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TITOLO TESI

Clinico-pathologic and molecular analysis of myxofibrosarcoma of the extremities.

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1. INTRODUCTION

Myxofibrosarcoma (MFS) is one of the most common soft tissue sarcomas (STS) in elderly patients. It represents approximately 5% of all STS diagnoses. [1] It primarily affects the extremities and girdles, especially in the lower limbs [2]. Considering the prevalence of this sarcoma in late age, the incidence of this neoplasia is expected to increase in our aging society. Males are affected slightly more often than females.[1] The tumor, as most STS, commonly arises as a slow-growing, painless mass. It may show a singular or multinodular appearance.

The incidence of local recurrence (LR) (ranging 32-60%) [3,4] is definitely higher than other STS, even in the case of low grade lesions and lesions widely excised. [5] However, the prognosis of MFS is relatively good compared to other STS [6], with a risk of distant metastases (DM) ranging between 20% and 25% [3] and a sarcoma-specific survival (OS) of 83%. [7]

1.1 SIZE AND LOCATION

According to the tumour location, MFS is categorized in superficial (dermal/subcutaneous) and deep (intramuscular/subfascial). The former seems to infiltrate through the fascia the cutaneous and subcutaneous tissues, whereas the latter tends to form a discrete mass. [1,4] Deep-seated MFS are usually associated with a worse OS and an increased risk of DM.[8]

Moreover, deeply seated MFS are usually larger than superficial ones at diagnosis. The tumour size seems to be correlated with OS. Lee et al. [9] found a worse OS at 5 years in patients with larger MFS (maximum diameter >10cm). Similarly, Look-Hong et al.[10] found an association between larger size of MFS and worse OS. Several other Authors support this correlation.[6,7,11-13]

1.2 HISTOLOGY AND INFILTRATIVE PATTERN

Diagnosis of MFS is based on the presence of alternating hypocellular, myxoid areas and hypercellular, fibrous areas, pleomorphism and aggregation of neoplastic cells around curvilinear vessels.

It has a characteristically infiltrative pattern [8,14-16], leading tumours to extend along vascular and fascial planes when deeply seated [14,15] or to infiltrate the dermis when superficial.[16] Even if many STS usually grow as a round or oval mass with defined borders, MFS often shows infiltrative margins, both microscopically and macroscopically, with the subsequent invasion along the normal anatomical structures, especially the fascial planes and vessels, or the surrounding tissues.[8,17] The result is that the microscopic clusters of neoplastic cells could be found far from the primary mass, predisposing to LR.

1.3 MAGNETIC RESONANCE IMAGING

Tumour features at magnetic resonance imaging (MRI) can help in identifying risk classes of LR. [8,14-16,18] Preoperative tumor evaluation with MRI has been shown to be difficult as well, leading to frequent underestimation of tumor extension due to the specific infiltrative pattern of MFS.[17,19] Recognition of the infiltrative tumour spread extending from the primary mass on MRI is essential to plan adequate surgical margins, thus facilitating complete excision of the tumour. On MRI, the infiltrative spread of the tumour along the fascial plane could be manifested with curvilinear shape, commonly defined as "tail", a pathologic tumor-extension along normal tissue planes for more than 2 mm from the main mass. This sign was found in 83% of superficial tumours and in 24% of deep-seated tumours. [20,21] The "tail sign" should not be considered diagnostic for MFS, since other STS, like undifferentiated pleomorphic sarcoma (UPS), can show the same infiltrative behavior. [20] Nonetheless, Lefkowitz et al. [17] in 2013 suggested that finding a well-defined, sharp or tapering, pointed curvilinear projection at least 1.0 cm in length from the main mass, with a signal similar to those of water and having contrast enhancement, could be considered suggestive for MFS with moderate sensitivity (64-77%) and high specificity (79-90%). A significative correlation between anatomopathological findings and the MRI-pattern was reported in many papers, ranging from 87% to 100%. [2,17,19,21-24]

Similarly to other myxoid-predominant tumors, MFS usually presents low signal intensity on T1 weighted sequences (lower than normal muscle) and variable high signal intensity on water-sensitive sequences (e.g. T2 weighted, Short Thau Inversion Recovery; STIR) depending on the content of myxoid matrix.[8,25] Even if the T2-weighted sequences are considered effective to investigate the tumor spread, sometimes it could be difficult for radiologists to differentiate neoplastic component from oedema, since both show a high-signal intensity on these sequences. MRI sequences after Gadolinium administration is helpful to distinguish between a true "tail sign" and peritumoral soft tissue oedema. In fact, the "tail" usually shows the same grade of enhancement to the primary mass [2,8,17,19] Moreover, a high grade of intratumoral contrast enhancement has found to be significantly associated with worst OS and with a higher risk of LR after MFS excision.[8]

Although it is still not clear if the whole tail is completely made of tumour or not, it is well assessed that MFS with a tail-sign appearance on MRI have a higher risk of local recurrence, strongly suggesting the presence of tumoral cells inside the tail.[8,22] Yoo et al. [22] observed an association between LR rate and the presence of "tail sign" in a series of MFS and UPS. In a recent large series of MFS, the "tail sign" detected on pre-operative MRI was significantly associated with a higher risk of LR.[8] Previous studies on MFS showed similar results, confirming a strong

association between “tail sign” and LR risk after surgery. In recent years, the tail sign has been considered as a risk factor not only for LR but also for possible distant metastasis. [26] The importance of detecting the tail is not only related to its diagnostic value but also for the pre-operative planning. [17]

To differentiate MFS from UPS, a major criterion includes the percentage of intratumoral myxoid matrix.[9] There is a controversy about the exact percentage of myxoid component to use for this cut-off point, with a range variable from 10% to 50%.[4,5,9,27] The percentage of myxoid matrix has been related to patients’ outcome in some studies. Several clinicopathological analyses found that patients with higher myxoid matrix percentage have better disease-specific survival and distant recurrence-free survival for both MFS and UPS. [4,5,9,27] A recent large and homogeneous series of MFS found a significant association between high percentage of myxoid matrix (evaluated on preoperative MRI) and an increased risk of LR. [8]

1.4 GRADE

Tumor grade correlates with the rate of metastases and tumor-related death but not the local failure in MFS. [7,28] A large series of 229 patients affected by primary and localized MFS of the extremities reported that grade 3 MFS are associated with the worst prognosis. On the other hand, patients affected by grade 1 MFS have the lowest risk of developing a local recurrence. [29] Moreover, high percentage (15–38%) of locally recurrent MFS progress to a higher-grade disease with an attendant increase in metastatic potential.[2]

1.5 SYSTEMIC INFLAMMATION

Systemic inflammation was reported to be indicative of aggressive tumor characteristics in STS.[30-32] Nakamura et al. [33] and Szkandera et al [31] observed that elevated C-reactive protein (CRP) and neutrophil-lymphocyte ratio (NLR) values can be associated with inferior survival rates and an increased risk of LR in patients affected by STS. However, these series were very heterogeneous, particularly regarding histologic subtypes and sites of the tumor.

Myxofibrosarcoma is particularly rich in inflammation, with the highest median macrophage infiltration and T-cell infiltration among STS. [34,35] Ogura et al. [36] observed that in MFS average fraction of infiltrated CD8+ T-cell was significantly higher in the better prognostic cluster. Hu et al. [37] reported that a high level of CD4+ T-cell infiltration was associated with significantly better relapse-free survival. [38]

This is not surprising because it is known that systemic inflammation can be associated with cancer development and progression.[39] The molecular basis of the relationship between elevated plasma

CRP levels and poor clinical outcome in cancer patients has not been fully elucidated, and several possible explanations have been postulated. First, tumor growth can induce tissue inflammation and hence increase the CRP levels. [40] Second, CRP could represent an indicator of an immune response of the host to tumor antigens. [41] Third, cancer cells and inflammatory cells of the tumor microenvironment could increase the production of inflammatory cytokines, which may induce CRP production in cancer patients.[40,42]

Because both the infiltrative pattern and the systemic inflammation are associated with a worse prognosis in neoplastic patients, Nakamura et al. investigated if the MRI-infiltrative pattern was related to blood inflammatory markers, such as C-reactive protein and neutrophil-lymphocyte-ratio. They found a strong association between the infiltrative neoplastic spread and an increase of the inflammatory markers. [33] The reason could be that the tumor infiltrative growth causes a tissue inflammation with the secondary increase of C-reactive protein and neutrophils. [33,40]

Recently, it was suggested the potential prognostic utility of preoperative plasma CRP and NLR levels as independent prognostic markers in MFS patients who underwent curative surgical resection.[43]

1.6 TREATMENT

Surgery is the mainstay of treatment in MFS. Still, the infiltrative growth pattern observed in MFS can make it difficult to highlight the real extent of the tumor in pre-operative, intraoperative and post-operative settings (specimen examination).[2,8,44] In most STS, surgeons can excise with "safe margins" quite easily [22,45,46]; nonetheless, it remains unclear whether standard margins assessment [46,47] is adequate for MFS.[6] Berner et al.[48] reported that staged resections of MFS with definitive reconstruction delayed until clear margins are obtained lead to a better local control. The infiltrative growth pattern of MFS is like that observed in other STS, such as UPS, dermatofibrosarcoma protuberans, epithelioid sarcoma and angiosarcoma, which showed a higher percentage of inadequate margins than other histotypes of STS. [6,49] A careful preoperative evaluation of the real extension of the tumor and wider resection including the tail observed on MRI is mandatory to reduce the risk of local recurrence. Moreover, the relevance of a total neoplastic excision is due also to the fact that low-grade lesions could become more aggressive with a higher grade of malignancies in recurrencies, with metastatic potential.[2]

The role of adjuvant therapies in MFS still needs to be investigated. Only few studies reported about the use of chemotherapy (ChT) in MFS.[50] In these papers, ChT was mostly administered in advanced cases. The role of radiotherapy (RTE) in MFS is still unclear.[7,51,52] Some Authors suggested a possible radiosensibility of MFS cells to RTE. However, other studies

supposed MFS to be a radioresistant sarcoma [6,7]. Imanishi et al. [26] retrospectively evaluated 8 MFS and found a very low mean necrosis (45%, range, 0%-90%) after pre-operative RTE. They found viable cells in more than half of the tails. Moreover, RTE represents a real challenge in the attempt to ensure that all potential microscopic disease is encompassed in the planning target volume. [23]

1.7 MARGINS

Dadrass et al. [53] in a homogeneous series of MFS, found an association of LR with positive margin resection. Interestingly, the rate of LR in negative margin resections was relatively high compared to other STS subtypes. Sambri et al. [6] observed no correlation between margin adequacy and LR in MFS.

Other series highlighted that specific infiltrative STS can highly recur even if excised with wide margins. [54,55] Although negative margins may be achieved during resection, the irregular histologic pattern of MFS can still reveal microscopically positive margins on histopathology, more often than would be expected in other histotypes. [53]

Fujiwara et al. [56] demonstrated that in the case of MFS and UPS neither the R-classification nor the R1-classification were able to stratify the risk of LR in patients with negative margins, indicating that these classification systems are not sufficiently sensitive to stratify what constitutes an adequate margin of resection for infiltrative STS. Nonetheless, the Authors observed that a margin more than 10 mm was associated with the lowest LR risk. In addition, LR risk in patients with closer margins between 0.1 and 9.9 mm with fascia/ periosteum was found to be equivalent to the risk of LR with resection margin greater than 10 mm with any margin quality [57]. Therefore, a margin comprised of fascia may be equivalent to a metric margin of at least 10 mm.

Goertz et al. [58] observed that only the quality of surgical margins, but not the negative margin width had a prognostic influence. Iwata et al. [59] suggested that a resection margin of 2 cm away from the end of the radiological tumour infiltration would be the ideal margin in MFS. Nevertheless, such wider resection can increase the demand for plastic reconstruction surgery, risk of wound complication, and functional deterioration.

1.8 BIOLOGY

Myxofibrosarcoma is currently regarded as a distinct fibroblastic/myofibroblastic tumor type defined by cellular pleomorphism, a curvilinear vascular pattern, and a myxoid stromal component. [60]

Genomic instability is a hallmark of all human tumors [61,62] and can be related to gene mutations, gene copy number alterations and structural chromosomal abnormalities such as translocations, telomere dysfunction, and whole-chromosome aneuploidy that later results in chromosome instability.[62]

Different studies have shown that MFS is among the most highly complex sarcoma types. [2,63] Molecular cytogenetic studies have identified a high level of genomic complexity with a recurrent amplification of the chromosome 5p and 7q regions, the biologic significance of which is unknown.[64,65]

Clonal heterogeneity may be evident within single samples, but can also be observed between different tumour regions within the same primary site (so-called "regional heterogeneity") or even between primary and metastatic sites.[66] This was recently highlighted also in MFS by Lohberger et al. [67]

Many Authors attempted to identify novel molecular markers, not only for prognostication in MFS, but also to serve as possible therapeutic targets, and thereby improve clinical outcomes. However, the molecular pathogenesis of MFS remains incompletely understood.[38]

INTEGRINα10

The role of integrin-α10 in human cancer is context-dependent, [68,69] but its role in sarcomagenesis remains unknown.

Okada et al. [70] analyzed the gene expression profiles of 64 primary high-grade MFS and found that expression of ITGA10, which encodes integrin-α10, was amplified on chromosome 5p and overexpressed in about 50% of MFS. In addition, 42% MFS possessed co-amplification of TRIO and RICTOR and had worse outcomes.

Heitzer et al.[71], in a series of 25 MFS, observed that while 44% of G3 tumors showed a co-amplification of TRIO and RICTOR, the same phenomenon was observed in only one G1 tumor (10%). However,over-representation of RICTOR alone was found in 40% of G1 tumors indicating that TRIO amplification is significantly more abundant in G3 tumors and therefore a late event.

Okada et al. [70] also carried on functional studies in patient-derived MFS cell lines. Knockdown of integrin-α10 specifically inhibited growth and decreased RAC and AKT activation in MFS cells but not in normal mesenchymal cells, which suggests that integrin-α10 signals through RAC and AKT in a tumor-specific manner. The authors hypothesized that integrin-α10 signals through TRIO for the control of RAC and through RICTOR for the control of AKT. Also, cells overexpressing integrin-α10 exhibited greater migration and invasion than the control cells.

Hu et al. [37] evaluated 61 MFS and UPS. They found that increased ITGA10 and decreased PPP2R2B expression had independent prognostic value. PP2R2B encodes the regulatory B subunit of protein phosphatase 2A. It directly interacts with PDK1 and suppresses its activation. Therefore, both ITGA10 and PPP2R2B might act as upstream regulators of AKT. Somatic mutation was not common in this genes, but DNA methylation showed a profound effect on their expression.
Activation of the AKT/mTORC2 pathway was correlated with histologic grade and tumor progression by Takahashi et al.[72] in a series including 68 primary MFS.
Integrin-mediated signaling is also known to cross-talk with MET.[73,74]

NF1

Regarding NF1 gene, Ogura et al. [36] found 4 homozygous deletions and 9 somatic mutations in 116 MFS.
These data suggest that a distinctive pattern of NF1 aberrations may play a role in MFS tumorigenesis, similarly to other cancers.[75] Loss-of-function mutations in NF1 gene were also reported by Barretina et al. [76] in 5 out of 35 MFS. Heitzer et al. [71] were unable to inform about the NF1 mutation status since they used a hotspot panel for mutation analysis which did not cover NF1. However, loss of NF1 was observed in two out of 25 patients.

EZRIN

Ezrin functions as a linker between the plasma membrane and cortical actin cytoskeleton.[77] Increased ezrin levels have been reported to be associated with the high metastatic propensity in different cancers, including rhabdomyosarcoma and osteosarcoma. [78-80] Its underlying mechanism may be ascribed to increased activated ezrin to modulate pleiotropic cellular phenotypes related to cancerous states, such as substrate adhesion, cell survival, cell migration, and formation of cell–cell junctions.[79,80]
Huang et al. [81] evaluated the expression of ezrin in 78 primary localized MFS with immunoistochemistry (HIC). Ezrin overexpression was present in approximately a half of tumors (49%), and it was significantly associated with important variables related to tumor aggressiveness, including necrosis and FNCLCC grading. Additionally, ezrin overexpression was an independent poor prognosticator for survival.
They also measured Ezrin mRNA expression level in in two MFS cell lines, through Real-Time RT–PCR and Western blot test. [82,83] Ezrin mRNA expression level of MFS cell lines was apparently lower than that of normal fibroblasts. However, active ezrin (phosphorylated form at the residue of Thr567) was only detectable in MFS cells, but not in fibroblasts.

AMACR

α-methylacyl coenzymeA racemase (AMACR) is a peroxisomal and mitochondrial enzyme encoded on chromosome 5p13.3, which acts as a gatekeeper for the β-oxidation of dietary branched-chain fatty acids and bile acid synthesis.[84] It was identified as a protein which drive tumor growth in prostate cancer and other neoplasia, because most malignancies increase the need for fatty acids as an energy source. [84-86]

AMACR role in MFS was recently investigated by Li et al. [87] in a series of 105 primary MFS and in two cell lines. AMACR amplification was found in 21% MFS by FISH and was strongly correlated with HIC overexpression. AMACR overexpression was correlated to FNLCC grade and to worse survival. However, approximately 40% of AMACR-overexpressing MFS lacked gene amplification. Thus, involvement of alterative regulatory mechanisms was likely in a subset of MFS.

The Authors also found that in MFS cell lines and xenografts, AMACR overexpression could increase cyclinD1 expression at the mRNA and protein levels, whereas the mechanisms underlying this regulatory link remain to be elucidated.

MAGE-A3

Melanoma-associated antigen 3 (MAGE-A3) protein was found to be overexpressed in multiple cancers. [88]

Conley et al. [89] explored expression of MAGE-A3 among a diverse number of sarcomas and sarcoma cell lines. [90,91]

MAGE-A3 was overexpressed in 41% of MFS and UPS, significantly higher than in other STS histotypes. High expression was more likely to be seen in recurrences than in primary tumours and was associated with an adverse survival. Immunotherapies targeting MAGE-A3 have shown both positive and negative results in the treatment of various cancers. [92]

SKP2

S-phase kinase-associated protein 2 (SKP2) is a negative regulator of p27kip1 cell-cycle inhibitor. Its overexpression was associated to metastatic propensity in common carcinomas, such as prostatic and esophageal cancers.[93,94]

SKP2 role in MFS was evaluated in two consecutive series [95,96] which included a total 82 cases. SKP2 gene amplification was detected in 38% of cases. Its amplification strongly correlated with SKP2 overexpression in HIC and to FNLCC higher grades. However, 14% of such tumors and

SKP2-expressing cell line lacked gene amplification. Both SKP2 protein overexpression and gene amplification were highly predictive of worse outcomes.

CD109 and TGF- β

CD109 is a cell surface glycoprotein that is expressed on endothelial cells, activated T-lymphocytes, platelets, and a subpopulation of bone marrow CD34 cells. CD109 is a TGF-β co-receptor that regulates TGF-β receptor endocytosis and degrading, thus inhibiting TGF-β signaling. [97] The roles of TGF- β in remodeling the tumor microenvironment, by suppressing T cell differentiation and activity, inducing fibrosis and angiogenesis, have been extensively characterized.[98] TGF-β also appears to have a role in chemotherapy resistance, as reported in several tumors including breast cancer and squamous cell carcinoma.[99-101]

CD109 has been found to be overexpressed in various cancers, including STS. [102,103] Emori et al. [104] evaluated 37 MFS with HIC and found an overexpression of the CD109 protein in 10% of patients, which was significantly associated with decreased survival.

De Vita et al. [105] analyze three patient-derived high-grade MFS cell cultures. Immunohistochemistry analysis showed that tumor cells were strongly positive for CD109. Conversely, CD109 was weakly expressed in normal tissue. Also, higher levels of CD109 were observed with gene expression analysis in all primary MFS cultures with respect to normal tissue.

CD44

The tyrosine kinase receptor MET and its ligand hepatocyte growth factor (HGF), splice variants of CD44 and ezrin, cooperate. [106] CD44 are ubiquitously expressed on all cell types, where they act as receptors for hyaluronic acid. They play various roles and are involved in cellular differentiation, cellular migration, and cell-cell contact.[107] Abnormal expression of CD44 may promote cells invasion and were correlated to more aggressive behavior in various cancers, including STS [108-110].

Matuschek et al. [111] analyzed with polymerase chain reaction (PCR) 4 variants of CD44 (CD44, CD44s, CD44v6, and CD44v8) in 34 MFS. They found a significant difference in tumor related survival only for CD44s and CD44v6, with increased CD44s and decreased CD44v6 expression associated to better prognosis. CD44v6 was shown to be strictly required for MET activation by HGF in rat and human carcinoma cells, in established cell lines as well as in primary keratinocytes.[106] This is consistent with prostate cancer, in which CD44s is a tumor suppressor but certain CD44 variants are oncogenes and promote growth. [112-114]

More recently, Tsuchie et al.[115] evaluated the HIC expression of CD44s in 44 MFS. Overall expression rate of CD44s in all patients was relatively high. The Authors found that high expression of CD44s was associated with LR but did not affect survival.

MET

MET encodes a transmembrane receptor tyrosine kinase, which constitutes the only known high-affinity receptor of HGF. Through combination with HGF, MET could activate RAS-MAPK or PI3K-AKT signaling pathway to promote cell motility and proliferation.[74] Besides mitogenic and antiapoptotic activities common to many growth factor receptors, enhanced MET activation can stimulate cell–cell detachment, migration, invasiveness and angiogenesis.[116-119]

Lee et al.[73] firstly investigated the role of MET in 86 primary localized MFS. Approximately two-thirds of MFS displayed MET overexpression at HIC, which correlated with adverse clinicopathological factors (tumor size and mitotic rate) and was independently predictive of shorter survival. The Authors quantified MET transcript by real-time reverse transcription PCR (RT–PCR) for 16 laser-microdissected tumors and two MFS cell lines. Nine (56%) specimens showed apparently upregulated MET transcript, suggesting their frequent upregulation in MFS. Lee et al. [73] found wild type MET oncogene in both cell lines. The Authors suggested that, as proven in a variety of cancers, MET protein expression might be upregulated by several small non-coding microRNAs. [113,114,120]

More recently, Ma et al.[121] confirmed the relationship between MET and MFS. They used HIC and fluorescence in situ hybridization (FISH) to detect the MET expression and gene status in 30 MFS. The Authors observed MET overexpression in 14 cases (46.7%), with a correlation with FNLCC grade and mitotic rate. They also found a polysomy of chromosome 7 in 11 of these cases, thus suggesting that chromosome 7 polysomy, rather than MET amplification, led to the overexpression of MET protein. Patients with MET overexpression or chromosome 7 polysomy also had a high risk of local recurrence and metastasis.

These findings [73,121] strengthen the possible causative function of MET in conferring an aggressive phenotype, implying the potentiality of HGF/MET as an attractive target of therapeutics in MFS. [122-124]

TP53 AND CELL CYCLE REGULATORS

Ogura et al.[36] analyzed a total of 106 MFS. They found frequent alterations in genes related to p53 signaling (51%), along with those associated with the cell cycle checkpoint (43%), including

RB1, CDKN2A/CDKN2B, CCND1, and CDK6. Alterations of any of cell cycle regulators were associated with poorer overall survival.

Heitzer et al.[71] evaluated MFS samples from 25 patients. Somatic mutations were identified in only 11 (44%) patients. Grade 3 tumors showed a higher amount of somatic copy number variations (CNV) than grade 1. All these 11 patients showed at least one somatic Tp53 mutation. Tp53 mutations are relatively common in sarcomas with non-specific genetic aberrations and complex karyotypes compared to sarcomas with reciprocal specific translocations.[125] Moreover, they identified focal amplification/deletion in a variety of known cancer driver genes such as CDKN2A, CCND1, CCNE1, EGFR, EPHA3, EPHB1, FGFR1, JUN, NF1, RB1, RET, in particular in grade 3 tumors. In addition, grade 3 areas of the same tumor showed novel emerged focal amplifications compared to the grade 1 areas. Many of these, such as BRAF, EGFR, FGFR, KIT or RET, are indeed actionable targets and are actively being used for precision medicine in different tumor entities.

Ogura et al. [36] also identified novel recurrently mutated genes such as GNAS (9%), ATRX (9%), KRAS (7%) and JAK1 (4%). The presence of GNAS mutations was significantly associated with LR-free survival. [36]

2. AIM

The aim of this study was to evaluate genetic profile of myxofibrosarcoma of the extremities, assess immunohistochemistry expression and to verify whether histologic and molecular characteristics of MFS influence prognosis of the disease.

3. MATERIALS AND METHODS

From January 1992 to June 2017 a total of 473 patients affected by MFS underwent surgical excision at a single Institution (Istituto Ortopedico Rizzoli, Bologna, Italy).

The study was approved by the Ethical Committee and informed consent was collected from all patients.

Inclusion criteria included primary tumors not treated elsewhere before, location in the limbs and girdles, specimen stored in the local biobank and presence of >90% viable tumor in the specimen. One hundred and sixty-six adult patients (>18 years) with primary MFS of the extremities (hand and foot excluded) were selected from the patient population.

All cases were histologically revised and classified according to the 2013 World Health Organization classification of STS [49] by experienced sarcoma pathologists of our Institute. Diagnosis of MFS was based on the presence of alternating hypocellular, myxoid areas and hypercellular, fibrous areas, pleomorphism and aggregation of neoplastic cells around curvilinear vessels. Immunostains were used to exclude other entities that may mimic MFS. A 3-step system (FNCLCC) was used to assess MFS grade. [126]

Tumor size was assessed on surgical specimens using the larger diameter as a reference and depths were divided into superficial (above the muscle fascia) and deep (below the muscle fascia), according to preoperative imaging (MRI). All patients underwent operation to obtain limb-sparing, function-sparing surgery with negative surgical margins, according to the Musculoskeletal Tumor Society (MSTS) classification. [127]

The use of RTE and ChT was decided at the discretion of a multidisciplinary team, which included orthopedic surgeon, radiotherapist, and oncologist. Radiation therapy was administrated according to STS guidelines.[128] Anthracycline-based drug regimen was used and, in most patients, incorporated ifosfamide.

3.1 IMMUNOISTOCHEMISTRY EVALUATION

Samples were obtained from biopsy or surgical specimens, fixed in 10% formalin, processed, and paraffin embedded. Hematoxylin and eosin (H&E)–stained slides of samples were reviewed by the pathologist to choose representative areas for tissue microarray (TMA) construction using TMA

Master System (EurocloneSpA, Milano, Italy). Tissue cores measuring 4 μm in diameter were placed on slides in triplicate, for a total of 80-sample TMA blocks, with some duplicated samples from the same patient. TMA slides were used for HIC analysis of anti-MET 51067 (1:50, Abcam); anti- RET 134100 (1:50, Abcam); anti-p53 M7001 (DO-7, Dako) and anti-RB 1F8 (1:10, Histoline).

For MET and RET analysis streptavidin–biotin peroxidase DAB rabbit/mouse (Dako) was used as the detection system. For p53 and RB antibody detection was performed using the UltraView Universal DAB Detection Kit and UltraView. Universal Alkaline Phosphatase Red Detection Kit (Ventana Medical Systems) on Ventana BenchMark following the manufacturer's guidelines (Ventana Medical Systems, Tucson, AZ, USA). Specimens with a diffused, high, or moderate immunostaining were considered as positive. Both negative and positive appropriate controls for immunostaining were performed.

3.2 NGS ANALYSIS – DETECTION OF SOMATIC HOTSPOT MUTATIONS

Twenty samples with a different clinical outcome (10 with a LR and 10 disease-free at final follow-up) were analyzed for the presence of genetic changes using the Cancer Hotspot Panel v2 (Thermo Fisher Scientific, Waltham, MA, USA), which include 2800 COSMIC mutations related to 50 oncogenes and tumor suppressor genes. 10 ng of DNA per sample was processed using the Ion AmpliSeqTM Library Kit 2.0 (Thermo Fisher Scientific). Ion Chef (Thermo Fisher Scientific) was used for automated template preparation and chip loading on Ion 318™ Chip Kit v2 BC, then loaded on the Ion Torrent PGM platform (Thermo Fisher Scientific). Data analysis was performed using both the Ion ReporterTM Server System and a specific somatic workflow from the SEQNEXT application (JSI medical systems GmbH Germany) for the mapping, alignment, and variant detection of NGS data. Pathogenic variant calls resulting from both software with a mutant allele frequency >5 were considered and evaluated, whereas genetic variants resulting from a single software were considered only if confirmed by Sanger sequencing analysis. Variants were annotated according to the nomenclature used by the Human Genome Variation Society (HGVS).

3.3 TP53 MUTATION SCREENING

To evaluate the Tp53 mutation status, the sequencing analysis was extended to all Tp53 coding regions (exons 2-11), also including the intron-exon boundaries. 84 samples, containing the 20 previously analyzed with NGS, were analyzed by Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and the ABI PRISM 3500XL Genetic Analyzer (Thermo Fisher Scientific).

3.4 EVALUATION OF SOMATIC CNV IN TP53 AND MET GENES

Somatic CNV play critical roles in activating oncogenes and inactivating tumor suppressor. Thus, we assessed for their presence with a digital PCR technology, due to its capability in detecting low-frequency CNV differences in a complex background.

To evaluate somatic CNV status in TP53 and MET genes, a Microfluidic Chip-Based Digital PCR reaction was performed using the QuantStudioTM3D Digital PCR System (Thermo Fisher Scientific – US). Digital-PCR analysis was feasible in 83 cases for TP53 and 79 cases for MET gene, because of inadequate RNA quality of the neoplastic tissue in the remaining cases. Two Taqman copy number assays (Hs06423639_cn and Hs02323823_cn) were selected to cover approximately the central part of both genes. RNase P gene was chosen as a reference locus. Polymerase chain reaction amplification was carried out on a ProFlex ™ 2 X Flat PCR System (Applied Biosystems). The fluorescence data were read and analyzed using QuantStudio 3D Analysis Suite Cloud Software. Results are expressed as copies per microliter and compared as a ratio of target (FAM)/Total (FAM + VIC) expressed in percentage. In case of a regular biallelic status, we expect this value to be around 50%. Both probes were previously validated on 20 DNA with a regular biallelic status of the Tp53 and MET genes, confirming the absence of CNV.

As Tp53 is an oncosuppressor gene, it needs a «double hit» to be considered a «driver gene». A «double hit» was defined as CNV<33,3% or as the association of CNV and a somatic point mutation. [129] However, it was recently observed that a single mutant dimer would prevent the Tp53 tetramer from binding DNA. This indicates that the “Achilles' heel” of Tp53 is in its dimer-of-dimers organization, thus the tetramer activity can be negated by mutation in only one allele followed by tumorigenesis.[130] Regarding Met (oncogene), copy number gains of gene drive expression of oncogenes.[131]

3.5 STATISTICAL ANALYSIS

Patients’ characteristics are presented by frequencies and percentages for categorical variables, median and

range for continuous variables. Unpaired two-tailed Student t tests were used to compare groups for independent samples. The Kaplan-Meier method was used to estimate OS and LR. Local recurrence-free survival interval was defined as the time between surgery and the first LR or last follow up available, whichever came first. Similarly, OS interval was defined as the time between surgery and death or last follow-up, whichever came first. Patients who died of other causes were censored. Differences in survival rates were assessed by the log-rank test. P values <0.05 were considered significant.

All analysis was completed using the Statistical Package for Social Science (IBM Corp. Released 2013. IBM
SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

4. RESULTS

Median age at the time of surgery was 70 years (range, 18 to 93); 95 patients (57.2%) were male, 71 (42.8%) were female.

Fifteen patients (9.0%) had metastasis at presentation (all but one to the lungs, in one case to lymph nodes).

Patients' characteristics are reported in Table 1.

Characteristic		N (%)
Age (median, range)		70 years (18-93)
FNLCC grade	1	6 (3.6%)
	2	15 (9.0%)
	3	145 (87.4%)
Sex	Male	95 (57.2%)
	Female	71 (42.8%)
Site	Upper Limb	40 (24.1%)
	Lower Limb	126 (75.9%)
Size	<5cm	37 (22.3%)
	5-10cm	58 (34.9%)
	>10cm	71 (42.8%)
Depth	Superficial	39 (23.5%)
	Deep	127 (76.5%)
Adjuvant therapies	Chemotherapy	34 (20.5%)
	Radiotherapy	107 (4.5%)

Table 1. Patients' characteristics.

4.1 IMMUNOHISTOCHEMISTRY EVALUATION

MET protein was successfully scored for 136/166 cases (81.9%). Thirty-two cases (19.3%) were positive for p53 and 150 (90.4%) cases for Ret. Immunohistochemistry for Rb1 was negative in most of the cases (148, 89.2%).

Positive immunohistochemistry for both Met and P53 were correlated to high FNLCC grades. In particular, none out of the 6 grade 1 MFS tested was positive for p53 HIC. (Table 2)

Characteristic		P53 HIC positive (n - %)	P*	MET HIC positive (n - %)	P*
	TOTAL	32 (19.3%)		136 (81.9%)	
FNLCC grade	1 (n=6)	0 (0%)	0.048	2 (33.3%)	0.007
	2 (n=15)	2 (13.3%)		13 (86.7%)	
	3 (n=145)	30 (20.9%)		121 (83.4%)	
Sex	Male (n=95)	18 (19.0%)	0.727	77 (81.0%)	0.749
	Female (n=71)	14 (19.7%)		59 (83.0%)	
Site	Upper Limb (n=40)	5 (12.5%)	0.256	26 (65.0%)	**0.002**
	Lower Limb (n=126)	27 (21.4%)		110 (87.3%)	
Size	<5cm (n=37)	5 (13.5%)	0.609	30 (81.0%)	0.496
	5-10cm (n=58)	12 (20.7%)		43 (74.1%)	
	>10cm (n=71)	15 (21.1%)		63 (88.7%)	
Depth	Superficial (n=39)	5 (12.8%)	0.175	29 (74.4%)	0.233
	Deep (n=127)	27 (21.3%)		107 (84.3%)	

Table 2. Characteristics of patients according to P53 and MET overexpression on HIC.

*chi-square

4.2 DETECTION OF SOMATIC HOTSPOT MUTATIONS

Tp53 pathogenic or likely pathogenic point mutations were observed mainly among locally recurred cases. (Table 3)

Mutations	**Recurrences (n=10)**	**No recurrences (n=10)**
TP53 (various)	50%	20%
KRAS (p.K117N, «pathogenic»)		10%
JAK3 (p.V722l, «conflicting»)	-	10%
ATM (p.V410A, «uncertain»)	-	10%
IDH2 (p.R140Q, «pathogenic», «likely pathogenic»)	-	10%

Table 3. Point mutations observed in NGS sequencing in 20 cases.

4.3 TP53 MUTATIONS AND CNV

Overall, point mutations in Tp53 were observed in 21/83 (25.3%) cases. Point mutations alone were observed in only two cases (2.3%), whereas in 19 (22.9%) they were associated with a gene deletion. Most of the cases (52, 61.9%) presented a Tp53 gene deletion. A “double hit” on Tp53 gene was observed in 43 (51.8%) of the cases, mostly in FNLCC higher grades (p=0.045). (Table 4) No correlation was found with HIC overexpression of p53.

Characteristic		Tp53 “double hit” (n - %)	P*	Tp53 alteration§ (n - %)	P*
	TOTAL	43 (51.8%)		71 (84.5%)	
FNLCC grade	1 (n=5)	0 (0%)	0.046	4 (80.0%)	0.936
	2 (n=7)	3 (42.8%)		6 (85.7%)	
	3 (n=71)	40 (56.3%)		61 (85.9%)	
Sex	Male (n=46)	17 (37.0%)	0.231	38 (82.6%)	0.300
	Female (n=37)	26 (70.2%)		33 (89.2%)	
Site	Upper Limb (n=18)	10 (55.6%)	0.464	15 (83.3%)	0.509
	Lower Limb (n=65)	33 (50.8%)		56 (86.2%)	
Size	<5cm (n=18)	7 (38.9%)	0.114	13 (72.2%)	0.132
	5-10cm (n=30)	20 (66.7%)		28 (93.3%)	
	>10cm (n=35)	16 (45.7%)		30 (85.7%)	
Depth	Superficial (n=20)	10 (50.0%)	0.528	18 (90.0%)	0.405
	Deep (n=63)	33 (52.3%)		53 (84.1%)	

Table 4. Characteristics of patients according to Tp53 “double hit” and Tp53 alterations.

*chi square

§Point mutation or CNV

4.4 MET COPY NUMBER VARIATIONS

Met gene amplification was observed in 55 (69,6.3%) of the analyzed cases, with no correlation with other clinical parameters. (Table 5)

Characteristic		MET amplification (n - %)°	P*
	TOTAL	54 (68.4%)	
FNLCC grade	1 (n=5)	4 (80.0%)	0.857
	2 (n=5)	3 (60.0%)	
	3 (n=69)	47 (68.1%)	
Sex	Male (n=43)	26 (60.5%)	0.145
	Female (n=36)	28 (77.8%)	
Site	Upper Limb (n=16)	10 (62.5%)	0.389
	Lower Limb (n=63)	44 (69.8%)	
Size	<5cm (n=17)	12 (70.6%)	0.963
	5-10cm (n=27)	18 (66.7%)	
	>10cm (n=35)	24 (68.6%)	
Depth	Superficial (n=18)	13 (72.2%)	0.463
	Deep (n=61)	41 (67.2%)	

Table 5. Characteristics of patients according to MET amplification.

*chi square

4.5 SURVIVAL ANALYSIS

Local recurrence

In 36 cases (21.7%) a LR occurred after a median period of 19 months (range, 2 to 107).

Local recurrence rate was 21.2% (C.I. 95% 23.8-28.6) at 3 years and 33.3% (C.I. 95% 25.0-41.6) at 5 years.

No LR were observed among grade 1 MFS. Grade 2 and 3 tumors has a significantly ($p<0.001$) increased risk of LR (36.8% and 34.8% at 5 years, respectively). (Figure 1)

Regarding HIC evaluation, cases with overexpression of MET had a higher risk of LR (33.4% vs 14.3% at 5 years, p=0.047), whereas P53 immunostaining did not correlate with LR risk (p=0.850). Among cases analyzed for gene alterations, Tp53 "double hit" showed a significantly increased risk of LR (37.2% vs 15.0% at 5 years, 0.020). Tp53 point mutations increased the risk of LR (45.6% vs. 20.0%, p=0.032). However, no increase in the risk of LR was observed in cases with a Tp53 deletion (p=0.592).

No correlation was found with MET gene amplification and LR risk (p=0.207). None of the other factors analyzed was found to correlated with the risk of LR.

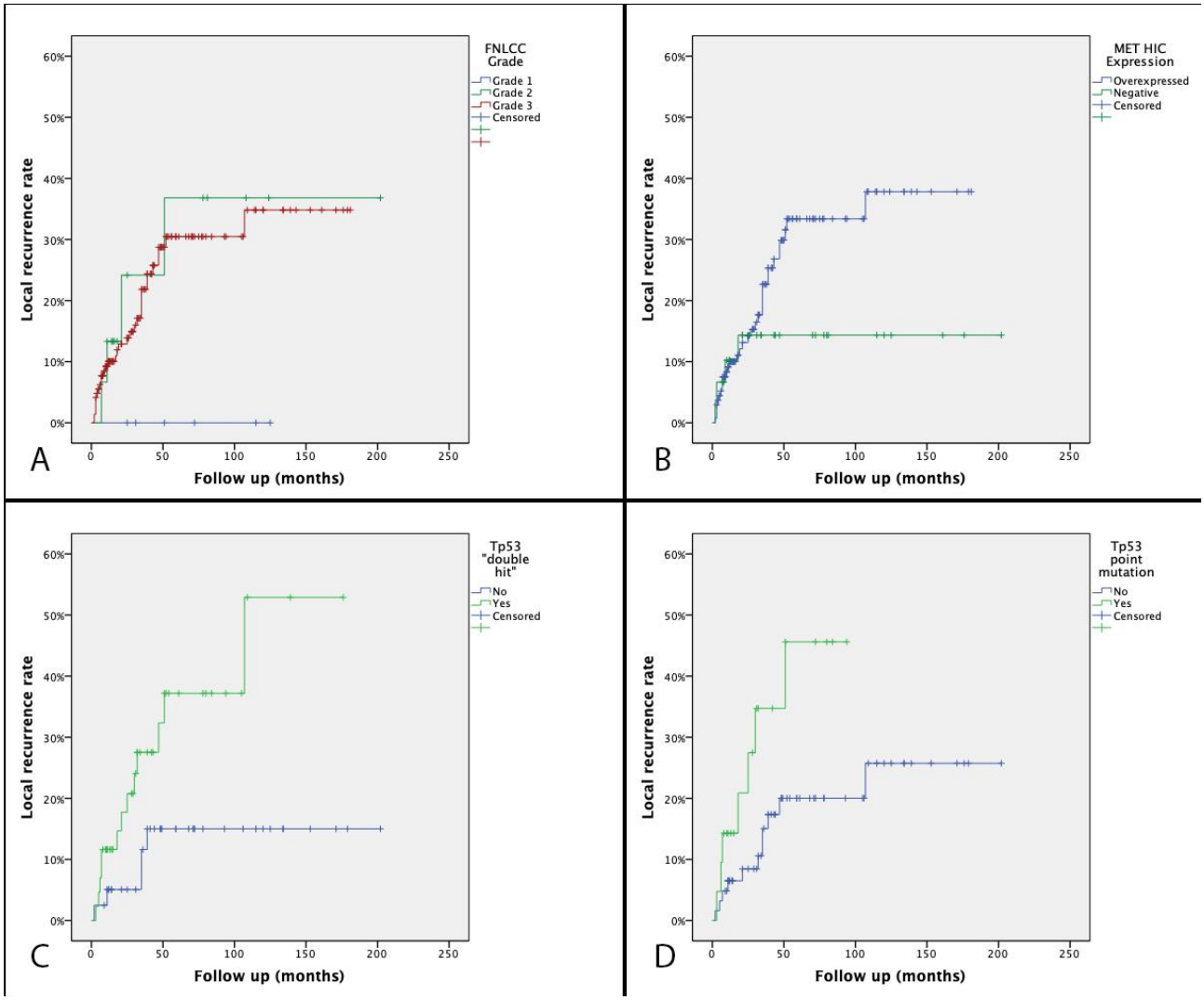

Figure 1. Kaplan-Meier survival analysis local recurrence curve according to FNLCC grade (A), MET HIC expression (B), Tp53 "double hit (C) and Tp53 point mutations (D).

Overall Survival

At the last follow-up (median 38 months, range 4 to 202), 89 patients (53.6%) were alive, 19 patients (11.4%) died of other causes. Fifty-eight (34.9%) patients died of the disease after a median time of

23 months (range, 4-174).

Kaplan-Meier analysis with OS as a primary endpoint showed an estimated survival of 75.7% (C.I. 95% 68.7-82.7%) at 3 years and 60.7% (C.I. 95% 51.7-69.7%) at 5 years.

A significant worse OS was observed in high grade MFS (OS at 3 years 100% for grade 1, 75.8% in grade 2 and 77.3% in grade 3, p=0.022). (Figure 2) A worse OS was also observed in deep tumors (70.3% vs 87.0% at 3 years, p=0.040) and in MFS larger than 10 cm (p=0.048).

Regarding HIC evaluation, cases with overexpression of MET had a tendency toward a worse OS (72.9% vs 78.9% at 3 years, p=0.058), whereas P53 overexpression did not correlate to OS (p=0.258). Among cases analyzed for gene alterations, no correlations were found neither between T53 "double hit" nor MET amplification and OS (p=0.872 and p=0.0734, respectively). However, Tp53 point mutations significantly decreased OS (71.4% vs 79.7% at 3 years, p=.0047). None of the other factors analyzed was found to correlated with the risk of OS.

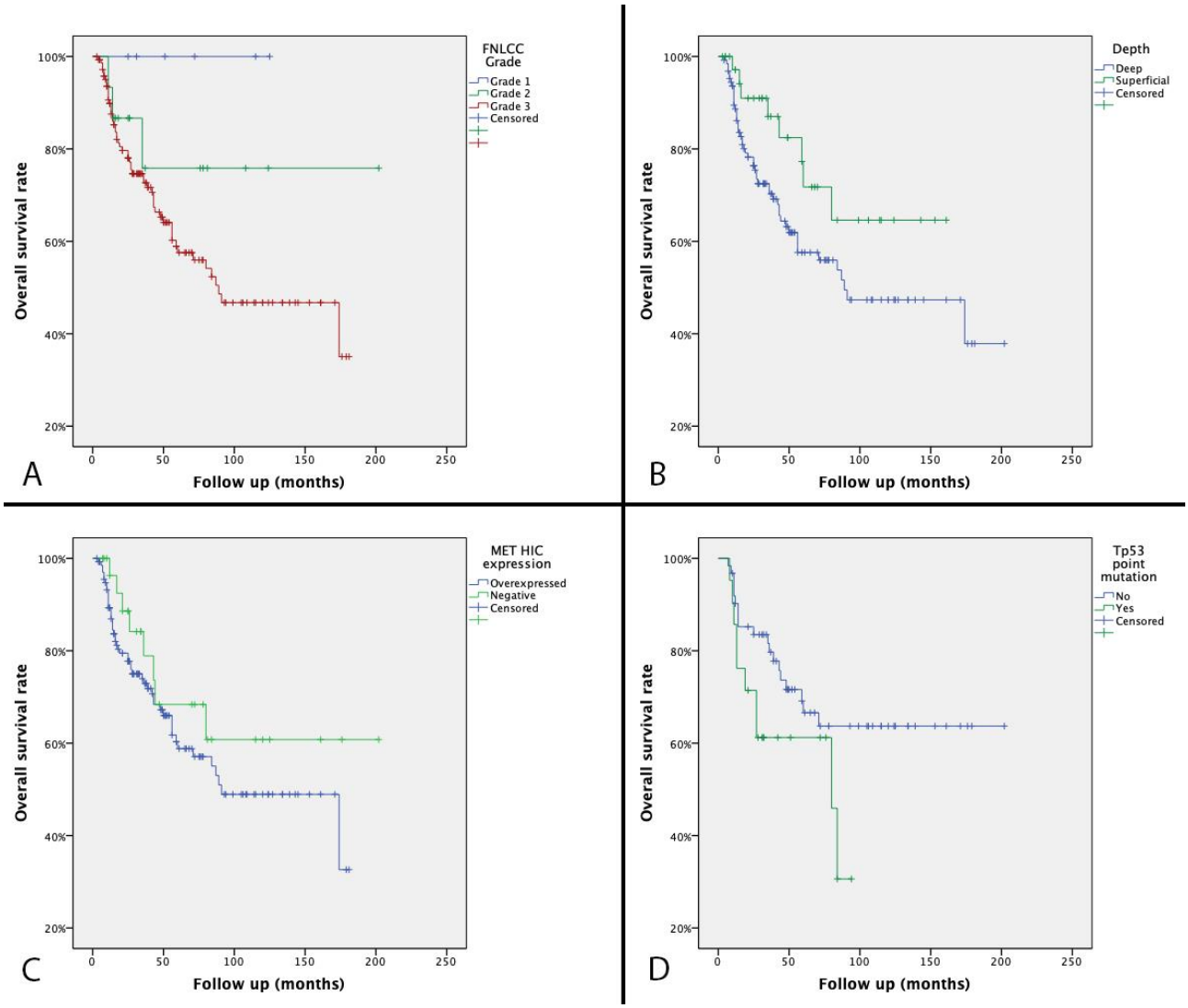

Figure 2. Kaplan-Meier survival analysis overall survival curve according to FNLCC grade (A), tumor depth (B), MET HIC expression (C) and Tp53 point mutations (D).

5. DISCUSSION

Even though histology-tailored chemotherapeutic regimens have not yielded results superior to those of standard ChT, [132] growing evidence suggests that different therapeutic approaches must be undertaken for molecularly distinct STS subtypes.[133] The prospect of genomic profiling with derived targeted therapies is now prompting increasing efforts to characterize cancer genomes.[131] However, developing targeted therapies for MFS has been challenging because these tumors characteristically harbor vast numbers of CNV and do not contain a defined fusion product or gene mutation.

Different studies have shown that MFS is among the most highly complex STS. [2,63] Molecular cytogenetic studies have identified a high level of genomic complexity with a recurrent amplification of the chromosome 5p and 7q regions, the biologic significance of which is unknown.[64,65] Many Authors attempted to identify novel molecular markers, not only for prognostication in MFS, but also to serve as possible therapeutic targets, and thereby improve clinical outcomes. However, the molecular pathogenesis of MFS remains incompletely understood.[38]

To the best of our knowledge, no previous large series tried to correlated protein expression and genetic profile to patients' prognosis in primary MFS of the extremities.

In the present series, Tp53 point mutations were observed in 25.3% (21/83) of the analyzed cases. Heitzer et al. [71] previously found a higher prevalence of somatic mutations in Tp53 (44%) but in a series which was smaller and heterogeneous in terms of grade of MFS.

We found that 21.4% MFS had both a point mutation and a Tp53 gene deletion, 61.9% only a gene deletion and 2.3% only a point mutation in Tp53. Tp53 was affected by both mutations and CNV in 74% of cases in a series by Scheipl et al. [134] A recent analysis by The Cancer Genome Atlas found that the most common CNV in MFS involve Tp53 and RB1.[135]

Li et al. [136] in a heterogeneous series which included 64 MFS and 30 UPS, identified deletions of Tp53 in 47 samples (50%). Of the 80 samples with sufficient quality and quantity of paired normal and tumor DNA for NGS, loss-of-function mutations were identified in the Tp53 gene in 27 samples (33%). They observed that CNV correlated with HIC staining of p53 among the 88 samples for which paraffin-embedded tissue was available. In our series, positive p53 HIC was observed in only 19.3% of the cases.

A «double hit» in Tp53, which made it a possible "driver gene", was observed in 43 (51.2%) cases, but we did not find any correlation between p53 HIC and the presence of a "double hit" in Tp53. This might be partially explained because of the type of antibody used for HIC. Interestingly, Tp53 «double hit» was associated to high FNLCC grade (p=0.045), with no "double hit" observed among

grade 1 MFS. These data suggest that Tp53 alterations might be an early event and act as a driver for tumor progression in a subset of MFS. Heitzer et al. previously observed that grade1 areas of MFS had a higher number of total focal events than grade 3 areas of the same tumor. [71] Intriguingly, patients with a "double hit" in Tp53 had an increased risk of LR, even though it seemed not to influence OS. Nonetheless, Tp53 point mutations strongly correlated with both a worse local control and worse OS. Ogura et al. observed that the alteration of any of cell cycle regulators, including Tp53, was associated with poorer overall survival. [36] However, the Authors didn't differentiate among different genes altered nor the type of alteration (CNV, point mutations). A single mutation might prevent the Tp53 tetramer from binding DNA, thus losing its function. [130]
Other series had previously reported further prognostic genes including CDK6, AMACR, SKP2, EZR, and MET, which were also significantly upregulated in MFS. [36,73,81,87,137] Met gene encodes a proto-oncogene which has been shown to be over-expressed in many tumors and its overexpression usually correlated with a poor prognosis. [73] In human cancers, aberrant c-Met signaling has been shown to result from diverse HGF ligand dependent and independent mechanisms, including activating mutation, autocrine/paracrine HGF stimulation, and overexpression with or without gene amplification.[138,139]
We found MET immunohistochemistry overexpression in 81.8%, mostly in high FNLCC grade. The prevalence of HIC overexpression was higher than reported by Ma et al. [121] (46.7% in 30 MFS), who also reported overexpression of Met among FNLCC grade 3 tumors. We found that HIC expression of MET protein was significantly higher in MFS with unfavorable prognosis in terms of LR and a tendency toward worse OS, similarly to previous reports of Lee et al. [73] Several Authors have previously reported polysomy of the MET gene locus on chromosome 7q in MFS and linked MET overexpression to an aggressive MFS biology.[73,121,137]
By Digital PCR analysis, we found that 64.3% of the analyzed cases had an amplification of MET proto-oncogene. This was mostly observed among high FNLCC grade, even though MET was also amplified in all (4/4) grade 1 tumors. Ma et al. [121] also reported that MET overexpression or chromosome 7 polysomy might have a high risk of LR and metastasis, but their series included only 15 cases.

Discrepancies between MET copy number gains and HIC expression may be due to several factors. It has become more explicit that the upregulatory mechanisms of MET protein expression appears more complex than earlier thought and that most mechanisms identified thus far, such activation of other oncogenes and transcription factors, inactivation of Tp53 tumor suppressor, hypoxia, and so on, are known to increase MET gene transcription. [117,120,139] More recently, several small non-coding microRNAs, including miR-34b, miR-34c, miR-199a*, miRNA-1, and

miRNA-206, have been proved to upregulate MET protein expression in a variety of cancers by either targeting MET mRNA for degradation or repressing its translation post-transcriptionally.[114,139] The series by Scheipl et al.[134] frequently displayed alterations in genes regulating DNA packaging, accessibility to transcription factors and other epigenetic modulators. These recurrent mutations in epigenetic modulators indicate a role of epigenetic regulation in sarcoma pathogenesis.[135] Ogura et al. [36] also identified recurrent mutations in regulators of the epigenome, represented by ATRX, TET2, SETD2, HIST1H3B, and WT1 (16%). Other heterogeneous STS series findings indicate the presence of epigenetic differences across primary and Metastatic/ Recurrent tissue samples.[140] However, no specific methylation pattern was identified for MFS, which showed the largest variation in methylation levels compared to any other STS and which was independent of FNLCC grade 3. [140]

The study has limitations that must be acknowledged. First, the study was retrospective and, therefore, it was subject to inherent limitations and biases. Second, different sample size as well as the analysis of samples from different areas of the same tumor were analyzed to look for mutations and CNV analysis and HIC evaluation. This should partially account for the discrepancy in implications between MET and p53 protein expression and their gene dosage [66] as well as the correlations with clinicopathological factors. The limited follow up in some patients might affect the results although the statistical methods used have taken this into account. Also, the present analysis was exclusively performed on MFS at a single timepoint, thereby providing only a snapshot of the disease. To study tumor evolution and to identify progression from early stage to advanced disease, or to detect early local recurrence, longitudinal studies are required. Finally, other mutations detected by NGS (such as KRAS and PIK3CA) and previously reported in other series [36] have not investigated further.

Nevertheless, we report data from a selected cohort of patients including only adult patients affected by primary, MFS of the extremities. Further studies may be required to confirm these results and a validation should be performed on a large-scale independent database prospectively.

The present series confirms that Tp53 and MET are frequently altered genes in myxofibrosarcoma of the extremities. Both CNV and point mutations appear to play important roles in MFS tumorigenesis. However, point mutations in Tp53 seem to influence the risk of both local recurrence and survival.

Both Tp53 and MET are potentially drugable targets and are actively being used for precision medicine in different tumor entities. [141] Thus, patients suffering from advanced myxofibrosarcoma might benefit from expanded molecular evaluation to detect drug-able targets which may also differ from stage to stage of the same disease.

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