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Isolation of Genes Differentially Expressed between the Yoshida Sarcoma and Long-survival Yoshida Sarcoma Variants: Origin of Yoshida Sarcoma Revisited

Haruo Tsuchiya, Yuri Tsuchiya, Toshiyuki Kobayashi, Yasushi Kikuchi and Okio Hino[1]

Department of Experimental Pathology, Cancer Institute, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170

The Yoshida sarcoma (YS) is characterized by growth as "free cells" in ascites. Long-survival Yoshida sarcoma (LY) variants, which develop after transplantation of YS into immunologically conditioned Donryu rats, in contrast, form "islands" in ascites. A representational difference analysis (RDA) approach was adopted to isolate genes differentially expressed between YS and LY variants to elucidate the molecular mechanism of their development. Fifteen clones presenting differences in expression were characterized. Nine genes (those encoding for the high-affinity IgE receptor γ chain, pJG116 repetitive sequence, non neuronal enolase, proteasome subunit RC1, cytotoxic T lymphocyte-associated gene transcript CTLA-1, interleukin-2 receptor γ chain, and three unknown sequences) expressed mRNA in YS, but showed lower or no expression of mRNA in LYs. The mRNAs of the other six genes (those encoding for cytokeratin 8, cytokeratin18 (Endo B), TIMP2 and three unknown sequences) were not found in YS, but were present in LYs. Interestingly, CTLA-1 is a non-epithelial (hematopoietic) cell-specific gene in terms of transcription, while cytokeratin 8 and cytokeratin 18 are both epithelium-specific genes. Immunohistochemically, YS expressed T-cell specific antigens CD2 and CD3, and T cell receptor β and γ chain genes were rearranged in YS, but not in LYs. Moreover, using restriction fragment length polymorphism probes, we found that LYs exhibited different cell lineage from YS. Thus, our present findings, unexpectedly, raise fundamental questions concerning the cellular origins of YS and LY variants rather than pointing to any specific mechanism to explain the LY phenomenon.

Key words: Yoshida sarcoma — Long-survival Yoshida sarcoma — Representational difference analysis — cDNA subtraction

The Yoshida sarcoma (YS) has been an epoch-making cell line in cancer research, including studies on cancer metastasis and chemotherapy. YS, an ascites tumor of a rat (unknown strain) fed *o*-aminoazotoluene (4′-amino-2,3′-dimethylaminoazobenzene) for 3 months and then painted on the back with KAs_2O_2 solution, was established by Tomizo Yoshida in 1943 and is characterized by growth as "free cells" in ascites and in an exclusively sarcomatous fashion in tissues.[1)] Cell lines designated as long-survival Yoshida sarcoma (LY) variants, which develop after transplantation of YS into immunologically conditioned Donryu rats, in contrast, demonstrate the formation of "islands" in ascites, and present an epithelial character. This phenomenon, the so-called "LY transformation," was first reported by Satoh in 1964[2)] and subsequently confirmed by Ishidate and Isaka.[3)] LY variants grown in host tissue showed adenocarcinomatous histological patterns and considerably slower growth than with YS, as revealed by longer survival of their hosts.[4)] At the present time, YS is widely accepted to have a hepatic cell origin, based on accumulated findings including this LY transformation,[4)] even though it was initially postulated to have been derived from a reticuloendothelial Kupffer satellite cell or peritoneal monocyte.

As an explanation for the mechanism of LY transformation, Tomizo Yoshida suggested that the "masked" epithelial cell nature of YS, was "unmasked" by unknown factors so that the original island or epithelial nature could become apparent.[4)] As mentioned above, LY variants are less malignant than the original YS and therefore, it is important to elucidate the molecular mechanisms underlying this LY transformation. Enzyme biochemical studies on YS and LY variants were extensively performed in the 1960's with Ono reporting no difference between them,[5)] although Sugimura did find variation in the hexokinase isozyme type.[6)] In the present study, in an attempt to elucidate the molecular basis for LY transformation, we identified genes specifically expressed in YS and in LY variants by cDNA subtraction using a modified representational difference analysis (RDA) method.[7,8)] Our present findings, unexpectedly, raise fundamental questions concerning the cellular origin of YS.

MATERIALS AND METHODS

Cell culture Two YS lines were obtained; one kindly provided by Prof. T. Suzuki (Fukushima Medical College, Department of Pathology) and the other from the Cancer Cell Repository, Tohoku University. LY variants

[1] To whom correspondence should be addressed.

(LY6 and LY336) were also obtained from the Cancer Cell Repository, Tohoku University. These cell lines were kept stable through *in vivo* passage in ascites fluid of Donryu rats. We also established an *in vitro* culture system for YS and LY variants. YS and LY variants (LY6 and LY336) were cultured in RPMI 1640 containing 10 to 20% fetal calf serum (FCS). The latter proved slower-growing than YS *in vitro* (data not shown), as had been found *in vivo*.[9]

cDNA library construction RNAs of YS and LY336 were extracted from *in vitro* culture cells and/or cells grown in ascitic fluid, respectively. cDNAs were synthesized with the aid of a λ ZAPII cDNA synthesis kit and cloned into λ ZAPII (Stratagene). Oligonucleotides were synthesized using a Cyclone Plus DNA synthesizer (Milligen/Biosearch, Division of Millipore) and purified by denatured acrylamide gel electrophoresis.[10]

Synthesis of subtracted probe by modified RDA method To avoid host cell contamination of ascites, cDNAs were made from YS and LY336 mRNAs extracted from cells in *in vitro* culture, with a cDNA synthesis kit (Boehringer) using 28 mer oligo dT [5′-GAGAGATC-$(T)_{18}$(G/A/C)N-3′], with an internal Sau3A1 site, as a primer. The RBA-1 (5′-AGCACTCTCCAGCCTCTC-ACCGAG-3′) and 2 (5′-GATCCTCGGTGA-3′) adaptors were ligated to the Sau3A1-digested "tester" and "driver" cDNAs (LY336 or YS). After filling in the ends, cDNAs were amplified by polymerase chain reaction (PCR) using the adaptor (RBA-1) as a primer; the products are known as amplicons.[7,8] Before hybrid subtraction, "driver" amplicons were digested with Sau3A1 and adaptors were removed by Suprec-02 (Takara). The NBA-1 (5′-AGGCAACTGTGCTATCCGAGGGAG-3′) and 2 (5′-GATCCTCCCTCG-3′) adaptors were ligated to the Sau3A1-digested "tester" amplicon (YS or LY336) and amplified by PCR using NBA-1 as a primer. The procedure used for hybrid subtraction was essentially the same as the original method[7] except for the primers. After the 1st round of hybrid subtraction, tester DNAs were digested with Sau3A1 and the adaptor (NBA-1, 2) was removed. Then, JBA-1 (5′-ACCGACG-TCGACTATCCATGAACG-3′) and 2 (5′-GATCCG-TTCATG-3′) adaptors were ligated to the digested "tester" DNAs, followed by the 2nd round of hybrid subtraction, after which the "tester" DNAs were digested with Sau3A1 and the adaptors were removed. Next, NBA-1 and 2 adaptors were ligated to the digested "tester" DNAs, followed by the 3rd round of hybrid subtraction. Finally, amplified products of "tester" cDNA were labeled by the random oligonucleotide-priming procedure. The method described above will be described in detail elsewhere (T. Kobayashi and O. Hino, in preparation).

Screening cDNA libraries About 10^4 independent phage clones of cDNA libraries of YS and LY variants were plated on 15 cm-NZCYM plates, transferred onto nylon filters (Biodyne B; Pall) and hybridized with appropriate PCR-derived subtracted tester cDNA probes (1×10^6 cpm/ml), overnight at 65°C in a solution containing 0.2 *M* $NaHPO_4$ (pH 7.2), 1 m*M* EDTA (pH 8.0), 1% bovine serum albumin, and 7% SDS.[10] The filters were washed in 2×SSC at room temperature for 15 min, followed by 0.1×SSC, 0.1% SDS at 65°C for 30 min. Positive phage clones were subcloned into pBluescriptSK(−) using *in vivo* excision. Subcloned cDNAs (about 10 ng) were spotted onto nylon membranes (Biodyne B; Pall) for DNA dot blotting to identify the same clones. Total RNAs of YS and LY variants (LY336 and LY6) were electrophoresed on 1.0% agarose gels and transferred onto nylon membranes for Northern blotting to check the expression patterns and size of transcripts of these subcloned cDNAs.

Sequence analysis cDNA clones were sequenced by the dideoxy method and a homology search performed with the GenEMBL (VAX system).

Immunostaining Monoclonal antibodies [anti-rat T lymphocyte and thymocyte monoclonal antibody (CD2 equivalent) (CD2, Cedarlane), and anti-rat ε-T3 α complex (CD3) monoclonal antibody (CD3, Cedarlane)] were used for immunohistochemistry. These antibodies were used at dilutions of 1:100–1000. Anti-mouse Ig, biotinylate species-specific whole antibody (Amersham) was used as the second antibody, which was diluted 1:400. Avidin-biotinylated peroxidase complex (ABC) was provided by Vector Laboratories. Paraffin sections of the cell block of YS were dewaxed with xylene and hydrated with a graded ethanol series, then incubated with methanol containing 0.3% H_2O_2 for 30 min at room temperature, rinsed with 10 m*M* phosphate-buffered saline (PBS, pH 7.4), and incubated with the antibodies overnight at 4°C in a moist chamber. The next day, they were rinsed with PBS and incubated with the second monoclonal antibody and ABC for 30 min at 37°C in a moist chamber. Diaminobenzidine treatment was done as described previously.[11]

Southern blotting and hybridization Genomic DNAs were extracted from normal rat liver, YS, LY336 and LY6 cells. They were digested with restriction enzymes and electrophoresed on a 1.0% agarose gel (10 μg per lane). Rat aldolase B gene, mouse protamine 1 gene and rat MEL14 (gift of Dr. T. Watanabe; University of Tokyo) were used as restriction fragment length polymorphism (RFLP) probes. Mouse T cell receptor (TCR) β[12] and γ[13] probes were kindly provided by Dr. T. Yamada (Sasaki Institute). Blotting, hybridization and washing were performed as described above.

Transplantation of YS and LY336 cells to nude mice YS (10^7 cells) LY336 (10^7 cells) were subcutaneously trans-

planted to nude mice (ICR Nu/Nu, Charles River Japan, Inc.). Tissues were fixed in 10% formalin and a routine histological examination was carried out on hematoxylin and eosin (HE)-stained paraffin sections.

RESULTS

Isolation of YS or LY336 specific cDNAs We isolated 36 positive clones from the YS cDNA library and 30 positive clones from the LY336 cDNA library. Some were revealed to be the same by DNA dot blotting. Thus, 16 independent clones were obtained from the YS cDNA library and 9 independent clones from the LY336 cDNA library. The expression levels of these clones in YS and LY variants were compared with each other, as shown in Fig. 1A and 1B. Since some were expressed in both types of cells at the same level, we finally characterized 9 YS- and 6 LY336-specific cDNA clones (Table I and Table II). YS32 gene was not expressed in LY336, but was expressed in LY6 (Fig. 1A). As mentioned below, LY336 and LY6 seem to be of different cell origins, although both were of epithelial origin. LY336 and LY6 both expressed α-fetoprotein (AFP), but YS did not (data not shown). YS32 gene (proteasome subunit RC1) is apparently not a tissue-specific gene.

Subtracted genes As shown in Table I, YS expressed the high-affinity IgE receptor γ chain, interleukin-λ (IL-2) receptor γ chain and cytotoxic T-cell-associated gene transcript CTLA-1. The high-affinity IgE receptor γ chain and IL-2 receptor γ chain are known to be expressed in many immune cells, including cytotoxic T-cell and mast cells,[14] while CTLA-1 is restricted to activated cytotoxic T-cells, thymocytes and mast cells.[15] Thus, YS expresses T-cell/mast cell specific genes (non-epithelium-specific genes). On the other hand, LY336 and LY6 were found to

Table I. Identification of YS-specific Clones

Clone	Name[a]	Transcript size (kb)	Number of clones[b]
YS2	Mouse CTLA-1	1.4 & 1.1	13
YS4	Human IL-2 receptor γ chain	1.45	3
YS9	Unknown	1.6	2
YS19	Unknown	1	3
YS22	Unknown	0.9	3
YS32	Proteasome subunit RC1	1	2
YS1	IgE receptor γ chain	0.57	1
YS10	pJG116 repetitive sequence	2.6	1
YS15	Non neuronal enolase	1.7	1

a) Use of human or mouse was due to lack of rat clones in the Gene Bank.
b) "Number of clones" indicates the cloning frequency of each clone.

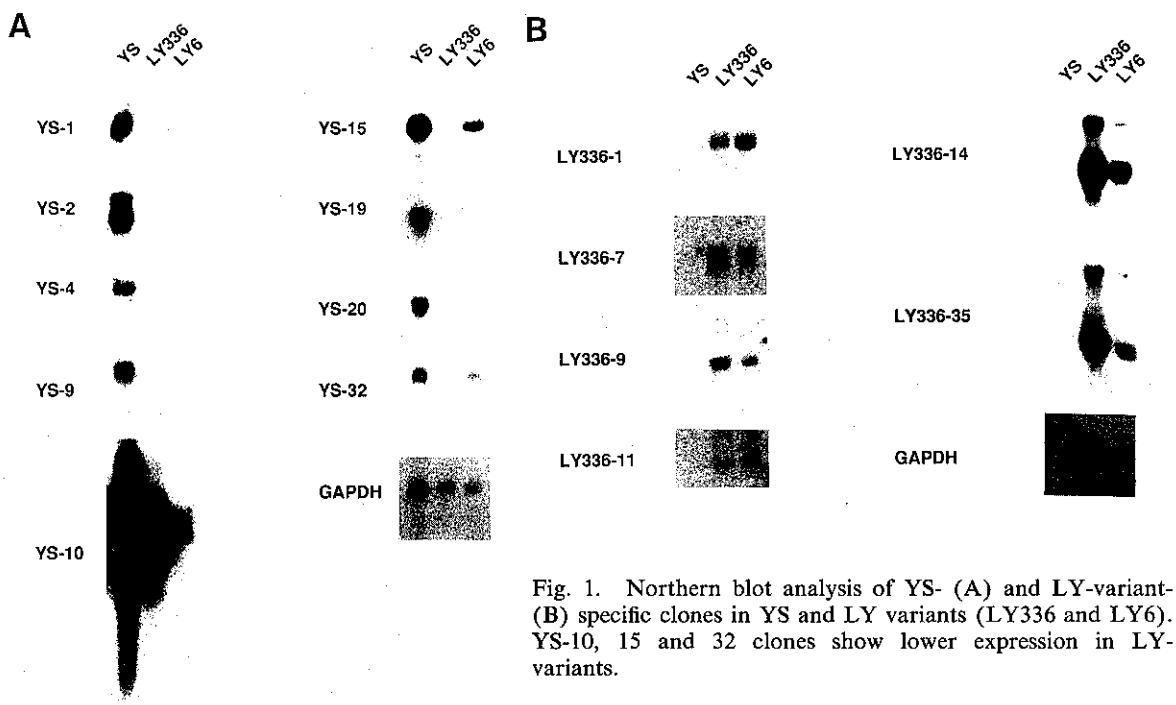

Fig. 1. Northern blot analysis of YS- (A) and LY-variant- (B) specific clones in YS and LY variants (LY336 and LY6). YS-10, 15 and 32 clones show lower expression in LY-variants.

express cytokeratin 8 and 18 (epithelium-specific genes) (Table II).

Expression of CD2 and CD3 antigens in YS To examine whether YS expresses other T cell-specific genes (CD2 and CD3), we performed immunohistochemistry. YS expressed CD2 (Fig. 2) and CD3 (data not shown) YS did not show metachromasia on toluidine blue staining for mast cells (data not shown).

Rearrangements of TCR β and γ chain genes in YS Since YS showed the expression of T cell-specific genes, we

Table II. Identification of LY-variants (LY336 and LY6) Specific Clones

Clone	Name[a)]	Transcript size (kb)	Number of clones[b)]
LY1	Cytokeratin 8	1.4	17
LY7	Unknown	1.5	2
LY9	Mouse cytokeratin 18	1.4	5
LY11	Unknown	6.5	1
LY14	Unknown	2.3 & 1.2	1
LY35	Mouse TIMP2	6.0 & 1.7	1

a) Use of human or mouse was due to lack of rat clones in the Gene Bank.
b) "Number of clones" indicates the cloning frequency of each clone.

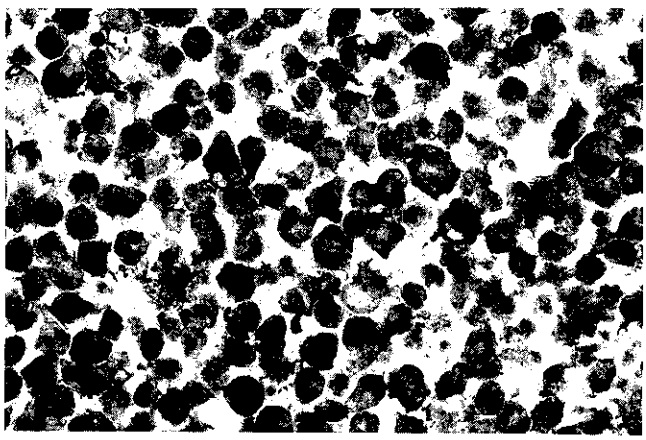

Fig. 2. Immunohistochemistry for CD2. CD2 was positive on the cell membrane surface of YS. ×20.

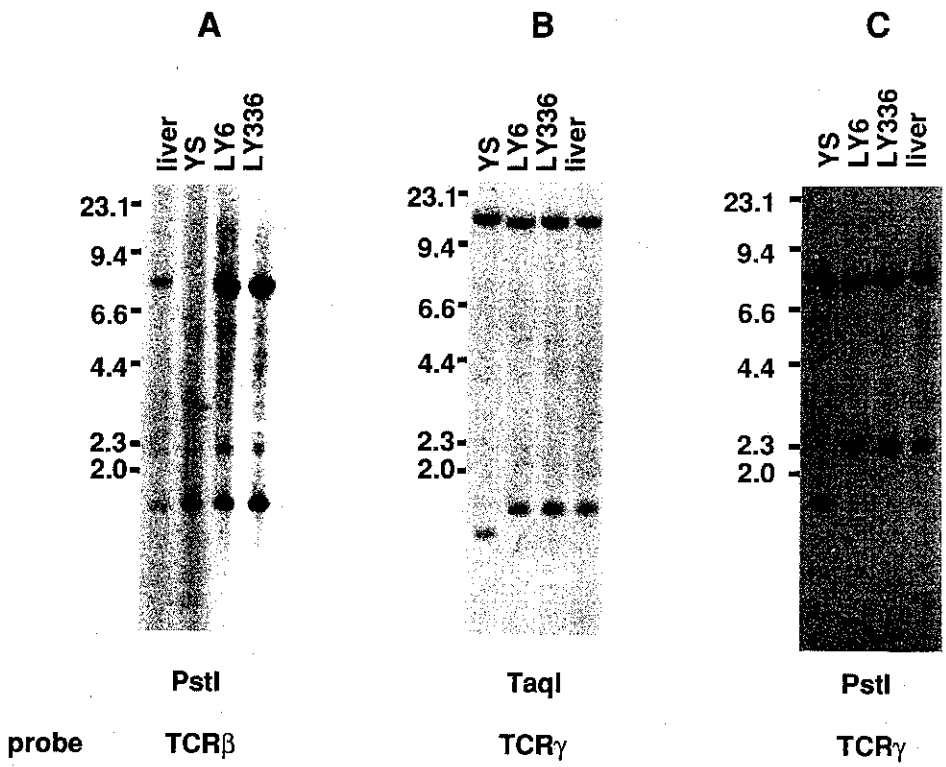

Fig. 3. Representative Southern blot analysis of rat normal liver, YS, LY6 and LY336 using TCR β chain (A) and TCR γ chain (B and C) gene probes. TCR β and γ chain genes are rearranged in YS, but not in a normal liver or in LYs. The sizes of DNA molecular weight markers (kb) are indicated on the left.

examined TCR gene rearrangements in YS and LYs using 6 different restriction enzymes (*Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I and *Taq*I). As shown in Fig. 3, we detected TCR β and γ chain gene rearrangements in YS, but not in LYs.

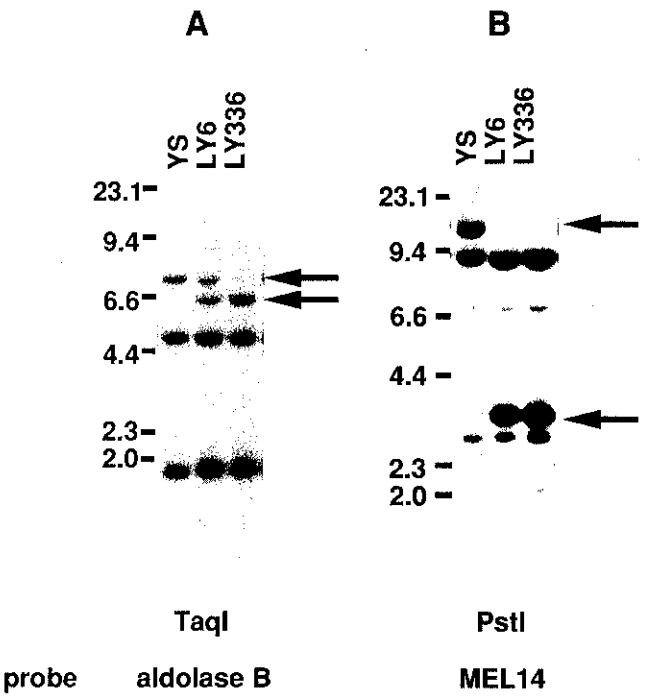

Fig. 4. Southern blot analysis of YS, LY6 and LY336 using RFLP probes; aldolase B and MEL 14. Arrows indicate polymorphic bands (unpublished data). YS and LYs show different RFLP patterns. The sizes of DNA molecular weight markers (kb) are indicated on the left.

YS, LY336 and LY6 show different RFLP patterns We performed RFLP analysis using aldolase B, mouse protamine and rat MEL14 as probes. As shown in Fig. 4, YS, LY336 and LY6 clearly showed different RFLP patterns. These results indicate that YS and LYs are genetically different from each other. Aldolase B gene (located on rat chromosome 5) showed different RFLP patterns (*Taq*I digestion) between LY6 and LY336 as shown in Fig. 4A. Protamine gene (located on rat chromosome 10) also showed different RFLP patterns (*Eco*RI digestion; data not shown), These results indicate that LY6 and LY336 were from different animals, since Donryu rats were not inbred at that time. These bands were not due to partial digestion because they were reproducible.

Histology of transplanted YS and LY336 Tumors were resected at 12 days (YS) or 25 days (LY336) after transplantation. Fig. 5 shows that LY336 was an epithelial tumor showing solid, trabecular and glandular structures, while YS was non-epithelial and lymphomatous.

DISCUSSION

Although YS has sarcomatous features, its origin has been considered to be a hepatocyte, based on the following evidence: I) the appearance of LY variants showing epithelial features[2,3]; II) some YS variants produce AFP *in vitro*[16]; III) the original rat, from which YS was established, had a small hepatoma nodule in addition to an ascites tumor[1]; IV) a series of rat ascites hepatomas (hepatocellular carcinomas) demonstrating free cell growth and sarcoma morphology could not be distinguished from YS.[4]

The present cDNA subtraction study clearly showed that YS and LY variants express genes that are normally specific for non-epithelial (hematopoietic) cells and epi-

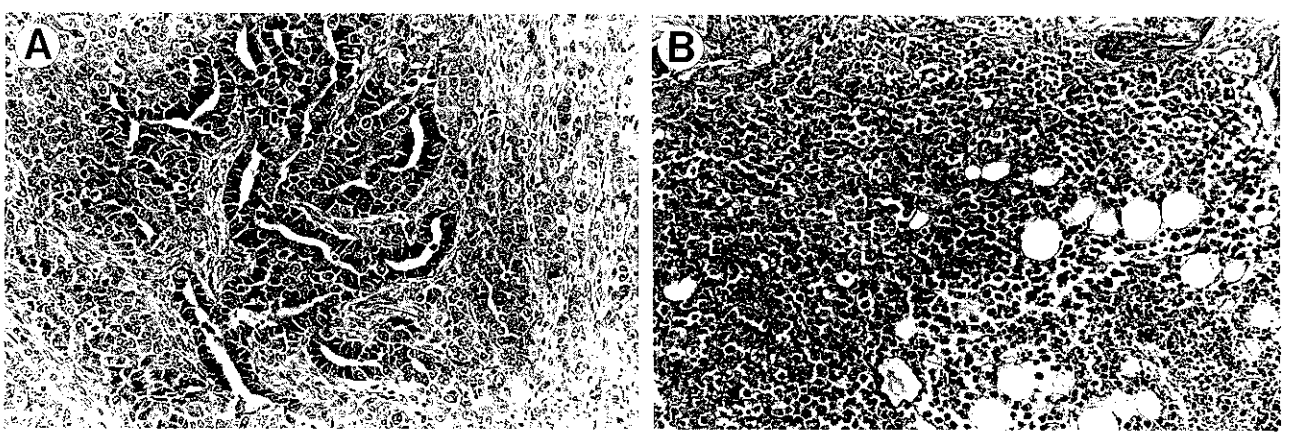

Fig. 5. Histology of transplanted tumors of LY336 (A) and YS (B). HE, ×20.

thelium, respectively. The normal strict regulation is not always maintained in tumor cells. Further, we can not exclude the remote possibility that expression of CTLA-1, IgE receptor γ and IL-2 receptor γ, and repression of cytokeratin 8 and cytokeratin 18 might occur in YS accompanying a lymphomatous nature, while the reverse is associated with adenocarcinomatous nature upon "LY transformation." However, we found that YS expressed CD2 and CD3 immunohistochemically, and TCR β and γ chain genes were rearranged in YS, but not in LYs. Moreover, LYs were revealed to be genetically different from YS by using RFLP probes. AH44 ascites hepatoma ("free cell" type) did not express CTLA-1 (data not shown). Thus, our findings indicate that the cellular origins of both YS and LY variants are different: we can conclude that YS is of non-epithelial origin, while LYs are of epithelial origin.

At present, the reason why the LY phenomenon occurs in YS-bearing rats is unknown. Recently, the development of Wilms' tumors in newborn rats, which were immunosuppressed with anti-thymocyte sera and xenografted with various human tumors (lymphomas and melanomas), was reported.[17] Although the age of the rats and type of developing tumor are different, this phenomenon seems quite similar to "LY transformation."

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