Characterization of the 12q Amplicons in Lipomatous Soft Tissue Tumors by Multiplex Ligation-dependent Probe Amplification-based Copy Number Analysis

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Abstract. *Background/Aim: Well-differentiated liposarcoma (WDLPS) and de-differentiated liposarcoma (DDLPS) are characterized by amplified sequences derived from the long arm of chromosome 12. The goal of the present study was to identify, besides the well-known candidate genes, novel relevant genes in these large, complex 12q amplicons. Materials and Methods: Using multiplex ligation-dependent probe amplification, genetic alterations in 19 different genes of 12q12-24 were evaluated in 77 lipomatous soft tissue tumors (including lipomas, WDLPS, DDLPS and pleomorphic liposarcomas). Results: We recorded several amplified genes of 12q13-15, including miR-26a-2, a gene not well studied in liposarcoma, and the well-known and previously described genes murine double minute 2 (MDM2), YEATS domain-containing protein 4 (YEATS4), high-mobility AT-hook 2 (HMGA2), cyclin-dependent kinase 4 (CDK4) and tetraspanin 31 (TSPAN31). Interestingly, the amplification profiles of these six genes were found to be significantly different between WDLPS and DDLPS, more frequently having a high-level status in DDLPS than in WDLPS. In addition, DDLPS were found to have significantly higher mean amplification ratios compared to WDLPS. Moreover, we identified additional genes exclusively amplified in DDLPS in 12q13, 12q21 and 12q24, including glioma-associated oncogene homolog 1 (GLI1), mitogen activated protein kinase kinase kinase 12 (MAP3K12), cyclin-dependent kinase 2 (CDK2), ALX homeobox 1 (ALX1) and T-box 5 (TBX5). Conclusion: Differences in amplification profiles among WDLPS and DDLPS may be related to progression/de-differentiation in liposarcomas and show how in the future amplification profiles could provide an adjunctive tool in characterizing progression to DDLPS. In addition, we identified additional genes exclusively amplified in DDLPS, which may play a role in liposarcomagenesis, particularly in the de-differentiation process.*

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The cytogenetic hallmark of well-differentiated liposarcoma (WDLPS) and de-differentiated liposarcoma (DDLPS) is a supernumerary ring or giant rod marker chromosome, either found as the sole anomaly or in combination with other more or less complex aberrations (1-3). Such supernumerary rings and giant rod marker chromosomes are composed of amplified sequences derived from the long arm of chromosome 12 (12q13-q15), which can be identified with fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization (CGH) (4-6). Genes within the 12q region that are commonly amplified in WDLPS and DDLPS include murine double minute type 2 (*MDM2*) (12q15), glioma-amplified sequence-41 (*GAS41* or *YEATS4*) (12q15), high-mobility AT-hook 2 (*HMGA2*) (12q14.3), cyclin-dependent kinase 4 (*CDK4*) (12q14.1) and tetraspanin 31 (*TSPAN31* or *SAS*) (12q14.1) (1-3, 5, 7-9). Furthermore, glioma-associated oncogene homolog 1 (*GLI1*) (12q13.3) and DNA-damage-inducible transcript (*DDIT3* or *CHOP*) (12q13.3) are rarely amplified, out of which *GLI1* is amplified in DDLPS-alone (5, 8). *MDM2* is the most frequently amplified gene, close to 100%, and *CDK4* is amplified in over 90% of cases. Co-amplification of *MDM2* and *CDK4* is a common feature in WDLPS and DDLPS and is thought to be the initiating 'driving' factor in fat tumorigenesis, resulting in proliferation through combined effects upon *p53* (by inactivating tumor protein p53 (TP53)) and the cell cycle (by Retinoblastoma 1 (*RB1*) phosphorylation), respectively (7).

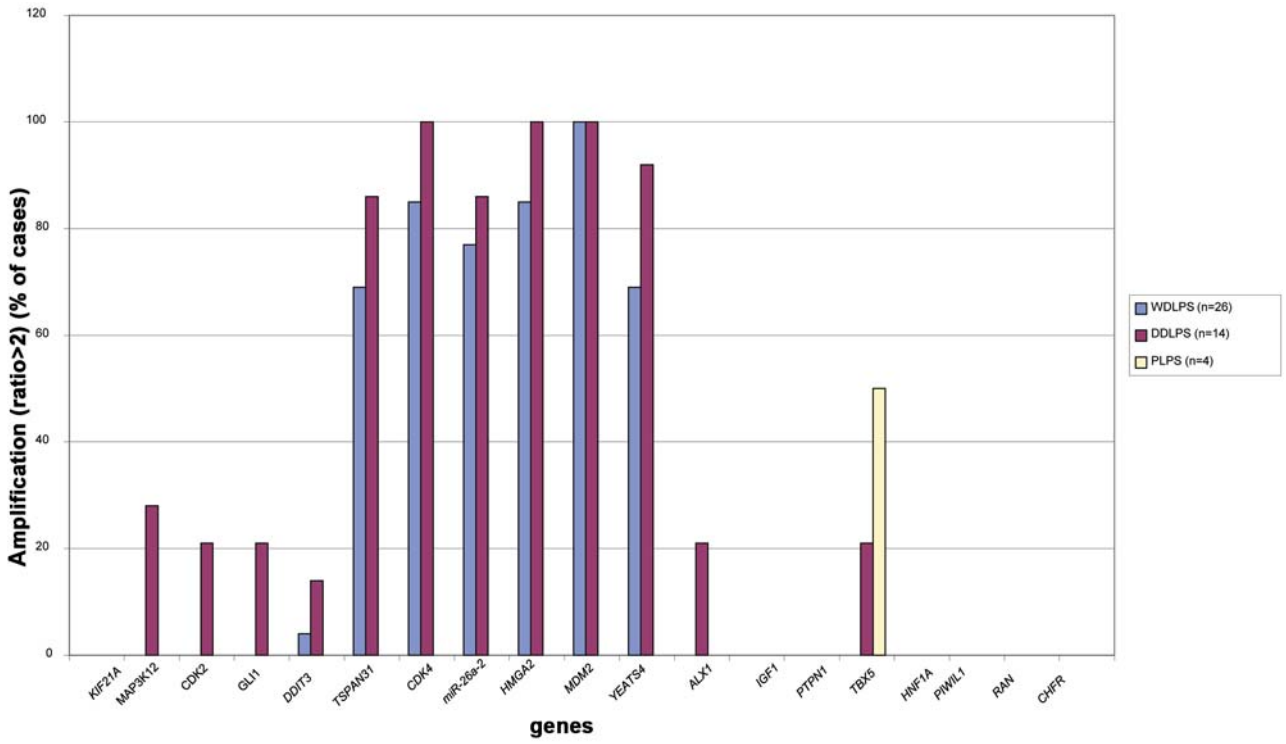

Figure 1. *Frequencies of amplification (ratio>2.0) of the 19 analyzed genes of 12q12-12q24 in well-differentiated liposarcoma (WDLPS), dedifferentiated liposarcoma (DDLPS) and pleomorphic liposarcoma (PLPS).*

Moreover, identifying *MDM2* amplification by FISH is proven to be an adjunctive tool in the diagnosis of lipomatous neoplasms, especially in the diagnosis of a WDLPS, because *MDM2* amplification is absent from 'ordinary' lipomas, and in the diagnosis of DDLPS, especially in cases of a high-grade sarcomas without an atypical adipocytic component (6, 9, 10).

The term DDLPS was first introduced by Evans in 1979 to describe a liposarcoma containing a WDLPS component juxtaposed to areas of high-grade non-lipogenic sarcoma and was believed to arise from WDLPS after several years (11). However, the concept of de-differentiation has undergone evolution in the past several years and the traditional views have been modified by the concept of low-grade de-differentiation in DDLPS and by the fact that about 90% of DDLPS arise *de novo* (synchronous), while only 10% occur in recurrence (metachronous) (4). The risk of de-differentiation is higher in deep-seated tumors, particularly those in the retroperitoneum, and is probably a time-dependent phenomenon (4). Most cases of DDLPS arise in the retroperitoneum. Several recent studies have reported that most sarcomas diagnosed as poorly-differentiated sarcomas and arising in the retroperitoneum are in fact DDLPS and can now be diagnosed as such on the basis of *MDM2* amplification, even in challenging cases of a non-lipogenic undifferentiated sarcoma without an atypical adipocytic component (12-15). Recently, Le Guellec *et al*. stated that peripheral (extremities, trunk wall, head/neck) undifferentiated pleomorphic sarcomas (without areas of a WDLPS component) are actually DDLPS, based on similarities in their clinical characteristics, morphology, genomic profile and follow-up with the group of peripheral conventional DDLPS (16). Since *MDM2* and *CDK4* amplifications are present in both WDLPS and DDLPS, the presence of these amplifications are not triggers for de-differentiation in liposarcomas.

The specific genetic changes that distinguish between WDLPS and DDLPS are still poorly understood. As a group, DDLPS exhibit more complex chromosomal aberrations than WDLPS. Chromosomal imbalances additionally to 12q13-q15 amplicon, including amplifications in 1p32 (including jun proto-oncogene (*JUN*), 1q21-q24 or 6q23 (including the activation of apoptosis signal-regulating kinase 1 (*ASK1*) or mitogen-activated protein kinase kinase kinase 5 (*MAP3K5* gene), have been reported to be more frequent in DDLPS than in WDLPS (5). Co-amplification of 1p32 and 6q23 are mutually exclusive and never seen in WDLPS (4, 17-21). Over-representation of 12q24 has also been observed in DDLPS and other types of high-grade sarcomas, namely malignant peripheral nerve sheath tumors, but is mostly absent from WDLPS (18, 22). However, the specific genes of importance in the 12q24 region are unknown.

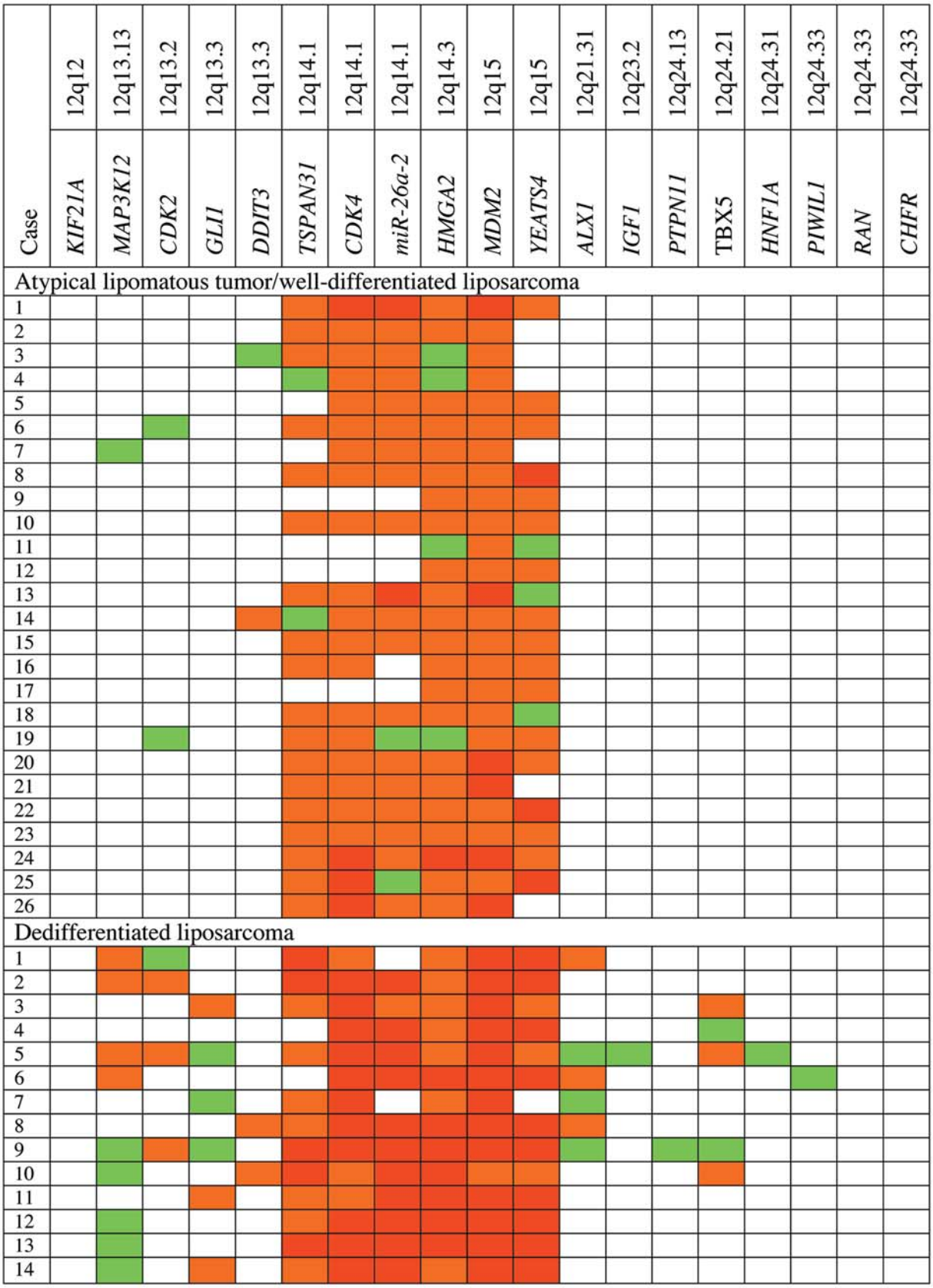

Figure 2. *A detailed multiplex ligation-dependent probe amplification analysis of 19 genes in the q12-24 region of chromosome 12. Green box, ratio 1.3-2.0 (gain); orange box. ratio 2.0-6.0 (amplification); red box. ratio >6.0 (high-level amplification).*

Table I. *MLPA CDK4-HMGA2-MDM2 probe mix containing 12 reference probes and probes detecting copy number changes of 19 different genes located in the 12q12-12q24 region of chromosome 12.*

GENE	LOCATION	MLPA PROBE FOR
KIF21A	12q12	exon 38
MAP3K12	12q13.13	exon 2
CDK2	12q13.2	exon 1
GLI1	12q13.3	exon 4 and exon 11
DDIT3	12q13.3	exon 3
TSPAN31	12q14.1	exon 2
CDK4	12q14.1	exon 3 and exon 8
miR26a 2	12q14.1	exon 1
HMGA2	12q14.3	exon 1, exon 2, exon3, exon 4 and exon 5
MDM2	12q15	exon 1, exon 7, exon 8 and exon 9
YEATS4	12q15	exon 1
ALX1	12q21.31	exon 1
IGF1	12q23.2	exon 3
PTPN11	12q24.13	exon 1
TBX5	12q24.21	exon 9
HNF1A	12q24.31	exon 9
PIWIL1	12q24.33	exon 21
RAN	12q24.33	exon 3 and exon 5
CHFR	12q24.33	exon 1
PAX8 (reference probe)	2q13	
POU1F1 (reference probe)	3p11.2	
PROS1 (reference probe)	3q11.2	
GNRHR (reference probe)	4q13.2	
IL4 (reference probe)	5q31.1	
RET (reference probe)	10q11.2	
RPGRIP1 (reference probe)	14q11.2	
OCA2 (reference probe)	15q13.1	
TGFB1I1 (reference probe)	16p11.2	
RNMT (reference probe)	18q11.21	
NPC1 (reference probe)	18q11.2	
SDHAF1 (reference probe)	19q13.12	

Multiplex ligation-dependent-probe amplification (MLPA) has recently been described as a new, multiplex Polymerase Chain Reaction (PCR) method which detects abnormal copy numbers of up to 50 different genomic DNA or RNA sequences and is able to distinguish differences as few as one nucleotide, allowing for copy number assessment of multiple chromosomal locations in the same PCR, which would normally require several FISH analyses (23-25). This saves time and reduces the amount of sample required. For an MLPA reaction, only 20 to 200 ng of sample DNA are required. Moreover, MLPA requires only small quantities of short DNA fragments, which makes it very suitable for analysis of paraffin-embedded material (26, 27). DNA samples from both fresh-frozen tumors and highly fragmented ones from formalin-fixed, paraffin-embedded tumor samples yield reliable results with MLPA. MLPA has already been applied for identifying *MDM2* and *CDK4* amplifications in lipomatous soft-tissue tumors (28).

In the present study, we performed MLPA analysis on a series of 77 primary lipomatous soft-tissue tumors comprising of 27 lipomas, 28 atypical lipomatous tumors (ALT)/WDLPS, 18 DDLPS and four pleomorphic liposarcomas (PLPS). We used 48 designed MLPA probes targeting 19 different genes in the 12q12-q24 region to identify, besides the well-known candidate genes (*MDM2*, *CDK4*, *HMGA2*, *TSPAN31*, *YEATS4*, *GLI1*, *DDIT3*), novel relevant genes from this region, previously not considered to play a role in 'lipo-sarcomagenesis', in particular the de-differentiation process.

Materials and Methods

Patient material. A total of 27 benign lipomatous tumors (19 lipomas, four intramuscular lipomas and four deep-seated lipomas), 28 ALT/WDLPS (24 lipoma-like, two inflammatory and two sclerosing subtypes), 18 DDLPS and four PLPS, were retrieved from the files of the Pathology Department of the University Hospital of Maastricht. It is important to emphasize that ALT and WDLPS are synonyms describing lesions which are identical both morphologically and karyotypically. Use of the term ALT is determined principally by tumor location and resectability (mostly in peripheral locations such as the extremities and trunk). In sites such as the retroperitoneum and mediastinum, it is usually impossible to obtain a wide tumor-free surgical excision margin of more than 2 cm. In such cases, local recurrence (often repeated and ultimately uncontrolled) is almost inevitable and often leads to death, even in the absence of dedifferentiation or metastasis. At these sites, the term WDLPS is therefore used rather than ALT.

The extremities (n=47) (including 23 lipomas, 15 ALT/WDLPS, five DDLPS and four PLPS) were the most prevalent site of presentation, followed by the retroperitoneum (n=23). The 23 retroperitoneal tumors consisted of 12 WDLPS (including the two inflammatory and two sclerosing subtypes) and 11 DDLPS. Other less common anatomic sites were the trunk (four lipomas), the paratesticular region (one WDLPS and one DDLPS) and the mediastinum (one DDLPS).

The average age at initial diagnosis was 55 (range=41-83) years. The male to female ratio was 43/34. All 77 tumors were primary tumors. The 18 DDLPS presented in a synchronous fashion, *i.e.* the DDLPS was present at the time of first diagnosis (*de novo* DDLPS).

Histologically, the samples of the majority of the DDLPS cases in this study (n=14) (nine in the retroperitoneum, four in the extremities, and one in the paratesticular region) consisted of only the dedifferentiated areas. The absence of a WDLPS component in the majority of DDLPS samples may be explained by inappropriate sampling, disappearance of the WDLPS component, or even by absence of the well-differentiated component in the *de novo* DDLPS cases. In the other DDLPS cases (n=4) (two in the retroperitoneum, one in the mediastinum, and one in the extremities), the samples contained a mixture of both components in which the well-differentiated component was much smaller compared to the dedifferentiated component and was irregularly (heterogeneously) intermingled between the dedifferentiated areas. Therefore it was impossible in these DDLPS samples to macrodissect the well-differentiated and dedifferentiated areas separately. The morphology of the selected DDLPS in our study resembled high-grade pleomorphic sarcoma, high-grade spindle cell

sarcoma, high-grade myxofibrosarcoma or low-grade spindle cell sarcoma (desmoid-type fibromatosis-like).

The material was fixed in 4% formalin and was processed for paraffin embedding. Paraffin sections were stained with hematoxylin and eosin (HE). Detailed histopathological classification of the HE-stained section was performed according to the criteria of the World Health Organization (29). The diagnosis of the cases was confirmed by a combination of histology and dual-color FISH, as golden standard, using *MDM2*- and *CDK4*-specific probes together with a centromere-specific probe for chromosome 12 (CEP12) (Abbott Molecular, Des Plaines, IL, USA) (performed by David Creytens and Ernst-Jan Speel) (9).

Detecting copy number changes of 19 genes located in the 12q12-q24 region of chromosome 12 by MLPA. The design of the MLPA probes (*CDK4-HMGA2-MDM2* probe mix) was as described by Schouten *et al*. (23). (Table I)The probe mix contains 48 probes detecting copy number changes of chromosome 12, including one probe for kinesin family member 21A (*KIF21A*) (12q12; exon 38), one for mitogen-activated protein kinase kinase kinase 12 (*MAP3K12*) (or *DLK*, dual leucine zipper-bearing kinase) (12q13.13; exon 2), one for *CDK2* (12q13.2, exon 1), two for *GLI1* (12q13.3; exon 4 an exon 11), one for *DDIT3* (12q13.3, exon 3), one for *TSPAN31* (12q14.1; exon 2), two for *CDK4* (12q14.1; exon 3 and exon 8), one for *miR-26a-2* (12q14.1; exon 1), five for *HMGA2* (12q14.3; exon 1, exon 2, exon 3, exon 4 and exon 5), s four for *MDM2* (12q15; exon 1, exon 7, exon 8 and exon 9), one for YEATS domain containing 4 (*YEATS4*) (12q15; exon 1), one for ALX homeobox 1 (*ALX1*) (12q21.31; exon 1), one for insulin-like growth factor 1 (*IGF1*) (12q23.2; exon 3), one for tyrosine-protein phosphatase non-receptor type 11 (*PTPN11*) (12q24.13; exon 1), one for T-box 5 (*TBX5*) (12q24.21; exon 9), one for HNF1 homeobox A (*HNF1A*) (12q24.31; exon 9), one for piwi-like RNA-mediated gene silencing 1 (*PIWIL1*) (12q24.33; exon 21), two for RAS-related nuclear protein (*RAN*) (12q24.33; exon 3 and exon 5), and one for Checkpoint with fork-head associated and ring finger (*CHFR*) (12q24.33; exon 1) with amplification products between 122 and 456 nucleotides. Twelve reference probes are included in this probe mix detecting 12 different autosomal chromosome locations, targeting chromosomal regions in which copy numbers changes are not expected in the group of lipomatous tumors (*PAX8*-2q13, *POU1F1*-3p11.2, *PROS1*-3q11.2, *GNRHR*-4q13.2, *IL4*-5q31.1, *RET*-10q11.2, *RPGRIP1*-14q11.2, *OCA2*-15q13.1, *TGFB1I1*-16p11.2, *RNMT*-18q11.21, *NPC1*-18q11.2, *SDHAF1*-19q13.12).

MLPA reagents were obtained from MRC-Holland (Amsterdam, the Netherlands) and the reactions were performed according to the manufacturer's instructions. All 77 cases of lipomatous soft tissue tumors were deparaffinized. After deparaffinization, all tissue sections were pretreated with a 30% solution of Oncor pre-treatment solution and digested with Proteinase K (10 mg/ml) at 37˚C for 10 minutes. After centrifugation, this DNA solution was used in the MLPA analysis. SALSA MLPA buffer (1.5 μl) and the MLPA probes were added to the DNA solution and after a brief incubation for 1 minute at 95˚C allowed to hybridize to their respective targets for 16 hours at 60˚C in a total of 8 μl. Ligation of the hybridized probes was performed for 15 minutes by reducing the temperature to 54˚C and adding 32 μl Ligase-65 mix. After inactivating the enzyme for 5 minutes at 98˚C, 10 μl of the ligase mix was diluted with 30 μl PCR Buffer. Universal PCR primers and SALSA Polymerase were added. All experiments were performed in duplicate.

The PCR reaction was performed using Biometra thermocyclers (Biometra, Goettingen, Germany). PCR amplification of the ligated MLPA probes was performed for 35 cycles (30 s at 95˚C, 30 s at 60˚C and 1 min at 72˚C). The PCR fragments were separated by length and analyzed by using capillary gel-electrophoreses CEQ2000 (Beckman Coulter, Fullerton, CA, USA) or stored at 4˚C until further use. Genomic human DNA (Promega, Fitchburg, WI, USA) and DNA extracted from normal fat tissue were used as references in the data analysis.

Data analysis was performed with Coffalyser NET software (MRC-Holland, Amsterdam, the Netherlands). The peak areas achieved using gene-specific probes for each patient sample were first normalized by the average of peak areas achieved by control probes specific for locations different from the 12q chromosomal arm. A corresponding calculation was performed for DNA from five reference normal samples (normal fat tissue). A final ratio was then calculated by dividing the value from the patient sample by that from the pool of normal reference samples. Finally, the median of all the ratios obtained was taken as the final ratio. Peak values below 0.7 were defined as loss, between 0.7 and 1.3 as normal, between 1.3 and 2.0 as gain, and values above 2.0 as amplified, as previously established in MLPA studies (30, 31). High-level amplification was defined as a ratio higher than 6.

Statistical analysis. Data were analysed using the Mann-Whitney test, chi-square test or Fisher's exact test, when appropriate. Continuous values are reported as the mean±SD. All tests were two-sided and significance was accepted when $p<0.05$.

Results

Frequencies of amplifications (ratio >2.0) for the 19 analyzed genes of the 12q12-12q24 in lipomas, WDLPS, DDLPS and PLPS are depicted in Figure 1. Figure 2 shows the amplification status for each gene in each tumor, with gains depicted in green, amplifications in orange, and high-level amplifications (ratio>6) in red. As illustrated in Figure 2, the amplification level of the six genes located between 12q14.1 and 12q15 (*TSPAN31, CDK4, miR-26a-2, HMGA2, MDM2* and *YEATS4*) were more frequently found at a high-level status in DDLPS than in WDLPS ($p\leq0.0056$). Considering amplified tumors, all six of these genes had a higher mean ratio in DDLPS compared to WDLPS ($p\leq0.001$). The number of amplified genes was also higher in DDLPS than in WDLPS, *i.e*. 6.9±1.3 *versus* 4.8±1.3 ($p<0.0001$). Except for *MDM2*, which was amplified in all cases in both WDLPS and DDLPS, all examined genes between 12q14.1 and 12q15 were more frequently amplified in DDLPS than in WDPLS. However, significance was only reached for *MAP3K12* ($p=0.0225$; $p\geq0.0616$ for the other genes). In our analysis of 19 genes, amplifications of *MAP3K12, CDK2, GLI1, ALX1* and *TBX5* were also only noted in DDLPS, being absent from WDLPS. Interestingly, we detected amplification of *TBX5* in two out of the four PLPS. No amplification of the 19 genes analyzed was seen in lipomas.

As expected, tumor localization was correlated with tumor type, *i.e*. DDLPS occurred more frequently in the retroperi-

toneum, while WDLPS was more localized within extremities. Therefore, it was not possible to investigate whether a statistically significant association exists between tumor localization and amplification status and level of the genes studied independently of the tumor type. However, no obvious differences in amplification status and level of these genes were seen between the group of peripheral WDLPS (ALT) and the abdominal/retroperitoneal WDLPS.

Discussion

Analysis of the 12q13-15 amplifications in WDLPS and DDLPS by MLPA confirmed that the three major amplified genes in this region are *CDK4*, *HMGA2* and *MDM2*. Consistent with previous literature (6, 8), *MDM2* was consistently amplified in WDLPS and DDLPS and was not amplified in lipomas and PLPS, emphasizing that amplification of the *MDM2* gene has an obvious major contribution to the formation of WDLPS and DDLPS, with has a high sensitivity and specificity in diagnosing WDLPS and DDLPS, but does not seem to be sufficient to induce de-differentiation by itself. Amplification of *CDK4* and *HMGA2* was more frequent in DDLPS compared to WDLPS, in line with previous PCR and FISH studies (6, 8, 32, 33).

In our patient population, the number of amplified genes was significantly higher in DDLPS than in WDLPS. This finding is consistent with an earlier array CGH study on WDLPS and DDLPS, in which a higher average number of chromosomal copy number aberrations was observed in DDLPS compared to WDLPS (5). All amplified genes, except for *MDM2*, which was amplified in all cases of WDLPS and DDLPS, were more frequently amplified in DDLPS than in WDPLS. Moreover, *MAP3K12* and *CDK2*, genes proximal (more centromeric) to *CDK4*, were only amplified in DDLPS, although in a small number of cases. *MAP3K12*, a member of the serine/threonine protein kinase family, is involved in the control of cell growth and differentiation and functions as an upstream activator of the *JNK* pathway (34). To our knowledge, *CDK2* amplification in DDLPS has not been recognized before. Overexpression of CDK2, a cyclin-dependent kinase quite similar to *CDK4*, interferes with the Rb mediated pathway through phosphorylation of Rb and inactivation of its cell growth-suppressive effect, promoting proliferation (35). In addition to the major amplifications in 12q13-15, we also detected amplification of the *ALX1* gene (12q21.31) in DDLPS, which to the best of our knowledge has not been previously reported. The precise function of *ALX1* in cancer development is unknown. Recently, *ALX1* was reported to contribute to the promotion of epithelial-mesenchymal transition and the acquisition of malignant characteristics, such as invasion, metastasis and resistance to chemotherapy, in ovarian cancer cells (36).

In this MLPA study, amplification of six of the studied genes located between 12q14.1 and 12q15 (*TSPAN31, CDK4, miR-26a-2, HMGA2, MDM2* and *YEATS4*) was found to be significantly different between WDLPS and DDLPS, with a more frequently high-level status in DDLPS than in WDLPS. In addition, DDLPS were found to have significantly higher mean ratios for the amplified *TSPAN31, CDK4, miR-26a-2, HMGA2, MDM2* and *YEATS4* genes comparing to WDLPS. These findings are in line with *MDM2* FISH ratio data in literature and suggest that increased amplification of these genes may be related to progression to DDLPS (37). MicroRNA *miR-26a-2*, known to target the tumor-suppressor genes phosphatase and tensin homolog (*PTEN*) and *RB1*, is not well studied in liposarcoma and could be an interesting potential target gene (38). Recently, Lee *et al*. described frequent amplification and overexpression of *miR-26a-2* in liposarcoma (39), suggesting its oncogenic role in liposarcoma.

In particular, we found amplification of *TBX5* (12q24) in DDLPS, previously not considered to play a role in liposarcomagenesis. Interestingly, we also detected amplifications of *TBX5* in PLPS in two out of the four studied cases. *TBX5*, a member of the T-box family of transcription factors, plays key roles in cardiac muscle development and limb identity (40). Germline mutations in *TBX5* occur in the Holt-Oram syndrome (41). Recently, Rosenbluh *et al*. a *β-catenin-YAP1-TBX5* complex identified in cancer cell lines essential to the transformation and survival of β-catenin-driven cancer (42). However, the function of *TBX5* in cancer development is largely unclear. These findings are in accordance with those of the study of Rieker *et al*., in which gain of 12q24 was seen in a subset of DDLPS, while being absent from WDLPS (17). However, similar to the 12q13-15 region, the 12q24 region also displayed a very discontinuous pattern and no gains or amplifications in another five genes of the 12q24 region (*PTPN11, HNF1A, PIWIL1, RAN* or *CHFR*) were observed.

In conclusion, our MLPA study confirmed that WDLPS and DDLPS display several amplified genes of 12q13-15, including the well-known and previously described genes *MDM2, YEATS4, HMGA2, CDK4* and *TSPAN31*, supporting the overlap in genetic etiologies of these two tumor types and differentiating them from lipomas and PLPS. Consistent amplification of *MDM2* and frequent *CDK4* co-amplification highlight the clinical and diagnostic importance of these genes in WDLPS and DDLPS. In addition, we described frequent amplification of miR-26a-2 (12q14.1), a novel candidate gene of potential diagnostic and therapeutic importance in WDLPS and DDLPS. Moreover, this MLPA study showed that the group of DDLPS characteristically more frequently has high-level amplification levels and higher mean ratios of different genes in the 12q13-15 region (including *TSPAN31, CDK4, miR-26a-2, HMGA2, MDM2* and *YEATS4*) compared to the group of WDLPS, suggesting that increased amplification of these genes may be related to progression to DDLPS and showing how amplification profiles could

provide an adjunctive tool in characterizing progression to DDLPS. Moreover, in this large, complex and discontinous 12q amplicon, we identified additional genes exclusively amplified in DDLPS, such as *GLI1* (12q13.3), *MAP3K12* (12q13.13) and genes previously not implicated in liposarcomas, including *CDK2* (12q13.2), *ALX1* (12q21.31) and *TBX5* (12q24.21). These additional amplification events in DDLPS may play a role in liposarcomagenesis, particularly in the de-differentiation process. However, the exact pathogenic role of these genes remains to be elucidated and their potential value in diagnosis and understanding the pathogenesis of DDLPS should be further elaborated. Further MLPA studies on larger number of DDLPS and correlation with other molecular methods, such as FISH and array CGH, may help confirm our findings.

Conflicts of Interest

The Authors declare no conflicts of interest with regard to this study.

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