individuals compared with results obtained in a similarly conducted 1997 survey (1). In general, approximately one-third of patients with RCC present with metastatic disease (mRCC), which has a 5 year survival rate of 10% (2).

The vascular endothelial growth factor receptor-tyrosine kinase inhibitors (VEGFr-TKI) sorafenib and sunitinib are now approved in Japan, Europe and the USA for the treatment of unresectable or metastatic RCC based on their demonstrated benefit in clinical trials (3,4). These targeted agents have become the standard therapy for mRCC. Until recently, however, no standard therapy existed for patients with mRCC that progressed after treatment with VEGFr-TKI-targeted agents.

Everolimus is an orally administered inhibitor of the mammalian target of rapamycin (mTOR) and was approved by the United States Food & Drug Administration (US FDA) in March 2009, the European Medicines Agency (EMEA) in August 2009 and in Japan in January 2010 for the treatment of unresectable or metastatic RCC. mTOR is a cytoplasmic serine/threonine kinase that acts as an integration point for three key inputs: (i) extracellular stimulation by growth factors including VEGF; (ii) nutrient availability; and (iii) intracellular energy status. The convergence of these upstream signals, combined with positive and negative feedback mechanisms, determines whether mTOR is activated. Once activated, mTOR initiates a downstream cascade that triggers the cell's translational machinery to produce proteins required for a variety of cellular functions, including metabolism, growth, proliferation and angiogenesis. Dysregulation of signaling elements both upstream and downstream of mTOR has been implicated in many cancers (5–8).

In a Phase II trial, everolimus demonstrated antitumor activity in patients with mRCC who experienced disease progression after treatment with cytokines, chemotherapy or erlotinib and bevacizumab (9). Everolimus was subsequently evaluated in RECORD-1 (Renal Cell cancer treatment with Oral RAD001 given Daily), the pivotal, Phase III, randomized, placebo-controlled trial of patients with mRCC who had progressed on VEGFr-TKI. Results of this trial, which enrolled patients from 10 countries, including Japan, led to the US FDA and EMEA approval of everolimus. RECORD-1 demonstrated that treatment with everolimus plus best supportive care (BSC) prolonged progression-free survival (PFS) compared with placebo plus BSC (4.9 vs. 1.9 months, respectively; $P < 0.001$; hazard ratio [HR], 0.33; 95% confidence interval [CI], 0.25–0.43) (10). Consequently, everolimus represents a viable treatment option for patients with VEGFr-TKI-refractory disease.

Results of a Phase I clinical and pharmacokinetic study of everolimus in previously treated Japanese patients with advanced solid tumors demonstrated that its pharmacokinetics and tolerability were similar to those observed in previous studies with large populations of Caucasian patients, for whom the most common drug-related toxicities included rash, stomatitis and fatigue. No dose-limiting toxicities were noted (11). The current analysis was initiated to assess the efficacy and safety of everolimus in the Japanese subgroup of patients who participated in RECORD-1.

PATIENTS AND METHODS

Patients

The RECORD-1 inclusion and exclusion criteria have been described in detail previously (12). Briefly, patients ≥18 years of age were eligible for enrollment if they had been diagnosed with metastatic carcinoma and had histologic or cytologic confirmation of clear cell RCC, had measurable disease, showed disease progression on or within 6 months of treatment with sunitinib, sorafenib or both, and exhibited adequate Karnofsky performance status (≥70%), blood counts and serum chemistry. Prior therapy with bevacizumab and cytokines was permitted. Written informed consent was obtained from each patient before screening procedures were initiated.

Study Design

RECORD-1 was a prospective, randomized, double-blind, placebo-controlled, international, multicenter, parallel-group Phase III trial (NCT00410124). It contained five phases: (i) screening/baseline; (ii) blinded treatment; (iii) open-label treatment; (iv) follow-up; and (v) extension treatment. The study design incorporated two planned interim analyses and a final analysis. The protocol specified that the interim analyses were to be carried out when ~30 and 60% of the 290 PFS events (per central radiology) required for the final analysis had been reached. The protocol and all amendments issued prior to or during the study were reviewed by the local independent ethics committee or institutional review board for each center, and the study was conducted according to the ethical principles of the Declaration of Helsinki.

Patients were randomized in a 2:1 ratio to receive everolimus 10 mg/day ($n = 277$) or matching placebo ($n = 139$) in conjunction with BSC. Randomization was stratified by the number of prior VEGFr-TKI therapies (1 or 2) and Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic criteria for patients with previously treated mRCC (favorable, intermediate or poor) (10,13). Treatment cycles were 28 days in length.

Patients continued on blinded treatment until tumor progression or unacceptable toxicity, death or discontinuation for any other reason. Dose modifications were permitted for clinically significant hematologic or other adverse events, as described previously (12). Patients randomized to receive placebo who demonstrated evidence of progression by investigator assessment were unblinded and permitted to cross over to receive open-label everolimus (12).

Efficacy Analyses

The primary efficacy endpoint was PFS by central review. Tumor response was assessed at scheduled intervals with the

Response Evaluation Criteria in Solid Tumors (RECIST) (14), based on imaging studies by the investigators and independent central radiology review (12). Secondary efficacy endpoints included overall survival (OS) and tumor response.

Safety Analyses

Safety analyses were carried out as described previously (12). Briefly, all adverse events were monitored and recorded, and laboratory parameters, vital signs, physical examinations and concomitant therapies were assessed regularly and recorded. The National Cancer Institute's Common Terminology Criteria (NCI-CTC) version 3.0 were used to grade adverse events and laboratory abnormalities (12). To detect radiologic lung changes suggestive of pneumonitis, a central radiology review of chest computed tomography scans and chest X-rays was performed.

Statistical Analyses

The full analysis set included all randomized patients; the safety population consisted of all patients who received at least one dose of study drug and who had at least one post-baseline safety assessment.

PFS and OS curves in each treatment group were estimated with Kaplan–Meier analysis. HR for the Japanese subpopulation was obtained from an unstratified Cox proportional hazard model. As previously reported for the overall population (12), PFS and OS were statistically compared among the groups with a stratified, one-sided log-rank test, adjusting for strata defined by the MSKCC risk criteria. HRs were obtained from a stratified Cox proportional hazards model, using the strata defined by the MSKCC risk criteria.

The blinded phase of RECORD-1 was stopped on 28 February 2008, based on the efficacy of everolimus shown in the second interim analysis (10). Compared with data for the second interim analysis (collected up to 15 October 2007; 191 PFS events), data for the final analysis were based on 75 additional PFS events, 6 additional patients accrued and 4.5 months of additional blinded follow-up. The cutoff date of follow-up for OS was 15 November 2008.

RESULTS

Patient Demographics and Disposition

Of the 416 patients randomized to treatment in the overall trial, the subpopulation of Japanese patients in the final analysis included 15 patients who received everolimus and 9 patients who received placebo. The baseline characteristics and prior therapies of these 24 Japanese patients and the 416-patient overall population are shown in Table 1 (10). The Japanese subpopulation was 79% male and the median age was 63.5 years. The Japanese subpopulation was similar to the overall trial population, with the exceptions that the predominant VEGFr-TKI therapy of the Japanese patients was sorafenib instead of sunitinib and that almost all Japanese patients had received prior treatment with interferon.

Table 1. Baseline characteristics and prior therapies in RECORD-1 (10)

Characteristic	Overall population		Japanese patients	
	Everolimus ($n = 277$)	Placebo ($n = 139$)	Everolimus ($n = 15$)	Placebo ($n = 9$)
Sex, *n* (%)				
Female	61 (22)	33 (24)	1 (7)	4 (44)
Male	216 (78)	106 (76)	14 (93)	5 (56)
Median age (range), year	61 (27–85)	60 (29–79)	65 (48–77)	62 (46–73)
KPS, ≥90/≤80, %	64/36	68/32	80/20	100/0
% MSKCC risk				
Favorable/ intermediate/ poor	29/56/14	28/57/15	33/60/7	44/44/11
Sites of metastases, %				
Lung	73	81	87	89
Bone	37	30	33	11
Liver	33	38	20	33
Prior therapies, %				
Nephrectomy	97	96	100	100
Radiotherapy	31	27	20	11
VEGFr-TKI therapy				
Sunitinib	45	43	13	22
Sorafenib	29	31	80	78
Both	26	26	7	0
Other systemic therapy				
Immunotherapy	65	67	100	89
Chemotherapy	13	16	13	11
Hormone therapy	2	4	0	0
Other	5	3	0	0

KPS, Karnofsky performance status; MSKCC, Memorial Sloan-Kettering Cancer Center; VEGFr-TKI, vascular endothelial growth factor receptor-tyrosine kinase inhibitor.

By the end of the double-blind phase, five Japanese patients in the everolimus group had discontinued (disease progression, $n = 4$; withdrew consent, $n = 1$) and eight patients in the placebo group had discontinued (all due to disease progression). These eight patients from the placebo group then crossed over to receive open-label everolimus. All 24 Japanese patients were included in the full analysis set and in the safety population. In the overall population, 202 patients in the everolimus group and 133 patients in the placebo group had discontinued therapy, with the primary reasons being disease progression and adverse events in the

everolimus group ($n = 137$ and $n = 36$, respectively) and disease progression and death for the placebo group ($n = 124$ and $n = 4$, respectively).

Treatment Administration

The median duration of treatment in the Japanese subpopulation was 135 days in the everolimus group and 96 days in the placebo group (overall population: 141 and 60 days, respectively). The median cumulative dose of everolimus was 1160 mg, the median dose intensity was 9.9 mg/day and the median relative dose intensity was 0.99 (overall population: 1252.5 mg, 10.0 mg/day and 1.0, respectively). In the Japanese subpopulation, dose reduction and/or interruption was necessary in eight patients in the everolimus group (53.3%; for five of eight patients, this was due to an adverse event) and in no patients in the placebo group; corresponding rates for the overall population were 46 and 15%, respectively, most commonly for an adverse event in both groups (39 and 9%, respectively).

Efficacy Assessment

Everolimus appeared to prolong PFS compared with placebo in the Japanese subpopulation. By independent central radiology review, median PFS was 5.75 months (95% CI, 4.90 months to not reached) with everolimus and 3.61 months (95% CI, 1.91–9.03 months) with placebo (Fig. 1A). The HR was 0.19, with a 95% CI of 0.05–0.83. In the overall population, median PFS was 4.90 months (95% CI, 3.98–5.52 months) with everolimus and 1.87 months (95% CI, 1.84–1.94 months) with placebo, translating into a highly significant HR of 0.33 (95% CI, 0.25–0.43; $P < 0.001$; Fig. 1B).

Median OS in the Japanese subpopulation was not reached in the everolimus group and was 14.9 months (95% CI, 11.0–16.8 months) in the placebo group at the cutoff date of 15 November 2008 (Fig. 2A). The HR was 0.30, with a 95% CI of 0.07–1.27. There were no on-treatment deaths among Japanese patients in either treatment group. One Japanese patient who had been treated with everolimus died during the follow-up period, over 6 months after the last dose. In the overall population, median OS was 14.8 months with everolimus and 14.4 months with placebo, translating into a non-significant HR of 0.87 (95% CI, 0.65–1.17; $P = 0.16$; Fig. 2B) that was influenced by the 80% rate of crossover from placebo to open-label everolimus (10).

The best overall response based on central radiologic assessment was stable disease (SD) in 14 of 15 patients who received everolimus (the response of 1 patient was unknown) and SD in 6 of 9 patients who received placebo (the other 3 patients had progressive disease). No patient achieved a partial response (PR) at the final analysis, but in a subsequent efficacy assessment made in an open-label extension phase only for Japanese subjects, one patient achieved a PR as an investigator-assessed best overall response. In the overall population, the SD rates were 185 of 277 (67%) with everolimus and 45 of 139 (32%) with placebo, with an additional 5 patients in the everolimus group (1.8%) achieving a PR.

Safety

As summarized in Table 2, stomatitis, infections and rash were the predominant adverse events among Japanese patients in the everolimus group during the blinded-study phase, with incidences that were notably higher than the incidences in the overall population. Most adverse events were mild (Grade 1) to moderate (Grade 2) in severity and were resolved with dose interruption and/or reduction. In the Japanese subpopulation, there were two reports of Grade 2 interstitial lung disease and two reports of Grade 1 pneumonitis during everolimus therapy, for a total on-treatment occurrence of pneumonitis (based on grouped terms) of 27%. The corresponding pneumonitis incidence among the everolimus group in the overall population was 14%, including Grade 1/2 ($n = 27$) as well as Grade 3 ($n = 10$) events. None of the Japanese patients stopped therapy permanently because of pneumonitis. With respect to the two Japanese cases of Grade 2 pneumonitis (reported as interstitial lung disease), study treatment was interrupted, corticosteroid therapy was instituted and everolimus was resumed at a reduced dose, with eventual resolution of the interstitial lung disease. Despite increased reporting of non-infectious pneumonitis for Japanese patients compared with the overall study population, central review of chest computed tomography scans for radiologic lung changes suggestive of pneumonitis found a similar incidence of radiologic changes in the Japanese and the overall population.

Treatment-related Grade 3/4 adverse events were infrequently reported, with no specific type reported in >1 patient in the Japanese subpopulation (all of which were Grade 3) and incidences of individual events typically <5% in the overall population (with a few exceptions with respect to laboratory abnormalities). No Grade 4 adverse events were reported. In the Japanese subpopulation, no adverse event led to study discontinuation in the double-blind phase, whereas the adverse event-related discontinuation rates in the overall population were 13.9 and 2.9% for the everolimus and placebo groups, respectively. The most common adverse events resulting in everolimus discontinuation were dyspnea and pneumonitis ($n = 7$ [3.6%] for each).

DISCUSSION

The results of RECORD-1 established that daily treatment with oral everolimus prolongs PFS in patients with mRCC that has progressed on VEGFr-TKI therapies and generally is well tolerated, fulfilling an unmet medical need in this patient population. The results of this subgroup analysis of patients in RECORD-1 suggest that a similar benefit is expected for Japanese patients.

The Japanese subpopulation analysis was limited by the small number of patients in the trial ($n = 24$); however, the PFS results in the Japanese subgroup aligned with the PFS

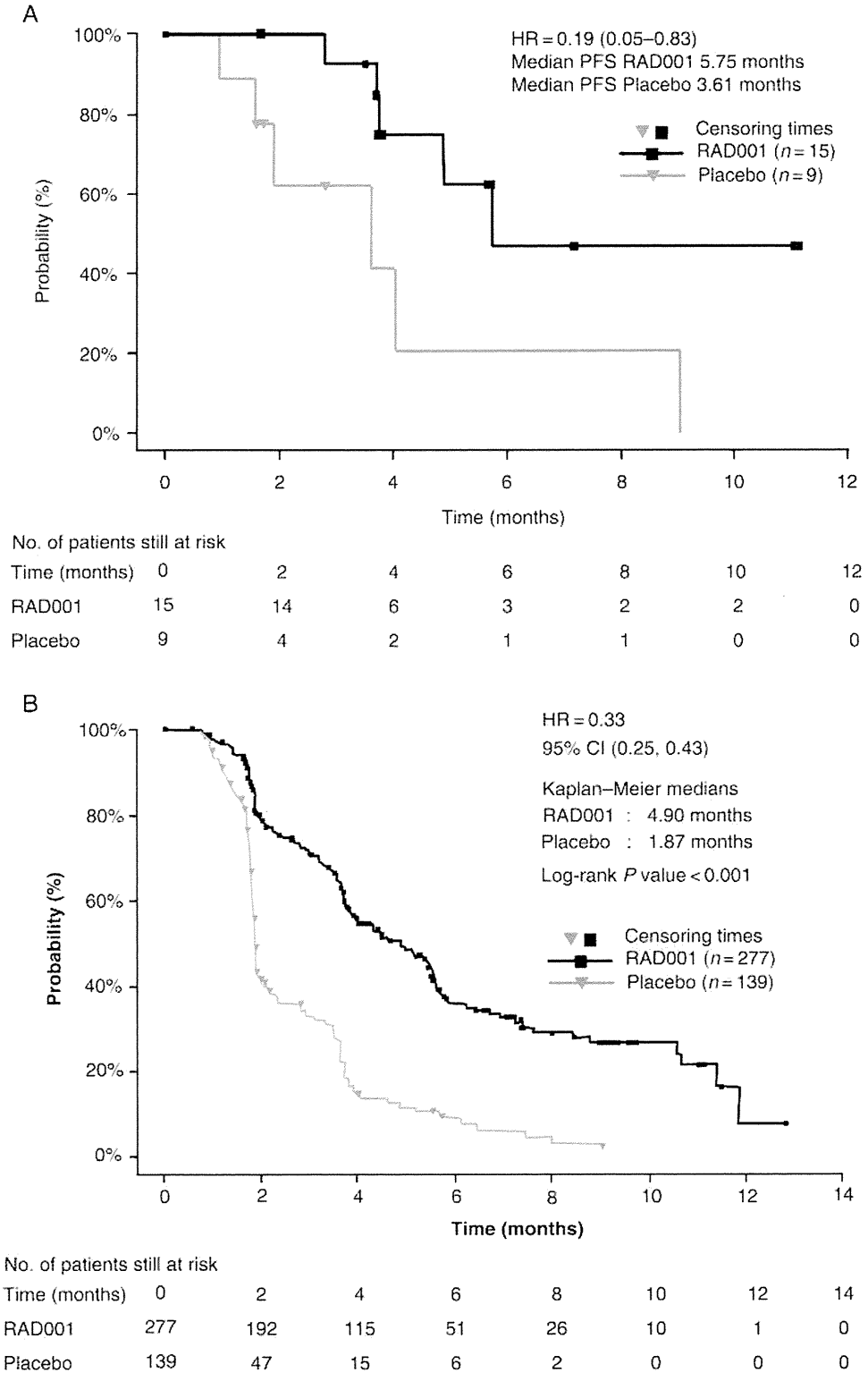

Figure 1. Median progression-free survival in the (A) Japanese subpopulation and (B) overall population (10) of RECORD-1 by central radiology review. Figure 1B reprinted with permission from Wiley & Sons. Copyright 2010. All rights reserved (10).

results obtained in the overall trial population. It was speculated that the longer PFS of both everolimus and placebo groups in the Japanese subpopulation compared with the overall population may have been caused by the better overall condition of patients enrolled in the study. Although OS was likely confounded by the crossover design of the trial, results trended toward a benefit for everolimus compared with placebo, similar to findings in the overall trial population.

In addition, the types of adverse events occurring in the Japanese patients were similar to those occurring in

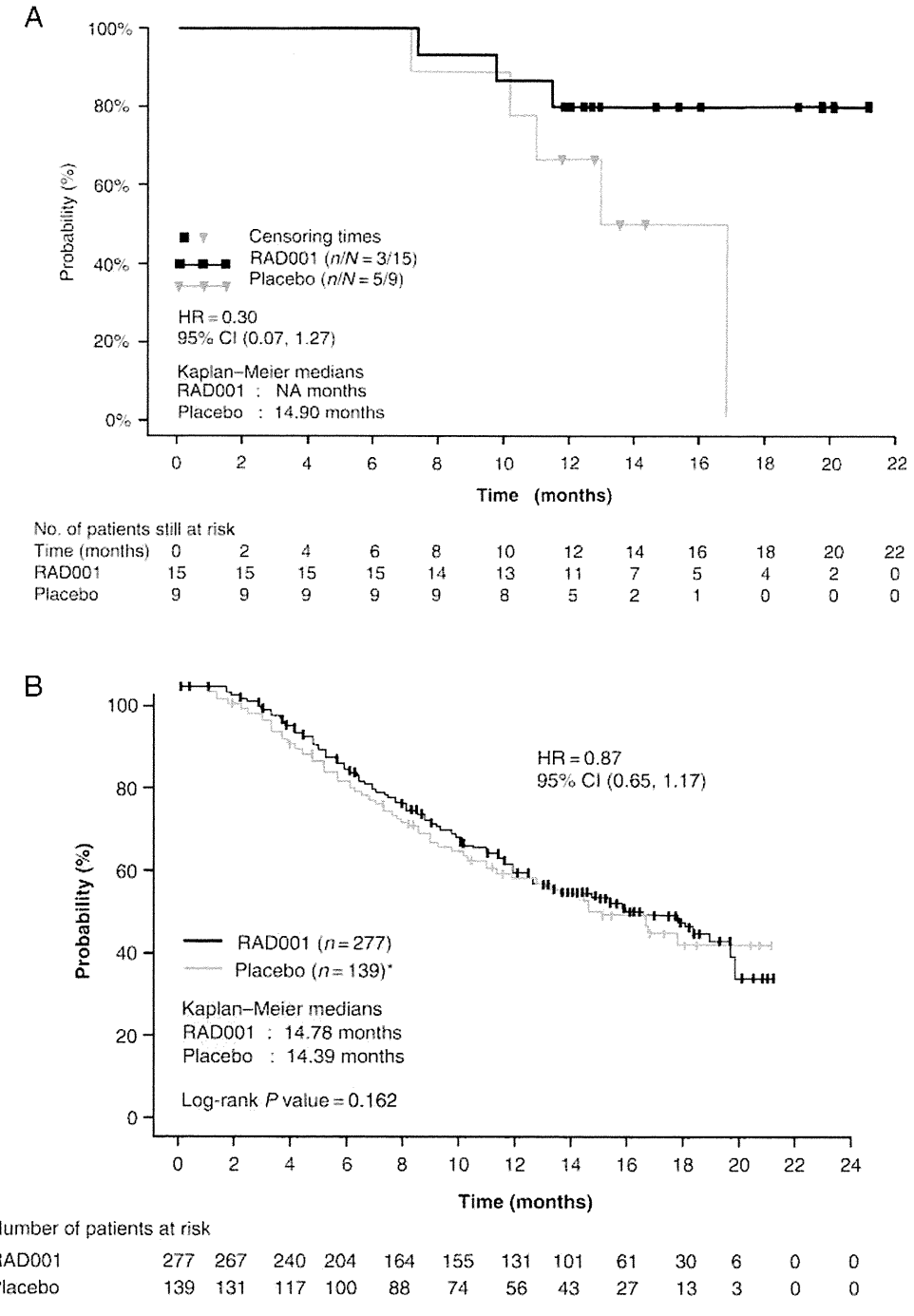

Figure 2. Median overall survival in the (A) Japanese subpopulation and (B) overall population (10) of RECORD-1. Figure 2B reprinted with permission from Wiley & Sons. Copyright 2010. All rights reserved (10).

the overall trial population. The most common event in the everolimus group was rash, followed in decreasing order by stomatitis, dysgeusia, diarrhea, epistaxis, cough and peripheral edema. These events also occurred with a relatively high frequency (≥10% incidence) in the overall population of RECORD-1. The pharmacokinetic profile of everolimus in a Phase I study in previously treated Japanese patients with advanced solid tumors was similar to that observed in previous studies with large populations of Caucasian patients (11), suggesting no difference in treatment exposure between these two ethnic populations. The increased incidence of adverse events may be due to differences in ethnicity, stricter investigation of the Japanese physicians in identifying adverse events, or the small number of Japanese patients in this study. Most adverse events were mild/moderate in severity and there was no evidence of worsening adverse events. The majority of adverse events requiring treatment resolved after dose interruption and/or reduction, and patients were able to continue receiving everolimus.

Table 2. Incidence of adverse events, irrespective of relationship to treatment and laboratory abnormalities in the Japanese subpopulation of RECORD-1

	Overall population				Japanese patients			
	Everolimus ($n = 274$), n (%)		Placebo ($n = 137$), n (%)		Everolimus ($n = 15$), n (%)		Placebo ($n = 9$), n (%)	
	All Grades	Grade 3/4	All Grades	Grade 3/4	All Grades	Grade 3/4	All Grades	Grade 3/4
Adverse event[a] (total)	265 (97)	142 (52)/36 (13)	128 (93)	32 (23)/7 (5)	15 (100)	6 (40)/0	5 (56)	0/0
Stomatitis[b]	120 (44)	11 (4)/1 (<1)	11 (8)	0/0	11 (73)	0/0	1 (11)	0/0
Infections[c]	101 (37)	19 (7)/7 (3)	25 (18)	2 (1)/0	10 (67)	1 (7)/0	2 (22)	0/0
Rash	80 (29)	3 (1)/0	9 (7)	0/0	10 (67)	0/0	0	0/0
Dysgeusia	28 (10)	0/0	3 (2)	0/0	7 (47)	0/0	0	0/0
Epistaxis	49 (18)	0/0	0	0/0	6 (40)	0/0	0	0/0
Diarrhea	81 (30)	4 (1)/0	9 (7)	0/0	6 (40)	0/0	0	0/0
Cough	82 (30)	2 (<1)/0	22 (16)	0/0	5 (33)	0/0	2 (22)	0/0
Edema peripheral	68 (25)	2 (<1)/0	11 (8)	1 (<1)/0	5 (33)	0/0	0	0/0
Pneumonitis[d]	37 (14)	10 (4)/0	0	0/0	4 (27)	0/0	0	0/0
Nail disorder	14 (5)	0/0	0	0/0	4 (27)	0/0	0	0/0
Constipation	53 (19)	1 (<1)/0	24 (18)	1 (<1)/0	4 (27)	0/0	1 (11)	0/0
Anorexia	69 (25)	4 (1)/0	19 (14)	1 (<1)/0	3 (20)	0/0	0	0/0
Cheilitis	4 (1)	0/0	0	0/0	3 (20)	0/0	0	0/0
Eyelid edema	11 (4)	0/0	0	0/0	3 (20)	0/0	0	0/0
Arthralgia	28 (10)	3 (1)/0	14 (10)	2 (1)/0	3 (20)	0/0	1 (11)	0/0
Hemorrhoid	15 (6)	0/0	1 (<1)	0/0	3 (20)	0/0	0	0/0
Nausea	72 (26)	4 (1)/0	26 (19)	0/0	3 (20)	0/0	0	0/0
Vomiting	56 (20)	6 (2)/0	16 (12)	0/0	3 (20)	0/0	1 (11)	0/0
Fatigue	84 (31)	15 (6)/0	37 (27)	4 (3)/1 (<1)	3 (20)	0/0	1 (11)	0/0
Pyrexia	54 (20)	2 (<1)/0	12 (9)	0/0	3 (20)	0/0	0	0/0
Laboratory abnormality								
Hematology								
Hemoglobin decreased	253 (92)	33 (12)/3 (1)	108 (79)	7 (5)/1 (<1)	14 (93)	0/0	6 (67)	0/0
Lymphocytes decreased	139 (51)	43 (16)/6 (2)	39 (28)	7 (5)/0	6 (40)	2 (13)/0	3 (33)	0/0
Platelets decreased	64 (23)	3 (1)/0	3 (2)	0/1 (<1)	4 (27)	1 (7)/0	0	0/0
Neutrophils decreased	37 (14)	0/1 (<1)	5 (4)	0/0	4 (27)	0/0	1 (11)	0/0
Biochemistry								
Cholesterol increased	212 (77)	12 (4)/0	48 (35)	0/0	13 (87)	0/0	7 (78)	0/0
Triglycerides increased	200 (73)	2 (<1)/0	46 (34)	0/0	9 (60)	1 (7)/0	4 (44)	0/0
Glucose increased	157 (57)	42 (15)/1 (<1)	34 (25)	2 (1)/0	8 (53)	1 (7)/0	0	0/0
Creatinine increased	137 (50)	4 (1)/0	46 (34)	0/0	6 (40)	0/0	3 (33)	0/0
Phosphate decreased	102 (37)	17 (6)/0	11 (8)	0/0	6 (40)	0/0	2 (22)	0/0
Aspartate transaminase increased	68 (25)	1 (<1)/1 (<1)	9 (7)	0/0	4 (27)	0/0	0	0/0
Alanine transaminase increased	58 (21)	3 (1)/0	5 (4)	0/0	5 (33)	0/0	0	0/0
Bilirubin increased	8 (3)	2 (<1)/1 (<1)	3 (2)	0/0	0	0/0	0	0/0

[a]Includes events that occurred in ≥3 patients in the Japanese subpopulation and corresponding data for the overall population. Data for the overall population are not all inclusive, only capturing the most common events in the Japanese subpopulation.
[b]Includes aphthous stomatitis, mouth ulceration and tongue ulceration.
[c]Includes all infections.
[d]Includes interstitial lung disease, lung infiltration, pneumonitis, pulmonary alveolar hemorrhage, alveolitis, pneumopathy and pulmonary toxicity.

Non-infectious pneumonitis is a known class effect of rapamycin and its derivatives, possibly representing a hypersensitivity reaction; however, its etiology has not been fully characterized (15,16). A diagnosis of non-infectious pneumonitis should be considered in patients presenting with non-specific respiratory signs and symptoms (to include pyrexia, cough or dyspnea) and in whom infectious, neoplastic and other non-medicinal causes have been excluded by appropriate investigation. According to the data reported here for everolimus in mRCC, the incidence of non-infectious pneumonitis was increased among Japanese patients, at 27 vs. 11% for the overall population; the impact of the small sample size on this disparate finding is unknown. However, it was noted during the central review of results that the radiological changes observed did not support the higher incidence of non-infectious pneumonitis reported in Japanese when compared with the overall population. Nonetheless, it is encouraging that the four Japanese cases were limited to Grade 1/2 severity and all were successfully managed, resulting in the resolution of the toxicity and the ability to continue treatment (with treatment interruption and dose reduction for the 2 patients with Grade 2 events).

In conclusion, the results of this subgroup analysis suggest that the benefits of everolimus in Japanese patients with mRCC are similar to those observed in the overall pivotal Phase III trial population. These findings, along with those of previous studies of everolimus in Japanese patients, suggest that everolimus is a valuable treatment option for Japanese patients with mRCC that has progressed on VEGFr-TKI therapy.

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Conflict of interest statement

Drs Takeshi Tajima, Akio Kasuga, Yoshie Fujita are employed by Novartis Pharma K.K. Dr Andrea Kay is employed by and owns stock in Novartis Pharma K.K. Drs Hideyuki Akaza, Hiro-omi Kanayama, Hirotsugu Uemura and Nobuo Shinohara declare the following potential conflict of interest: Medical advisor of everolimus on Novartis Pharma K. K. (compensated); Seminar presentation in the seminar hosted by Novartis Pharma K.K. (compensated).

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Appendix

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

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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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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

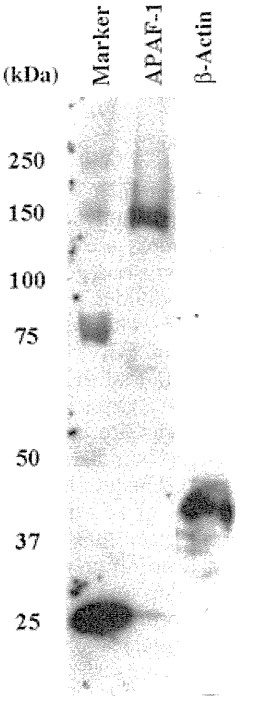

Fig. 1. Western blot analysis of a seminoma tumor showed a specific band for apoptotic protease activating factor-1 (APAF-1).

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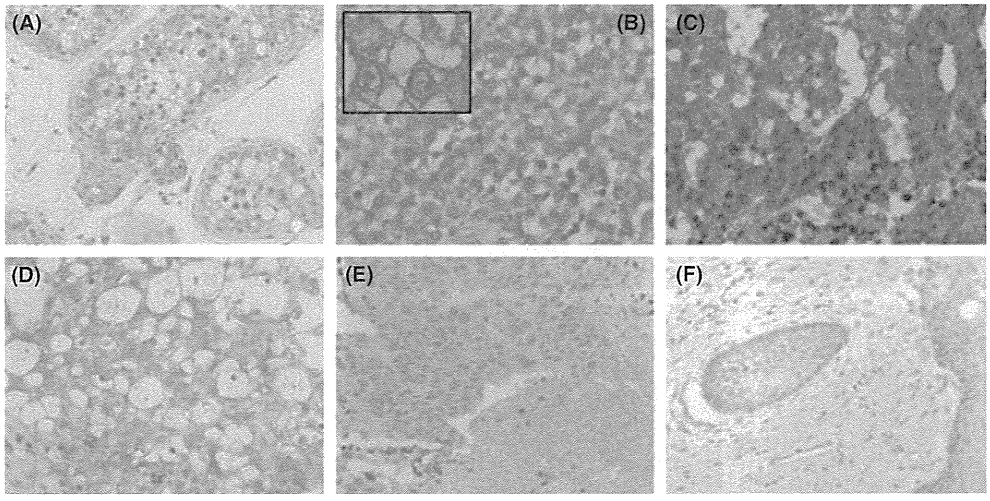

Fig. 2. Immunohistochemistry of apoptotic protease activating factor-1 for normal testis and representative testicular germ cell tumors. (A) Seminiferous tubules of normal testis. (B) Seminoma. Inset, membranous staining of tumor cells (×400). (C) Embryonal carcinoma. (D) Yolk sac tumor. (E) Choriocarcinoma.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

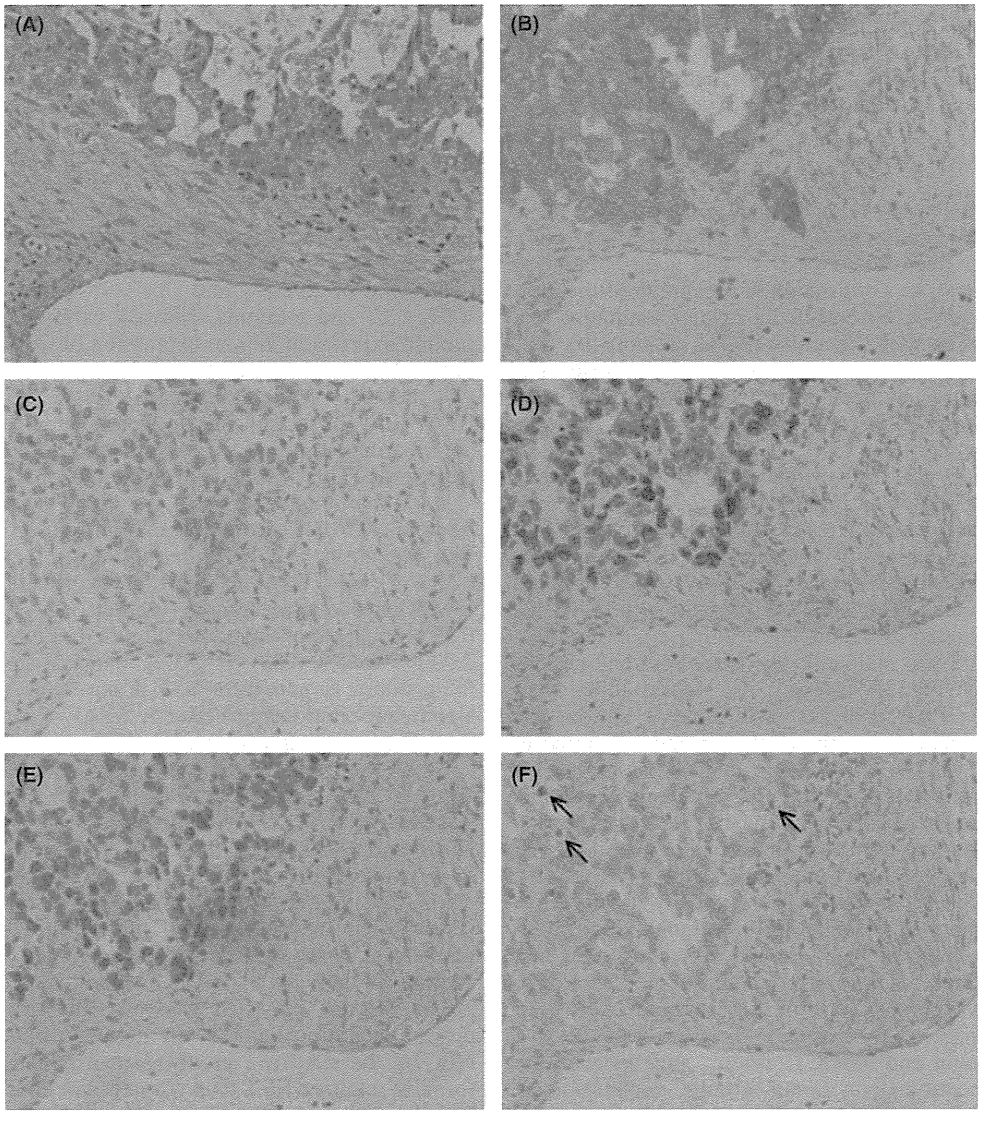

Fig. 3. Immunohistochemistry of various markers in an embryonal carcinoma case. (A) HE staining. (B) Apoptotic protease activating factor-1. (C) p53. (D) Ki-67. (E) Oct-3/4. (F) Cleaved caspase-3 (arrows). Magnification, ×100.

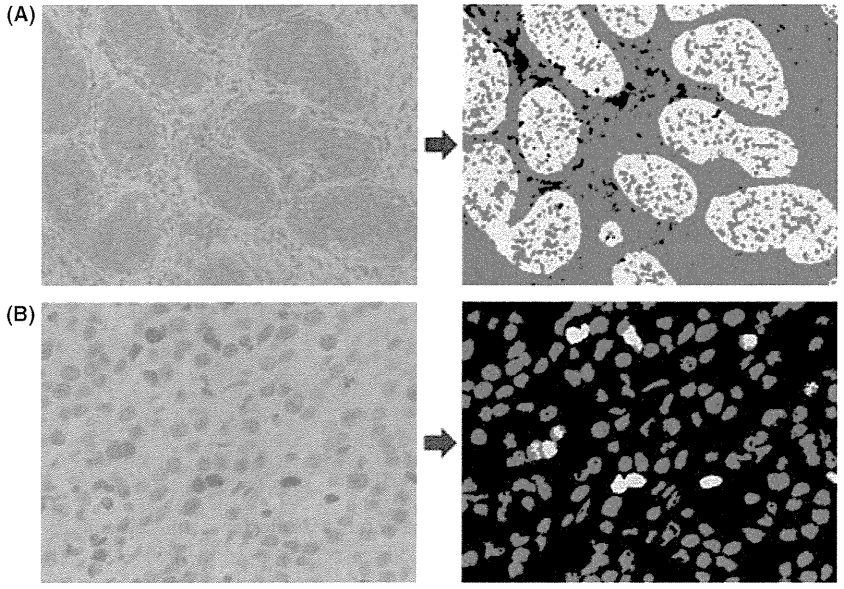

Fig. 4. Images taken by a digital camera and converted to digital data by Image Processor for Analytical Pathology software. (A) Cytoplasmic staining of APAF-1 in a teratoma. (B) Nuclear staining of p53 in an embryonal carcinoma. Magnification, ×100.

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

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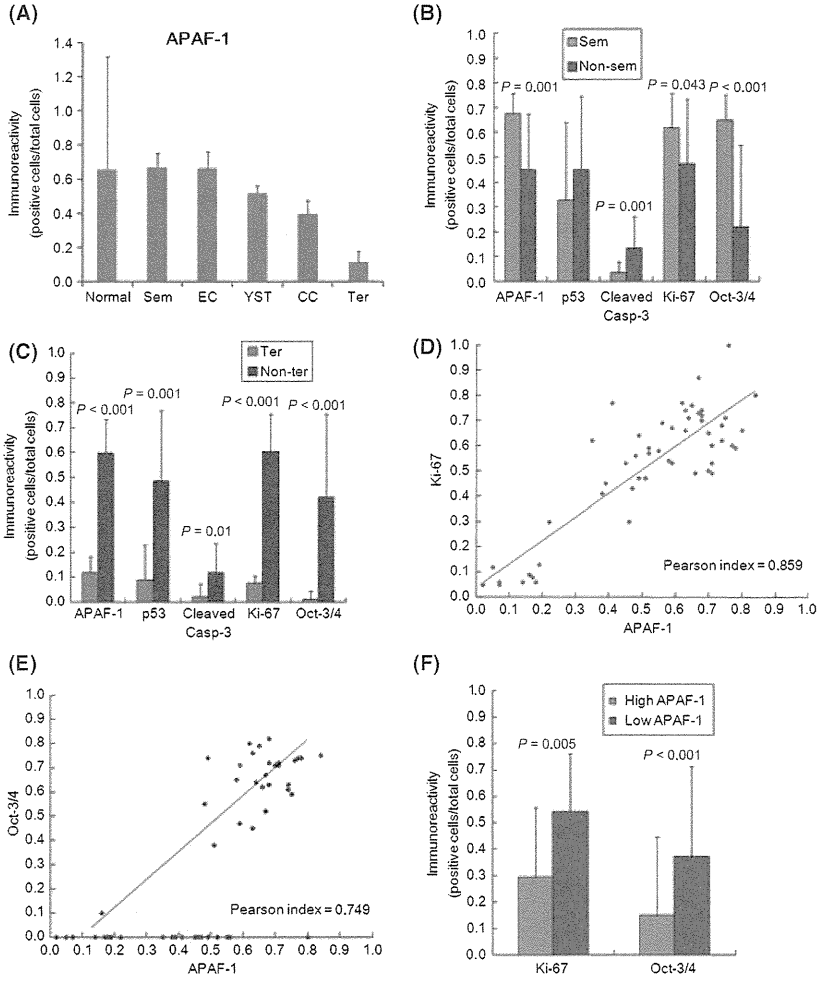

Fig. 5. Comparison of immunohistochemical results. (A) Expression of apoptotic protease activating factor-1 (APAF-1) protein in normal testis and testicular germ cell tumors (TGCT). (B) Expression of different marker proteins (seminoma [Sem] *vs* non-seminoma). (C) Expression of different marker proteins (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

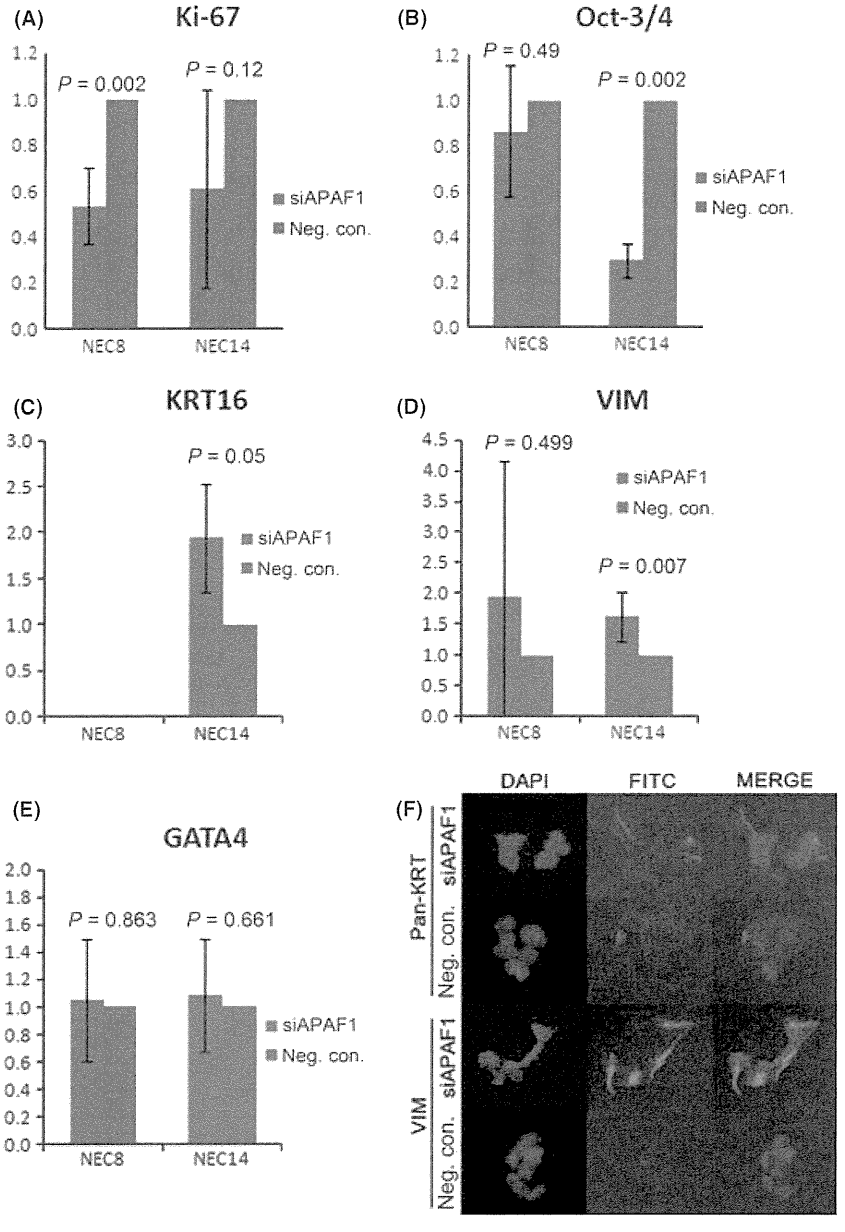

Fig. 6. Downregulation of apoptotic protease activating factor-1 (*APAF-1*) in the NEC8 and NEC14 testicular germ cell tumor cell lines. (A) Decrease of *Ki-67* after *APAF-1* downregulation. (B) Decrease of *Oct-3/4* after *APAF-1* downregulation. (C) Increase of *keratin16* (*KRT16*) after *APAF-1* 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

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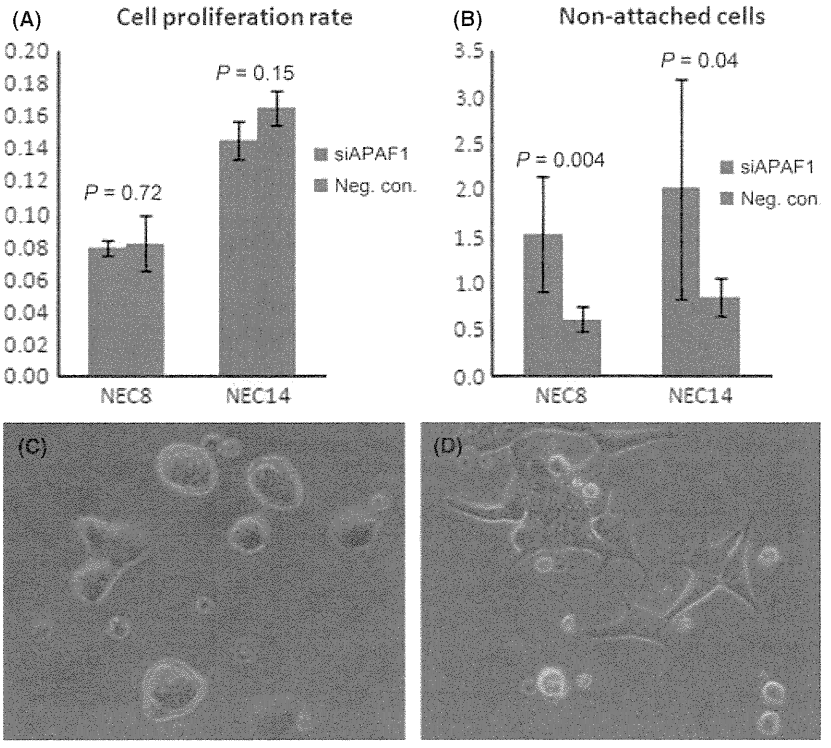

Fig. 7. (A) Decrease of the cell proliferation rate in NEC14 testicular germ cell tumor cells after apoptotic protease activating factor-1 (*APAF-1*) downregulation. (B) Non-attached cells increased in siAPAF1-transfected cells as compared with the negative control. (C) Morphology of NEC8 cells 48 h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

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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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RESEARCH COMMUNICATION

Weight Gain and Family History of Prostate or Breast Cancers as Risk Factors for Prostate Cancer: Results of a Case-control Study in Japan

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Abstract

The increase in the incidence rate of prostate cancer may be associated with changes in lifestyle in Japanese men. Accordingly, we conducted a case-control study to assess risk factors. A total of 117 (82.3%) of the 142 prostate cancer patients asked filled out the self-administrated questionnaires which included items about their lifestyle habits over the period of one or two years before their diagnosis. Four controls per case, namely 468, were randomly selected from resident registries with age and address matched with each case, and 318 controls (69.5%) filled out the same questionnaire as the cases. Data for 277 controls were used for the analysis, excluding 41 subjects with a history of previous cancer. The conditional logistic regression model was utilized for analyzing the individually age and address-matched data, and odds ratios (ORs) and their 95% confidence intervals (95%CIs) were calculated for potential risk factors. Higher body mass index at 20 years of age was marginally significantly associated with a decreased risk (P for trend=0.051), and larger weight gain in adult age was significantly associated with an increased risk (P for trend=0.041). History of prostate cancer in fathers or brothers was significantly associated with an increased risk (OR=9.71, 95%CI 3.59, 26.27), and history of breast cancer in mothers or sisters was also significantly associated with an increased risk (OR=2.70, 95%CI 1.12, 6.49). The recent increase in the incidence rate of prostate cancer may possibly be brought about by an increased proportion of Japanese men with large weight gain in adult age.

Keywords: Prostate cancer - case-control study - family history - weight gain - body mass index

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Introduction

Age-adjusted incidence rates of prostate cancer (PCa) have been reported to be lower among Asian populations than among Western populations (Parkin et al., 2002). However, the age-adjusted incidence rate of prostate cancer has increased in Japan, and became as high as 27.3 per 100,000 in 2003 based on data from 13 population-based cancer registries standardized to the World model population (Matsuda et al., 2009). Furthermore, the age-adjusted incidence rates of PCa among Japanese migrants to Hawaii or California have been shown to be much higher than those among Japanese people in Japan, and to be closer to those among Caucasians in Hawaii and California (Parkin et al., 2002).

Environmental and genetic factors have been suggested to be associated with the risk of PCa (Hayes, 2001; Plats and Giovannucci, 2002). History of PCa in first-degree relatives is a well-known risk factor (Stanford, 2001). African Americans have been shown to carry the highest incidence of PCa compared to other races (Parkin et al., 2002). However, it is still unclear whether this difference is due to genetic or environmental factors.

Increased prevalence of obese males has also been reported in Japan. According to the National Survey on Health and Nutrition in Japan, the proportion of men whose body mass index (BMI) was more than or equal to 25.0 aged 40 years to 59 years of age increased from about 25% in 1987 to about 34% in 2007 (Health and Welfare Statistics Association, 2009). Consequently, it is possible that the recent increasing trend of PCa incidence in Japan was brought about by the increased prevalence of obese males in Japan if obesity is associated with risk of PCa. We have suggested that the recent decrease in having a traditional Japanese diet (Mori et al., 2009), especially the decrease in the intake of soybean products (Nagata et al., 2007) in Japanese men, may be associated with the increase in the incidence rate of PCa in Japan. Decrease in the intake of soybean products might be one of the

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most prominent changes in Japanese dietary habits, as we reported the significantly decreased trend of intake of soybean isoflavones in the younger generation (Fujimoto et al., 2008). Therefore, we conducted a case-control study to assess the risk factors of PCa in Hokkaido, Japan, with special reference to dietary habits, obesity and family history of cancer.

Materials and Methods

There were 168 PCa patients who were newly pathologically diagnosed at the Department of Urology, Sapporo Medical University from January, 2007 to April, 2008. We excluded 26 patients from the study because 5 patients died, 19 patients were in too poor health, 1 subject moved out of the Hokkaido Prefecture and 1 subject was not Japanese. Among the residual 142 PCa patients, informed consent was obtained from 117 patients (82.3%).

The self-administrated questionnaire consisted of various inquiries over the period of one or two years before their diagnosis such as followings; frequencies of consumption for 28 foods or beverages such as beef, pork, ham, sausages, soybean products, vegetables, fruits, milk, green tea, coffee and black tea; physical activities such as walking hours per day and sports activities per week; cigarettes smoking and alcohol drinking; body height, body weight at 20 years old and a year before diagnosis, maximum body weight in lifetime, and weight gain in adult age defined as maximum body weight in lifetime minus body weight at 20 years old; own medical history and cancer histories in parents and siblings. All 117 patients filled out the self-administrated questionnaire.

The average age at the time of diagnosis of the 117 patients with PCa was 69.4 years (standard deviation or SD; 6.4 years; Range; 53-82 years). The mean and median interval between the date of the diagnosis and filling out the questionnaire were 1.8 and 1.7 years, respectively (SD; 1.1 years). Their distribution of clinical stages as classified

Table 1. Odds ratios (ORs) and their 95% Confidence Intervals (95%CI) for Prostate Cancer with Regard to Body Stature

Items	Contents	Cases No.	(%)	Controls No.	(%)	OR	95%CI
Body height	<160.0cm	15	(12.8)	31	(11.2)	1.00	
	160.0-164.9cm	37	(31.6)	82	(27.6)	0.90	0.43, 1.89
	165.0-169.9cm	39	(33.3)	97	(35.0)	0.86	0.42, 1.76
	≥170.0	26	(22.2)	67	(24.2)	0.87	0.40, 1.90
	P for trend =0.716						
Body weight	<55.0kg	7	(6.0)	24	(8.7)	1.00	
	55.0-64.9kg	52	(44.4)	125	(45.1)	1.49	0.57, 3.85
	65.0-74.9kg	45	(38.5)	95	(34.3)	1.74	0.65, 4.64
	≥75.0kg	13	(11.1)	33	(11.9)	1.64	0.55, 4.91
	P for trend =0.345						
Body mass index (BMI)	<21.0	14	(12.0)	39	(14.1)	1.00	
	21.0-22.9	29	(24.8)	87	(31.4)	1.05	0.50, 2.21
	23.0-24.9	41	(35.0)	77	(27.8)	1.63	0.77, 3.45
	≥25.0	33	(28.2)	74	(26.7)	1.39	0.66, 2.96
	P for trend=0.071						
Body weight at 20 years of age	<50.0kg	3	(2.6)	7	(2.5)	1.00	
	50.0-59.9kg	70	(59.8)	138	(49.8)	1.16	0.28, 4.51
	60.0-69.9kg	38	(32.5)	104	(37.6)	0.84	0.21, 3.41
	≥70.0kg	6	(5.1)	28	(10.1)	0.46	0.09, 2.44
	P for trend =0.063						
BMI at 20 years of age	<21.0	54	(46.2)	108	(39.0)	1.00	
	21.0-22.9	46	(39.3)	104	(37.6)	0.85	0.52, 1.39
	23.0-24.9	11	(9.4)	46	(16.6)	0.47*	0.22, 0.98
	≥25.0	6	(5.1)	19	(6.9)	0.58	0.22, 1.52
	P for trend =0.051						
Maximum body weight in lifetime	<60.0kg	6	(5.1)	23	(8.3)	1.00	
	60.0-69.9kg	55	(47.0)	122	(44.0)	1.70	0.67, 4.32
	70.0-79.9kg	41	(35.0)	86	(31.1)	1.72	0.64, 4.58
	≥80.0kg	15	(12.8)	46	(16.6)	1.36	0.48, 3.85
	P for trend =0.923						
Maximum BMI in lifetime	<23.0	20	(17.1)	55	(19.9)	1.00	
	23.0-24.9	32	(27.4)	72	(26.0)	1.31	0.66, 2.59
	25.0-26.9	35	(29.9)	78	(28.2)	1.16	0.57, 2.33
	≥27.0	30	(25.6)	72	(26.0)	1.22	0.61, 2.41
	P for trend =0.746						
Weight gain in adult age	<5kg	18	(15.4)	65	(23.5)	1.00	
	5.0-9.9kg	24	(20.5)	76	(27.4)	1.22	0.58, 2.55
	10.0-14.9-	43	(36.8)	55	(19.9)	3.55***	1.71, 7.39
	≥15.0kg	32	(27.4)	81	(29.4)	1.73	0.83, 3.59
	P for trend =0.041						

*P<0.05; ***P<0.001; Weight gain in adult age = (Maximum body weight in lifetime) - (Body weight at 20 years of age)

as 28 of stage I, 72 of Stage II, 16 of Stage III and 1 of Stage IV. Also, their distribution of Gleason scores was classified as 45 of Score 6, 43 of Score 7, 17 of Score 8 and 12 of Score 9. Among them, 79 patients had been operated on with a radical prostatectomy, 34 patients had been treated with radiation and 14 patients had been treated with hormone therapy.

Four controls per case, namely, 468 controls, were randomly selected from resident registries as age and address (ward, city or town) matched with each case in March, 2009. Excluding 9 subjects (2 dead, 7 moved out), 318 control subjects (69.5%) gave us informed consent and filled out the same questionnaire as a patient with PCa. Data of 277 control subjects were used for the analysis excluding 41 subjects with previous cancer history. As we did not collect sera from the controls, we could not estimate the risk of the personal characteristic of equol producer or non-producer itself. However, we conducted a stratified analysis according to personal status of equol production among the PCa patients.

The conditional logistic regression model was utilized for analyzing the individually age and address-matched data (Breslow and Day, 1980), and odds ratios (ORs) and their 95% confidence intervals (95%CIs) of potential risk factors for PCa were calculated using the software SAS System (SAS Institute Inc., 1995). The significance level was set at the 5% level. This study was approved by the Institutional Review Board of Sapporo Medical University.

Results

ORs and their 95%CIs for PCa with regard to body statue are shown in Table 1. BMI at 20 years of age of between 23.0 and 24.9 was found to be significantly associated with a reduced risk of PCa (OR=0.47, 95%CI 0.22, 0.98), and a higher BMI at 20 years of age was marginally significantly associated with a decreased risk of PCa (P for trend, P=0.051). Weight gain in adult age between 10.0kg and 14.9kg was noted to be significantly associated with an elevated risk of PCa (OR=3.55, 95%CI 1.71, 7.39), and larger weight gain in adult age was significantly associated with an increased risk of PCa (P for trend, P=0.041).

ORs (their 95%CIs) for PCa with regard to family cancer history are shown in Table 2. History of prostate cancer in fathers or brothers was significantly associated with an increased risk of PCa (OR=9.71, 95%CI 3.59, 26.27), and history of breast cancer in mothers or sisters was also significantly associated with an increased risk of PCa (OR=2.70, 95%CI 1.12, 6.49). Although history of any cancer in fathers was significantly associated with an increased risk of PCa (OR=2.11, 95%CI 1.27, 3.49), this elevated risk became insignificant when history of PCa in fathers was excluded from the analysis (OR=1.53, 95%CI 0.91, 2.57).

Table 2. Odds ratios (ORs) and 95% Confidence Intervals (95%CI) for Prostate Cancer with Regard to Family Cancer History

Items	Content	Cases No.	(%)	Controls No.	(%)	OR	95%CI
Any cancer in fathers							
	No	77	(65.8)	218	(78.7)	1.00	
	Yes	40	(34.2)	59	(21.3)	2.11**	1.27, 3.49
Any cancer in mothers							
	No	88	(75.2)	222	(80.1)	1.00	
	Yes	29	(24.8)	55	(19.9)	1.20	0.72, 2.00
Prostate cancer in fathers or brothers							
	No	97	(82.9)	272	(98.2)	1.00	
	Yes	20	(17.1)	5	(1.8)	9.71***	3.59, 26.3
Breast cancer in mothers or sisters							
	No	106	(90.6)	266	(96.0)	1.00	
	Yes	11	(9.4)	11	(4.0)	2.70*	1.12, 6.49
Uterine cancer in mothers or sisters							
	No	110	(94.0)	266	(96.0)	1.00	
	Yes	7	(6.0)	11	(4.0)	1.24	0.47, 3.28
Colorectal cancer in parents or siblings							
	No	100	(85.5)	254	(91.7)	1.00	
	Yes	17	(14.5)	23	(8.3)	1.88	0.97, 3.64
Pancreatic cancer in parents or siblings							
	No	111	(94.9)	266	(96.0)	1.00	
	Yes	6	(5.1)	11	(4.0)	1.31	0.46, 3.71
Bile duct cancer in parents or siblings							
	No	114	(97.4)	276	(99.6)	1.00	
	Yes	3	(2.6)	1	(0.4)	7.29	0.74, 71.7

*P<0.05; **P<0.01; ***P<0.001

Frequency of consumption of soybean products, such as tofu and natto, as well as the other surveyed foods or beverages were not associated with risk of PCa. None of the other variables, such as physical activities, cigarette smoking, alcohol drinking or various foods consumption were associated with risk of PCa, either.

The Spearman's correlation coefficients (r) in clinical status and risk factors among the 117 PCa patients are shown in Table 3. Age was significantly negatively correlated with history of PCa in fathers or brothers (r=-0.196, P<0.05). The Gleason score was significantly positively correlated with clinical stage (r=0.229, P<0.05). Weight gain in adult age was significantly negatively

Table 3. Spearman's Correlation Coefficients for Clinical Status and Risk Factors among 117 Prostate Cancer Patients

	1. AG	2. ST	3. GS	4. BMI20	5. WG	6. PCaFB
1. Age (AG)	1.000					
2. Stage (ST)	-0.032	1.000				
3. Gleason score (GS)	-0.071	0.229*	1.000			
4. BMI at 20 years (BMI20)	0.166	0.075	0.161	1.000		
5. Weight gain in adult age (kg) (WG)	-0.144	0.048	0.069	-0.275*	1.000	
6. Prostate cancer in fathers or brothers[1]	-0.196*	-0.119	-0.085	-0.063	0.054	1.000
7. Breast cancer in mothers or sisters[2]	-0.019	0.049	0.001	0.052	-0.272**	0.001

[1](Number) (PCaFB); [2](Number) (BCaMS); *P<0.05; **P<0.01

correlated with BMI at 20 years of age (r=-0.275, P<0.05) as well as history of breast cancer in mothers or sisters (r=-0.272, P<0.01).

Discussion

We found that higher BMI at 20 years old was associated with a marginally significantly reduced risk of PCa, and larger weight gain in adult age was significantly associated with an increased risk for PCa. Similarly, Write at al. (2007) also showed that higher baseline BMI was associated with a significantly reduced incidence of PCa, and larger weight gain from 18 years old was significantly positively associated with a risk of PCa mortality in a large cohort study.

As total energy intake was shown to be positively associated with risk of PCa (Andersson et al., 1996; Grönberg et al., 1996a; Hsieh et al., 2003; Kristal et al., 2003) increased risk of PCa by larger weight gain in adult age might be achieved through high total energy intake and low physical activity. However, we did not get information of total energy intake. Association of physical activities with reduced PCa risk was not observed in our brief questions either.

Some studies (Bradbury et al., 2005; Porter and Stanford, 2005) have suggested a significantly negative association of BMI with PCa risk. On the contrary, other studies (Grönberg et al., 1996; Rodriguez et al., 2001; Dal Maso et al., 2004; Engeland et al., 2003) have indicated a significantly positive association of BMI with risk of PCa. Difference of stage, grade or aggressiveness may provide an explanation of the conflict in these results of the relationship between obesity and PCa risk (Gong et al. 2006), (MacInnis et al., 2003; Littman et al., 2007; Rodriguez et al., 2007). Most of the reports have shown that BMI is inversely associated with the risk of nonmetastatic and low-grade PCa, but BMI is positively associated with the risk of metastatic or high-grade PCa. However, clinical stage or Gleason's score were not correlated with BMI at 20 years of age or weight gain in adult age in our study, as shown in Table 3.

The complex relationship of weight gain in adult age or BMI at 20 years of age with PCa risk has been suggested to be caused by conflict in hormonal role of development of PCa (Rodriguez et al., 2001; 2007; Dal Maso et al., 2004; Gong et al., 2006; Wright et al. 2007). Larger weight gain in adult age may be associated with higher insulin, leptin, free IGF-I and lower sex hormone-binding globulin concentrations, which could possibly increase the risk of PCa. Conversely, higher BMI at 20 years of age may be associated with lower circulating testosterone concentrations and higher estrogen concentrations, which could decrease the risk of PCa.

History of PCa in first-degree relatives is an established risk factor for PCa (Ghadirian et al., 1991; Carter et al., 1992; Grönberg et al., 1996b; Lesko et al., 1996; Ghadirian et al., 1997; Cerhan et al., 1999; Eldon et al., 2003; Karakiewicz et al., 2003; Negri et al., 2005). The reported range of risk ratios in family history of PCa varied from 1.7 to 8.7. The reason of a higher odds ratio (OR=9.71) in our study than in other studies is not able to be clearly explained, but it may be a part of the reason that nature of the control group was not hospital-based, but population-based. However, some extent of recall bias in the study subjects cannot be ruled out.

History of PCa in fathers or brothers was inversely correlated with age at onset in our study, as it has been similarly reported that risk of family history of PCa is prominent in younger age groups (Eldon et al., 2003; Carter et al., 1992; Grönberg et al., 1996b; Lesko et al. 1996). Although Spangler et al. (2005) suggested that family history of PCa may be associated with predictors of deteriorated clinical outcome, family histories of PCa were not correlated with clinical stage or Gleason's score in our study. Although Giovannucci et al. (2003) a found significantly inverse correlation between BMI and a family history of PCa, either of BMI at weight or gain in adult age was not correlated with family history of PCa in our study, as shown in Table 3.

History of breast cancer in first-degree relatives has also been reported as a significant risk factor for PCa (Cerhan et al., 1999; Grönberg et al., 2000; The Breast Cancer Linkage Consortium, 1999), although the other studies have shown no association between family history of breast cancer and PCa risk (Eldon et al., 2003; Karakiewicz et al., 2003; Negri et al. 2005). In a report from the Breast Cancer Linkage Consortium (1999), the risk ratio of PCa in BRCA2 carriers was 4.65 (95%CI 3.48, 6.22). A significantly inverse correlation between weight gain in adult age and history of breast cancer in mothers or sisters was observed in our study, although similar result have not been reported in previous studies.

To our knowledge, this is the first case-control study of PCa using population-based controls in Japan. However, there were some limitations in our study. First, the sample size of the study was not large enough, especially, to assess the interaction between the host and environmental factors. Although we previously reported the preventive effects of soybean products or isoflavones for PCa (Nagata et al. 2007), we could not detect any risk or preventive dietary factors in this study. The reason for such null findings might be from the small sample size in addition to the methodological limitation of the survey that we did not collect information on the amount of food consumptions. Second, response from the study subjects was not perfect. However, a relatively high response rate was obtained not only from the cases (82.3%), but also from the controls (69.5%). Third, we used self-reported weight, height and family history of cancer, all of which are subject to error. However, it has been shown that self-reported weight and height were highly valid compared with measured values (Stevens et al. 1990), and self reported cancer in a first-degree relative was relatively accurate (Whittemore et al., 1995).

In conclusion, the recent increase in the incidence rate of PCa may have possibly been brought about by an increased proportion of Japanese men with larger weight gain in adult age.

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