Homologous deletion frequency and expression levels of the CDKN2 gene in human sarcomas – relationship to amplification and mRNA levels of CDK4 and CCND1

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Summary Homologous deletions of the putative tumour-suppressor gene CDKN2, which encodes an inhibitor of cdk4, have been detected in a high percentage of cancer cell lines of various histological types. In the present study, 109 human sarcomas were examined for homologous deletions and for mRNA expression levels of the CDKN2 gene. Altogether, deletions were found in only eight (7%) of the cases, but, interestingly, in two (of eight) malignant Schwannomas and in two (of five) rhabdomyosarcomas. In comparison, such deletions were seen in only one (of 21) osteosarcomas and in none of 20 MFHs and 21 liposarcomas. Notably, highly elevated CDKN2 mRNA levels were found in 33% of the sarcomas, whereas no detectable transcript was present in 12 normal tissues. Amplifications of CDK4 and CCND1 (cyclin D1) were observed in 11% and 4% of the sarcomas respectively, but never in tumours with CDKN2 deletions. The level of CDK4 mRNA expression was increased in nine tumours in addition to the 12 samples with CDK4 amplification. Increased levels of the cyclin D1 transcript was found in 37 cases, four with and 33 without amplification. The data indicate that aberrations of these functionally related genes, or in regulation of the expression of the kinase, the activator or the inhibitor, may participate in sarcoma development. Furthermore, the data suggest that homologous CDKN2 deletions may be of dissimilar significance in different sarcoma subtypes.

Keywords: MTS1; p16\textsuperscript{INK4}; chromosome 9p21; pRb; cyclin D1

Identification of various molecules involved in cell cycle control has demonstrated a close association between regulation of the cell cycle and neoplastic transformation. Thus, derangements in the cell cycle machinery may play a critical role in oncogenesis and contribute to uncontrolled cell growth.

Recent results indicate that an inhibitor of the cell cycle, the p16 protein, may be a new tumour suppressor. The gene encoding p16, denoted either MTS1 (multiple tumour suppressor 1) (Kamb et al., 1994), CDK41 (Nobori et al., 1994) or CDKN2 (The HUGO Nomenclature Committee designation), is localised to human chromosome segment 9p21, a region frequently found to contain cytogenetic abnormalities in several types of cancer, including malignant melanomas, gliomas, lung carcinomas and leukaemias (Kamb et al., 1994; Nobori et al., 1994). Moreover, the gene has been found to be homozgyously deleted or mutated in a high percentage of cell lines derived from tumours of various histological types (Kamb et al., 1994). On this background CDKN2 has been suggested to be involved in the formation of malignancies originating from a wide range of tissues (Kamb et al., 1994).

p16 was originally identified when searching for proteins able to associate with the cell cycle regulating enzyme cyclin-dependent kinase 4 (cdk4) (Serrano et al., 1993). Cdk4 is, when activated by cyclin D1, able to phosphorylate the retinoblastoma tumour-suppressor protein (pRb), resulting in release of pRb-mediated G\textsubscript{1} arrest. Since p16 can bind to cdk4 and thereby inhibit the catalytic activity of the cyclin D1–cdk4 complex, the protein seems to participate in a regulatory pathway together with cdk4, cyclin D1 and pRb.

Alterations of cyclin D1 and cdk4 have also been suggested to be involved in oncogenesis (Khatib et al., 1993; Motokura and Arnold, 1993). Thus, translocations involving the q13 segment of chromosome 11, harbouring the cyclin D1 gene (CCND1), have frequently been observed in parathyroid adenomas and B-cell lymphomas (Motokura and Arnold, 1993). In addition, CCND1 has been found amplified and overexpressed in breast (Lammie et al., 1991) and oesophageal carcinomas (Jiang et al., 1992). Similarly, the amplification and overexpression of CDK4, localised to chromosome band 12q13, has been suggested to contribute to deranged growth control in some sarcomas (Khatib et al., 1993; Forus et al., 1995).

As most studies on CDKN2 so far have been performed on cell lines, it is still unclear to what extent deletions and mutations of this gene are a result of in vitro cell cultivation or represent a critical step in cancer development. Preliminary reports indicate that p16 aberrations are not as frequent in biopsed tumour material as first anticipated (Cairns et al., 1994; Spruck et al., 1994). In an attempt to determine the possible involvement of CDKN2 in sarcoma tumorigenesis, we screened a panel of 109 tumours for homologous deletions of CDKN2. In parallel, the amplification frequency of the functionally related genes CDK4 and CCND1 was studied. Furthermore, the mRNA levels of the three genes were determined to examine to what extent the DNA status of the tumours was reflected at the transcriptional level, or if aberrant gene expression could be observed in tumours without detectable deletions or amplifications. It was also of interest to analyse whether a consistent co-variation might exist between the mRNA levels of the genes encoding the kinase (cdk4), the activator (cyclin D1) and the inhibitor (p16).

Materials and methods

Specimens

The panels and xenografts were harvested from patients with sarcomas following ethical approval both in the USA (National Cancer Institute xenograft programme) and in Europe (European Organisation for Research and Treatment of Cancer). Sarcoma tissue of different histological subtypes was obtained from 77 patients and from 27 human tumour xenografts in nude mice. In five cases, both patient and xenograft material was available. In addition, five human sarcoma cell lines and a panel of 12 normal tissue samples representing mononucleated cells from peripheral blood, kidney, colon, liver, salivary gland, brain, lung, placenta, striated muscle, breast gland, ovary and skin were studied. The different sarcoma subtypes were represented by two chondrosarcomas, 21 osteosarcomas, two carcinosarcomas, five fibrosarcomas, one haemangiopericytoma, 13 leiomyosarcomas, 21 liposarcomas, 20 malignant fibrous histiocytomas.
(MFHs), eight malignant Schwannomas, five rhabdomyosarcomas and 11 non-classified sarcomas. The last group included one undifferentiated, one neuroectodermal and one monocytic-like sarcoma. Immediately upon surgery, the tumour tissue was frozen into liquid nitrogen and subsequently stored at −135°C.

Southern blot analysis

Genomic DNA from sarcoma tissue was isolated by standard methods (Maniatis et al., 1982). Aliquots (7 µg) of DNA were digested with HindIII, separated on 0.8% agarose gels and transferred by alkaline blotting onto Hybond N* membranes (Amersham, Amersham, UK), according to the manufacturer’s manual. After UV cross-linking for 5 min, the blots were prehybridised for 2 h and subsequently hybridised with DNA probes labelled with 32P by the random primer technique (Feinberg and Vogelstein, 1983). The hybridisation was carried out in 50% formamide, 6 x standard saline citrate (20 x SSC = 3.0 M sodium chloride, 0.3 M sodium citrate), 0.5% sodium dodecyl sulphate (SDS), 1.5 x Denhardt’s (50 x Denhardt’s = 1% Ficoll, 1% bovine serum albumin, 1% polyvinylpyrrolidone) and 100 µg ml−1 denatured salmon sperm DNA at 42°C over night as described by Maniatis et al. (1982). After hybridisation, the membranes were washed for 20 min at 65°C subsequently in 2 x SSC/0.5% SDS, 1 x SSC/0.5% SDS and 0.5 x SSC/0.5% SDS. For multiple hybridisations, the bound probe was removed by incubating the filters for 15 min at room temperature in 100 mM sodium hydroxide and 1 mM sodium EDTA.

Samples with a signal weaker than 25% when compared with the signal from a reference lane were scored as having a homologous deletion of the corresponding gene. A signal at least 3-fold more intense than signals from samples with a normal copy number of the gene was scored as an amplification. Denstomietronic analysis of the autoradiograms was used to decide in cases that were not obvious. To adjust for unequal amounts of loaded DNA, the blots were rehybridised to a control probe encoding apolipoprotein B, located on chromosome 2.

Northern blot analysis

Total cellular RNA was prepared by the guanidinium thiocyanate–cesium chloride method described by Maniatis et al. (1982). Samples of 5 µg of total RNA were separated by 1% agarose–formaldehyde gel electrophoresis and blotted onto Hybond N* membranes (Amersham, Amersham, UK) according to the manufacturer’s manual. After baking for 2 h and subsequent ultraviolet cross-linking, the filters were hybridised with DNA probes labelled with 32P by the random primer method (Feinberg and Vogelstein, 1983). The hybridisations were carried out in 0.5 M sodium phosphate (pH 7.2), 7% SDS and 1 mM sodium EDTA overnight at 65°C as described by Church and Gilbert, (1984). The membranes were subsequently washed three times for 15 min in 40 mM sodium phosphate (pH 7.2) and 1% SDS. For multiple hybridisations the bound probe was removed by incubating the filters twice for 5 min in 0.1 x SSC and 0.1% SDS at 95–100°C.

To correct for uneven amount of RNA loaded in each lane, the filters were rehybridised with a kinase-labelled (Maniatis et al., 1982) oligonucleotide probe specific for human 18S rRNA. The mRNA expression levels were classified as follows: ‘−’/‘+’, undetectable/low expression; ‘++’ and ‘+++’, high or very high expression.

Probes

The following probes were used: CCND1 cDNA kindly provided by Dr D Beach, Howard Hughes Medical Institute, Cold Spring Harbor, NY, USA; and CDK4 cDNA by Dr P Meltzer, National Institute of Health, Bethesda, MD, USA. As probe for CDKN2 a 929 bp PCR product was used, amplified from a plasmid encoding the CDKN2 cDNA (Dr D Beach) with primers suggested by Kamb et al. (1994). The APOB clone pB27, kindly provided by Dr J Breslow, Rockefeller University, New York, NY, USA, and a human specific oligonucleotide probe complementary to nucleotides 287–305 of 18S rRNA were used as control probes for the Southern and Northern blots respectively.

Results

Southern blot analysis

DNA from 109 human sarcomas of various histological subtypes was analysed for homologous deletions of the putative tumour-suppressor gene CDKN2 and for amplification of CDK4 and CCND1 (Table I and Figure 1). Homologous deletion of CDKN2 was found in eight tumours (7%), including two rhabdomyosarcomas, two malignant Schwannomas, one chondrosarcoma, one leiomyosarcoma, one osteosarcoma and one non-classified sarcoma. Six of these samples were obtained directly from patients (6/77 = 8%), one specimen was a xenograft (1/27 = 4%) and one was a cultured cell line (1/5 = 20%).

Amplification of CDK4 was observed in 12 cases, including four liposarcoma, two osteosarcoma and in one haemangiopericytoma, one malignant Schwannoma, rhabdomyosarcoma and a non-classified sarcoma. CCND1 amplification was found in only four cases: one osteosarcoma, one haemangiopericytoma, one liposarcoma and one non-classified sarcoma. Interestingly, none of the tumours with CDKN2 deletions showed amplification of any of the two other genes, whereas three of the four cases with amplified CCND1 also had amplification of CDK4. When adding up the alterations involving the CDKN2, CDK4 and CCND1 genes, the total number of aberrations in this pathway increases to 21 cases (Table I).

Northern blot analysis

To examine the association between DNA status and gene expression at the mRNA level, total RNA was extracted from 100 of the 109 tumours and from a panel of 12 different normal tissue samples.

As expected, no CDKN2 mRNA was detected in tumours with homologous deletion of the gene, whereas these cases expressed variable amounts of mRNA for both cdk4 and cyclin D1 (Figure 2 and Table I). Interestingly, although none of the 12 normal tissues examined expressed detectable levels of the CDKN2 transcript, 33 of the sarcomas (33%) showed high to very high mRNA levels (‘++’ or ‘+++’), of which 18 were patient biopsies (14 primary tumours and four metastatic lesions, i.e. 26% of the patient biopsies), whereas 13 originated from xenografts (48% of the xenografts) and two were in vitro cell lines (50%) (Table II).

All 12 tumours with CDK4 amplification demonstrated a high (‘++’ or ‘+++’) transcript level, and 11 of these tumours showed a concomitant high expression of one of the two other genes (Figure 2 and Table I). Moreover, high (‘++’ or ‘+++’) CDK4 expression was found also in nine tumours without amplification of the gene (Table II). Altogether, elevated CDK4 transcript levels were present in biopsies from 11 patients (16%), in seven sarcoma xenografts (26%) and in three in vitro cell lines (75%).

The expression levels in normal tissues varied. Thus, specimens obtained from kidney, lung, ovary and breast gland showed a high (‘+++’) expression, whereas the other tissues demonstrated a low, but detectable (‘+’) CDK4 mRNA level.

Except for lung and skin, the mRNA level of CCND1 was generally low in the normal tissues. In contrast, high expression levels were found in 37 sarcomas (37%), of which 24 were tumour biopsies (35% of the patient biopsies), 12 xenografts (44%) and one of four cultured cell lines (Table II). Unlike the results of the other transcripts, the expression patterns of CCND1 differed in the two main groups of sarcomas. Thus, whereas 42% of the soft-tissue tumours
expressed high levels of cyclin D1 mRNA, only 22% of the osteogenic sarcomas did so.

**Relationship between the mRNA levels of CDKN2, CDK4 and CCND1**

Of the 33 tumours found to express high levels of mRNA encoding the cdk4 inhibitor, 25 (76%) had low or undetectable level of the CCND1 transcript. Conversely, of the 37 tumours with high CCND1 expression, 29 (78%) did not express detectable amounts of the inhibitor mRNA. Taken together, these results suggest an inverse relationship between the mRNA levels of the kinase inhibitor and the kinase activator (two-sided Fischer exact test, \( P = 0.08 \)).

In 10 of the 33 tumours the elevated inhibitor expression was accompanied by a high level of CDK4 mRNA, including nine cases with amplification of the kinase gene. Only five tumours, all with amplification of CDK4, showed high expression of all three genes.

**Discussion**

The CDKN2 gene, encoding an inhibitor of cdk4 activity, was recently reported to be homozygously deleted in a high percentage of human cancer cell lines (Kamb et al., 1994; Nobori et al., 1994). Based on these results it was suggested that the inhibitor, p16, may be an important new tumour-suppressor protein (Kamb et al., 1994). By binding to cdk4, p16 inhibits the formation of the cyclin D1–cdk4 complex, which is known to phosphorylate and thereby inactivate the retinoblastoma protein (Serrano et al., 1993). The loss of p16 expression caused by gene deletion may result in increased cdk4-induced phosphorylation of pRb, thus releasing the G1 cell cycle block.

To investigate whether CDKN2 aberrations might be involved in the tumorigenesis of human sarcoma, a panel of more than 100 tumours of various histological subtypes was analysed for homozygous deletions of the gene. It was found that only eight of the sarcomas showed such deletions. Notably, no deletion of CDKN2 was seen in any of the 21 liposarcomas and 20 MFHs studied, and in only one of 21 osteosarcomas. The low deletion frequency stands in contrast to the 25–87% homozygous deletions found in various human tumour cell lines, including 60% of the human osteosarcoma cell lines studied (Kamb et al., 1994; Nobori et al., 1994). Interestingly, however, we found that two of eight malignant Schwannomas and two of five rhabdomyosarcomas had lost CDKN2, suggesting that homozygous deletions of CDKN2 might be of different importance in the development of various sarcoma subtypes. The overall low incidence of deletions found here is in accordance with the results of Spruck et al. (1994) and Cairns et al. (1994), who observed homozygous deletions that included the CDKN2 gene only in about 10–20% of bladder, brain, head and neck and lung carcinomas. Conceivably, the high deletion frequency observed in cell lines might be a result of *in vitro* cell cultivation. Our data do not permit any conclusions on this, but it was found that one of five sarcoma cell lines had lost the CDKN2 gene, whereas only one of 27 (4%) sarcomas

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**Table I** Tumours with DNA* deletion or amplification affecting either of the CDKN2, CDK4 or CCND1 genes. Relationship to the mRNA levels

| Tumours with DNA aberrations (total no. of tumours of each subtype) | CDKN2 DNA | CDKN2 mRNA | CDK4 DNA | CDK4 mRNA | CCND1 DNA | CCND1 mRNA |
|---|---|---|---|---|---|---|
| Osteosarcoma (n = 21) | | | | | | |
| O56x | N | + (+) | A | ++ | N | (–) |
| OS11p | D | – | N | +++ | N | + |
| OSAcl | N | +++ | A | +++ | A | +++ |
| Chondrosarcoma (n = 2) | | | | | | |
| CS2p | D | – | N | (+) | N | + |
| Fibrosarcoma (n = 5) | | | | | | |
| FS1p | N | + | A | +++ | N | + |
| FS2p | N | (+) | A | + | N | + |
| Haemangiopericytoma (n = 1) | | | | | | |
| HP1x | N | +++ | A | +++ | A | +++ |
| Leiomyosarcoma (n = 13) | | | | | | |
| LMS8p | D | – | N | (+) | N | +++ |
| Liposarcoma (n = 21) | | | | | | |
| LS2p | N | (+) | N | (+) | A | +++ |
| LS11p | N | + | A | +++ | N | + |
| LS21p | N | + | A | +++ | A | + |
| LS22p | N | (+) | A | ++ | N | + |
| LS28x | N | +++ | A | +++ | N | + |
| Malignant Schwannoma (n = 8) | | | | | | |
| MS2x | D | – | N | + | N | + |
| MS7p | D | – | N | + | N | + |
| MS8p | N | + | A | +++ | N | + |
| Rhabdomyosarcoma (n = 5) | | | | | | |
| RMS3p | D | – | N | + | N | – |
| RMS4cl | D | – | N | + | N | + |
| RMS13cl | N | + | A | +++ | N | + |
| Non-classified sarcoma (n = 11) | | | | | | |
| NCS2x | N | +++ | A | +++ | A | +++ |
| NCS7p | D | – | N | + | N | (+) |

*No. of tumours with DNA alterations | 8 | 12 | 4

*\( ^N \), normal; \( ^D \), deletion; \( ^A \), amplification. *Expression levels as described in Materials and methods. *\( ^c \), cell line; \( ^p \), patient biopsy; \( ^x \), xenograft. Altogether 109 tumours.
Figure 1  Representative Southern blot analysis demonstrating homozygous deletion of CDKN2 (a and b) and amplification of CDK4 and CCND1 (a). The DNA (7 μg in each lane), digested with HindIII, was subsequently hybridised with probes encoding the three different genes and, as a control, with an APOB probe. Samples with DNA aberrations, scored as described in Materials and methods, are indicated by a D (deletion) or A (amplification) below the corresponding panel. For the MS8 tumour, two additional bands emerged with the CCND1 cDNA probe owing to cross-hybridisation of cyclin D2 which is amplified in this tumour.

Figure 2  Northern blot analysis demonstrating the mRNA levels of CDKN2, CDK4 and CCND1 in the same sarcomas as listed in Figure 1a and the OSA cell line. Five micrograms of total RNA in each lane was subsequently hybridised with probes encoding the three different genes, and as a control with an 18 S rRNA oligonucleotide probe.

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grown as xenografts in nude mice harboured such lesions. When tumour material and xenografts established from the same patient could be examined, identical results on CDKN2 aberrations were obtained, indicating that the process of xenografting does not induce homozygous deletion of the gene, in accordance with the results of Caldas et al. (1994).

The possibility exists that the somewhat contradicting reports concerning the incidence of homozygous CDKN2 deletions might in part be related to methodological factors. Despite careful dissection of the tumour tissue before freezing, infiltration of normal cells in the tumour biopsy could result in a low observed deletion frequency. However, Southern blot analysis, as used here, should be less sensitive to a moderate contribution from normal tissue than the PCR analysis used by others (Cairns et al., 1994; Kamb et al., 1994; Nobori et al., 1994; Spruck et al., 1994).

Furthermore, it should be noted that part of our sarcoma material has previously been analysed for loss of heterozygosity of the TP53 gene without any indications of normal cell DNA affecting the results (Andreassen et al., 1993). It cannot be excluded that the CDKN2 gene may be inactivated by mechanisms other than loss of the entire gene, including point mutations and small deletions, as have been reported in cases of oesophageal (Mori et al., 1994), non-
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Table II Tumours with elevated mRNA expression

| Source of sample | No. analysed | CDKN2 | CDK4 | CCND1 |
|------------------|-------------|-------|------|-------|
| Tumour tissue    | 69          | 18    | 11   | 24    |
| Xenografts       | 27          | 13    | 7    | 12    |
| Cell lines       | 4           | 2     | 3    | 1     |
| Total            | 100         | 33 (33%) | 21 (21%) | 37 (37%) |

*Scored as described in Materials and methods.
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