exclusive as has been described previously[38], indicating their

complimentary roles in the same pathway, with *CDKN2A*(p16) being an upstream regulator of Rb[39]. Again, the benefit of using WGS for studying complex genomes such as USTSs is reflected in the nature of mutations in this pathway, as 28% of aberrations in *RB1* and/or *CDKN2A* are mediated by structural variants which would be missed with exome or targeted sequencing.

Replicative immortality is a hallmark of cancer and is achieved in many cancers through dysregulation of telomere maintenance[40]. This is achieved mainly through mutations in the promoter region of the telomerase gene *TERT* or commonly through activation of the alternative lengthening of telomeres pathway through inactivating mutations in *ATRX* or *DAXX*[40].The phenomenon of telomere crisis, induced by telomere dysfunction can lead to extensive genomic instability, and has been linked with three complex genomic events[41]. The first of these is breakage fusion bridge cycles; the fusing of telomeres from two chromosomes which are then broken apart as each of the two centrosomes from the parent chromosomes are pulled to opposite poles of the cell during mitosis[42]. Secondly, chromothripsis; the shattering and random reconstitution of a chromosome following chromosome missegregation and micronucleus enucleation[43]. Lastly, kataegis; localised clusters of SNV mutations thought to be mediated by APOBEC enzyme activity[44]. However, this state of telomere crisis and its associated instability induces senescence, halting a tumour cells proliferation[45]. Therefore, both to immortalize the cell, and to escape telomere crisis, cancer cells must reactivate telomere maintenance. Mutations in *ATRX* and *DAXX* are seen in 31% of USTSs[13,15] (Figure 3A), however, some samples show promoter hypermethylation of *TERT*, the active subunit of telomerase, leading to activation of telomere maintenance[46]. Furthermore, through joint RNA sequencing and whole genome sequencing, it was observed that some rearrangements within the vicinity of *TERT* lead to increased *TERT* expression, ostensibly through enhancer capture[13,40,47]. This observation has not only profound implications for routes to telomere maintenance in USTS, but more broadly suggests that the large number of rearrangements seen in these tumours may have wide-ranging and poorly understood effects on gene expression independent of their disruptive effect when seen within tumour suppressor genes or when generating fusion genes.

The third most prevalently altered pathway is the mTOR signalling pathway, predominantly through biallelic *PTEN* mutations observed in 10% of samples overall[13,15] (Figure 3A). Considering other genes in the pathway including *RICTOR* and *PIK3CA* increases the alteration rate to 23%. Of note is the finding of amplification of *RICTOR* which is the second most common alteration in the mTOR pathway. It has previously been shown that in osteosarcomas both *TERT* and *RICTOR* are co-amplified in the same sample, which does not seem to be the case in USTSs[48].

Over the last decade defects in DNA repair pathways has gained renewed interest with the advent of synthetically lethal targets and immunotherapy that both aim to exploit the deficiency of fidelity in DNA repair in some tumours[49,50]. More specifically, DNA mismatch repair defects have increasingly been understood to be more common across cancer than previously known[51,52]. Indeed, USTSs, which were once believed to have a universally low mutational load, are hypermutated in

approximately 10% of patients[13]. Much of this is mediated by defects in mismatch repair proteins leading to somatic microsatellite instability. Rarely, DNA glycosylase genes are mutated and contribute to the high mutational load in USTS[13]. *MBD4* loss of function mutations lead to a predominance of C>T mutations through failure to repair spontaneous deamination[53], and *NTHL1* loss leads to retention of reactive oxygen species mutations with a distinctive mutational pattern[54,55]. Whether these rare causes of hypermutation are recurrent or enriched in USTSs requires the collection and study of larger sample sets of fully elucidate. Of note, three of the seven patients with hypermutated tumours had potentially pathogenic heterozygous germline variants in DNA repair genes, with the second hit occurring through somatic loss of the wild-type allele in the tumour; this highlights the importance of examining the germline genetics of DNA repair genes to identify the potential contributor to high mutational loads in USTSs. A subsequent study using targeted sequencing and immunohistochemistry for mismatch proteins has revealed that approximately 2.3% of sarcomas harbour mismatch repair deficiency[56].

Focusing on driver copy number events, analysis of the combined datasets revealed recurrent deletions around *CDKN2A*, *RB1* and *TP53* (Figure 3B, blue line), recapitulating earlier cytogenetic findings rearrangement patterns[12,13], and recurrent amplifications around *JUN* and *RICTOR* (Figure 3B, red line). Multiple peaks of deletion are seen in telomeric regions such as on chromosomes 2, 4 and 7, which may indicate losses due to telomere attrition, as hinted at by the observation of telomeric associations in cytogenetic studies[32]. There remain peaks of recurrent copy number alteration, such as the deletion peak on 1q that have not been linked to a definite driver gene in USTS.

Molecular subgroups

There has been a failure to identify clinically meaningful genetic subgroups of USTS through cytogenetic or array-based studies. As a result, molecular subclassification of USTS remains a key goal for new omic based studies in the personalised medicine era. An illustrative example of where other omic studies could be of value is the proposal by the TCGA study that some high grade MFS variants share transcriptional patterns with USTS and may represent one end of the spectrum of these tumours[15]. This conclusion was reached using RNA sequencing primarily. Interestingly however, these two tumour types could also not be separated by clustering based on copy number alterations, miRNA expression, methylation profiling, protein profiling and pathway analysis. Moreover, classification based on all of the above datasets combined found no cluster that was private to either MFS or USTS. Further, there appeared to be no driver mutations that were only seen in one or the other tumour types. This raises the possibility that some MFS variants and USTS are one and the same, lying along a spectrum of the myxoid phenotype. Therefore, for the purposes of this review data from the TCGA MFS samples have been combined and analysed with USTS.

Based on consensus clustering, TCGA split USTS into two distinct groups, however, the differences between these groups remains poorly defined[15]. In contrast, Steele *et al.* found no evidence from either methylation profiling or RNA sequencing of subgroups of USTS[13]. This lack of a clear distinction is recapitulated here when

performing principal components analysis on the combined RNA datasets (Figure 4, left) or on the combined methylation datasets (Figure 4, right), with tumour samples falling into a single group in each analysis (orange and green points), separated from the normal adjacent tissue samples (blue). This discrepancy between the two studies, which may be a consequence of different sample selection criteria or divergent methodology for identifying subgroups, remains as an open question as to whether USTS can be split into distinct biological entities with possibly divergent life histories or cells of origin.

A pattern of mutual exclusivity between SNV/indel instability and structural instability has been described over the last decade[15]. The interrogation of WGS data allows for this to be investigated comprehensively. In USTS, a similar pattern is seen, with tumours having either a high mutational load (>5 mutations/Mb; mutHi), a high rearrangement load (>100 rearrangements; rearrHi) or neither of the two (mutLo-rearrLo; Figure 5A). No sample was found with a high rearrangement and mutational load (mutHi-rearrHi). The mutHi group comprised 10-15% of samples that are hypermutated at an SNV/indel level with driver losses of DNA repair machinery, that will increase the base rate of mutation accumulation in mutHi samples (Figure 5B, red), leading to a gradual separation between the mutation load of mutLo (Figure 5B, blue) and mutHi samples over time. The rearrHi group comprised ~60% of samples with an elevated level of structural variation in the genome and was enriched in chromothriptic events. In contrast to hypermutation, chromothripsis is an example of punctuated evolution, which will greatly increase the rearrangement burden of a tumour cell in a single event (Figure 5C, red), but may not increase the base rate of rearrangement accumulation over time outside of this event compared to rearrLo samples (Figure 5C, blue). The remaining ~20% of samples harboured a relatively low amount of both SNVs/indels and rearrangements, in comparison to other USTSs (mutLo-rearrLo). Importantly, patients with hypermutated tumours were found to have an improved prognosis[13].

Punctuated evolution of USTSs

Given the known karyotypic complexity of undifferentiated sarcomas[31], to fully understand the aetiology and life-history of USTS it is imperative to understand the structural alterations, and resulting copy number changes that lead to such complexity. A gene-by-gene approach, as is performed whilst identifying driver genes, is insufficient for this purpose, as it ignores the overall picture of copy number change. To remedy this a strategy to identify common patterns of copy number that are shared across samples has been implemented[13]. This method is analogous to methods that have been developed for identifying SNV mutational signature over the last decade[57], where the repertoire of mutations in a cancer is summarised as the counts of each of 96 triplet context mutation classes, which are then deconvoluted from multiple tumours into distinct mutational patterns shared across tumours. These SNV mutational signatures have increasingly been linked to endogenous mutational processes such as DNA mismatch repair deficiencies[58], and exogenous mutational processes such as tobacco[59] or ultraviolet radiation[57] exposure.

In order to extract a copy number signature in an analogous fashion, an allele-specific copy number profile (Figure 6A, top left; red=major allele, green=minor allele) is summarised by categorising its segments by their LOH status, total copy number and segment size (Figure 6A, top right). Non-negative matrix factorisation is then performed on multiple such profile summaries, identifying any number of copy number patterns that can be recombined to reconstitute a given copy number profile (Figure 6A, bottom). Performing the copy number profile summarisation across multiple datasets (Figure 6B, copy number categories displayed on y-axis, samples on x-axis) demonstrates that USTS are extremely heterogenous in their copy number profiles, both at an intratumoural (multiple copy number categories represented in each sample) and intertumoural (unique distributions of each copy number category in each tumour) level[13,15]. Hierarchical clustering on these samples (clusters denoted by brown and purple boxes in top annotation) shows multiple distinct copy number flavours in USTS with similar distributions of copy number classes shared between samples.

By implementing a copy number signature (CNS) framework (Figure 6A), 7 copy number signatures were identified in USTS[13] (Figure 6C) that broadly corresponded to: CNS1=mostly heterozygous segments with copy number 5 or more with some LOH segments of copy number 3 or more, inferred to be twice genome doubled from a hypodiploid background; CNS2=heterozygous segments with copy number 3-4 with LOH segments of copy number 3-4, inferred to be a single genome doubling on a hypodiploid background; CNS3=mostly heterozygous copy number neutral segments with some copy number 1 segments, inferred to be a hypodiploid state; CNS4=copy number neutral LOH segments; CNS5=mostly small copy number 5+ segments, correlated with chromothriptic events; CNS6=mixture of heterozygous and homozygous copy number states; CNS7=heterozygous segments with copy number 3-4 without LOH, inferred to be a relatively simple genome doubling event. Here we synthesise the 7 copy number signatures into three major routes of copy number alteration (Figure 6D), where a precursor diploid cell (grey), may lose and gain some content becoming hypodiploid, from which point it may undergo a single or multiple genome doubling events to become hypotetraploid or hypooctoploid respectively, which we term the ploidy route (blue). At any point along the ploidy route, a cell may be subject to chromothripsis, leading to a highly complex genome (purple). Alternatively, the cell may undergo extensive genomic loss before genome doubling to become near-haploid (green). Whether this loss occurs in a punctuated or sequential fashion remains unknown, however, these events are invariably followed by genome doubling leading to a pseudohaploid genome, and thus completing the pseudohaploid route. Note that many of the events that define the paths through these routes are examples of punctuated evolutionary events, with large scale alterations likely occurring simultaneously rather than sequentially over time.

Looking more closely at the ploidy route, WGD events have been associated with increased proliferation rates and poorer prognosis across a range of cancers[60], and are thought to increase the evolutionary space within which a tumour is free to evolve[61]. Punctuated evolution through WGD in USTSs is the norm rather than the exception with 71% and 88% of samples in Steele *et al.*, and TCGA respectively

having undergone at least one WGD event[13,15], over twice the rate seen in advanced cancers as a whole[60]. Image cytometry analysis, providing a readout of nuclear DNA content at a single cell level, has revealed that the majority of USTS harbour multiple subclones within a single tumour with distinct ploidy levels (Figure 7A, ploidy clones represented by peaks) providing further evidence for the sequential nature of genome doubling in these tumours[13]. Additionally, timing of driver mutations in relation to genome doubling in USTSs has shown that driver mutations occur predominantly before any genome doubling event, and are likely extremely early events in the life of the tumour[13]. This allows for a rough understanding of the clonal evolutionary dynamics of USTS over time, with early *TP53/RB1* or other driver mutations followed by complete clonal expansion and subsequent sequential genome doubling events with incomplete clonal expansion (Figure 7B). Given the role of *TP53* in "policing" the genome, these mutations may be obligate before the extensive copy number alteration seen in USTS can be tolerated. However, image cytometry analysis necessarily removes any spatial information as it is performed on nuclear suspensions, so it remains unknown whether the higher ploidy subclones are formed of multiple independent genome doubled subclones distributed sporadically throughout the tumour (Figure 7C, top, darker polygons represent a simulation of higher ploidy cells), or if they form as localised patches within the tumour with clonal expansion of a single progenitor (Figure 7C, bottom). The former is more likely in view of the typical histologies of these tumours which often display cells with increased nuclear area that are dispersed throughout the tumours singly rather than forming clusters (see Figure 1C); this suggests either the distinct origin of multiple high ploidy subclones or a high degree of mobility of such giant cells. Single-cell spatial analysis of these tumours will be required to elucidate the precise history of these events.

As described previously, chromothripsis following the missegregation of chromosomes during anaphase[62] is an important method of punctuated evolution in USTSs, occurring in 58% of samples[13]. In USTSs this is predominantly following genome doubling and so lacks the classical alternating pattern of LOH and heterozygosity, although, this does not preclude chromothripsis before genome doubling. See Figure 7D for an example of a canonical chromothripsis event on chromosome 8 of a USTS, with oscillating copy number of the minor allele between 0 and 1 (red), an unaltered copy number state of the major allele (blue), and structural variant deletions (++), tandem duplications (--) and inversions (+-, -+) distributed across the chromothriptic region (grey arcs). Given the methods used, there is some uncertainty as to whether breakage fusion bridge cycles (BFB) may be responsible for some of the chromothripsis-like events seen in USTSs; it is becoming clear that the two events are not as separate as originally believed, in that BFB events can lead to chromothripsis[63]. The chromothriptic events observed in USTS often lead to amplification of oncogenes, such as *JUN* (Figure 3A), through the generation and multiplication of double minute chromosomes, however, chromothripsis-induced tumour suppressor knockout, as seen in canonical chromothripsis is less common in USTS due to the preceding genome doubling.

Both chromothripsis and genome doubling via endoreduplication have previously been linked to telomere crisis[64–66]. The prevalence of both of these processes in

USTSs, in conjunction with the recurrent activation of *TERT* through rearrangements or methylation alterations, and recurrent activation of alternative lengthening of telomeres through abrogation of *ATRX* or *DAXX* suggests telomere crisis is a common event in the life history of USTSs providing a strong selective pressure to reactivate telomere maintenance[13,15]. Indeed, such telomere crisis can directly lead to mitotic errors, which are observed frequently in H&E stained slides of USTS (Figure 7E), and may be the proximal cause of many of the punctuated evolutionary events seen in USTSs.

The haploidy seen in the pseudohaploid route is thought to originate through anaphase missegregation, and can lead USTS with as much as 90% of the genome as LOH as presaged by earlier cytogenetic findings[11]. Such a copy number profile is seen in Figure 7F, with the minor allele (red) having CN 0 across the majority of the genome, and the major allele (blue) having CN 2 across much of the genome, suggesting an early near-haploid state, followed by doubling of the single remaining copy of the genome, as described in the pseudohaploid route. The near-haploid phase of this process is likely to induce senescence or severely reduce fitness of the cell, evidenced by strong negative selection of mutations in key genes in LOH regions[61], however, this is invariably rescued by genome doubling. Given the presumed loss of fitness in the near-haploid state, these events must be compensating in some way; this may be through unmasking multiple deleterious mutations in tumour suppressor genes at once, or perhaps through reducing neoantigen burden of the cell drastically in one event so as to evade the immune system.

It is worth noting here that these events of genome doubling, genomic losses and gains, and chromothripsis are likely occurring repeatedly in different cells within the tumour throughout its life, similar to ongoing mitotic errors seen in colorectal cancer[67]. The majority of these will be deleterious and lead to the death of the cell, however, some small percentage of events will occur in just the right region of the genome to provide an advantage to the cell, so will be propagated through the tumour via selection. The patterns of change that we see in the final tumour are necessarily the end product of this tumult of alteration, death and survival, and can enlighten us as to events that are commonly selected for or against in USTSs, as seen not only through recurrent mutations (Figure 3A), copy number alterations (Figure 3B) and rearrangements, but also through regions of the genome that have an under-representation of alteration such as chromosomes 5p and 7p[13]. To obtain a full understanding of the dynamics of these events, single cell analysis of USTS genomes will be necessary.

TRANSLATIONAL RELEVANCE OF GENOMIC PROFILING

Molecular testing in the form of RT-PCR, fluorescence in-situ hybridisation and/or RNA sequencing panels are used routinely for sarcoma diagnostics, but less so for treatment selection apart from some subtypes. In contrast, genomic profiling is being conducted routinely for patients with melanoma, colorectal and lung cancer for patient stratification[68].

Amongst sarcomas, gastrointestinal stromal tumour (GIST) is probably the most common sarcoma subtype which harbours *c-KIT* or *PDGFRα* mutations and has been one of the few success stories for targeted therapies[69,70]. Another case in point is the recognition that the TRK inhibitor Larotrectinib is effective in sarcomas with *NTRK3* fusions e.g. infantile fibrosarcoma[71]. Further, ALK translocations in inflammatory myofibroblastic tumours sensitise these tumours to the targeted therapeutic Crizotinib [72], TSC1 and TSC2 mutations in PEComas are amenable to mTOR inhibitors[73], and CSF1 fusions in tenosynovial giant cell tumours sensitize to CSF1R inhibitors [74]. Lastly, patients with DDLPS may be eligible to enrol in clinical trials to target MDM2 and CDK4/6 amplification[75]. The role for targeted therapies for USTS however is not so clear cut.

Patients with inoperable or metastatic soft tissue sarcoma have a poor prognosis with overall survival rates of approximately a year with the first line currently being palliative chemotherapy[7]. In recent years there have been multiple failures of phase III randomised clinical trials for sarcoma patients[76]. A case in point is the recent withdrawal of the platelet derived growth factor alpha (PDGFRα) inhibitor Olaratumab from the market for sarcoma patients based on poor Phase III results[77]. The reason behind the failure of many of these trials is still not completely clear but some contributory factors include the lack of biomarkers for appropriate stratification of patients in the first instance. The “lumping together” of histological types in order to fulfil recruitment criteria without a biomarker is clearly a problem and, in some instances, may be considered unethical given what we now know about the inter- and intratumoural heterogeneity of sarcomas[78]. Some trials are also being conducted without sufficient evidence from preclinical studies which could prove informative. What these failures have highlighted is a desperate need for more translational research in sarcomas to identify objective stratification markers for patient selection and the need to rethink clinical trial design in order to improve outcomes, particularly for patients with rare subtypes.

Genomic profiling of USTS for clinical benefit

USTS show extensive inter- and intratumoural genomic heterogeneity[13,15,31]. Unlike most other common tumour types their heterogeneity is underpinned by copy number aberrations rather than single point mutations. The vast majority of point mutations identified in USTS are clonal[13] and the subclonal architecture is dominated by aberrations in ploidy and large-scale copy number alterations (Figure 6B, 7A). However, it is important to note that there is a lack of data on multi-regional and temporal studies in USTS that could provide better resolution of the extent of heterogeneity and inform on cancer evolution.

It has recently been proposed that in ovarian cancer which shows extensive genome complexity, that using the metrics from aberrations in the cancer genome as a whole rather than single mutations as biomarkers may be feasible[79]. For instance, the identification of mutational processes such as homologous recombination deficiency in ovarian cancer in the absence of *BRCA* mutations is a viable route for patient selection for PARP inhibitor therapy[80]. Moreover, quantifying the copy number

processes active in ovarian cancer yields prognostic and predictive information[79]. The identification of *BRCA*ness has proven to be of benefit in some sarcomas[81,82]. The jury is still out with regards to doing similar with USTS due to the small numbers of cases examined at a whole genome level thus far, but it is likely with the advent of routine genome profiling and whole genome sequencing in particular that this data will soon be forthcoming. Importantly though, there is evidence that the quantification of single point mutational load may be important in a small subset of patients with USTS and could be used as a biomarker for selection for immunotherapy related trials[13]. The identification of the mutational processes that generate these mutations (mutational signatures) is beneficial to understanding cancer biology, identification of cancers of unknown primaries and rational therapeutic selection in many other cancer types[83]. Despite there being only a relatively small number of USTS genomes published thus far, the data from these studies suggest that mutational signatures will prove informative in this heterogenous sarcoma type.

Also, the identification that 26% patients harbouring aberrations in the *mTOR* pathway and similarly the 15% that harbour mismatch repair deficiency is suggestive that routine genomic profiling may be of benefit for some USTS patients, particularly in centres where basket trials for drugs targeting these pathways are open.

Immunotherapy and USTS

Immune evasion is another important hallmark of cancer[84]. Two phase 2 clinical trials of immune checkpoint inhibitors in sarcomas have shown clinically meaningful response rates of between 16-18%[25,26]. These responses were seen across a few histology types including USTS, with as high a response rate as 40% in USTS. In fact ¾ of the USTS that stained positive for PD-L1 appeared enriched for responders, compared to none of the non-USTS PD-L1 positive tumours [25]; this suggests IHC staining for PD-L1 may be particularly promising in USTS for stratifying patients that may respond to immunotherapy. More recently, it has been proposed that the antigen MAGE-A3 may be relevant target for immunotherapies[85] based on its strong expression in USTS.

Characterisation of the tumour microenvironment in soft tissue sarcomas is still in its infancy. In one study 81 formalin fixed paraffin embedded (FFPE) sarcomas of various histologies were assessed by performing gene expression profiling using the Nanostring instrument and via T cell receptor sequencing[86]. Interestingly this study revealed that USTS and leiomyosarcoma expressed high levels of antigen presenting cell related genes. Additionally, T cell receptor sequencing showed that USTS had significantly higher levels of T cell infiltration compared to other sarcoma subtypes. Moreover, the T cell infiltrates were more oligoclonal in nature compared to other STS types suggestive of a selection process driven by neoantigen formation. In another study based on TCGA samples, it was shown using a novel clustering method of gene expression data that USTS associated with strong intratumoral immune responses[15]. A score for immune cell infiltrate was calculated based on the gene expression signature of various immune cells. In addition to the T cell score, the natural killer cell score and dendritic cell score also correlated with

improved patient survival in myxofibrosarcoma and USTS. This suggests that both antigen dependent and antigen non-dependent effectors may have roles in the immunological response in STS and subsets of USTS in particular. Whilst this field hold promise for some USTS patients, the vast majority of USTS are immune “cold” and more work is needed to understand the immune evasion mechanisms that may be operative in these tumours.

Future work

From the few genomic studies that have been performed on USTS to date it is clear that DNA and RNA sequencing is likely to be transformative in understanding the biology of these tumours. However, the path to using these tools as predictive markers is less clear, especially in the absence of a comprehensive and appropriately funded translational research framework that is aligned to clinical trials. It is likely that over the next few years many more immunotherapy related trials will be initiated for sarcomas and the encouraging results seen in USTS thus far suggest that more work should be done in this area to identify the biomarkers of response that could be applied for patient stratification. More targeted therapies are also likely to be developed and a translational research strategy is required to understand the successes and failures of those trials. Moreover, there is going to be a need to critically re-evaluate the commonly used double blinded randomised trial design and develop innovative trials for rare cancers such as sarcomas. It is likely that basket and/or umbrella trials may prove to be informative in this regard. Notwithstanding these challenges, the post-genomic era we are entering is promising and hold much potential and hope for sarcoma patients.

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FIGURE LEGENDS

Figure 1: Pathology and genetics of USTS

A. Macroscopic specimen of resected USTS in a superficial (above fascial) soft tissue location. Small ellipse of skin is preserved on the surface and suture is present to aid the surgical orientation of the specimen.
B. Cut section of tumour showing a variegated appearance with haemorrhagic and necrotic foci interspersed between fleshy cream areas of solid tumour.
C. Typical histological appearance showing irregular fascicles of cytologically atypical pleomorphic and spindle cells in a fibrous stroma. Scalebar=250μm
D. Circos plot visualisation of a whole genome sequence. The outer ring represents positions on the different chromosomes (1-22) and the sex chromosomes (X,Y). The subsequent rings, in order from outer to inner, comprise representations of the somatic mutations: single nucleotide variants, insertions and deletions, copy number changes, rearrangements and their genomic locations. The barplots represent the counts of the various mutational classes across the genome.

Figure 2: Historical timeline of major changes in nomenclature, classification and advances in biological understanding of USTS. MFH=malignancy fibrous histiocytoma, UPS=undifferentiated pleomorphic sarcoma, WHO=World Health Organisation,MFS=myxofibrosarcoma,USARC=undifferentiated sarcoma

Figure 3. USTS driver genes and pathways. A) Driver single nucleotide variants/indels, structural variants, and copy number alterations from two studies of USTS. Driver genes are organised into pathways; stab=DNA stability, CC=cell cycle, tel=telomere maintenance, mTOR=mTOR signalling, men=MENIN pathway, repair=DNA repair. Copy number alterations are shown as full blocks and are derived from ASCAT copy number calls for both TCGA (Reference 13) and Steele et al. (Reference 12) data; Hom Del=homozygous deletion, Amp=amplification, LOH=loss of heterozygosity. SNVs and indels are shown as small rectangles for somatic variants and circles for germline variants; 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. B) Recurrent copy number alterations for combined copy number profiles. Log10(q) is shown across the genome for deletions (blue) and amplifications (red), with key driver genes labelled.

Figure 4. Transcriptional and epigenetic molecular subgroups of USTS. A) Combined data from Ref. 12 and 13 fails to show any clear subgroups at either a gene expression (left) or methylation (right) level using principal components analysis.

Figure 5. Genetic subgroups of USTS. A) Molecular subgroups of USTS defined by mutational (y-axis) and rearrangement (x-axis) burden. Percentages of samples in Ref 12. in each category are displayed. B) Hypothetical mutation load over time for USTS that are mutation high (mutHi, red) or mutation low (mutLo, blue). The rate of mutation accumulation increases in mutHi after loss of function of a gene involved in DNA repair (dashed line). C)

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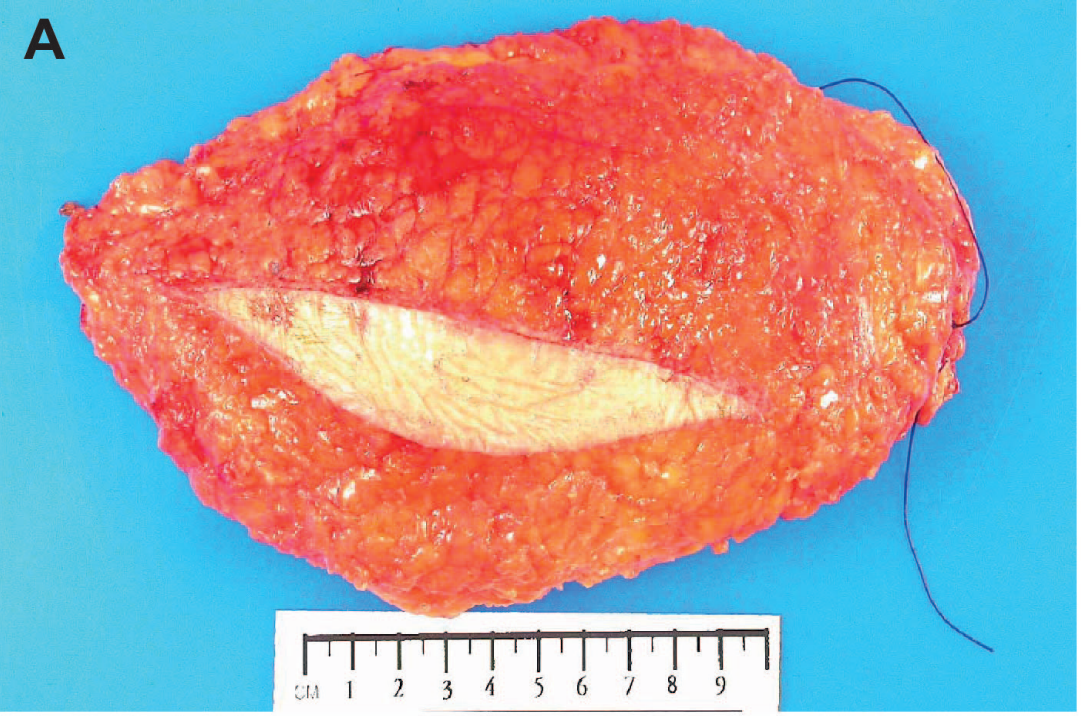
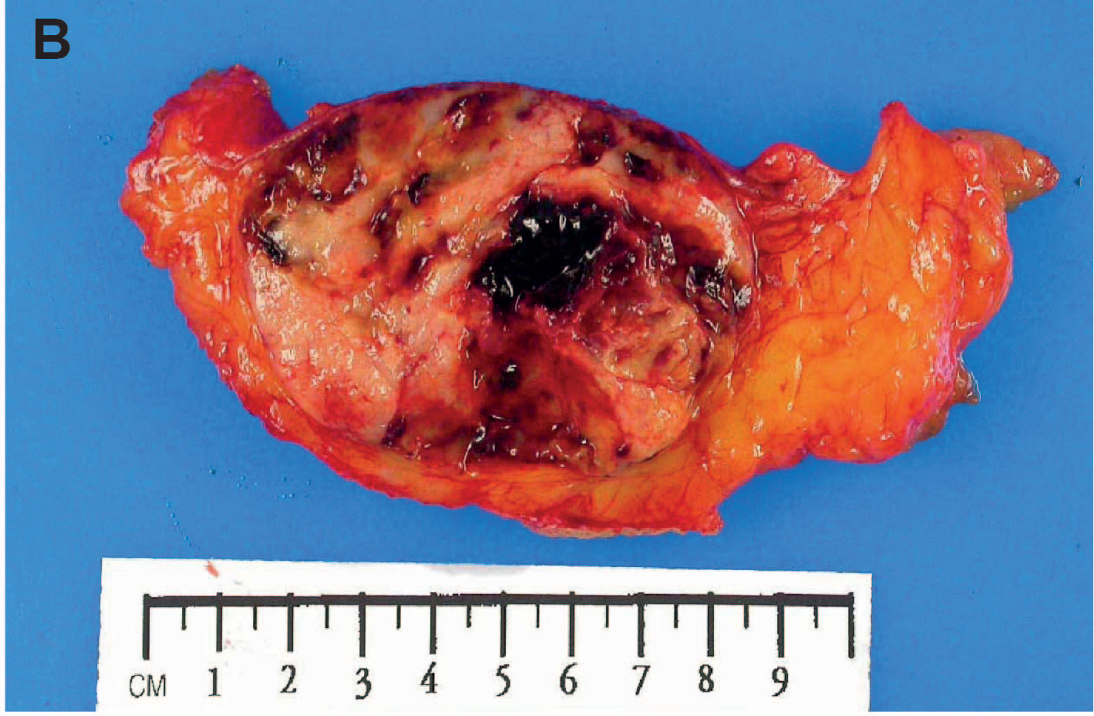
TABLES

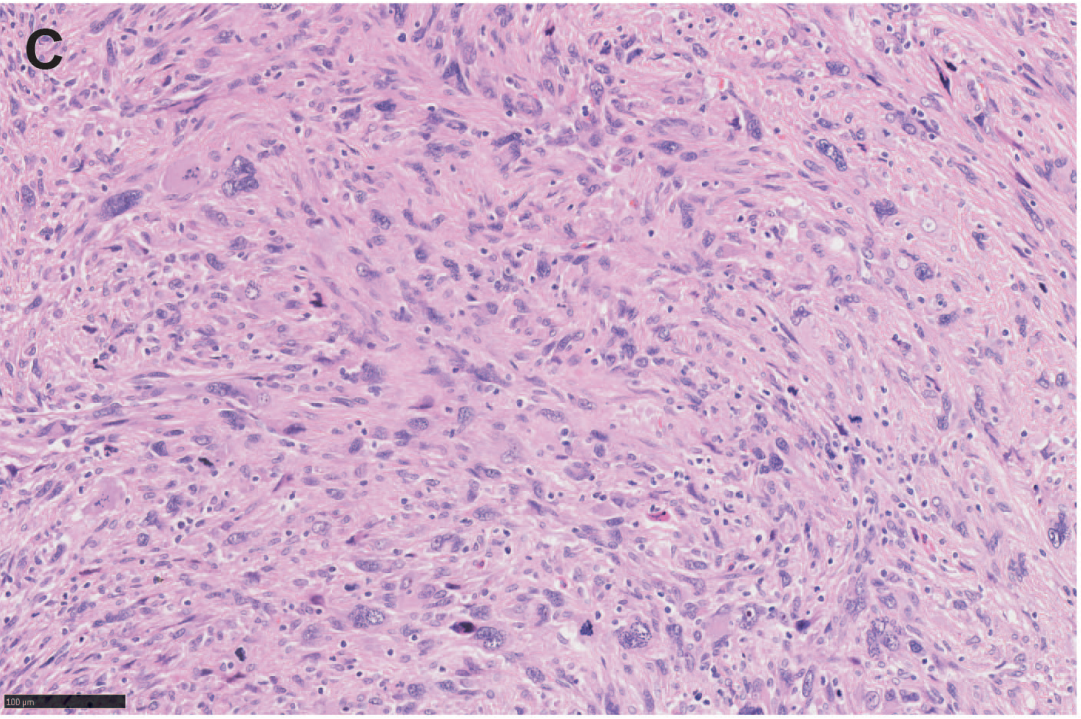
Diagnosis	Pathological /histological features	Immunohistochemistry	Genetics
Poorly differentiated or spindle cell carcinoma	Associated with a well differentiated carcinoma component.	Cytokeratin expression, p63, p40 EMA	
Melanoma	Wide ranging histological patterns and can mimic many other cancer types.	S100, HMB45, Melan A, MITF	*BRAFV600E* mutations, *MITF* amplifications, high mutational load, Ultraviolet light mutational signature.
Malignant peripheral nerve sheath tumour	In a typical case – alternating hyper and hypocellular areas, geographic necrosis, nuclear palisading, whorls. Association with a nerve. Setting of neurofibromatosis.	S100 (positive in 50% of cases). H3K27me3 loss, scattered desmin positivity in Triton variant. Loss of neurofibromin.	*NF1* and, PRC2 complex mutations
Pleomorphic leiomyosarcoma	Smooth muscle differentiation. Association with blood vessel wall in rare cases.	Smooth muscle actin, h-caldesmon, desmin	
Pleomorphic liposarcoma	Sometimes requires extensive tissue sampling to identify pleomorphic lipoblasts.		
Dedifferentiated liposarcoma	High index of suspicion if tumour is retroperitoneal. A well differentiated lipomatous component or previous history	MDM2, CDK4 staining.	*MDM2* or *CDK4* gene amplification.

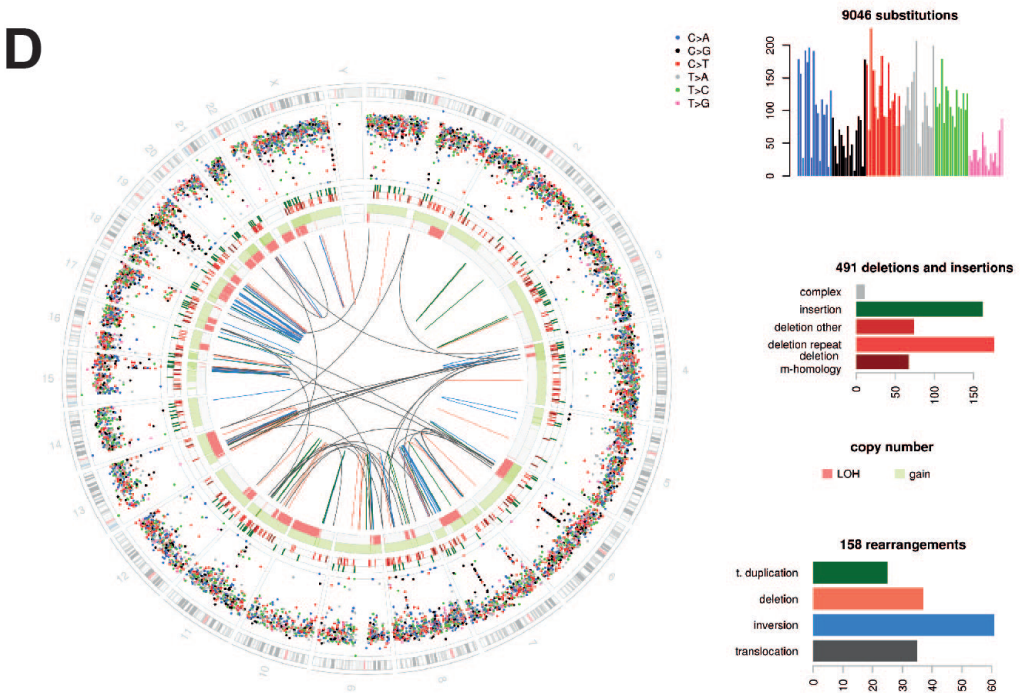
	may be informative. Can be non-lipogenic.		
Pleomorphic rhabdomyosarcoma		Desmin, myogenin	
Myxofibrosarcoma	Myxoid stroma, multilobulated growth, vascularity		
Spindle cell sarcomas (paediatric)	Usually monomorphic histology	Focal and patchy smooth muscle actin	*NTRK* and *BRAF* fusions

Table 1. Overview of histological mimics of USTS and the criteria that can be used to distinguish them.


A
CM 1 2 3 4 5 6 7 8 9

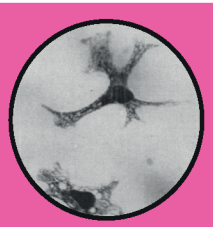

B
CM 1 2 3 4 5 6 7 8 9


C
100 µm


D
C>A
C>G
C>T
T>A
T>C
T>G
9046 substitutions
0
50
100
150
200
491 deletions and insertions
complex
insertion
deletion other
deletion repeat
deletion
m-homology
0
50
100
150
copy number
LOH
gain
158 rearrangements
t. duplication
deletion
inversion
translocation
0
10
20
30
40
50
60

The evolving classification of undifferentiated sarcomas

1960s



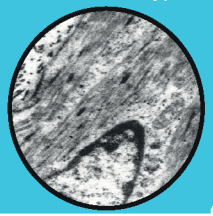
MFH introduced by Stout based on "histiocytic" properties in cell culture.

Malignant fibrous histiocytoma (MFH)

1970s

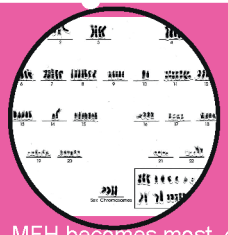
MFH or fibroxanthosarcoma

MFH usage enters pathology lexicon. Proposal that a subset of MFH should be renamed fibroxanthosarcoma based on histology.



Controversy as Fletcher puts out landmark study disputing nature of MFH and proposes that most cases are pleomorphic subtypes of other sarcomas.

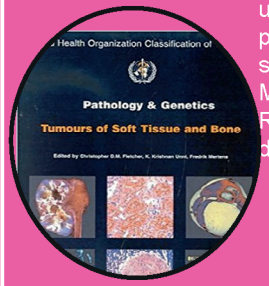
Coincides with widespread use of electron microscopy, immuno-histochemistry and cytogenetics.



MFH becomes most common sarcoma entity. Five variants: myxoid, giant cell,inflammatory, angiomatoid and pleomorphic. Complex karyotype.

Is MFH a distinct entity?

1980s



1990s

MFH is overdiagnosed

2006

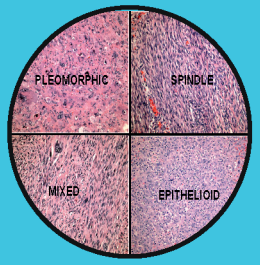
Pleomorphic MFH is reclassified as undifferentiated pleomorphic sarcoma (UPS) but MFH term is retained. Regarded as a diagnosis of exclusion.

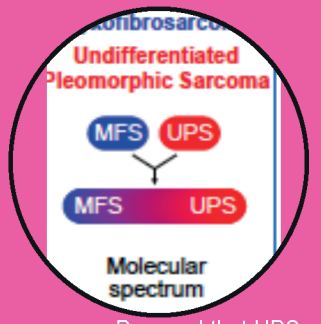
W.H.O classification is revised

2013

MFH is removed from WHO classification

Four variants of undifferentiated sarcoma are described: Spindle,pleomorphic, epithelioid and mixed types.





Proposal that UPS and myxofibrosarcoma are different ends of a molecular spectrum based on exome and RNA-seq data.

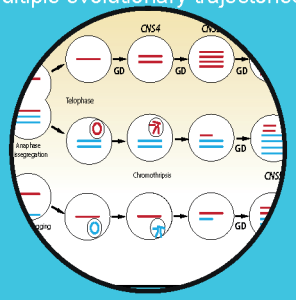
TCGA study of sarcoma

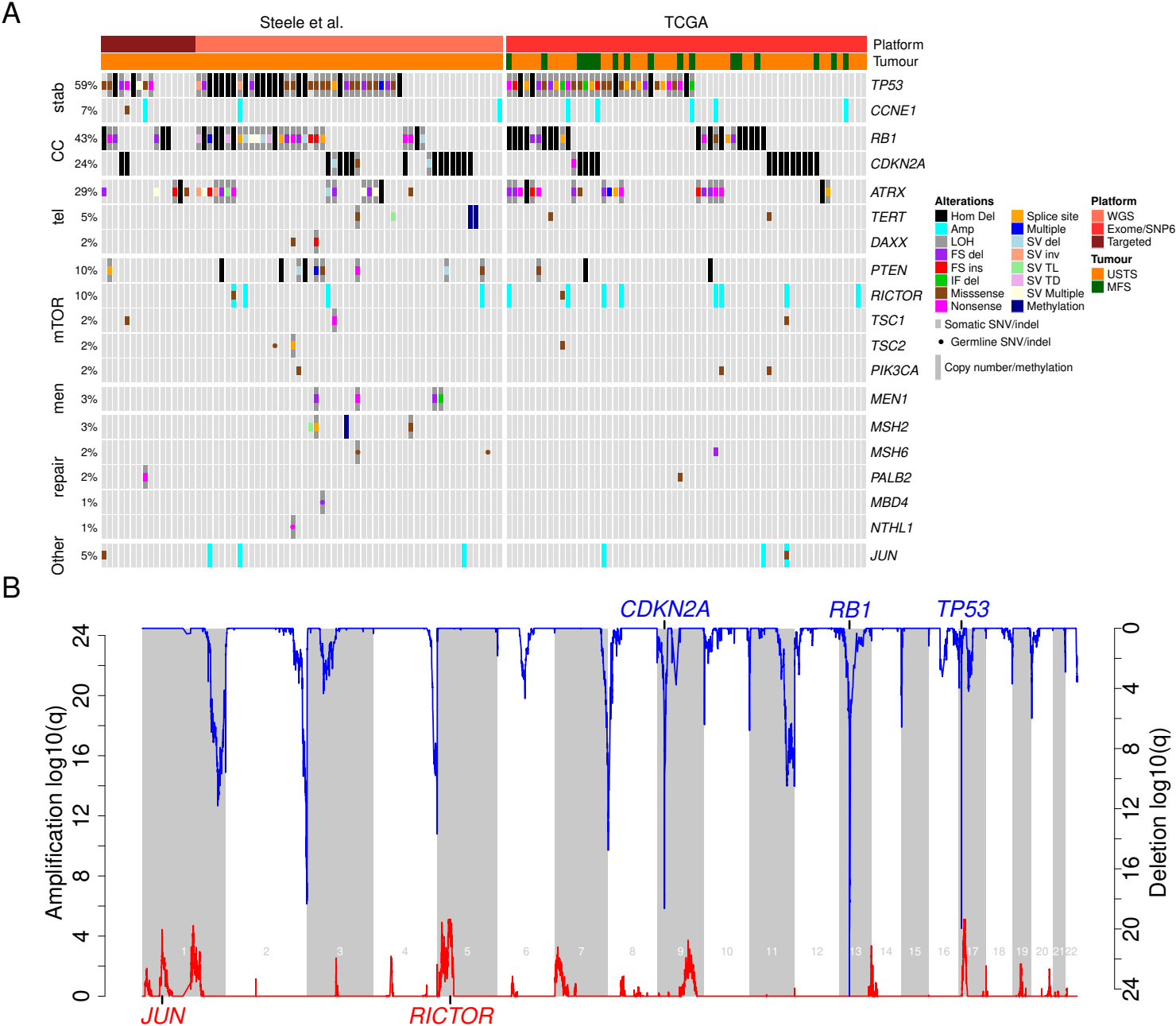
2017

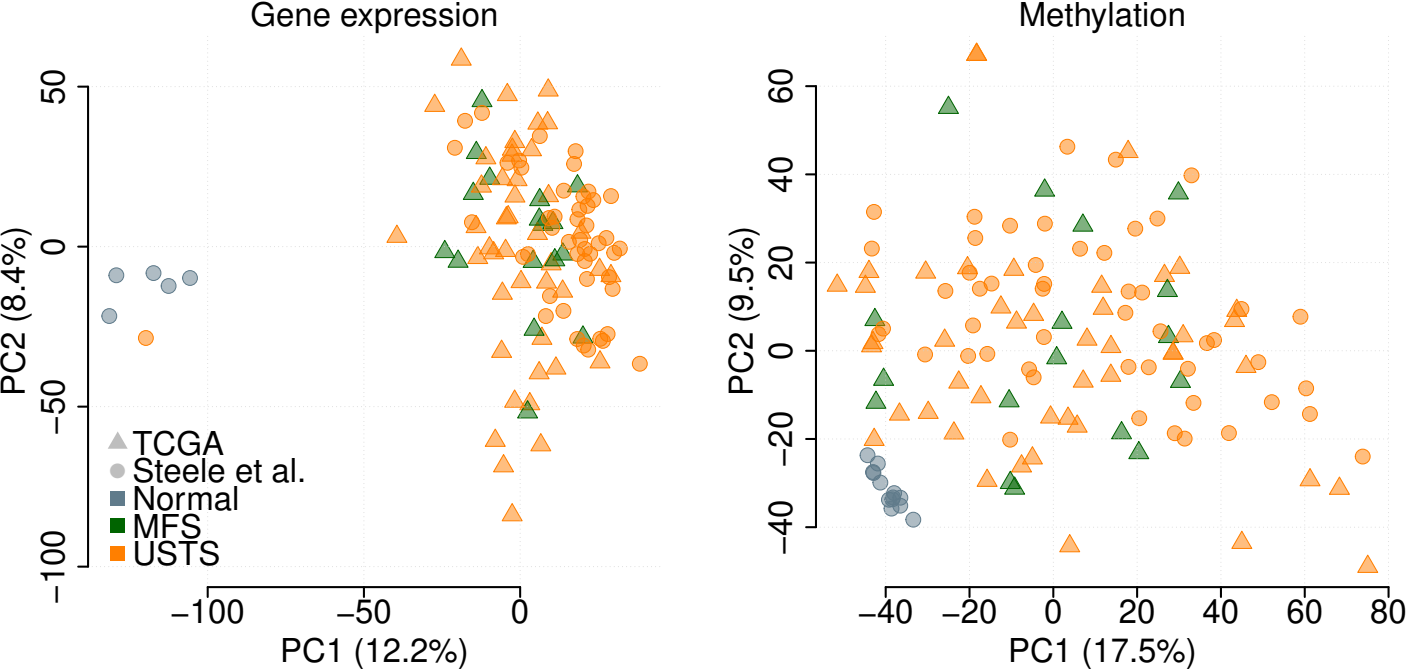
2019

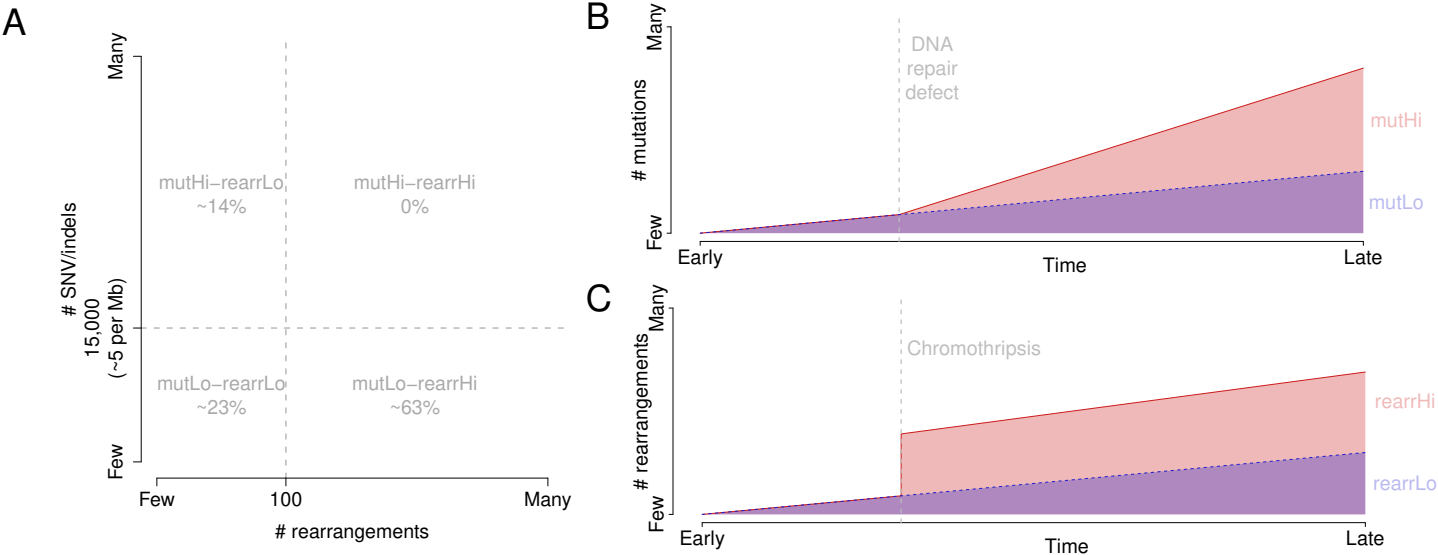
First whole genome study of undifferentiated sarcoma.**(USARC)**

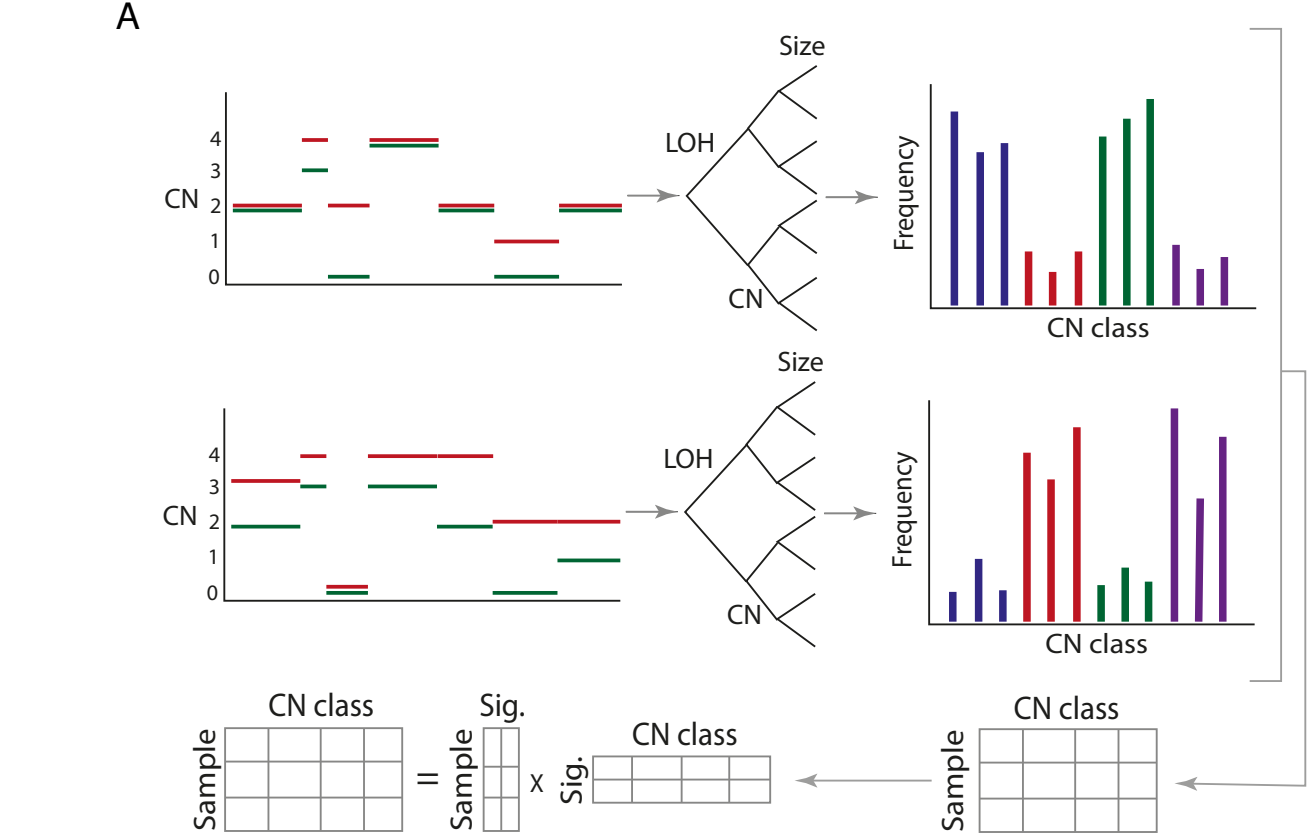
Clinically relevant molecular classification. Copy number signatures to deconvolute complex karyotype. Multiple evolutionary trajectories identified.

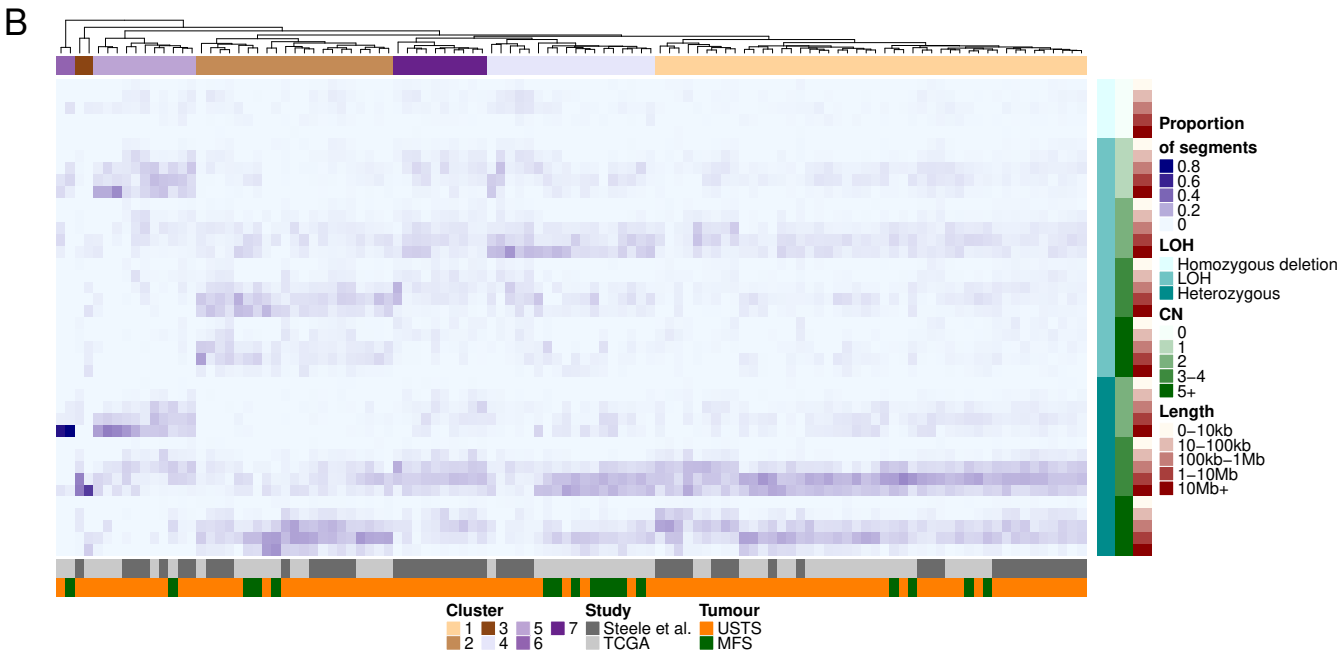



A
Steele et al.
TCGA
Platform
Tumour
stab
CC
tel
mTOR
men
repair
Other
59%
TP53
7%
CCNE1
43%
RB1
24%
CDKN2A
29%
ATRX
5%
TERT
2%
DAXX
10%
PTEN
10%
RICTOR
2%
TSC1
2%
TSC2
2%
PIK3CA
3%
MEN1
3%
MSH2
2%
MSH6
2%
PALB2
1%
MBD4
1%
NTHL1
5%
JUN
Alterations
Hom Del
Amp
LOH
FS del
FS ins
IF del
Misssense
Nonsense
Splice site
Multiple
SV del
SV inv
SV TL
SV TD
SV Multiple
Methylation
Somatic SNV/indel
Germline SNV/indel
Copy number/methylation
Platform
WGS
Exome/SNP6
Targeted
Tumour
USTS
MFS
B
CDKN2A
RB1
TP53
Amplification log10(q)
Deletion log10(q)
0
4
8
12
16
20
24
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
JUN
RICTOR


Gene expression
Methylation
PC2 (8.4%)
PC1 (12.2%)
PC2 (9.5%)
PC1 (17.5%)
TCGA
Steele et al.
Normal
MFS
USTS
50
0
−50
−100
60
40
20
0
−20
−40
−100
−50
0
−40
−20
0
20
40
60
80

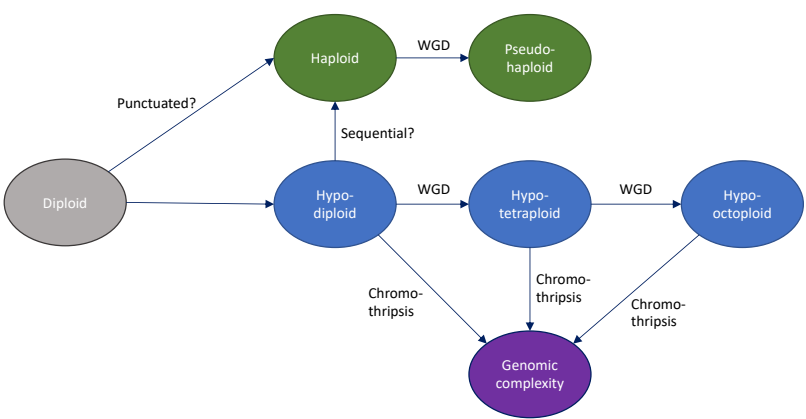

A
Many
SNV/indels
15,000
(~5 per Mb)
Few
mutHi–rearrLo
~14%
mutHi–rearrHi
0%
mutLo–rearrLo
~23%
mutLo–rearrHi
~63%
Few
100
Many
rearrangements
B
Many
mutations
Few
DNA
repair
defect
mutHi
mutLo
Early
Time
Late
C
Many
rearrangements
Few
Chromothripsis
rearrHi
rearrLo
Early
Time
Late





C

Copy number signature	Description	Associations	Cluster
CNS1	Hypo-octoploid	2×WGD; TP53 mutant	2
CNS2	Hypo-tetraploid		1
CNS3	Hypo-diploid		5
CNS4	Neutral LOH	TMB; PTEN mutant	4
CNS5	Small amplifications	Chromothripsis	
CNS6	Complex		7
CNS7	Tetraploid		3



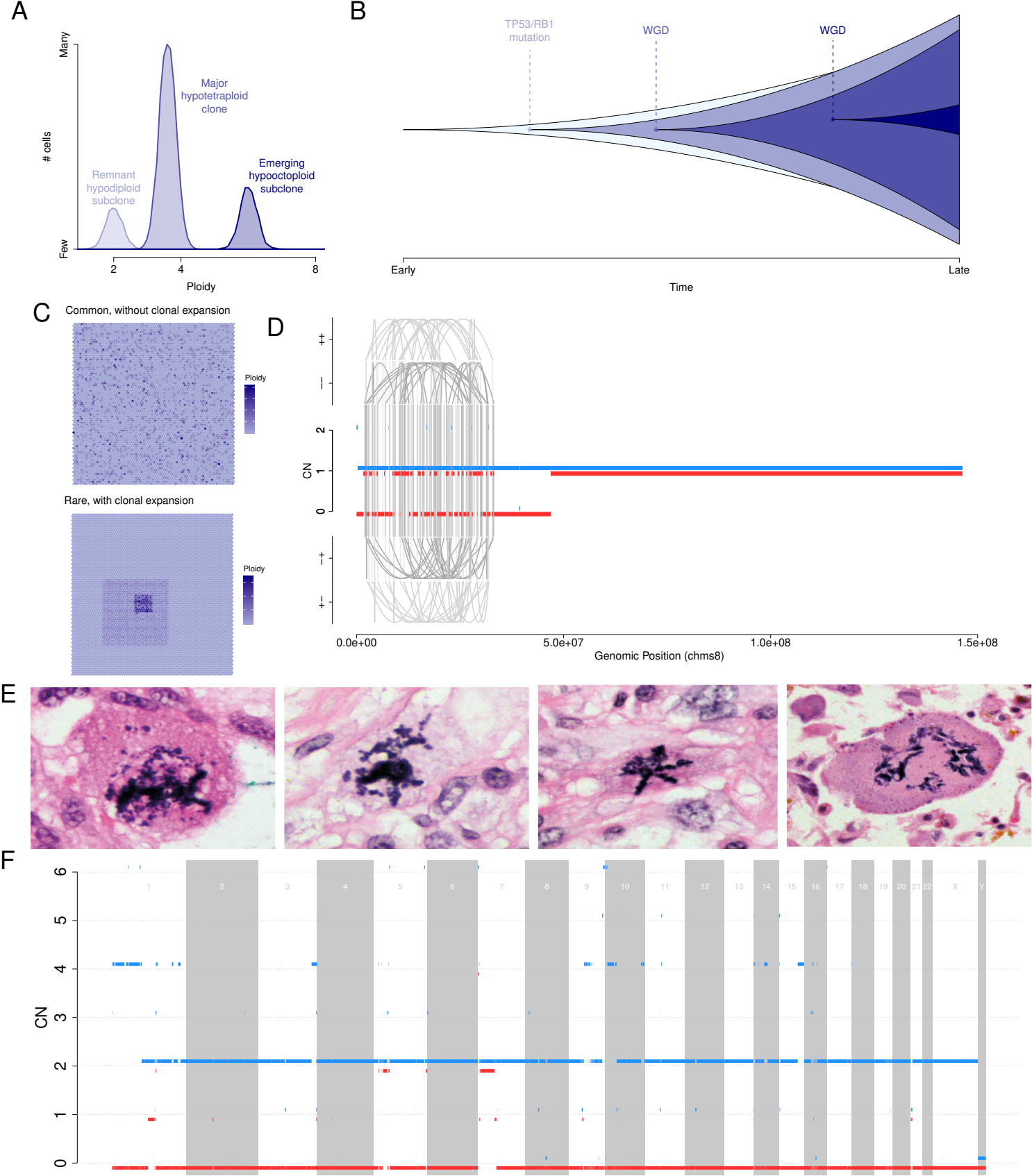

A
Many
cells
Few
Major
hypotetraploid
clone
Remnant
hypodiploid
subclone
Emerging
hypooctoploid
subclone
2
4
8
Ploidy
B
TP53/RB1
mutation
WGD
WGD
Early
Late
Time
C
Common, without clonal expansion
Ploidy
Rare, with clonal expansion
Ploidy
D
++
+−
CN
2
1
0
−+
−−
0.0e+00
5.0e+07
1.0e+08
1.5e+08
Genomic Position (chms8)
E
F
CN
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y

Figure Legends

Figure 1: Pathology and genetics of USTS

A. Macroscopic specimen of resected USTS in a superficial (above fascial) soft tissue location. Small ellipse of skin is preserved on the surface and suture is present to aid the surgical orientation of the specimen.
B. Cut section of tumour showing a variegated appearance with haemorrhagic and necrotic foci interspersed between fleshy cream areas of solid tumour.
C. Typical histological appearance showing irregular fascicles of cytologically atypical pleomorphic and spindle cells in a fibrous stroma. Scalebar=250μm
D. Circos plot visualisation of a whole genome sequence. The outer ring represents positions on the different chromosomes (1-22) and the sex chromosomes (X,Y). The subsequent rings, in order from outer to inner, comprise representations of the somatic mutations: single nucleotide variants, insertions and deletions, copy number changes, rearrangements and their genomic locations. The barplots represent the counts of the various mutational classes across the genome.

Figure 2: Historical timeline of major changes in nomenclature, classification and advances in biological understanding of USTS

MFH=malignancy fibrous histiocytoma, UPS=undifferentiated pleomorphic sarcoma, WHO=World Health Organisation,MFS=myxofibrosarcoma,USARC=undifferentiated sarcoma

Figure 3. USTS driver genes and pathways. A) Driver single nucleotide variants/indels, structural variants, and copy number alterations from two studies of USTS. Driver genes are organised into pathways; stab=DNA stability, CC=cell cycle, tel=telomere maintenance, mTOR=mTOR signalling, men=MENIN pathway, repair=DNA repair. Copy number alterations are shown as full blocks and are derived from ASCAT copy number calls for both TCGA (Reference 13) and Steele et al. (Reference 12) data; Hom Del=homozygous deletion, Amp=amplification, LOH=loss of heterozygosity. SNVs and indels are shown as small rectangles for somatic variants and circles for germline variants; 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. B) Recurrent copy number alterations for combined copy number profiles. Log10(q) is shown across the genome for deletions (blue) and amplifications (red), with key driver genes labelled.

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