Oncogene (2004) 23, 8552–8556

www.nature.com/onc

Aggrus: a diagnostic marker that distinguishes seminoma from embryonal carcinoma in testicular germ cell tumors

Yukinari Kato[1,2], Isoji Sasagawa[3], Mika Kaneko[2], Motoki Osawa[2], Naoya Fujita[1] and Takashi Tsuruo*[,1,4]

[1]*Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan;* [2]*Department of Experimental and Forensic Pathology, Yamagata University School of Medicine, 2-2-2, Iida-nishi, Yamagata 990-9585, Japan;* [3]*Department of Urology, Yamagata University School of Medicine, 2-2-2, Iida-nishi, Yamagata 990-9585, Japan;* [4]*Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 1-37-1, Kami-Ikebukuro, Toshima-ku, Tokyo 170-8455, Japan*

Aggrus (also known as T1α/podoplanin) is a membrane sialoglycoprotein whose function in tumors is unknown. We recently determined that Aggrus possessed the ability of inducing platelet aggregation and that its expression was frequently upregulated in colorectal tumors. Thus, Aggrus expression might be associated with tumor-induced platelet aggregation and tumor metastasis. Here we show, by means of cancer profiling array and real-time PCR, that *aggrus* mRNA expression is frequently upregulated in testicular germ cell tumors when compared with the surrounding normal tissue. Immunohistochemical staining revealed that Aggrus protein expression was detected in 10 of 11 seminomas (90.9%), but its expression was not observed in embryonal carcinomas (0/4; 0%). Specific markers for seminomas have not been reported, and Aggrus is a potential diagnostic marker for seminomas and may be associated with malignancies of the testis.
Oncogene (2004) **23,** 8552–8556. doi:10.1038/sj.onc.1207869
Published online 13 September 2004

Keywords: Aggrus; seminoma; embryonal carcinoma; germ cell tumor

Testicular germ cell tumors (GCTs) comprise less than 2% of all human malignancies and are the most frequent cancer in young males between the ages of 20 and 40 years (Moller, 1993; Adami *et al*., 1994). Over the past several decades, the incidence of GCTs has been increasing in the Western world (Bergstrom *et al*., 1996). GCTs comprise large numbers of histologically different forms and can be practically divided into two broad categories: seminoma and nonseminomatous germ cell tumors (NSGCTs) (Moller, 1993; Adami *et al*., 1994). NSGCT is an umbrella designation that includes tumors of one histological type, such as embryonal carcinoma, as well as those with more than one histological type. However, there are combined or mixed GCTs that are composed of elements from both seminomas and NSGCTs. Chromosomal and molecular studies have shown that seminoma is capable of differentiation into embryonic (embryonal carcinoma, teratoma) and extraembryonic (yolk sac tumor, choriocarcinoma) tissues (de Jong *et al*., 1990; Fogel *et al*., 1990). GCTs of all types are frequently associated with carcinoma *in situ* (CIS). Therefore, both seminoma and NSGCTs are thought to arise from cytologically identical CIS lesions, indicating a common cell for all GCTs.

Seminoma and NSGCTs present with distinctive clinical features, but they also differ with respect to therapy and prognosis (Moller, 1993; Adami *et al*., 1994). Seminoma, which is extremely radiosensitive and tends to remain localized for long periods, has the best prognosis. More than 95% of patients with stage I or II disease can be cured. Among NSGCTs, histologic subtype does not influence the prognosis significantly, and hence they are treated as one group. Although NSGCTs do not share the same prognosis as seminoma, 90% of patients with NSGCTs can achieve complete remission with aggressive chemotherapy, and most can be cured (Moller, 1993; Adami *et al*., 1994). With the availability of effective treatment, it is important to clarify histological subtype in testicular tumors. However, clinical differentiation between various types of testicular GCTs is imperfect because there are no specific clinical features of the testicular masses produced by tumors of different histological types. Hence, the immunohistochemical staining after total orchiectomy is very important for diagnosis, and the distinction between seminoma and NSGCT is essential for treatment of testicular tumors.

Mouse Aggrus is a 44-kDa sialoglycoprotein (gp44), which is expressed on the tumor cell surface with a platelet aggregation-inducing ability (Watanabe *et al*., 1988). Our generated 8F11 monoclonal antibody-recognizing mouse Aggrus could neutralize tumor-induced platelet aggregation (Watanabe *et al*., 1988). Recently, we revealed that mouse Aggrus is identical to

*Correspondence: T Tsuruo, Laboratory of Cell Growth and Regulation, Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan; E-mail: ttsuruo@iam.u-tokyo.ac.jp
Received 21 December 2003; revised 4 May 2004; accepted 4 May 2004; published online 13 September 2004

T1α/podoplanin, whose function in tumors is unknown (Kato *et al*., 2003). The molecules have putative extracellular and transmembrane domains and a short cytoplasmic tail with putative PKC and cAMP phosphorylation sites (Ma *et al*., 1998). Aggrus is highly *O*-glycosylated and contains sialic acid (Ma *et al*., 1998), and its expression was found in lymphatic endothelium or alveolar type I cells (Wetterwald *et al*., 1996; Williams *et al*., 1996). Sequence analysis indicates that Aggrus does not share common domains with other protein families, of known function, that predict its function. Therefore, the physiological role of Aggrus has yet to be determined. Recently, Ramirez *et al*. (2003) generated *T1α* null mice containing a targeted mutation in the *T1α* gene locus. Homozygous null mutant mice died immediately after birth, due to respiratory failure (Ramirez *et al*., 2003). The T1$\alpha^{-/-}$ mice also had defects in lymphatic vasculature formation and were characterized by congenital lymphedema (Schacht *et al*., 2003).

We previously cloned human Aggrus (Kato *et al*., 2003; GenBank Accession No. AB127958). Although mouse and human Aggrus proteins have only 39%

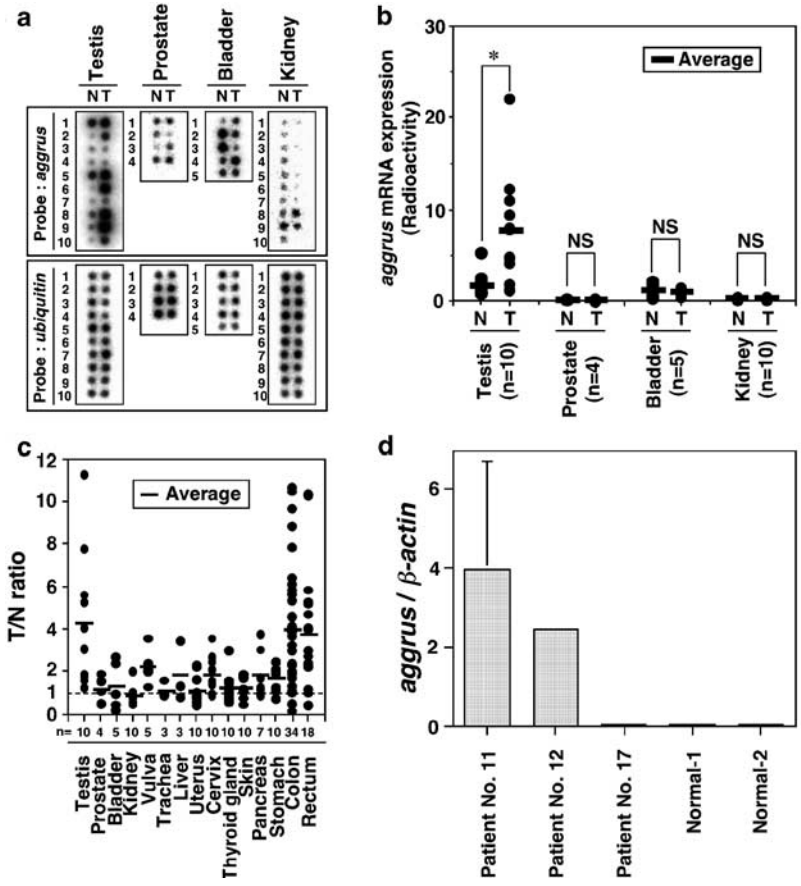

Figure 1 Increased *aggrus* mRNA expression in testicular tumors. (**a**–**c**) Human *aggrus* mRNA expression in tumor (*T*) and normal (*N*) tissues in the same patient was assessed by hybridizing ^{32}P-labeled human *aggrus* cDNA to the Cancer Profiling Array or Cancer Profiling Array II (BD Biosciences) as described previously (Kato *et al*., 2003). The radioactivity of each dot was visualized (**a**) and quantified (**b** and **c**). (**b**) The relative *aggrus* mRNA expression level in each dot was normalized by measuring the radioactivity of each dot after hybridization with ^{32}P-labeled *ubiquitin* cDNA. (**c**) The *T*/*N* ratio was calculated by dividing normalized radioactivity of tumor samples with that of normal tissues in the individual patients. Bars, average of the human *aggrus* mRNA expression (**b**) and *T*/*N* ratio in each tissue (**c**). Statistical significance of *aggrus* mRNA expression in normal and tumor tissues was performed using paired *t*-test. Asterisk indicates the significant difference: $*P<0.05$. The expression level of *aggrus* mRNA of tumor *versus* normal tissues in the prostate, bladder, and kidney was not significantly different (NS). (**d**) The *aggrus* transcripts in testicular tumor tissues (patients 11, 12, and 17) and normal testicular tissues (Normal-1 and -2) were quantified by performing real-time PCR. Total RNAs were prepared from frozen sections of patients 11, 12, and 17 employing an RNeasy mini prep kit (Qiagen Inc.). The first-strand cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase (USB Corp.) via priming nine random oligomers according to the manufacturer's instructions. PCR was performed using the human *aggrus* sense (5′-GGAAGGTGTCAGCTCTGCTC-3′), human *aggrus* antisense (5′-CGCCTTCCAAACCTGTAGTC-3′), human *β-actin* sense (5′-ACTCTTCCAGCCTTCCTTCCTG-3′), and human *β-actin* antisense (5′-ATCTCCTTCTGCATCCTGTCGG-3′) oligonucleotides. Real-time PCR was carried out using the QuantiTect SYBR Green PCR (Qiagen). PCR conditions were 95°C for 15 min (one cycle), followed by 40 cycles of 94°C for 15 s, 55°C for 25 s, and 72°C for 20 s. Subsequently, a melting curve program was applied, with continuous fluorescence measurement. Standard curves for *aggrus* and *β-actin* templates were generated by serial dilution of the PCR products (1×10^8 copies/μl to 1×10^3 copies/μl). The expression level of *aggrus* was normalized by estimating the quantity of the *β-actin* transcript. The vertical bars represent s.d. values of duplicate determinations

amino-acid identity, human Aggrus also had the ability to induce platelet aggregation (Kato *et al*., 2003). Moreover, the rat Aggrus homolog had platelet aggregation-inducing capability (Kato *et al*., 2003). Thus, under pathological conditions, Aggrus could be involved in the platelet aggregation induced by tumor cells. In most patients with intestinal tumors, Aggrus mRNA and protein expression was enhanced (Kato *et al*., 2003). However, the role of Aggrus in GCTs has never been reported. In the present study, we investigated the expression of Aggrus in the testicular tumors.

Expression of *aggrus* mRNA in testicular GCTs

We estimated human *aggrus* expression in human tumors using the Cancer Profiling Array II. In this array, normalized cDNA pairs from 160 primary tumor tissues and corresponding normal tissues from individual patients were immobilized on a nylon membrane. The increase in *aggrus* mRNA expression was observed in almost all testicular GCTs, compared with the corresponding normal testicular tissue (Figure 1a and Table 1). After hybridization with ^{32}P-labeled *ubiquitin* cDNA, human *aggrus* expression level in each dot was normalized (Figure 1b). The average expression levels of human *aggrus* mRNA (relative *aggrus*/*ubiquitin* radioactivity) in testicular GCTs and normal tissue were about 7.6 and 1.8, respectively (Figure 1b). The *aggrus* mRNA expression of tumor *versus* normal tissues in testis was significantly increased ($P<0.05$). In contrast to testicular GCTs, *aggrus* mRNA expression was significantly lower in prostate, bladder, and kidney tumors (Figures 1a and b). Calculating T/N (tumor/normal) ratios of *aggrus* mRNA expression in the individual patients confirmed the considerably elevated expression in testicular GCTs (Figure 1c). The average T/N ratio was about 4.3 ($n=10$). We also estimated human *aggrus* expression in intestinal tumors (colon and rectum) using the Cancer Profiling Array and found the elevated expression of *aggrus* in intestinal tumors when compared with corresponding normal tissues (Figure 1c). This result was consistent with our previous report using Cancer Profiling Array II (Kato *et al*., 2003). The average T/N ratios of human *aggrus* in colon ($n=34$) and rectum ($n=18$) tumor patients were about 4.0 and 3.72, respectively. In contrast, T/N ratio in prostate ($n=4$), bladder ($n=5$), kidney ($n=10$), vulva ($n=5$), trachea ($n=3$), liver ($n=3$), uterus ($n=10$), cervix ($n=10$), thyroid gland ($n=10$), skin ($n=10$), pancreas ($n=7$), and stomach ($n=10$) varied from patient to patient (Figure 1c), and the average expression ratios in these organs were about 1.2, 1.3, 0.8, 2.2, 1.1, 1.9, 1.1, 1.8, 1.3, 1.3, 1.8, and 1.7, respectively. Thus, *aggrus* might be a marker for not only intestinal tumors but also testicular GCTs.

To confirm the result of array analysis, we performed real-time PCR with cDNA prepared from the frozen tissues of three testicular tumor patients who underwent surgery for testicular GCT in the Yamagata University Hospital (patients 11, 12 and 17, Table 1) and normal testicular tissues (Figure 1d). Two normal testis specimens (Normal-1 and -2) were obtained from patients who underwent bilateral orchiectomy (castration). Quantitative PCR analysis revealed that human *aggrus*

Table 1 Patient information of the samples used in this study

Patient	*Year*	*Race*	*Stage*	*Type*	*T/N*	*TT679*
1	Unknown	Caucasian	T1N0M0	Seminoma	1.76	N/D
2	22	Asian	T2N0M0	Seminoma	5.19	N/D
3	45	Caucasian	T1N0M0	EC	1.53	N/D
4	69	Caucasian	T1N0M0	Seminoma	1.88	N/D
5	32	Caucasian	T1N0M0	Seminoma	3.07	N/D
6	54	Caucasian	T1N0M0	Seminoma	7.72	N/D
7	54	Caucasian	T1N0M0	Seminoma	1.20	N/D
8	42	Caucasian	Unknown	Seminoma	5.56	N/D
9	38	Caucasian	Unknown	Seminoma	11.20	N/D
10	28	Caucasian	T3N0M0	Seminoma	4.00	N/D
11	31	Asian	T2N2M0	Seminoma	N/D	Positive
12	27	Asian	T1N1M1	Seminoma	N/D	Positive
13	34	Asian	Unknown	Seminoma	N/D	Positive
14	34	Asian	T2N0M0	Seminoma	N/D	Positive
15	28	Asian	T2N2M0	Seminoma	N/D	Negative
16	35	Asian	T1N0M1	Seminoma	N/D	Positive
17	18	Asian	T2N3M1	EC	N/D	Negative
18	35	Asian	T1N0M0	EC	N/D	Negative
19	33	Asian	T1N0M1	EC	N/D	Negative
20	16	Asian	T2N3M0	EC	N/D	Negative
21	31	Asian	T1N0M0	Seminoma	N/D	Positive
22	61	Asian	T1N0M0	Seminoma	N/D	Positive
23	34	Asian	T1N0M0	Seminoma	N/D	Positive
24	34	Asian	T2N0M0	Seminoma	N/D	Positive
25	47	unknown	T1N0M0	Seminoma	N/D	Positive

EC: embryonal carcinoma, N/D: not determined

mRNA expression was clearly enhanced in testicular GCTs derived from patients 11 and 12, but not from patient 17. In the normal testicular tissue, almost no *aggrus* mRNA expression could be detected. The average amounts of *aggrus* mRNA in samples from patients 11, 12, and 17 were about 80, 50, and 0.43 times as much as those in normal testicular samples (Normal-1 and -2).

Expression of Aggrus protein in seminoma tissue

As shown in Figure 1d, *aggrus* mRNA expression was detected in seminoma patients (patients 11 and 12, Table 1), but not in an embryonal carcinoma patient (patient 17, Table 1). Thus, we checked the immunohistochemical reactivity of an anti-Aggrus polyclonal antibody (TT679) to specimens from testicular GCTs. We stained 15 testicular tumors (11 seminomas and four embryonal carcinomas) (Table 1). As shown in Table 1, this study included 14 patients who underwent surgery for testicular GCT (from 1990 to 2002) in the Yamagata University Hospital (patients 11–24) and one additional human testicular tumor specimen purchased from Novagen (patient 25). We found that 10 of the 11 (90.9%) seminomas showed strong immunoreactivity to TT679 (Figure 2a and Supplementary Figure 1). Only patient 15 seminoma sample showed negative TT679 reactivity (Table 1 and Supplementary Figure 1). Although seminomas are divided into three histological subtypes (classical, anaplastic, and spermatocytic), all the 11 seminomas studied in this study were diagnosed as classical subtypes. Thus, its negative TT679 reactivity was not dependent on histological subtypes. The reason why patient 15 sample showed negative TT679 reactivity should be solved in future. In contrast, no immunoreactivity was detected in the embryonal carcinomas (Figure 2a and Supplementary Figure 1). Furthermore, Aggrus was not found in other NSGCTs, such as yolk sac tumors ($n=1$) and teratomas ($n=4$) (data not shown). Western blot analysis confirmed that Aggrus protein was expressed in seminomas but not in embryonal carcinomas or normal testicular tissue (Figure 2b).

Aggrus is identical to lymphatic marker podoplanin (Wetterwald *et al*., 1996). We thus stained lymphatic vessels in normal colorectal tissues by TT679 antibody in combination with an antibody to lymphatic marker Prox1 protein. Prox1-positive lymphatic endothelial cells were strongly stained by TT679 antibody (Supplementary Figure 2). This result suggests that lymphatic vessels expressed Aggrus/podoplanin on their surface. Although seminoma tissues used in this study could be stained by TT679 antibody, they could not be stained by an anti-Prox1 antibody (data not shown). Moreover, seminoma tissues showed no immunoreactivity to antibodies against other lymphatic marker proteins LYVE-1 and VEGFR-3 (data not shown). These results indicate that TT679 stained seminomas but not lymphatic vessels in seminoma tissues. NSGCTs have

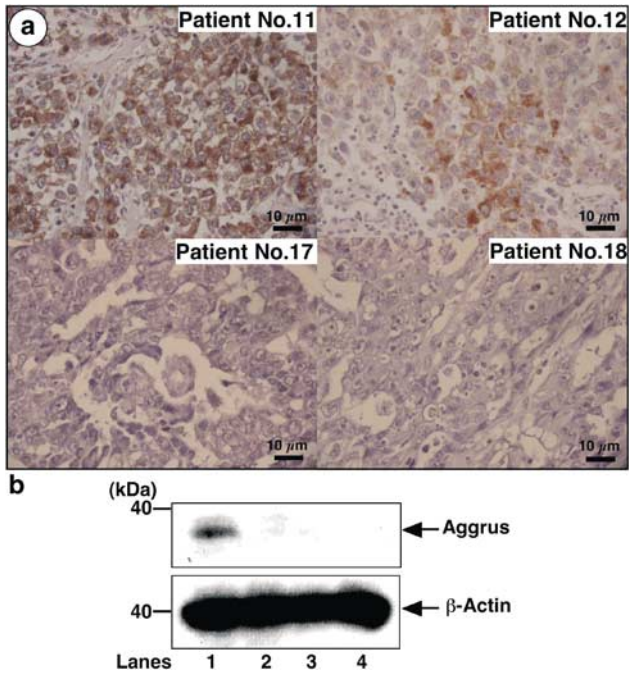

Figure 2 Increased Aggrus protein expression in testicular tumors. (**a**) Immunohistochemical staining of Aggrus in testicular tumors. A polyclonal antibody to human Aggrus (TT679) was added to the deparaffinized and rehydrated specimens (1 μg/ml) for 4 h at 23°C. Specimens were further developed using the DAKO CSA system (DakoCytomation Co.). Seminoma tissues from patients 11 and 12, and embryonal carcinoma tissues from patients 17 and 18 (Table 1). Bars, 10 μm. (**b**) Western blot analysis of Aggrus protein in testicular tumors was performed as described previously (Kato *et al*., 2003). Tumor tissues from patient 11 (lane 1) and patient 17 (lane 2), and normal testicular tissues of two prostate tumor patients (Normal-1, lane 3; Normal-2, lane 4) were solubilized and immunoblotted with an anti-human Aggrus polyclonal TT679 antibody (upper panel) or an anti-β-Actin antibody (Sigma, lower panel)

clinically useful, specific markers such as AFP or HCG. Seminomas used in this study could not be stained by anti-HCG and anti-AFP antibodies (Figures 3b and c, respectively). These results indicate that Aggrus could be a diagnostic marker for seminoma.

According to Izquierdo *et al*. (1995), c-kit protooncogene is highly expressed in 28 of the 28 seminomas. However, nine of 29 NSGCTs (32%) also have immunoreactivity to an anti-c-kit antibody. The tumor antigen MAGE-A4, which can be recognized by tumor-specific cytolytic T lymphocytes, has been reported to be expressed in seminomas but not in NSGCTs (Richie, 2003). However, Cheville and Roche (1999) and Jungbluth *et al*. (2000) reported that MAGE-A4-positive seminomas were only 42 and 70%, respectively. Our results suggest that the Aggrus protein was specifically expressed in seminomas (10/11, 90.9%) but not in embryonal carcinomas (0/4, 0%). Thus, Aggrus might have an important role in the malignancies of testis and be useful for diagnosis of seminoma. Further studies, including careful clinical follow-ups of seminoma patients, are needed to evaluate the significance of Aggrus in disease prognosis.

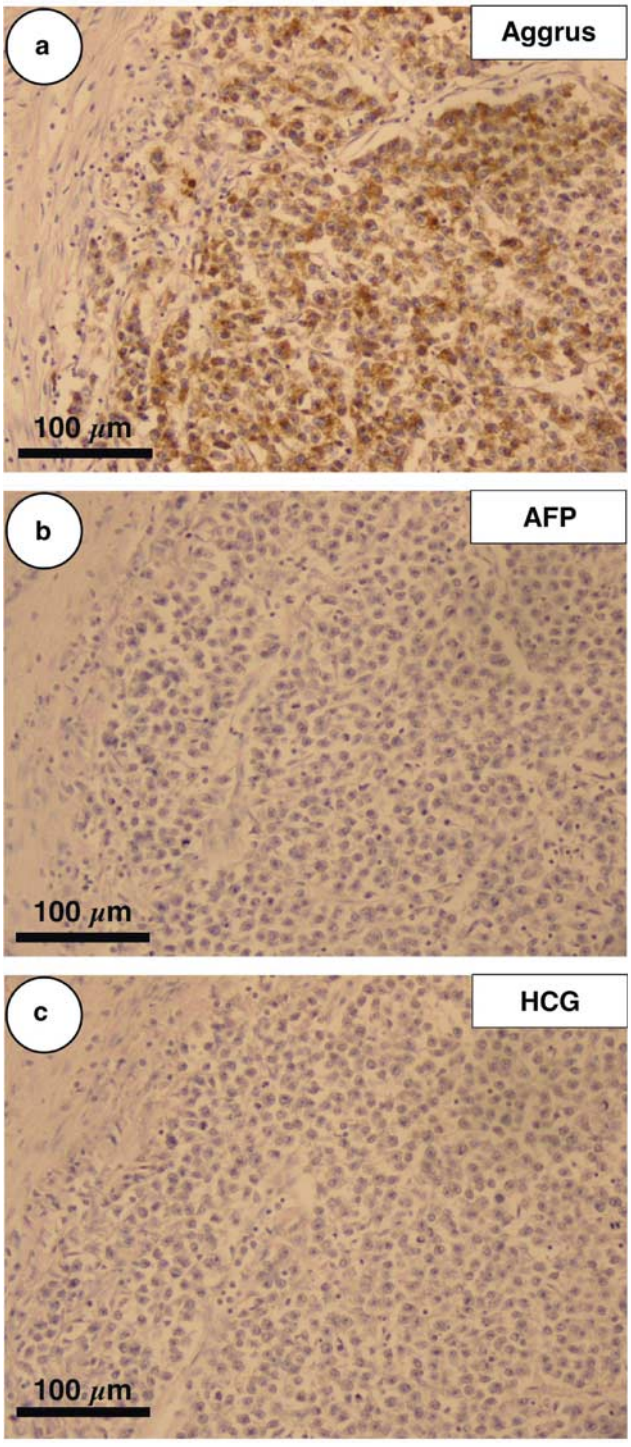

Figure 3 Immunohistochemical staining of Aggrus, AFP, and HCG in seminomas. Seminoma specimens of patient 11 were stained with an anti-human Aggrus antibody (TT679, **a**), an anti-AFP antibody (DakoCytomation, **b**), and an anti-HCG antibody (DakoCytomation, **c**) at a concentration of 1 μg/ml for 4 h at 23°C. Then the specimens were further developed using the DAKO EnVision System (DakoCytomation). Bars, 100 μm

Acknowledgements
This study was supported in part by a special grant for Advanced Research on Cancer from the Ministry of Education, Culture, Sports, Science, and Technology, Japan. We are grateful to Dr Horiuchi, Department of Experimental and Forensic Pathology, Yamagata University School of Medicine, Japan, for kind assistance.

References

Adami HO, Bergstrom R, Mohner M, Zatonski W, Storm H, Ekbom A, Tretli S, Teppo L, Ziegler H, Rahu M, Gurevicius R and Stengrevics A. (1994). *Int. J. Cancer*, **59,** 33–38.

Bergstrom R, Adami HO, Mohner M, Zatonski W, Storm H, Ekbom A, Tretli S, Teppo L, Akre O and Hakulinen T. (1996). *J. Natl. Cancer Inst.*, **88,** 727–733.

Cheville JC and Roche PC. (1999). *Mod. Pathol.*, **12,** 974–978.

de Jong B, Oosterhuis JW, Castedo SM, Vos A and te Meerman GJ. (1990). *Cancer Genet. Cytogenet.*, **48,** 143–167.

Fogel M, Lifschitz-Mercer B, Moll R, Kushnir I, Jacob N, Waldherr R, Livoff A, Franke WW and Czernobilsky B. (1990). *Differentiation*, **45,** 242–249.

Izquierdo MA, Van der Valk P, Van Ark-Otte J, Rubio G, Germa-Lluch JR, Ueda R, Scheper RJ, Takahashi T and Giaccone G. (1995). *J. Pathol.*, **177,** 253–258.

Jungbluth AA, Busam KJ, Kolb D, Iversen K, Coplan K, Chen YT, Spagnoli GC and Old LJ. (2000). *Int. J. Cancer*, **85,** 460–465.

Kato Y, Fujita N, Kunita A, Sato S, Kaneko M, Osawa M and Tsuruo T. (2003). *J. Biol. Chem.*, **278,** 51599–51605.

Ma T, Yang B, Matthay MA and Verkman AS. (1998). *Am. J. Respir. Cell Mol. Biol.*, **19,** 143–149.

Moller H. (1993). *Eur. Urol.*, **23,** 8–13.

Ramirez MI, Millien G, Hinds A, Cao Y, Seldin DC and Williams MC. (2003). *Dev. Biol.*, **256,** 61–72.

Richie JP. (2003). *Urol. Oncol.*, **21,** 91.

Schacht V, Ramirez MI, Hong YK, Hirakawa S, Feng D, Harvey N, Williams M, Dvorak AM, Dvorak HF, Oliver G and Detmar M. (2003). *EMBO J.*, **22,** 3546–3556.

Watanabe M, Okochi E, Sugimoto Y and Tsuruo T. (1988). *Cancer Res.*, **48,** 6411–6416.

Wetterwald A, Hoffstetter W, Cecchini MG, Lanske B, Wagner C, Fleisch H and Atkinson M. (1996). *Bone*, **18,** 125–132.

Williams MC, Cao Y, Hinds A, Rishi AK and Wetterwald A. (1996). *Am. J. Respir. Cell Mol. Biol.*, **14,** 577–585.