Gene Expression Profiling of Early- and Late-Relapse Nonseminomatous Germ Cell Tumor and Primitive Neuroectodermal Tumor of the Testis

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ABSTRACT

Purpose: **To better understand the molecular mechanisms that underlay the development and progression of nonseminomatous germ cell tumor of testis (NSGCTT) as well as malignant transformation of teratoma and primitive neuroectodermal tumor (PNET).**

Experimental Design: **We studied the gene expression profiles of 17 retroperitoneal NSGCTTs (10 yolk sac tumors, 3 embryonal carcinomas, 4 teratomas) and 2 PNETs obtained from patients with two clinical outcomes. Tissue samples were obtained from the Indiana University. One group of NSGCTT and PNET patients developed metastases within 2 years (early-relapse) of initial successful treatment, and the other group developed metastases after 2 years (late-relapse). Gene expression in these groups of patients was quantified using cDNA microarrays and real-time relative quantitative PCR.**

Results: **We demonstrate that the gene expression profiles of these tumors correlate with histological type. In addition, we identify type-specific genes that may serve as novel diagnostic markers. We also identify a gene set that can distinguish between early-relapse and late-relapse yolk sac tumors. The expression differences of these genes may underlie the differences in clinical outcome and drug response of these tumors.**

Conclusion: **This is the first study that used gene expression profiling to examine the molecular characteristics of the NSGCTTs and drug response in early- and late-relapse tumors. These results suggest that two molecularly distinct forms of NSGCTTs exist and that the integration of expression profile data with clinical parameters could enhance the diagnosis and prognosis of NSGCTTs. More importantly, the identified genes provide insight into the molecular mechanisms of aggressive NSGCTTs and suggest intervention strategies.**

INTRODUCTION

Testicular cancer is a relatively rare malignancy; however, an estimated 7,400 new cases were reported in the United States in 1999, and it is the most common cancer of men between the ages of 15 and 34 (1, 2). Based on morphological features, germ cell tumors are divided into seminoma, yolk sac tumor, embryonal carcinoma, choriocarcinoma, and teratoma according to the WHO International Histological Classification of Testicular Tumors (3). Non-germ-cell malignancies such as primitive neuroectodermal tumors (PNETs) arise in testicular germ cell teratoma and are associated with an increased number of relapses and a decreased relapse-free and overall survival (4). Teratoma is pluripotential tissue that can differentiate along ectodermal, endodermal, or mesodermal elements. PNET represents malignant transformation of teratoma along mesodermal lines.

Testicular cancer has a 95% cure rate, and most relapses occur within the first 2 years of therapy. Only 2–3% of patients treated for testicular cancer have late-relapse (defined as recurrence $\geq$2 years after initial successful therapy). Metastatic germ cell tumors have remarkably high cure rates. However, those with late relapse are found to be refractory to chemotherapy and carry a poor prognosis (5). Cytogenetic studies suggest most testicular tumors contain extra copies of the short arm of chromosome 12, and this genomic alteration may be responsible for the development of testicular neoplasms (6). Unfortunately, many of these additional differences remain unknown. However, differences in chemosensitivity and survival are most likely related to additional differences in underlying genetic and biological characteristics.

Microarray technology has provided new insights into the underlying molecular mechanisms of many types of cancers. The gene expression profiles from microarrays can identify the molecular signatures of cancers and can be used to distinguish histological types and to discover novel types. Classification of tumors by gene expression may reflect heterogeneity in transformation mechanisms, cell types, or aggressiveness among tumors. Furthermore, several studies have identified prognostic sets of genes that may underlie the heterogeneity in tumor aggressiveness (7–10). The identification of such genes may lead to the discovery of new potential targets for cancer diagnosis and therapy. Recently, differentially expressed genes on chromosome 17 in 18 testicular samples (15 germ cell tumors;

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Table 1 Differentially expressed genes in yolk sac tumor

Accession ID	Gene name	Fold change[a]	*P*
N62394	*Gap junction protein, β 1, 32kD*	6.5	0.0047
H59861	*Thrombomodulin (THBD)*	6.4	0.0350
AA775872	*Glypican 3*	4.2	0.0300
AA148737	*Syndecan 4 (amphiglycan, ryudocan)*	4.1	0.0062
R10382	*Serine (or cysteine) proteinase inhibitor, clade A, member 5*	4.1	0.0220
AA431347	*GATA-binding protein 4*	3.9	0.0017
H77652	*GATA-binding protein 6*	3.9	0.0036
AA858026	*Serine (or cysteine) proteinase inhibitor, clade A, member 5*	3.9	0.0230
N26536	*ATPase, Cu++ transporting, beta polypeptide*	3.2	0.0023
N38992	*GATA-binding protein 4*	3.1	0.0066
N53172	*G protein-coupled receptor*	3.1	0.0058
AA457700	*Stearoyl-CoA desaturase*	3.0	0.0044
AA452840	*Fibulin 2*	2.9	0.0120
AA001614	*Insulin receptor*	2.8	0.0180
AA487149	*Numb (Drosophila) homolog*	2.8	0.0088
R02036	*KIAA1543 protein*	2.7	0.0370
AA406266	*Hypothetical protein FLJ23309*	2.2	0.0170
AA490216	*CK2 interacting protein 1; HQ0024c protein*	−2.4	0.0180
AA701933	*CUG triplet repeat, RNA-binding protein 2*	−2.4	0.0290
AA459394	*LIS1-interacting protein NUDE1, rat homolog*	−2.6	0.0500
AA863383	*pim-2 oncogene*	−2.7	0.0360
N39240	*Homo sapiens cDNA FLJ11918 fis, clone HEMBB1000272*	−3.1	0.0034
AA455925	*Four and a half LIM domains 1*	−3.1	0.0190
AA047478	*Coronin, actin-binding protein, 1A*	−3.5	0.0220
AA157813	*Interferon, α inducible protein 27*	−3.6	0.0096
R06438	*Homo sapiens cDNA: FLJ22300 fis, clone HRC04759*	−3.7	0.0036
H73590	*IMMUNOGLOBULIN HEAVY CHAIN-RELATED*	−4.0	0.0086
T67053	*Immunoglobulin λ-like polypeptide 1*	−6.6	0.0091
AA663981	*Immunoglobulin heavy constant γ 3*	−6.9	0.0460
AA488070	*Immunoglobulin κ constant*	−7.4	0.0260
N92646	*Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain*	−7.4	0.0330

[a] Fold change indicates yolk sac tumors have relatively higher or lower expression of this fold change compared to other types of nonseminomatous germ cell tumor of testes and primitive neuroectodermal tumors studied.

3 normal testes) were identified using a microarray containing 636 cDNA. *Growth factor receptor-bound protein 7* and *junction plakoglobin* at the 17q11–q21, *lethal giant larvae homologue 2*, and *phosphodiesterase 6G* at the 17q24–qter were consistently the most up-regulated in the testicular tumors (11, 12). However, no study has identified a prognostic set of genes.

In this study, we characterized the molecular signature of 17 metastatic regions of nonseminomatous germ cell tumors of testes (NSGCTTs) and two PNETs by using 19,968 cDNA microarrays to elucidate their underlying molecular mechanisms. Furthermore, we sought to identify a gene set that can distinguish between early-relapse and late-relapse tumors and correlate with differences in survival and chemosensitivity.

MATERIALS AND METHODS

Patient Information and Tumor Samples. Tissue samples were obtained from 19 patients with metastatic lesions (NSGCTTs and PNETs) of the testis. The patients had undergone retroperitoneal lymph node dissection after chemotherapy at Indiana University. Nine patients had early relapse (within 2 years of initial management), whereas 10 had late relapse (after 2 years of initial management). Informed consent was obtained from patients to use their removed tissue specimens and clinicopathological data for research purposes. Approval was obtained by the Indiana University Internal Review Boards. The samples were rendered anonymous before the study. Part of each tumor sample was frozen immediately after the operation and stored at −80°C. Total RNA was isolated from the frozen tissue by using TRIzol reagent (Invitrogen, Carlsbad, CA). For all samples, total RNA was purified with 2.5 M final concentration of LiCl. The remaining parts of tumors were fixed with 10% buffered formalin, and the paraffin sections were stained with H&E. The WHO International Histological Classification of Tumors was used for histological evaluation of the specimens (3). Union Internationale Contre le Cancer Tumor-Node-Metastasis classification and stage groupings were also used (13).

cDNA Microarray Fabrication and Procedures. Microarray production was performed as described previously (14, 15) with slight modification. Briefly, 19,968 cDNA clones were PCR-amplified directly from bacterial stocks purchased from Research Genetics (Huntsville, AL). Following ethanol precipitation and transfer to 384-well plates, clones were printed onto aminosilane-coated glass slides using a custom-built robotic microarrayer. Normal human testicle total RNA (Ambion, TX) purified with 2.5 M final concentration of LiCl was used as a reference. For all samples, 50 μg of total RNA from tumors and reference were reverse-transcribed with oligodeoxythymidylic acid primer and Superscript II (Invitrogen) in the presence of Cy5-dCTP and Cy3-dCTP (Amersham Pharmacia Biotech, Pea-

Table 2 Differentially expressed genes in embryonal carcinoma

Accession ID	Gene name	Fold change[a]	*P*
N92646	*Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain*	12.1	0.0260
AA663981	*Immunoglobulin heavy constant γ 3*	10.1	0.0430
AA419251	*Interferon induced transmembrane protein 1*	9.4	0.0003
AA488070	*Immunoglobulin κ constant*	8.2	0.0470
W73144	*Lymphocyte cytosolic protein 1 (L-plastin)*	8.0	0.0004
AA863383	*pim-2 oncogene*	7.2	0.0007
H73590	*IMMUNOGLOBULIN HEAVY CHAIN-RELATED*	7.2	0.0005
AA047478	*Coronin, actin-binding protein, 1A*	7.0	0.0004
T67053	*Immunoglobulin λ-like polypeptide 1*	6.5	0.0300
N53214	*RIBONUCLEASE INHIBITOR-RELATED*	6.4	0.0066
AA406020	*Interferon-stimulated protein, 15 kDa*	5.8	0.0002
AA058323	*Interferon-induced transmembrane protein 1*	5.7	0.0003
H77597	*Metallothionein 1H*	5.2	0.0067
AA157813	*Interferon, α-inducible protein 27*	5.0	0.0042
N30205	*ESTs*	4.4	0.0120
AA599094	*KELCH-RELATED*	−3.7	0.0021
AA453170	*Hypothetical protein FLJ14299*	−3.9	0.0032
W87528	*Nuclear factor I/B*	−3.9	0.0180
R42421	*Cdc42 effector protein 2*	−4.0	0.0120
H12784	*ESTs*	−4.2	0.0012
AA488636	*KIAA0878 protein*	−4.9	0.0005
AA148737	*Syndecan 4 (amphiglycan, ryudocan)*	−5.0	0.0170
AA496878	*Hypothetical protein FLJ22041 similar to FK506 binding proteins*	−5.1	0.0002
N53172	*G protein-coupled receptor*	−5.4	0.0110
AA464250	*Keratin 19*	−6.5	0.0280
AA131299	*Hypothetical protein PRO1489*	−7.3	0.0029
W60582	*Uncharacterized hypothalamus protein HBEX2*	−7.5	0.0071
H77652	*GATA-binding protein 6*	−8.9	0.0015
N62394	*Gap junction protein, β 1, 32kD*	−9.0	0.0092
AA775872	*Glypican 3*	−11.0	0.0021

[a] Fold change indicates embryonal carcinomas have relatively higher or lower expression of this fold change compared to other types of nonseminomatous germ cell tumor of testes and primitive neuroectodermal tumors studied.

pack, NJ). The Cy5- and Cy3-labeled cDNA probes were mixed with probe hybridization solution containing formamide and hybridized to pre-warmed (50°C) slides for 20 h at 50°C. Following hybridization, slides were washed, dried by snap centrifugation, and scanned immediately using Scan Array Lite operating at 532 and 635 nm (GSI Lumonics, Billerica, CA).

Data Analysis. Images were analyzed by using the software GENEPIX PRO 3.0 (Axon Instruments, Foster City, CA). Spots showing no signal or obvious defects were excluded from the analysis. The local background was subtracted from the remaining spots, and the ratios of net fluorescence from the Cy5-specific channel to the net fluorescence from the Cy3-specific channel were calculated for each spot, representing tumor RNA expression relative to the normal testicular total RNA.

Microarray Analysis. Two sources of systematic bias were reduced in the data by normalization. Loess regression was performed on the log-transformed data to eliminate the intensity-dependent variation in the ratio of the spot intensities that has been observed in gene expression data (16, 17). This normalization was performed in a pin-dependent fashion to further reduce systematic variation related to the physical properties of each pin of the slide-spotting robot. Following loess normalization, the data were median-centered and rescaled using the median absolute deviation as described elsewhere (18, 19). These normalizations were carried out using the Bioconductor package[7] for the R statistical analysis framework (20). Unsupervised clustering was performed on the normalized data using CLUSTER and visualized using TREEVIEW.[8] The significance threshold was set at $P < 0.05$, and only those genes for which data were available in 75% of all 19 cases (*i.e.*, at least 15 in all) were included in the analysis (4,569 genes). Because the cases initially clustered according to histological type, indicating unique genetic signatures among the types, it was decided that the histological types should not be combined for the purpose of determining possible genetic predictors of outcome (early-relapse *versus* late-relapse). However, with the exception of yolk sac tumors, there were not enough cases within each histological type to perform this analysis. Therefore, the analysis of genes related to outcome was restricted to the yolk sac tumors.

Differentially expressed genes were identified using the Cluster Identification Tool application (21), which compares the mean expression in each group and uses random permutation to estimate *P*. Because of the possibility of false discovery, we selected the two differentially expressed genes in late-relapse NSGCTTs and four differentially expressed genes in late-re-

[7] http://www.bioconductor.org.
[8] MB Eisen; http://rana.lbl.gov.

Table 3 Differentially expressed genes in teratoma

Accession ID	Gene name	Fold change[a]	*P*
AA155913	*Matrix Gla protein*	7.4	0.0120
AA459308	*Elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)*	7.1	0.0003
R06438	*Homo sapiens cDNA: FLJ22300 fis, clone HRC04759*	7.0	0.0059
AA126989	*Myosin, heavy polypeptide 11, smooth muscle*	6.9	0.0020
AA459394	*LIS1-interacting protein NUDE1, rat homolog*	6.1	0.0018
AA156947	*Sirtuin 4*	5.4	0.0017
AA488073	*Mucin 1, transmembrane*	4.7	0.0190
N91811	*ESTs*	4.4	0.0200
AA134871	*Fibulin 1, transcript variant D*	4.3	0.0120
AA455925	*Four and a half LIM domains 1*	4.3	0.0140
R09729	*Serum deprivation response*	4.2	0.0025
AA496022	*Microfibrillar-associated protein 4*	4.1	0.0091
AA070226	*Selenoprotein P, plasma, 1*	4.0	0.0027
AA463926	*Protein phosphatase 1, regulatory (inhibitor) subunit*	4.0	0.0050
R67283	*ESTs*	3.9	0.0005
AA478066	*Membrane-associated tyrosine- and threonine-specific*	−2.7	0.0002
N70010	*Homo sapiens, similar to RIKEN cDNA 2610036L13 gene*	−2.7	0.0078
AA873159	*Apolipoprotein C-I*	−2.7	0.0460
AA663995	*Minichromosome maintenance deficient (mis5, S. pombe)*	−2.8	0.0012
AA682321	*NIMA (never in mitosis gene a)-related kinase 2*	−2.8	0.0054
AA071486	*Serine/threonine kinase 12*	−2.9	0.0013
AA629262	*Polo (Drosophia)-like kinase*	−2.9	0.0056
AA457034	*V-myb avian myeloblastosis viral oncogene homolog-like 2*	−2.9	0.0280
H59260	*Cell division cycle 25A*	−3.0	0.0063
AA448261	*High-mobility group protein*	−3.0	0.0130
AA419177	*Solute carrier family 7, member 5*	−3.0	0.0490
AA446462	*Budding uninhibited by benzimidazoles 1*	−3.1	0.0024
N30069	*ESTs*	−3.1	0.0150
AA136566	*Forkhead box M1*	−3.4	0.0014
R00707	*Stearoyl-CoA desaturase (Δ-9-desaturase)*	−3.7	0.0140

[a] Fold change indicates teratomas have relatively higher or lower expression of this fold change compared to other types of nonseminomatous germ cell tumor of testes and primitive neuroectodermal tumor studied.

lapse yolk sac tumors identified by this method and confirmed their expression level using real-time PCR.

Comparative Genomic Microarray Analysis Algorithm. To identify regional gene expression biases, gene expression values that map to a given chromosomal arm are collected, and a binomial test is used to determine if a significant upward or downward bias is present. First, sequence comparisons are used to map microarray probe sequences (the sequences that are placed on the microarray) to predicted Ensembl transcripts (Ensembl version 10.2; Ref. 22). Included in the Ensembl transcript annotations are chromosomal mapping locations at base-pair resolution. Expression values from multiple probes that map to the same gene are condensed to a single value by averaging. To apply the binomial test to expression data, of n non-zero expression values that map to a given chromosomal arm, r gene expression values are scored as "up" if the $\log_2$(R/G) value is positive and $(n - r)$ "down" if the log-transformed ratio is negative. This binomial test assumes that in a cytogenetically normal genomic region, the probability of a gene expression value being positive (p) is equal to the probability of the expression value being negative (q) such that $p = q = 0.5$. For the binomial test, the probability of obtaining r "up" observations with a probability p and $n - r$ "down" observations with a probability q is

$$P(r)_{\text{binomial}} = {}_nC_r{*}p^r{*}q^{n-r} = (n!p^rq^{n-r}/(r!(n-r)!).$$

In the cases where $n > 25$ and $n{*}p{*}q > 10$, the binomial probability density function is approximated using the normal probability density function, and a z-statistic for the chromosomal arm is computed such that $z = (2r - n)/\text{sqrt}(n)$. Therefore, a large positive z-statistic indicates a significant positive expression bias (*i.e.*, genomic gain), and a large negative z-statistic indicates the presence of a negative expression bias (*i.e.*, genomic loss). A set of chromosomal arm z-statistics can be plotted as a heat map to identify and summarize predicted cytogenetic features (23).

Real-Time Relative Quantitative PCR (RT-PCR). RT-PCR was performed in triplicate using the ABI PRISM 7700 Sequence Detection System according to the manufacturer's instructions, and these data were averaged. The two primers and the TaqMan probe were specifically designed for the following six genes: glutathione *S*-transferase θ1 (GSTT1); fatty acid synthase (FASN); phospholipase A2 group IIA (PA2IIA); trinucleotide repeat containing 3 (TNRC3); glypican 3 (GPC3); and glutaredoxin (GLRX) using Primer Express v1.5a (Applied Biosystems, Foster City, CA).

One hundred ng of each cDNA were amplified using PCR Master Mix according to the following PCR conditions: 50°C for 2 min; 95°C for 10 min; followed by 40 cycles of 95°C for 15 s; and 60°C for 1 min. Because 18S rRNA resulted in the least variation throughout the samples among a total of 11 housekeeping genes using Taqman Human Endogenous Control Plate (Applied Biosystems), this gene was used as the endogenous control. Each threshold cycle (C_T), which indicates the cycle at which an increase in reporter fluorescence just goes over the optimal value line, was determined. The C_T value of

Table 4 Differentially expressed genes in primitive neuroectodermal tumor

Accession ID	Gene name	Fold change[a]	*P*
AA683041	*Neuronal pentraxin II*	12.0	0.0110
AA670438	*Ubiquitin carboxyl-terminal esterase L1*	10.1	0.0052
H24007	*KIAA1878 protein*	9.8	0.0040
AA129861	*KIAA1878 protein*	9.4	0.0051
AA019748	*Aryl-hydrocarbon receptor nuclear translocator 2*	6.3	0.0052
AA670382	*H.sapiens mRNA for 3′UTR of unknown protein*	5.5	0.0170
N21633	*ESTs*	5.3	0.0160
AA435948	*Heat shock 40kD protein 1*	5.0	0.0230
AA454215	*NDRG family, member 4*	5.0	0.0160
AA775957	*ATPase, Na+/K+ transporting, α 3 polypeptide*	4.8	0.0170
AA137072	*ESTs*	4.8	0.0052
N50428	*Hypothetical protein FLJ10582*	4.4	0.0039
AA463251	*Nucleosome assembly protein 1-like 3*	4.4	0.0096
N66104	*Kinesin family member 5C*	4.2	0.0100
R74171	*KIAA0871 protein*	4.1	0.0052
AA099820	*ESTs*	−4.8	0.0240
AA465521	*SMC (mouse) homolog, Y chromosome*	−5.3	0.0170
AA412053	*CD9 antigen (p24)*	−5.6	0.0310
AA431347	*GATA-binding protein 4*	−5.9	0.0062
T73468	*Glutathione S-transferase A2*	−5.9	0.0180
H06273	*Keratin 8*	−6.1	0.0100
AA872020	*Protease, serine, 8 (prostasin)*	−6.1	0.0052
W37780	*Hypothetical protein*	−7.0	0.0038
T73090	*Plasminogen*	−7.4	0.0280
AA430665	*Claudin 4*	−7.5	0.0330
AA148737	*Syndecan 4 (amphiglycan, ryudocan)*	−7.6	0.0076
AA598517	*Keratin 8*	−9.4	0.0052
N62394	*Gap junction protein, β 1, 32kD*	−9.6	0.0190
N53214	*RIBONUCLEASE INHIBITOR-RELATED*	−12.3	0.0048
AA458849	*Serine protease inhibitor, Kunitz type, 2*	−14.1	0.0041

[a] Fold change indicates primitive neuroectodermal tumors have relatively higher or lower expression of this fold change compared to other types of nonseminomatous germ cell tumor of testes studied.

18S rRNA was subtracted from each C_T value of tumor or normal for normalization, and the ratio of tumor to normal testicular RNA expression was calculated to compare with microarray data.

RESULTS

Gene Expression Profiling. We analyzed the gene expression profiling data in two ways. First, we compared the gene expression of each tumor sample with normal testicular RNA to identify gene expression alterations that occur in NSGCTT and PNET. In addition, we identified particular subsets of genes that most strongly distinguished between late- and early-relapse patients.

Differentially expressed cDNAs in each type of tumors are listed in Tables 1-4. They are significantly up-regulated or down-regulated in each type of tumors compared to other types of tumors. A patient dendrogram based on a set of differentially expressed genes in each of the tumors compared with all other types of NSGCTTs (or PNETs) studied was shown in Fig. 1*A*. All differentially expressed cDNA in late-relapse tumors compared to early-relapse tumors and all differentially expressed cDNA in late-relapse yolk sac tumors compared to early-relapse yolk sac tumors are listed in Tables 5 and 6. A patient dendrogram based on the whole differentially expressed 13 cDNA set that might distinguish the late-relapse group from the early-relapse group is shown in Fig. 1*B*.

Real-Time Relative Quantitative PCR. Six genes were selected to confirm the microarray results with RT-PCR. To compare the gene expression ratios obtained by microarray to the RT-PCR data, the expression ratio of tumor to noncancerous testis was calculated by RT-PCR (Fig. 2). RT-PCR showed a significantly higher expression ratio of *GSTT1* ($P = 0.016$) in late-relapse yolk sac tumors (Fig. 2*A*) but not *FASN* ($P = 0.222$, Fig. 2*B*). *PA2IIA* and *TNRC3* were significantly under expressed in late-relapse yolk sac tumors ($P = 0.032$ and 0.016, respectively; Fig. 2, *C* and *D*), and *PA2IIA* was more highly expressed in early-relapse yolk sac tumors. *GPC3* and *GLRX* did not have significant changes ($P = 0.310$ and 0.548, respectively; Fig. 2, *E* and *F*).

Chromosomal Changes. We also used the gene expression profiles to infer chromosomal abnormalities in these samples. Chromosomal changes identified in our study are shown in Fig. 3. Frequent chromosomal changes of 6 and 12, as reported previously in NSGCTTs were confirmed. In addition, a novel chromosome change of 14q was frequently indicated by comparative genomic microarray analysis among late-relapse tumor in this study.

DISCUSSION

Differentially Expressed Genes in Each Type of NSGCTT. Identifying differentially expressed genes based on histological diagnosis may lead to the discovery of new markers for differ-

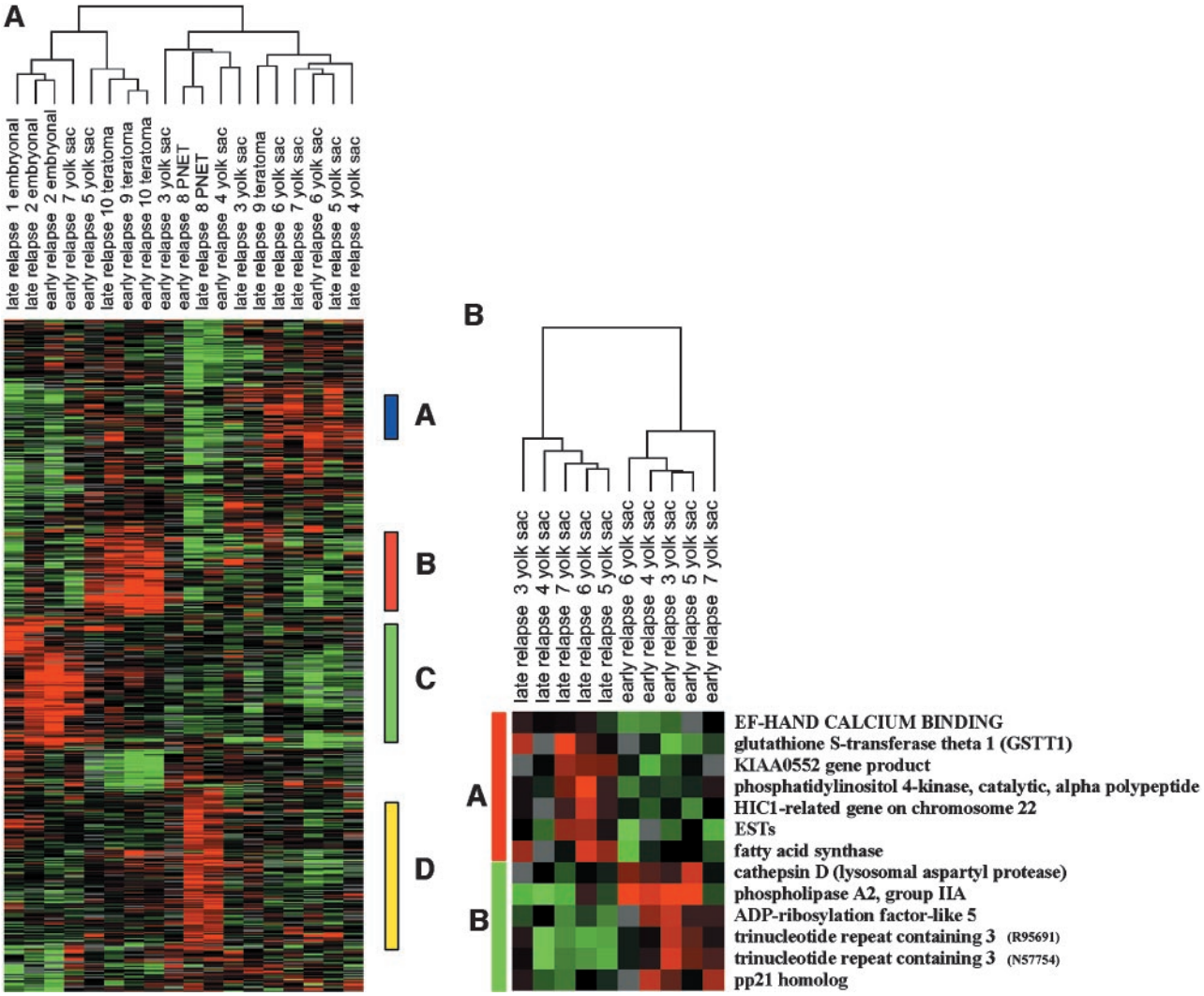

Fig. 1 *Panel A*, hierarchical clustering of 19 testicular tumors. Hierarchical clustering of the gene expression profiles of testicular tumors. *Rows* represent individual cDNAs, and *columns* represent individual tumor samples. The *color of each square* represents the median-polished, normalized, log-transformed gene expression ratio (tumor *versus* reference). Expression levels greater than the median are shaded in *red*, those below the median, *green*; equal to the median, *black*; and inadequate or missing data, *gray*. The *color saturation* indicates the degree of divergence from the median. Clustering is based on a combined set of genes that are unique to each subtype (total 543 cDNA). Basically, there are two main groups; one group consists of embryonal carcinomas and teratomas, and the other consists of yolk sac tumors and primitive neuroectodermal tumor. The highly-expressed genes from each tumor type are shown using *sidebars with different colors* (*A*, yolk sac tumor; *B*, teratoma; *C*, embryonal carcinoma; *D*, primitive neuroectodermal tumor). *Panel B*, clustering of 10 yolk sac tumors. Clustering based on 13 differentially expressed cDNAs that discriminate between late-relapse and early-relapse samples is shown in Fig. 1*B*. *A*, genes mostly over-expressed in tumors with the late relapse yolk sac tumors. *B*, genes mostly under-expressed in tumors with the late relapse yolk sac tumors.

ential diagnosis. Because global clustering analysis using 4,569 cDNAs showed that each type of NSGCTT had distinct molecular signatures, we sought to identify the differentially expressed genes contributing to these distinctions. However, the combination of teratoma and yolk sac tumor is quite common as is the combination of embryonal carcinoma and yolk sac tumor. These combinations may affect gene expression, therefore we will require a more detailed pathological review of those specimens that cluster with other tumor types.

Generally, while some immunoglobulin genes and immunological-related genes are differentially expressed in yolk sac tumors (Table 1), a large number of immunoglobulin genes, including IFN-related genes, are found in embryonal carcinomas (Table 2). The possibility exists that immunological response to the tumor may be important or that embryonal carcinoma is more immunogenic than yolk sac tumors. Differentially expressed genes in teratoma (Table 3) include proteins such as mucin, elastin, matrix protein, and fibulin, all of which are structural proteins and would be consistent with the type of histological characterization of well-differentiated teratoma. Similarly, the differentially expressed genes in PNET (Table 4) do not display any patterns other than relatedness to neuronal tissue (see below).

If we concentrate on individual genes, *gap junction protein*

Table 5 Differentially expressed genes in late relapse nonseminomatous germ cell tumor of testes (NSGCTT)s

Accession ID	Gene name	Fold change[a]	*P*
R02346	*Small nuclear ribonucleoprotein 70kD polypeptide*	4.3	0.0200
AA775872	*Glypican 3*	4.1	0.0360
H50323	*Fatty acid synthase*	3.9	0.0045
AA858026	*Serine (or cysteine) proteinase inhibitor, clade A member 5*	3.3	0.0500
AA233070	*KIAA0552 KINESIN-RELATED*	3.0	0.0066
AA844124	*ESTs*	2.5	0.0200
T64469	*p8 protein (candidate of metastasis 1)*	−2.2	0.0140
AA497051	*Sialyltransferase*	−2.3	0.0390
AA456975	*Apolipoprotein D*	−3.3	0.0450
AA447978	*Aldehyde dehydrogenase 1 family, member A2*	−3.6	0.0290
AA291163	*Glutaredoxin (thioltransferase)*	−4.0	0.0036

[a] Fold change indicates late relapse NSGCTTs and primitive neuroectodermal tumors have relatively higher or lower expression of this fold change compared to early relapse NSGCTTs and primitive neuroectodermal tumors studied.

β 1 (*GJB1*, *connexin 32*) is the most over-expressed gene in yolk sac tumors (Table 1). Mutations in the *GJB1* gene are responsible for the majority of cases of X-linked Charcot-Marie-Tooth disease. *Connexin* expression is frequently decreased in neoplasia and may contribute to defective growth control and loss of differentiated functions (24).

The genes of *acyl-CoA dehydrogenase* (C-2 to C-3 short chain; *ACADS*) are the most differentially under-expressed in yolk sac tumors. The *acyl-CoA dehydrogenases (ACDs*) are a family of mitochondrial flavoenzymes required for fatty acid β-oxidation and branched-chain amino acid degradation. The defects of *ACDs* in isoleucine and valine catabolism have been proposed in clinically diverse patients with an abnormal pattern of metabolites in their urine. In *ACDs* deficiency, the maternal serum and amniotic fluid concentrations of α-fetoprotein were elevated (25). This is a very interesting observation because there is a similar elevation of serum α-fetoprotein in yolk sac tumors.

ACADS and *immunoglobulin heavy constant* γ3 are the most over-expressed in embryonal carcinomas. Interestingly, *ACADS* is the most under-expressed gene in yolk sac tumors but is the most up-regulated gene in embryonal carcinomas. Immunoglobulin heavy chain has a relationship with multiple myeloma and plasma cell leukemia (26). Immunoglobulin genes including *immunoglobulin heavy constant* γ3 may play important roles in embryonal carcinoma.

Differentially under-expressed genes in the embryonal carcinoma are *GPC3* and *gap junction protein* β1. *GPC3* is a membrane-bound heparin sulfate proteoglycan. The *GPC3* gene is located at Xq26, frequently deleted in advanced ovarian cancer cell lines (27). Expression of *GPC3* is also silenced in human breast cancer (28) and decreased in human gastric cancer (29). In contrast, the expression of *GPC3* in Wilms' tumor, hepatoblastoma, and hepatocellular carcinoma were increased (30, 31). In our study, expression of *GPC3* and *Gap junction protein* β1 in yolk sac tumors was increased. These results show some of the molecular genetic differences between yolk sac tumors and embryonal carcinomas.

Matrix Gla protein (*MGP*) is the most over-expressed gene in teratomas (Table 3). *MGP* is a vitamin-K-dependent protein and is synthesized in a variety of tissues such as lung, heart, kidney, cartilage, and bone. *MGP* mRNA levels have been found to be elevated in a breast cancer cell lines, 600 polyethylenimine, primary renal-cell carcinomas, prostate carcinomas, and testicular germ-cell tumors (32, 33).

PNET is a malignant, small, round cell tumor that exhibits neuroepithelial differentiation. The histological designation of PNETs in the testis (neuroblastoma or medulloepithelioma) did

Table 6 Differentially expressed genes in late relapse yolk sac tumors

Accession ID	Gene name	Fold change[a]	*P*
H99813	*Glutathione S-transferase θ 1 (GSTT1)*	6.0	0.0160
AA844124	*ESTs*	4.0	0.0430
H50323	*Fatty acid synthase*	3.9	0.0320
AA233070	*KIAA0552 gene product*	3.7	0.0190
T67442	*HIC1-related gene on chromosome 22*	3.0	0.0082
AA421269	*Phosphatidylinositol 4-kinase, catalytic, α polypeptide*	2.8	0.0380
AA196393	*EF-HAND CALCIUM BINDING*	2.4	0.0250
AA485373	*Cathepsin D (lysosomal aspartyl protease)*	−2.5	0.0330
H96654	*pp21 homolog*	−3.0	0.0400
AA424568	*ADP-ribosylation factor-like 5*	−4.3	0.0088
R95691	*Trinucleotide repeat containing 3*	−5.3	0.0300
N57754	*Trinucleotide repeat containing 3*	−5.4	0.0170
T61271	*Phospholipase A2, group IIA*	−9.7	0.0150

[a] Fold change indicates late relapse yolk sac tumors have relatively higher or lower expression of this fold change compared to early relapse yolk sac tumors studied.

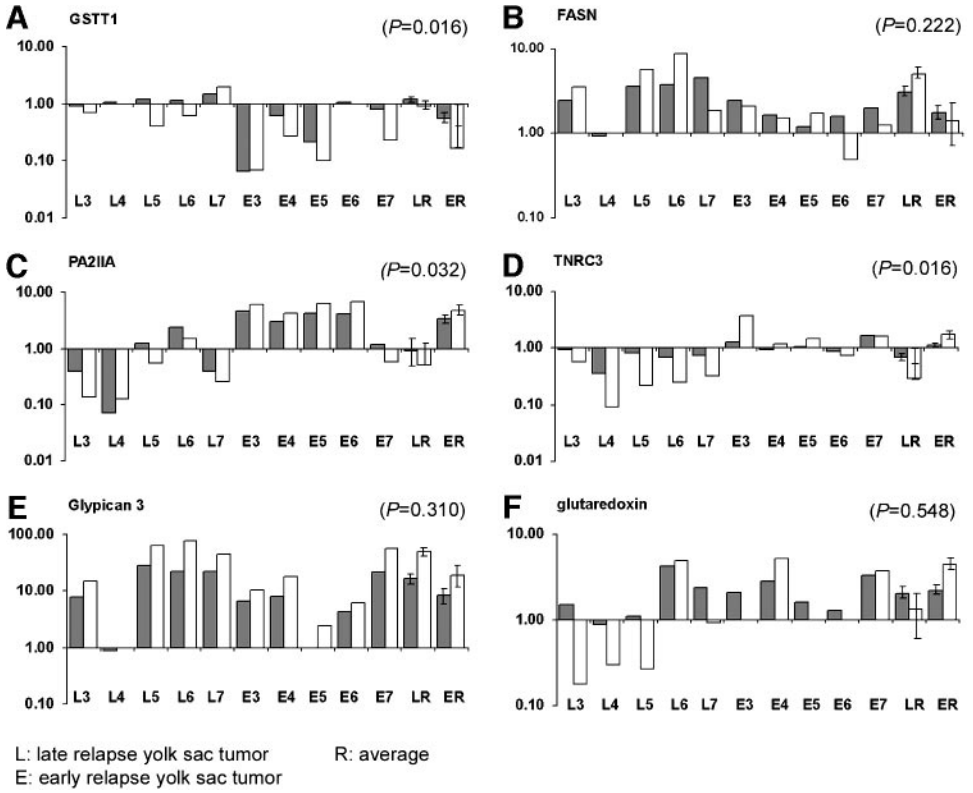

Fig. 2 Real-time relative quantitative PCR analysis of *GSTT1* (*A*), *FASN* (*B*), *phospholipase A2 group IIA* (*C*), *TNRC3* (*D*), *glypican 3* (*E*), and *glutaredoxin* (*F*). The *left side bar in gray* of each sample indicates the expression ratio of tumor to normal testis by real-time relative quantitative PCR and the *right side bar in white* indicates the expression ratio by microarray experiments. Data grouped based on clinical parameters are given as the mean and the SE. They are evaluated statistically using the Mann-Whitney *U* test. All real-time relative quantitative PCR data were consistent with microarray data. *GSTT1* was significantly highly expressed in late-relapse yolk sac tumors ($P = 0.016$; *A*), but *FASN* was not significantly different between late- and early-relapse yolk sac tumors ($P = 0.222$; *B*). *PA2IIA* and *TNRC3* were significantly under-expressed in late-relapse yolk sac tumors ($P = 0.032$ and 0.016, respectively; *C* and *D*). However, *glypican 3* and *glutaredoxin* also were not significantly different between the two groups. $P = 0.310$, *E*; $P = 0.548$, *F*.

not predict which tumor metastasized. Extratesticular PNETs in patients with testicular germ-cell tumors are usually fatal, but patients with neuroblastomatous metastases may have a more prolonged course (4).

Neuronal pentraxin II and *ubiquitin COOH-terminal esterase L1* (*UCHL1*) are the most over-expressed genes in the PNETs (Table 4). The existence of a family of pentraxin proteins that are expressed in the brain, and other tissues may play important roles in the uptake of extracellular material (34). *Protein gene product 9.5 (PGP 9.5*), most likely identical to *UCHL1*, is a major constituent of cytoplasmic polypeptides in neurons. There is immunoreactive *PGP 9.5* in many neuroendocrine cells, in spermatogonia, Leydig cells of the testis, ova, and some cells of the corpus luteum (35). *PGP 9.5* is also highly expressed in peripheral PNET cell lines and embryonic tumors (36). These findings are also consistent with our gene expression profiling results.

Serine protease inhibitor Kunitz type 2 (*SPINT2*) and *RNase inhibitor-related* are the most differentially under-expressed genes in the PNETs. *SPINT2* is down-regulated in normal human fibroblast MRC-5 cells expressing an activated H-*ras* oncogene and in the human fibrosarcoma cell line HT1080 (37). The human RNase inhibitor 2 is expressly limited to testis and may play critical roles in human spermatogenesis (38).

Differentially Expressed Genes in Each Prognosis: Late-Relapse and Early-Relapse NSGCTTs. To identify differentially expressed genes in late-relapse NSGCTTs and PNETs compared to early-relapse NSGCTTs and PNETs, we first identified genes that were at least 2-fold up- or down-regulated in at least 75% of the tumors. Next, we identified genes that could distinguish between early- and late-relapse samples. The six up-regulated and five down-regulated genes that met the above criteria are summarized in Table 5. Both previously known and unknown genes were found to be significantly up- or down-regulated in 19 tumors, but the change values, both up and down, were small. The most over-expressed gene in the late-relapse group is *small nuclear ribonucleoprotein 70kD polypeptide*. The U1–70kD small nuclear ribonucleoprotein auto antigen is a major target of B-cell responses in patients with connective tissue diseases (39).

Glutaredoxin (GRX) and *aldehyde dehydrogenase* (*ALDH*)*-1 family member A2* are the most differentially under-expressed genes in the late-relapse group. GRX, also known as

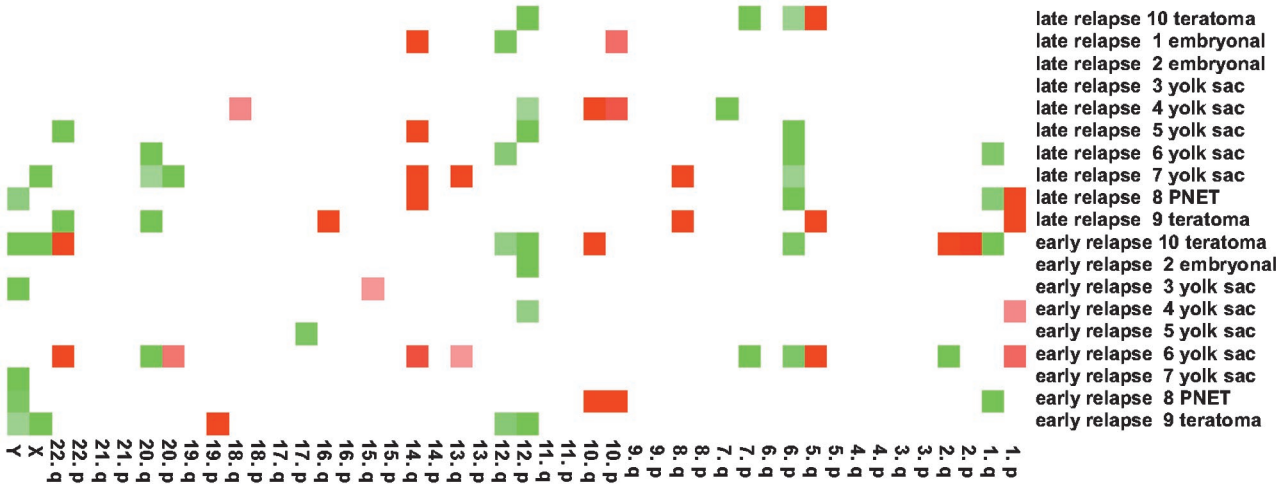

Fig. 3 Chromosomal changes in nonseminomatous germ cell tumor of testis (or primitive neuroectodermal tumors) are shown. *Red* corresponds to a gain of chromosome, and *green* corresponds to a loss of chromosome. Frequent changes of chromosome 6 and 12 as reported previously in nonseminomatous germ cell tumor of testes were confirmed.

thioltransferase, is a small dithiol protein that has been shown to be involved in regulating various cellular functions (40). ALDHs are a group of enzymes that catalyze the conversion of aldehydes to the corresponding carboxylic acids. The expression of *ALDH-1* is higher in radioresistant cervical squamous cell carcinomas cells (41). Generally, genes related to the cell function may play some role between the two categories.

Differentially expressed genes in late-relapse yolk sac tumors are shown in Table 6. The seven up-regulated and six down-regulated genes that met the criteria are summarized in Table 6. The most over-expressed gene in late-relapse yolk sac tumors is *GSTT1*. The *glutathione S-transferases* (*GSTs*) have been implicated as susceptibility genes for a number of cancers. The homozygous deletions of *GSTT1* [*GSTT1*(−)] group had a worse prognosis than either the homozygous or heterozygous for *GSTT1* [*GSTT1*(+)] group in acute myeloid leukemia (42). On the other hand, the *GSTT1*(−) genotype conferred the reduction in risk of relapse compared to the *GSTT1*(+) in children with acute lymphoblastic leukemia (43, 44). Furthermore, *GSTT1*(−) was associated with decreased time to the next primary tumor presentation in the basal cell carcinoma (45). Our results suggest that *GSTT1* may play an important role for the relapse frequencies of yolk sac tumors.

Phospholipase A2 group IIA (*PLA2IIA*) is the most differentially under-expressed gene in late-relapse yolk sac tumors. On the other hand, it is the most differentially over-expressed gene in early-relapse yolk sac tumors. This enzyme releases free fatty acids through catalysis of membrane in mammalian cells. The product arachidonic acid is metabolized to produce prostaglandins and leukotrienes that mediate a diverse array of biological activities including inflammation, mitogenesis, and tumor cell invasion. *PA2IIA* can generate arachidonate from cellular phospholipids. The expression of *PA2IIA* is elevated in the prostatic cancer, and dysregulation of this enzyme may play a role in prostatic carcinogenesis and progression (46, 47). Furthermore, breast cancer patients with high PLA2 expression have significantly shorter disease-free and overall survival than those patients with low PLA2 expression (48). Therefore, *PLA2IIA* may play some roles in the relapse of yolk sac tumors.

Real-Time Relative Quantitative PCR. All real-time PCR data were consistent with microarray data (Fig. 2); however, in some cases the expression ratios calculated by real-time PCR were different from those obtained by microarray. Spot saturation and competitive hybridization during the microarray experiments and the amplification methods of PCR may result in these discrepancies.

Initial cisplatin-based combination chemotherapy is curative for many patients with metastatic testicular cancer. Subsequent second-line chemotherapy with high-dose carboplatin plus etoposide will cure approximately half of the relapsing patients. However, patients who experience a late relapse are rarely cured with any type of chemotherapy (49). Our results suggest that yolk sac tumor, with over-expressions of *GSTT1* and under-expressed *PLA2IIA*, will have a risk of late-relapse and drug resistance. For this group of patients, we need careful follow-up on the patients after 2 years of initial management. In contrast, yolk sac tumors with *GSTT1* under-expression, and *PLA2IIA* over-expression will have relapsed early, but cisplatin-based combination chemotherapy has proven more effective.

In conclusion, we have identified gene expression alterations that are specific for each type of NSGCT and PNET. The identified genes may give insight into tumorigenesis and progression of these tumors. We have also identified gene expression alterations that distinguish between early- and late-relapse yolk sac tumors. This may have important clinical implications because the late relapse patients may require a different approach. In addition, some of these discriminating genes may give potential insights into new drug targets.

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