ORIGINAL ARTICLE

Cytogenetic analysis of myxoid liposarcoma and myxofibrosarcoma by array-based comparative genomic hybridisation

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Aim: To investigate overall chromosomal alterations using array-based comparative genomic hybridisation (CGH) of myxoid liposarcomas (MLSs) and myxofibrosarcomas (MFSs).

Materials and methods: Genomic DNA extracted from fresh-frozen tumour tissues was labelled with fluorochromes and then hybridised on to an array consisting of 1440 bacterial artificial chromosome clones representing regions throughout the entire human genome important in cytogenetics and oncology.

Results: DNA copy number aberrations (CNAs) were found in all the 8 MFSs, but no alterations were found in 7 (70%) of 10 MLSs. In MFSs, the most frequent CNAs were gains at 7p21.1–p22.1 and 12q15–q21.1 and a loss at 13q14.3–q34. The second most frequent CNAs were gains at 7q33–q35, 9q22.31–q22.33, 12p13.32–pter, 17q22–q23, Xp11.2 and Xq12 and losses at 10p13–p14, 10q25, 11p11–p14, 11q23.3–q25, 20p11–p12 and 21q22.13–q22.2, which were detected in 38% of the MFSs examined. In MLSs, only a few CNAs were found in two sarcomas with gains at 8p21.2–p23.3, 8q11.22–q12.2 and 8q23.1–q24.3, and in one with gains at 5p13.2–p14.3 and 5q11.2–5q35.2 and a loss at 21q22.2–qter.

Conclusions: MFS has more frequent and diverse CNAs than MLS, which reinforces the hypothesis that MFS is genetically different from MLS. Out-array CGH analysis may also provide several entry points for the identification of candidate genes associated with oncogenesis and progression in MFS.

The recent development of cancer cytogenetics has helped to clarify the mechanisms underlying the tumorigenesis of soft-tissue sarcomas. Myxoid liposarcoma (MLS) is the second most common subtype of liposarcoma, accounting for more than one third of all liposarcomas and representing about 10% of all adult soft-tissue sarcomas.¹ It constitutes one of the major myxoid malignancies of the soft tissue along with myxofibrosarcoma (MFS). It is sometimes difficult to distinguish MLS from other myxoid tumours, particularly MFS. A unique chromosome translocation, t(12;16)(q13;p11), resulting in a fusion of the segments of the *DDIT3* gene (also known as *CHOP* or *GADD153*) on chromosome 12 and the *FUS* (also referred to as *TLS*) on chromosome 16, is the key genetic aberration in MLS.² More than 90% of MLSs are cytogenetically characterised by this translocation.³

MFS, also known as a myxoid variant of malignant fibrous histiocytoma (MFH), is one of the most common sarcomas known to affect elderly people. The karyotypic abnormalities in MFH, including its myxoid variant, are usually complex, and multiple numerical and structural rearrangements are frequently found in this type of soft-tissue sarcoma.⁴ However, no chromosomal aberrations specific to MFH or MFS have been identified so far, although telomeric associations, ring chromosomes and dicentric chromosomes are often seen in MFH.⁵

Conventional chromosomal comparative genomic hybridisation (CGH) can detect chromosomal imbalances of the whole genome by a single analysis, and it is also useful in obtaining a bird's eye view of the DNA copy number alterations in the whole tumour genomes. Knowledge of DNA copy number aberrations (CNAs) may facilitate identification of oncogenes and CNAs can be used for tumour classification.⁶ The recently developed, array-based CGH

technique allows a high throughput analysis of copy number changes at high resolution throughout the whole genome and also provides information on genomic regions near the centromere or telomere, which are often difficult to detect by conventional CGH.⁷ Moreover, a gain or loss of DNA copy number detected by array CGH may directly reflect increased or decreased expression of the corresponding gene, and the loci or regions showing high frequencies of copy number gains or losses suggest the presence of oncogenes or tumour suppressor genes. Although the most frequent approaches used to screen tumours for genomic dosage alterations are fluorescence in situ hybridisation and CGH, the application of these two methods is limited, partly because of their poor resolution and the need for high-quality metaphase spreads.

No detailed genomic profiles of MFS or MLS using array-based CGH have been so far available in the literature. We investigated the overall DNA copy number alterations of MLS and MFS by using array-based CGH.

MATERIALS AND METHODS

Materials and DNA extraction

Cases of MLS and MFS were retrieved from the files of the Department of Pathology and Oncology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan. The diagnosis of each case was made according to currently used morphological criteria.¹ In brief, MLSs were diagnosed by the presence of lipoblasts at varying stages of differentiation and stellate or spindle mesenchymal cells, associated with a prominent plexiform capillary network and an abundant myxoid matrix (fig 1A). MFSs were

Abbreviations: CGH, comparative genomic hybridisation; CNAs, copy number aberrations; MDM2, murine double minutes 2; MFH, malignant fibrous histiocytoma; MFS, myxofibrosarcoma; MLS, myxoid liposarcoma

Figure 1 Representative histological features of (A) myxoid liposarcoma and (B) myxofibrosarcoma. (A) Myxoid liposarcoma is composed of spindle or stellate cells and lipoblasts with a plexiform capillary network and an abundant myxoid matrix. (B) Myxofibrosarcoma is made up of atypical and pleomorphic tumour cells with no evidence of lipoblastic differentiation.

characterised by variable myxoid stroma, cellular pleomorphism and a distinctively curvilinear vascular pattern (fig 1B). We found 10 patients with MLSSs and 8 with MFSs. Table 1 summarises their clinical features. Four patients with MLSSs were proved to have the *FUS-DDIT3* fusion gene, by nested reverse transcription-polymerase chain reaction, or *t*(12;16)(q13;p11), by cytogenetic analysis.^{8,9}

Genomic DNA was obtained from snap-frozen tumour tissue specimens according to standard procedures using proteinase K digestion and phenol-chloroform extraction. Normal DNA was isolated from the lymphocytes of healthy men and women donors as a reference for two-colour hybridisations.

DNA microarray slide

We used commercially available genomic DNA microarray slides (MacArray Karyo1400, MacroGen, Seoul, South Korea) that were developed for use in microarray-based CGH assays. The microarray contains 1440 target DNA clones (bacterial artificial chromosome clones, including 356 cancer-related genes) covering the entire human genome and representing regions important in cytogenetics and oncology. Approximately 2 ng of each bacterial artificial chromosome DNA clone comprising the desired target sequences are arrayed in target spots of 17.5×26 mm diameter, and each clone is represented by three target spots.

Fluorescence labelling of sample DNA and hybridisation

Array-based CGH was carried out according to the manufacturer's protocols (MacroGen Inc., Seoul, South Korea). Briefly, tumour DNA and reference DNA were labelled with Cy3-2'-deoxyuridine 5'-triphosphate (Amersham, Tokyo, Japan) and Cy5-2'-deoxyuridine 5'-triphosphate (Amersham), by the random-priming method using a Random Priming Labelling Kit (Invitrogen, Tokyo, Japan). DNAs labelled with fluorochromes were mixed with Cot-1 DNA, denatured at 75°C for 15 min, and then incubated at 37°C for 1 h. The hybridisation mixture was then introduced into the hybridisation chamber of the microarray slide, which was incubated at 37°C for 72 h. After removing the

Table 1 Clinical data of patients with myxoid tumours

	Sex	Age (years)	Site	Size (cm)	Specimen	Histological subtype
Myxoid liposarcoma						
Case 1*	M	38	Thigh	5×2	Recurrence	Myxoid
Case 2*	M	25	Thigh	5×3	Recurrence	Myxoid
Case 3	M	48	Thigh	10×4	Recurrence	Myxoid
Case 4†	F	48	Retroperitoneum	17×10	Recurrence	Myxoid
Case 5	M	34	Shoulder	15×15	Primary	Myxoid
Case 6	F	46	Thigh	7×6	Primary	Myxoid
Case 7	F	48	Thigh	13×9	Primary	Myxoid and round cell
Case 8	M	50	Thigh	3×2	Recurrence	Myxoid
Case 9†	M	73	Thigh	18×11	Primary	Myxoid
Case 10	M	61	Thigh	3×5	Recurrence	Myxoid
Myxofibrosarcoma (myxoid MFH)						
Case 11‡	M	69	Retroperitoneum	12×9	Primary	Intermediate grade
Case 12‡	M	44	Lower leg	4×2	Primary	High grade
Case 13	M	81	Hand	3×2	Primary	Intermediate grade
Case 14	F	66	Lower leg	7×4	Primary	Low grade
Case 15	F	46	Elbow	5×4	Primary	Intermediate grade
Case 16	M	61	Lower leg	4×3	Primary	Intermediate grade
Case 17	F	60	Lower leg	4×3	Primary	Intermediate grade
Case 18	M	61	Upper arm	2×1	Recurrence	Low grade

F, female; M, male; MFH, malignant fibrous histiocytoma.

*The *FUS-DDIT3* fusion gene was detected in these cases by nested reverse transcription-polymerase chain reaction.

†Two cases with myxoid liposarcoma were proved to have *t*(12;16)(q13;p11) by cytogenetic analysis using short-term cultures of tumour cells.

‡The *FUS-DDIT3* fusion gene was not detected in either of the two cases by nested reverse transcription-polymerase chain reaction.

Figure 2 (A) Whole genomic profile of myxofibrosarcoma (case 16) and (B) detailed profile of chromosome 8. Thresholds for copy number gains and losses are shown at $\log_2$ ratios of 0.25 and -0.25 , respectively. Amplicons of distal 8q are present in combination with proximal amplicons to this region (B).

hybridisation chamber from the microarray slide, the slides were rinsed twice in washing solution (50% formamide/ $2\times$ SSC) at 46°C and were then transferred into $2\times$ SSC solution and air dried.

The hybridised and washed microarray slides were then captured with a universal microarray reader system, Genepix 4000A (Axon Instruments, Foster City, California, USA) and analysed with an analysis software specifically developed for the present array-based CGH (ArrayAnalysis, Macrogen). Test (Cy3):reference (Cy5) fluorescence ratio of each sample was determined automatically by using the software. The increase and decrease of sample DNA copy number were determined as thresholds set at $\log_2$ ratios of 0.25 and -0.25 , respectively, according to the standard protocol provided by the manufacturer (Macrogen; fig 2). Amplification (high-level gain) was defined by a $\log_2$ ratio threshold >0.5 as described previously.¹⁰

RESULTS

Table 2 and fig 3 summarise the array CGH findings in myxoid tumours analysed in our study. CNAs were found in all of the eight MFSs and three MLSs. The remaining seven MLSs lacked detectable genomic imbalances.

In MFS, the CNAs detected most often were gains at 7p (the minimal common region 7p21.1–p22.1) and 12q (12q15–q21.1) and a loss at 13q (13q14.3–q34). These alterations were seen in 4 (50%) MFSs. The second most frequent CNAs were gains at 7q (the minimal common region 7q33–q35), 9q (9q22.31–q22.33), 12p (12p13.32–pter), 17q (17q22–q23), Xp (Xp11.2) and Xq (Xq12), and losses at 10p (10p13–p14), 10q (10q25), 11p (11p11–p14), 11q (11q23.3–q25), 20p (20p11–p12) and 21q (21q22.13–q22.2), which were detectable in 3 (38%) MFSs. Several recurrent chromosomal imbalances in MFS were found in every histological grade. Tables 3 and 4 depict the clones with gains or losses for the putative cancer-related genes in at least three MFSs. In addition, 42 clones containing cancer-related genes, including murine double minutes 2 (MDM2), PDGFRA and MET, previously described

as candidate genes of MFH associated with oncogenesis or progression, were gained in 2 of the 8 MFSs. Table 5 shows the amplifications (high-level gain) for all of the clones in the cases with MFS.

In two MLSs, CNAs detected were gains at 8p21.2–p23.3, 8q11.22–q12.2 and 8q23.1–q24.3. Gains at 5p13.2–p14.3 and 5q11.2–5q35.2 and a loss at 21q22.2–qter were seen in the other MLSs. No high-level amplifications were observed in the MLSs.

DISCUSSION

MFS and MLS should be distinguished from each other because of their distinct biological behaviours and potentially different clinical treatments. More than 90% of MLSs are cytogenetically characterised by a unique chromosome translocation, t(12;16) (q13;p11).³ However, in a small number of previous CGH studies of myxoid or round-cell liposarcomas, genomic imbalances were observed less frequently than in other subtypes of liposarcoma.^{11–13}

Schmidt *et al*¹¹ reported that only 8 of the 17 patients with myxoid or round-cell liposarcomas showed changes in DNA copy number, and 4 of the 8 tumours had gains of 13q without any high-level gain (amplification). The other CGH studies of myxoid or round-cell liposarcomas reported an amplification of chromosome 8 as the most frequent chromosomal aberration.^{12–13} These observations were in line with ours: 3 (30%) of the 10 MLSs showed DNA copy number changes in our study, and 2 of them had copy number changes in chromosome 8, with no high-level gains in any. These findings suggest that MLS is mainly characterised by structural aberrations that occur at a chromosome level, but may not disrupt the genomic DNA balance.

By contrast, complex cytogenetic changes are frequently detected in MFH.^{14–16} Some recurrent changes and possible candidate genes associated with MFH have been identified in previous cytogenetic and CGH studies. It has also been suggested that 7q32 is a possible sign of an adverse prognosis and that the loss of chromosome 13 is the most common

Table 2 Areas of gain and loss

	Gain	Loss
Myxoid liposarcoma		
Case 1	–	–
Case 2	–	–
Case 3	–	–
Case 4	–	–
Case 5	–	–
Case 6	5p13.2–p14.3, 5q11.2–5q35.2	21q22.2–qter
Case 7	8p21–p23, 8q	–
Case 8	–	–
Case 9	–	–
Case 10	8p21.2–p23.3, 8q11.22–q12.2, 8q23.1–q24.3	–
Myxofibrosarcoma (myxoid MFH)		
Case 11	1q21.2–q25.2	–
Case 12	1q23, 7q33–q35, 15q14–q15.2	1q31–qter, 2p12–pter, 2q14.2–qter, 3p11–p22, 3q12.1–q21.3, 3q24–qter, 4p , 4q, 5p13.1–p15.33, 8p11, 8p21–p23, 10q21.1–q26.2, 11p11.1–p15.4, 11q22.2–qter, 12p11.2–p13.3, 12q14.2–q24.21, 13q, 16q, 17q11.2–q12, 20p 13q12.11–q34, Yq11.2
Case 13	–	20p11.21–p12.3, 20q12–q13.2, 21q21.3–q22.2, 22q11
Case 14	7p21–p22.1, 9q33–q34, 12p13.32–pter, 12q13–qter, 17p11.2, 17q12–qter, 19p, 19q13, Xp11.2, Xq12	10p11.23–p14, 11p11–p14, 14q31
Case 15	4q13–q25, 9q22.31–q22.33, 10q24.1–q26.1, 11p15.4–pter, 13q14.3–q22.2, 21q22.3, 22q11.1–q11.23	2q32.3–q37.3, 4q12–q35.2, 6q13–q16, 10p12.31–pter, 11p11–p14, 11q23.3–q25, 12p12.3–p13.31, 13q14.3–qter, 14q24–q31, 20p, 22q12.1
Case 16	1p13.3–p32.1, 1p36–pter, 4p15–pter, 5p, 5q11.2–q22.3, 7p21–p22, 8p, 8q11.22–q21.12, 8q24–qter, 12p13.32–pter, 12q15–q21.1, 12q24.12–q24.23, 14q32.3, 18p11.21–pter, 19q13, 20q, 21q22.3, Xp11.2–p22.3, Xq12–q26.3, Xq28–qter	10q25–q26, 17q11, 21q11.2–q22.2
Case 17	3p11.2–p14.2, 4q31–qter, 7p13–p22, 7q22.3–q31.31, 9q22.31–q32, 12q13.3–q21.32, 14q24–q31, 16q12–q22, 17q22–q23.3	1p22.3–p35, 3p25.3–pter, 3q27–qter, 4q32–qter, 6p12.1–p21.2, 10p13–pter, 10q21.1–q26.1, 11q13.5–q22, 11q23–q25, 13q11–q34, 15q11–q21, 16q22.2–q23.2, 18p, 18q12.1–qter, 21q22.13–q22.3, Yq
Case 18	1p36, 3p12, 3q12.3–q13.21, 5p12–p13.3, 5q11.2–q14.3, 6p24.3–p25, 7p, 7q21, 7q32.2–qter, 8p11–p23, 8q11–q21.2, 9p11.2–p21.1, 9q21.33–q33.1, 11q22, 12p11–p12.1, 12p13.32–pter, 12q13–qter, 14q, 15q22–q26, 17p11.2–p13.3, 17q21.33–qter, 20q13.13, 22q13.1–q13.31, Xp11.2–p22.2, Xq12–q13, Xq24–qter	

–, none; MFH, malignant fibrous histiocytoma.

chromosomal imbalance.^{14–15} However, the cytogenetic information on MFS is still limited except for a single CGH study by Idbaih *et al*,¹⁶ who reported that the most frequent gains affecting chromosome subregions or pericentromeric regions of 1, 5p, 19p, 19q and 20q and losses in 1q, 2q, 3p, 4q, 10q, 11q and 13q were also recurrently found.

In our study, MFSSs were shown to have recurrent chromosome imbalances; the most frequent gains were seen at 7p and 12q, and a loss at 13q. In chromosome 12q, tumour-associated genes or oncogenes such as sarcoma amplification sequence, human homologue of MDM2, cyclin-dependent kinase 4 and high-mobility group protein IC have been shown to be amplified in MFH.¹⁷ MDM2

presumably functions as a cellular regulator and mediator of the tumour suppressor protein p53, and increased levels of MDM2 may functionally inactivate p53.¹⁸ Support for this hypothesis comes from studies showing that one third of MFHs had MDM2 amplification and one third had mutations of p53, including homozygous loss, rearrangements and point mutations.^{19–20} In our analysis, the gain of DNA containing high-mobility group protein IC was identified in 3 of the 8 MFSSs, and that of MDM2 in 2. No clone representing cyclin-dependent kinase 4 was included in the array used. In chromosome 13q, we found at least three putative tumour suppressor genes: endothelin receptor type B (13q22), retinoblastoma 1 (13q14) and breast cancer susceptibility

Figure 3 Summarised gains and losses of the DNA copy number in 10 myxoid liposarcomas, as shown by thin dotted lines, and 8 myxofibrosarcomas shown by thick lines. The losses are shown on the left and gains on the right. Each line represents a genetic aberration seen in one sample.

gene (13q12). Alterations to retinoblastoma 1 are a frequent finding in a variety of cancers, including MFH.²¹ Stratton *et al*¹⁹ reported that abnormalities of the *p53* gene were found in several classes of soft-tissue sarcoma, including MFH, and also showed that abnormalities of the retinoblastoma 1 suppressor gene and of the *p53* gene often occur together, thus indicating that the coincidental inactivation of more than one tumour suppressor gene may, in some cases, be required for tumour development.¹⁹ Thus, MFS is often associated with the genomic imbalance of various chromosomal regions, which may harbour genes important for oncogenesis and progression.

Because of the high resolution of the array CGH used, we could detect previously undescribed alterations in the chromosomal regions, such as 7p21.1–p22.1 and 7q33–q35, in MFS. These regions include several genes that are likely candidate genes in other malignant neoplasms. The *TIM1* gene is located in 7q33–q35, which was one of the most frequently gained regions in our study. *TIM1* belongs to the Dbl family of guanine nucleotide exchange factors for ρ -guanine-5' triphosphatases and is specifically expressed in breast tumours, suggesting its role in breast tumour progression. Genes at 7p21.1–p22.1, such as those encoding interleukin 6, which increases vascular endothelial growth

factors and angiogenesis in gastric carcinoma, *TWIST* as a critical regulator of prostate cancer cell growth and an ETS transcription factor *ER81*, which is regulated by oncogenic human epidermal growth factor receptor-2/Neu and activator of thyroid and retinoic-acid receptor in mammary tumorigenesis, may be related to the development of MFS.^{22–25} The genetic pathways and molecular targets associated with the development and progression of MFS are complicated and still remain controversial. Further detailed studies are necessary to clarify the genetic pathway of the development of MFS.

In conclusion, array-based CGH is a feasible and valuable technique that allows us to delineate clearly the genotypes of MLS and MFS. Our study has identified several recurrent genetic imbalances in MFSs, but not in MLSs. These results support the notion that MFS is a myxoid soft-tissue sarcoma

Table 3 Gain at chromosomal region and localised cancer genes in myxofibrosarcoma

Region	Cancer genes	Cases with gain (%)*
4q11	<i>AFP</i>	4/8 (50)
7q33–q35	<i>TIM1</i>	4/8 (50)
8p11.2	<i>POLB</i>	3/8 (38)
8q24.3	<i>TG</i>	3/8 (38)
12p11.2	<i>krag</i>	3/8 (38)
12p13	<i>CCND2</i>	4/8 (50)
12q15	<i>HMGIC</i>	3/8 (38)
17q23	<i>TBX2</i>	3/8 (38)
19p13.3	<i>SH3GL1</i>	3/8 (38)
Xp21.1	<i>OTC</i>	3/8 (38)
Xq12	<i>ED1</i>	4/8 (50)
Xq13.1	<i>TED</i>	4/8 (50)

*Alterations were defined by the log₂ ratio thresholds of 0.25 for copy number gain. Using this threshold, we generated a frequency table. Clones with amplifications in at least three tumours are shown, based on the order of their chromosomal position.

Table 4 Loss at chromosomal region and localised cancer genes in myxofibrosarcoma

Region	Cancer genes	Cases with loss (%)*
1q43	<i>Akt3</i>	5/8 (62)
2p12	<i>REG1A</i>	3/8 (38)
2p23	<i>ALK</i>	3/8 (38)
2q21	<i>CXCR4</i>	3/8 (38)
3p14.2	<i>FHIT</i>	3/8 (38)
3q26	<i>MDS1</i>	3/8 (38)
4q21–q25	<i>IBSP</i>	4/8 (50)
6q27	<i>PDCD2</i>	3/8 (38)
10p15.1	<i>DNMT2</i>	3/8 (38)
10q24	<i>PLAU</i>	3/8 (38)
10q24	<i>HOX11</i>	3/8 (38)
10q25.1–q25.2	<i>FACL5</i>	4/8 (50)
10q25.3–q26.1	<i>DMBT1</i>	4/8 (50)
11p11.2	<i>KAI1</i>	3/8 (38)
11q23	<i>PIG8</i>	3/8 (38)
11q25	<i>ADAMTS8</i>	3/8 (38)
12p13.3–p11.2	<i>LRP6</i>	3/8 (38)
12q15	<i>HMGIC</i>	3/8 (38)
12q22	<i>BTG1</i>	4/8 (50)
17q11.2–q12	<i>LIG3</i>	4/8 (50)
20p11	<i>JAG1</i>	3/8 (38)
Xq22	<i>PLP</i>	3/8 (38)

*Alterations were defined by the log₂ ratio thresholds of –0.25 for copy number loss. Using this threshold, we generated a frequency table. Clones with deletions in at least three tumours are shown, based on the order of their chromosomal position.

Table 5 High-level amplifications in myxofibrosarcoma

Region	Loci names	Cases with high-level amplifications (%) ^a
2p23.2	D2S1980	2/8 (38)
8p11	MOZ	2/8 (38)
8q11.22	SHGC-153 877	2/8 (38)
8q23.1	D8S1638	3/8 (38)
8q24.3	TG	2/8 (38)
12q24.12	SHGC-68 495	2/8 (38)
12q24.13	WI-12 136	2/8 (38)
17q21.2	HER-2 (ERBB2)	2/8 (38)
17q23	TBX2	2/8 (38)
21q21.2	SHGC-8256	2/8 (38)
Xq28	MECP2	2/8 (38)
Xp11.23	SHGC-144 705	3/8 (38)
Xq12	ED1	4/8 (50)

^aAlterations were defined by the log₂ ratio thresholds of 0.5 for high-level amplification. Using this threshold, we generated a frequency table. Clones with amplifications in at least two tumours are shown, based on the order of their chromosomal position.

with a distinct genetic background from MLS and may provide some entry points for the identification of candidate genes associated with the oncogenesis or progression of MFS.

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