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Deciphering WNT Signalling Vulnerabilities in Soft Tissue Sarcoma

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Soft tissue sarcoma · WNT signalling · Primary culture · β-catenin · Personalized treatment

Abstract
Introduction: Soft tissue sarcomas (STSs) are a group of rare malignancies with limited treatment options and a persistent lack of effective therapies. Despite the heterogeneous nature of STS, the canonical WNT/β-catenin signalling pathway has been associated with sarcomagenesis and also with the initiation and progression of other cancers. ***Methods:*** Eight patient-derived primary cultures representing different STS histological subtypes were characterized by immunohisto(cyto)chemistry, WNT/β-catenin activation and next-generation sequencing. ***Results:*** Elevated levels of active phospho-β-catenin and its nuclear localization were found in all STS primary cultures, with heterogeneous downstream WNT signalling activation. Genomic analysis of matched STS tumours identified pathogenic or likely pathogenic genetic variants in crucial signalling pathways, including WNT, DNA damage repair, and PI3K/AKT/mTOR-MAPK pathways. Interestingly, 50% of STS tumours studied carried genetic variants in *PIK3CA* and *PTEN* genes with potential clinical significance, listed as predictive biomarkers of response to specific drugs (FDA level 3). ***Conclusions:*** This study provides valuable insights into WNT signalling vulnerabilities in STS, offering a foundation for the development of targeted therapeutic strategies and the identification of potential biomarkers for personalized treatment approaches in this challenging group of malignancies.

Introduction

Soft tissue sarcomas (STSs) are rare malignant tumours of mesenchymal origin with over 70 histological subtypes, comprising less than 1% of all adult cancer [1, 2]. Despite increased incidence since the 1990s, STS mortality rates have remained unchanged, indicating a lack of effective treatments [3]. Currently, surgery is the mainstay of localized STS treatment, accompanied by radiotherapy or/and chemotherapy [4]. Although only 15% STS present metastasis at diagnosis, distant relapses are very frequent in high-grade tumours [5, 6]. For at least

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40 years now, doxorubicin still remains the standard first-line treatment for advanced STS [4, 7]. STS heterogeneity [1] and genomic and molecular complexity [8–10] represent big challenges to develop personalized STS treatment. Identifying biomarkers that reflect functional tumour alterations is a crucial need to guide future therapeutic strategies in STS.

The canonical WNT/β-catenin signalling pathway is an evolutionarily conserved pathway involved in cell cycle regulation, cellular proliferation, and differentiation [11, 12]. Aberrant activation of this pathway has been linked to cancer initiation and progression [13–15]. In some adenocarcinomas, WNT hyperactivation is often due to mutations in key regulators of the pathway, such as *APC*, *CTNNB1* (β-catenin), *AXIN1* or *AXIN2* [15–21]. Although aberrant activation of the WNT/β-catenin pathway has been demonstrated in STS [22–24], it is not typically linked to these mutations. Although studies on STS are scarce, two clinical trials are evaluating WNT signalling inhibition in STS patients [25, 26]. In this line, our group has demonstrated that combining doxorubicin with the WNT inhibitor foscenvivint (PRI-724) enhances treatment efficacy in STS cell lines [23, 24].

Most STS studies focused on the WNT signalling pathway use human immortalized cell lines [22, 23, 27], which may not accurately represent original tumour genetics. Thus, using patient-derived primary STS cultures can improve translation of pre-clinical findings. This study aimed to generate a collection of patient-derived STS cultures to analyse genetic alterations and WNT/β-catenin pathway activity, in order to contribute to the development of novel therapeutic strategies for STS.

Methods

Primary Tumour Samples

Patients from Son Espases University Hospital (Palma de Mallorca, Spain) with STS suspicion or previously biopsy-confirmed STS who underwent surgical resection of primary, relapsed, or metastatic tumours were eligible. Fresh tumour samples were provided by the IdISBa Biobank immediately after surgical resection. Histological subtypes and tumour grades were confirmed by an expert pathologist in this field, according to the latest World Health Organization (WHO) 5th edition classification from 2020 [28] and the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) histological grading system [29]. Further details on establishment of tumour-derived primary cultures and cell lines are listed in the online supplementary methods and online supplementary Figure 1 (for all online suppl. material, see https://doi.org/10.1159/000544933).

Immunohisto(cyto)chemistry

The detection of subtype-dependent STS markers was carried out at the Pathology Department of Son Espases University Hospital using the ultraView Universal DAB Detection Kit and the automated system Ventana® NexES. Primary antibodies for caldesmon (anti-caldesmon E89), desmin (anti-desmin DE-R-11), α-smooth muscle actin (anti-α-SMA 1A4), cytokeratin 7 (anti-CK7 SP52), epithelial membrane antigen (anti-EMA E29) and CD34 (anti-CD34 QBEnd/10) from Ventana Medical Systems, Inc. were used. Further details and negative controls for the staining are provided in online supplementary Figure 2.

Western Blotting

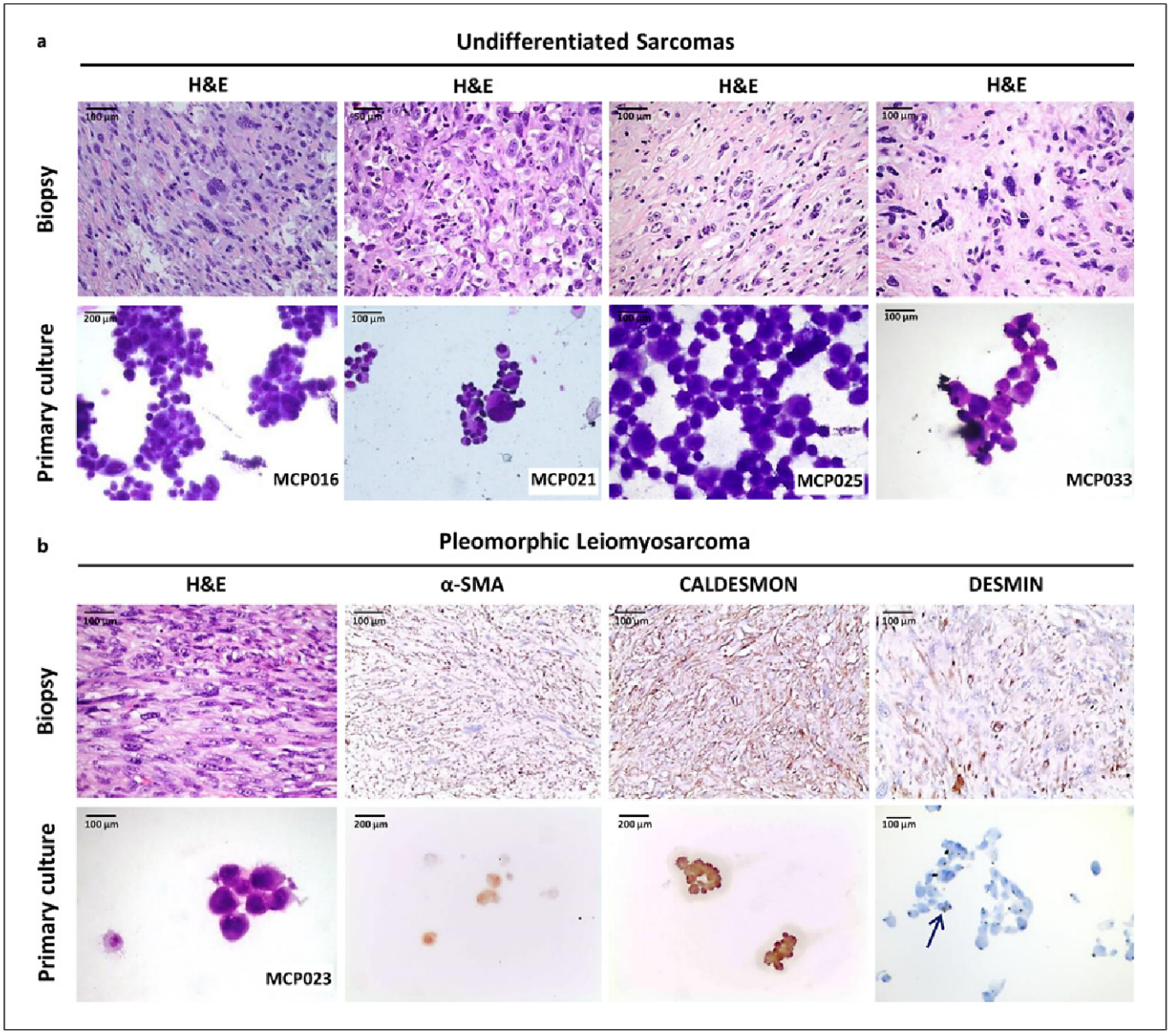
Whole-cell extracts were prepared by lysing cells with a Hielscher UP50H sonicator (Hielscher Ultrasonics GmbH, Teltow, Germany) using a lysis buffer [23]. Nuclear and cytoplasmic fractions were obtained using Nuclear Extract Kit (Active Motif, Waterloo, Belgium) and following the manufacturer's instructions. Primary and secondary antibodies used and corresponding dilutions can be found in online supplementary Table 1. The intensity of band immunoreactivity was analysed and quantified with the Odyssey CLx Imaging System and Image Studio™ from LI-COR.

Immunofluorescence

DAPI (Thermo Scientific™) was used to visualize the nuclei. Four representative images per well were taken using the confocal microscope, ZEISS LSM 710 (Carl Zeiss, Germany), with a ×40 objective and power of 512x512 pixels. ZEN Imaging software (Black edition) was used to quantify β-catenin staining intensity. Briefly, mean intensity values of the β-catenin channel in the nuclei (areas with DAPI and β-catenin colocalization) were analysed. Detailed information on primary and secondary antibodies can be found in online supplementary methods and online supplementary Table 1.

Transfection and Luciferase Reporter Gene Assay

WNT/β-catenin transcriptional activity was assessed using Dual Luciferase® Reporter Assay Kit (Promega, Madison, WI, USA), based on TOPflash/FOPflash reporter plasmid system, as described elsewhere [24].



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Quantitative Real-Time PCR

64 ng of RNA were reverse-transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Subsequent real-time PCR reactions were run on a CFX96 Real-Time System, C1000 Thermal Cycler (BIO-RAD) using the following TaqMan® probes: *CDC25A* (Hs00947994), *C-MYC* (Hs00153408), *CUL4A* (Hs00757716), and *CCND1* (Hs00765553) (Applied Biosystems). Relative gene expression was normalized to *β-2 microglobulin* (Hs99999907) using the 2-ΔΔCt method [30].

Flow Cytometry

Samples were incubated with fluorescence-conjugated antibodies APC/Cyanine7 anti-human CD45 (BioLegend, San Diego, CA, USA) and PE mouse anti-human CD140a (BD Biosciences, United States) and measured on a flow cytometer using the BD system Verse BD FACScan (Coulter Epics XL-MCL; Beckman Coulter, Fullerton, CA, USA) and FACSuit software (BD Bioscience, Franklin Lakes, NJ, USA). All further details on staining and gating strategy for the

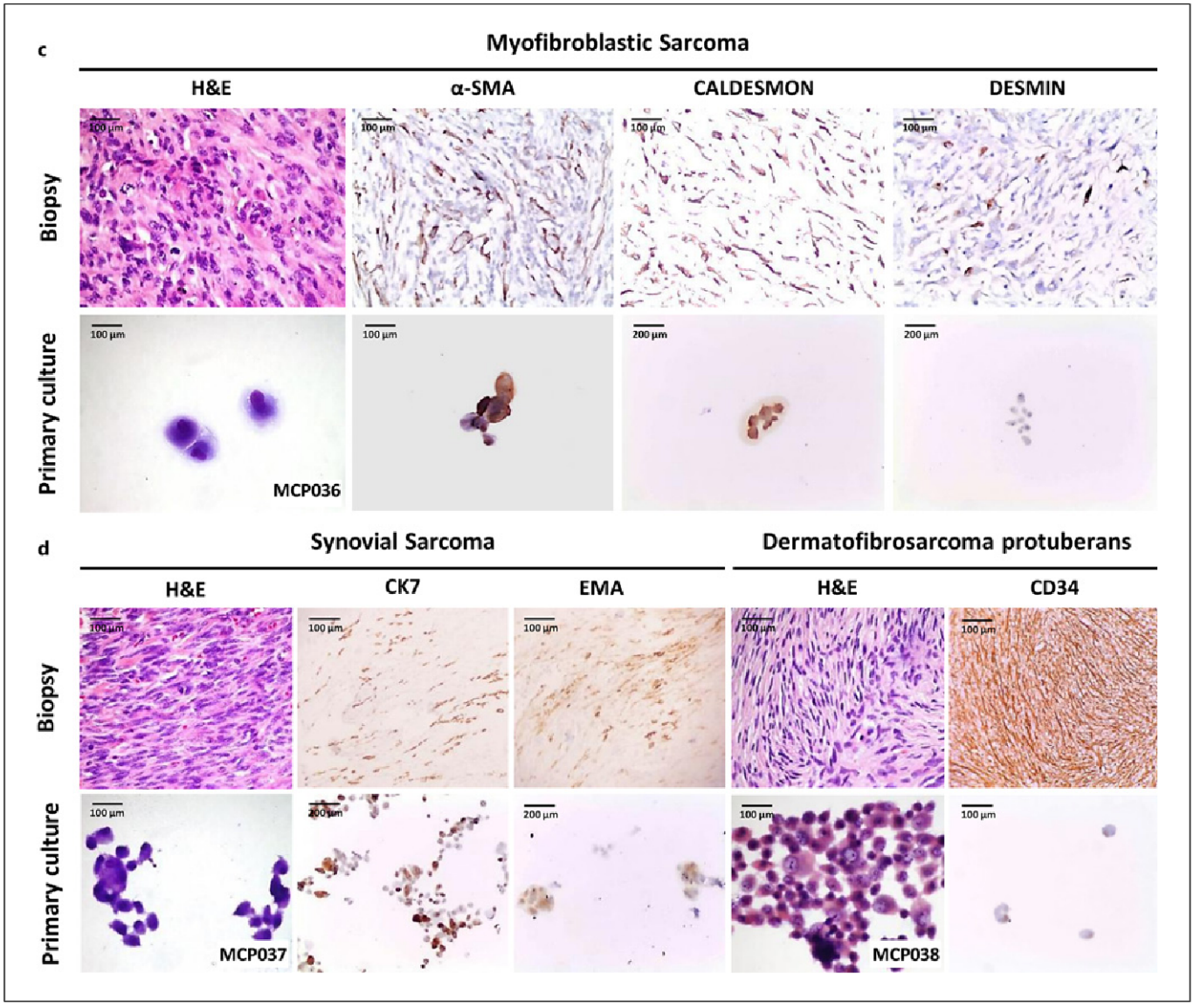


Fig. 1. a H&E-stained tumour biopsies of the four undifferentiated sarcoma cases alongside their matched Diff-Quik-stained primary cell cultures (MCP016, MCP021, MCP025, and MCP033 from left to right). Histologically, these neoplasms exhibit proliferation of markedly atypical cells with significant size and shape variability, diverse cellular morphologies including pleomorphic cells, multinucleated cells, spindle-shaped cells, or cells with intranuclear inclusions. These cells display eosinophilic cytoplasm, patternless arrangement, with abundant aberrant mitotic figures, associated inflammatory cellular infiltrates, and foci of necrosis (not visible in the image). **b** Histopathological staining of pleomorphic leiomyosarcoma biopsy and matched primary culture MCP023. Tumour biopsy and MCP023 cells showed α-SMA and caldesmon positivity. Desmin was focal positive in the biopsy and negative in MCP023 cells. **c** Histopathological staining of myofibroblastic sarcoma biopsy and matched primary culture MCP036. Biopsy and MCP036 cells showed α-SMA and caldesmon positivity. Desmin was focal positive in the biopsy and negative in MCP036 cells. **d** On the left, histopathological staining of synovial sarcoma biopsy and matched primary culture MCP037. Positivity for epithelial markers CK7 and EMA in both tissue biopsy and MCP037 cells confirmed a compatible diagnosis with synovial sarcoma. On the right, histopathological staining of dermatofibrosarcoma biopsy and matched primary culture MCP038. Both showed immunoreactivity for CD34. Images were taken with the Olympus BX41 microscope (DP74 camera). Upper micrographs are magnified ×200 or ×400 and lower micrographs are magnified ×100 or ×200 in **a**, **b**, **c**, and **d**. α-SMA, α-smooth muscle actin; CK7, cytokeratin 7; EMA, epithelial membrane antigen.

Table 1. Information related to primary cell cultures obtained from STS patient tumours

Primary culture	STS subtype	Histological grade	Tumour source	Tumour site	Age/ sex	Neoadjuvant treatment	Recurrence	Patient status
MCP016	Undifferentiated pleomorphic sarcoma	High	Primary	Lower limb	59/F	EPI/IFO 3x	No	Alive
MCP021	Undifferentiated pleomorphic sarcoma	High	Metastasis	Inguinal lymph node	76/ M	CIS/DXR 2x MTX 2x	Yes	Exitus
MCP023	Pleomorphic leiomyosarcoma	High	Primary	Upper limb	56/ M	No	No	Alive
MCP025	Undifferentiated pleomorphic sarcoma	High	Primary	Pelvis	49/ M	No	No	Alive
MCP033	Undifferentiated sarcoma fibrous histiocytoma-like	Intermediate	Primary	Lower limb	53/ M	No	Yes	Alive
MCP036	Myofibroblastic sarcoma	Intermediate with high-grade areas	Primary	Lower limb	71/ M	No	Yes	Exitus
MCP037	Monophasic synovial sarcoma	Intermediate-high	Primary	Lower limb	34/ M	EPI/IFO 3x	No	Alive
MCP038	Dermatofibrosarcoma protuberans	Intermediate	Primary	Abdomen	40/F	No	No	Alive

F, female; M, male; EPI/IFO, epirubicin/ifosfamide; x, cycles of chemotherapy; CIS/DXR, cisplatin/doxorubicin; MTX, methotrexate.

detection of the surface markers are provided in online supplementary methods and online supplementary Figure 3.

Genomic DNA Isolation and Targeted Sequencing

Tumour tissue sections were selected by an expert pathologist. Genomic DNA was isolated and purified from 3 freshly cut sections of FFPE tumour tissues, with a thickness of 10 μm, using GeneRead™ DNA FFPE Kit (QIAGEN, Germany) according to the manufacturer's instructions. Library construction and target enrichment was performed using components from QIAseq Targeted DNA Panels (QIAGEN) and following manufacturer's instructions. Libraries were sequenced on NextSeq 500 high-output platform (Illumina) with 800 M paired-end reads and 2500x mean coverage (based on 2 × 151 bp paired-end read) following the manufacturer's user manual. A detailed list of the analysed genes and more information can be found in online supplementary methods and online supplementary Table 2.

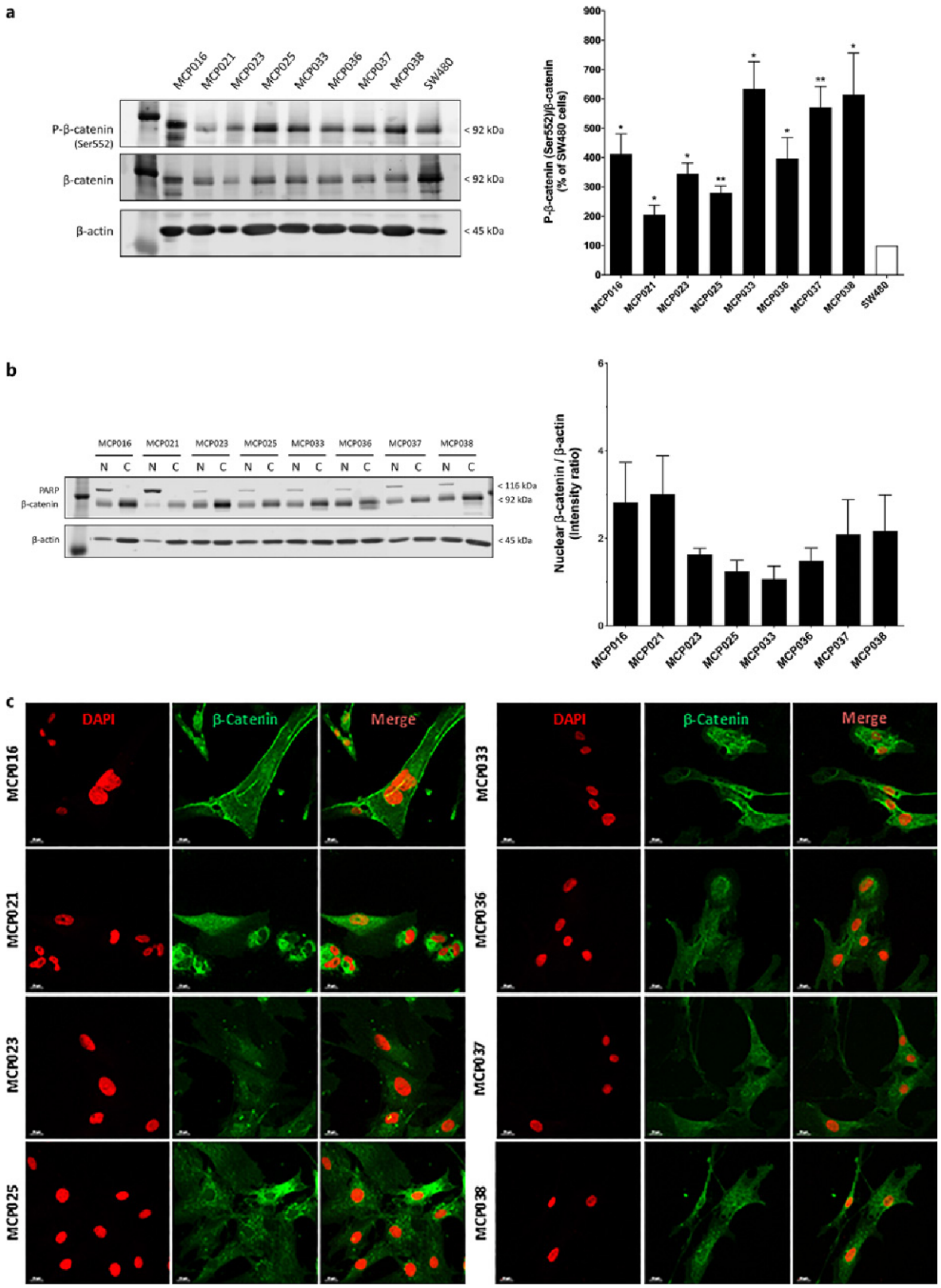
Statistical Analysis

All data are expressed as the mean ± SEM from n independent experiments performed in duplicate or triplicate. One-way analysis of variance followed by Dunnett's multiple comparison test was used for statistical evaluations. Differences were considered statistically significant at $p < 0.05$ and were indicated by: $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$. All calculations were done by using GraphPad Prism 8 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

STS Patient-Derived Primary Cultures Characterization

The neoplastic origin of cultured cells was confirmed by comparing histopathological features and immunohistochemical STS markers between tumour biopsies and their matched primary cultures. In both undifferentiated sarcoma biopsies and STS-derived primary cultures, neoplastic cells showed pleomorphism, anisocytosis, anisokaryosis, and multiple nuclei with large nucleoli (shown in Fig. 1a). Two cell types could be distinguished only in MCP021 staining, multinucleated giant cells, and smaller single-nucleus cells, suggestive of being leukocytes because of their size and round well-delimited nucleus without nucleoli [31]. Curiously, the origin of MCP021 is a metastatic tumour sample coming from an



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inguinal lymph node (Table 1), which could explain the presence of an immune cell population in this primary culture. Furthermore, flow cytometric analysis was conducted to evaluate the purity of the established patient-derived primary cultures and potential cross-contamination with other cell populations, which is a common occurrence in primary cultures [32]. The presence of CD45, a pan-leucocyte marker, and CD140a (PDGFRα), a cell surface marker typically associated with fibroblasts and cells of similar origin, was evaluated in primary cell cultures MCP016, MCP021, and MCP033 (online suppl. Fig. 3). This assessment was conducted as these cellular subpopulations are commonly observed in early patient-derived cell cultures. The MCP016 cells at passage 16 were found to be $CD45^-/CD140a^-$, indicating a pure cell culture. In contrast, MCP021 at passage 20 exhibited a predominant population of $CD45^+/CD140a^-$ cells (92%), substantiating the presence of leukocytes in this myxoid culture, and a minor population of $CD45^-/CD140a^-$ tumour cells (5%). In the case of the MCP033 culture, 69% of the cells were $CD45^-/CD140a^-$, while the remaining 31% were $CD45^-/CD140a^+$, indicating the presence of fibroblastic cells. Accordingly, the MCP033 culture was derived from an undifferentiated sarcoma fibrous histiocytoma-like tumour (Table 1).

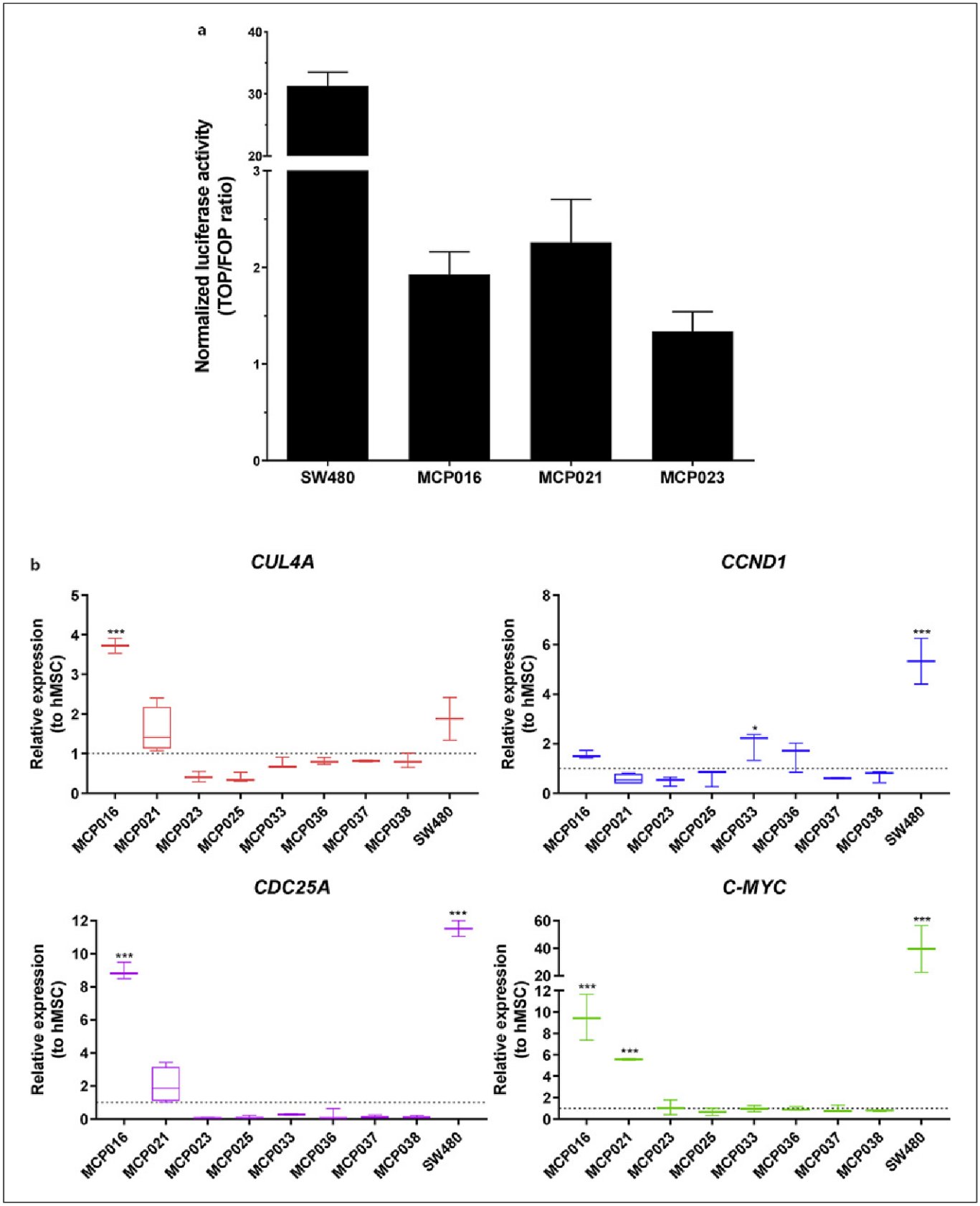
The rest of STS primary cultures also shared common histopathological features with the parental tumour tissues. Other characteristics of patients, tumour samples, and their matched primary cell cultures are collected in Table 1. Besides, expression of α-smooth muscle actin (α-SMA), a specific myofibroblast marker, caldesmon, a smooth muscle differentiation marker, and desmin, a smooth and striated muscle cell marker, were assessed in MCP023 and MCP036 cells by immunocytochemistry. Primary cells were positive for α-SMA and caldesmon as were their respective biopsies and negative for desmin unlike their biopsies that stained focal positive (shown in Fig. 1b,c). The focal positivity for desmin observed in biopsy samples suggests that only a subset of cells within the tumour tissue expresses this protein, likely representing the most differentiated cells. These desmin-positive cells may not survive or proliferate efficiently under culture conditions, potentially explaining their absence in immunocytochemistry experiments on primary cell cultures. The combination of epithelial markers CK7 and EMA is highly specific for diagnosing synovial sarcoma [33]. MCP037 cells stained strongly for CK7 and EMA, confirming the similarity between the synovial sarcoma tumour and its derived primary culture (shown in Fig. 1d). Finally, we tested CD34 expression in MCP038 cells as it is the mainly used diagnostic marker for dermatofibrosarcoma protuberans [34] and focal positive immunoreactivity was detected (shown in Fig. 1d).

Furthermore, cellular authentication of the primary cultures with their respective tumours of origin was performed in order to provide a more robust validation of shared genotypical features. The results demonstrated matching short tandem repeat profiles between STS primary cells and paired tumours, with slight karyotype differences mainly manifested by loss of heterozygosity, a phenomenon that normally occurs during cell passages (see online suppl. Table 3).

Assessment of the Functional Status of β-Catenin as the Main Effector of Canonical WNT Signalling Activation

To study the potential oncogenic activity of β-catenin in patient-derived STS primary cultures, we analysed the expression of the active form of β-catenin and its subcellular localization. Protein expression of total β-catenin and its active form phospho-β-catenin (Ser552) were detected in all STS primary cultures (shown in Fig. 2a). The human colorectal carcinoma cell line SW480 with a strong intrinsic WNT signalling activity [23] was used as a positive control. All studied STS primary cultures expressed higher active β-catenin levels than that of SW480 cells. The highest amounts of active β-catenin were found in the undifferentiated sarcoma MCP033, the synovial sarcoma MCP037 and the dermatofibrosarcoma MCP038

Fig. 2. a Panels show the immunoreactive bands of total and phosphorylated β-catenin (Ser552) in STS primary cells in representative immunoblots. β-actin was used as a loading control. Columns represent the phosphorylated-β-catenin ratio. Each column represents mean ± SEM of three independent experiments normalized to SW480 cells (taken as 100%). $^*p < 0.05$ and $^{**}p < 0.01$ versus SW480 cells. **b** Panels show the immunoreactive bands of total β-catenin in representative immunoblots of nuclear and cytoplasmic fractions of STS primary cells. PARP was used as a nuclear marker and β-actin as a loading control. Columns represent nuclear β-catenin normalized to loading control. Each column represents mean ± SEM of three independent experiments. Band immunoreactivity intensities in panels a and b were analysed and quantified with the Odyssey CLx Imaging System and Image Studio™ from LI-COR. **c** Representative immunofluorescence images of subcellular β-catenin localization in STS-derived primary cultures taken with the confocal microscope ZEISS LSM 710 at ×400 magnification. Scale bars 20 μm. Cells were fixed with methanol:acetone (1:1) and then incubated with primary antibody β-catenin (D10A8) (XP Rabbit mAb #8480, Cell Signalling Technology) and with secondary antibody (Alexa Fluor 488 Goat a-R A11008, Invitrogen). DAPI was used to visualize the nuclei.



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primary cultures, being 6.3 ± 0.9-fold increase in active β-catenin compared to control cells the maximum detected levels. On the contrary, MCP021 primary culture expressed lower levels of active β-catenin.

The intracellular localization of β-catenin was analysed by Western blot subcellular fractionation and immunofluorescence. All STS primary cultures showed β-catenin expression in both nuclear and cytosolic fractions (shown in Fig. 2b), also confirmed by immunofluorescence results (shown in Fig. 2c). According to the total β-catenin quantification in the nuclear fractions, the highest nuclear β-catenin levels were found in MCP016, MCP021, MCP037, and MCP038 cultures (shown in Fig. 2b). Immunofluorescence images showed nuclear β-catenin staining in all STS primary cultures (immunofluorescence quantification range: 25–40 intensity/mm^2), with MCP023 (28 intensity/mm^2) and MCP025 (25 intensity/mm^2) showing weaker β-catenin staining (shown in Fig. 2c). Interestingly, 2 β-catenin staining patterns were found in the MCP021 cell culture: strong β-catenin staining in the nucleus of a subpopulation of large, elongated cells, and weak or absent nuclear β-catenin staining in a subpopulation of round cells (shown in Fig. 2c). These round cells were initially postulated to be leukocytes based on their size and round, well-defined nuclei without nucleoli (online suppl. Fig. 1, 3). Taken together, these results demonstrate that STS primary cultures express high levels of active β-catenin and that this protein is also present in the nucleus, where it can trigger transcriptional activation of several signalling pathways, such as the canonical WNT signalling pathway.

Canonical WNT Signalling Pathway Transcription Is Activated in Some STS Primary Cultures

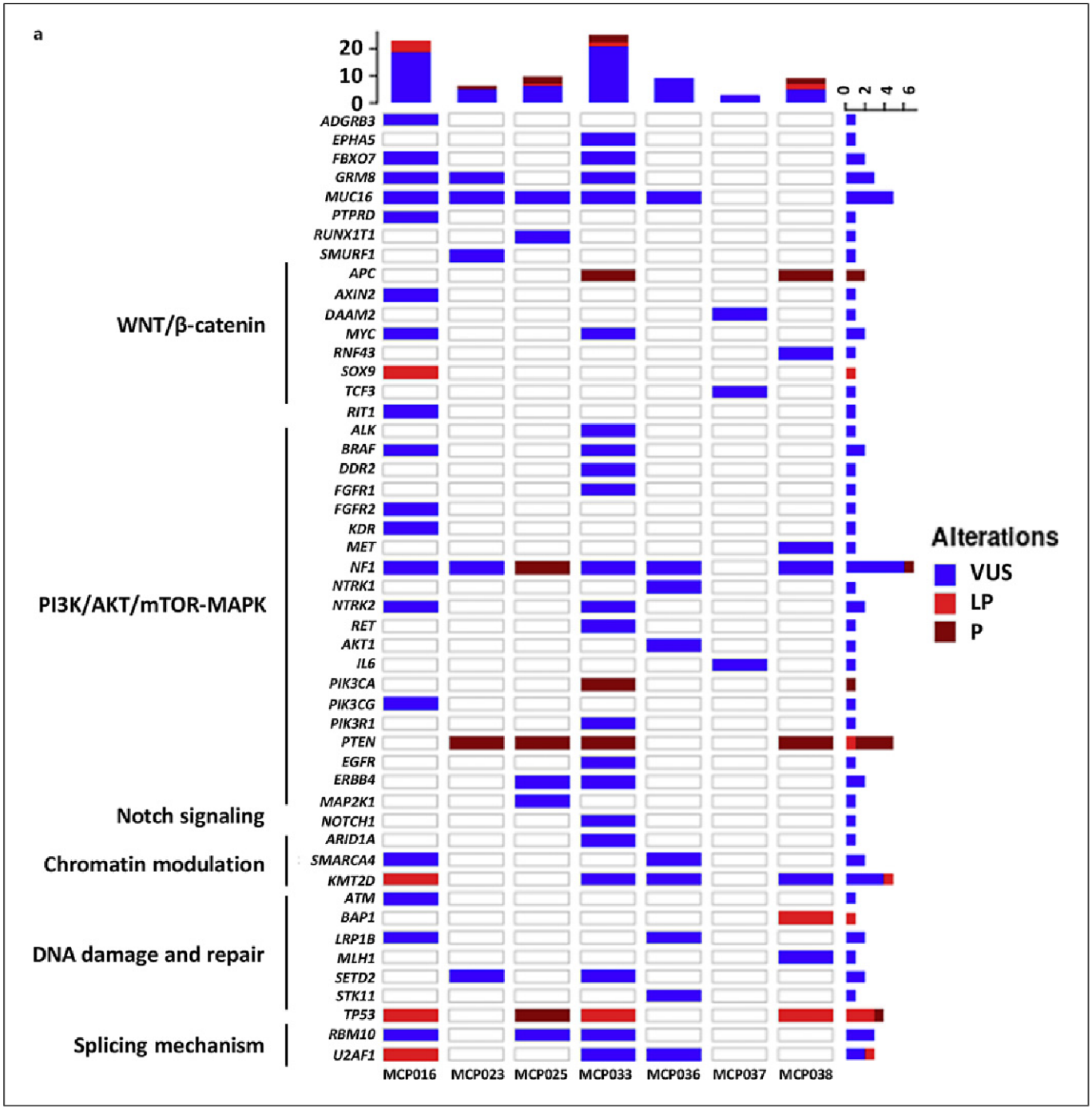
TCF/β-catenin-mediated transcriptional activity and the expression of the WNT target genes *CUL4A*, *CCND1*, *CDC25A*, and *C-MYC* were quantified next. Moderate increased basal TCF reporter activity was found in STS primary cell cultures MCP016, MCP021, and MCP023, similar to our previous results obtained in sarcoma cell lines [23], but less pronounced than that of SW480 colon cancer cells (shown in Fig. 3a). In 2 undifferentiated sarcoma-derived primary cultures, MCP016 and MCP021, basal levels of *CUL4A*, *CDC25A*, and *C-MYC* were higher than those expressed in human mesenchymal stem cells (hMSCs) (shown in Fig. 3b), a reference cell line with low WNT signalling activity [35]. Furthermore, in MCP016 cells, expression levels of *CUL4A* and *CDC25A*, but not *C-MYC*, were higher or similar than those of positive control cells. On the contrary, upregulation of *CUL4A*, *CDC25A*, and *C-MYC* could not be detected in the rest of the STS primary cultures (shown in Fig. 3b). Regarding *CCND1*, both hMSC and all STS primary cell cultures showed high expression levels (online suppl. Table 3). Therefore, high levels of *CCND1* in STS cells were not found increased when represented relative to those in hMSCs (shown in Fig. 3b), suggesting that hMSCs were not a good negative control for this target gene. These results demonstrate upregulated WNT signalling in MCP016 and MCP021 cultures, as evidenced by high levels of activated β-catenin, which was able to transactivate all WNT target genes studied, and by increased TCF reported activity. In 6 out of 8 primary cultures, only the *CCND1* gene showed elevated expression, whereas *CUL4A*, *CDC25A*, and *C-MYC* gene expression was not significantly upregulated.

Identification of Genetic Variants with Clinical Significance in STS Patients

We further evaluated the landscape of gene mutations of the 8 STS tumours from which primary cultures were derived (Table 1). According to the results of the next-generation sequencing analysis, a total of 102 genetic variants were identified in 87.5% (7/8) of the STS parental tumour samples. Among these variants, 81.4% (83/102) were classified as variants of unknown significance, 7.8% (8/102) were likely pathogenic alterations, and 10.8% (11/102) were known pathogenic variants (shown in Fig. 4a). Variants of unknown significance were excluded from further analyses.

From all samples, 62.5% (5/8) harboured at least one genetic variant classified as pathogenic or likely pathogenic, with an average of 3.8 variants per sample. The dermatofibrosarcoma protuberans sample MCP038

Fig. 3. a TCF luciferase reporter activity. Confluent cells were transfected with TOP/FOPflash luciferase reporter plasmids. Luciferase reporter activities were measured 24 h after transfection and normalized to Renilla luciferase activities. Results are represented as the ratio of TOP/FOP luciferase activity. Each column represents mean ± SEM of two independent experiments performed in triplicate. The human colorectal carcinoma SW480 cell line was used as positive control. **b** Expression analysis of TCF/β-catenin target genes CUL4A, CCND1, CDC25A, and C-MYC were performed following the TaqMan probes method described in the Materials and Methods section. β-2 microglobulin was used as an internal control for normalization. Results are mean ± SEM of three independent determinations performed in duplicate and normalized values relative to those in hMSCs (negative control of TCF/β-catenin-mediated transcriptional activity) are represented.



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exhibited the highest number of variants, reaching a maximum of 5. Pathway-specific analyses within each sarcoma subtype revealed that a number of pathways important in carcinomas were infrequently altered in sarcoma, including TGFβ and Notch. By contrast, the DNA damage repair pathway and the PI3K/AKT/mTOR-MAPK pathways showed the highest frequency of known pathogenic or likely pathogenic variants (*TP53*, 80% and *PTEN*, 80%, respectively), followed by the WNT signalling pathway (*APC*, 40%), the chromatin modulation

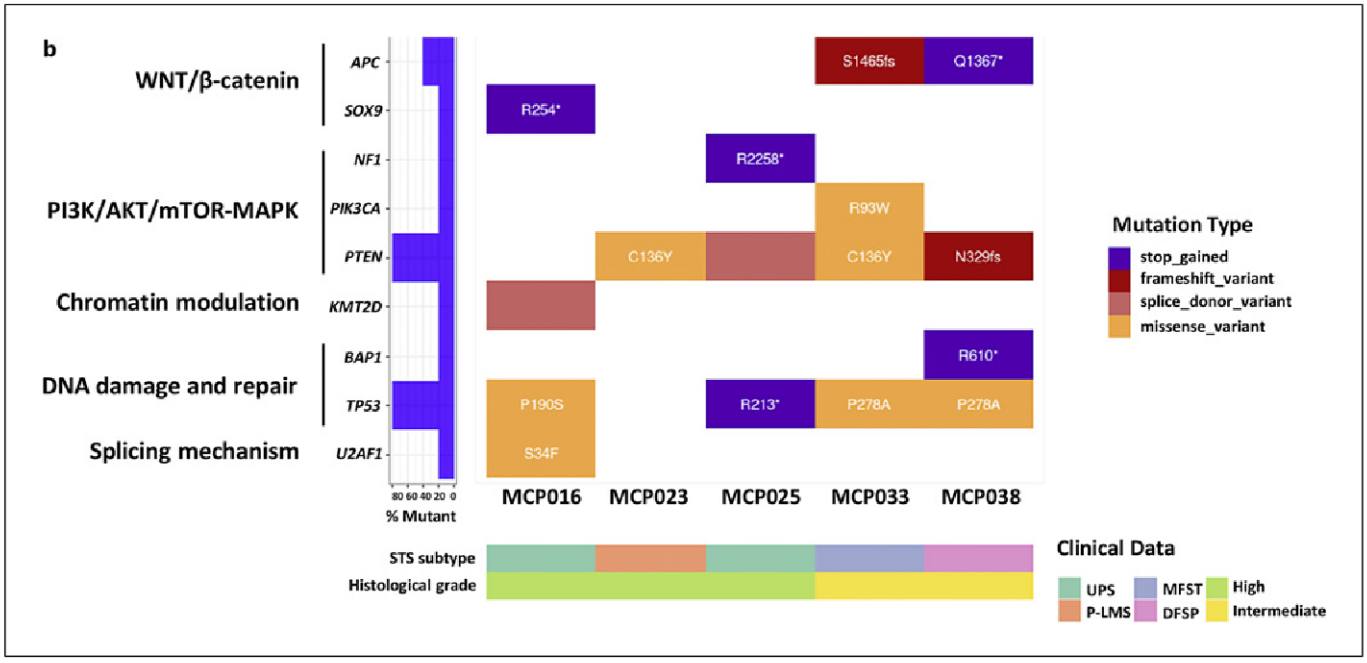

Fig. 4. a Landscape of genomic alterations in the 7/8 parental STS tumour samples from which primary cultures were established. Each row represents a gene, and each column represents a tumour sample. The histogram on top represents the number or variants identified per sample. The histogram on the right side represents the number of variants per gene. Conflicts arising from two different variants in the same gene/sample cell were resolved by keeping the pathogenic among the likely pathogenic or VUS. VUS, variant of unknown significance; LP, likely pathogenic; P, pathogenic. **b** Likely and known pathogenic variants identified in the 5/8 parental STS samples from which primary cultures were established. Each row represents a gene, and each column represents a tumour sample. The mutational spectrum is grouped according to the histological grade and subtype. Conflicts arising from two different variants in the same gene/sample cell were resolved by keeping the most deleterious as defined by their order in the legend. The histogram on the left side represents the mutation frequency of each gene. UPS, undifferentiated pleomorphic sarcoma; P-LMS, pleomorphic leiomyosarcoma; MFST, undifferentiated sarcoma fibrous histiocytoma-like; DFSP, dermatofibrosarcoma protuberans.

(*KMT2D*, 20%), and the splicing mechanism (*U2AF1*, 20%) pathways (shown in Fig. 4). As expected, *TP53* gene was significantly altered across STS subtypes which harboured pathogenic variants, whereas the WNT/β-catenin pathway displayed a low rate of pathogenic variants. The variants found in *APC* and *SOX9* genes were pathogenic or likely pathogenic variants, while variants detected in the rest of the pathway genes were variants of unknown significance.

To assess the potential clinical impact of these findings, we utilized OncoKB (http://oncokb.org), a publicly available genetic variant database recognized by the FDA. OncoKB provides information about the therapeutic, diagnostic, or prognostic implications of genetic aberrations as well as their corresponding FDA-levels. Among all the genetic variants identified, the variant R93W in *PIK3CA* was found only in the MCP033 tumour sample, whereas the genetic variant C136Y detected in *PTEN* gene was present in MCP033, MCP023, MCP025, and MCP038 tumour samples (shown in Fig. 4b). Supported by solid biological evidence, these two genetic variants have been listed as predictive biomarkers of response to specific drugs. Furthermore, regarding FDA categorization, both are considered to have potential clinical significance (FDA level 3).

Discussion

Many pre-clinical studies on anti-cancer therapies for STS patients use established commercial cancer cell lines [23, 24, 36], which often provide less representative and reproducible results when translated to other pre-clinical models and to clinical practice [37, 38]. In this study, we generated a collection of patient-derived STS primary cultures (Table 1) and performed an analysis of their histopathological features and molecular alterations. Our collection resembled the typical subtype distribution of

STS, since 5 of the 8 selected for this study belong to the most common sarcoma types found in adults, i.e., undifferentiated sarcomas and leiomyosarcoma [39], of which, moreover, only few commercial cell lines exist.

As we previously demonstrated that the WNT signalling pathway was constitutively activated in a broad range of STS cell lines [23, 24], we have now explored the endogenous activation of this pathway in patient-derived STS primary cultures, to uncover potentially actionable therapeutic targets for STS precision therapy. In accordance with previous studies [22–24], β-catenin showed elevated levels of its phosphorylated and transcriptionally active form (Ser552) in all STS primary cell cultures. More interesting, cytoplasmic and nuclear presence of β-catenin was also found in all patient-derived STS cultures, being the undifferentiated pleomorphic sarcoma MCP016 and MCP021 cells the ones with higher nuclear β-catenin levels (shown in Fig. 2b,c). In line with these findings, TCF reporter activity and expression of WNT/β-catenin signalling target genes (*CUL4A*, *CCND1*, *CDC25A*, and *C-MYC*) were also increased (shown in Fig. 3), indicating constitutive activation of the canonical WNT/β-catenin pathway in those 2 cultures. High levels of the phosphorylated β-catenin (Ser552) did not stringently correlate with high levels of nuclear β-catenin localization and vice versa in some of these primary STS cultures, suggesting that multiple tissue-specific regulatory factors might influence β-catenin stability, subcellular localization, nuclear abundance, and, in particular, its transcriptional activity. In the context of our results, we cannot discard that nuclear β-catenin in different STS subtypes might regulate tumorigenic events through tissue-specific interactions. As it has been demonstrated before, nuclear β-catenin can interact with other transcriptional partners in the absence of TCF/LEF, such as FOXO, SOX17, OCT4, or YAP1/TBX5, to promote the expression of different sets of genes associated with pluripotency, pro-survival and tumour metabolism [40–43].

It is well known that WNT/β-catenin is involved in crosstalk with PI3K/AKT and DNA damage and repair pathways promoting dysregulated cell cycle progression, increased cell proliferation, apoptosis escape, and resistance to cancer treatment [44–46]. Accordingly, within the landscape of gene mutations found in the 8 patient's tumour samples, the highest frequency of known pathogenic or likely pathogenic variants were detected in the DNA damage and repair pathway (*TP53*, 80%) as well as in the PI3K/AKT/mTOR-MAPK pathways (*PTEN*, 80%), followed by the WNT signalling pathway (*APC*, 40%) (shown in Fig. 4). Focusing on the WNT pathway, 2 tumour samples, MCP033 and MCP038, carried pathogenic variants in the *APC* tumour suppressor gene, whereas the sample MCP016 showed a likely pathogenic alteration in the *SOX9* gene, a repressor transcription factor of the WNT/β-catenin pathway (shown in Fig. 4a). Both genes carried loss-of-function mutations (shown in Fig. 4b) repressing their anti-tumour activity. These mutations align with higher levels of active phospho-β-catenin (MCP033 and MCP038) and/or nuclear β-catenin (MCP016 and MCP038), or with WNT/β-catenin transcriptional activation (MCP016 only) (shown in Fig. 2, 3). Anti-tumour therapy for *APC*-mutated patients is limited, with few clinical trials (NCT02175914, NCT06005974, and NCT05552755) for treating *APC*-mutated familial adenomatous polyposis or *AXIN1/APC*-mutated unresectable advanced/metastatic tumours. In addition, two genetic variants with FDA-3 level clinical implication and listed as predictive biomarkers of response to targeted drugs were found in *PIK3CA* and *PTEN* genes in 4 STS tumour samples. The known gain-of-function variant *PIK3CA*-R93W [47, 48] was found together with an *APC* alteration in MCP033 sample, which expressed the highest levels of phospho-β-catenin (Ser552). These findings are in agreement with other works in which the modulation of the AKT/GSK-3β phosphorylation status regulated the WNT signalling activation in different carcinomas [49, 50]. Since PI3Kα is the most frequently alterated kinase in all cancers [51], RLY-2608 and RLY-5836, both pan-mutant and isoform-selective PI3Kα inhibitors [52], are currently being evaluated in clinical trials (NCT05216432; NCT05759949). Our results suggest that the therapeutic use of these PI3Kα inhibitors, designed to overcome the toxicity of the traditional inhibitors, might be beneficial for a subset of STS patients harbouring the *PIK3CA* gain-of-function alteration. Finally, the *PTEN*-C136Y loss-of-function variant was found in samples MCP023, MCP025, MCP033, and MCP038 (shown in Fig. 4b), suggesting that these tumours could be susceptible to β-isoform-selective PI3K-targeted inhibitors, such as GSK2636771 and AZD8186 agents.

Since the discovery of TP53's role in cancer, significant research has focused on developing strategies to target TP53 alterations [53, 54]. However, few TP53-targeting therapies have advanced to late-stage clinical trials or gained FDA approval [55]. Strategies developed to address *TP53* alterations include restoring wild-type conformation in missense mutations, rescuing nonsense mutations, promoting degradation of altered p53 proteins, and exploiting vulnerabilities created by *TP53* alterations.

While our collection of patient-derived STS primary cultures represents the most common adult sarcoma subtypes, the number of primary cell cultures per subtype limits confident association of specific molecular or genetic alterations with a particular subtype. Although patient-derived primary STS cultures offer advantages in translating pre-clinical findings, this remains an in vitro study that lacks the complex tumour microenvironment; hence, missing information of how immune cells and surrounding stroma interact with and influence tumour cells. To address these limitations and gain a more detailed understanding of STS biology for effective therapy development, it would be highly valuable in the future to establish 3D cultures and in vivo models using these patient samples.

In summary, the findings in the present study emphasize the heterogeneity of WNT signalling activation among different STS patient-derived primary cultures. The identification of potentially actionable mutations, such as genetic variants in *PIK3CA* and *PTEN*, opens avenues for personalized treatment approaches in STS. Future research should explore the clinical significance of identified biomarkers and develop new therapies targeting WNT signalling and related pathways in STS to improve patient outcomes.

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Statement of Ethics

This study protocol was reviewed and approved by the Ethical Committee of Clinical Investigation of the Balearic Islands (CEIC-IB, Palma, Spain), Approval No. 4047/19 PI. Written informed consent was obtained from all patients. All procedures were performed according to the principles of the Helsinki Declaration.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.O.-H., M.P.-C., E.M.-F., and R.A. performed the study conceptualization. M.P.-C., E.M.-F., M.R.M., and R.A. conceived and carried out experiments. E. P. performed pathology analyses. M.P.-C. and E.M.-F. performed data curation, data analysis, and visualization. R.R. and R.S.M. provided sample resources and informed consents. M.P.-C., E.M.-F., R.A., O.V., and A.O.-H. wrote the original draft. A.O.-H. and R.A. supervised all the work. All authors approved the final version of the manuscript.

Data Availability Statement

All data generated or analysed during this study are included in this article and its online supplementary material files. Further enquiries can be directed to the corresponding author.

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