Clinical and Biological Significance of *CDK4* Amplification in Well-Differentiated and Dedifferentiated Liposarcomas

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Abstract

Purpose: The *MDM2* and *HMGA2* genes are consistently amplified in well-differentiated/dedifferentiated liposarcomas (WDLPS/DDLPS) whereas *CDK4* is frequently but not always amplified in these tumors. Our goal was to determine whether the absence of *CDK4* amplification was (*a*) correlated to a specific clinico-histopathologic profile; and (*b*) compensated by another genomic anomaly involving the *CCND1/CDK4/P16INK4a/RB1/E2F* pathway.

Experimental Design: We compared the clinical characteristics of a series of 143 WDLPS/DDLPS with amplification of both *MDM2* and *CDK4* (*MDM2+*/*CDK4+*) to a series of 45 WDLPS/DDLPS with *MDM2* amplification and no *CDK4* amplification (*MDM2+*/*CDK4-*). We used fluorescence *in situ* hybridization, real time quantitative reverse transcription PCR, and immunohistochemistry to explore the status of *CCND1, P16INK4a, P14ARF,* and *RB1*.

Results: We found that *MDM2+/CDK4-* WDLPS/DDLPS represent a distinct clinical subgroup with favorable prognostic features, including low-grade lipoma-like histology, peripheral location, and lower rate of recurrence. By using fluorescence *in situ* hybridization, we found that genomic aberrations expected to be alternative mechanisms for compensating the lack of *CDK4* amplification, such as *RB1* and *CDKN2A* deletions or *CCND1* amplification, were very uncommon. In contrast, by using real time quantitative reverse transcription PCR and immunohistochemistry, we observed that overexpression of *P16INK4a* (and *P14ARF)* and *CCND1* and reduced expression of *RB1* were very frequent, independently of the *CDK4* status.

Conclusions: Our results underscore the complex coordinated regulation of the RB and p53 growth-control pathways in WDLPS/DDLPS. Because the absence of *CDK4* amplification is not specifically counterbalanced by a genomic alteration of the *CCND1/CDK4/P16INK4a/RB1/E2F* pathway, *CDK4* amplification may only represent a "*MDM2-HMGA2*-helper" in WDLPS/DDLPS tumorigenesis. (Clin Cancer Res 2009;15(18):5696–703)

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The *CDK4* gene encodes a 33-kD protein that plays an important role in the regulation of the G_1-S transition of the cell cycle (1). CDK4 forms molecular complexes with members of the cyclin D family, such as cyclin D1 (CCND1). The phosphorylation of RB1 protein by the CDK4-CCND1 complex leads to the release of the E2F transcription factor, which up-regulates gene expression required for progression through the S-, G_2-, and M-phases (1). The activity of CDK4 is negatively regulated by p16INK4a, which prevents its binding to CCND1 (Fig. 1). p16INK4a is the product of the *CDKN2A* gene that also encodes p14ARF (2). Alterations of genes involved in the CCND1/CDK4/p16INK4a/RB1/E2F pathway play a crucial role in the pathogenesis of many tumor types (1, 3).

We and others have shown that well-differentiated/dedifferentiated liposarcoma (WDLPS/DDLPS) cells contain supernumerary ring or giant marker chromosomes composed of highly

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Received 12/8/08; revised 5/12/09; accepted 6/15/09; published OnlineFirst 9/8/09.

Grant support: Programme Hospitaller de Recherche Clinique (PHRC National 2003; Nice University Hospital), Comité des Alpes-Maritimes de la Ligue National Contre le Cancer, Conseil Général des Alpes-Maritimes, Association pour la Recherche sur le Cancer (ARC# 4080 to FP), and Institut National du Cancer (projet libre GENOSTT).

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doi:10.1158/1078-0432.CCR-08-3185

Translational Relevance

Well-differentiated/dedifferentiated liposarcoma (WDLPS/DDLPS) is the most frequent sarcoma subtype in adult patients. WDLPS/DDLPS are characterized by a consistent amplification of the 12q14-15 chromosomal region containing the *MDM2* and *HMGA2* genes in all the cases, whereas the *CDK4* gene is only inconsistently amplified. CDK4 and other cyclin-dependent kinases play a crucial role in the control of the cell cycle progression and are the target of several anticancer drugs in clinical development. We report here the first study investigating the clinical significance of CDK4 amplification in WDLPS/DDLPS and the genomic aberrations involving the *CCND1/CDK4/P16INK4a/RB1/E2F* pathway in this tumor type.

amplified sequences from the 12q14-15 chromosomal region (4, 5). Although the *MDM2* and *CDK4* genes were initially considered to be the two main targets of the 12q14-15 amplicon in WDLPS/DDLPS, we have recently shown (6) that only *MDM2* (12q15) and *HMGA2* (12q14.3) were consistently amplified in WDLPS/DDLPS. The *CDK4* gene (12q14.1) belongs to a distinct inconsistent amplicon that is not present in about 10% of cases (6). The concomitant amplification of *MDM2* and *HMGA2* seems therefore to be the crucial event in WDLPS/DDLPS pathogenesis. However, considering the important function of CDK4 in the cell cycle, *CDK4* gene amplification and overexpression very likely play an important role in the tumorigenic process. This may have clinical implications because several anticancer drugs belonging to the group of "CDK inhibitors" are currently in preclinical development or under clinical trials (7).

Further clarification of the role of *CDK4* amplification in WDLPS/DDLPS is needed for a better understanding of the tumorigenesis of such tumors. We report here a study which aimed at determining whether the absence of *CDK4* amplification in WDLPS/DDLPS is (*a*) correlated with specific clinicopathologic features, and (*b*) compensated by another genomic event involved in the CCND1/CDK4/p16INK4a/RB1/E2F pathway.

Patients and Methods

Patients. From 1991 to 2008, 45 cases of WDLPS/DDLPS, for which amplification of *MDM2* but no amplification of *CDK4* was detected (*MDM2+/CDK4-*), were retrieved from the respective databases of the Laboratory of Solid Tumor Genetics (Nice, France) and of the Department of Pathology of the Bergonié Institute (Bordeaux, France). The clinicopathologic characteristics of these cases were compared with those of a series of 143 WDLPS/DDLPS with amplification of both *MDM2* and *CDK4* (*MDM2+/CDK4+*) analyzed during the same period. In all the cases, the diagnosis of WDLPS/DDLPS was established according to the WHO Classification of Tumors (4). The *MDM2/CDK4* status of all the cases was assessed by fluorescence *in situ* hybridization (FISH) and/or quantitative PCR as previously described (8).

Molecular cytogenetic analysis. FISH analysis was done in 30 cases (18 *MDM2+/CDK4-*, 12 *MDM2+/CDK4+*) with available formalin-fixed, paraffin-embedded blocks, in order to determine the genomic

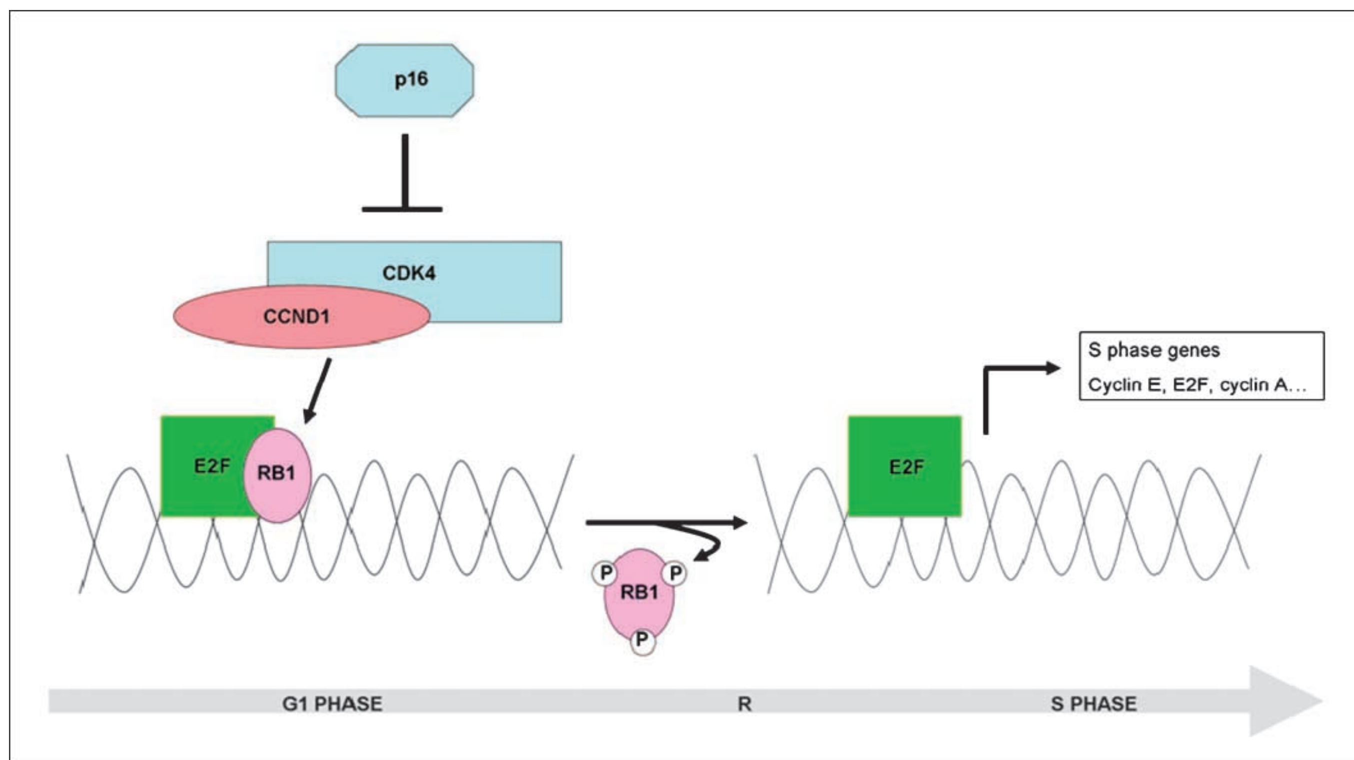

Fig. 1. Schematic involvement of the CCND1/CDK4/P16INK4a/RB1/E2F pathway in the G_1-S transition of the cell cycle. Accumulation of the CCND1-CDK4 complex leads to phosphorylation of the retinoblastoma protein (RB1), allowing E2F to promote expression of genes that leads to the progression from the G_1 phase to the S phase of the cell cycle. The activity of the CCND1-CDK4 complex is negatively regulated by P16INK4a that inhibits CDK4.

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status of the *CDKN2A* (9p21.3), *CCND1* (11q13.3), and *RB1* (13q14.2) genes. Five-micrometer sections were deparaffinized, rehydrated, and incubated with pepsin by using the Histology FISH Accessory Kit (Dako) according to the manufacturer's instructions. Three FISH probes were used in this study: the p16/CEP9 Dual Color Probe (Abbott Molecular), and the bacterial artificial chromosome probes RP11-156B3 (11q13.3, *CCND1*) and RP11-839E5 (13q14.2, *RB1*). BAC clones from the Roswell Park Cancer Institute library were selected according to their location on the University of California Santa Cruz database (http://genome.ucsc.edu/; March 2006 release), were obtained from the Children's Hospital Oakland Research Institute (CHORI) (http://bacpac.chori.org/), and were prepared as probes for FISH analysis according to standard procedures. Microscopic analysis was done using a DM6000B microscope (Leica) and images were processed using the ISIS software (MetaSystems). At least 100 nuclei per slide were analyzed. Amplification was defined as the presence of ≥10 fluorescent signals per cell in >1% of cells. Deletion was defined as >50% nuclei with only one fluorescent signal.

Array comparative genomic hybridization analysis. DNA from frozen material of three cases of *MDM2*+/*CDK4*-WDLPS/DDLPS was extracted and processed for array comparative genomic hybridization (array-CGH) as previously described (9). The microarray used contained 3,342 sequence-validated BAC with an average resolution of 1 Mb (9).

Quantitative reverse transcription-PCR analysis. Quantitative reverse transcription-PCR (qRT-PCR) was used to determine the expression levels of *p16INK4a*, *p14ARF*, *CCND1*, and *RB1* in 16 WDLPS/DDLPS cases (7 *MDM2+/CDK4-*, 9 *MDM2+/CDK4+*) for which frozen material was available and in the 4 *MDM2+/CDK4+* WDLPS/DDLPS cell lines 93449, 94778, 95T1000, and 98T1430 (6). Total RNAs were extracted from WDLPS/DDLPS and normal subcutaneous adipose tissue (NSAT) using either the RNeasy lipid tissue minikit (Qiagen; frozen fragments) or Trizol (Invitrogen; WDLPS/DDLPS cell lines) according to the manufacturer's protocol. The RNA samples were treated by DNA-free (Applied Biosystems). One microgram of total RNA was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and each qRT-PCR experiment was done twice in duplicate with the ABI PRISM 7300 Detection System using either FAM dyes (Applied Biosystems) for *CCND1*, *RB1*, and *p14ARF* or SYBRGreen dye for *p16INK4a* according to the manufacturer's protocol. *RPLP0* (the large P0 subunit of the acidic ribosomal phosphoprotein) was used as endogenous control for normalization. QRT-PCR was done using the following TaqMan gene expression assays (Applied Biosystems): Hs99999189_m1 *(p14ARF)*, Hs 00277039_m1 (*CCND1*), Hs 01078066_m1 (*RB1*), and Hs99999902_m1 (*RPLP0*). The reaction mix consisted of 10 μL of TaqMan master mix 2X, 1 μL of TaqMan gene expression mix, and 5 μL of 1/10 cDNA in a final volume of 20 μL. For SYBRGreen experiments, primer sequences were the following: *RPLP0* forward: TGCATCAGTACCCCATTCTATCAT, *RPLP0* reverse: AAGGTGTAATCCGTCTCCACAGA, *p16INK4a* forward: GGGGGCACCAGAGGCAGT, and *p16INK4a* reverse: GGTTGTGGCGGGGGCAGTT. The reaction mix consisted of 12.5 μL of SYBR Green PCR Master Mix (Applied Biosystems), 300 nmol/L forward and reverse primers and 5 μL of 1/10 cDNA in a final volume of 25 μL. Amplification of specific transcripts was confirmed by melting curve profiles generated at the end of the PCR program.

The PCR conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The comparative Ct (threshold cycle) method was used to achieve relative quantification of gene expression. The mRNA levels of genes of interest (R) were normalized to the mRNA levels of RPLP0: $\Delta Ct = Ct_R - Ct_{RPLP0}$. The relative amount of mRNA between controls (NSAT) and WDLPS/DDLPS was given by $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = \Delta Ct_R$ of WDLPS/DDLPS - mean of ΔCt_R of controls (NSAT). The ΔCt values of the controls were homogenous.

Immunohistochemistry. Immunohistochemical staining was done on representative slides from each case, following the manufacturer's instructions with the following primary antibodies: CDK4 (clone DCS-31, Biosource International), P16 (CINtech P16-INK4A kit, clone E6H4, Dako North America, Inc.), and CYCLIND1 (clone SP4, Neomarkers). Appropriate positive and negative controls were used.

Statistical analysis. Descriptive statistics were used to show the distribution of variables in the population. Differences between groups were evaluated by χ^2 test or Fisher's exact test for categorical variables and *t* test for continuous variables.

Results

MDM2+/CDK4- WDLPS/DDLPS are more frequently low-grade lipoma-like lesions occurring in the limbs and have more favorable outcome than MDM2+/CDK4+ WDLPS/DDLPS. The clinicopathologic characteristics of the patients are described in Table 1. In comparison with *MDM2+/CDK4+* WDLPS/DDLPS, *MDM2+/CDK4-* WDLPS/DDLPS were more frequently low-grade lipoma-like lesions (64% versus 40%; *P* = 0.0039) and occurred in the majority of cases in the deep soft tissues of the extremities (71% versus 47%; *P* = 0.0045). They were very rarely located in the retroperitoneum (7%), whereas the *MDM2+/CDK4+* tumors were retroperitoneal lesions in 35% of cases (*P* = 0.0002). Follow-up data were available for 56 patients with primary lesions at diagnosis (32 *MDM2+/CDK4+*, 24 *MDM2+/CDK4-*). The mean follow-up was 61 months for *MDM2+/CDK4+* and 46 months *MDM2+/CDK4-* patients. The local recurrence rate was significantly higher in patients with *MDM2+/CDK4+* tumors than in patients with *MDM2+/CDK4-* tumors (47% versus 12.5%; *P* = 0.0064). The distant recurrence rate was not statistically different between the two groups (12.5% versus 9.5%; *P* = 0.71). Ten deaths related to the disease were

Table 1. Patient characteristics (*N* = 188)

	MDM2+/CDK4+ **(%) (*n* = 143)**	***MDM2+/CDK4-*** **(%) (*n* = 45)**
Median age at diagnosis, y (range)	60 (24-83)	66 (36-89)
Sex (%)		
Male	66 (46)	25 (55)
Female	77 (54)	20 (45)
Presentation at diagnosis (%)		
Primary lesion*	111 (78)	43 (96)
Recurrence	32 (22)	2 (4)
Tumor size (mm)		
Median	160	150
Mean	165	157
Range	10-500	30-340
Location (%)		
Upper limbs	10 (7)	3 (7)
Lower limbs*	57 (40)	29 (64)
Retroperitoneum*	50 (35)	3 (7)
Paratesticular area	6 (4)	2 (4)
Pelvis	4 (3)	1 (2)
Others	16 (11)	7 (16)
Histologic subtype (%)		
Lipoma-like*	59 (41)	25 (56)
Sclerosing	23 (16)	4 (9)
Inflammatory	2 (1)	2 (4)
Spindle cell	0	2 (4)
Dedifferentiated*	59 (41)	12 (27)
Grade (%)		
1	88 (62)	31 (69)
2	37 (26)	7 (16)
3	14 (10)	4 (9)
Unknown	4 (3)	3 (7)

*$P < 0.05$ (statistically significant difference).

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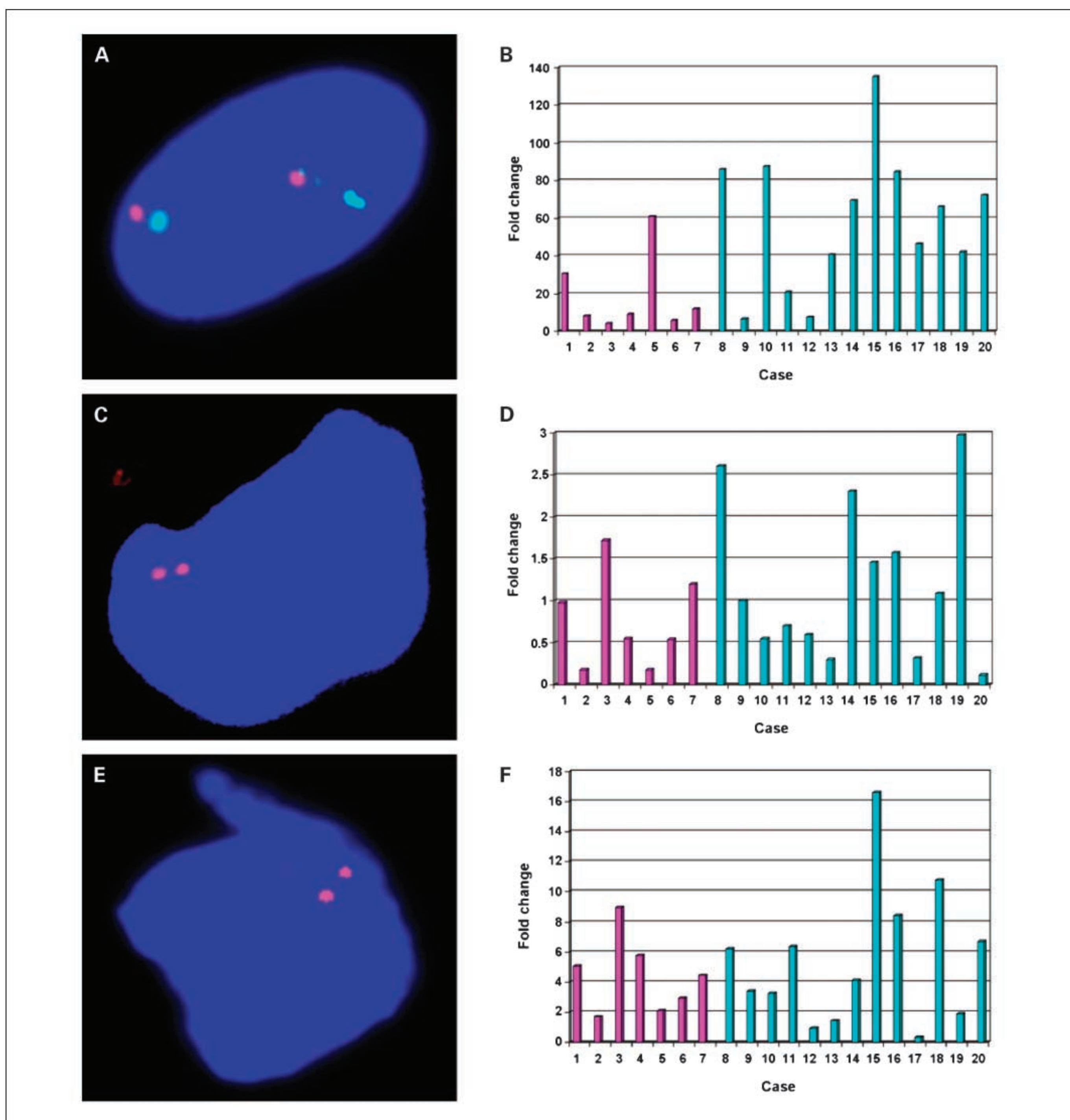

Fig. 2. Status of *P16INK4a, RB1* and *CCND1* in *MDM2+/CDK4+* and *MDM2+/CDK4-* WDLPS/DDLPS. *A,* dual-color FISH assays using *p16* (*red*) and chromosome-9 centromere (CEP9, *green*) showing balanced disomy. *C,* FISH analysis with BAC probe RP11-839E5 (RB1) showing balanced disomy. *E,* FISH analysis with BAC probe RP11-156B3 (CCND1) showing balanced disomy. *B, D, F,* qRT-PCR analysis measuring *P16INK4a, RB1*, and *CCND1* expression in 7 *MDM2+/CDK4-* (*purple bars*) and 13 *MDM2+/CDK4+* (*light blue bars*) WDLPS/DDLPS. Gene expression was quantified by qRT-PCR, normalized to levels of *RPLP0* mRNA and expressed as fold changes relative to s.c. normal adipose tissue.

observed in the *MDM2+/CDK4+* group and only one in the *MDM2+/CDK4-* group (31% versus 4%; $P = 0.0116$). In all the cases but three, deaths related to the disease were observed in patients with retroperitoneal DDLPS.

CDK4 ***is not overexpressed in*** **MDM2+/CDK4-** ***WDLPS/DDLPS.*** QRT-PCR analysis showed a strong overexpression of *CDK4* in the *MDM2+/CDK4+* cases analyzed whereas no significant overexpression was observed in the *MDM2+/CDK4-* cases (mean level compared with NSAT in *MDM2+/CDK4+* WDLPS, 16.9; mean level compared with NSAT in *MDM2+/CDK4-* WDLPS, 1.76; P = 0,0005). Four cases of *MDM2+/CDK4-* WDLPS/DDLPS were also analyzed by

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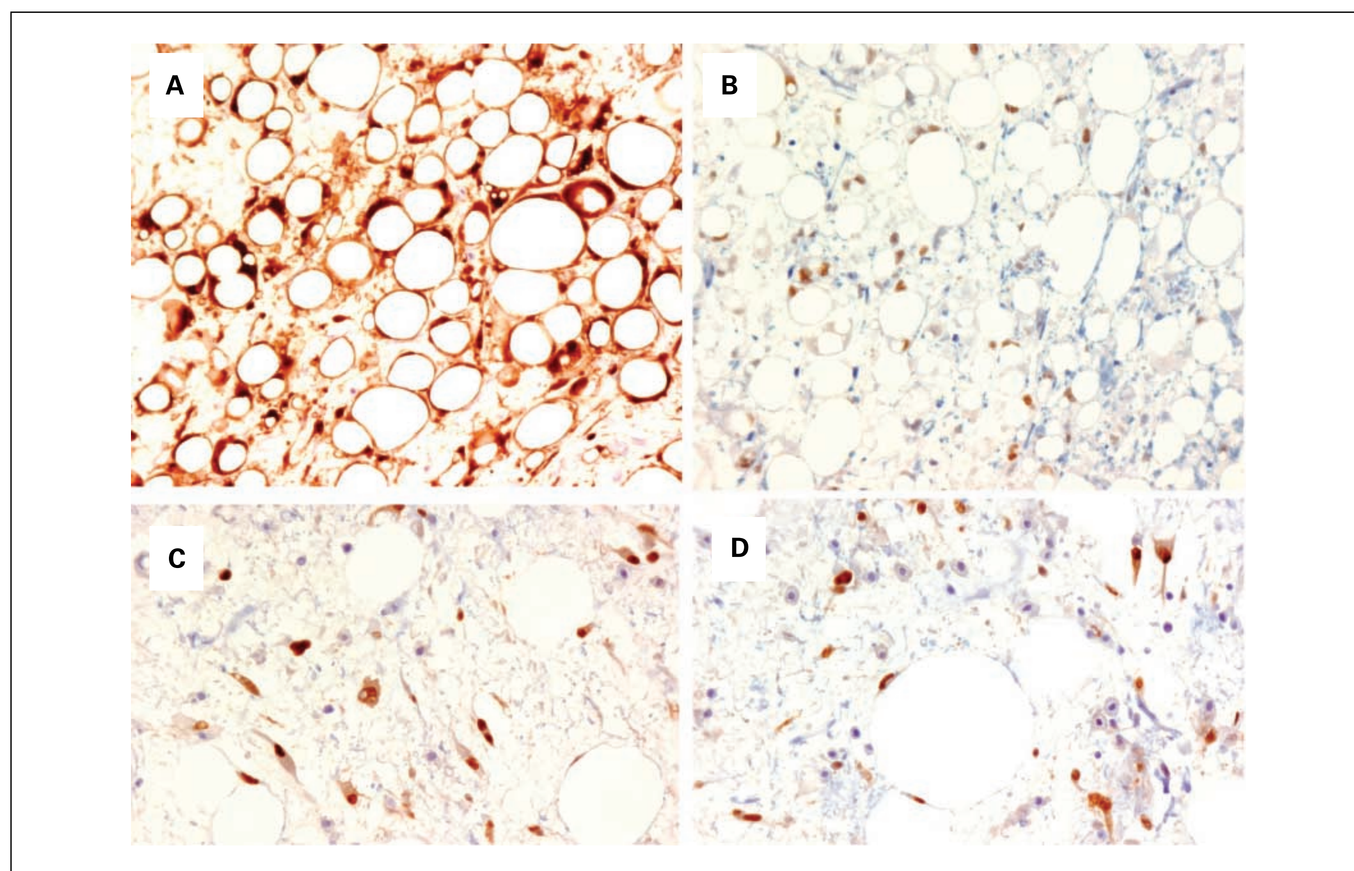

Fig. 3. Status of P16INK4a and CCND1 assessed by immunohistochemistry in *MDM2+/CDK4+* and *MDM2+/CDK4-* WDLPS/DDLPS. *A* and *B,* P16INK4a and CCND1 immunohistochemistry in a case of *MDM2+/CDK4+ WDLPS* showing positive staining for P16INK4a (*A*) and CCND1 (*B*). *C* and *D,* P16INK4a and CCND1 immunohistochemistry in a case of *MDM2+/CDK4- DDLPS* showing positive staining for P16INK4a (*C*) and CCND1 (*D*).

immunohistochemistry for *CDK4* expression. All the four cases were negative for CDK4. We also analyzed the expression status of *CDK6* which encodes a protein that is also a kinase partner of CCND1. A trend for a higher expression of *CDK6* was observed in *MDM2+/CDK4-* WDLPS/DDLPS (mean level compared with NSAT in *MDM2+/CDK4-* WDLPS, 7.6; range, 0.7-40.6) in comparison with *MDM2+/CDK4+* WDLPS/DDLPS (mean level compared with NSAT in *MDM2+/CDK4+* WDLPS/DDLPS, 1.9; range, 0.7-4.5). However, this trend in the series was only due to a single case of *MDM2+/CDK4-* WDLPS/DDLPS showing a strong overexpression of *CDK6* (fold change 40.6). It was not statistically significant ($P = 0.16$).

The* CDKN2A *locus is neither deleted nor gained in most WDLPS/DDLPS cases, independently of the* CDK4 *amplification status (Fig. 2). Twenty-seven cases (10 *MDM2+/CDK4+*; 17 *MDM2+/CDK4-*) evaluated by FISH exhibited euploidy for chromosome 9 and *CDKN2A*. Heterozygous deletion of *CDKN2A* was found in only one case of WDLPS/DDLPS (*MDM2+/CDK4+*), and *CDKN2A* gain (3 to 5 copies) related to polysomy for chromosome 9 was observed in two cases (one *MDM2+/CDK4+*; one *MDM2+/CDK4-*).

P16INK4a *is consistently overexpressed in both* MDM2+/CDK4+ *and* MDM2+/CDK4- *WDLPS/DDLPS (Figs. 2 and 3).* QRT-PCR analysis showed overexpression of *P16INK4a* in all the 20 cases analyzed (13 *MDM2+/CDK4+* cases including the case with heterozygous deletion of *CDKN2A* and 7 *MDM2+/CDK4-* cases; Fig. 2). The level of overexpression was significantly higher in cases with amplification of *CDK4* (mean level compared with NSAT in *MDM2+/CDK4+* WDLPS, 58.85; range: 6.5-135; mean level compared with NSAT in *MDM2+/CDK4-* WDLPS, 18.7; range: 4.1-60.9; $P = 0.0043$). Nineteen cases (7 *MDM2+/CDK4+* WDLPS/DDLPS and 12 *MDM2+/CDK4-* WDLPS/DDLPS) were also analyzed by immunohistochemistry for P16 protein expression. A positive labeling for P16 was observed in all the cases but three (one *MDM2+/CDK4+* WDLPS/DDLPS and two *MDM2+/CDK4-* WDLPS/DDLPS).

P14ARF was also overexpressed in all the cases analyzed (mean level compared with NSAT in *MDM2+/CDK4+* WDLPS, 35.8; range, 10.8-84.9; mean level compared with NSAT in *MDM2+/CDK4-* WDLPS, 12.6; range, 6.7-22.9; $P = 0.0064$).

CCND1 *is neither deleted nor gained in most WDLPS/DDLPS cases and is frequently overexpressed in WDLPS/DDLPS independently of the CDK4 amplification status (Figs. 2 and 3).* Twenty-nine cases (12 *MDM2+/CDK4+*, 17 *MDM2+/CDK4-*) evaluated by FISH exhibited disomy for *CCND1*. Gain (3 to 5 copies) of *CCND1* was found in only one case of WDLPS/DDLPS (*MDM2+/CDK4-*). QRT-PCR analysis showed overexpression of *CCND1* in 14 of 20 cases analyzed, independently of the *CDK4* status (9 *MDM2+/CDK4+*, 6 *MDM2+/CDK4-*; 69% versus 86%; $P = 0.42$; mean level compared with NSAT in *MDM2+/CDK4+* WDLPS/DDLPS, 7.3; range, 0.9-16.6; mean level compared with NSAT in *MDM2+/CDK4-* WDLPS/DDLPS, 4.9; range, 1.7-8.9;

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P = 0.13). A reduced expression of *CCND1* was found in one case of *MDM2+/CDK4+* WDLPS/DDLPS (fold change compared with NSAT, 0.33). Nineteen cases (7 *MDM2+/CDK4+* WDLPS/DDLPS, 12 *MDM2+/CDK4-* WDLPS/DDLPS) were also analyzed by immunohistochemistry for CCND1 protein expression. A positive labeling was observed in 15 cases, a negative labeling in 3 cases (3 *MDM2+/CDK4-* WDLPS), and the result was not interpretable in 1 case.

RB1 *gene is neither deleted nor gained in most WDLPS/DDLPS cases and frequently shows a reduced expression independently of the CDK4* amplification status (Fig. 2). Twenty-nine cases (12 *MDM2+/CDK4+*, 17 *MDM2+/CDK4-*) evaluated by FISH exhibited disomy for *RB1*. Gain (3 to 5 copies) of *RB1* was found in only one case of WDLPS/DDLPS (*MDM2+/CDK4-*). QRT-PCR analysis showed a reduced expression (≥ 50%) of *RB1* in 8 of the 20 cases analyzed independently of the CDK4 status (4 *MDM2+/CDK4+*, 4 *MDM2+/CDK4-*; 57% versus 31%; *P* = 0.25; mean level compared with NSAT in *MDM2+/CDK4+* WDLPS/DDLPS, 0.32; range,0.3-2.9; mean level compared with NSAT in *MDM2+/CDK4-* WDLPS/DDLPS, 0.36; range, 0.18-1.7). In all the cases but one, reduced expression of *RB1* and overexpression of *CCND1* were mutually exclusive.

Detection of 1q21-23 amplification in* MDM2+/CDK4- *WDLPS/DDLPS. Three cases of *MDM2+/CDK4-* WDLPS/DDLPS were analyzed by array-CGH in order to detect specific genomic imbalances. The results are summarized in Table 2. Besides the 12q14-15 region, the sole recurrently amplified region was 1q21-23 in the three cases. No aberration of the *CDKN2A*, *CCND1* and *RB1* loci were detected in these three cases in accordance with the FISH results.

Table 2. Array-CGH analysis of three cases of MDM2+/CDK4- WDLPS/DDLPS

	Aberration	Chromosomal location	Size (Mb)
Case 1	+ 1q23.2-1q23.3	157.467-160.713	3.2
	+ 2q14.3-2q21.1	127.839-131.237	3.4
	+ 2q24.1	155.745-159.178	3.4
	+ 8p11.1-8p12	30.172-43.787	13.6
	+ 12q13.2-12q13.3	52.098-53.203	1.1
	+ 12q15-12q21.31	67.347 79.526	12.2
	+ 12q21-31	78.745 80.543	1.8
Case 2	+ 1q21.1-1q21.3	144.480 153.108	8.6
	+ 1q23.2-1q25.1	157.578 173.190	15.6
	+ 2p11.1-2p11.2	88.679 91.303	2.6
	+ 7q11.21	57.980 62.015	4
	- 9q12-13	67.862 70.514	2.6
	+ 12q13.13-12q13.2	52.696 53.730	1
	+ 12q15	67.347 67.926	0.6
	+ 12q21.2-12q21.31	74.117 79.607	5.5
	+ 12q21.31	79.699 83.470	3.8
	+ 12q21.31-12q21.32	82.333 87.375	5
Case 3	+ 1p36	16.581-16.880	0.3
	+ 1p21	101.859-106.510	4.7
	+ 1q21-1q31	145.676-197.012	51.5
	- 3p21	50.188-50.533	0.4
	+ 5q21-5q31	98.438-131.910	33
	+ 12q14-12q23	63.785-102.490	39
	+ 22q11.2	19.570-20.213	0.7

Discussion

We recently reported that *HMGA2* and *MDM2* were always coamplified in WDLPS/DDLPS whereas *CDK4* belonged to a distinct inconsistent amplicon (6). These results suggested that the amplification of *CDK4* might not be as indispensable as the amplification of *HMGA2* and *MDM2* for WDLPS/DDLPS tumorigenesis.

In the present study, we found that WDLPS/DDLPS lacking *CDK4* amplification were significantly associated with peculiar clinicopathologic features. Indeed, most of these lesions were low-grade lipoma-like WDLPS located in the limbs. Their rates of local recurrence and lethality were lower than those of *MDM2+/CDK4+* lesions that were significantly more often retroperitoneal. Although a correlation between amplification/overexpression of *CDK4* and a bad prognosis has been described in several tumor types (10–15), our results may also reflect the fact that free surgical margins are more easily obtained for lesions of the extremities than for retroperitoneal ones. Indeed, the disease-related mortality, typically associated with complications of multiple local recurrences, is higher for patients with retroperitoneal tumors than for those with peripheral tumors. Therefore, because our data are retrospective, the independent prognostic value of the *CDK4* amplification remains to be confirmed in a prospective way.

The alternative occurrence of *CDK4*, *p16INK4a*, *CCND1*, or *RB1* aberrations has been described in several tumor types (16–24). Indeed, the losses of *p16INK4a* or *RB1* functions as well as overexpression of *CCND1* represent alternative mechanisms for *CDK4* amplification (25). In our study, however, we did not find any significant aberrations of the *CDKN2A*, *CCND1*, or *RB1* genes that were confined to *MDM2+/CDK4-* WDLPS/DDLPS only. The *CDKN2A* gene encodes two tumor suppressor proteins, p16INK4a and p14ARF, by a mechanism of alternative splicing of the first exon. By using FISH, we did not find deletions of *CDK2N2A*. We observed a strong overexpression of *P16INK4a* and *P14ARF* in both *MDM2+/CDK4+* and *MDM2+/CDK4-* WDLPS/DDLPS. Such an overexpression of tumor suppressor genes in malignant tumor cells is very intriguing. In this regard, it is interesting to note that *P16INK4a* overexpression was significantly higher in *MDM2+/CDK4+* WDLPS/DDLPS than in *MDM2+/CDK4-* WDLPS/DDLPS. *P16INK4a* overexpression has been reported in several tumor types such as gliomas (26), myxoid liposarcomas (27), thyroid carcinomas (28), and cervical carcinomas (29). *P16INK4a* overexpression might be a secondary event to RB1 inactivation, because RB1 repressed *P16INK4a* at the transcriptional level (30, 31). In our series, *RB1* expression was significantly reduced in 40% of the cases, independently of the *CDK4* status. This suggests that in at least a subset of WDLPS/DDLPS, inactivation of RB1 and CDK4 overexpression may act as synergic or additional events to enhance the G_1-S transition and that the expressions of *CDKN2A/P16* and *RB* are not always inversely related (32). An alternative explanation for *P16INK4a* overexpression in WDLPS/DDLPS might be related to the role of HMGA2. Although there are several lines of evidence indicating that *HMGA2* should be considered an authentic oncogene (33), recent findings have shown that *HMGA2* may also exhibit antioncogenic properties by playing a role in cellular senescence (34). Interestingly, Narita et al. have shown that high levels of *HMGA2* expression induce *P16INK4a* expression in human diploid fibroblast and that the expression of *MDM2* or *CDK4* allowed such cells to bypass *HMGA2*-induced proliferation arrest (34). Therefore,

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the high level of *P16INK4a* in WDLPS/DDLPS might be related to the overexpression of *HMGA2*, the antiproliferative activity of which is circumvented by the consistent amplification of *MDM2*. Data about the respective expression of *P14ARF* and *P16INK4a* in tumor cells are scarce. The strong overexpression of both *MDM2* and *P14ARF* in WDLPS/DDLPS suggests that p53 inactivation in WDLPS/DDLPS is the result of a complex regulation. Indeed, the physiologic function of p14ARF protein is to bind to the MDM2 protein and to inhibit its ubiquitin ligase activity, increasing the levels of the p53 protein (35). The expression of *p14ARF* is directly activated by E2F1 (36). Therefore, the overexpression of *p14ARF* in WDLPS/DDLPS may result from several oncogenic events that could deregulate the E2F1 activity, such as RB1 inactivation and CDK4 or CCND1 overexpression. We have shown that *CCND1* is consistently overexpressed in WDLPS/DDLPS whatever the *CDK4* status. Besides forming active complexes with CDK4 or CDK6 that promote cell cycle progression, CCND1 also functions as a transcriptional modulator by regulating the activity of several transcription factors and histone deacetylase (HDAC3; ref. 37). This latter activity that may play an important role in tumorigenesis is independent of CDK4. Although up-regulation of CCND1 protein in cancer frequently results from translational and/or posttranslational mechanisms (38), *CCND1* mRNA is also overexpressed in several tumor types including nonadipose soft-tissue tumors (39, 40).

In order to obtain a global overview of genomic imbalances in *MDM2+/CDK4-* WDLPS/DDLPS, we analyzed three of these cases by array-CGH. As previously reported in *MDM2+/CDK4+* WDLPS/DDLPS, two to five regions were coamplified with 12q14-15 in all the cases (41, 42). The sole recurrently region to be coamplified with 12q14-15 in *MDM2+/CDK4-* cases was 1q21-23. This aberration cannot be considered as specific to *MDM2+/CDK4-* WDLPS/DDLPS because it has also been described in *MDM2+/CDK4+* WDLPS/DDLPS (41, 42). Although several genes located at 1q21-q23, including *ATF6*, *DUSP12*, *COAS1*, *COAS2*, *COAS3*, *PRUNE*, and *FASLG*, have previously been reported to be amplified in supernumerary ring and giant markers in WDLPS/DDLPS as well as in other sarcomas (42–45), the targets of the 1q21-23 amplicon, which are supposed to play an effective role in WDLPS/DDLPS tumorigenesis, are still unknown.

In summary, we have shown here that *MDM2+/CDK4-* WDLPS/DDLPS represent a distinct clinical subgroup with favorable prognostic features. Although *CDKN2A* deletion is a frequent event in human cancers, our results show that this aberration is not involved in the pathogenesis of WDLPS/DDLPS even in those lacking *CDK4* amplification Moreover, we have shown a complex pattern of expression for *CCND1*, *P16INK4a*, *P14ARF*, and *RB1* that is not dependent on the *CDK4* status. These results underscore the complex coordinated regulation of the RB and p53 growth-control pathways in WDLPS/DDLPS. The knowledge of the way by which WDLPS/DDLPS cells regulate the mitogenic and antimitogenic signaling pathways during the cell cycle is of crucial importance for the design of new therapeutic strategies. Our results represent an important basis for further investigations with the aim of determining how the activities of the actors of the CCND1/CDK4/P16INK4a/RB1/E2F pathway are coordinated in WDLPS/DDLPS.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Marion Marty for her assistance with the immunohistochemistry experiments and Mrs. Stephanie Bonnafous for expert technical assistance.

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