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Amplification and overexpression of *PRUNE* in human sarcomas and breast carcinomas – a possible mechanism for altering the nm23-H1 activity

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***PRUNE*, the human homologue of the *Drosophila* gene, is located in 1q21.3, a region highly amplified in human sarcomas, malignant tumours of mesenchymal origin. Prune protein interacts with the metastasis suppressor nm23-H1, but shows impaired affinity towards the nm23-H1 S120G mutant associated with advanced neuroblastoma. Based on these observations, we previously suggested that prune may act as a negative regulator of nm23-H1 activity. We found amplification of *PRUNE* in aggressive sarcoma subtypes, such as leiomyosarcomas and malignant fibrous histiocytomas (MFH) as well as in the less malignant liposarcomas. *PRUNE* amplification was generally accompanied by high mRNA and moderate to high protein levels. The sarcoma samples expressed nm23-H1 mostly at low or moderate levels, whereas mRNA and protein levels were moderate to high in breast carcinomas. For the more aggressive sarcoma subtypes, 9/13 patients with *PRUNE* amplification developed metastases. A similar situation was observed in all breast carcinomas with amplification of *PRUNE*. Infection of NIH3T3 cells with a *PRUNE* recombinant retrovirus increased cell proliferation. Possibly, amplification and overexpression of *PRUNE* has the same effect in the tumours. We suggest that amplification and overexpression of *PRUNE* could be a mechanism for inhibition of nm23-H1 activity that affect the development or progression of these tumours.** *Oncogene* (2001) **20,** 6881–6890.

Keywords: PRUNE; nm23; sarcoma; breast

Introduction

Sarcomas comprise a large, heterogeneous group of tumours of mesenchymal origin (Enzinger and Weiss, 1995). Although infrequently occurring, these tumours have been widely studied at the molecular level, and this has provided insight into mechanisms of importance for tumour development in general. Notably, amplification and overexpression of *MDM2* and *CDK4*, representing alternative pathways for inactivation of the tumour suppressors p53 and pRb, were first described for this group of tumours (Oliner *et al*., 1992; Khatib *et al*., 1993).

Comparative genomic hybridisation (Kallioniemi *et al*., 1992) revealed novel amplified regions that seem to be important for the development of sarcomas, in particular 1q21-q22 (Forus *et al*., 1995a,b; Szymanska *et al*., 1996). A variety of solid tumours show amplification of 1q21-q22, e.g. 70–80% of hepatocellular carcinomas and 25–30% of ovarian cancers (Tapper *et al*., 1998; Wong *et al*., 1999; Zimonjic *et al*., 1999). Although gain of the whole long arm (1q) is most frequent in breast cancers, local amplification affecting 1q21-q22 has been observed (Tirkkonen *et al*., 1998). Studies of renal clear cell and hepatocellular carcinomas revealed a higher frequency of 1q21-q23 gains in metastatic tumours (Gronwald *et al*., 1997; Qin *et al*., 1999), and gains of the 1q21-q25 region have been associated with short overall survival in high-grade osteosarcomas and neuroblastomas (Hirai *et al*., 1999; Tarkkanen *et al*., 1999).

We have characterised the 1q21-q22 amplicon in sarcomas by analysing copy numbers of known genes and genomic clones (Forus *et al*., 1998). None of the genes showed the high copy numbers and frequent amplification expected for a candidate gene, but two partly overlapping YACs (Yeast Artificial Chromosomes) located proximal to the S100-cluster (Marenholz *et al*., 1996) were frequently amplified.

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Recently, the human homologue of the *Drosophila Prune* gene was cloned (Banfi *et al*., 1996; Timmons and Shearn, 1996; Volorio *et al*., 1998; Reymond *et al*., 1999). *PRUNE* is located on chromosome 1q21.3, in the amplified chromosomal region covered by the two YACs. Mutation of *Drosophila Prune* reduces the red pigment and changes the colour of the eye to a brownish purple (Lindsley and Zimm, 1992). The metabolic defects in the *Prune* fly do not affect viability or fertility, however, the homo- or hemizygous *Prune* mutant is lethal in the presence of a single copy of a gain-of-function mutation in the abnormal wing disc gene (awd), dubbed 'Killer-of-Prune' ($awd^{K\text{-}pn}$). The flies die at the third larval instar stage, developing melanotic (pseudo) tumours (Orevi and Falk, 1975; Biggs *et al*., 1988).

The human protein nm23-H1 is one of the human homologues of *Drosophila awd* (Biggs *et al*., 1990; Leone *et al*., 1991, 1993). Eight different genes of this family have been identified in humans (Lombardi *et al*., 2000), and their expression is linked to suppression of tumour metastasis, differentiation, apoptosis, proliferation and DNA mutation (de la Rosa *et al*., 1995). *NM23-H1* and *-H2* are nucleoside diphosphate (NDP) kinases, and metastasis suppression was observed in several tumour cell lines transfected with nm23-H1 (Leone *et al*., 1991; Russell *et al*., 1998). Recently, it was reported that increased nm23-H1 expression, induced by an inhibitor of DNA methylation, reduced cell motility but had little effect on cell proliferation (Hartsough *et al*., 2001). Other investigators have reported that nm23-H1 may also play a role in the regulation of cell structure and shape, as it has been shown to be a constituent of the centrosome (Roymans *et al*., 2001).

In colon, breast, gastric and ovarian carcinomas, as well as in melanomas, an inverse association between nm23-H1 expression and metastasis has been found (Hennessy *et al*., 1991; Florenes *et al*., 1992; Martinez *et al*., 1995; Srivatsa *et al*., 1996; Muller *et al*., 1998). Nevertheless, the opposite has also been observed: in neuroblastoma, high levels of a mutated *NM23-H1* mRNA was often associated with advanced stages of disease and poor patient survival (Chang *et al*., 1994), and in sarcomas, nm23-H1 expression was found to increase with the metastatic potential for some tumours, but some aggressive cases also showed loss of expression (Royds *et al*., 1997).

We have shown that prune protein binds nm23-H1 (Reymond *et al*., 1999) and nm23-H2 (D'Angelo *et al*., unpublished results) both *in vitro* and *in vivo*. Moreover, prune interacts with the nm23-H1 mutant P96S, that mimics the activity of the $awd^{K\text{-}pn}$ mutant, but shows an impaired affinity towards the gain-of-function mutant nm23-H1 S120G associated with advanced neuroblastoma (Reymond *et al*., 1999). We suggested that prune may act as a negative regulator of nm23-H1 activity, and the gene is therefore an interesting candidate for the 1q21-q22 amplifications. We have analysed a panel of sarcomas with 1q21-q22 amplification and some advanced breast carcinomas for *PRUNE* amplification, and correlated this to mRNA and protein expression levels of both prune and nm23-H1. *PRUNE* amplification and/or overexpression was found in tumours that expressed nm23-H1, and was frequent in aggressive, metastasising tumours as well as in tumours of borderline malignancy. This is similar to what has been reported for amplification of *MDM2* in sarcomas (Forus *et al*., 1993; Berner *et al*., 1996; Pedeutour *et al*., 1999). These results could suggest that for some tumours, inactivation of nm23-H1 by amplification and/or overexpression of *PRUNE* may be important for development, and in other tumours, it will affect the progression or the metastatic potential.

Results

PRUNE *amplification*

PRUNE copy numbers were analysed in a panel of sarcomas where the 1q21 amplification status has been previously defined (Forus *et al*., 1995a, 1998; Simons *et al*., 2000). Among the 19 samples analysed, 16 showed moderate amplification (3–9 signals) of *PRUNE* in more than 50% of the cells, and of those, five showed high-level amplification ($\geqslant$10 signals) in 25–60% of the cells (Figure 1). In two additional samples, amplification of *PRUNE* was moderated, and in one, copy numbers were normal.

Five of the six primary cases of advanced breast carcinomas showed moderate amplification of *PRUNE*, the last one had normal copy numbers (Figure 1). Amplification was found in 33–83% of the nuclei. For all samples with amplification, the average copy number of *PRUNE* was always considerably higher than the average copy numbers of centromere 1.

mRNA expression of PRUNE *and* NM23-H1

Expression of *PRUNE* was examined in 17 sarcoma samples (LMS14 and MFH 49 were not available and from LS2, LS6, LS9 data is not shown) by Northern blot analysis. In general, samples with moderate or high amplification of the gene showed moderate to high mRNA expression (Figure 2 and Table 1), with a few exceptions: MFH25, which showed normal copy numbers, showed very high expression levels of *PRUNE*, in MFH9 and LMS2x, expression was low or absent, although the gene was amplified in both samples. The latter could be an artefact due to the small amount of RNA present on the blot (not shown). Overall, the well differentiated liposarcomas (grade 1) showed low mRNA expression of *PRUNE* compared to the high mRNA expression levels observed for the more aggressive sarcoma (grade 3–4; LMS, MFH and MS).

Five of the six breast cancer samples showed high expression levels of *PRUNE*, including Ma15, which showed normal copy numbers of the gene (Table 1 and Figure 2). Only Ma51 showed low to moderate

Sample	Cen	CGH
LMS2x	2.4	1q21-q23
LMS14	ND	1q21-q23
LMS15	2.8	1q21-q23
LS2	2.3	1q21-q22
LS3x	2	1q21-q23
LS6	2.1	1q21-q22
LS9	3.7	1q21-q22
LS13	2.2	1q21-q23
LS43	2	ND
MFH3x	2.9	No ampl
MFH9	3.5	No ampl
MFH19	2	1q21-q23
MFH36	2.4	1pter-q22
MFH48	2.2	1p22-q21
MFH49	2.7	1p31-qter
MFH52	2.3	1p31-q25
MFH56	2.1	1q10-q23
MS8x	2.1	1q21-q22
MFH25	2.2	No ampl
Ma10	2.3	ND
Ma40	2.2	ND
Ma51	2.3	ND
Ma201	3.6	ND
Ma215	2.7	ND
Ma15	2	ND

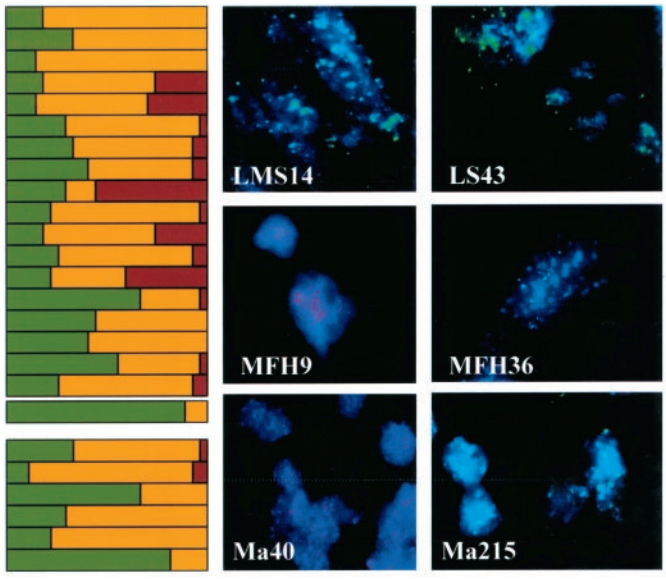

Figure 1 FISH analyses of *PRUNE* amplification in selected sarcomas and breast carcinomas. LMS = Leiomyosarcoma, LS = Liposarcoma, MFH = Malignant Fibrous Histiocytoma, MS = Malignant Schwannoma (Malignant Peripheral Nerve Sheath Tumour), Ma = Mammary Carcinoma. Left panel: The coloured bars display the fraction of nuclei that show normal copies (two signals, green), moderate amplification (3–9 signals, yellow) and high-level amplification (⩾10 signals, red) of *PRUNE*. The samples MFH25 had no detectable amplification of 1q21-q22 and was included as control. Cen = average number of signals using a probe for centromere 1. The CGH column show the amplified region on chromosome 1. ND = not done, No ampl = apparently normal. Right panel: Copy numbers of *PRUNE* on interphase nuclei from frozen tissue and paraffin embedded tissue. Signals were detected by avidin conjugated Cy3 (red), anti-digoxygenin-labelled FITC (green) or streptavidin-conjugated FITC (green). All these samples showed amplification of *PRUNE* relative to centromere 1, but copy numbers were variable

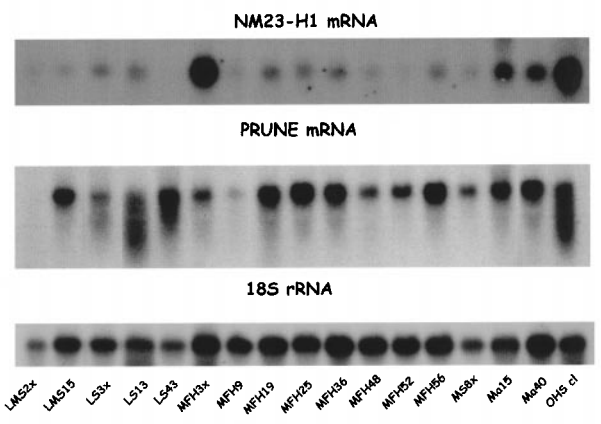

Figure 2 Northern analyses of *PRUNE* and nm23-H1-expression. Representative Northern blot showing expression of nm23-H1 and *PRUNE* in 14 sarcomas and two mammary carcinomas from which RNA was available. A probe for 18S rRNA was used as loading control. Quantitation of expression levels is shown in Table 1. OHS is an osteosarcoma cell line that was included as a loading control here. This cell line was not examined for *PRUNE* amplification or prune/nm23-H1 protein expression

expression. The breast carcinomas with high level of *PRUNE* mRNA were classified as moderate and high-grade tumours.

Expression of *NM23-H1* was also variable. Most sarcoma samples showed low mRNA levels, and moderate to high expression levels were only detected in LMS14 and MFH3x. In contrast, five out of six breast cancers showed moderate to high expression levels of *NM23-H1* (Table 1 and Figure 2), and only Ma51 showed low expression levels.

prune and Nm23-H1 protein expression

Expression of prune and nm23-H1 proteins was examined by immunohistochemistry on paraffin embedded tissues from 14 of the samples that were analysed by FISH, and in 16 additional LS and MFH cases (Table 1 and Figure 3b,c). In sarcomas, samples with increased copy numbers of *PRUNE* in more than 40% of the nuclei generally showed moderate to high expression of the protein, with some exceptions: in MS8x, LMS2x and LMS15, no protein could be detected, and in LS3x, only very low levels. The lack of protein signal was due to improper fixation of the tissues (NR in Table 1). In general, most of the LS and MFH cases examined showed moderate to high levels of prune, and more variable expression levels of nm23-H1.

The five breast cancer samples with amplification of *PRUNE* had low or moderate expression of the protein, although mRNA levels were generally high. Expression of nm23-H1 protein was moderate to high in most of the sarcoma and breast carcinoma samples, except for Ma215, where no expression could be detected (Table 1 and Figure 3).

Table 1 Clinical data, mRNA and protein expression

							PRUNE		*Nm23H1*	
Sample	*Stage*	*Grade*	*Subtype*	*Location*	*Metastatic disease*	*Status*	*mRNA*	*Protein*	*mRNA*	*Protein*
LMS2x	Rec	3		arm	+	DOD	0	NR	1	NR
LMS14	Prim	4		lung	+	D	NA	2 (80%)	2	2 (70%)
LMS15	Prim	3		abdomen	+	D	3	NR	1	NR
LS2	Prim	1	WDLPS	thigh	–	CR	1-2	NA	1	NR
LS3x	Prim	4	pleomorphic	abdomen	–	D-UK	2	NR	1	NR
LS6	Prim	1	WDLPS	gluteus	–	DOD	1-2	3 (90%)	1	3 (90%)
LS9	Prim	1	WDLPS	thigh	–	CR	1-2	NA	1	NA
LS13	Rec	1	WDLPS	thigh	–	CR	1-2	NA	1	NA
LS32	Prim	3	variable	thigh	–	CR	NA	1 (60%)	NA	2 (60%)
LS33	Prim	1	WDLPS	thigh	–	CR	NA	1 (80%)	NA	2 (50%)
LS34	Prim	2	myx + r	thigh	+	D	NA	2 (70%)	NA	1 (40%)
LS37-2	Met	2	myx	groin	+	D	NA	3 (90%)	NA	2 (50%)
LS39	Prim	1	WDLPS + myx	thigh	–	CR	NA	2 (90%)	NA	1 (40%)
LS40	Prim	2	myx	thigh	–	CR	NA	2 (50%)	NA	2 (70%)
LS42	Rec	4	myx + r	groin	–	DOD	NA	2 (80%)	NA	1 (40%)
LS43	Prim	1	WDLPS	thigh	–	CR	3	2 (90%)	0	1 (50%)
LS44	Prim	2	myx	arm	–	CR	NA	2 (90%)	NA	2 (80%)
LS47	Rec	1	WDLPS + dd	thigh	–	CR	NA	2 (80%)	NA	2 (70%)
MFH2	Rec	4		back	+	D	NA	3 (90%)	NA	1 (50%)
MFH3x	Prim	4		abdomen	+	D	2	2 (90%)	3	2 (60%)
MFH9	Rec	4		thigh	–	CR	1	2 (90%)	1	2 (70%)
MFH11	Prim	3		thigh	–	DOD	NA	2 (70%)	NA	1 (80%)
MFH19	Rec	3		thigh	–	CR	3	2 (80%)	1	2 (70%)
MFH25	Prim	4		thigh	–	CR	3	2 (90%)	1	2 (60%)
MFH36	Prim	4		shoulder	+	D	3	NA	1	NA
MFH42	Prim	4		arm	+	D	NA	2 (70%)	NA	2 (40%)
MFH44	Rec	4		thigh	–	DOD	NA	2 (90%)	NA	1 (40%)
MFH45	Prim	4		arm	–	CR	NA	2 (90%)	NA	1 (30%)
MFH46[1]	Prim	4		thigh	–	CR	NA	2 (70%)	NA	3 (80%)
MFH47[1]	Prim	4		femur	+	D	NA	2 (80%)	NA	1 (70%)
MFH48	Prim	4		tibia	+	D	2	2 (80%)	1	1 (50%)
MFH49	Rec	4		tibia	–	CR	NA	NA	NA	NA
MFH52[2]	Prim	4		abdomen	+	D	2	2 (80%)	0	2 (80%)
MFH56	Prim	4		arm	+	CR	3	3 (90%)	1	2 (40%)
MS8x	Prim	3		thigh	+	D	3	NR	1	NR
Ma10	Prim	3	ductal		+	D	3	2 (70%)	3	3 (50%)
Ma15	Prim	2	ductal		+	D	3	NA	2	NA
Ma40	Prim	2	ductal		+	A	3	2 (70%)	2	2 (40%)
Ma51	Prim	2	ductal		+	D	1-2	1 (30%)	1	2 (60%)
Ma201	Prim	3	ductal		+	D	3	1 (70%)	2-3	1 (20%)
Ma215	Prim	3	ductal		+	D	3	1 (60%)	3	0

LMS = Leiomyosarcoma, LS = Liposarcoma, MFH = Malignant Fibrous Histiocytoma, MS = Malignant Schwannoma (Malignant Peripheral Nerve Sheath Tumour), Ma = Mammary Carcinoma, WDLPS = Well-differentiated liposarcoma, x = xenograft, variable = variable differentiation (round cell, myxoid and WDLPS), myx = myxoid liposarcoma, r = round cell, dd = regions of dedifferentiation. Stage: Prim = Primary, Rec = Recurrence, Met = metastasis. Status: D = Dead of cancer, DOD = Death of other disease, D-UK = dead of unknown cause, CR = Complete remission, A = alive. mRNA expression: 0 = not detectable, 1 = low, 2 = moderate, 3 = high. Protein expression: 0 = negative (expression >10% of the cells), 1 = weakly positive, 2 = moderately positive, 3 = strong. In parentheses the percentage of immunoreactive cells. NA = Not analysed, NR = Not immuno-reactive, the sample was negative for antibodies against prune, nm23H1 and the control (vimentin), indicating that the fixation of the sample was improper. [1]The patient received preoperative radiation therapy. [2]This patient also suffered from cancer in the lung and kidneys

Cellular proliferation assay

To further understand the role of prune overexpression we have tested its cell proliferation activity in retroviruses infected NIH3T3 cells. Prune recombinant retrovirus increases cellular proliferation fourfold compared to the control (Figure 4a). Similarly, almost twofold increase in proliferation (measured after 48, 72 and 96 h) was observed in transient transfection experiments (48 h post transfection) see Figure 4c. The same is true for a positive control gene (RET/PTC3, Santoro *et al*., 1994) used in the assay (not shown).

Discussion

Amplification of 1q21-q22 is among the most common chromosomal aberrations in a variety of human cancers. In some tumour types, this aberration is more

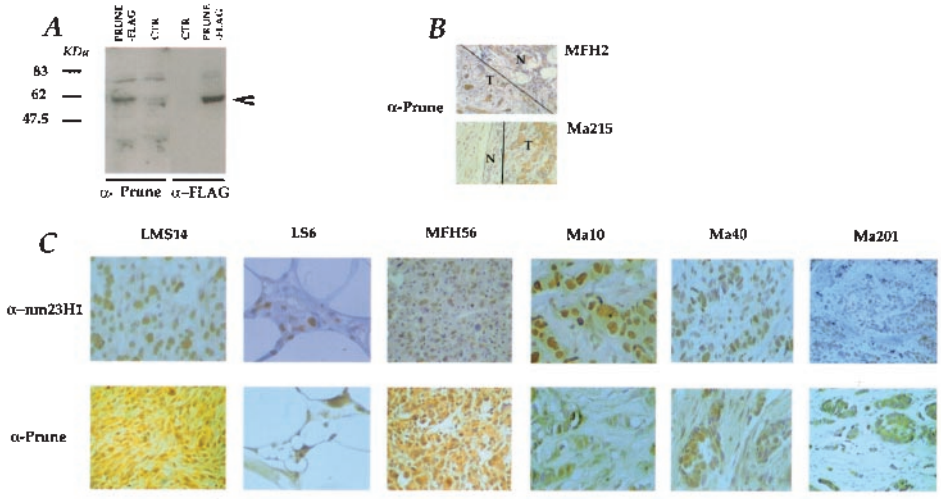

Figure 3 Protein expression of nm23-H1 and prune. (**a**) Western blot analysis of crude protein extracts derived from Cos-7 cells transient transfected cells with pCDNA vector containing *PRUNE* cDNA-*FLAG* gene under a CMV promoter. Lanes 1 and 2 were revealed with the α-Prune polyclonal antibody, Lanes 3 and 4 were revealed with α-FLAG monoclonal antibody as described in Materials and methods. An arrow shows the specific immuno-reactivity against 60 kDa prune protein. (**b**) Representative IHC analyses using antibodies recognising prune protein (α-prune) of MFH2 (upper panel) and Ma215 (lower panel) cases. T = tumour tissue; N = normal tissue. Upper panel: On the left side some MFH2 cells (T) with strong nuclear and cytoplasmic immunoreactivity, on the right side (N) adipocytes, inflammatory cells and vascular endothelial cells with little or no immunoreactivity. Lower panel: On the right side, Ma215 cells (T) with strong nuclear and cytoplasmatic immunoreactivity, on the left side (N) terminal ductal lobular unit, with little or no immunoreactivity. 400 × magnification. (**c**) Representative IHC analyses panel using antibodies recognising nm23-H1 and prune protein. Evaluation of protein levels are shown in Table 1

frequent in metastatic than in primary lesions, associated with short overall survival (Hirai *et al*., 1999; Tarkkanen *et al*., 1999), and/or chemotherapy resistance (Kudoh *et al*., 1999). These findings suggest that possible target genes could be involved in metastasis-related processes, for instance by inhibiting possible metastasis suppressor proteins, or in mechanisms of drug resistance.

It has been suggested that prune, a putative phosphoesterase, may act as a negative regulator of the NDPKinase and putative metastasis suppressor nm23-H1. Prune is ubiquitously expressed in human adult tissues (Reymond *et al*., 1999), while nm23-H1 expression is higher in normal than neoplastic tissues. Accordingly, one would expect that the expression level of prune and nm23-H1 is balanced and tightly regulated in normal cells. In neoplastic tissues, however, we propose that amplification and overexpression of *PRUNE* could shift this balance and abrogate the possible regulatory function of nm23-H1. To be significant, this would require co-expression of the two proteins. Such a mechanism could be comparable to the effect of amplification of *MDM2* and *CDK4*/*CCND1* on p53 and pRb1, respectively. Increased expression of these oncoproteins, caused by increased copy numbers of the respective genes, has been shown to inhibit the function of the two tumour suppressors (Momand *et al*., 1992; Oliner *et al*., 1992; Khatib *et al*., 1993; He *et al*., 1995; Pilotti *et al*., 1998).

We found amplification of *PRUNE* at various levels in 18 out of 19 sarcoma samples. This is the very first indication that amplification and increased expression of an oncogene could be a mechanism for inhibition of nm23-H1 activity. *PRUNE* is also the first candidate gene for the 1q21-q22 amplicon in sarcoma that may affect a possible suppressor of metastasis or proliferation. In the more aggressive sarcoma types, LMS, MFH and MS, *PRUNE* amplification was generally accompanied by moderate to high mRNA levels, and corresponding protein levels. All these samples expressed nm23-H1 mRNA and protein, mostly at low or moderate levels. Thirteen of the patients in this group showed amplification of *PRUNE*, and among those, nine developed metastases, and seven of those died of cancer. For the patients with an unknown copy number of *PRUNE* (16 LS and MFH cases) there was no clear correlation between prune and/or nm23-H1 protein expression and prognosis. Nevertheless, the four patients that developed metastases and died all showed moderate to high expression levels of prune, and low to moderate levels of nm23-H1. The number of patients analysed is too low to draw any conclusion on the association of *PRUNE* amplification and overexpression with metastasis events or disease outcome, but it is remarkable that the expression levels of the gene is generally higher in aggressive tumours than in the relatively benign well-differentiated liposarcomas (WDLPS).

Likewise five out of six breast carcinomas showed moderate amplification of *PRUNE*. In these tumours, mRNA levels of both *PRUNE* and *NM23-H1* were generally high, although protein levels were low to moderate for prune and moderate to high for nm23-H1. Some reports have suggested an association

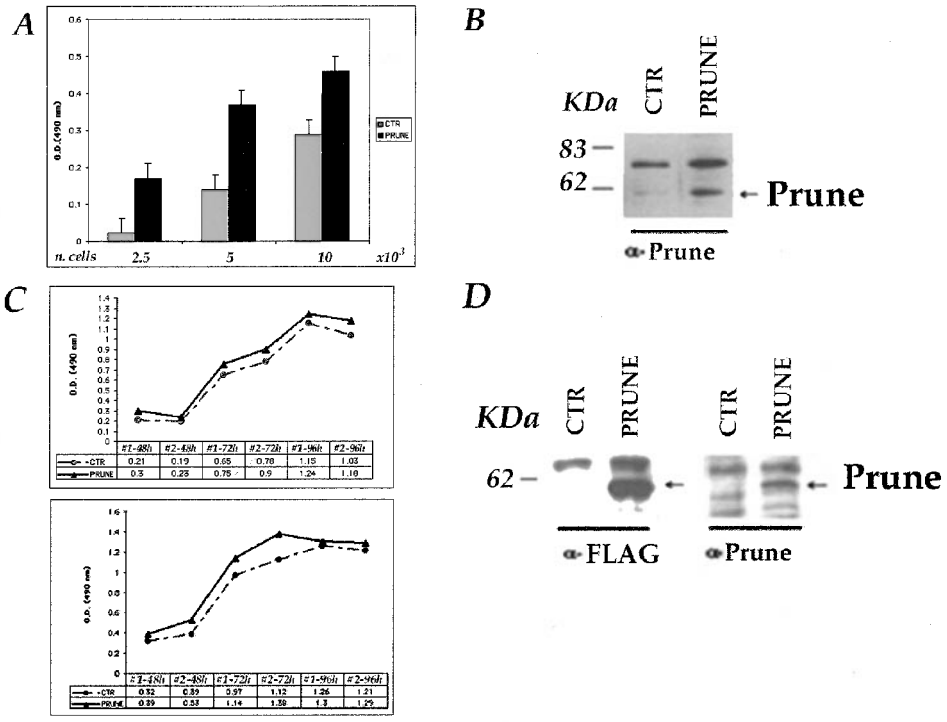

Figure 4 MTS cellular proliferation assay and prune protein content. (**a**) NIH3T3 cells have been infected with both PRUNE cDNA retrovirus and empty virus, and stabilised for 4 days under puromycin antibiotic selection. Each column correspond to the average absorbance value (O.D. = Optical Density) obtained in 12 independent wells with the corresponding standard deviation (s.d.). Black columns values correspond to *x* retrovirus stable NIH3T3 cells, while white columns correspond to control p-Babe retrovirus infected NIH3T3 cells. (**b**) Western blot analyses of infected clones. NIH3T3 protein extracts (3 μg/lane) were analysed by using α-Prune Abs at 1 : 5000 dilution (West Femto substrate). (**c**) NIH3T3 cells transfected by PRUNE cDNA as described in Materials and methods, analysed 48 h after transfection. MTS assays were performed 48, 72 and 96 h later. In the upper and the bottom panel 5000 cells/well and 10 000 cells/well were analysed, respectively. Time points have been scored for 12 independent wells (MTS assay) corresponding to two independent replica clones (#1, #2). (**d**) Western blot analyses. NIH3T3 transfected protein extracts (6 μg/lane) were analysed 96 h after transfection by using α-Prune and α-FLAG Abs at 1 : 5000 and 1 : 6000 dilution (West Femto substrate) respectively

between nm23-H1 negativity and poor prognosis (Royds *et al*., 1993), whereas other reports show that nm23-H1 expression increases in breast carcinoma tissue (Sastre-Garau *et al*., 1992), thus, the results are conflicting. It would be interesting to examine how amplification and overexpression of *PRUNE* correlates with nm23-H1 expression and prognosis in a larger series of breast carcinomas and sarcomas of different grading, since we could not find an unequivocal correlation due to the modest number of samples studied here.

In tumours with expression of nm23-H1 and amplification of *PRUNE* we speculate that the increased levels of prune may interfere with the proposed suppressor activities of nm23-H1. Accordingly, one would expect to find *PRUNE* amplification mainly in tumours that express a normal nm23-H1, and tumours with the nm23-H1-S120G mutation would probably not have a selective advantage from *PRUNE* amplification since this mutant protein has a diminished affinity towards prune (Reymond *et al*., 1999). This model would also explain why some tumours behave aggressively despite their high levels of (normal) nm23-H1, as has been reported for instance in gastric carcinomas (Muller *et al*., 1998).

The finding that *PRUNE* is amplified and over-expressed both in aggressive tumours and tumours of borderline malignancy is similar to what has been reported for *MDM2* in sarcomas (Forus *et al*., 1993; Berner *et al*., 1996; Pedeutour *et al*., 1999). For instance, *MDM2* is amplified and over-expressed in almost all WDLPS cases reported to date, although these tumours are of borderline malignancy, and hardly ever metastasise. Furthermore, although one important role for mdm2 over-expression is to inactivate p53, there is no clear correlation between *MDM2* amplification and tumour aggressiveness.

At present, we have no evidence that prune inhibits the suggested anti-metastatic activity of nm23-H1. However, we show that (over)expression of prune increases proliferation in NIH3T3 cells. Possibly, the amplification and overexpression of the gene may have a similar effect in the tumours. This would explain why amplification of the gene could provide a selective advantage also in the relatively benign WDLPS, which

are tumours that tend to recur, but hardly ever metastasise. However, we do not know at present if this function is regulated by nm23-H1, or via other mechanisms. Furthermore, the data correlating low activity of nm23-H1 to tumour aggressiveness are rather conflicting, and the exact role of nm23-H1 as a metastasis suppressor is not clear. There are several reports that link nm23-H1 activity to cell growth and proliferation. In some cases, decreasing levels of nm23-H1 is associated with increased proliferation (Caligo *et al*., 1997), whereas in other studies, the opposite has been shown (Cippolini *et al*., 1997). Interestingly, Hartsough *et al*. (2001) recently showed that increased Nm23-H1 expression as induced by DNA methylation inhibitors reduced cell motility, thus suggesting a direct function for Nm23-H1 in one aspect of metastasis. We suggest that for some tumours, inactivation of nm23-H1 by amplification and/or over-expression of *PRUNE* may be important for development, and in other tumours, it will affect the progression or the metastatic potential.

Recently, Subramanian *et al*. (2001) reported that the Epstein-Barr virus encoded protein EBNA-3C interacts specifically with nm23-H1, and that this interaction reversed the ability of nm23-H1 to suppress migration of Burkitts lymphoma and breast carcinoma cells. This is the first evidence that a DNA tumour virus may target a cellular protein associated with metastasis. Furthermore, Tseng *et al*. (2001) have reported that an interaction between Rad, a novel Ras-related GTPase, and nm23-H1 may regulate growth and tumorigenicity of breast cancer cells. Since nm23-H1 seems to be involved in a variety of cellular processes, inhibition of the nm23-H1 protein may involve various oncoproteins that target different nm23-H1 protein functions. It will be important to identify other nm23-H1 interacting and regulating proteins in order to fully understand the role of this protein in tumorigenesis and metastasis.

The 1q21-22 region is large and gene rich (Marenholz *et al*., 1996; White *et al*., 1999), and contains several genes with a suggested role in tumour development or progression. The other major amplicon in sarcomas, 12q13-15, contain at least three candidate genes with a proposed role in tumour development, *MDM2, HMGIC* and *CDK4* (Momand *et al*., 1992; Oliner *et al*., 1992; Forus *et al*., 1993; Khatib *et al*., 1993; Berner *et al*., 1996, 1997). The frequency of amplification of the different genes may vary among different tumour types. It is quite likely that *PRUNE* amplification, in a similar way, may be particularly important in some sarcoma subtypes, whereas other genes may be the driving force in other tumours.

Materials and methods

Material

Nineteen sarcomas were first analysed by FISH and Northern analysis: three leiomyosarcomas (LMS), six liposarcomas (LS), nine malignant fibrous histiocytomas (MFH) and one malignant peripheral nerve-sheath tumour (malignant Schwannoma, MS). All tumours except for one were previously analysed by CGH (Forus *et al*., 1995a; Simons *et al*., 2000). Six primary mammary carcinomas (Ma) from patient with advanced stage were included. The tissues were collected immediately after surgery, snap-frozen in liquid nitrogen and stored at -80°C. Fourteen of the sarcomas, the six Ma samples and 16 additional paraffin embedded LS and MFH (tissue array) samples were analysed by immunohistochemistry. All the tumours were classified according to the WHO International Histological Classification of Tumours (Weiss, 1994).

Fluorescent in situ *hybridisation (FISH) to interphase nuclei*

Preparation of interphase nuclei from frozen tissues and cell lines was done as described (Forus *et al*., 1998). One of the samples, LMS14 was available only as paraffin embedded tissue. Preparation of the sections was done by standard procedures.

Probes PAC 279H19, containing the human *PRUNE* gene, was from the human PAC library RPCI-5 pools. The centromere 1 probe was a biotin-labelled D1Z5 α-satellite (P5001-B.5, Oncor, Gaithersburg, MD, USA).

Preparation of probes DNA from 279H19 was labelled with biotin-14-dATP or digoxygenin-11-dUTP (Boehringer-Mannheim, Germany) by nick translation. For each hybridisation, 200–500 ng of labelled DNA was pre-hybridised with 50–100-fold excess of human Cot-1 DNA. The probes were denatured at 80°C for 10 min, snap-cooled on ice and pre-hybridised at 37°C for 30–60 min. The centromere 1 probe was used as recommended by the manufacturer.

FISH hybridisation to interphase nuclei Slides were treated as described (Forus *et al*., 2001), hybridisation and washes were according to standard procedures. Fluorescein isothiocyanate (FITC)-conjugated sheep anti-digoxygenin (Boehringer Mannheim, Germany) followed by FITC- or ALEXA 488-labelled donkey anti-sheep (Molecular Probes, Leiden, The Netherlands) was used for detection of digoxigenin, andavidin-conjugated Cy3 (Amersham Life Science, Little Chalfont, UK) for detection of biotin.

FISH hybridisation to paraffin sections Slides were treated as described (Kononen *et al*., 1998). Detection of signals was done by tyramide amplification (TSA-Indirect kit for ISH, NEN, Boston, MA, USA) followed by a streptavidine-FITC antibody (NEN, Boston, MA, USA) (Forus *et al*., 2001).

Evaluation of results Hybridised slides were examined visually using a Zeiss Axioplan microscope equipped with appropriate filter for exitation of DAPI, DAPI/FITC, DAPI/Rhodamine (Cy3), and DAPI/FITC/Rhodamine(Cy3). For each probe, the number of hybridisation signals was counted in at least 150 nuclei per slide.

Prune retrovirus production and cellular proliferation assay

pBABE puro-PC3 vector was modified to include a FLAG epitope at the carboxy-terminal region of the protein and an SP6 promoter and primer site at the 3′ terminus in the poly-

linker sequence at the *Eco*RI site. The vector was used to subclone the entire full-length PRUNE cDNA and used in transient transfection of BOSC 23 ecotropic retroviruses cells as described (Pear *et al*., 1993). High titer retrovirus supernatant was collected and used to infect NIH3T3 cells lines, a generous gift from Dr Mario Chiariello (Università degli Studi Federico II, Naples, Italy). After 4 days on puromycin (2 μg/ml) selection the cells were tested by a cellular proliferation and viability assay 'MTS protocol' (Cell-Titer 96™ AQ_{ueous} proliferation assay-Promega). Cells were plated in a 96 well plate at concentrations ranging from 2.5, 5 and 10×10^3 cells per well and assayed after 48 h. A colorimetric reaction was performed after incubation of the cells for 2 h at 37°C measuring the absorbance (O.D.) at 490 nm using a Packard 96-well reader spectrophotometer. The results presented are the mean of four replicate assay series.

Transient transfection and cellular proliferation assay in NIH3T3 cells

1×10^6 NIH3T3 cells were transfected by Lipofectamine reagent (Gibco-BRL) with 10 μg of pCDNA vector containing *PRUNE* cDNA-*FLAG* gene under a CMV promoter. After 48 h, transfected cells were plated in a 96 well plate and measured by MTS protocol (48, 72 and 96 h later).

Northern analyses

RNA expression levels were analysed by Northern blot analysis. Total cellular RNA was isolated from frozen tissue or cell lines, electrophoresed and blotted onto membranes essentially as described (Forus *et al*., 1993). Quantitation of signal intensities was carried out by two-dimensional densitometry on a laser densitometer (Molecular Dynamics), and expression levels were calculated using the ImageQuant analysis software. A *PRUNE* cDNA clone and a specific cDNA probe for *nm23-H1* as described (Reymond *et al*., 1999) were sequentially hybridised to the filter. The net signal values were corrected for uneven loading by calibration relative to signals from an 18S-oligonucleotide control probe. Expression levels were grouped as follows: No expression (0), weak (1), moderate (2) and high (3).

Immunohistochemistry

Construction of a tissue array Thirty of the sarcoma samples have been included in a tissue-array (Forus and Kononen, unpublished) and protein analyses of these samples was done using this array. All the samples were from archived formalin-fixed, paraffin embedded material. Two tissue cylinders (0.6 mm) were punched from representative tumour areas on each tissue block, and both samples were evaluated in the protein analyses. The construction of the arrays was done as described previously (Kononen *et al*., 1998; Schraml *et al*., 1999). For the remaining samples, 4 μ sections were prepared from formalin-fixed, paraffin embedded material after standard procedures.

Production of prune antibodies The entire full-length PRUNE cDNA and almost two thirds of the entire cDNA was sub-cloned in both pMaltose and PGEX4X plasmid to produce *E. Coli* fused protein (inducible expression systems) respectively. *E. Coli* competent cells were transformed and induced by IPTG for 3 h at 37°C. Cells were harvested and lysed with low stringent detergent solution, and protein purification was performed as described (New England Biolabs Inc.; Pharmacia Biotech). Columns with amylose and glutathione-derived resin were used to further purify the fused protein, and a total of 1.3 mg of pure recombinant protein was injected in rabbits to obtain polyclonal antibodies. The antibodies were purified according to manufacturer's protocols. Western blotting (Figure 3a) and immuno-fluorescence analysis on paraffin-embedded human tissue sections were used to determine the affinity and specificity of the prune polyclonal antibody.

Western blotting Standard procedures were followed. The prune polyclonal antibody was used at 1 : 500 dilution. The control, anti-FLAG monoclonal antibody M2 Ab (Sigma), was used at 1 : 4000 dilution. ECL detection system (Amersham), West Femto substrate (Pierce) and PVDF transfer membranes (Millipore) were used according to manufacturer's protocols.

Antibody staining All IHC analyses were done on paraffin sections from individual samples or the tissue array. The ABC procedure was used for immunohistochemistry (VECTASTAIN from Vector, Vector Laboratories, Inc). The monoclonal antibody anti-NDP kinase:nm23-H1, clone NM301 (from Santa Cruz USA) was used at 1 : 25 dilution. The prune polyclonal antibody was used at 1 : 300 dilution and the monoclonal anti-vimentin antibody (clone Vim 3B4, Boehringer-Mannheim) was used at 1 : 300 dilution. Colorimetric reaction was performed by using DAB (Vector, Vector Laboratories, Inc). Microwave pretreatment was used for nm23-H1 and vimentin detection for 15′ at 90°C in 10 mM NaCitrate pH6. The primary antibody was omitted for negative controls. Positive controls included sections from tissues known to be positive. The controls gave satisfactory results.

Evaluation of results We have observed nm23H1 protein with a prevalent nuclear localisation and with some cytoplasmic staining, whereas prune immunoreactivity was mainly cytoplasmic and in few cases nuclear as already described by Reymond *et al*. (1999). Tumours were considered positive if at least 10% of the tumour cells were stained. Signal intensity was grouped as follows: Negative (0, expression in $<10\%$ of the cells), weakly positive (1), moderately positive (2) and strong (3).

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