Modern Pathology (2010) 23, 1379–1392

Prognostic implication of MET overexpression in myxofibrosarcomas: an integrative array comparative genomic hybridization, real-time quantitative PCR, immunoblotting, and immunohistochemical analysis

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It remains obscure in myxofibrosarcoma about the basis of tumorigenesis, progression, and metastasis. Chromosome 7 gains are common in some sarcomas, including myxofibrosarcoma, whereas the specific oncogenes are yet to be characterized. We performed an integrative study of *MET* gene at 7q31.2 to elucidate its implication in myxofibrosarcoma. Focused on candidate oncogenes on chromosome 7, 385K array comparative genomic hybridization was used to profile DNA copy number alterations of 12 samples. *MET* transcript was successfully quantified by real-time RT–PCR for 16 laser-microdissected tumors and two myxofibrosarcoma cell lines (NMFH-1, OH931). MET immunoexpression was assessable in 86 primary localized tumors with follow-up. To analyze endogenous MET expression and activation, NMFH-1 and OH931 cells, both with wild-type *MET* gene, were subjected to Western blotting and hepatocyte growth factor-treated NMFH-1 cells were evaluated for the kinetics of MET tyrosine phosphorylation. Non-random large-scale gains on 7q were detected in five cases, delineating three recurrent amplicons, 7q21.11–7q21.3, 7q22.1–22.3, and 7q31.1–7q32.3, in which the locus of *MET* displayed increased copy number, among others. *MET* mRNA was upregulated in OH931, NMFH-1, and nine tumors (56%), whereas neither gene dosage nor mRNA expression of *MET* was associated with clinicopathological factors. In contrast, MET protein overexpression, present in 67% of cases, was highly related to deep location ($P=0.004$), higher grades ($P=0.001$), and more advanced stages ($P<0.001$). Importantly, MET overexpression independently portended inferior metastasis-free survival ($P=0.004$) and overall survival ($P=0.0221$). Expressing activating phospho-MET at Tyr^{1234}/Tyr^{1235}, OH931 cells had more abundant total MET than NMFH-1 cells, whereas the latter became promptly phosphorylated on stimulation of

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This work has been presented in part in the 98th annual meeting of United States and Canadian Academy of Pathology, Boston, USA, 7–13 March 2009
Received 31 March 2010; revised and accepted 10 May 2010; published online 16 July 2010

hepatocyte growth factor. In primary myxofibrosarcomas, MET overexpression, as a frequent event, is likely driven by 7q gains with mRNA upregulation, associated with important prognosticators, and independently predictive of worse outcomes, highlighting its possible causative function in tumor aggressiveness and potentiality as a therapeutic target.
Modern Pathology (2010) **23,** 1379–1392; doi:10.1038/modpathol.2010.128; published online 16 July 2010

Keywords: HGF; MET; myxofibrosarcoma; 7q gain

Myxofibrosarcoma is a common sarcoma characterized histologically by multinodular growth of spindle to polygonal sarcoma cells within variably myxoid stroma containing long curvilinear vessels.[1] Clinically, increased tumor grading and staging are frequently seen in myxofibrosarcomas after relentless local recurrences, which may eventually lead to metastatic diseases.[1–3] Especially for those at the low-grade end, it seems challenging to predict acquisition of invasive and metastatic properties for individual cases by histological evaluation.[1–3] Furthermore, metastatic myxofibrosarcomas are often refractory to current treatment strategies and constitute the primary cause of sarcoma-related death.[1,3,4] To develop novel therapeutic interventions, it is highly desirable to elucidate the molecular determinants correlated with tumor invasion and metastatic spread in myxofibrosarcoma progression.

Somatic alterations in cellular DNA underlie most human cancers. Gains of gene copy number drive expression of oncogenes, whereas decreased gene dosage by hemizygous and/or homozygous deletion results in inactivation of tumor suppressor genes.[5] The prospect of genomic profiling with derived targeted therapies is now prompting increasing efforts to characterize cancer genomes.[5] There is mounting evidence that regional gains and/or high-level amplifications on chromosomal arm 7q are recurrently found in various types of bone and soft tissue sarcomas,[6–12] including myxofibrosarcomas.[8] However, as for myxofibrosarcomas, the pathogenetically relevant target oncogenes on 7q and their prognostic implications remain largely unknown.

Compared with conventional comparative genomic hybridization (CGH), array CGH (aCGH) is promising to narrow down imbalanced chromosomal regions.[5,8,13,14] In this series, we applied ultrahigh-resolution tiling-path aCGH to pinpoint breakpoints of DNA copy number alterations (CNAs) in chromosome 7 for 12 myxofibrosarcomas, so as to identify candidate oncogenes implicating its tumorigenesis. Special attention was given to chromosomal segments harboring the established oncogenes, such as hepatocyte growth factor (*HGF*) at 7q21 and *MET* at 7q31. Acting as a multi-functional cytokine on cells, HGF is typically secreted by mesenchymal cells as a single-chain propeptide that requires proteolytic cleavage to generate an active α/β heterodimer.[15–17] *MET* encodes the 170-kd precursor of a transmembrane receptor tyrosine kinase, which constitutes the only known high-affinity receptor of HGF after cleavage into the 50-kd α subunit and 140-kd β subunit and linkage by disulfide bonds.[15–17]

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.[15–19] A subset of common carcinomas is known to harbor amplified *MET* gene (eg gastric cancers, 10–20%; lung cancers, 7%; metastatic colorectal cancers, 9–18%),[15,17,20] which is therapeutically relevant in prediction of response to selective MET tyrosine kinase inhibitors.[20] Despite more frequent MET overexpression in higher-grade lesions, *MET* gene amplification was not found by Southern blots in 35 various soft tissue tumors tested,[21] unlike that observed in 7,12-dimethylbenz(a)anthracene-induced rat sarcomas.[21,22] Given its crucial function in tumor biological function, MET has emerged as a promising target for cancer therapy.[15,17,23] In this context, we conducted by far the first systematic study to elucidate the underlying molecular aberrations and mRNA abundance of MET in selected fresh tumor samples and its protein expression and prognostic significance in 86 archival primary localized myxofibrosarcomas. Furthermore, the endogenous expression level and activation status of MET were examined by Western blotting for two myxofibrosarcoma cell lines (OH931 and NMFH-1), with the lower MET-expressing NMFH-1 cells also evaluated for the kinetic alteration of MET phosphorylating activation in response to exogenous HGF treatment.

Materials and methods

Cell Culture

The OH931 and NMFH-1 myxofibrosarcoma cell lines were established and kindly provided by Dr Bridge[24] and Dr Ogose,[25] respectively. Both sarcoma cell lines were grown in RPMI 1640 medium supplemented with fetal bovine serum (20% for OH931; 10% for NMFH-1), 1% penicillin/streptomycin, and 1% L-glutamine. In addition, 1% HEPES was also added to the RPMI1640 medium to culture OH931 cells. The CCD966SK dermal fibroblast cell line was purchased from the repository of Taiwan BCRC and cultured in minimum essential medium (Eagle) in Earle's BSS containing 10% of heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, and 1% of non-essential amino acids.

Table 1 Clinicopathological characteristics of 12 myxofibrosarcomas subjected to genomic profiling by array comparative genomic hybridization

Cases	*Depth*	*Size (cm)*	*Myxoid areas (%)*	*Mitotic rate*[a]	*Atypical mitosis*	*Tumor necrosis*	*FNCLCC grade*	*AJCC stage*	*Status*
MFSa1b	Deep	≥5, <10	10	13	Yes	Yes	2	3	Recurrent
MFS6c	Deep	≥5, <10	10	24	Yes	Yes	3	3	Recurrent
MFS7b	Deep	≥5, <10	55	12	No	No	2	3	Recurrent
MFS8b	Deep	≥10	35	10	Yes	Yes	2	3	Primary
MFS19b	Superficial	<5	60	2	No	No	1	2	Primary
MFS64	Deep	≥5, <10	60	8	Yes	Yes	2	3	Primary
MFS84b	Deep	≥5, <10	40	31	Yes	No	2	3	Recurrent
MFS86	Superficial	<5	45	6	Yes	No	1	2	Recurrent
MFS49a	Superficial	≥5, <10	50	45	Yes	Yes	3	2	Primary
MFS96b	Deep	≥5, <10	75	17	No	Yes	2	3	Recurrent
MFS99b	Deep	≥5, <10	10	24	Yes	Yes	3	3	Primary
MFS101	Deep	<5	95	1	No	No	1	1	Primary

[a]Per 10 higher power fields.

Sequencing of *MET* Gene

Both OH931 and NMFH-1 myxofibrosarcoma cells were extracted for genomic DNA to perform extensive sequencing of *MET* gene, covering the extracellular semaphorin domain, IPT repeats, juxtamembrane domain, and tyrosine kinase domain, based on the earlier reported mutation frequency and methodologies with minor modifications.[16] The sequences of primer pairs will be provided on request.

Patients and Tumor Materials

The criteria of histopathological diagnosis and assessment for various parameters were elaborated in our earlier publications.[3,4,26] Histological grading and staging were classified according to updated *French Federation of Cancer Centers (FNCLCC) scheme*[27] and the 6th edition of *American Joint Committee on Cancer (AJCC)* system, respectively. Retrospective clinical data collection and tissue procurement were in accordance with the local guidelines of research ethics (97-1110A3). For genomic profiling, aCGH was used to evaluate somatic CNAs in 12 fresh tumor specimens (six primary, six recurrent). To evaluate the reliability of CNAs obtained from aCGH profiling, 17 selected fresh specimens of myxofibrosarcomas were subjected to laser capture microdissection (LCM) for quantification of *MET* mRNA expression by real-time reverse-transcription PCR (RT–PCR). To perform MET immunostain, we retrieved representative paraffin-embedded blocks of primary localized myxofibrosarcomas of 86 patients from the archives of three tertiary medical centers. In the absence of preoperative neoadjuvant radiation or chemotherapy, these 86 patients, all with available clinical follow-up, received surgical excision with curative intent between 1986 and 2005. Postoperative adjuvant radiotherapy ($n=21$) and/or chemotherapy ($n=7$) were randomly given in a minor subset of high-grade or recurrent cases without consistent guidelines. Details on clinicopathological characteristics of cohorts used in aCGH analysis and immunoexpression were summarized in Tables 1 and 2.

DNA Preparation, Hybridization, and Data Analysis of aCGH

On the basis of H&E sections, regions suitable for DNA extraction were dissected from 12 fresh specimens after trimming necrotic and fibrous areas to assure higher than 80% of tumor content. The reference DNAs were obtained from adjacent normal tissues available in five cases and from normal lymphocytes of gender-matched donors in the remaining seven cases. For each case, 1 μg of total genomic DNA at a concentration of 500 ng/μl was extracted from fresh tissue by using 'recoverall DNA Purification kits' (Ambion, Austin, TX, USA) for hybridization against oligonucleotide microarrays. To ensure DNA integrity, each sample should have an $A_{260/280}$ ratio of 1.7–2.0 and no sign of degradation, as evaluated by gel electrophoresis. Each tiling-path whole genomic microarray had 385K oligonucleotide probes with a median spacing of 6 kb and variable length to achieve a melting temperature of 76°C (NimbleGen Systems, Madison, WI, USA). The procedures of DNA labeling, hybridization, normalization of oligonucleotide arrays, window averaging of contained probes, and data acquisition were carried out by the facility of manufacturer as earlier reported.[13] Briefly, genomic DNAs of tumor and reference samples were sonicated to a size range of 500–2000 bp before labeling by random priming with fluorescent Cy3 and Cy5, respectively. After combining the data of signal intensity with information of the genomic coordinates, the Cy3 and Cy5 signal intensities are

Table 2 Clinicopathological characteristics and associations with MET immunoexpression in 86 primary localized myxofibrosarcomas

	Number of cases (%)	*MET expression index*	P-*value*
MET expression			
Low expression (<30%)	28 (32.6)		
Overexpression (≥30)	58 (67.4)		
Sex			0.917
Male	47 (54.7)	44.47 ± 29.456	
Female	39 (45.3)	45.13 ± 28.435	
Age (years) (median: 64, range 16 to 84)			0.268, $r=0.053$
≤40	10 (10.5)		
41–50	9 (10.5)		
51–60	15 (17.4)		
61–70	29 (33.7)		
>70	23 (26.7)		
Location			0.961
Extremity	66 (76.7)	44.85 ± 29.560	
Axial	20 (23.3)	44.50 ± 26.994	
Tumor depth			0.004*
Superficial	33 (38.4)	33.94 ± 27.776	
Deep	53 (61.6)	52.31 ± 27.269	
Tumor size			0.003*, $r=0.323$
<6 cm	42 (50.6)		
≥6 cm	41 (49.4)		
Unknown	3		
Mitotic rate (per 10 high power fields)			0.008*, $r=0.285$
≤9	50 (58.1)		
10–19	20 (23.3)		
≥20	16 (18.6)		
% of tumor necrosis			<0.001*, $r=0.391$
0%	52 (60.5)		
<10%	7 (8.1)		
10–49%	25 (29.1)		
≥50%	2 (2.3)		
% of myxoid area			<0.001*, $r=$-0.452
10–25%	8 (9.3)		
25–49%	28 (32.6)		
50–74%	25 (29.1)		
≥75%	25 (32.6)		
FNCLCC grade			0.001*
Grade 1	39 (45.3)	32.05 ± 26.732	
Grade 2	34 (39.5)	53.82 ± 27.110	
Grade 3	13 (15.1)	59.23 ± 26.904	
AJCC stage			<0.001*
Stage I	15 (18.1)	20.67 ± 18.504	
Stage II	34 (41.0)	41.32 ± 27.367	
Stage III	34 (41.0)	59.71 ± 26.854	
Unknown	3		

*Statistically significant.

normalized to one another using Qspline normalization, a robust non-linear normalized method for two-color experiments. The circular binary segmentation algorithm proposed by Olshen *et al*,[28] was used for segmentation of the averaged $\log_2$ ratio data. Each segment was then assigned a $\log_2$ ratio that was the median of 10 contained probes, and the data were centered by the tallest mode in the distribution of the segmented values. To finely delineate the breakpoints of whole array probes, we defined gains and losses as $\log_2$ ratios of $\geq +0.20$ or ≤ -0.20, respectively.

Real-Time RT–PCR to Measure *MET* mRNA Expression Level in Fresh Samples and Cell Lines of Myxofibrosarcoma

To circumvent the contamination of the surrounding non-neoplastic cells, we adopted LCM technology to

isolate pure tumor cells. Three 7-μm sections each were serially cut from 17 snap-frozen tumor tissues, placed onto a PEN-membrane slide, and stained with HistoGene LCM Staining kit to isolate cells of interest by using the Veritas automated LCM system (Arcturus Engineering, Mountain view, CA, USA). Approximately 1500 cells were collected onto the Capsure Macro cap, extracted by Picopure RNA isolation kit at 42°C for 30 min with 50 μl of extraction buffer, and then eluted by purification column (Arcturus Bioscience, Mountain view, CA, USA). The adjacent normal fibromuscular tissues from three snap-frozen specimens were also microdissected in parallel and extracted for RNA to serve as calibrator controls. The amount and concentration of RNA obtained were measured by a nanodrop spectrophotometer (SSP-3000, infinigen, City of Industry, CA, USA). Following the manufacturer's instructions, total RNAs were extracted with RNeasy Mini kit (Qiagen, Valencia, CA, USA) from OH931, NMFH-1, and CCD966SK cells. By using ImProm-II Reverse transcription system (Promega, Madison, WC, USA), total RNA was reverse transcribed in a final volume of 40 μl with the following conditions: 2 μl of total RNA from LCM-isolated cells of each tissue sample (or 2 μg of total RNA from each cell line), 0.5 mmol/l dNTPs, 25 U of RNase inhibitor, 16 μl of RNA eluant, and 4 μl of random primers. The reactions were performed at 42°C for 60 min, followed by inactivation of the enzyme at 70°C for 15 min. Real-time PCR assays to quantify the expression levels of *MET* transcript were performed using the LightCycler instrument 2.0 (Roche molecular diagnostics, Mannheim, Germany). The intron-spanning primers and LON probes for cDNAs of target and housekeeping (*POLR2A*, a.k.a. RPII) genes were ordered from Universal Probelibrary and their corresponding nucleotide sequences were *MET*: sense, agtgggaattctagacacatttca, UPL probe no. 31 (tggtggaa), and antisense, cattcaagaatactgtttgacacactt; *POLR2A*: sense, gcatcatgaacagcgatgag, UPL probe no. 69 (ggaggaag), and antisense, tcatccatcttgtccaccac. Amplification was conducted in duplicate for each reaction for fresh specimens and in triplicate for cell lines with LightCycler TaqMan MasterMix, using 2 μl of cDNAs (or 1000-fold diluted for cell line samples), 100 nmol/l of the probes, and 200 nmol/l of the primers in a final 20 μl of reaction mixture. After 2 min incubation at 40°C to allow for uracil *N*-glycosylase cleavage, Taq DNA polymerase was activated by incubation for 10 min at 95°C. Each reaction of the 45 PCR cycles consisted of 10 s of denaturation at 95°C and hybridization of the probe and primers for 30 s at 60°C. After normalization to *POLR2A*, the relative expression fold of *MET* transcript was then given by $2^{-\Delta\Delta C_P}$, where $\Delta\Delta C_T = \Delta C_T$ ($_{\text{Sarcoma cells}}$)$-\Delta C_T$ ($_{\text{CCD966SK}}$) or $= \Delta C_T$ ($_{\text{tumor specimens}}$)$-\Delta C_T$ ($_{\text{normal fibromuscular tissue}}$), and ΔC_T represented the C_T of *MET* subtracted from the C_T of *POLR2A*. Only samples with C_T value <33 for *POLR2A* were considered to have acceptable RNA quality and included in the analyses.

Immunohistochemistry

For each of the 86 cases tested, MET immunohistochemistry was performed on one 4-μm-thick, formalin-fixed tissue section from a representative block. The tissue sections were then deparaffinized in xylene, rehydrated in graded alcohols, quenched with 3% H_2O_2, microwave heated for antigen retrieval in 0.01 M citrate buffer at pH 6.0 for 15 min, and incubated in 10% normal goat serum for 30 min to block non-specific immunoreactivity. Afterwards, the tissue sections were incubated at 4°C overnight with a primary antibody against total MET (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), washed with PBS, and detected by using the streptavidin-biotin immunoperoxidase method. Diaminobenzidine was used as the chromogen and Mayer's hematoxylin was as the counterstain. One of the authors (JCL), blind to clinicopathological data, independently evaluated the slides. The labeling intensity of MET was classified as negative, weak, moderate, and strong. MET reactivity was predominantly seen in the cytoplasm, with only few cases also exhibiting distinct membranous staining. Therefore, the percentage of tumor cells with moderate or strong cytoplasmic immunoreactivity, with or without membranous staining, was recorded as expression index in 5% increment. After testing a series of cutoff values, MET protein was construed as overexpressed when the expression index was $\geq$30% of tumor cells.

Western Blotting Assays to Assess the Endogenous Protein Expression and Activation of MET in Myxofibrosarcoma Cell Lines

Equal amounts of total protein (25 μg) extracted from OH931, NMFH-1, and CCD966SK cell lines were separated on 4–12% gradient SDS–PAGE gel NuPAGE (Invitrogen, Carlsbad, CA, USA), transferred to PVDF membranes (Amersham Biosciences, Buckinghamshire, UK), and then blocked with 5% skimmed milk in TBST buffer at room temperature for 1 h. Afterwards, the membranes were probed with antibodies at 4°C overnight against total MET (1:200, 3D4; Zymed, South San Francisco, CA, USA), phosphorylated MET at the tyrosine residues 1234/1235 (Tyr^{1234}/Tyr^{1235}, 1:200, D26; Cell Signaling Technology, Beverly, MA, USA), and GAPDH (1:10000, MAB347, Chemicon) for a loading control and then incubated with the secondary antibody at room temperature for 1.5 h. Enhanced chemiluminescence reagents (Amersham Biosciences) were used to visualize the targeted proteins, which were then semi-quantitatively measured by densitometry. The endogenous expression level and activation status of MET in OH931 and NMFH-1

cells were compared with those of CCD966SK fibroblasts.

Evaluation of the Kinetic Alteration of Tyrosine Phosphorylation in Response to Exogenous HGF Stimulation in NMFH-1 Cells

To evaluate the tyrosine phosphorylation of MET, NMFH-1 cells grown in 6-cm dishes were serum starved overnight, cultured with fresh minimal essential medium containing non-essential amino acids, and treated with either 50 ng/ml recombinant human HGF (H9661, Sigma, St Louis, MO, USA) or serum-free medium alone for indicated time intervals. For Western blotting assay, total protein (40 μg each) was extracted from NMFH-1 cells harvested from various batches with or without HGF stimulation at different time intervals, with β-actin (1:3000, MAB1501, Chemicon) serving as the loading control.

Follow-Up and Statistical Analyses

Statistical analyses were performed using SPSS 14 software package. Associations and comparisons of MET mRNA expression or immunoexpression with various clinicopathological parameters were evaluated by Student's *t*-test, one-way ANOVA, Kruskal–Wallis, or Pearson's correlation coefficient test as appropriate. In the 86 patients tested for MET immunoexpression, the observation intervals of follow-up ranged from 2 to 229 months with the median duration of 44.8 months as of January 2008, forming the basis for prognostic analysis. The end points evaluated for survival analyses were overall survival (OS) and metastasis-free survival (MeFS). The cutoffs of various clinicopathological parameters for prognostication were described earlier.[3,4,26] In univariate survival analysis, Kaplan–Meier curves were plotted for each variable and the difference between groups compared by the log-rank test. In Cox multivariate regression model, all significant parameters identified at the univariate level were entered to compare the independent prognostic impact. Student's *t*-test was used to analyze the results of real-time quantitative RT–PCR for cell line samples. For all analyses, two-sided tests of significance were used with $P<0.05$ considered significant.

Results

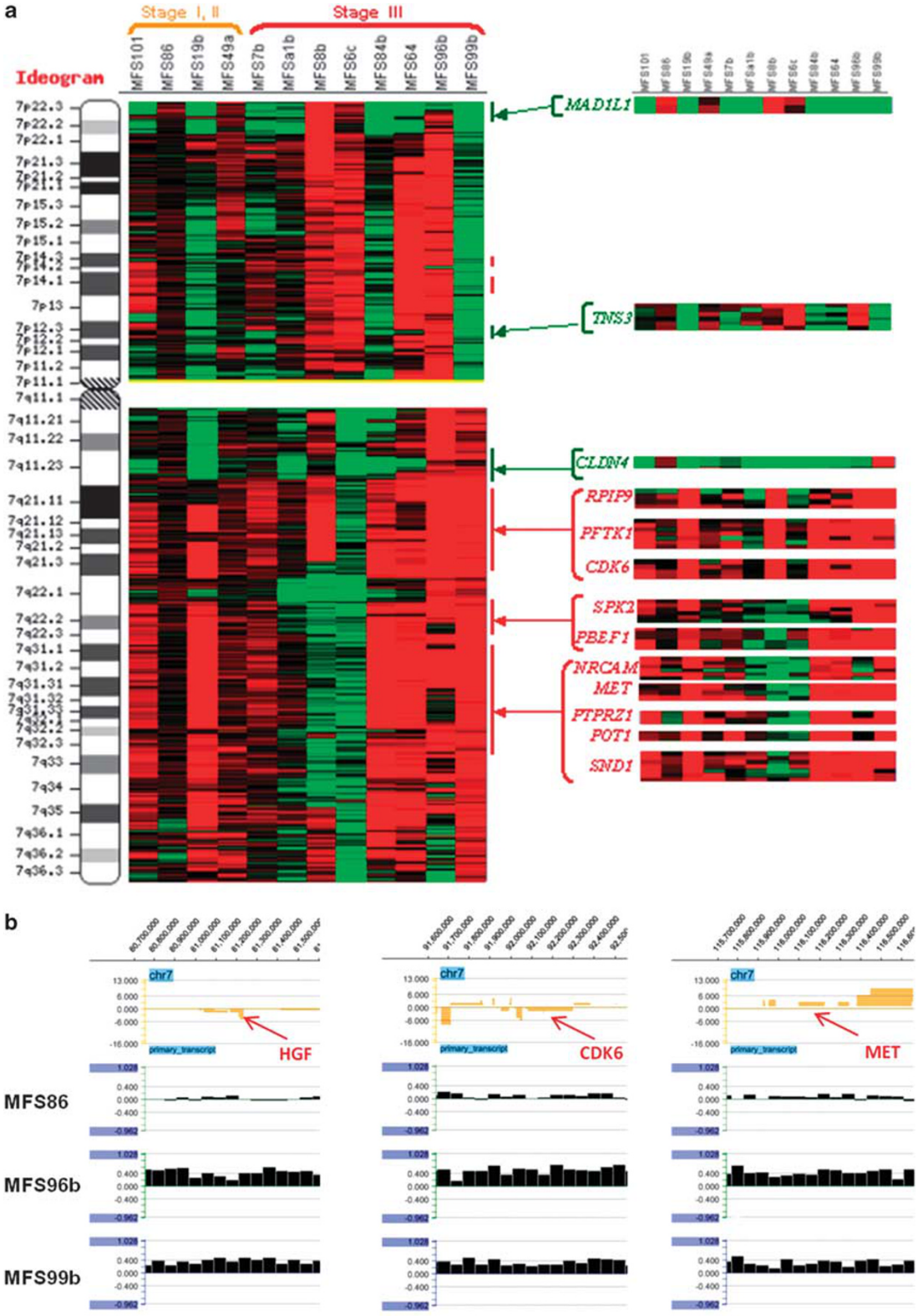
Genomic Profiling of Chromosome 7 by Ultrahigh-Resolution aCGH

Chromosomal imbalances of varying size were detected in all 12 samples subjected to aCGH profiling. Examples of aCGH profiles are shown in Figure 1. Although large-scale chromosomal gains were only seen in two (MFS64, MFS96b) cases on 7p and five cases on 7q (MFS19b, MFS64, MFS84b, MFS96b, MFS99b), non-contiguous segmental copy number gains interspersed by small interstitial regions of no alteration were frequently detected on at least one of both arms. The later finding might be attributed to the higher resolution of current aCGH platform to localize exact breakpoints. Besides, there was no apparent correlation of CNAs of chromosome 7 with the tumor grading, staging, or primary *vs* recurrent status of myxofibrosarcoma. To unravel causal genes showing copy number-driven deregulated expression, we filtered the imbalanced chromosomal regions with non-random alterations at the identical DNA segments in at least 40% of samples. Accordingly, recurrent regions of gain and/or amplification were mapped to 7p14.3 and 7p14.1 on the p arm and 7q21.11–7q21.3, 7q22.1–22.3, and 7q31.1–7q32.3 on the q arm. Among the three amplicons on the q arm, we found that nine, three, and six loci of DNA gains in the chromosomal regions 7q21.11–7q21.3, 7q22.1–22.3, and 7q31.1–7q32.3, respectively, are known to harbor reported putative or established oncogenes implicating tumorigenesis. These included *RPIP9* (7q21.12, a.k.a, *RUNDC3B*),[29] *PFTK1* (7q21.13),[14] *CDK6* (7q21.2),[30] *SRPK2* (7q22.3),[31] *PBEF1* (7q22.3),[32] *NRCAM* (7q31.1),[33] *MET* (7q31.2),[15,20,23] *PTPRZ1* (7q31.32),[34] *POT1* (7q31.33),[35] and *SND1* (7q32.1).[36] However, complete genomic DNA gains at the probes spanning *HGF* were only observed in three cases (Figure 1). On the other hand, DNA losses tended to be small focal changes and recurrently mapped to 7p22, 7p12.3, and 7q11.23 in which three earlier reported candidate tumor suppressor genes, that is *MAD1L1* (7p22.3),[37] *TNS3* (7p12.3),[38] and *CLDN4* (7q11.23),[39] were located.

Figure 1 Array comparative genomic hybridization (aCGH) profiling of myxofibrosarcoma showed a colorimetric gene copy number matrix of chromosome 7. Intensities of red and green coloration generated by Java TreeView indicate an increased or decreased signal ratio for each averaged window of 10 probes, respectively. Each row corresponds to an individual average window and each column represents the aCGH profile of chromosome 7 in a tumor sample. Cytoband pattern of chromosome 7 is shown to the left. Recurrent regions of alterations are denoted by vertical lines in the middle, with gains and deletions of candidate oncogenes and tumor suppressor genes indicated in green and red brackets, respectively. The close-up views of loci harboring genes of most interest are shown to the right. (**b**) Horizontal karyograms of chromosomal region mapped to candidate oncogenes, *HGF*, *CDK6*, and *MET*, in three representative myxofibrosarcoma samples. MFS86 (upper panel) showed no apparent CNAs across these three genes. High-level DNA gains were seen in MFS96b (middle panel) at the loci harboring *CDK6* and *MET*, but not so for *HGF*. In addition to *CDK6* and *MET* genes, MFS99b (lower panel) showed slight DNA gains at the locus of HGF.

MET Transcript Was Frequently Upregulated in Myxofibrosarcoma Specimens but not Associated with Clinicopathological Factors

Next, we addressed whether the mRNA expression of *MET* gene was upregulated in at least a subset of myxofibrosarcoma specimens as inferred from the aCGH findings and whether there was association between the expression level of *MET* transcript and clinicopathological factors. Real-time RT–PCR quantification could be successfully determined with



sufficient RNA yields in LCM-isolated pure tumor cells (Figure 2a) of 16 fresh myxofibrosarcoma samples, including four grade 1, seven grade 2, and five grade 3 cases. As compared with the normal adjacent fibromuscular tissues, we found that 9 out of 16 cases tested showed more than threefold higher expression of *MET* transcripts, suggesting their frequent upregulation in human myxofibrosarcomas. However, there was no significant difference among myxofibrosarcoma specimens regarding various clinicopathological characters, including grading (Figure 2b), staging, and primary *vs* recurrent status, which was generally in keeping with the aCGH findings.

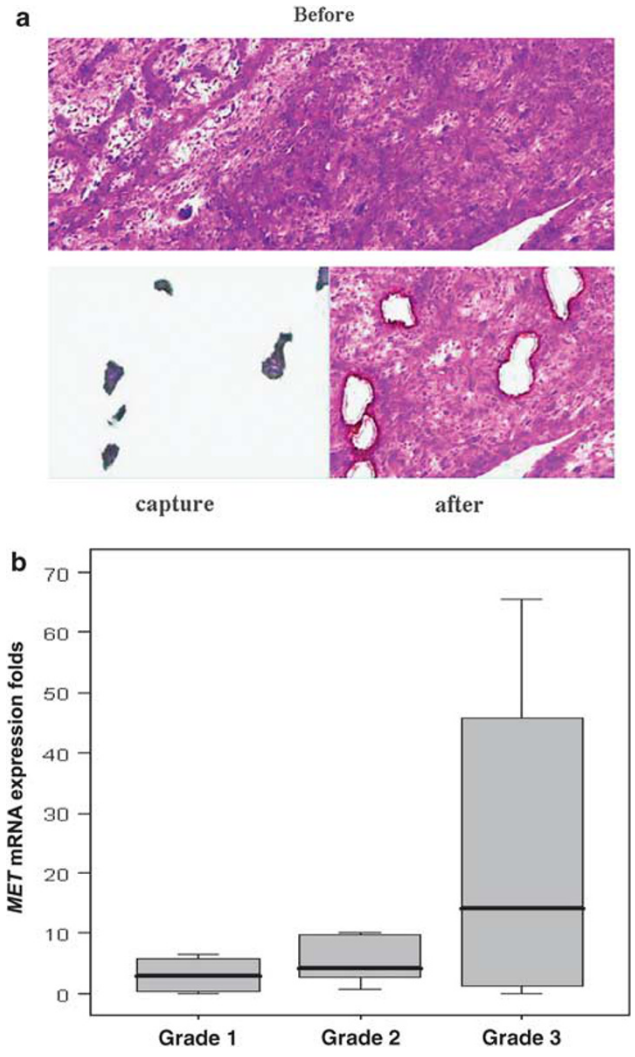


Figure 2 Pure myxofibrosarcoma cells were precisely isolated by LCM technology (**a**) without contamination of surrounding vasculature or inflammatory cells for downstream quantitative real-time RT–PCR assay (**b**) that showed frequent overexpression of *MET* mRNA, but no apparent difference among cases of various grades ($P = 0.115$ by Kruskal–Wallis test).

Expression and Activation of Endogenous MET Kinase in Myxofibrosarcoma Cell Lines

Given activating *MET* mutations identified in few cancer types,[15–17,23] we extensively sequenced the genomic DNAs of OH931 and NMFH-1 myxofibrosarcoma cell lines and found wild-type *MET* gene in both (data not shown). Real-time RT–PCR quantification in triplicate measurements (Figure 3a) showed that the expression levels of *MET* mRNA were significantly higher in two myxofibrosarcoma cell lines than in the non-transformed CCD966SK fibroblasts ($P < 0.001$). In addition, OH931 myxofibrosarcoma cells also had significantly more abundant *MET* mRNA when compared with NMFH-1 ($P < 0.001$). It has become explicit that the Tyr^{1234}/Tyr^{1235} residues of MET kinase must be first activated by phosphorylation to relay MET-mediated signaling, a prerequisite critical for tumor growth and progression.[15,19,23,40] We, therefore, further compared the endogenous expression and activation status of MET protein in myxofibrosarcoma cells *vs* CCD966SK fibroblasts. By Western blotting, OH931 myxofibrosarcoma cells showed high endogenous expression of total MET, whereas it was comparatively lower in abundance in NMFH-1 cells and barely detectable in CCD966SK fibroblasts (Figure 3b, row 2). However, endogenous phosphorylation of MET at Tyr^{1234}/Tyr^{1235} was only detected in OH931 myxofibrosarcoma cells (Figure 3b, row 1).

Tyrosine Phosphorylation of MET in Response to Recombinant HGF Stimulation in NMFH-1 Myxofibrosarcoma Cells

As NMFH-1 cells showed relatively lower endogenous expression of MET protein without apparent phosphorylating activation, we further examined its kinetics of tyrosine phosphorylation of MET in response to exogenous HGF ligand. As shown in Figure 3c, phosphorylation of Tyr^{1234}/Tyr^{1235} was observed at 5 min after HGF treatment and lasted until 10 min. Afterwards, expression of phospho-$MET^{Tyr1234/Tyr1235}$ started to diminish. The total amount of MET protein was decreased at 60 min after HGF treatment, probably because of the degradation by proteasome after internalization.

Correlations of MET Immunoexpression with Clinicopathological Factors in Primary Myxofibrosarcomas

MET protein in primary myxofibrosarcomas was successfully scored for 86 cases (Table 2), including 47 males and 39 females with a median age of 64 years (range, 16–84). On the basis of *FNCLCC* grading scheme, 39 cases were classified as grade 1 (Figure 4a), 34 as grade 2 (Figure 4b), and 13 as grade 3 (Figure 4c). Fifteen (18%), 34 (41%), and 34 (41%)

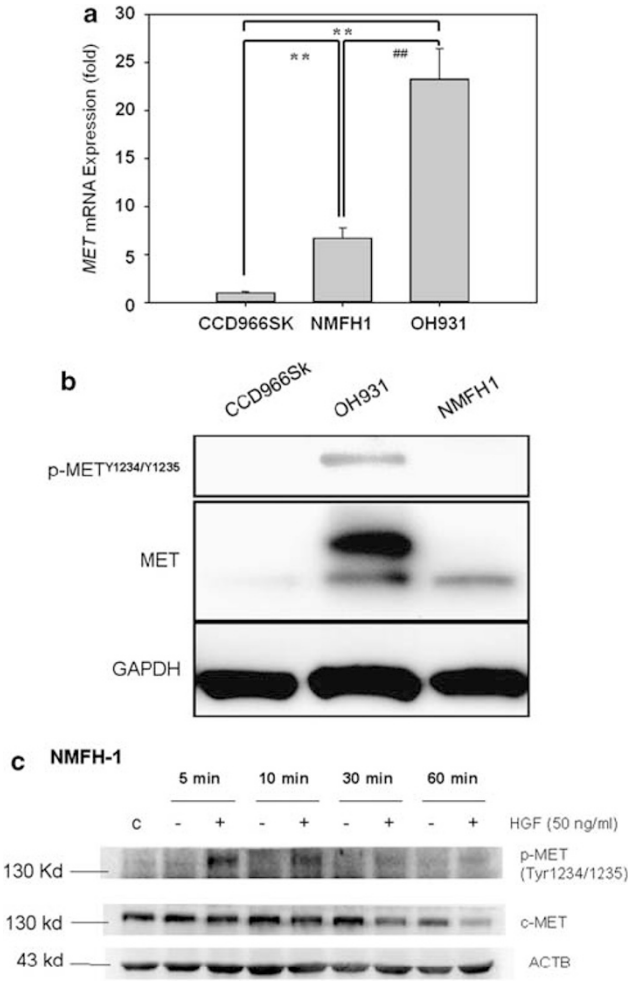


Figure 3 (**a**) Real-time RT–PCR to quantify the MET mRNA expression level: $^{**}P<0.001$ for comparison of OH931 or NMFH-1 myxofibrosarcoma cell line with CCD966SK fibroblasts; $^{\#\#}P<0.001$ for comparison between OH931 and NMFH-1 cells. (**b**) Western blotting assays: when compared with CCD966SK fibroblasts, MET protein overexpression was apparent with activating phosphorylation in OH931 myxofibrosarcoma cells as seen by probing with phospho-MET$^{Tyr1234/1235}$, whereas there was only modest expression of total MET protein in NMFH-1 cells without phosphorylation. (**c**) Kinetics of tyrosine phosphorylation of MET in NMFH-1 myxofibrosarcoma cells in response to exogenous HGF treatment. Cells grown in 6-cm dishes were serum starved overnight, left unstimulated or stimulated with 50 ng/ml HGF for indicated time periods, and then probed with indicated antibodies.

cases were AJCC stage I, stage II, and stage III, respectively. However, the tumor staging of three remote cases could not be determined due to the lack of data of tumor size. As shown in Table 2 and Figures 4d–f, MET was overexpressed in 58 cases (67%) with an expression index $\geq 30\%$, whereas MET expression in the remaining 28 cases was either absent ($n=10$) or low ($n=18$, expression index $\geq 5\%$ but $<30\%$). MET immunolabeling was significantly higher in expression index in those myxofibrosarcomas that were deep seated ($P=0.004$), classified as *FNCLCC* grade 2 or grade 3 ($P=0.001$), and more advanced in AJCC staging ($P<0.001$). In addition, MET expression index was positively related to increased tumor size ($P=0.003$), mitotic rate ($P=0.008$), and percentage of tumor necrotic area ($P<0.001$), but inversely associated with the proportion of myxoid component ($P<0.001$). However, we could not substantiate the associations of MET overexpression with age, gender, and tumor location.

Survival Analyses

At last follow-up, 15 patients died of the tumors, 7 died of other causes, 14 were alive with tumors, and 50 were alive without evidence of tumor. Correlations of patient survivals with immunohistochemical and various clinicopathological parameters at the univariate level are shown in Table 3 and Figure 5. The cumulative 5-year rates of OS and MeFS were 62.7 and 50.1%, respectively, in patients with primary localized myxofibrosarcomas. In agreement with our and others' earlier reports,[1,2,4] several clinicopathological factors significantly correlated with adverse outcomes with respect to both end points, including the deep locations (OS, $P=0.0321$; MeFS, $P=0.0162$), high mitotic count (OS, $P<0.0001$; MeFS, $P<0.0001$), higher histological grades (OS, $P=0.0437$; MeFS, $P=0.0030$), and AJCC stage III (OS, $P=0.0354$; MeFS, $P=0.0056$). However, older age ($P=0.0333$), larger tumor size ($P=0.0124$), and remarkable tumor necrosis ($P=0.0010$) were only significantly related to worse MeFS. More importantly, MET overexpression was found not only significantly associated with inferior OS ($P=0.0032$, Figure 5a), but also strongly predictive of worse MeFS ($P=0.0007$, Figure 5b).

In multivariate analysis (Table 4), MET overexpression remained as an independent adverse prognosticator for both OS ($P=0.0221$) and MeFS ($P=0.0040$), along with high mitotic count ($P<0.0001$ for both OS and MeFS). In addition, MET overexpression also identified patients at more than six- and ninefold higher risks of worse OS and MeFS rates, respectively. However, an older age ($P=0.0003$) at presentation was independently predictive of worse MeFS alone. Other significant factors at the univariate level lost their statistical significance, including histological grading and tumor staging.

Discussion

The management of myxofibrosarcoma is complicated by the lack of good prognosticators to accurately determine tumor invasion and metastatic spread. However, it is perceived that increased clinical aggressiveness of myxofibrosarcoma is accompanied by phenotypic change from predominantly loose myxoid to more cellular solid histology,[1–3] which provides a unique model of exploring

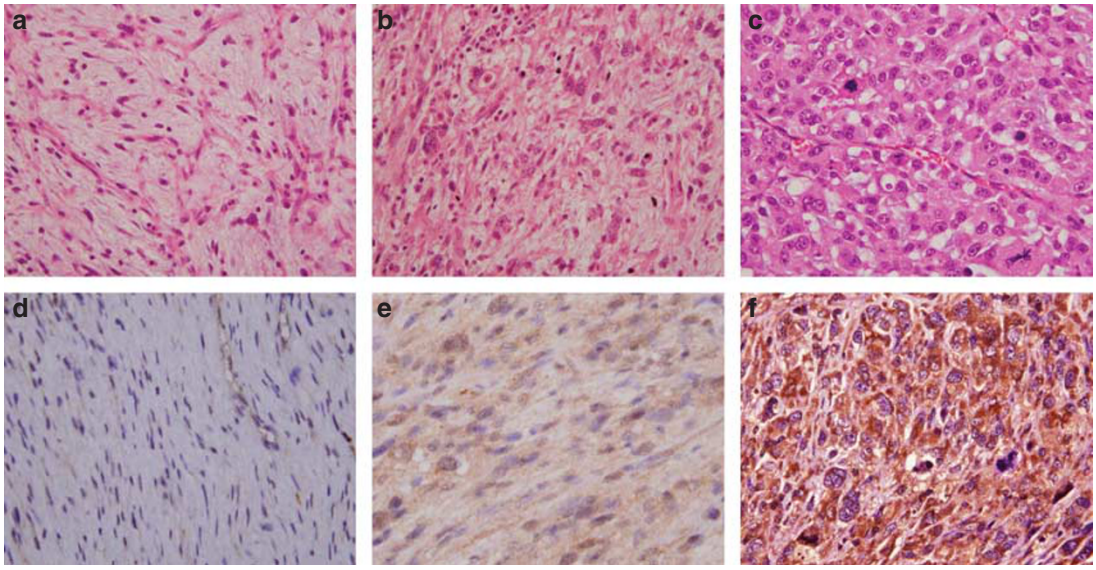


Figure 4 MET immunohistochemical overexpression preferentially detected in myxofibrosarcomas of higher grades. Histological features of one each representative myxofibrosarcoma classified as FNCLCC grade 1 **(a)**, grade 2 **(b)**, and grade 3 **(c)**. The grade 1 lesion showed no staining of MET **(d)**, whereas overexpression of MET was readily found in grade 2 **(e)** and grade 3 **(f)** lesions.

Table 3 Univariate log-rank analyses for prognostic factors in 86 patients with follow-up

Parameters	*Category*	*No. of patients with follow-up*	*OS*		*MeFS*	
			No. of event	P-*value*	*No. of event*	P-*value*
Sex	Male	47	12	0.7680	15	0.3483
	Female	39	10		15	
Age	<60 years	32	7	0.1517	8	0.0333*
	≥60 years	54	15		22	
Location	Extremity	66	17	0.6290	23	0.5839
	Axial	20	5		7	
Tumor size	<6 cm	42	9	0.1762	10	0.0124*
	≥6 cm	41	12		19	
Tumor depth	Superficial	33	5	0.0321*	7	0.0162*
	Deep	53	17		23	
Myxoid area	≥75%	25	3	0.1381	4	0.0576
	<75%	61	19		26	
Necrotic area	<10%	59	13	0.1378	15	0.0010*
	≥10%	27	9		15	
Mitotic count	<20/10 HPFs	70	13	<0.0001*	18	<0.0001*
	≥20/10 HPFs	16	9		12	
FNCLCC grade	Grade 1	39	5	0.0437*	6	0.0030*
	Grades 2 and 3	47	17		24	
AJCC stage	Stages I and II	49	9	0.0354*	12	0.0056*
	Stage III	34	12		17	
MET expression	Low expression (<30%)	28	1	0.0032*	2	0.0007*
	Overexpression (≥30%)	58	21		28	

*Statistically significant *P*-values.
OS, overall survival; MeFS, metastasis-free survival.

stepwise genetic alterations in soft tissue malignancies. Presenting as both continuous large-scale and segmental interstitial gains, DNA copy number gains across both arms of chromosome 7 were detected in our series using ultrahigh-resolution aCGH. However, the frequencies and regions of recurrent CNAs appeared somewhat different from those reported by Ohguri *et al*[8] using bacterial artificial chromosome-

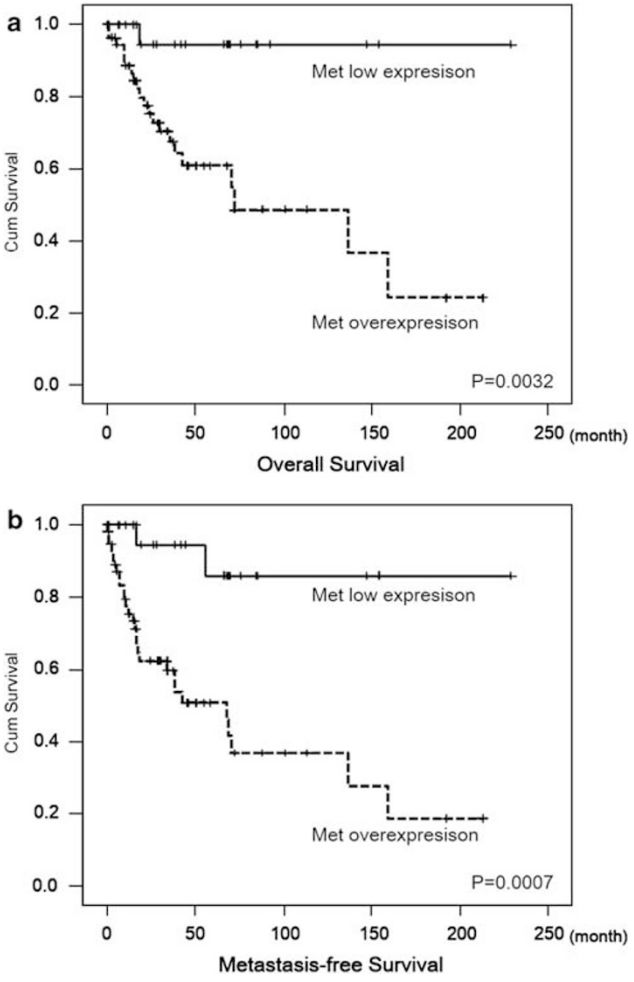


Figure 5 Kaplan–Meier plots to predict overall survival (**a**) and metastasis-free survival (**b**) according to MET immunoexpression status.

based aCGH,[8] which comprised only 1440 target DNA clones to cover the whole genome. Despite being one of the most common CNAs (4/8, 50%) reported by Ohguri *et al*, gains at 7p21.1-p22.1 were present in a minor proportion of our cases, not fulfilling our threshold to define non-random aberrations in aCGH profiling. In this study, three amplicon cores were mapped to 7q21.11–7q21.3, 7q22.1–q22.3, and 7q31.1–7q32.3, respectively, which harbored established oncogenes on the q arm, including *CDK6* (7q21.2) and *MET* (7q31.2). However, the boundaries of these recurrent CNAs did not overlap 7q33–q35, the minimal common region of DNA gains on 7q in the series of Ohguri *et al*.[8]

By using RNA extracted from LCM-isolated tumor cells for validation, 56% of 16 myxofibrosarcoma specimens showed apparently upregulated *MET* transcript by real-time RT–PCR. This finding suggested that *MET* should represent a bona fide oncogene located within regions of chromosomal gain on 7q in myxofibrosarcoma, rather than a co-amplified 'passenger' unrelated to tumorigenesis. Although histological grade and tumor stage of myxofibrosarcoma were identified as univariate adverse prognosticators, neither of these was associated with increased copy number gains or mRNA expression levels of *MET* oncogene. Intriguingly, we found that immunoexpression of MET protein was significantly higher in myxofibrosarcomas with unfavorable features, such as deeper location, higher grades, more advanced stages, and so on. Regarding the correlations with clinicopathological factors, we consider that the relatively fewer case numbers used in aCGH profiling and real-time RT–PCR quantification should not entirely account for the discrepancy in implications between MET protein expression and its gene dosage and mRNA abundance. This argument was partly in keeping with the findings of a karyotypic study of 32 myxofibrosarcomas, showing that chromosomal aberrations were not restricted to higher-grade myxofibrosarcomas, but could also be observed in low-grade tumors, albeit less frequently.[41]

In many tumors, MET protein is actually expressed at levels much higher than in the normal counterpart.[16,17] However, it has become more explicit that the upregulatory mechanisms of MET protein expression appear more complex than earlier thought and that most mechanisms identified thus far, such activation of other oncogenes and transcription factors, inactivation of p53 tumor suppressor, hypoxia, and so on, are known to increase *MET* gene transcription.[15–17,21,23,42–44] 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.[43,45–47] These microRNAs may constitute a novel and critical mechanistic basis to modulate *MET* expression in tumor cells. Through binding of activated HGF to its extracellular domain, MET undergoes oligomerization with subsequent phosphorylation of multiple tyrosine residues at the intracellular domains of the β subunit, thereby modulating its internalization, catalytic activity, and docking of adaptor proteins.[15,18,19,21,23,40,44,48] The interaction between HGF and MET has vital functions in orchestrating the 'invasive growth program' acquired in cells involving various physiological processes, such as embryonic organ morphogenesis and tissue repair.[15,18,19,23,41,48] Besides mitogenic and antiapoptotic activities common to many growth factor receptors, heightened MET activation can stimulate cell–cell detachment, migration, invasiveness, and angiogenesis.[15,18,19,23,40,48]

However, the biological and prognostic implications of MET overexpression in human mesenchymal malignancies vary among series and may be tumor type-dependent.[42,44,49–51] MET ovexpression is

Table 4 Results of Cox multivariate analyses in relation to OS and MeFS

Outcome	*Variable*	*Category*	*RR*	*95% CI*	P-*value*
OS	Mitotic rate	<20/10 HPFs	1.000	—	<0.0001*
		≥20/10 HPFs	7.415	2.786–19.736	
	MET expression	Low expression (<30%)	1.00	—	0.0221*
		Overexpression (≥30%)	6.140	1.298–29.051	
	Tumor depth	Superficial	1.000	—	0.2781
		Deep	1.932	0.588–6.355	
	AJCC stage	Stages I and II	1.000	—	0.6763
		Stage III	0.792	0.264–2.371	
	FNCLCC grade	Grade 1	1.00	—	0.6970
		Grades 2 and 3	1.253	0.402–3.907	
MeFS	Mitotic rate	<20/10 HPFs	1.000	—	<0.0001*
		≥20/10 HPFs	18.289	5.506–60.751	
	Age	<60 years	1.000	—	0.0003*
		≥60 years	5.699	2.220 – 14.626	
	MET expression	Low expression (<30%)	1.00	—	0.0040*
		Overexpression (≥30%)	9.733	2.071–45.749	
	Necrotic area	<10%	1.00	—	0.0777
		≥10%	2.414	0.907–6.427	
	Tumor depth	Superficial	1.000	—	0.1159
		Deep	2.642	0.787–8.867	
	AJCC stage	Stages I and II	1.00	—	0.2020
		Stage III	0.451	0.132–1.533	
	Tumor size	<6 cm	1.00	—	0.5946
		≥6 cm	0.758	0.274–2.101	
	FNCLCC grade	Grade 1	1.00	—	0.9747
		Grades 2 and 3	1.021	0.279–3.739	

*Statistically significant
OS, overall survival; MeFS, metastasis-free survival; HPF, high power fields; RR, risk ratio; CI, confidence interval.

present in a high percentage of human rhabdomyosarcomas and osteosarcomas.[42,44,49] In rhabdomyosarcomas, MET overexpression not only correlates with alveolar histology and advanced group, but also has an important function in the dissemination to bone marrow,[49] whereas only expression of HGF enhances the resistance to radiochemotherapy.[48] Recently, the tumorigenic property of *MET* oncogene in osteosarcomas was further reinforced by a study showing that MET overexpression can drive the transformation of human primary cultured osteoblasts into bone-forming sarcoma cells, both *in vitro* and *in vivo*.[52] In synovial sarcomas, MET is expressed in approximately one-third of cases and co-expression of both MET and HGF, instead of MET expression alone, correlates with adverse outcomes.[51] Conversely, MET overexpression was frequently found in skull base chordomas, but significantly predictive of favorable prognosis.[50] In primary myxofibrosarcomas, we have identified for the first time that MET overexpression was independently predictive of shorter OS and MeFS as well as being associated with tumor size and mitotic rate. Our findings and the reported discrepancies in the implications of MET expression among various sarcoma types indicated its pleiotropic functions in tumor development, progression, and dissemination.

It has become clarified that activating mutations constitutively elicit MET signaling only in few tumor types, such as papillary renal cell carcinomas and gastric carcinomas.[15,17,23] We performed direct sequencing for OH931 and NMFH-1 myxofibrosarcoma cells and found wild-type *MET* oncogene in both cell lines. Actually, MET receptor tyrosine kinase in most cancers is more frequently activated by transcriptionally upregulated or amplification-driven protein overexpression.[15,17,23,43] In this study, NMFH-1 myxofibrosarcoma cells, with lower endogenous MET expression, were responsive to treatment of recombinant HGF ligand by showing phosphorylation at Tyr^{1234}/Tyr^{1235}, two tyrosine residues essential for HGF-dependent activation of MET kinase.[40] Although the activating phosphorylation induced by exogenous HFG ligand only lasted transiently, this finding suggested the possibility of an ectopic autocrine or paracrine loop in myxofibrosarcomas that mediates the HGF-MET signaling pathway. This scenario had been earlier exemplified in osteosarcomas, rhabdomyosarcomas, gliomas, and so on, when the tumors or surrounding microenvironments aberrantly express MET receptor or its HGF ligand.[18,19,48]

In conclusion, our work highlights the value of genomic profiling with ultrahigh-resolution aCGH. In myxofibrosarcomas, it shows recurrent regions of gain harboring oncogenes on 7q and the likelihood of an increased *MET* gene dosage in the induction of mRNA expression. Immunohistochemically, approximately two-thirds of primary localized tumors display MET protein overexpression, which correlates with adverse clinicopathological factors and independently portends worse MeFS and OS. Our

findings 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 myxofibrosarcoma. The latter approach seems conceivable given the availability of several HGF-neutralizing antibodies and MET-directed small molecule inhibitors undergoing clinical trials for other cancers.

Acknowledgements

This work was supported in part by grants from Chang Gung Memorial Hospital (CMRPG870751, CMRPG870752) and Chi-Mei Medical Center (CMFHR 9634). The authors are deeply indebted to Dr Julia Bridge and Dr Ogose for kindly providing OH931 and NMFH-1 cell lines. We are also grateful to genomic core laboratory (CMRPG880251) and tissue bank (CMRPG870461) of Chang Gung Memorial Hospital-Kaohsiung Medical Center for critical technical assistance.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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