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ORIGINAL ARTICLE

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Expression of cadherins and their undercoat proteins (α-, β-, and γ-catenins and p120) and accumulation of β-catenin with no gene mutations in synovial sarcoma

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Abstract E-cadherin, the major intercellular adhesion molecule of epithelial cells, is important in determining the architecture of sarcomas, especially those showing epithelioid features. In addition to its role in cell adhesion, β-catenin, a cadherin undercoat protein, has been shown to function as a downstream transcriptional activator of the Wnt/Wingless signaling pathway. In order to evaluate the significance of the cadherin cell adhesion system and the Wnt/Wingless signaling pathway in the morphogenesis and/or tumorigenesis of synovial sarcoma (a major type of sarcoma with epithelioid features), immunoreactivity for pan-cadherin, E-cadherin, and their undercoat proteins (α-, β-,and γ-catenins and p120) was evaluated in 15 synovial sarcomas. Immunoreactivity for pan-cadherin, E-cadherin, α-catenin, β-catenin, and p120 was observed in all 15 specimens. Immunoreactivity for pan-cadherin was stronger than that for E-cadherin. Expression of γ-catenin was detected in ten specimens. Although β-catenin was observed only at the cell–cell boundaries in four specimens, it was present in the nucleus and cytoplasm and at the cell–cell boundaries in the other 11, suggesting constitutional activation of the Wnt/Wingless signaling pathway in synovial sarcoma. Direct sequencing for exon 3 of the β-catenin gene, however, revealed no mutations in any of the 15 specimens. In conclusion, other types of cadherin besides E-cadherin, together with cadherin undercoat proteins, may play a role in cell adhesion in synovial sarcoma. Furthermore, mechanisms other than mutation of exon 3 of the β-catenin gene may activate the Wnt/Wingless signaling pathway in this type of tumor.

Keywords Synovial sarcoma · Cadherin · Catenin · p120 · Wnt/Wingless signaling pathway

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Introduction

E-cadherin is the prime mediator of intercellular adhesion in epithelial cells. This glycoprotein has a single transmembrane domain, the extracellular component of which mediates cell–cell adhesion through Ca^{2+}-dependent homotypic interactions [36]. The cytoplasmic domain associates with a group of undercoat proteins termed catenins (α-, β-, and γ-catenins) [12, 21]. Both β-catenin and γ-catenin bind directly via their Armadillo domains to the cytoplasmic domain of E-cadherin, while α-catenin links the bound β-catenin or γ-catenin to the actin microfilament network of the cellular cytoskeleton [12]. Since this binding is essential for stable cell–cell adhesion, α-catenin plays a crucial role in regulating the adhesive function of E-cadherin [14, 31]. The p120 protein, which was originally identified as a substrate of *Src* and several other receptor tyrosine kinases, belongs to the Armadillo family and binds directly to the cytoplasmic domain of E-cadherin [22, 23, 28].

In addition to its role in cell adhesion, β-catenin has been shown to function as a downstream transcriptional activator of the Wnt/Wingless signaling pathway, forming complexes with the DNA-binding proteins T-cell factor (Tcf) and lymphoid enhancer factor (Lef) [2, 3]. The adenomatous polyposis coli (APC) tumor suppressor gene product regulates β-catenin levels by cooperating with glycogen synthase kinase 3β (GSK-3β) to phosphorylate multiple serine/threonine residues, which are coded by exon 3 of the β-catenin gene [20, 25]. This phosphorylation is followed by degradation of β-catenin

through the ubiquitin–proteasome pathway [1, 25]. Constitutional activation of the Wnt/Wingless signaling pathway by stabilization and accumulation of β-catenin in the nucleus and cytoplasm (mainly caused by inactivating mutations in the APC gene or by activating mutations in exon 3 of the β-catenin gene) has been shown to be important in the development of colorectal [15, 35], hepatocellular [7, 19], uterine [10], esophageal [17], and thyroid [11] carcinomas and malignant melanoma [24].

Synovial sarcoma, which accounts for 5–10% of all soft tissue sarcomas, typically arises in the para-articular regions and occurs in two major forms, the biphasic and monophasic fibrous types [9]. Cytogenetic studies have revealed a characteristic chromosomal translocation, t(X; 18)(p11; q11), in more than 90% of synovial sarcomas [37]. Cloning of the translocation breakpoints showed that t(X; 18) results in fusion of the *SYT* (at 18q11) and *SSX* (at Xp11) genes [4]. In addition, the overall incidence of synovial sarcomas showing p53 alterations was approximately 30%, and p53 alterations were correlated with a shorter overall survival [27].

We have previously found that E-cadherin is expressed by synovial sarcomas, suggesting a possible role in epithelial differentiation [26]. Thus, we decided to analyze the expression of the cadherin undercoat proteins (α-, β- and γ-catenins and p120) in synovial sarcoma. Furthermore, we studied the accumulation of β-catenin in the nucleus and cytoplasm of the tumor cells and investigated whether this accumulation might be due to mutations in exon 3 of the β-catenin gene itself.

Materials and methods

Tissue specimens

We examined synovial sarcomas from 12 patients registered with the Clinical Laboratory Division, National Cancer Center Hospital, Tokyo, between 1983 and 1998, and from 3 further patients registered with the Department of Pathology, Tokai University School of Medicine, Kanagawa, between 1975 and 1998. Of the 15 specimens, 10 were also used in our previous study [26]. Clinicopathological data for the patients are summarized in Table 1. Their median age was 31.9 years and none had any history of other malignant tumors, including colorectal carcinoma and familial adenomatous polyposis (FAP).

The tissue samples used in this study were obtained from surgical specimens, fixed in 10% formalin, and embedded in paraffin. For routine histological observation, the tissue sections were stained with hematoxylin and eosin (H&E). The H&E-stained sections and original immunohistochemical slides were reviewed to confirm the histological diagnosis and tumor typing according to the World Health Organization (WHO) classification [38]: the monophasic fibrous type is composed exclusively of spindle-shaped fibrosarcomatous tumor cells, although epithelial differentiation is inferred from the presence of clusters of round cells with pale cytoplasm, whereas the biphasic type is characterized by epithelial glands situated within a spindled fibrosarcomatous stroma. For the biphasic type, histologic sections containing characteristic features of both epithelial and spindled elements were selected for immunohistochemical studies.

Antibodies

Rabbit polyclonal anti-pan cadherin antibody (Sigma, Mo.) was diluted 1:1000 and used to detect members of the cadherin family regardless of their type. An undiluted culture supernatant preparation of mouse anti-human E-cadherin monoclonal antibody (HECD-1) [30] was used as the primary antibody for E-cadherin detection. Mouse monoclonal antibodies against α- (clone 5), β- (clone 14), and γ-catenins (clone 15) and p120 (clone 98, Transduction Laboratories, Ky.) were diluted 1:100, 1:200, 1:500, and 1:500, respectively.

Immunohistochemistry

The paraffin-embedded tissue specimens were cut into 3-μm-thick sections, mounted on adhesive-coated slides, deparaffinized, and hydrated using xylene and ethanol. Endogenous peroxidase was blocked by soaking in 0.3% H_2O_2 in methanol for 30 min. The slides were placed into plastic jars containing 10 mM citrate buffer and heated in a H2800 microwave processor (Energy Beam Inc., Mass.) for 20 min (two times, 10 min each) to unmask the antigen. After cooling, the sections were preincubated in phosphate-buffered saline (PBS) containing 2% (v/v) normal swine serum (Dako, Denmark) for 10 min. The slides were then incubated at 4°C overnight with the primary antibody, washed with PBS, and incubated for 30 min with biotinylated anti-rabbit immunoglobulin (Ig)G or biotinylated anti-mouse IgG (Vector Laboratories, Calif.) as a second antibody. They were then incubated for 30 min with avidin-biotinyl-peroxidase complex (ABC) using a Vectastain ABC kit (Vector Laboratories). The peroxidase reaction was performed by incubating with 0.007% (v/v) hydrogen peroxidase in Tris-HCl buffer (pH 7.6) for 5–10 min, using 0.02% (w/v) 3.3'-di-

Table 1 Clinicopathological data for patients with synovial sarcoma

Patient	Age (years)	Gender	Site	Histological type
1	26	Male	Right thigh	Biphasic
2	11	Female	Neck	Biphasic
3	46	Female	Left thigh	Biphasic
4	27	Male	Left shoulder	Biphasic
5	47	Male	Right foot	Biphasic
6	17	Female	Right thigh	Biphasic
7	10	Female	Left elbow	Biphasic
8	37	Female	Lung metastasis	Biphasic
9	39	Female	Left foot	Biphasic
10	74	Female	Right foot	Monophasic fibrous
11	21	Male	Abdominal wall	Monophasic fibrous
12	14	Male	Right foot	Monophasic fibrous
13	30	Male	Left shoulder	Monophasic fibrous
14	62	Male	Right thigh	Monophasic fibrous
15	18	Female	Right buttock	Monophasic fibrous

aminobenzidine tetrahydrochloride as the chromogen. The nuclei were counterstained with hematoxylin. The slides were washed three times with PBS between each step.

Negative controls were generated by incubating tissue sections with normal rabbit Ig (Dako), and the same class of mouse Ig (Dako) instead of the primary antibody and yielded negative results in all cases. Normal epithelial cells, which appeared on the same slide as the immunostained tumor samples, served as internal positive controls. If no normal epithelial cells were visible on the same slide, normal epithelial cells from other paraffin blocks of the same sample were used as external positive controls. The degree of immunoreactivity was evaluated as follows: absent (–), 0% expression; a few (+), up to 20% of cells positive; moderate (2+), 21–50% of cells positive; and many (3+), more than 50% of cells positive. The intensity of the immunoreactivity was also categorized as follows: weak (W), weaker than that of normal epithelial cells used as positive controls; moderate (M), equal to that of normal epithelial cells; and strong (S), stronger than that of normal epithelial cells. In the biphasic type, epithelial and spindled elements were evaluated separately.

DNA samples

DNA was extracted from the formalin-fixed, paraffin-embedded specimens. Tissue sections (10-μm thick) were cut and reviewed microscopically, and tumor tissue was microdissected from areas composed of at least 80% tumor cells. Nontumorous tissue was also microdissected from the same specimens for comparison. DNA was extracted from the tissues as previously described [10, 18].

Direct sequencing of the β-catenin gene

Exon 3 of the β-catenin gene was amplified using the polymerase chain reaction (PCR) using the following previously described intronic primers: forward, 5'-atttgatggagttggacatggc-3'; reverse, 5'-ccagctacttgttcttgagtgaagg-3' [10, 18]. The purified PCR products were sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Conn.) and an Applied Biosystems 310 Genetic Analyzer (Perkin-Elmer). The data were collected and analyzed using Applied Biosystems sequencing analysis software (Perkin-Elmer), all according to the manufacturers' protocols. Sequencing was performed in both directions using the same primers used for the PCR.

Results

Immunohistochemistry

In normal epithelial cells of the epidermis, used as positive controls (H&E, Fig. 1A), immunoreactivity for pan-cadherin (Fig. 1B), E-cadherin (Fig. 1C), and α-catenin (Fig. 1D), β-catenin (Fig. 1E), and γ-catenin (Fig. 1F) and p120 (Fig. 1G) was localized or predominantly detected at the cell–cell boundaries.

Histological features of H&E-stained sections from the biphasic type (Fig. 2A) and monophasic fibrous type (Fig. 2B) are shown. The degree and intensity of immunoreactivities detected in the synovial sarcoma samples are summarized in Table 2. Cadherin expression was detected with the pan-cadherin antibody in all 15 tumor specimens. Pan-cadherin immunoreactivity was primarily localized at the cell–cell boundaries in both biphasic (Fig. 2C) and monophasic fibrous type (Fig. 2D) tumors. E-cadherin expression was observed at the cell–cell boundaries in all 15 synovial sarcomas. The results of immunostaining with HECD-1 have already been described in our previous report for 10 of the 15 tumors included in the present study [26]. In the biphasic type, the degree of immunoreactivity for HECD-1 was higher in epithelial elements than in spindle elements (Fig. 2E and Table 2). In the monophasic fibrous type, slightly larger

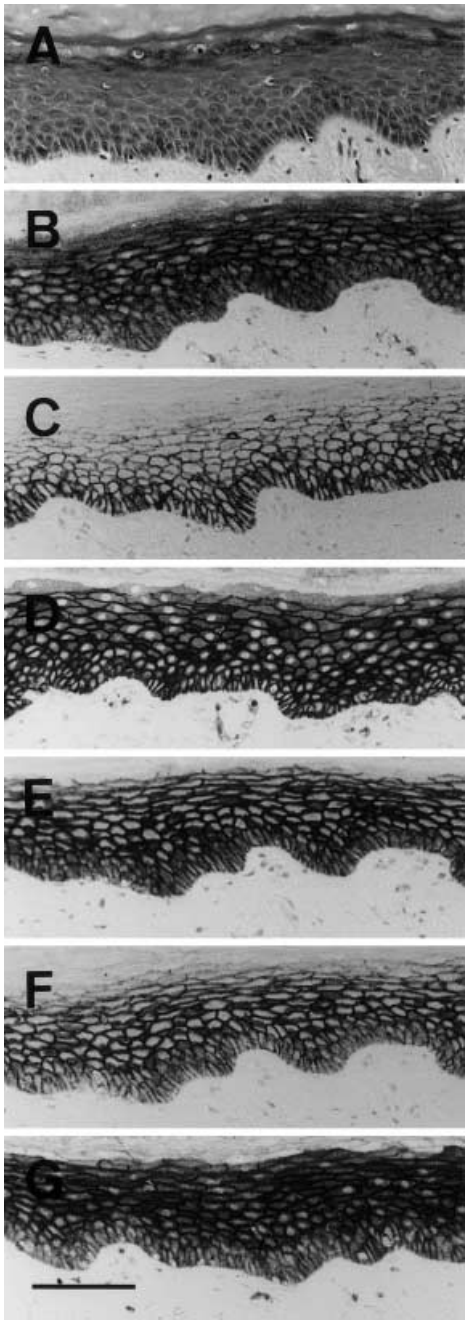

Fig. 1 Immunohistochemical staining in normal epithelial cells of the epidermis used as positive controls in synovial sarcoma cases. **A** Hematoxylin and eosin staining. Immunoreactivity for pan-cadherin (**B**), E-cadherin (**C**), α-catenin (**D**), β-catenin (**E**) and γ-catenin (**F**), and p120 (**G**) was localized or predominantly detected at the cell–cell boundaries. Original magnification, ×50. *Bar* 100 μm

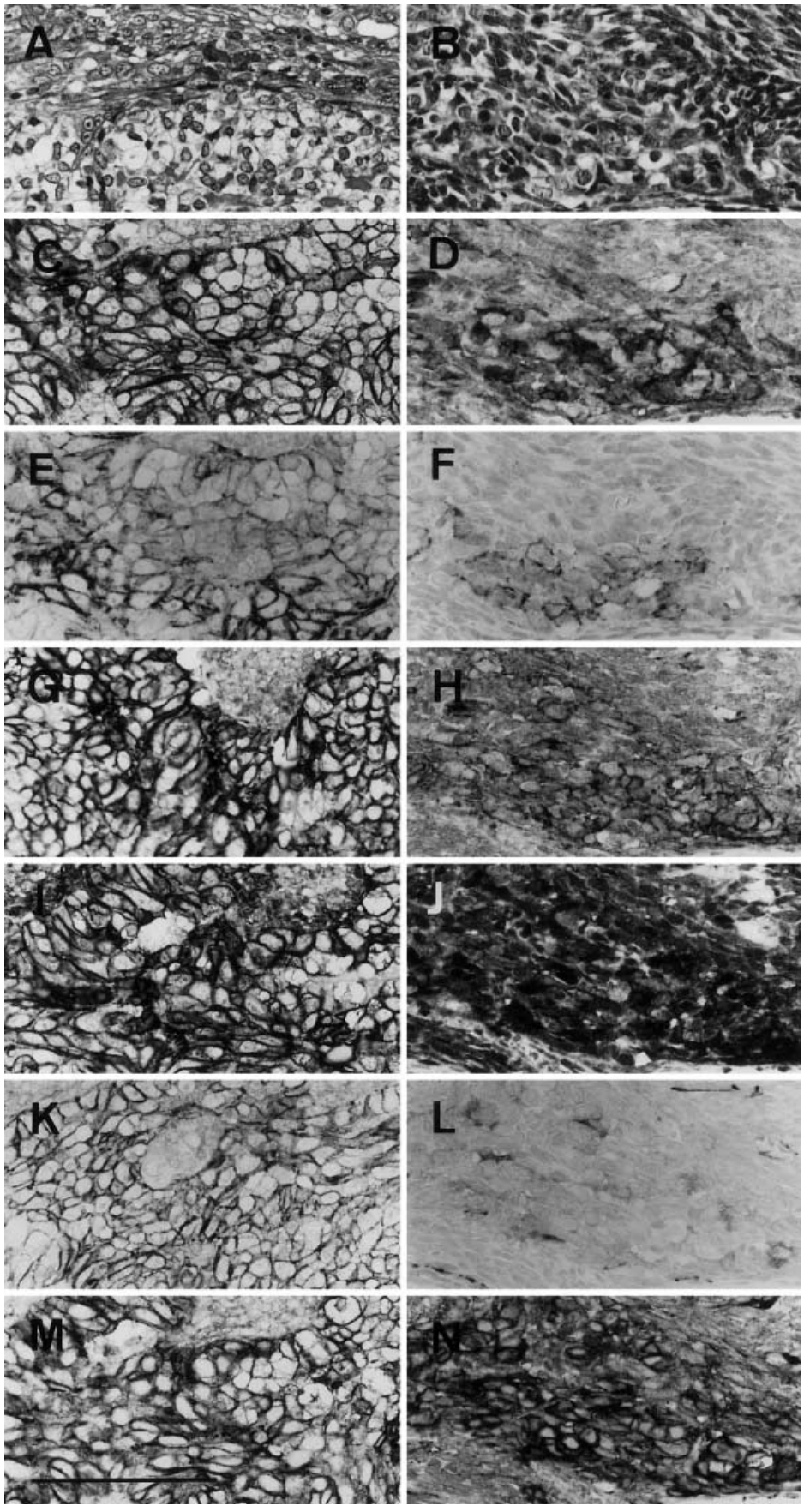

Fig. 2 Immunohistochemical staining in biphasic synovial sarcoma (patient 3; **A**, **C**, **E**, **G**, **I**, **K,** and **M**) and monophasic fibrous synovial sarcoma (patient 11; **B**, **D**, **F**, **H**, **J**, **L**, and **N**). **A** and **B** Hematoxylin-eosin staining. Expression of both pan-cadherin (**C** and **D**) and E-cadherin (**E** and **F**) was mainly observed at the cell–cell boundaries. Slightly larger and more ovoid or polygonal cells were positive for E-cadherin in patient 11 (**F**). Immunoreactivity for E-cadherin (**E** and **F**) was generally weaker than that for pan-cadherin (**C** and **D**). Expression of α-catenin (**G** and **H**) and p120 (**M** and **N**) was also predominantly observed at the cell–cell boundaries. Although immunoreactivity for β-catenin was detected only at the cell–cell boundaries in patient 3 (**I**), marked accumulation of β-catenin was apparent in the nucleus and cytoplasm in patient 11 (**J**). Expression of γ-catenin was generally observed at the cell–cell boundaries (**K**), but a few tumor cells showed weak immunoreactivity in patient 11 (**L**). Original magnification, ×100. *Bar* 100 μm

Table 2 Expression of cadherins, catenins, and p120 in synovial sarcoma specimens. Membranous β-catenin immunoreactivity was detected only at the cell–cell boundaries; accumulated pronounced β-catenin immunoreactivity was detected in the nucleus and cytoplasm. *Ep* epithelial element; *Sp* spindled element. – 0% expression; *1+* up to 20% of cells positive; *2+* 21–50% of cells positive; *3+* more than 50% of cells positive; *W* weaker than positive control; *M* equal to positive control; *S* stronger than positive control

Patient	Pan-cadherin	E-cadherin	α-Catenin	β-Catenin	γ-Catenin	p120
Biphasic type						
1 (Ep)	3+, M	2+, W	1+, W	3+ (Accumulated), S	–	3+, M
(Sp)	2+, M	1+, W	1+, W	3+ (Accumulated), S	–	1+, M
2 (Ep)	3+, M	2+, W	2+, W	2+ (Membranous), M	1+, W	3+, M
(Sp)	3+, M	1+, W	1+, W	2+ (Membranous), M	1+, W	2+, M
3 (Ep)	3+, M	3+, M	3+, M	3+ (Membranous), M	3+, W	3+, M
(Sp)	2+, M	1+, W	2+, W	3+ (Membranous), M	3+, W	3+, M
4 (Ep)	3+, M	1+, W	3+, M	3+ (Accumulated), S	1+, W	3+, M
(Sp)	1+, M	1+, W	2+, W	3+ (Accumulated), S	1+, W	3+, M
5 (Ep)	3+, M	2+, W	3+, M	2+ (Membranous), M	–	3+, M
(Sp)	2+, M	1+, W	3+, M	2+ (Membranous), M	–	3+, M
6 (Ep)	2+, M	2+, W	2+, W	2+ (Accumulated), S	1+, W	2+, M
(Sp)	1+, M	1+, W	2+, W	2+ (Accumulated), S	1+, W	2+, M
7 (Ep)	3+, M	2+, W	1+, W	3+ (Accumulated), S	1+, W	1+, W
(Sp)	2+, M	1+, W	1+, W	3+ (Accumulated), S	1+, W	1+, W
8 (Ep)	3+, M	2+, W	3+, M	3+ (Accumulated), S	1+, W	3+, M
(Sp)	2+, M	1+, W	3+, M	3+ (Accumulated), S	1+, W	3+, M
9 (Ep)	3+, M	2+, W	3+, M	3+ (Membranous), M	2+, W	3+, M
(Sp)	2+, M	1+, W	3+, M	3+ (Membranous), M	2+, W	3+, M
Monophasic fibrous type						
10	3+, M	1+, W	3+, M	3+ (Accumulated), S	–	3+, M
11	2+, M	1+, W	2+, W	3+ (Accumulated), S	1+, W	3+, M
12	3+, M	1+, W	2+, W	3+ (Accumulated), S	–	1+, M
13	3+, M	1+, W	2+, W	3+ (Accumulated), S	1+, W	2+, M
14	2+, M	1+, W	3+, W	3+ (Accumulated), S	–	2+, M
15	2+, M	1+, W	2+, W	3+ (Accumulated), S	1+, W	3+, M

and more ovoid or polygonal tumor cells were positive for E-cadherin (Fig. 2F). Immunoreactivity for E-cadherin was generally weaker than that for pan-cadherin, and even tumor cells which were negative for E-cadherin showed pan-cadherin immunoreactivity in both biphasic and monophasic fibrous tumors.

Immunoreactivity for α-catenin was detected in all 15 specimens, predominantly at the cell–cell boundaries (Fig. 2G and Fig. 2H). Immunoreactivity for β-catenin was also detected in all 15 specimens; however, while this was confined solely to the cell–cell boundaries in four samples (27%, membranous type, Fig. 2I), the remaining 11 (73%) showed prominent immunoreactivity in the nucleus and cytoplasm and at the cell–cell boundaries (accumulated type, Fig. 2J). All four samples, showing only a membranous distribution of β-catenin, were biphasic synovial sarcoma. Immunoreactivity for γ-catenin was observed in ten samples (67%, Fig. 2K), mainly at the cell–cell boundaries. As well as being less commonly detected than immunoreactivity for α-catenin and β-catenin, γ-catenin immunoreactivity was generally weaker (Fig. 2L and Table 2). Expression of p120 was detected in all 15 samples, predominantly at the cell–cell boundaries (Fig. 2M and Fig. 2N).

Direct sequencing of the β-catenin gene

Examples of the results of direct sequencing are shown in Fig. 3. Direct sequencing revealed no mutations in exon 3 of the β-catenin gene in any of the 15 synovial sarcomas or the corresponding nontumorous tissues.

Discussion

We have previously reported the expression of E-cadherin in synovial sarcoma [26]. The incidence of E-cadherin expression in synovial sarcomas in the present study (100%) was consistent with that found in our previous study. Immunoreactivity for E-cadherin was heterogeneous even within a single tumor but seemed to be associated with epithelial features and tight adhesion between tumor cells. Sarcomas other than synovial sarcoma have been shown to express cadherins other than E-cadherin, e.g., N-cadherin in rhabdomyosarcoma [33], and vascular-endothelial cadherin in epithelioid sarcoma [32]. Thus, in the present study, we carried out immunostaining with anti-pan-cadherin antibody. Immunoreactivity for pan-cadherin was stronger than that for E-cadherin in synovial sarcoma, and even tumor cells that were negative for E-cadherin showed pan-cadherin immunoreactivity at their cell–cell boundaries. To exclude

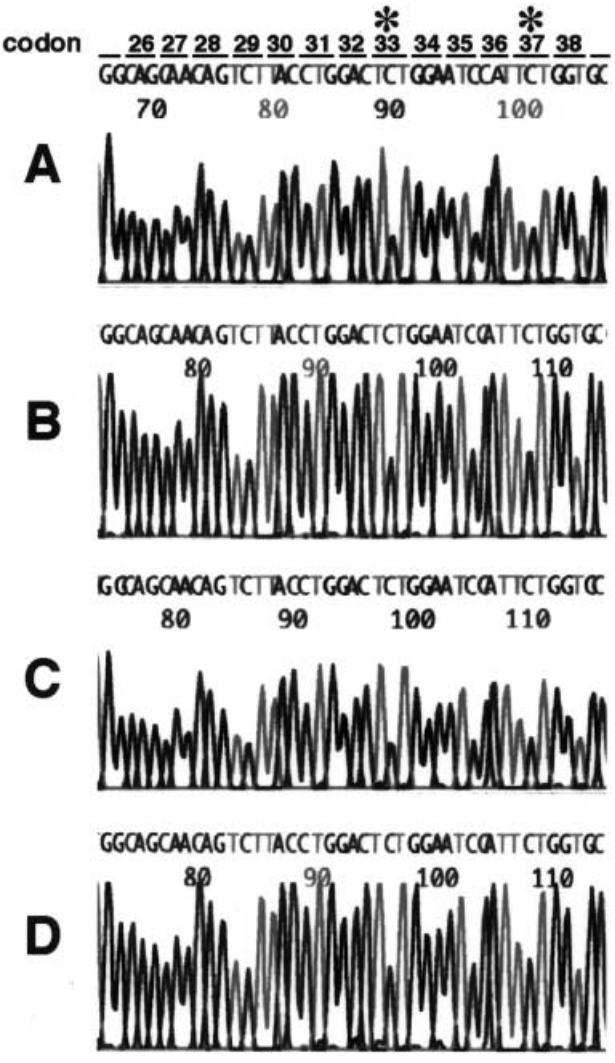

Fig. 3 Representative results of direct sequencing of exon 3 of the β-catenin gene in synovial sarcoma samples (**A** and **C**) and corresponding nontumorous tissues (**B** and **D**) from patients 3 (**A** and **B**) and 11 (**C** and **D**). Positions 288–330 (based on GenBank accession number NM001904) are shown. *Codon numbers* are indicated on the top row. Two codons encoding potentially phosphorylated serine residues are indicated with *asterisks*. No mutations were detected in any of the 15 tumor samples

the possibility that the epitope for HECD-1 was solely masked, we also performed immunostaining using another monoclonal antibody against E-cadherin (clone 36; Transduction Laboratories; 1:500 dilution) in all specimens. The results of immunohistochemistry using HECD-1 and clone 36 (data not shown) were almost in agreement. Therefore, it is suggested that other types of cadherin besides E-cadherin may be involved in the general constitution of synovial sarcoma. N-cadherin is one possible candidate, since the anti-pan-cadherin antibody showed especially strong immunoreactivity for N-cadherin in our western blotting using multiple human cultured cells (unpublished data). However, further studies are needed to identify which types of cadherin other than E-cadherin participate in the architecture of synovial sarcoma.

In addition to cadherins, immunostaining for undercoat proteins was also performed in the present study. The fact that α-catenin and β-catenin, which are essential for the cell-adhesion function of cadherins [12, 21], were co-expressed and co-localized with cadherins in all of the tumor samples strongly suggests that the cadherins expressed in synovial sarcomas are functional. Although five of the samples lacked γ-catenin immunoreactivity, exclusive association of β-catenin or γ-catenin with cadherins is known to occur [13], and the absence of γ-catenin expression does not indicate dysfunction of the cadherin cell adhesion system. It can be assumed that only an α-catenin/β-catenin/cadherin complex was present in these five tumors.

Expression of p120 was observed in all 15 synovial sarcomas. p120 is phosphorylated in response to several growth factors, and its tyrosine phosphorylation by *Src* tyrosine kinase is associated with cell transformation [6, 22, 28]. Although co-expression of E-cadherin and p120 has been confirmed in bladder carcinoma [29], p120 is expressed independently of α-catenin, β-catenin, and E-cadherin in breast carcinoma, suggesting the absence of coordinated regulation among these proteins [8]. Thus, further studies will be needed to determine whether p120 might be involved in the morphogenesis and/or tumorigenesis of synovial sarcoma, either in coordination with or independently of the cadherin cell adhesion system.

The most striking feature seen in this study was the frequent accumulation of β-catenin in the nucleus and cytoplasm of the synovial sarcoma samples. This suggests that the Wnt/Wingless signaling pathway may be constitutionally activated in synovial sarcomas, and that β-catenin localized in the nucleus might play a role as a transcriptional activator during the development of these tumors. However, no mutations in exon 3 of the β-catenin gene were observed. Recently, Iwao et al. reported a mutation in exon 3 of the β-catenin gene in only one (14%) of seven synovial sarcomas [16]. This resulted in an amino acid substitution next to a potentially phosphorylated serine. However, although this mutation may affect the protein conformation around a possible target residue for GSK-3β, its low frequency is essentially consistent with our present findings. None of the patients we examined had any history of colorectal carcinoma or FAP, and no mutations of the APC gene have been reported in synovial sarcoma to date. Therefore, mechanisms other than mutations in the β-catenin or APC genes must be responsible for β-catenin accumulation in synovial sarcoma.

Cytogenetic studies have revealed a characteristic chromosomal translocation, t(X; 18)(p11; q11), in more than 90% of synovial sarcomas [37]. The presence of this translocation as the sole cytogenetic abnormality suggests that it is the primary causal event in synovial sarcoma. In other solid tumors, t(X; 18), it is known to result in the formation of a chimeric protein that deregu-

lates transcription [34], and it has been proposed that this chimeric protein may also enhance the expression of specific target genes in synovial sarcoma [5]. The possibility that the β-catenin gene is one of these targets cannot be confirmed or discounted until further studies to identify the mechanisms responsible for aberrant β-catenin accumulation in synovial sarcoma have been performed.

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