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Gene expression patterns in AIDS versus non-AIDS-related diffuse large B-cell lymphoma

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Abstract

Diffuse large B-cell lymphoma (DLBCL) is more prevalent and more often fatal in AIDS patients compared to immune-competent individuals. Potential explanations for these differences include distinct tumorigenic mechanisms and/or altered cellular microenvironments. We previously discovered that the *TCL1* (*T-cell leukemia-1*) proto-oncogene is expressed in a high proportion of AIDS–DLBCL compared to DLBCL cases and that aberrant *TCL1* expression causes DLBCL in a new transgenic mouse model. Here, we continue to search for other genes that may contribute to the differential pathogenesis of DLBCL in AIDS. Gene subtraction yielded over 1800 potential AIDS–DLBCL candidates, of which about 50% were unknown and not further considered. The remaining 50% of genes were annotated and, when combined with miniarray screening from multiple patient samples, were reduced to 18 candidate genes for extended analysis. These 18 genes showed distinct patterns of expression in both AIDS–DLBCL and DLBCL samples. However, unlike *TCL1*, none of these genes was preferentially associated with either AIDS–DLBCL or DLBCL. Our data suggest that the increased incidence and severity of AIDS–DLBCL compared to DLBCL is likely due to crippled immune surveillance rather than to markedly different gene expression profiles.

Keywords: AIDS; Diffuse large B-cell lymphoma; TCL1; Suppression subtractive hybridization; Somatic hypermutation

Introduction

Diffuse large B-cell lymphoma (DLBCL)[1] encompasses a heterogeneous group of mature peripheral B-cell malignancies (Harris et al., 2000). DLBCL is 60 to 200-fold more likely to occur in individuals with AIDS than in the general HIV-negative population (Gaidano et al., 1998; Knowles and Pirog, 2001; Weiss, 1999). Unlike DLBCL, which has variable morbidity and mortality, AIDS–DLBCL almost always demonstrates an aggressive course and poor outcome. The reason(s) for the increased incidence and extreme severity of DLBCL in AIDS patients compared to others are not resolved but have been thought to depend on decreased host resistance and possibly unique mechanisms of tumor formation in AIDS–DLBCL.

In an initial study to identify genetic factors that differ between AIDS–DLBCL and DLBCL, we employed sup-

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[1] Abbreviations used: DLBCL, diffuse large B-cell lymphoma; *TCL1*, T-cell leukemia-1; SSH, suppression subtractive hybridization; GC, germinal center; PTLDL, posttransplant lymphoproliferative disorder lymphoma; HYP, HIV-infected hyperplastic lymph node; NHL, non-Hodgkin's lymphoma; SHM, somatic hypermutation.

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pression subtractive hybridization (SSH) to isolate differentially expressed genes between two patient samples (Teitell et al., 1999). We chose lymph-node-derived AIDS–DLBCL and DLBCL samples for SSH that were negative for herpes virus (EBV and HHV-8) infection and lacked lesions in proto-oncogenes or tumor suppressors previously associated with mature B-cell cancers. These tumors had no known molecular lesions to promote malignancy, originated from the same body location, and differed, as best as could be determined, only by the fact that one developed in a patient with AIDS while the other one did not. We found high expression of the *TCL1* proto-oncogene in the AIDS–DLBCL sample versus a complete lack of *TCL1* expression in the DLBCL sample (Teitell et al., 1999). Thereafter, we showed that a high proportion of AIDS–DLBCL tumors aberrantly expressed *TCL1* compared to a significantly lower frequency of DLBCL samples (Said et al., 2001; Teitell et al., 1999). We attribute the increased frequency of dysregulated *TCL1* expression in AIDS to destruction of critical immune regulatory cells and lymphoid organ architecture, resulting in profound disruption of signals that normally down-regulate *TCL1* expression in germinal center (GC) B-cells. Supporting this hypothesis, we have shown that signals relayed from the surrounding microenvironment, such as CD40L with IL-4 and IL-10, silence *TCL1* expression in normal tonsillar B-cells (Said et al., 2001) . In fact, the AIDS–DLBCL sample used in SSH demonstrated a more profound destruction of the surrounding nodal microenvironment, as evidenced by its lack of the dendritic cell chemokine *BCA-1,* compared to the abundance of *BCA-1* seen in the DLBCL sample (Teitell et al., 1999). Subsequently, we created a new transgenic mouse model in which dysregulated *TCL1* expression alone promoted a high rate of mature B-cell lymphoma, including DLBCL (Hoyer et al., 2002). These results confirm our strategy for isolating novel tumorigenic genes from AIDS–DLBCL patient samples by SSH. They also suggest that changes in the tumor cells themselves occur from altered microenvironment signaling and further lead us to predict that genes in addition to *TCL1* will be dysregulated by this mechanism.

Here, we explore this prediction by investigating additional differentially expressed genes that were isolated in the same SSH survey as *TCL1*. We obtained numerous uncharacterized gene fragments that will require further workup before their potential role(s) in tumor formation can be evaluated. We selected a moderate number of known annotated candidate genes and analyzed these in multiple AIDS–DLBCL and DLBCL samples. These selected genes showed no expression preference for either AIDS–DLBCL or DLBCL patient samples. Rather, the pattern of gene expression for the constellation of genes examined was unique for each AIDS–DLBCL and DLBCL patient sample, supporting the disease heterogeneity previously observed by traditional pathologic analyses and recently confirmed for DLBCL by gene expression profiling (Alizadeh et al., 2000; Shipp et al., 2002). The striking similarity between gene expression profiles for these AIDS–DLBCL and DLBCL samples strongly suggests that the distinct biological differences in incidence and outcome between DLBCL patients with and without AIDS are due to crippling of the immune system by HIV and not due to marked differences in gene expression.

Materials and methods

Patient samples, cDNA preparation, and SSH

Fresh-frozen normal and tumor biopsy samples were obtained from individuals with AIDS and those uninfected by HIV, in strict accordance with UCLA institutional policies for the protection of human subjects. These samples were chosen from a large tissue bank and were selected based upon the absence of EBV and HHV-8 infections, along with the lack of oncogenic mutations or alterations in *p53, RAS, c-MYC, BCL-2,* and *BCL-6* genes (Teitell et al., 1999). Samples were examined histologically and tissue blocks were trimmed to exclude areas of necrosis or surrounding nonlymphoid tissues. Microtome sections (5–10 μm) were placed into 5 ml of RNA STAT-60 (Tel-Test, Friendswood, TX) for total RNA extraction according to the manufacturer's instructions. cDNA was synthesized from 0.4 to 0.5 μg of total RNA (Superscript II, Gibco-BRL, Gaithersburg, MD) using the SMART PCR cDNA kit (Clontech, Palo Alto, CA) followed by PCR amplification under conditions recommended by the manufacturer, as has been previously reported (Teitell et al., 1999).

Bidirectional SSH was performed between an AIDS–DLBCL (AIDS IBLP 1 from Teitell et al., 1999) and a DLBCL (non-AIDS IBLP 1 from Teitell et al., 1999), a PTLDL (posttransplant lymphoproliferative disorder lymphoma), or a HYP (HIV-infected hyperplastic lymph node; HYP 2 from Teitell et al., 1999) using the PCR Select cDNA Subtraction kit (Clontech), as done previously (Teitell et al., 1999). Subtracted cDNA fragments were cloned into the plasmid pCR2.1 using the TOPO TA cloning kit followed by transformation into TOP10 competent *Escherichia coli* and plating (Invitrogen, Carlsbad, CA).

Miniarray preparation, probe screening, and fragment classification.

Approximately 4000 colonies from the AIDS–DLBCL minus DLBCL SSH direction were manually picked into 96-well plates. Bacterial cultures in 96-well plates were transferred using the Multi-Blot Replicator (V&P Scientific, San Diego, CA) into 96-well Cycleplates (Robbins Scientific, Sunnyvale, CA) and PCR amplified. The quality of the PCR-generated fragments was confirmed on 1% agarose gels stained with ethidium bromide and inserts were generally 300 to 800 bp (data not shown). PCR products were transferred to replicate MagnaCharge nylon filters (Micro

Table 1
Probe mixture of predominant gene fragments used in initial miniarray screen[a]

Annotation	Fragment name	GenBank Accession Numbers
Cell structure	HSC 70	P19120; P19378; Y00371
	HSP 90	D87666; P08238; P11499
Cytokines, receptors, and secreted proteins	β2-Microglobulin	P01884
	CD20	P11836
	CD20 receptor	X07203
	CD45	Y00638; P08575
	J-chain	M12759; P01591
	Igκ	X95747
	Igλ	(Various—18 distinct GenBank entries)
Energy metabolism	HMG-CoA reductase	P00347
	Acetyl-CoA acetyltransferase	JC2378
	ACADM	M16827
	MCAD	P11310
	NUDT5	AF155832
	ADH5	M81118
	ATP synthase	P18859; X60221; O75964; P00846
	Cytochrome *b*	S69130; P00156
	Cytochrome *c* oxidase	P00395; P24311
	UDPGP 1	O35156
	Glutaredoxin	P35754
	G3PD	P04406
	PP	AF154065
	LDH-B	P07195
	COII	U12693; P00403; M25171
	COG	P14854
	COIII	P00414
	COXII	X15759
	ND2	AF014882
	CI-B12	O43676
	CI-MLRQ	O00483
	NADH oxidoreductase	P03901; P03905
	SDHD	AB026906
	CI-MNLL	O75438
	Pyruvate dehydrogenase	X57778
	NAD(+) SSAD	P51649
	Thioredoxin peroxidase 2	Q06830
	UDPGP2	Q16851
	ATPase subunit	AF038954
	Quinone reductase	Q08257
Protein synthesis and degradation	TLERS	P41252
	12S rRNA	X93342
	16S rRNA	X99189
	TRNAs	V00660; V00710
	tRNA synthetase subunit	AF042346

[a] Gene fragments were identified from a SSH with AIDS–DLBCL (AIDS IBLP 1 from Teitell et al., 1999) minus DLBCL (non-AIDS IBLP 1 from Teitell et al., 1999) patient samples followed by PCR amplification and sequencing, as previously described (Teitell et al., 1999). Equivalent amounts of each PCR fragment listed were combined to generate a probe mixture of predominant genes and used to screen replicate stamped miniarray filters.

Separations, Westburough, MA) using the Multi-Blot Replicator. Several controls were spotted on the filters, including an irrelevant *Lemna gibba* RuBPCase plant gene, blank positions, and orientation markers. DNA on the filters was denatured, neutralized, and UV crosslinked in the Stratalinker (Stratagene, La Jolla, CA). Random-primed [α-^{32}P]ATP (NEN, Boston, MA)-labeled probes were generated with the Prime-It II Random Primer Labeling Kit (Stratagene) from a pool of predominant nontumorigenic cDNA fragments (Table 1) or subtracted cDNA populations. Filter hybridizations were performed in aqueous buffer (0.5 M $NaPO_4$ pH 7.0, 1 mM EDTA, 7% SDS, 1% BSA) at 62°C overnight and blots were washed with buffer (1.5 mM NaCl, 0.15 mM Na citrate, 0.1 × SSC, 0.1% SDS) at least three times for 15 min each at 60 °C. Hybridization signals were determined by autoradiography.

Identified cDNA fragments were determined by high-throughput automated sequencing of both DNA strands (Amgen Sequencing Core Facility, Thousand Oaks, CA). Sequences were searched in GenBank's nucleotide and protein databases using the BLAST algorithm (Altschul et al., 1997). Clones were assigned to a functional category based

upon GenBank annotations. cDNA gene fragments to be used as probes in Northern and "virtual" Northern blots were prepared by PCR amplification using Advantage cDNA Polymerase Mix (Clontech) with subsequent *Rsa*I enzyme digestion to remove linkers, followed by agarose gel purification (Millipore, Bedford, MA).

Cell culture and RNA preparation

Cell lines were maintained at 37°C in a humidified environment with 5% CO_2. RPMI 1640 medium with 4 mM Hepes (Omega Scientific, Tarzana, CA) was supplemented with 0.05 mM β-mercaptoethanol (Sigma, St. Louis, MO), 0.1 mM nonessential amino acids (Gibco-BRL), 1 mM sodium pyruvate (Gibco-BRL), 4 mM L-glutamine (Omega), 10 μg/ml penicillin (Omega), and 10 U/ml streptomycin (Omega). The RPMI medium was additionally supplemented with 5 to 10% fetal bovine serum (Omega) for the following human B-cell lines: 2F7, 10C9, RRBL, E, R, KS-2, SU-DHL-5, SU-DHL-6, SU-DHL-8, SU-DHL-9, NU-DHL-1, RL, HT, Ramos, and BL41. IMDM medium (Omega) was supplemented with the same reagents and additionally supplemented with 20% pooled human plasma containing 15 U/ml heparin sulfate, 28.5 mM $CaCl_2$, and 34.8 mM $MgCl_2$–$7H_2O$ (Sigma) for the following human DLBCL cell lines: OCI-LY-1, -3, -4, -7, -8, -10, -12, -13.2, -18, and -19. These lines were obtained from Eric Davis (NIH, Bethesda, MD) with the kind permission of Hans Messner (University of Ontario, Canada).

Total RNA was prepared from cell lines growing in log phase with the RNeasy Midiprep Kit (Qiagen, Carlsbad, CA) using the TissueTearor homogenizer (Bartlesville, OK). RNA concentrations were determined by UV spectroscopy and quality was confirmed on formaldehyde gels stained with ethidium bromide (data not shown).

Northern and "virtual" Northern blots

For Northern blots, 10 μg of total RNA was fractionated in 1% agarose, 0.67 M formaldehyde gels in 1× Mops running buffer (0.4 M Mops, 0.1 M NaOAc, 10 mM EDTA), transferred to Nytran nylon filters using the Turboblotter (Schleicher & Schuell, Keene, NH) overnight in 10× SSC, and UV crosslinked in the StrataLinker (Stratagene). Equal sample loading was verified by ethidium bromide gel staining (data not shown). Random-primed [α-^{32}P]ATP (NEN)-labeled probes were generated as above. Probe hybridizations were performed in Dendhardt's solution (5× Dendardt's buffer: 0.1% Ficoll 400, 0.1% polyvinylprrolidone, 0.1% BSA Fraction V; 5× SSC, 50% formamide, 1% SDS) at 42°C overnight. Filters were washed twice with 1× SSC, 0.1% SDS at 42°C for 15 min and twice with 0.1 × SSC, 0.1% SDS at 52°C for 15 min and hybridization signals were determined by autoradiography.

For "virtual" Northern blots, cDNA prepared from reverse-transcribed total RNA was fractionated in 1% agarose gels with 1× TBE running buffer, denatured, neutralized, transferred to Nytran nylon filters as described above, and baked at 80°C for 30 min. Equal sample loading was verified by ethidium bromide gel staining (data not shown). Hybridizations were performed with [α-^{32}P]ATP (NEN)-labeled probes as described above.

Somatic hypermutation analysis

The extent of somatic hypermutation was determined for the AIDS–DLBCL used in SSH from 165 independent subtracted clones. Each sequence was compared to germline V-region sequences (V-BASE) using the DNAPLOT program.

Results

Categorization of subtracted gene fragments by annotation

SSH was performed with histologically indistinguishable AIDS–DLBCL and DLBCL patient samples as previously described (Teitell et al., 1999). Both samples were selected because they were found to lack specific genetic lesions and herpes virus infections known to play a role in the development of this common non-Hodgkin's lymphoma (NHL) subtype (Teitell et al., 1999). The AIDS–DLBCL minus DLBCL subtracted cDNA population was cloned and PCR-generated cDNA inserts from ~4000 clones were initially prescreened by hybridization in replicate stamped miniarrays with a probe mixture of predominant gene fragments not thought to have a role in tumor formation (e.g., *Igλ, J-chain;* summarized in Table 1). Arrayed gene fragments that hybridized with this predominant probe mixture were excluded from further analyses. Roughly 1800 cDNA fragments did not hybridize to this mixed probe, were identified by automated sequencing, and were categorized according to GenBank database annotations into functional groups (Table 2).

A diverse collection of genes encompassing multiple functional categories was identified. Generally, categories with >100 of the 1800 sequenced cDNA fragments contained multiple isolates of specific genes. For example, within the category denoted Cytokines, Receptors, and Secreted Proteins, 264 cDNA fragments were identified, and over 85% of these were *Igλ* or *J-chain.* Although not rigorously assessed, most if not all of these fragments were from regions of *Igλ* and *J-chain* genes that were not included in the prescreening predominant probe mix. As previously reported for this specific SSH, multiple isolates of *TCL1* were identified from the AIDS–DLBCL patient sample (Teitell et al., 1999). This collection of fragments represented >300 known genes potentially involved in tumor formation from diverse categories, as assessed by their an-

Table 2
Classification of unique and repeated gene fragments isolated with SSH that did not hybridize with the probe mixture of predominant gene fragments

Annotation	No. of isolates	Annotation	No. of isolates
Unique	710	Signal transduction	36
Cytokines, receptors, and secreted proteins	264	Cell cycle	21
Protein synthesis and degradation	191	Proliferation	19
Unknown	136	Transcription	18
Energy metabolism	134	DNA replication	10
Cancer related	83	Known oncogenes	7
Miscellaneous	68	Cell life and death	5
Cell structure	66	Differentiation	4
Chromatin and nuclear structure	42	Grand total	1814

notated functions (data not shown). Strikingly, nearly half of the 1800 clones (47%) fell into categories with no annotations and uncharacterized functions (Table 2). These initial results suggest that a diverse set of mainly Unique and Unknown genes is potentially differentially expressed in AIDS–DLBCL compared with DLBCL.

Preliminary selection of genes by miniarray screening with subtracted probe populations

To further identify genes with a potential role in tumor formation that are differentially expressed in AIDS–DLBCL versus DLBCL, the >300 annotated gene fragments were screened on replicate filter-stamped miniarrays. Initially, radiolabeled cDNA pools from multiple patient samples were used as screening probes. However, hybridization signals with these probe pools were low and very few clones were identified at significant levels above background nonhybridizing *L. gibba* RuBPCase plant gene controls stamped on the filters (data not shown). This is reasonable since our strategy of SSH, followed by cloning and removal of predominant genes not likely involved in cancer formation, could yield a selection of genes that may be differentially expressed but also very rare, from low copy number mRNAs. Therefore, screening was subsequently performed with bidirectionally subtracted cDNA populations to enrich for signal intensities of differentially expressed genes. We used AIDS–DLBCL versus DLBCL, AIDS–DLBCL versus PTLDL, and AIDS–DLBCL versus HYP pools from single paired subtractions to create six probe sets for screening. As expected, 44% of cDNA gene fragments hybridized with cDNA probes from the AIDS–DLBCL minus DLBCL, PTLD, and/or HYP subtraction direction while only 11% of the stamped fragments hybridized with cDNA populations from the reverse subtraction direction (data not shown). Nearly one-third (29%) of the stamped positions did not differentially hybridize with any probe combination in either direction, reflecting the imperfect removal of common cDNAs with SSH in complex populations. Results from screening with these six probe sets were complicated (data not shown). We used the criteria that *TCL1* was differentially expressed in AIDS–DLBCL versus DLBCL and PTLDL samples to select additional candidate genes for further analysis in cell lines and additional patient samples. The rationale for this selection criteria was that a similar dual screening pattern identified *TCL1* as differentially expressed between AIDS–DLBCL and DLBCL in our prior studies (Teitell et al., 1999). However, one difference between this and the prior study was the requirement for a differential result between an AIDS–DLBCL and a PTLDL rather than a HYP (Teitell et al., 1999). We further reasoned that this was desirable since this would reduce the number of potential differentially expressed genes due to non-HIV-mediated immune suppression. Applying these criteria, we selected 18 genes including *TCL1* and 3 novel, recently identified genes that showed preferential hybridization to both AIDS–DLBCL minus DLBCL and AIDS–DLBCL minus PTLDL probe sets on replicate stamped miniarrays (Table 3). We also included for further analysis two genes due to their recently reported role(s) in tumor metastases, *Thymosin-β* and *RhoA*, which were identified during sequencing but did not exhibit this hybridization pattern (Clark et al., 2000; del Peso et al., 1997).

Of our 20 candidate genes, 11 have established functions in proliferation, DNA replication, cellular signaling, transcription, and cytoskeletal organization. The remaining 9 genes described below have poorly understood or unknown functions. Annotations from GenBank indicate that *ART4* is an adenocarcinoma antigen recognized by T-cells, that *Silencer element* is the human homologue of the chicken *Silencer element SCG10* gene, and that *GS3786* is a novel gene isolated from human primary-cultured osteoblast cells of unclear function. *RGS13* is an uncharacterized member of the family of regulator of G-protein signaling (RGS) which down-regulate signaling by heterotrimeric G-proteins as a mechanism of signal desensitization (Druey et al., 1996; Koelle, 1997). *LHFP* is a novel translocation partner of *HMGIC* in human lipoma (Petit et al., 1999). *UNR* (upstream of RAS) was identified by close physical linkage to *N-RAS* (Jeffers et al., 1990). *NGP-1* is an autoantigen of breast that localizes to the nucleolus and contains GTP-binding motifs (Racevskis et al., 1996). *LDF* (leukemia differentiation factor) is a differentiation-specific factor iso-

Table 3
Gene fragments identified by stamped miniarray screening that preferentially hybridized to AIDS–DLBCL minus DLBCL and AIDS–DLBCL minus PTLDL probe sets

AIDS–DLBCL (−) DLBCL	AIDS–DLBCL (−) PTLDL	Fragment name	Genbank Accession No.	Annotation
+	+	Cyclin G	U53328	Cell cycle
+	+	T-cell cyclophilin	Y00052	Miscellaneous
+	+	AMY1	AB007191	Transcription
+	+	TCL1	X82240	Cancer related
+	+	JAW-1	I38656	Cell structure
+	+	SMAD1/SETβ	S68987	Cancer related
+	+	Silencer element	D50375	Transcription
+	+	Stathmin	P16949	Cancer related
+	+	ART4	AB026125	Cancer related
+	+	HMG1	U51677	DNA replication
+	+	RGS13	O14921	Signal transduction
+	+	GS3786	D87120	Unknown
+	+	PAG	X67951	Proliferation
+	+	NGP-1	Q13823	Energy metabolism
+	+	LDF	S51027	Cancer related
+	+	UNR	P18395	Unknown
+	+	Moesin	P46150	Cell structure
+	+	LHFP	AF098807	Unknown
+		Thymosinβ		Cancer related, metastases
		Rho A		Cell cycle, metastases

lated from the myelogenous leukemia line HL-60 (Kostanyan et al., 1994). *JAW-1* is a developmentally regulated lymphoid gene that localizes to the endoplasmic reticulum, suggesting a role in vesicle targeting and fusion (Behrens et al., 1994). Thus, these incompletely characterized proteins in our isolates have predicted functions in cell signaling, transcription, differentiation, and protein targeting that may be involved in AIDS–DLBCL pathogenesis.

AIDS–DLBCL genes with enhanced expression were not confirmed in human B-lymphoma lines

To establish AIDS–DLBCL enhanced expression of the 18 genes identified by SSH and miniarray screening, total RNA from 4 AIDS–DLBCL, 15 DLBCL, 2 AIDS–BL, 3 BL, and 1 diffuse mixed lymphoma (DML) human cell lines was prepared and analyzed by Northern blot (data not shown). Cell lines rather than primary samples were chosen for initial analysis because ample, renewable materials could be generated. Three patterns of expression could be identified (summarized in Table 4). One pattern included those genes without detectable expression in any of the lymphoma cell lines tested (*AMY1, Moesin, SMAD1/SETβ, HMG1, LHFP, UNR,* and *GS3786*). Another pattern showed relatively equal expression in AIDS–DLBCL and DLBCL cell lines (*ART4, NGP-1, PAG, Cyclin G,* and *RhoA*). The final pattern exhibited moderately increased gene expression in DLBCL compared to AIDS–DLBCL cell lines (*TCL1, RGS13, JAW-1, Stathmin, LDF, T-cell cyclophilin,* and *Silencer element*). These data are unexpected for AIDS–DLBCL enhanced gene expression. For example, *TCL1* expression was not detected in AIDS–DLBCL cell lines unlike the pattern seen in multiple primary tumor samples. Several possibilities exist to explain this apparent discrepancy between primary lymphoma samples and cell lines. One is that it may be difficult to establish AIDS–DLBCL lines that express *TCL1,* thereby selecting against such lines. However, it is apparently not difficult to find and grow lines outside this classification that express minimal to abundant *TCL1* and an alternate reason for a selective absence in the lines tested may be a sampling artifact (Table 4). A third possibility is that cell lines evolve in culture and may have a distinct gene expression profile compared to when they were derived from the original diseased tissue. Other explanations are also possible, but the net effect is that we detect a general lack of concordance between gene expression levels in primary patient samples and established tumor cell lines. For this reason, determination of the pathogenesis of AIDS–DLBCL and DLBCL requires examination of tumor samples derived directly from patients.

AIDS–DLBCL and DLBCL annotated genes show similar expression patterns

Patient sample cDNA from 7 AIDS–DLBCL, 2 DLBCL, 1 PTLDL, and 1 HYP was analyzed by "virtual" Northern blot (summarized in Table 5). Two general expression patterns for the 20 annotated genes under investigation were observed. One pattern included genes that were expressed in both AIDS–DLBCL and DLBCL patient samples at moderate to robust levels (*Stathmin, Silencer element, PAG, LHFP, HMG1, RhoA, UNR, Cyclin G, T-cell cyclophilin,*

Table 4
Summary of gene expression levels in human B-cell lines by Northern blot[a]

Type	Cell line	*TCL1*	*RGS13*	*JAW-1*	*Stathmin*	*LDF*	*T-cell cyclophilin*	*Silencer element*	*PAG*	*ART4*	*NGP-1*	*Cyclin G*	*RhoA*
BL	RL	+++	+++	+++	+++	++	++++	++++	++++	++++	++	++	+
DML	HT	++	+++	++	+++	+++	+++	++++	++++	++++	++	++	++
BL	Ramos	++++	++++	+++	+++	+++	+++	++++	++++	++++	++	++	++
BL	BL41	+++	−	++	+++	++	+++	++++	++++	++++	++	+	+
AIDS–BL	2F7	++	+	++	+++	+++	+++	++++	++++	++++	++	++	++
AIDS–BL	10C9	−	−	−	+++	++	+++	++++	++++	++++	++	+	++
AIDS–DLBCL	RRBL	−	−	−	+	+	++	++	++	++++	+	++	+
AIDS–DLBCL	E	−	−	−	+	+	++	++	++	++++	+	++	+
AIDS–DLBCL	R	−	−	−	+	+	++	++	++	++++	+	++	+
AIDS–DLBCL	KS-2	+	−	−	+	+	++	++	+++	+++	+	++	+
DLBCL	SU.DHL.5	+	++++	++++	++	++	++	++++	+++	++++	+	++	++
DLBCL	SU.DHL.6	++	+++	+++	++	++++	++	++++	++++	+++	++	++	++
DLBCL	SU.DHL.8	++++	+	+	++++	++++	++	++++	++++	++++	++	++	++
DLBCL	SU.DHL.9	++++	++	++	+++	++++	++++	++++	+++	++++	+	++	++
DLBCL	NU.DHL.1	++	+	++	+	+	+	++	++++	+++	++	++	+
DLBCL	OCI.LY.1	++	++	++	+++	+++	++++	++++	++++	++++	++	++	++
DLBCL	OCI.LY.3	+	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
DLBCL	OCI.LY.7	++	++	++	++	+++	++	++++	+++	+++	+	++	++
DLBCL	OCI.LY.18	+++	−	++	+	+++	+	++++	+++	+++	++	++	++
DLBCL	OCI.LY.19	+++	++	+++	++	+++	+++	++++	++	+++	+	++	++
DLBCL	OCI.LY.8	+++	−	n/d	n/d	++	++	++	+++	n/d	++	n/d	n/d
DLBCL	OCI.LY.10	++	+	n/d	n/d	+	++	+++	+++	n/d	+	n/d	n/d
DLBCL	OCI.LY.12	−	−	+	+	+	++	+++	+++	+++	++	++	n/d
DLBCL	OCI.LY.13.2	−	−	n/d	n/d	n/d	++	++++	n/d	n/d	n/d	n/d	n/d
DLBCL	OCI.LY.4	−	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d

[a] Expression was scored − to ++++. Genes with undetectable expression in all lines examined include *AMY1, Moesin, SMAD1/Setβ, LHFP, GS3786, HMG1,* and *UNR. Thymosinβ* was not examined. BL, Burkitt's lymphoma; DML, diffuse mixed lymphoma; n/d, not determined.

ART4, and *LDF*). Another pattern included genes that were either very weakly expressed or undetectable in all patient samples examined (*JAW-1, AMY1, Moesin, SMAD1/Setβ, GS3786, NGP-1,* and *Thymosin-β*). A potential exception to these two patterns was *RGS13* which was undetectable in 3 of 5 AIDS–DLBCL examined but was detectably expressed in all DLBCL, PTLDL, and HYP samples tested. Another exception was the pattern of *TCL1* expression, as previously reported (Teitell et al., 1999). *TCL1* is the only differentially expressed gene identified in these subtractions that distinguishes AIDS–DLBCL and DLBCL patient samples. Exact *TCL1* expression levels in some patient samples varied modestly from those previously reported and confirmed and these differences are most likely attributable to biased PCR amplification during the manufacture of cDNA pools following SSH. With the exception of *TCL1*, the combined

Table 5
Summary of gene expression levels in patient tumor samples by "virtual" Northern blot[a]

Type	*TCL1*	*Stathmin*	*Silencer element*	*PAG*	*LHFP*	*HMG1*	*RhoA*	*RGS13*	*UNR*	*Cyclin G*	*T-cell cyclophilin*	*ART4*	*LDF*
AIDS–DLBCL[b]	++++	++++	+	++++	++	+/−	++	−	+	+++	++	+	+++
AIDS–DLBCL	++++	++	++	++	+++	+	+	−	+	+++	++++	++	+++
AIDS–DLBCL	+	−	n/d	+	+	n/d	n/d	n/d	n/d	+	n/d	n/d	+++
AIDS–DLBCL	−	+++	n/d	+++	++	n/d	n/d	n/d	n/d	+++	n/d	n/d	++++
AIDS–DLBCL	+++	++	+	+++	++	+	+	−	−	+	++++	++	++++
AIDS–DLBCL	−	++++	+++	+++	++	++	+	+	−	+++	++++	+	++++
AIDS–DLBCL	−	+	+	+++	+++	+	+	+++	−	+++	++++	++	++++
DLBCL[b]	−	+++	+	++++	+++	+	++++	+	++	+++	++++	++	++++
DLBCL	+	++++	+++	++++	+++	++	++++	+	++	+++	++++	++	++++
HYP	++	−	+/−	+	+	+	+	+	−	++	++++	+	++++
PTLDL	++++	+++	+	++++	++	++	+	+	−	++++	++++	+	++++

[a] Expression was scored − to ++++. Genes with undetectable expression in all samples examined include *JAW-1, AMY1, Moesin, SMAD1/Setβ, GS3786, NGP-1,* and *Thymosinβ.*

[b] AIDS–DLBCL and DLBCL samples used in SSH. n/d, not determined.

data indicate similar expression patterns for the 20 annotated genes identified by SSH and miniarray screening between AIDS–DLBCL and DLBCL patient samples.

Gene expression patterns demonstrate the heterogeneity of AIDS–DLBCL and DLBCL patient samples

Since the expression patterns of our identified annotated genes appeared similar between AIDS–DLBCL and DLBCL, we next examined the levels of expression within each of the AIDS–DLBCL and DLBCL patient samples. The 13 genes with detectable expression by virtual Northern blots (*TCL1, Stathmin, Silencer element, PAG, LHFP, HMG1, RhoA, RGS13, UNR, Cyclin G, T-cell cyclophilin, ART4,* and *LDF*) showed unique expression level patterns for each patient sample within AIDS–DLBCL and DLBCL categories (Table 5). This variation in gene expression among these patient samples is in agreement with the previously described heterogeneity for both AIDS–DLBCL and DLBCL pathogenesis (reviewed in Bower, 2001; Diebold et al., 1997; Gaidano et al., 2000; Knowles, 1999).

AIDS–DLBCL sample used in SSH exhibits markedly reduced SHM

Errors in the B-cell somatic hypermutation (SHM) mechanism that target non-Ig genes are considered a major driving force for GC- and post-GC-based mature B-cell malignancies including DLBCL (Pasqualucci et al., 1998, 2001; Shen et al., 1998). However, the general destructive effects of HIV on lymphoid organ cellularity, structure, and function make it uncertain whether this mechanism is optimally functioning within the B-lineage cells of AIDS–DLBCL. Therefore, we analyzed Ig V-regions from the AIDS–DLBCL sample used in SSH for SHM. The extent of SHM was determined from 165 subtracted and sequenced Ig cDNA clones from this AIDS–DLBCL and compared to germline V-region sequences in the GenBank database. One hundred of these sequences did not align with a germline V-region and likely represented C-region sequences. From 65 clones matching germline V-region sequences, 3 V_L loci and 1 V_H locus were observed (data not shown). V_L 2b2 sequences were 83% (54/65), V_L 2a2 sequences were 1.5% (1/65), and V_L 3r sequences were 11% (7/65) of the total clones examined. Most likely, the finding of multiple light chain loci suggests that this tumor is clonal for V_L 2b2 and contaminated by a minority of bystander nonmalignant B-cells expressing V_L 2a2 and V_L 3r sequences. The average mutation frequency per clone examined for V_L 2b2 was 1.4%, while that for V_L 3r from bystander B-cells was 4.8% (data not shown). Mutation frequencies ranging from 0 to 14.4% have been reported for AIDS-related lymphomas (Bessudo et al., 1996; Delecluse et al., 1999; Julien et al., 1999), and 0–13 mutations in V_H loci have been reported in AIDS–DLBCL specifically (Delecluse et al., 1999; Julien et al., 1999). Thus, the AIDS–DLBCL used for SSH here demonstrated minimal (1.4%) SHM. By contrast, DLBCL typically undergo high levels of SHM (12.7%) of IgV-regions (Pasqualucci et al., 2001). These data suggest that SHM and selection events were interrupted in this AIDS–DLBCL specimen compared to the typical DLBCL from immune-competent patients. This implies that in certain AIDS–DLBCL, the rate of Ig SHM and associated mistargeting of SHM to non-Ig genes may be markedly reduced and therefore potentially not as significant a factor in the evolution of specific AIDS–DLBCL.

Discussion

These studies were initiated to identify genes that were differentially expressed in AIDS–DLBCL versus DLBCL. The demonstration of such genes would establish that distinct tumorigenic mechanisms contribute to the radical differences in frequency and severity of outcome observed in these patient populations. Using a sensitive, PCR-based SSH strategy, we previously showed that the *TCL1* proto-oncogene was expressed in a higher proportion of AIDS–DLBCL compared to DLBCL and that this dysregulation could promote tumorigenesis in a new mouse transgenic model (Said et al., 2001; Teitell et al., 1999; Hoyer, et al., in press, 2002). To identify differentially expressed genes in addition to *TCL1* in AIDS–DLBCL samples, we expanded the analysis of the original AIDS–DLBCL minus DLBCL subtraction direction by coupling the output of this subtraction to miniarray screening on replicate stamped nylon filters with subtracted probe pools from three separate SSH. We screened ~4000 cDNA fragments and classified over 1800 sequenced cDNA clones according to GenBank annotations. Roughly half of the cDNA fragments (47%) lacked annotation and have no characterized functions. We then picked approximately 300 nonredundant and nonoverlapping fragments with a potential role in tumorigenesis based upon annotation for further analysis. We initially attempted to screen these fragments on miniarrays with probe pools from multiple patient sample cDNAs but could not obtain quantifiable signals (data not shown). One possible reason for this is that the nearly identical tumors in the SSH, and our removal of predominant nontumorigenic clones in a preselection step, eliminated major differences between the samples and, with the exception of *TCL1,* yielded differentially expressed cDNA fragments of very low abundance. In theory, SSH will isolate such low abundance transcripts that would be below the level of detection for nonamplified probe sets, such as those used in current gene-chip screening technologies (Alizadeh et al., 2000; Klein et al., 2001; Shipp et al., 2002). Therefore, we attempted to use amplified screening probe sets made from three paired SSH to enrich for low abundance, differentially expressed fragments. With criteria previously used to identify *TCL1,* we screened over 300 cDNA clones potentially involved in tumor development on miniarrays with amplified subtracted probe sets and

identified 18 candidates for further evaluation. Evaluation indicated that none of these 18 additional candidates was preferentially expressed in AIDS–DLBCL versus DLBCL in multiple patient samples or cell lines. To date we have only identified AIDS–DLBCL-preferential expression for *TCL1*. Nevertheless, the results of this comparison uncover several important points about the pathogenesis of AIDS–DLBCL and DLBCL.

The lack of differentially expressed genes between AIDS–DLBCL and DLBCL strongly suggests a marked similarity between the subtracted samples. In fact, the samples used for SSH were originally selected to minimize differences between AIDS–DLBCL and DLBCL in order to isolate genes expressed preferentially in the AIDS–DLBCL sample due to AIDS. The expectation is that differential expression would be found in genes that are more profoundly affected by signaling from the environment. The finding of differential *TCL1* expression is consistent with this hypothesis (Teitell et al., 1999). Also, transactivation by HIV TAT expression could potentially yield differentially expressed genes by this approach but none were identified.

Many of the genes we identified have not been previously implicated in DLBCL, and more than half of the candidate genes (13/20) used in our surveys were also not found on the Lymphochip (Alizadeh et al., 2000), implying their relatively unexplored roles in lymphocyte cancer biology. Genes that may be implicated in AIDS–DLBCL and DLBCL may be those with higher expression compared to PTLDL and/or HYP. However, lower expression in HYP may reflect the greater diversity of cell types in the lymph node, compared to DLBCL and PTLDL tissues that contain predominantly tumor cells. Stathmin, also called Op18, p18, p19, and prosolin, was expressed in 6 of 7 AIDS-DLBCL, 2 of 2 DLBCL, and 1 PTLDL sample, but not in HYP. Stathmin is expressed in most proliferating cells, including GC B-cells (Nylander et al., 1995; Rowlands et al., 1995), and is overexpressed in many acute leukemias and lymphomas, including B-cell lymphomas of centroblastic type (Roos et al., 1993). Stathmin is a cell-cyle-regulated phosphoprotein that interacts with tubulin controlling mitotic spindle formation (Belmont and Mitchison, 1996; Marklund et al., 1996). PAG, also known as NKEFA and MSP23, was identified in 7 of 7 AIDS-DLBCL, 2 of 2 DLBCL, PTLDL, and HYP, albeit very weakly. PAG, a stress-induced antioxidant protein, is an isoform of the peroxiredoxin family (Noh et al., 2001), takes part in cell signaling by inhibiting c-abl (Wen and Van Etten, 1997), and confers proliferative properties (Prosperi et al., 1993, 1998). LHFP, a translocation partner gene of HMGIC in lipomas, was expressed in 7 of 7 AIDS–DLBCL, 2 of 2 DLBCL, and the PTLDL. Silencer element was expressed in 5 of 5 AIDS–DLBCL, 2 of 2 DLBCL, and the PTLDL and has been annotated in GenBank as a potential human homologue to chicken SCG10. SCG10 is a stathmin family member (Okazaki et al., 1993), is considered a panneural membrane marker of neuronal differentiation (Anderson and Axel, 1985), and contains a transcriptional repressor motif that controls neuron-specific gene expression (Eggen and Mandel, 1997; Thiel et al., 1998). HMG1 was expressed in 3 of 5 AIDS–DLBCL, 2 of 2 DLBCL, and the PTLDL. HMG1 is abundant in all mammalian nuclei and acts as an architectural transcription factor (Calogero et al., 1999). Cyclin G was strongly expressed in 5 of 9 AIDS–DLBCL, 2 of 2 DLBCL, and the PTLDL compared to HYP. Cyclin G is constitutively expressed throughout the cell cycle (Okamoto and Beach, 1994), transgene overexpression has demonstrated a growth advantage (Smith et al., 1997), and it may abrogate the p53 checkpoint in some cancers (Reimer et al., 1999). Thus, a number of the genes isolated here are important regulators of replication, cell signaling, proliferation, and transcription which have not been previously associated with AIDS–DLBCL or DLBCL.

Since both AIDS–DLBCL and DLBCL showed similar gene expression patterns in this study, unique environmental factors, HIV infection, and the resulting severe immune suppression are strongly implicated in the increased incidence and extreme aggressiveness seen in AIDS–DLBCL compared to DLBCL. Immune suppression results in reduced surveillance that contributes to the outgrowth of proliferating B-cells which, when combined with impaired GC reactions such as that detected by decreased SHM in the AIDS–DLBCL sample reported here, lead to B-cell NHL. In fact, the presence of a seemingly intact immune system and activated CD4+ T-cells in biopsy samples of B-cell NHL predicted a better patient prognosis (Ansell et al., 2001). In contrast, patients that have a crippled immune system and lack functional CD4+ T-cells in biopsy samples have a generally poor outcome. Although the AIDS–DLBCL and DLBCL patient samples examined here showed distinct patterns and levels of gene expression within and between sample types, the constellation of genes expressed in each sample was surprisingly similar, despite the involvement of HIV and severe immune suppression in the AIDS-derived samples. These findings suggest that the main difference in incidence and aggressiveness between these types of AIDS–DLBCL and DLBCL is due to differences in immune function rather than unique gene expression profiles.

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