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Expression of *MYCN* in pediatric synovial sarcoma

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Synovial sarcoma accounts for between 6 and 10% of childhood sarcomas and histological diagnosis can be challenging, even for experienced pathologists. Several other tumors enter the differential diagnosis, including malignant peripheral nerve sheath tumor, Ewing sarcoma/primitive neuroectodermal tumor and undifferentiated sarcoma. Several recent reports utilizing expression array techniques have documented expression of the *MYCN* oncogene in synovial sarcoma. In order to more fully investigate this finding, a series of 12 synovial sarcomas and 29 other sarcomas (four malignant peripheral nerve sheath tumors, 15 Ewing sarcoma/primitive neuroectodermal tumors, 10 undifferentiated sarcomas) were examined for *MYCN* expression and gene amplification. By RT-PCR, nine of 12 synovial sarcomas (75%) expressed *MYCN.* Five synovial sarcomas (42%) expressed *MYCN* at high levels. Of the other sarcomas, one malignant peripheral nerve sheath tumor (25%) and five Ewing sarcoma/primitive neuroectodermal tumors (33%) expressed *MYCN* at low levels, and all other cases were negative for *MYCN*. None of the synovial sarcomas had genomic amplification, suggesting that high *MYCN* expression levels resulted from epigenetic phenomena. Examination of selected downstream targets of *MYCN* in synovial sarcoma revealed expression of *MCM7* (minichromosome maintenance protein 7) in all synovial sarcomas, and expression of nestin ($n = 10$; 83%), *ID2* (inhibitor of DNA binding protein 2) ($n = 6$; 50%) and *MRP1* (multidrug resistance protein 1) ($n = 1$; 8%) in a subset of synovial sarcomas. Expression of downstream targets did not correlate with expression of *MYCN*. Neither *MYCN* nor expression of downstream targets significantly correlated with metastases at presentation, progression-free survival or overall survival in this small series. In summary, high levels of *MYCN* expression was useful for distinguishing synovial sarcoma from other childhood-spindled cell sarcomas with specificity and sensitivity of 100 and 42%, respectively, in this series. The clinical and biological significance of this finding deserves further study.
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Synovial sarcoma is a soft tissue tumor of uncertain mesenchymal cell origin. It accounts for 2–6% of sarcomas in childhood[1] and up to 10% of sarcomas in late childhood and early adulthood,[2] with most cases occurring between the ages of 15 and 35 years.[2] The most common sites of occurrence are the extremities, usually around joints, with smaller numbers occurring in the head and neck, retroperitoneum and mediastinum.[1–3] Histologically, there are four major types; biphasic, monophasic spindled, monophasic epithelioid and poorly differentiated, with the former two being the most common.[1,2,4] Synovial sarcoma is associated with a specific balanced translocation t(X:18)(p11.2;q11.2) in more than 90% of cases.[4–6] The rearrangement fuses the *SYT* gene on chromosome 18q11.2 to one of the *SSX* gene family members (*SSX1*, *SSX2* or *SSX4*) located on Xp11.2.[7–10] Prognostic indicators of synovial sarcoma include age, size and morphology,[4] as well as molecular genetics, with *SSX2* translocations associated with a better outcome.[11]

The diagnosis of synovial sarcoma can be difficult to make, especially for the monophasic-spindled and poorly differentiated variants. The histological differential diagnoses include other childhood sarcomas, namely malignant peripheral nerve sheath tumor, Ewing sarcoma/primitive neuroectodermal tumor and undifferentiated sarcoma.[12,13] Malignant peripheral nerve sheath tumor can be particularly

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difficult to distinguish from synovial sarcoma, as they share several morphological, immunohistochemical and ultrastructural features.[13–15] Some authors have even reported the rare occurrence of a t(X;18) translocation in malignant peripheral nerve sheath tumor,[16] adding further difficulty in differentiating these two tumors. Thus, markers specifically expressed in either one tumor or the other would be extremely useful.

MYCN is a member of the *MYC* family of oncogenes, which also include *CMYC* and *LMYC*. Amplification of the *MYCN* gene is associated with advanced tumor stage, tumor progression and poor outcome in neuroblastomas.[17] Several downstream targets of *MYCN* have been described and include the genes encoding the *m*ultidrug *r*esistance *p*rotein 1 (*MRP1*),[18] the *m*ini*c*hromosome *m*aintenance protein 7 (*MCM7*),[19] the *i*nhibitor of *D*NA binding protein *2* (*ID2*)[20] and the intermediate filament nestin.[21]

Amplification and overexpression of *MYCN* has been described in tumors other than neuroblastoma, including alveolar rhabdomyosarcoma,[22] astrocytoma,[23] medulloblastoma,[24] retinoblastoma,[25] Wilms tumor[26] and breast carcinoma.[27] Two recent studies utilizing cDNA expression array analysis documented *MYCN* expression in synovial sarcoma.[28,29] With respect to malignant peripheral nerve sheath tumor, one study has documented *MYCN* expression,[29] whereas others have not.[30,31] *MYCN* expression has not been reported in Ewing sarcoma/primitive neuroectodermal tumor.[32,33] In order to investigate the potential diagnostic usefulness of these findings, the current study examined the expression pattern of *MYCN* in a series of 12 synovial sarcomas and 29 other pediatric sarcomas by RT-PCR. In addition, the status of *MYCN* gene amplification and expression of downstream targets of *MYCN* was assessed in synovial sarcoma.

Materials and methods

Tumor Selection

Twelve cases of synovial sarcoma diagnosed between 1990 and 2004 were selected from the files of the Hospital for Sick Children, Toronto. All tumors were positive for the *SYT/SSX* fusion transcript indicative of the t(X;18)(p11.2;q11.2) chromosomal rearrangement. A series of other sarcomas entering the differential diagnosis of synovial sarcomas were also tested and included four malignant peripheral nerve sheath tumors (S100 positive, t(X;18) negative), 15 Ewing sarcoma/primitive neuroectodermal tumors (CD99 positive, *EWS*-rearrangement positive) and 10 undifferentiated sarcomas (see Somers *et al*[12] for selection criteria). A series of neuroblastomas diagnosed during the same time period were used as a control group. The study design and implementation were approved by the Hospital for Sick Children Research Ethics Board.

RT-PCR for *SYT/SSX* Fusion Transcripts and *MYCN* and *MRP1* Gene Expression

RNA was extracted from fresh snap-frozen tissue using routine Trizol-based methods. RT-PCR for the *SYT/SSX1* and *SYT/SSX2* fusion transcripts was performed according to previously published protocols.[34] Semiquantitative RT-PCR for *MYCN* gene expression was performed using *MYCN* primers (*MYCN* forward 5′-CGA CCA CAA GGC CCT CAG TA-3′ and *MYCN* reverse 5′-CAG CCT TGG TGT TGG AGG AG-3′) and *PBGD* primers (*PBGD* sense 5′-CAT GTC TGG TAA CGG CAA TGC GGC TGC-3′ and *PBGD* antisense 5′-GAA CTC CAG ATG CGG GAA CTT TC-3′). The expression levels for *MYCN* were interpreted as high (similar to the level of expression in the neuroblastoma cell line NUB-7), low (similar to the level of expression in neuroblastoma cell line SK-N SH) or negative (normal tonsil RNA). Semiquantitative RT-PCR for *MRP1* gene expression was performed using *MRP1* primers (*MRP1* forward 5′-TCT CTC CCG ACA TGA CCG AGG-3′ and *MRP1* reverse 5′-CCA GGA ATA TGC CCC GAC TTC-3′) and *PBGD* primers as for the *MYCN* assay (see above). Expression levels of *MRP1* were interpreted as high (similar to expression IMR32 cell line) or low (similar to expression of RNA from tonsil).

PCR for *MYCN* Amplification

DNA was extracted from fresh snap-frozen tissue using routine methods. Primers and conditions for semiquantitative PCR amplification of the *MYCN* gene were performed according to previously published protocols.[35]

Chromogenic *In Situ* Hybridization for *MYCN* Gene Amplification

Chromogenic *in situ* hybridization (CISH) was performed using the Spotlight® CISH polymer detection kit (Zymed Laboratories, San Francisco, CA, USA) using the digoxigenin-labeled Spotlight® N-MYC probe (Zymed Laboratories) as described previously.[36] Scoring of the histological sections for amplification was performed according to previously published criteria.[36] Briefly, amplification was defined as greater than 10 signals per tumor cell nucleus, or the presence of homogenously stained regions within tumor cell nuclei.

Immunohistochemistry for *ID2*, *MCM7* and Nestin

Immunohistochemical analyses were performed on 4-μm thick sections using the Ventana DAB kit as per the manufacturer's instructions (Ventana Medical Systems, Tucson, AZ, USA). Antibodies tested were against *ID2* (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, USA), *MCM7* (1:100, Santa Cruz) and nestin (1:200, Chemicon International, Temecula,

CA, USA). Immunohistochemical stains were considered either positive or negative using the following scoring system. The sections were scored as per previously published criteria.[12] Briefly, scores for intensity were 1 for low, 2 for moderate and 3 for high, with the positive control used as the standard for high intensity. The distribution of staining was scored as 1 for $<10\%$ of cells positive, 2 for 11–50% of cells positive and 3 for $>50\%$ of cells positive. A combined score of 4 or more was considered positive; <4 was considered negative.

Clinical Data

The material reviewed included operative reports (to identify extent of tumor resection at diagnosis), medical charts and the Hematology/Oncology database at the Hospital for Sick Children. Clinical parameters obtained were progression-free survival, the presence or absence of metastases at presentation and overall survival. Progression-free survival was defined as absence of clinical and radiological recurrence at the primary site up to the time of most recent follow-up. Recurrence was defined as relapsed disease occurring after documented disease remission. Progression-free survival and overall survival were calculated from the start of treatment to the time of recurrence or death from any cause, respectively.

Statistical Analyses

Non-parametric data were compared using Fisher's exact test. For each clinical and morphological parameter, the association with progression-free survival and overall survival was characterized by univariate analysis using the Kaplan–Meier method and log-rank test. Statistical analyses were performed using SPSS version 13.0 software. Significance was defined as $P<0.05$.

Results

Tumor Histology and RT-PCR for t(X;18)

Twelve synovial sarcomas from nine male subjects and three female subjects were included in the study. Of the 12 synovial sarcomas, four had biphasic morphology, seven had monophasic-spindled morphology and one was poorly differentiated (Figure 1, Table 1). All tumors were positive for the characteristic *SYT/SSX* fusion transcript.

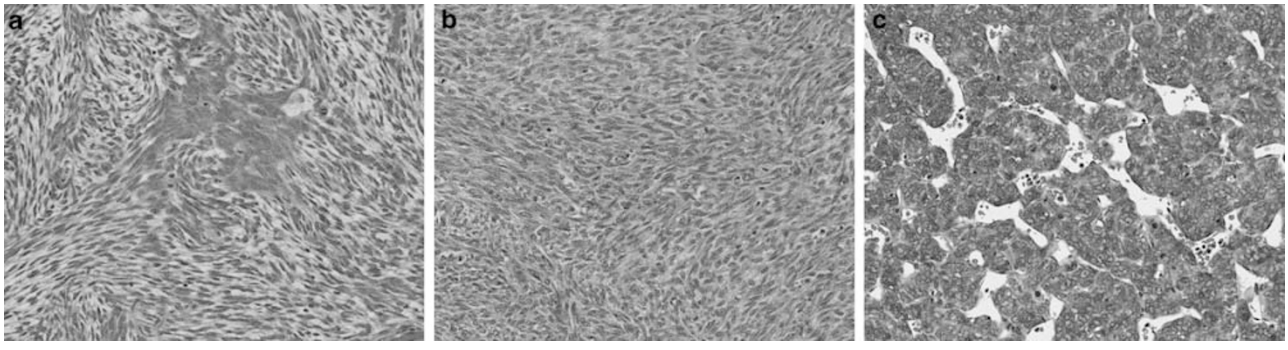

Figure 1 Examples of synovial sarcomas used in the study included biphasic (**a**), monophasic (**b**) and poorly differentiated variants (**c**). Hematoxylin and eosin, $\times 200$.

Table 1 Clinicopathological summary of the 12 t(X;18)-positive synovial sarcomas used in the current study

Age	*Sex*	*Histology*	*MYCN*	*ID2*	*Presentation*	*Recurrence*	*Overall survival*
6 months	M	Biphasic	++	POS	Localized	Yes	DOD
15 years	F	Biphasic	++	POS	Localized	Yes	AWD
11 years	M	Monophasic	++	POS	Localized	No	A
17 years	F	Monophasic	++	POS	Localized	No	A
12 years	M	Poorly-differentiated	++	NEG	Localized	No	A
18 years	M	Biphasic	+	POS	Localized	Yes	A
16 years	M	Biphasic	+	POS	Metastases	No	AWD
10 years	M	Monophasic	+	NEG	Localized	No	A
14 years	M	Monophasic	+	NEG	Localized	No	A
10 years	M	Monophasic	–	NEG	Metastases	No	DOD
13 years	M	Monophasic	–	NEG	Localized	No	A
14 years	F	Monophasic	–	NEG	Metastases	No	DOD

Abbreviations: ++, high levels MYCN expression; +, low levels MYCN expression; –, no MYCN expression; A, alive; DOD, died of disease; AWD, alive with disease.

MYCN Gene Expression and Amplification in Pediatric Sarcomas

RNA was extracted from 12 synovial sarcomas. Nine of the 12 synovial sarcomas (75%) showed some expression of *MYCN*, with five (42%; two biphasic, two monophasic, one poorly differentiated) showing high levels of expression and four showing low levels of expression (Table 1). The synovial sarcomas with overexpression had expression levels comparable to that seen in neuroblastoma controls with *MYCN* gene amplification (Figure 2). High levels of *MYCN* expression were not associated with a specific histologic subtype ($P = 0.8485$, Fisher's exact test). Of the other sarcomas tested, 5/15 Ewing sarcoma/primitive neuroectodermal tumors, 0/10 undifferentiated sarcomas and 1/4 malignant peripheral nerve sheath tumors expressed *MYCN*. All of

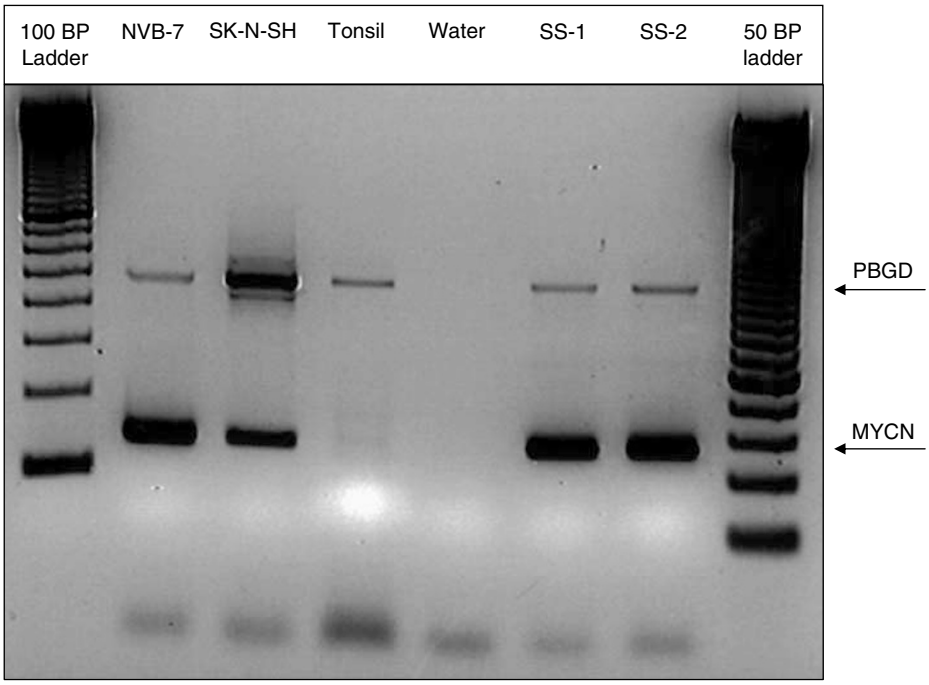

Figure 2 Semiquantitative RT-PCR analysis of the *MYCN* gene expression using RNA extracted from samples of synovial sarcoma (SS-1, patient 1; SS-2, patient 2). NVB-7, control for high *MYCN* expression; tonsil, control for negative *MYCN* expression; SK-N-SH, control for low *MYCN* expression; water control; SS-1 and SS-2, synovial sarcoma samples exhibiting high levels of *MYCN* expression.

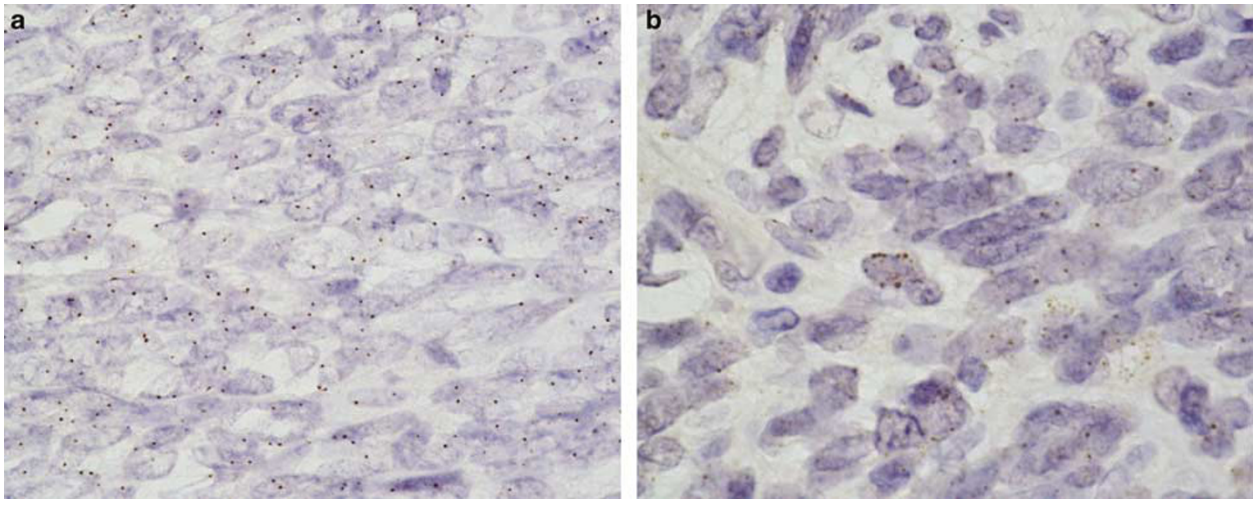

Figure 3 CISH for the *MYCN* gene showing one to two signals per nucleus, consistent with diploidy in paraffin sections (**a**). Focal aneuploidy was seen in some sections as scattered nuclei with greater than two signals (**b**), however, amplification (>10 signals in the majority of tumor cells) was not present. Hematoxylin counterstain, ×600.

the non-synovial sarcomas expressed *MYCN* at low levels. Thus, high levels of *MYCN* expression was useful for distinguishing synovial sarcoma from other childhood-spindled cell sarcomas with specificity and sensitivity of 100 and 42%, respectively, in this series (Table 2).

MYCN gene amplification in the series of synovial sarcomas was tested for by a combination of CISH (all cases) and PCR (four cases). None of the synovial sarcomas showed amplification of the *MYCN* gene by CISH (Figure 3) or PCR.

Table 2 Summary of *MYCN* expression status in all tumors

Tumor type	*Negative for MYCN*	*Low levels of MYCN expression*	*High levels of MYCN expression*
Synovial sarcoma	3	4	5
Malignant peripheral nerve sheath tumor	3	1	0
Ewing sarcoma/primitive neuroectodermal tumor	10	5	0
Undifferentiated sarcoma	10	0	0

MRP1 Expression by RT-PCR in Synovial Sarcoma

Of the 12 synovial sarcomas, only one tumor expressed *MRP* in the low-mid range; this was associated with high levels of *MYCN* expression. All other tumors had negligible levels of expression.

ID2, *MCM7* and Nestin Expression by Immunohistochemistry in Synovial Sarcoma

Examination of downstream targets of *MYCN* in synovial sarcomas revealed expression of *MCM7* in all cases of synovial sarcoma. Nestin was expressed in 10 cases of synovial sarcoma (83%) and *ID2* in six cases (50%) (Figure 4). Expression of downstream targets did not significantly correlate with high levels of *MYCN* expression; however, expression of *MYCN* at either low or high levels was associated with a trend toward expression of *ID2* ($P = 0.0909$, Fisher's exact test) (Table 1).

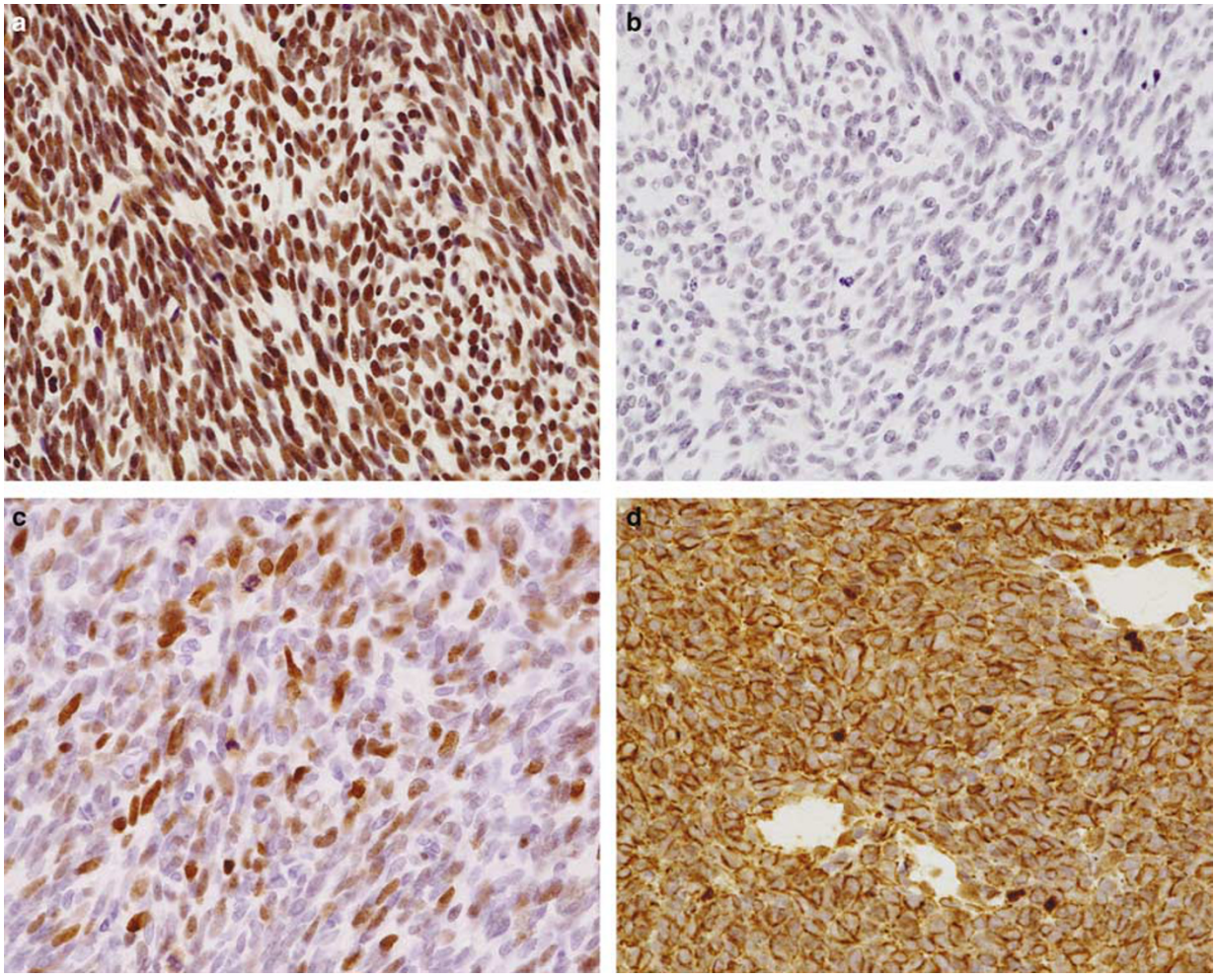

Figure 4 Immunohistochemistry for *ID2* (positive in **a**, negative in **b**), *MCM7* (**c**) and nestin (**d**). Both *ID2* and *MCM7* show nuclear staining, whereas nestin has a cytoplasmic pattern of staining. Hematoxylin counterstain, $\times 200$.

Prognostic Impact in Synovial Sarcoma

Expression of *MYCN*, *MRP1*, *MCM7*, *ID2* or nestin was not significantly associated with metastases at presentation, local recurrence or survival. However, tumors that recurred locally were all *ID2*-positive (3/3, 100%), compared with 3/9 (33%) tumors without local recurrence ($P = 0.1850$).

Discussion

According to previous expression array studies,[28,29] synovial sarcomas express the oncogene *MYCN*. Significant levels of expression of *MYCN* were not found in other sarcomas, including liposarcoma, clear cell sarcoma and fibrosarcoma, and *MYCN* thus served as one of the genes differentiating synovial sarcoma from other spindle cell tumors.[28] In the current study, the majority of synovial sarcomas expressed *MYCN* by RT-PCR and almost 50% expressed *MYCN* at high levels. No correlation between histological subtype and *MYCN* expression was found, which is in keeping with the findings of Nagayama *et al*.[29] Of the other sarcomas, a minority of malignant peripheral nerve sheath tumors and Ewing sarcoma/primitive neuroectodermal tumors expressed *MYCN* at low levels only; none of the non-synovial sarcomas expressed *MYCN* at high levels. The low levels of *MYCN* expression seen in malignant peripheral nerve sheath tumors is in keeping with a previous expression array study, where malignant peripheral nerve sheath tumors were found to express *MYCN* at levels lower than that seen in synovial sarcomas.[29] Thus, in the current series of pediatric sarcomas, only synovial sarcomas expressed *MYCN* at high levels and was a useful test for distinguishing synovial sarcomas from other sarcomas with a specificity of 100% but sensitivity of only 42%, as several cases of synovial sarcoma expressed *MYCN* at low levels or not at all.

None of the cases of synovial sarcoma showed *MYCN* gene amplification. The lack of genomic amplification of *MYCN* in synovial sarcoma suggests alternative mechanisms are responsible for the high *MYCN* expression, such as alterations in transcriptional activity[37] or dysregulation of protein degradative pathways, as described in neuroblastoma.[38,39] In neuroblastoma, the clinical significance of high levels of *MYCN* expression without gene amplification remains controversial; some studies suggest no clinical significance,[40,41] whereas others have reported a significant association with poorer outcome in a subset of older patients but not in infants.[42] In the present series of synovial sarcoma, a high level of *MYCN* expression in the absence of *MYCN* amplification was not associated with a significant difference in local recurrence, metastatic rate or overall survival.

The expression of several downstream targets of *MYCN* in synovial sarcoma was also assessed. *MRP1* encodes a member of the superfamily of ATP-binding cassette transporters, and functions as a multispecific organic anion transporter.[43] Increased expression of *MRP1* is associated with increased drug resistance and enhanced *MRP1*-mediated drug efflux.[18] *MCM7* is a DNA-binding protein essential for replication of DNA during the transition of G1 to S phase of the cell cycle,[44] and increased expression of *MCM7* has been demonstrated in proliferating tissues.[45] *ID2* is a helix-loop-helix transcription factor[46] that binds to and inactivates the *RB* protein, thus stimulating cell proliferation by inhibiting the *RB* tumor suppressor pathway.[20] Nestin is an intermediate filament and is thought to play a role in tumor development and aggressiveness.[47,48] *MYCN* has been shown to bind directly to the promoters of all four genes,[18–21] resulting in increased levels of expression of the resultant proteins in *MYCN*-amplified tumors. Although the current study did not find a significant correlation between high levels of *MYCN* expression and expression of downstream targets *MRP1*, *MCM7* and nestin, there was a trend toward significance with expression of *ID2*. The lack of statistical significance raises two possible explanations. Firstly, the number of tumors in the present series is relatively small, and a larger series may be required to show a statistically significant correlation. For example, *ID2* was expressed in 67% of synovial sarcomas with low or high levels of *MYCN* expression, but was not expressed at all in synovial sarcomas negative for *MYCN*. Secondly, the expression of such targets may be controlled by factors other than *MYCN*. For example, the *MCM7* protein has numerous transactivation sites for the *E2F* transcription factor,[49] and expression of *MCM7* is increased by activation of *E2F* in fibroblasts.[50] Furthermore, the nestin gene has several putative binding sites for the transcription factors *HIF-2α* and *GATA* within its first intron,[51] suggesting a role for multiple transcription factors in nestin expression.

The present study did not show a significant correlation between expression of *MYCN* or downstream targets with progression-free survival, overall survival or metastases at presentation. Nonetheless, all cases that recurred locally were *ID2*-positive, compared with only three of nine cases that did not recur. This result, although not statistically significant in our small series, is worthy of further investigation. Overexpression of *ID2* results in transformation of NIH 3T3 fibroblasts *in vitro*,[52] and embryonic fibroblasts from *ID2*-null mice show noticeably lower rates of division. Furthermore, the correlation of *MYCN* and *ID2* expression has been described in neuroblastoma,[52] and it has been suggested that expression of *ID2* is a better indicator of poor outcome than *MYCN* amplification. Such results provide a possible explanation for the oncogenic mechanism of *MYCN* overexpression

in vivo.[52] However, the prognostic significance of *ID2* expression has been refuted by others, with some reporting no such association between *ID2* expression and poor outcome in neuroblastomas and neuroblastoma cell lines.[53]

The current study identified a group of synovial sarcomas with high levels of *MYCN* expression. Such overexpression was not seen in other sarcomas entering the differential diagnosis of synovial sarcoma, and provides evidence that testing for *MYCN* expression may be a useful ancillary investigation in the differentiation of pediatric-spindled and monomorphic sarcomas. The biological and prognostic significance of such a finding is as yet unclear and deserves further study.

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