Cancer Science
The official journal of the Japanese Cancer Association

APAF-1 is related to an undifferentiated state in the testicular germ cell tumor pathway

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(Received March 24, 2010/Revised August 24, 2010/Accepted August 31, 2010/Accepted manuscript online September 17, 2010/Article first published online October 26, 2010)

Apoptotic protease activating factor-1 (*APAF-1*) is a key regulator gene of apoptosis, located downstream from *p53*. Loss of *APAF-1* expression is associated with chemorefractory malignant melanoma and neuronal cell differentiation. In order to make clear the function of APAF-1 in the carcinogenesis of germ cell tumors, we evaluated the expression levels of APAF-1 and several apoptosis and differentiation markers by immunohistochemistry in formalin-fixed paraffin-embedded samples from 43 cases of testicular germ cell tumor (TGCT) and six specimens of normal testis tissue. Expression of cleaved caspase-3, Oct-3/4, and Ki-67 were also examined by immunohistochemistry to evaluate apoptotic reactivity, tumor differentiation, and proliferation activity, respectively. *APAF-1* was downregulated in two TGCT cell lines by siRNA transfection, and subsequent expression of the *Ki-67* and *Oct-3/4* genes and differentiation markers of three embryonic germ layers including *keratin16 (KRT16)* for ectoderm, *vimentin (VIM)* for mesoderm and *GATA4* for endoderm were then tested. No significant relationship was found between APAF-1 expression and apoptotic activity in TGCTs. Expression of APAF-1, Oct-3/4, and Ki-67 was significantly higher in seminomas than in non-seminomas. In TGCTs, higher APAF-1 expression was correlated with higher proliferation (high Ki-67) and a lower degree of differentiation (high Oct-3/4). Interestingly, the expression of APAF-1 gradually decreased in accordance with tumor differentiation (seminoma and embryonal carcinoma > teratoma). Downregulation of *APAF-1* in TGCT cell lines resulted in a decrease of *Ki-67* and *Oct-3/4* and an increase of *VIM* and *KRT16* gene expression. These data show that higher expression of *APAF-1* is related to an undifferentiated state in the TGCT pathway. (*Cancer Sci* 2011; 102: 267–274)

Testicular germ cell tumor (TGCT) is the most common cancer in young men and accounts for 1% of all malignant neoplasms in males. These tumors are classified into two entities, seminoma, and non-seminomatous germ cell tumors (NSGCT). The latter are in turn subdivided into embryonal carcinoma (EC), yolk sac tumor (YST), choriocarcinoma (CC), and teratoma.[1] The incidence rate of TGCT has increased by 50–100% in recent decades.[2–6] The rate of mortality due to testis cancer in Japan is estimated to be 100 cases per year.[7]

Testicular germ cell tumors are unique as they form distinct morphological subtypes that simulate different lineages of the developing human embryo. The tetrahedron model of histogenesis represented the process of transformation of TGCTs from germ cells.[8–12] According to this model, seminoma, which resembles undifferentiated primitive germ cells, develops to EC and both of them can develop to YST and CC. In the next step, both EC and YST transform to immature teratoma. Finally, immature teratoma differentiates to mature teratoma.[8] Non-seminomatous germ cell tumors show varying embryonic and extra-embryonic patterns of differentiation. Embryonal carcinoma shows early zygotic or embryonal-like differentiation. Both YST and CC show extra-embryonal forms of differentiation, and teratoma shows somatic differentiation along several lineages. Seminoma and EC have pluripotent capabilities and may differentiate into YST, CC, or highly differentiated teratoma.[13,14]

Apoptotic protease activating factor-1 (*APAF-1*) is a gene located downstream of the tumor-suppressor gene *p53* and is the key regulator gene of the apoptosis pathway. After damage to the cell, cytochrome *c* leaks from mitochondria and binds to APAF-1 to form apoptosomes. Apoptosomes activate caspases and promote apoptosis.[15,16] Defects in the regulation of apoptosome function have been documented in various forms of human cancer, and may play a role in both carcinogenesis and chemoresistance.[17] Loss of APAF-1 expression has been reported in metastatic melanoma lesions and chemorefractory rectal tumors.[18,19]

A study of the methylation status of the promoter region of the *APAF-1* gene indicated epigenetic inactivation of *APAF-1* in all seminomatous and non-seminomatous TGCTs as well as in 60% of normal testicular tissue.[20] Moreover, *APAF-1* deletion and absence of *APAF-1* mRNA expression have been reported in some TGCT cell lines.[21] *APAF-1* knockout mice have been reported to show degeneration of spermatogonia, resulting in a virtual absence of sperm, and infertility.[22] Studies of neuronal cell lines have shown that cell differentiation is accompanied by a decrease of APAF-1 and activity of apoptosomes.[23]

This research was established to study APAF-1 and its relation to the differentiation and proliferation status in human TGCT. Downregulation of the *APAF-1* gene was also studied in TGCT cell lines.

Materials and Methods

Specimens and cell lines. Formalin-fixed and paraffin-embedded specimens from 43 cases of TGCT with differing histological subtypes and six samples of normal testis (obtained by castration for treatment of androgen-responsive prostate cancer) were selected from the archive of the Department of Pathology, Tsukuba University Hospital (Ibaraki, Japan), between 1996 and 2006 and examined by immunohistochemistry (IHC) (Table 1). All of the patients concerned gave informed consent to the use of their samples. For patients under 15 years of age, their parents provided informed consent. Two TGCT cell lines, NEC8 (embryonal carcinoma) and NEC14 (mixed type) (both from Riken, Tsukuba, Japan), were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% FBS.

Western blot analysis (WB). Protein was extracted from frozen tissue sections using T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL, USA) containing 1:10 (v/v) protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Twenty micrograms of extracted protein was electrophoresed in two

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doi: 10.1111/j.1349-7006.2010.01750.x

Table 1. Clinicopathological characteristics of 43 patients with testicular germ cell tumors and six patients from whom normal testis was removed by castration

Category	*n*	Mean age (years)	Metastasis *n* (%)
Seminoma	16	36.7 ± 7.5	6 (37.5)
Embryonal carcinoma	7	28.6 ± 9.4	6 (85.7)
Yolk sac tumor	4	18.5 ± 20.8†	2 (50.0)
Choriocarcinoma	2	28.5 ± 3.5	2 (100.0)
Teratoma	6	8.0 ± 13.0‡	1 (16.7)
Mixed tumor	8	27.0 ± 6.7	3 (37.5)
Normal testis	6	64.2 ± 19.1	NA

†One patient was newborn and another was 2 years old.
‡Four patients were newborns. NA, not applicable.

wells of a 7.5% SDS-PAGE gel (Bio-Rad Laboratories, Richmond, CA, USA) and blotted onto a nitrocellulose membrane. The membrane was then blocked with Super Block Blocking Buffer (Pierce) containing 0.05% Tween-20 and cut into two pieces. Each piece was treated with a 1:20 000 dilution of anti-APAF-1 antibody (Chemicon, Boronia, Australia) or a 1:10 000 dilution of anti-β-actin antibody (Sigma) at 4°C, overnight. After three rinses with PBS containing 0.05% Tween-20 for 5 min, the membranes were treated with a 1:10 000 dilution of goat anti-rat IgG-HRP (Jackson Immunoresearch Laboratories, West Grove, PA, USA) and a 1:2500 dilution of goat anti-mouse IgG-HRP (Pierce), respectively, at room temperature (RT) for 1 h. After rinsing with PBS-Tween, the visualization step was carried out using a SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific, Rockford, IL, USA).

Immunohistochemistry. Deparaffinization, antigen retrival, and peroxidase activity quenching were done according to conventional methods. For APAF-1 staining, sections were blocked with a Biotin Blocking System (Dako, Glostrup, Denmark), then with 1% BSA (Sigma) in PBS. Sections were then incubated with primary antibodies against APAF-1, p53, cleaved caspase-3, Ki-67, or Oct-3/4 (Table S1) for 1 h at RT, except for the anti-cleaved caspase-3 antibody, which was incubated overnight at 4°C. All primary antibodies were diluted in antibody diluent (Dako), except the anti-APAF-1 antibody, which was diluted in PBS containing 1% BSA (Sigma). After the primary antibody had been washed off, the anti-APAF-1 antibody was detected by incubating the sections with biotinylated rabbit anti-rat Ig (Dako) as a secondary antibody, then with HRP-conjugated streptavidin (Dako). Other primary antibodies were detected with an anti-mouse and anti-rabbit Envision System Labeled Polymer (Dako). The reactions were visualized by incubating the sections with DAB + chromogen (Dako) for 5–10 min. Washes between incubations were carried out with TBS containing 0.05% Tween-20, pH 7.6. Sections were then counterstained with hematoxylin. Class-matched negative control for APAF-1 was carried out using rat IgG2a, kappa antibody (Abcam, Cambridge, UK) 1:100. Slides were examined by a microscope. At least 1000 cells were counted and analysed with an Image Processor for Analytical Pathology (IPAP; Sumika Technos, Osaka, Japan). Samples were also assessed for APAF-1 semiquantitatively by one pathologist (Y.I.) according to the area and intensity of staining, and were categorized as having low (weak) or high (moderate or strong) APAF-1 expression. Each component of mixed tumors was evaluated as its relevant histological subtype.

siRNA transfection. Both NEC8 and NEC14 were transfected with three sets of *APAF-1* Stealth Select RNAi, including HSS141234, HSS141235, and HSS141236 (Invitrogen) using Lipofectamine RNAi MAX Reagent (Invitrogen) by the reverse method to downregulate *APAF-1*. All three siRNAs were able to efficiently downregulate *APAF-1*. Among them, HSS141236 was chosen for later experiments because it had the strongest effect. The control group was transfected with Negative Universal Control (Invitrogen). RNA was extracted using an RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA) after 24 and 48 h and 1 μg RNA was converted to cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Protein was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce) containing 1:10 (v/v) Protease Inhibitor Cocktail. Expression of *APAF-1* RNA and protein was tested by real-time PCR (7300 Real Time PCR System; Applied Biosystems) and WB, respectively.

Real-time PCR. Expression of RNA for *APAF-1*, *Oct-3/4*, *Ki-67*, *18s ribosomal RNA* (all from Takara, Otsu, Japan), and three differentiation markers of embryonic germ layers including *keratin16 (KRT16)* for ectoderm, *vimentin (VIM)* for mesoderm, and *GATA4* for endoderm differentiation (all from Fasmac, Tokyo, Japan) was also tested. Primer sequences are shown in Table S2. Results were normalized to 18s ribosomal RNA expression.

Indirect immunofluorescence. Transfected cells were cultured on 4-chamber slides for 48 h. Then cells were washed with PBS and fixed with methanol for 2 min at RT. The cells were rinsed with distilled water and incubated with 0.5% Casein (Sigma) blocking solution in TBS for 20 min. The cells were then probed with mouse anti-pan-keratin mAb (Roche, Mannheim, Germany) 1:200 or mouse anti-VIM mAb (Dako) 1:200 in antibody diluent at 4°C overnight. Indirect immunofluorescence labeling was carried out by exposure to FITC-conjugated donkey anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:100 in 0.5% casein/TBS at RT for 1 h. Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) was used to counterstain nuclei.

WST-1 analysis. Cell proliferation rate of transfected cells was determined using Cell Proliferation Reagent WST-1 (Roche) and the ratio of non-attached to attached cells was also

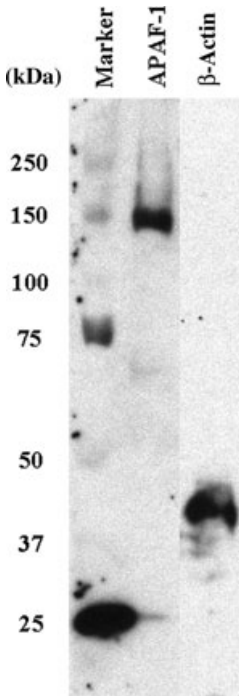

Fig. 1. Western blot analysis of a seminoma tumor showed a specific band for apoptotic protease activating factor-1 (APAF-1).

doi: 10.1111/j.1349-7006.2010.01750.x

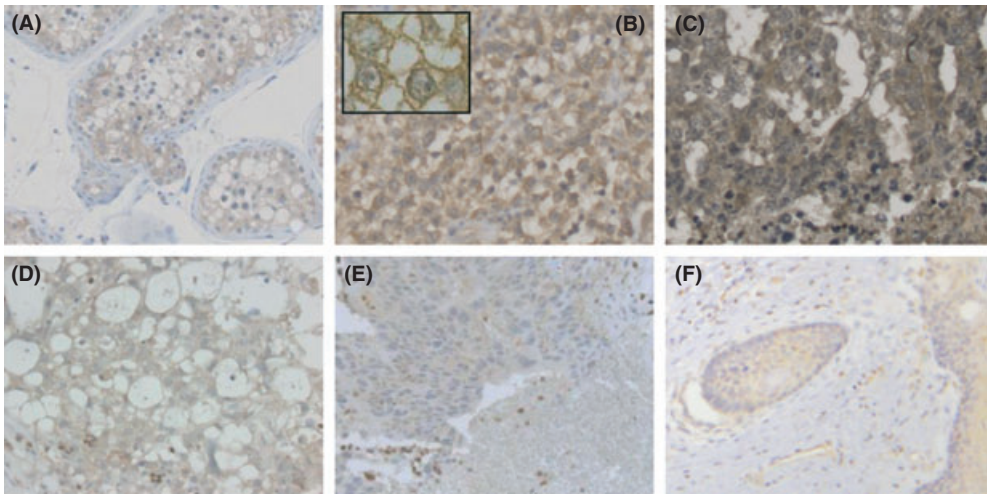

Fig. 2. Immunohistochemistry of apoptotic protease activating factor-1 for normal testis and representative testicular germ cell tumors. (A) Seminiferous tubules of normal testis. (B) Seminoma. Inset, membranous staining of tumor cells (×400). (C) Embryonal carcinoma. (D) Yolk sac tumor. (E) Choriocarcinoma. (F) Teratoma. Magnification, ×100.

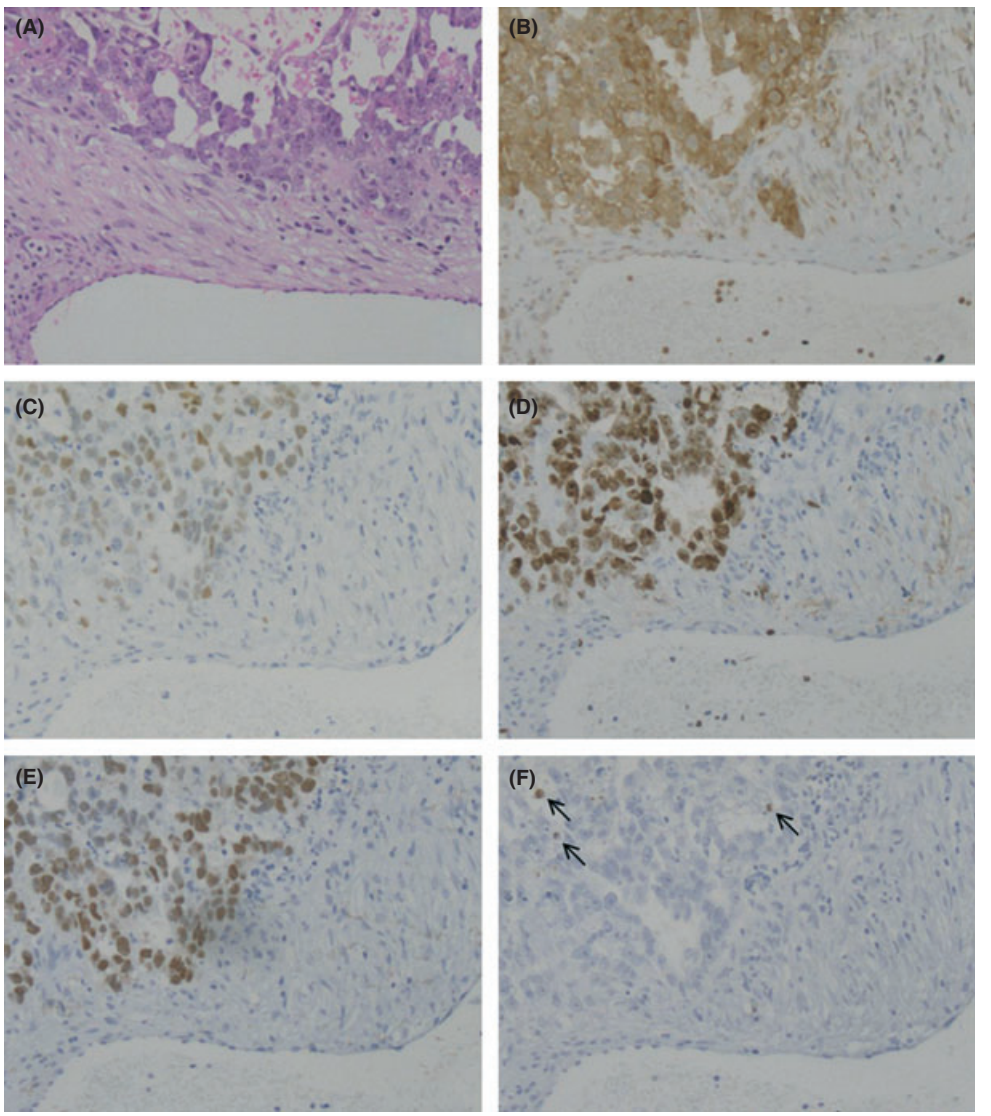

Fig. 3. Immunohistochemistry of various markers in an embryonal carcinoma case. (A) HE staining. (B) Apoptotic protease activating factor-1. (C) p53. (D) Ki-67. (E) Oct-3/4. (F) Cleaved caspase-3 (arrows). Magnification, ×100.

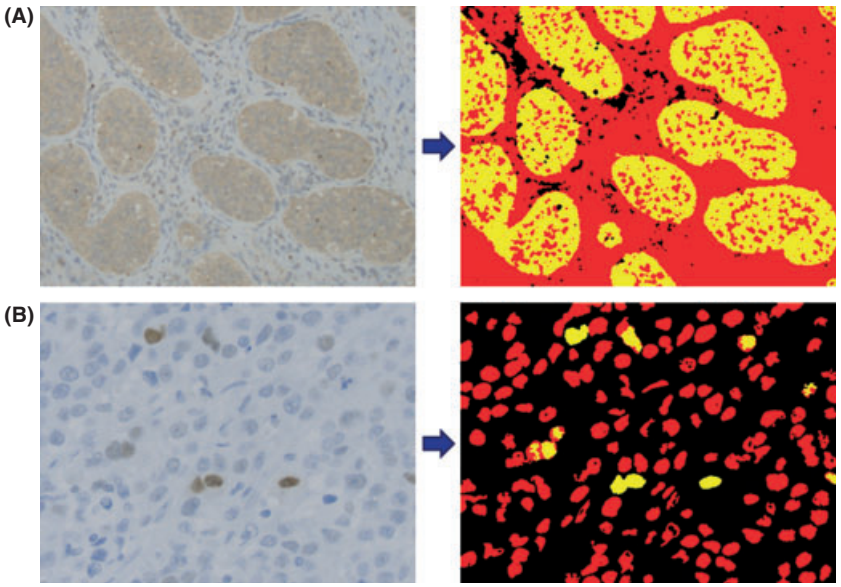

Fig. 4. Images taken by a digital camera and converted to digital data by Image Processor for Analytical Pathology software. (A) Cytoplasmic staining of APAF-1 in a teratoma. (B) Nuclear staining of p53 in an embryonal carcinoma. Magnification, ×100.

determined in case and control groups. Morphology of cells was observed using an inverted microscope.

Statistics. Statistical analysis was done using SPSS (SPSS, Chicago, IL, USA) and Dunkan's multiple range test.[24]

Results

Expression of APAF-1 was evaluated and compared with those of a cell proliferation marker (Ki-67), an anti-oncogene (p53), an apoptosis marker (cleaved caspase-3), and a differentiation marker (Oct-3/4). First, we confirmed the specificity of the anti-APAF-1 antibody by WB. We selected a case of seminoma that gave a positive reaction with the anti-APAF-1 antibody by IHC (data not shown). Western blot analysis was then carried out using the protein extracted from frozen material of the same case. As Figure 1 shows, a single specific band of 141 kDa was detected.

APAF-1 expression was then evaluated in normal testis and various subtypes of testicular tumors (Figs 2,3A,B). The cell cytoplasm of all cases of normal testes and tumors were stained, although with various degrees of intensity and various percentages of tumor cells. Cell membrane staining was also observed in some seminoma cases (Fig. 2B). The proteins of p53, Ki-67, and Oct-3/4 were stained in the nuclei of tumor cells (Fig. 3C–E). Cleaved caspase-3 was stained in the cytoplasm and perinuclear region of cells (Fig. 3F, arrows). All stained slides were analyzed using the IPAP analyzer. As Figure 4 shows, the stained areas were scanned into the computer and converted to digital data (Fig. 4, yellow color). To calculate the degree of positivity, the proportion of positive cells relative to total cells was determined on the basis of two different parameters. ''Area'' was applied for cytoplasmic staining (Fig. 4A) and ''Number'' was applied for nuclear staining (Fig. 4B). Interestingly, the expression of APAF-1 gradually decreased in accordance with tumor differentiation (seminoma and EC > teratoma) (Fig. 5A, Table 2). The expression rates of YST and CC were between those of seminoma and EC, and teratoma. Figure 5(B,C) shows comparisons of the average expression levels of APAF-1, p53, cleaved caspase-3, Ki-67, and Oct-3/4 between the seminoma and NSGCT groups, and between the teratoma and non-teratomatous tumor groups. Expression of APAF-1, Ki-67, and Oct-3/4 was significantly higher in seminoma than in NSGCTs (Fig. 5B). All five markers were expressed more strongly in non-teratomatous germ cell tumors than in teratomas (Fig. 5C). Both seminoma and teratoma showed lower expression of p53 and cleaved caspase-3 than the other tumors (Fig. 5B,C). In TGCTs, the expression of APAF-1 showed no correlation with that of p53 or cleaved caspase-3 (data not shown); however, the level of APAF-1 expression was correlated with a high Ki-67 labeling index (LI), which is a marker of proliferation (Fig. 5D), and with high expression of Oct-3/4, which is a marker of undifferentiation (Fig. 5E). There were two patterns of correlation between APAF-1 and Oct-3/4 expression: one group, including seminoma and EC, showed correlated expression of APAF-1 and Oct-3/4; the other group, including YST, CC, and teratoma, showed various degrees of APAF-1 protein expression, but no expression of Oct-3/4 (Fig. 5E). Similarly, the results of IHC for APAF-1 carried out by the pathologist showed that the expression levels of both Oct-3/4 and the Ki-67 LI were significantly higher in samples with high (moderate or strong) APAF-1 expression than in those with low (weak) expression (Fig. 5F), which was in line with the results of IPAP.

As Table 3 indicates, we found no significant relationship between APAF-1 expression and metastasis. However, Ki-67 LI was higher in TGCTs with metastasis than in those without (stage 1). The level of APAF-1 expression was significantly lower in pediatric tumors (patient age 0–2 years; APAF-1 0.28 ± 0.22) than in adult tumors (patient age 18–51 years; APAF-1 0.55 ± 0.20) ($P < 0.02$, data not shown) because all of the pediatric tumors were YST or teratoma. We did not find any significant relationship between APAF-1 expression and serum markers, including hCG, bhCG, AFP, and LDH.

Small interfering RNA transfection efficiently downregulated *APAF-1* in both cell lines (data not shown). Following downregulation of *APAF-1*, a tendency for decreased expression of *Ki-67* and *Oct-3/4* was noted in both cell lines. In particular, *Ki-67* was significantly downregulated in the NEC8 cell line and *Oct-3/4* was significantly downregulated in the NEC14 cell line (Fig. 6A,B). In NEC14, two out of the three differentiation markers of embryonic germ layers, *KRT16* and *VIM*, showed a significant increase in siAPAF1 compared to negative control group (Fig. 6C,D). *GATA4* was not changed after *APAF-1* downregulation (Fig. 6E). In indirect immunofluorescence of NEC14, the siAPAF1 group seemed to have a stronger signal for VIM and pan-KRT than the negative control group (Fig. 6F). In immunocytochemistry, we did not detect membranous staining in any TGCT cell line (data not shown). In NEC14, the cell proliferation rate was significantly decreased after transfection

doi: 10.1111/j.1349-7006.2010.01750.x

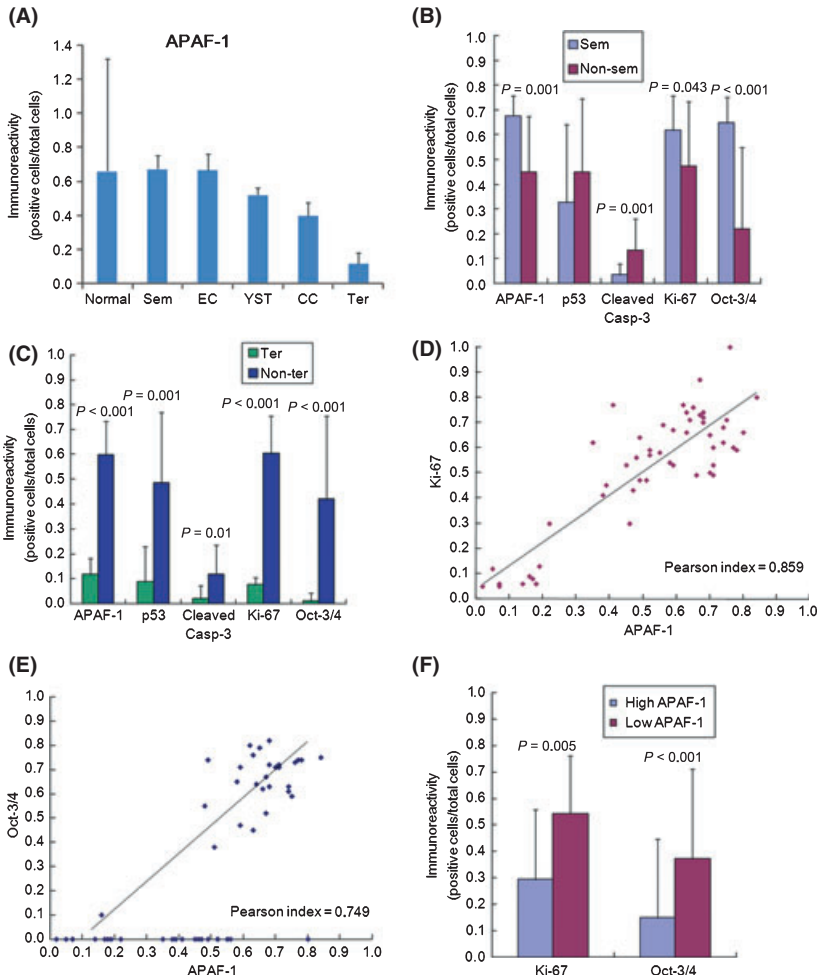

Fig. 5. Comparison of immunohistochemical results. (A) Expression of apoptotic protease activating factor-1 (APAF-1) protein in normal testis and testicular germ cell tumors (TGCT). (B) Expression of different marker proteins (seminoma [Sem] *vs* non-seminoma). (C) Expression of different marker proteins (teratoma [Ter] *vs* non-teratoma). (D) Correlation of protein expression levels between APAF-1 and Ki-67 labeling index in TGCT. (E) Correlation of protein expression levels between APAF-1 and Oct-3/4 in TGCT. (F) Comparison of Ki-67 and Oct-3/4 expression between low and high APAF-1 groups. Casp-3, caspase-3; CC, choriocarcinoma; EC, embryonal carcinoma; YST, yolk sac tumor.

Table 2. Details of expression levels of markers in testicular germ cell tumors and germinal epithelium of normal testes

Category	APAF-1	*P*	p53	*P*	Cleaved caspase-3	*P*	Ki-67	*P*	Oct-3/4	*P*
Seminoma	0.67 ± 0.08	a	0.33 ± 0.32	bc	0.03 ± 0.04	b	0.62 ± 0.14	ab	0.65 ± 0.10	a
EC	0.67 ± 0.10	a	0.60 ± 0.24	ab	0.22 ± 0.12	a	0.70 ± 0.09	a	0.63 ± 0.22	a
YST	0.52 ± 0.04	b	0.60 ± 0.15	a	0.10 ± 0.06	b	0.55 ± 0.15	b	0	b
CC	0.40 ± 0.08	c	0.61 ± 0.17	ab	0.18 ± 0.12	a	0.52 ± 0.15	b	0	b
Teratoma	0.12 ± 0.06	d	0.09 ± 0.14	cd	0.02 ± 0.05	b	0.08 ± 0.03	c	0.01 ± 0.03	b
Normal	0.66 ± 0.66	a	0.02 ± 0.03	d	0.02 ± 0.01	b	0.16 ± 0.07	c	0	b

Means with at least one similar letter are not significantly different at the 0.05 level according to Duncan's multiple range test.[24]
CC, choriocarcinoma; EC, embryonal carcinoma; Normal, normal germinal epithelium of normal testis; YST, yolk sac tumor.

Table 3. Protein expression level in cases of testicular germ cell tumor with and without metastasis

Protein	Without metastasis		With metastasis		*P*-value
	Expression level	*n*	Expression level	*n*	
APAF-1	0.49 ± 0.24	30	0.56 ± 0.2	24	0.28
p53	0.35 ± 0.3	24	0.49 ± 0.3	21	0.15
Cleaved caspase-3	0.07 ± 0.07	30	0.15 ± 0.14	24	0.006*
Ki-67	0.45 ± 0.24	30	0.59 ± 0.22	24	0.03
Oct-3/4	0.29 ± 0.34	30	0.43 ± 0.35	24	0.15

*<0.05, statistically significant.

with *APAF-1* siRNA (Fig. 7A). Moreover, transfected cells showed a higher rate of non-attachment in comparison to the negative control (Fig. 7B). Morphologically, the number of attached cells decreased and their shape changed from a dendritic to a round form (Fig. 7C,D).

Discussion

APAF-1 is the transcriptional target of p53 in DNA damage-induced apoptosis. Absence of normal p53 may decrease the endogenous expression of APAF-1 and thus create an apoptosis-resistant cell.[25] We evaluated the expression of APAF-1, as

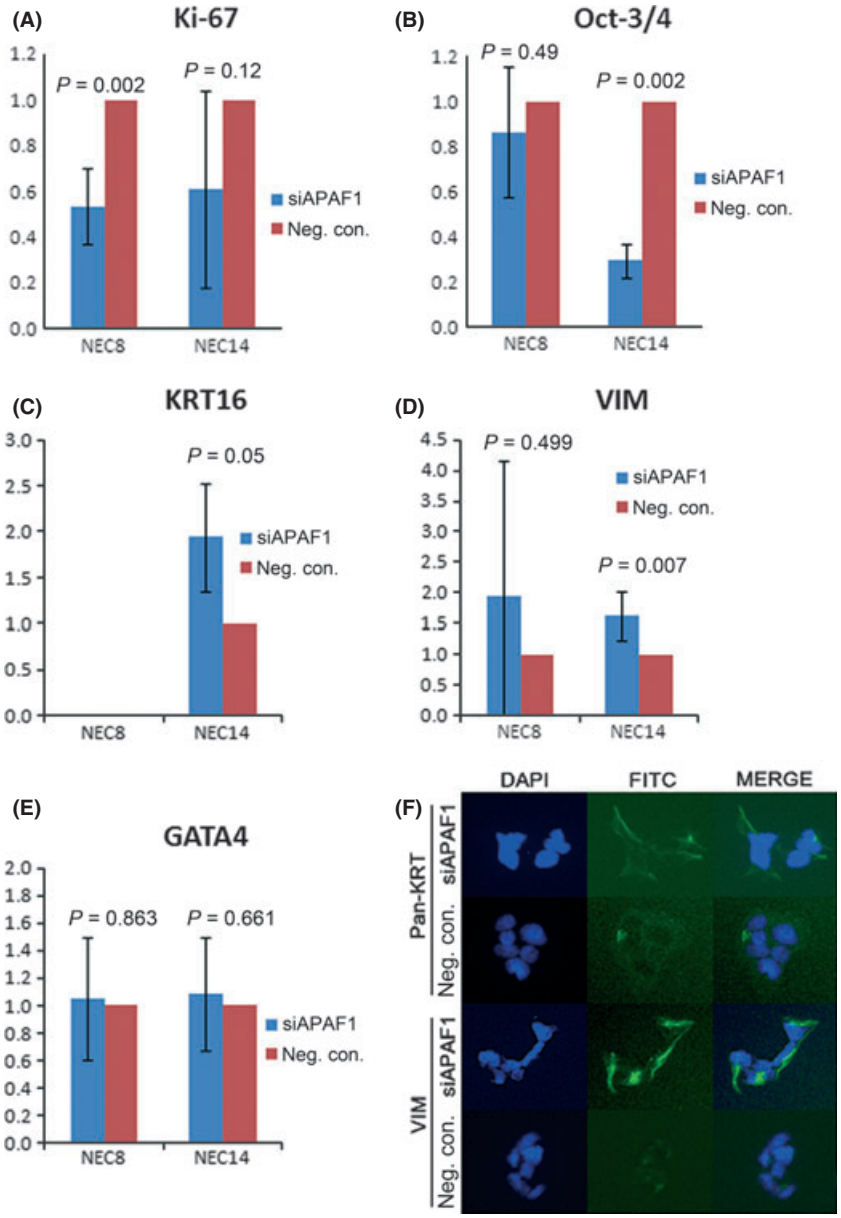

Fig. 6. Downregulation of apoptotic protease activating factor-1 (*APAF-1*) in the NEC8 and NEC14 testicular germ cell tumor cell lines. (A) Decrease of *Ki-67* after *APAF-1* downregulation. (B) Decrease of *Oct-3/4* after *APAF-1* downregulation. (C) Increase of *keratin16* (*KRT16*) after *APAF-1* downregulation. (D) Increase of *vimentin* (*VIM*) after *APAF-1* downregulation. (E) *GATA4* did not change after *APAF-1* downregulation. (F) Immunofluorescent staining of pan-keratin and VIM in the NEC14 cell line.

well as that of markers of apoptosis, proliferation, and differentiation, in TGCTs and normal testis using IHC. APAF-1 was expressed in the cytoplasm and cell membrane of cells (Fig. 2). To our knowledge, membranous staining of APAF-1 has not been reported previously, and could be due to a connection between this protein and some proteins present in the membrane. This interaction with membrane proteins might be associated with apoptosis or differentiation. We did not find any significant correlation between APAF-1 and apoptosis-related proteins, including p53 and cleaved caspase-3. Our finding was in line with another study of tissue-specific regulation of Apaf-1 expression by p53,[25] which showed that in mouse testis, APAF-1 is not regulated by p53. Moreover, *p53* gene mutation is rare in TGCT,[26,27] whereas *Fas* gene mutation is frequently detected in TGCT.[28] Although mutation analysis of the *p53* gene was not done in the present study, the high rate of APAF-1 expression and the low rate of caspase-3 expression in TGCTs indicated that APAF-1 is not closely related to apoptosis of tumor cells. These data suggest that in TGCT, apoptosis might be executed through the extrinsic (Fas signaling) pathway rather than the intrinsic (APAF-1) pathway. However, the expression of cleaved caspase-3 was lower in teratoma than in non-teratomas (Fig. 5C). Cleaved caspase-3 is the final executioner in both the intrinsic and extrinsic apoptosis pathways, and an indicator of apoptosis activity. As teratoma is a relatively chemoresistant tumor, the low level of cleaved caspase-3 in this tumor type might explain its resistance to chemotherapy.

Lindholm and Arumäe[16] indicated that APAF-1 was highly expressed in undifferentiated PC-12 cells and sympathetic neuronal cells, but that the expression dropped dramatically following the differentiation of neuronal cells, and this drop in APAF-1 expression rendered the mature neurons resistant to cytochrome *c*-dependent apoptosis. According to the tetrahedron model of histogenesis,[8] EC originates from seminoma, and both have a capacity for pluripotency and differentiation to other types of TGCTs. We showed that seminoma and EC, which are pluripotent and less differentiated, expressed high levels of APAF-1 protein, whereas teratoma with somatic differentiation had the lowest level of APAF-1 protein expression (Fig. 5A, Table 2). The expressions of APAF-1 in YST and CC were

doi: 10.1111/j.1349-7006.2010.01750.x

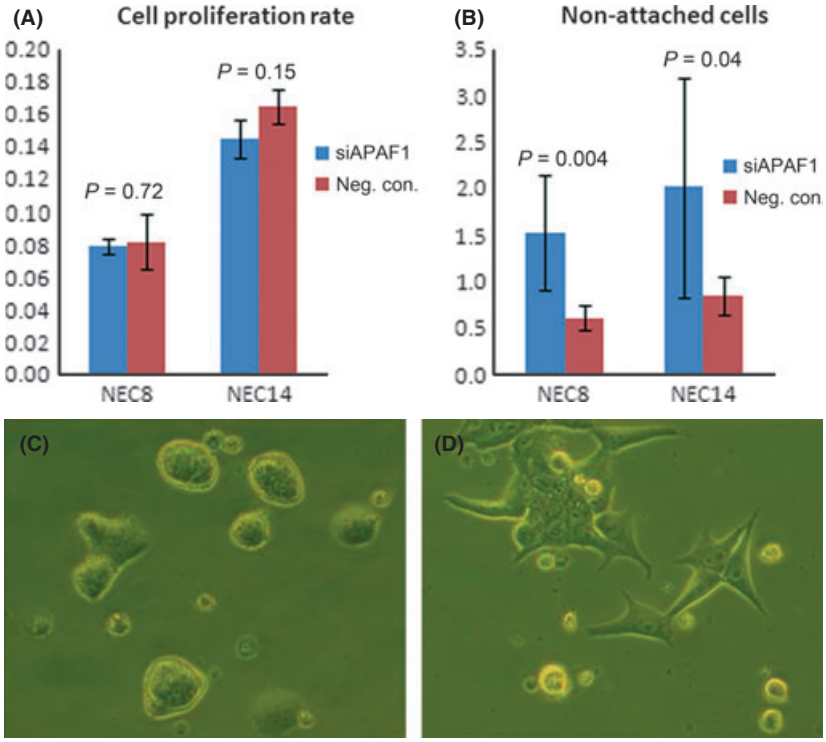

Fig. 7. (A) Decrease of the cell proliferation rate in NEC14 testicular germ cell tumor cells after apoptotic protease activating factor-1 (*APAF-1*) downregulation. (B) Non-attached cells increased in siAPAF1-transfected cells as compared with the negative control. (C) Morphology of NEC8 cells 48 h after siAPAF1 transfection (×100). (D) Morphology of NEC8 cells in the negative control group (×100).

between those of seminoma and EC, and teratoma (Fig. 5A, Table 2). This result may indicate that YST and CC are more differentiated tumors than seminoma and EC but still have a capacity of differentiation compared to teratoma. Moreover, the expression level of APAF-1 was positively correlated with Ki-67 LI, which is a marker of cell proliferation (Fig. 5D). Although Ki-67 does not directly reflect cell undifferentiation, its expression has been shown to accompany cell undifferentiation. For instance, Ki-67 overexpression has been shown to be negatively correlated with cell differentiation in gastric carcinoma.[29] In the present study, the expression level of APAF-1 was also positively correlated with that of Oct-3/4, which is a marker of undifferentiated cells (Fig. 5E). The presence of two distinct tumor types was of considerable interest. One type included seminoma and EC, showing a positive correlation between the expression of APAF-1 and that of Oct-3/4. The other included YST, CC and teratoma, and showed no expression of Oct-3/4 irrespective of the degree of APAF-1 expression. We speculate that seminoma and EC have a capacity for pluripotency, and show functional activation of APAF-1. Using the siRNA method, we confirmed that downregulation of *APAF-1* in NEC8 and NEC14 decreased the expression of *Ki-67* and *Oct-3/4* (Fig. 6A,B). Moreover, among differentiation markers of the three embryonic germ layers, ectoderm and mesoderm markers increased significantly after *APAF-1* downregulation (Fig. 6C,D). This increase in differentiation markers was seen in NEC14, which also showed a significant decrease of *Oct-3/4* after *APAF-1* downregulation, thus confirming the findings of IHC. Investigation of the cell proliferation rate using WST-1 reagent confirmed that *APAF-1* was related to cell proliferation and surface attachment ability (Fig. 7A,B). This evidence suggests that, through the TGCT pathway, *APAF-1* is related to an undifferentiated and highly proliferative cell state. The molecular mechanism underlying *APAF-1*-related differentiation and proliferation is unknown and needs to be revealed. Overexpression or downregulation of *APAF-1* in embryonic stem cell lines might help to uncover this mechanism.

In conclusion, our present investigation of APAF-1 protein expression in TGCTs and normal testis tissues has revealed no significant relationship between APAF-1 and markers of apoptosis. However, a correlation was found between APAF-1 and markers of cell proliferation and undifferentiation. On the basis of these results, we speculate that *APAF-1* is closely related to an undifferentiated state in the TGCT pathway.

References

1 van de Geijn GJ, Hersmus R, Looijenga LH. Recent developments in testicular germ cell tumor research. *Birth Defects Res C Embryo Today* 2009; **87**(1): 96–113.
2 Walschaerts M, Huyghe E, Muller A, Bachaud JM, Bujan L, Thonneau P. Doubling of testicular cancer incidence rate over the last 20 years in southern France. *Cancer Causes Control* 2008; **19**(2): 155–61.
3 Clemmesen J. A doubling of morbidity from testis carcinoma in Copenhagen, 1943–1962. *Acta Pathol Microbiol Scand* 1968; **72**(2): 348–9.
4 Bergström R, Adami HO, Möhner M *et al.* Increase in testicular cancer incidence in six European countries: a birth cohort phenomenon. *J Natl Cancer Inst* 1996; **88**: 727–33.
5 Richiardi L, Bellocco R, Adami HO *et al.* Testicular cancer incidence in eight northern European countries: secular and recent trends. *Cancer Epidemiol Biomarkers Prev* 2004; **13**: 2157–66.
6 Kawai K, Hinotsu S, Oikawa T *et al.* Treatment outcome of metastatic testicular cancer at a single institution in Japan, a country with low incidence of germ cell tumor. *Jpn J Clin Oncol* 2006; **36**(11): 723–30.
7 Nakata S, Takahashi H, Ohtake N *et al.* Chronological and regional differences of testicular cancer – epidemiological analysis of incidence in Gunma prefecture and estimated data of national survey, and of death in Japan. *Nippon Hinyokika Gakkai Zasshi* 2002; **93**(1): 1–6.
8 Thomas M, Ulbright MD, Mahul B, Amin MD, Robert H, Young MD. *Tumors of the Testis, Adenex, Spermatic Cord, and Scrotom*, 3rd edn. Washington DC: AFIP (Armed Forces Institute of pathology) 1999; 41–55 (Atlas of tumor pathology; vol 25).
9 Damjanov I, Solter D. Experimental teratoma. *Curr Top Pathol* 1974; **59**: 69–130.
10 Pierce GB, Dixon FJ Jr. Testicular teratomas. I. Demonstration of teratogenesis by metamorphosis of multipotential cells. *Cancer* 1959; **12**(3): 573–83.

11 Kleinsmith LJ, Pierce GB Jr. Multipotentiality of single embryonal carcinoma cells. *Cancer Res* 1964; **24**: 1544–51.
12 Pierce GB, Verney EL. An in vitro and in vivo study of differentiation in teratocarcinomas. *Cancer* 1961; **14**: 1017–29.
13 Lind GE, Skotheim RI, Lothe RA. The epigenome of testicular germ cell tumors. *APMIS* 2007; **115**(10): 1147–60.
14 Reubinoff BE, Pera MF, Fong CY *et al.* Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol* 2000; **18**(4): 399–404.
15 Twiddy D, Brown DG, Adrain C *et al.* Pro-apoptotic proteins released from the mitochondria regulate the protein composition and caspase-processing activity of the native Apaf-1/caspase-9 apoptosome complex. *J Biol Chem* 2004; **279**(19): 19665–82.
16 Lindholm D, Arumäe U. Cell differentiation: reciprocal regulation of Apaf-1 and the inhibitor of apoptosis proteins. *J Cell Biol* 2004; **167**(2): 193–5.
17 Fadeel B, Ottosson A, Pervaiz S. Big wheel keeps on turning: apoptosome regulation and its role in chemoresistance. *Cell Death Differ* 2008; **15**(3): 443–52.
18 Niedojadło K, Łabedzka K, Łada E *et al.* Apaf-1 expression in human cutaneous melanoma progression and in pigmented nevi. *Pigment Cell Res* 2006; **19**(1): 43–50.
19 Zlobec I, Vuong T, Compton CC. The predictive value of apoptosis protease-activating factor 1 in rectal tumors treated with preoperative, high-dose-rate brachytherapy. *Cancer* 2006; **106**(2): 284–6.
20 Christoph F, Kempkensteffen C, Weikert S *et al.* Frequent epigenetic inactivation of p53 target genes in seminomatous and nonseminomatous germ cell tumors. *Cancer Lett* 2007; **247**(1): 137–42.
21 Bala S, Oliver H, Renault B. Genetic analysis of the APAF1 gene in male germ cell tumors. *Genes Chromosomes Cancer* 2000; **28**(3): 258–68.
22 Honarpour N, Du C, Richardson JA. Adult Apaf-1-deficient mice exhibit male infertility. *Dev Biol* 2000; **218**(2): 248–58.
23 Wright KM, Linhoff MW, Potts PR, Deshmukh M. Decreased apoptosome activity with neuronal differentiation sets the threshold for strict IAP regulation of apoptosis. *J Cell Biol* 2004; **167**(2): 303–13.
24 Nouri MS, Komatsu S. Comparative analysis of soybean plasma membrane proteins under osmotic stress using gel-based and LC MS/MS-based proteomics approaches. *Proteomics* 2010; **10**(10): 1930–45.
25 Ho CK, Bush JA, Li G. Tissue-specific regulation of Apaf-1 expression by p53. *Oncol Rep* 2003; **10**(5): 1139–43.
26 Peng HQ, Hogg D, Malkin D *et al.* Mutations of the p53 gene do not occur in testis cancer. *Cancer Res* 1993; **53**(15): 3574–8.
27 Schenkman NS, Sesterhenn IA, Washington L *et al.* Increased p53 protein does not correlate to p53 gene mutations in microdissected human testicular germ cell tumors. *J Urol* 1995; **154** (2 Pt 1): 617–21.
28 Takayama H, Takakuwa T, Tsujimoto Y *et al.* Frequent Fas gene mutations in testicular germ cell tumors. *Am J Pathol* 2002; **161**(2): 635–41.
29 Chen L, Li X, Wang GL, Wang Y, Zhu YY, Zhu J. Clinicopathological significance of overexpression of TSPAN1, Ki67 and CD34 in gastric carcinoma. *Tumori* 2008; **94**(4): 531–8.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of primary antibodies used for immunohistochemistry.

Table S2. List of primers used for real-time PCR and their sequences.

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doi: 10.1111/j.1349-7006.2010.01750.x