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Expression of Cyclin-dependent Kinase Inhibitor p27/Kip1 and AP-1 Coactivator p38/Jab1 Correlates with Differentiation of Embryonal Rhabdomyosarcoma

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Cyclin-dependent kinase (CDK) inhibitor p27/Kip1 (p27) is a diagnostic and prognostic marker of various malignancies. Low expression of p27 reflects poor differentiation and poor prognosis, and an inverse correlation between the expression of p27 and degree of tumor malignancy has been reported. Because p27 mutation is extremely rare in human tumors, it is important to study the expression of p27 and its inactivator, p38/Jab1 (JAB1). Here we analyzed the expression of p27 and JAB1 by immunohistochemistry in embryonal rhabdomyosarcoma (E-RMS). We first confirmed the expression of p27 and JAB1 in normal human tonsillar epithelium, and observed a coordinated expression pattern depending on cell differentiation. Subsequently, specimens of eight poorly- and three well-differentiated E-RMS were examined for the expression of p27 and JAB1. The analyses revealed that four out of eight poorly-differentiated E-RMS were negative for p27, with positivity for nuclear JAB (NJAB) (−/+ for p27/NJAB) in three and negativity for any JAB-1 expression (−/−) in one. The remaining four poorly-differentiated E-RMS expressed p27 in the nuclei, together with predominant NJAB (+/+). In three well-differentiated E-RMS, only one expressed nuclear p27 and all of these three expressed no NJAB (+/− for p27/NJAB), but expressed predominant cytoplasmic JAB1 (CJAB). These findings suggest that JAB1 may play an important role in determining the differentiation stage of rhabdomyosarcoma cells by modulating the activity of CDK inhibitor p27.

Key words: Cyclin-dependent kinase inhibitor p27/Kip1 — p38/Jab1 — Ubiquitin/proteasome pathway — Embryonal rhabdomyosarcoma — Immunohistochemistry

p27/Kip1 (p27) is a member of the Cip/Kip family of cyclin-dependent kinase (CDK) inhibitors and negatively regulates cell growth by inactivating G1 stage-specific CDK-cyclin complexes.[1)] Haploinsufficiency of p27 expression predisposes to cancer development in experimental animals.[2)] Low expression of p27 is associated with poor prognosis and high malignant grading in a variety of human tumors including colorectal cancer,[3–5)] breast cancer,[3, 6, 7)] gastric carcinoma,[8)] lung cancer,[9)] prostate adenocarcinoma,[10)] oral carcinoma,[11)] epithelial ovarian cancer,[12)] thyroid tumor,[13)] and malignant lymphoma.[14, 15)] These findings suggest that disruption of the cell cycle regulatory mechanism by p27 is closely related to the development of neoplasia. However, this is not always the case, since an inverse correlation between the expression of p27 and degree of tumor malignancy has been reported in some highly proliferative human breast cancers[3)] and B-cell chronic lymphocytic leukemia.[16)] These recent findings suggest that some epigenetic modulation might be involved in counteracting the CDK-inhibitory activity of p27.

Ubiquitination is the principal mechanism regulating p27 protein degradation.[17)] Recent studies showed that p27 is exported from the nucleus by p38/Jab1 (JAB1),[18)] which was originally identified as a molecule stabilizing complexes of c-Jun or Jun D with AP-1 sites and increasing the specificity of target gene activation by AP-1 proteins.[19)] p27 is subsequently degraded through the ubiquitin/proteasome pathway.[18)] This biochemical finding of possible cell cycle regulation by JAB1 through p27 raises a question as to whether the expression of p27 and JAB1 correlates with differentiation of cells under normal or pathological conditions.

Cells undergoing differentiation contain high levels of p27 protein.[20)] p27 regulates differentiation of mouse embryo skeletal muscles,[21)] rat oligodendrocytes[22)] and mouse osteoblasts.[23)] In mouse embryo skeletal muscles, functional assays showed that expression of p27 by trans-

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duction of p27-expression vector enhances the efficiency of MyoD-initiated muscle differentiation. These findings prompted us to investigate the role of p27 and its regulatory protein JAB1 in cellular differentiation and tumor progression in embryonal rhabdomyosarcoma (E-RMS).

Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma in children, consisting of a heterogeneous group of histological subtypes, i.e., alveolar and embryonal forms.[24–27] The alveolar RMS occurs in adolescents and young adults, and usually is located in the musculature of the extremities. It is characterized by small round cells, being held together by strands of intercellular collagen. The other, more frequent histological group is E-RMS, which occurs mostly in infancy and childhood. E-RMS has a broad spectrum of histologic appearances, ranging from a highly cellular neoplasm arranged in fascicles or sheets to an extremely paucicellular lesion within an abundant myxoid matrix. Cells of E-RMS are characterized by uniformly distributed nuclear chromatin with varying degrees of myogenic differentiation of the cytoplasm.[25]

Here we present our data on immunohistological analyses of p27 and JAB1 expression in E-RMS. We also analyzed those in normal human squamous epithelial cells. Our findings suggest that the expression of p27 in the differentiation of E-RMS cells needs to be evaluated together with that of JAB1 protein.

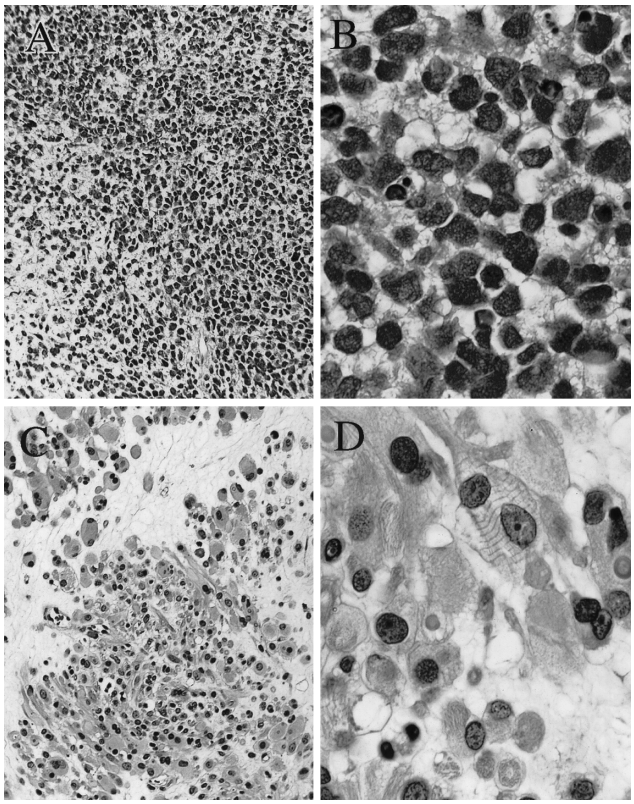

Fig. 1. A, B: Hematoxylin and eosin-stained sections of poorly-differentiated E-RMS (case 6) consisting of diffuse proliferation of small round tumor cells. C, D: Hematoxylin and eosin-stained sections of well-differentiated E-RMS consisting of rhabdomyoblastoid tumor cells with eosinophilic cytoplasm and cross-striation (case 11) (magnification: A and C, ×100; B and D, ×1000).

MATERIALS AND METHODS

Patient characteristics and tissue evaluation Tissue samples were obtained from E-RMS patients who had undergone biopsy or surgical resection before any treatment at the National Children's Hospital, Tokyo, between March 1983 and July 1999. Clinical follow-up data were available from all patients included in this study, and outcome data were collected from hospital charts. Patients who died of causes other than RMS were not included in the study (Table I). The histological diagnosis and classification of RMS were based on WHO criteria.[24] According to these criteria, RMS in our study was classified into E-

Table I. Clinical Profiles of the Patients Studied

Case No.	Age (y)/Sex	Primary organ	Material	Outcome (survival period: month)
1	3/F	urinary bladder	bx	deceased (51)
2	12/F	rt. thigh	bx	deceased (24)
3	4/M	prostate	bx	no evidence of disease (133)
4	10/F	lt. nasal cavity	bx	deceased (33)
5	2/F	rt. nasal cavity	bx	no evidence of disease (52)
6	1.5/F	bile duct	op	deceased (19)
7	3/M	urinary bladder/prostate	bx	no evidence of disease (117)
8	1.3/M	peritoneum	op	no evidence of disease (119)
9	3/F	vagina	bx	no evidence of disease (51)
10	4/M	urinary bladder	bx	no evidence of disease (131)
11	2/M	retroperitoneum	bx	no evidence of disease (124)

bx, biopsy sample; op, surgically resected sample. The starting point of survival calculation is the date of diagnosis.

Table II. Histological Features, p27 and JAB1 Expression in the Tissues

Case No.	Differentiation	p27	%	NJAB	%	CJAB	%
1†	poorly	–	0.0	–	0.2	–	0.0
2†	poorly	–	0.0	+	34.2	–	0.7
3	poorly	–	0.6	+	13.1	+	50.0 (W)
4†	poorly	–	0.3	+	45.5	–	5.1
5	poorly	+	57.0	+	85.1	–	0.1
6†	poorly	+	33.2	+	72.2	–	0.8
7	poorly	+	41.7 (S)	+	74.6	–	0.0
8	poorly	+	13.2 (S)	+	57.4 (W)	–	4.2
9	well	–	1.3	–	0.0	+	37.5
10	well	–	6.0	–	0.3	+	22.4 (S)
11	well	+	22.2 (W)	–	0.0	+	92.0 (S)

%, percentage of positive cells.
–, less than 10% of the cells were positive; +, more than 10% of cells were positive.
† deceased; (S), strongly positive; (W), weakly positive.

RMS and alveolar RMS, and no pleomorphic RMS was identified.

Eleven initial diagnosis specimens were enrolled in this study. The patients were aged from 1.3 to 12 years old. These E-RMS samples were further classified into two groups based on the differentiation stage of tumor cells.[25, 26] The "poorly-differentiated type" consisted of E-RMS with predominantly immature and small round cells with clear and scanty cytoplasm (Fig. 1, A and B). Eight samples were classified as "poorly-differentiated type." The "well-differentiated type" consisted of E-RMS with predominantly rhabdomyoblastoid tumor cells with abundant eosinophilic cytoplasm (Fig. 1, C and D), and 3 samples were diagnosed as this type (Table II).

Immunohistochemical staining Immunohistochemical study was carried out according to the method described previously.[28] The intensity of expression was expressed as "strong" or "weak" compared with tonsillar squamous epithelium, which was used as a positive control. For the negative control, phosphate-buffered saline (PBS) alone was used as a substitute for the primary antibody to check the possibility of false-positive responses from the second antibody. No evidence of non-specific staining was obtained. For double immunohistochemical staining, samples were first incubated with anti-p27 antibody (Transduction Laboratories, Lexington, KY) followed by incubation with biotinylated anti-mouse IgG (Dako Japan, Inc., Kyoto). After the staining with streptavidin-biotin-peroxidase complex (Dako Japan, Inc.), samples were treated with chromogen, cobalt chloride-diaminobenzidine tetrahydrochloride. Anti-JAB1 antibody was generated as described before.[18] Secondary antibody for JAB1 was biotinylated anti-rabbit IgG (in 1:1000 dilution, 1.5 mg/ml, Vector Laboratories, Inc., Burlingame, CA) and the streptavidin-biotin-alkaline phosphatase/First Red system (Histofine, Nichirei, Inc., Tokyo) was employed. Using this staining method, p27 appeared dark blue and JAB1 stained purple red.

Semi-quantitative analysis of stained sections Four fields from each tissue section were randomly selected for microscopic evaluation at high magnification (×400) and 2000 cells were scored for the staining of p27 or JAB1. p27 is a nuclear protein and is expressed in the nucleus as described previously,[5] while JAB1 is expressed in the nucleus and/or the cytoplasm. The JAB1 expression pattern was classified as nuclear or cytoplasmic. When positive cells constituted more than 10% of the total population, the specimen was classified as positive (+), while when these cells were less than 10% of the total, the specimen was classified as negative (−). Sections were examined independently by two pathologists and the final classification was established by consensus. A *P*-value below 0.005 was considered significant.

The prognostic significance of the staining pattern The prognostic significance of the staining pattern for p27 and JAB1 expression was analyzed by relating the pattern to the survival period after the initial diagnosis of each patient. Due to the small sample size, statistical analysis was not applicable.

RESULTS

Expression of p27 and JAB1 in normal squamous epithelium In order to understand the expression pattern of p27 and JAB1 in normal cell differentiation, human tonsillar squamous epithelium specimens were stained for p27 (Fig. 2A) and JAB1 (Fig. 2B). In the basal layer of the epithelium, where the most immature epithelial cells reside and proliferate, p27 was completely absent (Fig. 2, A-1), but JAB1 was expressed both in the nucleus (NJAB) and cytoplasm (CJAB) (Fig. 2, B-4, arrowhead), though less intensely in the latter (−/+ for p27/NJAB). In the

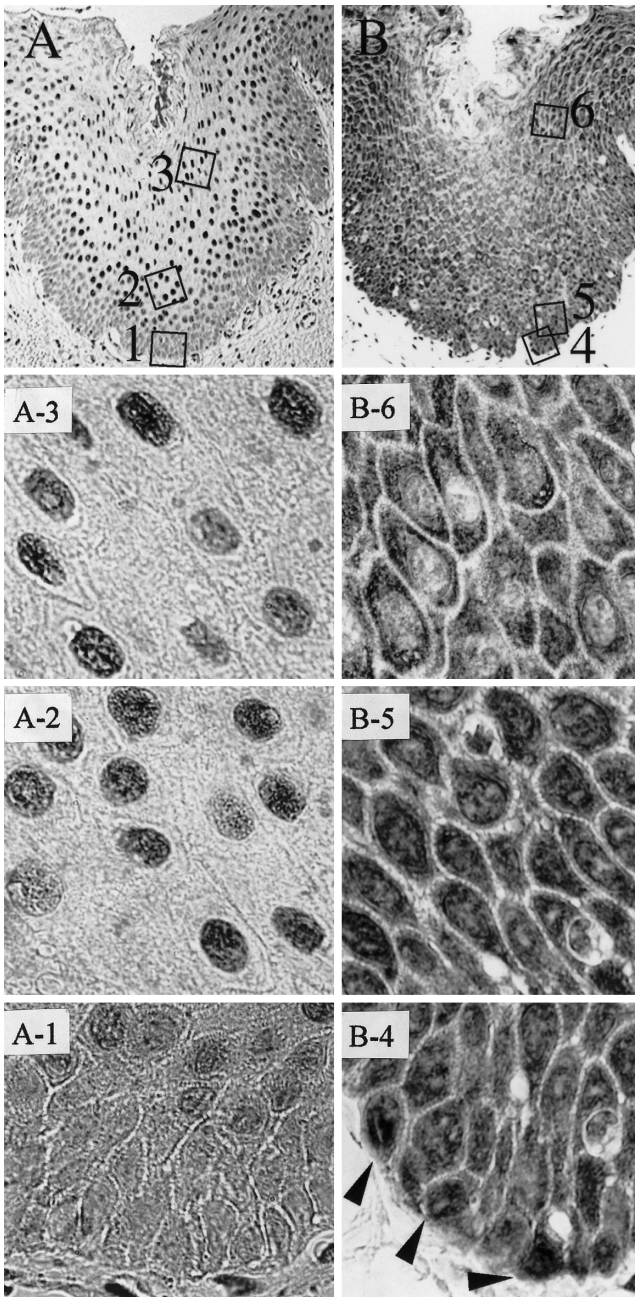

Fig. 2. p27 (A and A-1, A-2, A-3) and JAB1(B and B-4, B-5, B-6) expression in normal squamous epithelium of the tonsil. A-1, A-2, A-3 and B-4, B-5, B-6 are magnified pictures of each square of A and B, respectively. A-1 and B-4, basal cell layers; A-2 and B-5, lower middle cell layers; A-3 and B-6, upper cell layers. In the basal cell layer, p27 is completely absent and JAB1 is predominantly expressed in the nucleus (arrowhead). Note that nuclear JAB1 expression gradually decreases in the upper middle layer of epithelium, whereas nuclear p27 expression did not change (magnification: A and B, ×100; A-1–A-3, ×1000; B-4–B-6, ×1000).

lower middle layer of the epithelium, p27 was expressed in the nucleus (Fig. 2, A-2) in association with nuclear and cytoplasmic expression of JAB1 (+/+ for p27/NJAB) (Fig. 2, B-5). It was noted that in the upper layers of the epithelium, where nuclear p27 expression was maintained, nuclear JAB1 is absent and JAB1 is expressed exclusively in the cytoplasm (+/− for p27/NJAB) (Fig. 2, A-3, B-6). Since p27 induces cell cycle arrest leading to cell differentiation while NJAB counteracts or degrades p27, the coordinated pattern of p27 and NJAB expression suggests that these two proteins are integrated in the regulation of differentiation steps in normal tissue development.

p27 and JAB1 expression in RMS cells The results of immunohistochemical analysis of E-RMS samples are summarized in Table II. Among eight specimens classified as poorly-differentiated E-RMS, four expressed no p27 but were associated with the expression of NJAB (−/+), except for one case (case 1). Case 1 did not express p27 or JAB1, though predominant NJAB was seen in 0.2% of cells, thus being classified as −/− according to our criteria. The remaining four poorly-differentiated E-RMS expressed p27 concomitantly with NJAB (+/+). In three well-differentiated E-RMS, one expressed p27 and all of these three expressed CJAB but not NJAB (+/−). Double immunostaining for p27 and JAB1 expression was carried out in several samples. In the well-differentiated E-RMS, nuclear p27 and CJAB were demonstrated by immunohistochemical staining (Fig. 3). In immature round tumor cells, the nucleus was stained positively with both anti-p27 and anti-JAB1 antibodies by immunofluorescence assay (data not shown).

Correlation of cell numbers positive for p27, NJAB and CJAB is shown in individual cases by a bar graph (Fig. 4). Including 7 additional post-treatment samples (data not shown), the positive cell percentages for p27, NJAB and CJAB in poorly-differentiated tumor cells were 16.5%, 50.3% and 12.3%, respectively, while in well-differentiated tumor cells they were 30.1%, 6.0% and 61.0%, respectively. The difference between poorly- and well-differentiated tumor cells with regard to NJAB and CJAB expression was statistically significant (P=0.002, P=0.003, respectively) by t distribution statistical analysis, although the difference of p27 expression between poorly- and well-differentiated tumor cells was not (Fig. 5).

Prognostic significance of p27 and JAB1 expression The prognostic significance of p27 and JAB1 expression was verified in 11 patients whose pre-treatment specimens were studied by immunohistochemical analysis. Among four patients (cases 1–4) having poorly-differentiated E-RMS with −/+ expression for p27/NJAB, three died at 51 (case 1), 24 (case 2), and 33 (case 4) months after the diagnosis. Among four patients having poorly-differentiated E-RMS with +/+ expression for p27/NJAB, one patient died at 19 months (case 6) and the remaining three

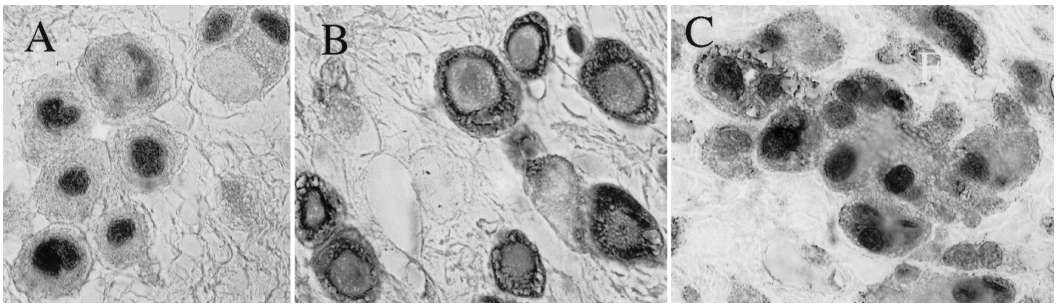

Fig. 3. Nuclear p27 (A) and cytoplasmic JAB1 (B) expression in well-differentiated E-RMS (case 11). Simultaneous immunohistochemical staining for p27 and JAB1 (C). magnification: ×1000.

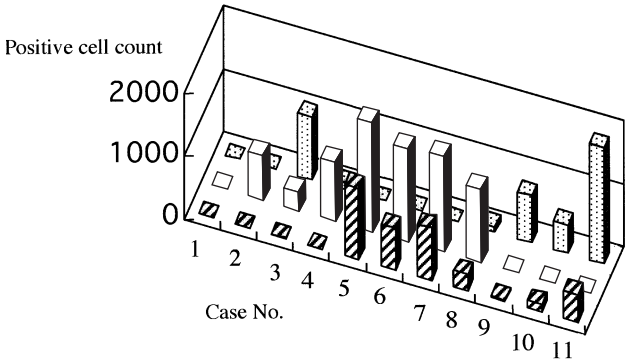

Fig. 4. Correlation of positive cell counts of p27, NJAB and CJAB in each case. Cases 1–8, poorly-differentiated E-RMS; cases 9–11, well-differentiated E-RMS. A slash bar ▨ is for p27-positive cell percentage, a white bar ▭ is for NJAB and a dotted bar ⬚ is for CJAB.

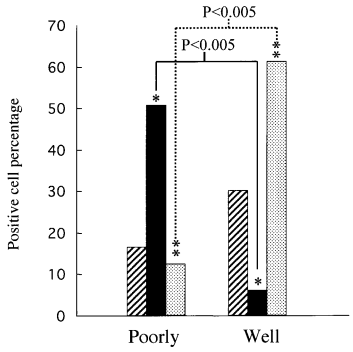

Fig. 5. Statistical analysis of total positive cell percentages with expression of p27/Kip, NJAB and CJAB for poorly- and well-differentiated E-RMS cells. A slash bar ▨ is for p27-positive cell percentage, a black bar ■ is for NJAB and a dotted bar ⬚ is for CJAB. The difference between poorly- and well-differentiated tumor cells for NJAB is P=0.002 and that for CJAB is P=0.003. These values are statistically significant by t distribution statistical analysis (P<0.005), although the difference for p27 expression between poorly- and well-differentiated tumor cells is not.

patients are alive at 52 (case 5), 117 (case 7) and 119 (case 8) months. All three patients having well-differentiated E-RMS with +/+ or −/+ expression for p27/CJAB are alive at 51 (case 9), 131 (case 10), and 124 months (case 11).

DISCUSSION

JAB1 binds p27 and controls the activity of p27 by facilitating its degradation. Binding of JAB1 directs the movement of p27 from the nucleus to the cytoplasm. The export of p27 is controlled in a proteasome-dependent manner but the precise mechanism of this translocation is not known.[18)]

In three out of four poorly-differentiated E-RMS without p27 expression (cases 1, 2, 3, and 4), NJAB was expressed with no (cases 2 and 4) or scanty (case 3) CJAB, which may correspond to the expression pattern of most immature squamous epithelial cells (−/+ for p27/NJAB). In this context, it is not clear at present whether p27 had already been exported from the nucleus to the cytoplasm then degraded by JAB1, or whether only a small amount of p27 was synthesized. Since JAB1 was originally identified as a coactivator of c-Jun and JunD, and is known to selectively potentiate transactivation by c-Jun or JunD, it is possible that NJAB activates genes targeted by transcription factor AP-1 proteins,[19)] which would suggest that NJAB itself stimulates cell proliferation. This original function of NJAB may be compatible with our observation in poorly-differentiated cells.

It was rather unexpected that the remaining four poorly-differentiated E-RMS showed nuclear p27 expression. The nuclear p27 in these cases was, however, associated with predominant NJAB expression, a pattern that might resemble that in the lower middle cell layer of squamous epithe-

lium (+/+ for p27/NJAB), which was close to maturation. These findings suggest an important function of JAB1; concomitant nuclear expression of JAB1 may more or less inactivate p27 before nuclear exportation. This interpretation is consistent with the initial finding of JAB1-induced cell cycle regulation in transfection experiments.[18] In this context, we postulate that the p27 expression profile by itself may not be useful, but rather the coordinated expression pattern of nuclear JAB1 and p27 may more intimately correspond to the differentiation of E-RMS.

Four out of eight patients with poorly-differentiated E-RMS died between 19 and 51 months after the diagnosis, while all three patients with well-differentiated E-RMS are still alive and have had no evidence of disease for more than 3 years. These findings are consistent with the previous notion that the differentiation stage of RMS can predict prognosis.

Poorly-differentiated E-RMS could be classified into two groups depending on p27 expression. Three cases out of 4 with no p27 expression died, while only one of 4 with p27 expression died, despite the pathological diagnosis of poorly-differentiated E-RMS. Since seven out of these 8 had NJAB expression, these findings suggest that differentiation status depends on the expression of NJAB, but the clinical response may depend more on the expression of p27 in the poorly-differentiated E-RMS. These findings call for further studies to examine JAB1 expression in poorly-differentiated E-RMS.

Our results also showed no p27 expression in two samples of well-differentiated E-RMS (cases 9 and 10). In these samples, expression of JAB1 was predominantly cytoplasmic and none was detected in the nucleus, suggesting that some relationship may exist between differentiation and CJAB expression.

One patient (case 1) was classified as poorly-differentiated E-RMS based on the biopsy sample at the initial diagnosis. In this case, p27 was not expressed, and nuclear JAB1 expression was seen in 0.2% of cells. This was classified as negative for NJAB expression using our classification criteria. This is a case for which interpretation of the expression pattern of p27 and JAB1 was difficult.

p27 is post-transcriptionally controlled by phosphorylation and subsequent ubiquitination.[17] p27 is ubiquitinated by F-box protein SKP-2 following phosphorylation, and this leads to an acceleration of proteasome-mediated degradation of p27.[29] Thus, the machinery required to degrade p27 via molecules other than JAB1 may also play an important role in determining the characteristics of proliferation of human tumors by modulating the activity of CDK inhibitors. Such a scenario may well explain the histological features of p27-positive poorly-differentiated E-RMS. It might also explain why p27 mutation is extremely rare in human tumors.[7, 30]

Sui *et al*. recently reported that JAB1 expression is inversely correlated with p27 expression levels, and suggested that JAB1 may be associated with the progression and poor prognosis of epithelial ovarian tumors.[31] These findings overall indicate that p27 by itself is not sufficient to predict cell proliferation and differentiation status, and additional molecules which modulate p27 protein and its function need to be taken into consideration. Thus, further studies to elucidate the regulatory systems of cell-cycle inhibitors may eventually help to improve the diagnosis and predict the outcome in cancer patients.

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