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Gene Expression Profile of Serial Samples of Transformed B-Cell Lymphomas

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SUMMARY: Follicular lymphoma (FL) is characterized by a continuous rate of relapse and transformation to a high-grade lymphoma, usually diffuse large B-cell lymphoma (DLBCL), associated with a dismal prognosis and a poor response to conventional chemotherapy. The progression of indolent to aggressive FL is accompanied by the successive accumulation of recurrent chromosomal defects, but the resultant alterations of gene expression are largely unknown. To expand the understanding of the pathogenesis of FL transformation, we initially performed oligonucleotide microarray analyses using Affymetrix HuFL chips on five cases with matched snap-frozen lymph nodes before and after transformation. Expression data were analyzed using the Affymetrix Microarray Suite 4.0 and Genespring 4.0. Thirty-six genes with increased expression and 66 genes with decreased expression associated with transformation were identified and functionally classified. The expression of differentially expressed genes was confirmed by real-time quantitative RT-PCR (QRT-PCR) using a total of seven matched pairs and an additional five FL and five unrelated DLBCL. In addition, selected genes were further analyzed by QRT-PCR or immunohistochemistry using a large, unrelated series of FL (grades 1 to 3) as well as transformed and de novo DLBCL (total of 51 samples). The microarray results correlated with the protein expression data obtained from samples at the time of initial diagnosis and transformation. Furthermore, the expression of 25 candidate genes was evaluated by QRT-PCR with a 78% confirmation rate. Some of the identified genes, such as nucleobindin, interferon regulatory factor 4, and tissue inhibitor of metalloproteinases 1, are already known to be associated with high-grade non-Hodgkin's lymphoma. Novel candidate genes with confirmed increased and decreased expression in transformed DLBCL include ABL2 and NEK2, and PDCD1 and VDUP1, respectively. In summary, this study shows that transformation of FL to DLBCL is associated with a distinct set of differentially expressed genes of potential functional importance. (*Lab Invest 2003, 83:271–285*).

Follicular lymphoma (FL), one of the most common non-Hodgkin's lymphoma (NHL) in the west, is characterized by a long median survival of 8 to 10 years and a continuous pattern of relapse (Horning, 2000). The probability of transformation to a higher grade lymphoma is 22% at 5 years and 31% at10 years after diagnosis (Bastion et al, 1997), with a median duration of survival after transformation of 22 months (Yuen et al, 1995) because of refractoriness to therapy (Bastion et al, 1997; Horning, 1994).

Similar to oncogenesis in solid tumors, lymphomagenesis is considered to be a multistep process. After transformation, FL has a variable histology and phenotype, with diffuse large B-cell lymphoma (DLBCL) being most common, followed by Burkitt-like lymphoma (Aventin et al, 1990; Yano et al, 1992) and blastic morphology (Come et al, 1980; Weiss and Warnke, 1985). Clonality studies strongly suggest that the majority of DLBCLs arising in FL are clonally related to the initial lymphoma (Matolcsy et al, 1999; Traweek et al, 1993; Zelenetz et al, 1991). The translocation (14;18)(q32;q21), the genetic hallmark of FL, is detectable in some normal individuals (Limpens et al, 1995) and is not sufficient to cause FL (Strasser et al, 1990), suggesting that additional genetic events are required (Knutsen, 1997; McDonnell and Korsmeyer, 1991). Several molecular or genetic events have been associated with transformation of FL, including p53 mutations (Lo Coco et al, 1993; Sander et al, 1993), MYC (De Jong et al, 1988; Yano et al, 1992), and REL

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gene activation (Goff et al, 2000; Houldsworth et al, 1996; Joos et al, 1996; Rao et al, 1998), rearrangements of both mbr and mcr regions of BCL-2 (Nomdedeu et al, 1998), somatic mutations of the translocated BCL-2 gene (Matolcsy et al, 1996; Ottensmeier et al, 1998; Stamatopoulos et al, 2000), as well as deletions of 9p21 involving p15 (INK4b) and p16 (INK4a) (Elenitoba-Johnson et al, 1998; Pinyol et al, 1998; Stranks et al, 1995). Cytogenetic studies identified nonrandom chromosomal abnormalities in FL that correlated with survival, response to treatment, and risk of transformation (Tilly et al, 1994). Also, comparative genomic hybridization showed an unexpected high incidence of chromosomal imbalances and gene amplifications in FL (Bentz et al, 1996). Gene expression profiling has been very useful for studying NHL. For example, *Clusterin* has been identified as a new diagnostic marker for anaplastic large-cell lymphomas (Wellmann et al, 2000). Also, de novo DLBCLs has been separated into two subgroups, a *germinal center-like* DLBCL with a better prognosis and an *activated B-cell-like* DLBCL with a worse clinical outcome (Alizadeh et al, 2000). In addition, microarray profiling demonstrated that the t(14;18) defines a unique set of DLBCL with a germinal center B-cell expression profile (Huang et al, 2002).

Taken together, different pathways can lead to transformation of FL. Oligonucleotide microarray analysis is a robust technique to examine multiple cellular pathways. Using initially matched, microdissected samples that evolved into a high-grade lymphoma from the same individuals, we profiled gene expression associated with progression from low- to high-grade lymphomas. Changes in gene expression were validated in a large number of genes using both quantitative real-time PCR (QRT-PCR) and immunohistochemistry of an extensive collection of well-characterized lymphomas, resulting in the identification of cellular pathways that may become aberrant during progression of FL.

Results

Distribution of Gene Expression in Transformed B-Cell Lymphoma

Table 1 shows the distribution of differentially expressed genes in transformed B-cell lymphomas calculated with the Genespring software. Twenty-seven genes showed at least a 3-fold increased expression in at least three of five cases to an average difference value of five times the background numbers. Sixty-three genes showed at least a 3-fold decreased expression in at least three of five cases from an average difference value of five times the background numbers. Together with genes that were labeled *increased* or *decreased* by the Affymetrix software, we selected a total of 36 genes with increased expression and 66 genes with decreased expression including 3 duplicate genes. All selected genes are shown as a hierarchical cluster in Figure 1. Before concentrating on the above group of highly selected genes, we confirmed four additional genes (ABL2/ARG, HOXA1, NEK2, and TPRC/PRCC) that are also shown in Figure 1.

Classification of Differentially Expressed Genes in Transformed B-Cell Lymphomas

Table 2 summarizes the list of differentially expressed genes according to the above strict selection criteria. Thirteen T cell–specific genes and four dendritic cell–specific genes showed decreased expression. We found 28 genes with increased expression that are associated with cell proliferation and metabolism. This is in concordance with a higher cell proliferation rate in the high-grade lymphomas. Three transcription factors were increased and six apoptosis-related genes decreased. In addition, a mixed group of 6 genes with increased expression and 30 genes (3 duplicates) with decreased expression included potential candidate genes. A statistical analysis of this data set is constrained by the low case numbers and the nonuniform changes for many of the identified genes. Genes with a significance of $p < 0.05$ are indicated.

Correlation of Diagnostic Immunohistochemistry Results and Microarray Gene-Expression Data

All lymphomas expressed BCL2 and CD20 with the exception of Case 1, which lost CD20 expression after transformation. The microarray results correlated well with the protein expression data obtained at the time of initial diagnosis and transformation, exemplified by vascular endothelial growth factor, CD20, CD22, and c-MYC expression in Case 1 (Table 3). Because of limited amounts of available tissue, no material for

Table 1. Distribution of differentially expressed genes in matched samples of transformed B-cell lymphomas before and after transformation to a high-grade NHL

	Up			Down		
Fold change	×10	×3	×2	÷2	÷3	÷10
Frequency						
5 of 5 cases	0	0	2	1	1	1
4 of 5 cases	0	7	41	53	25	7
3 of 5 cases	25	135 (27[a])	367	380	190 (63[a])	77

Twenty-seven genes showed at least a 3-fold increased expression in at least three of five cases to an average difference value of five times the background numbers. Sixty-three genes showed at least a 3-fold decreased expression in at least three of five cases from an average difference value of five times the background numbers. Data restriction: absolute raw data restriction for differentially expressed genes is at least one times or 5-fold ([a]) of background value.

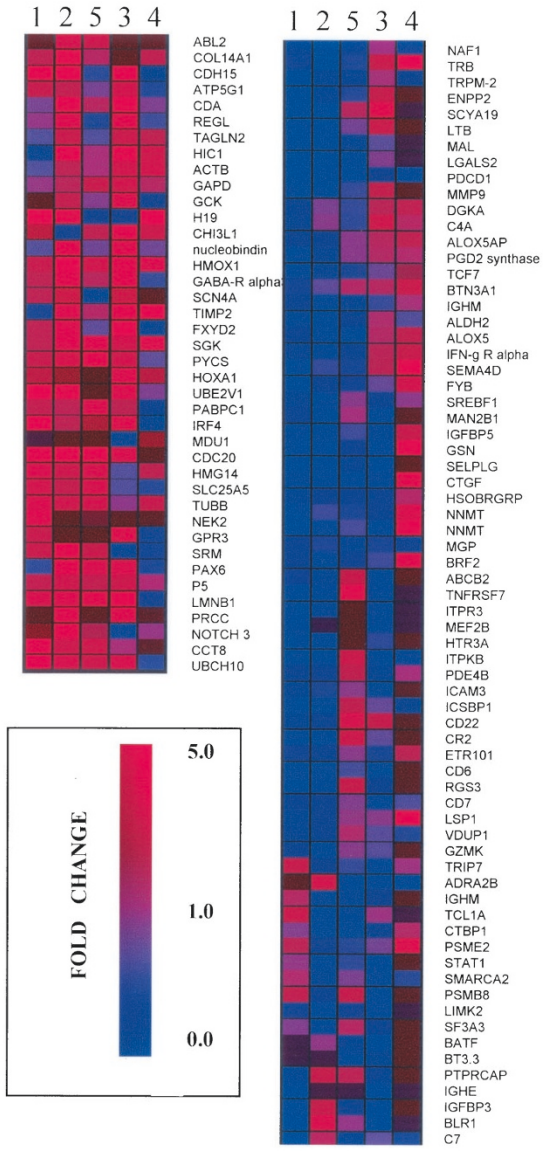

Figure 1.

Hierarchical clustering of differentially expressed genes in matched samples of transformed B-cell lymphomas before and after transformation to a high-grade non-Hodgkin's lymphoma (NHL). Four cases of follicular lymphoma (FL) (nos. 1–4) and one case of marginal zone lymphoma (no. 5) transformed to a high-grade NHL. Shown are the fold changes of all cases, calculated by two chip experiments per patient. Selection criteria were at least 3-fold changes in at least three of five transformations. The left column shows a cluster of genes with increased expression, and the right column shows a cluster of genes with decreased expression. Red = increased expression; Blue = decreased expression.

further immunohistochemistry was available for Cases 2, 6, and 7.

Confirmation of Microarray Data with QRT-PCR

For all genes tested, we generated standard curves and graphs comparing the microarray data and QRT-PCR. The RT-PCR analysis included two additional matched cases of FL transformation (nos. 6 and 7) for which not enough RNA was available for microarray studies, five unrelated FL, and five cases of de novo DLBCL. The average correlation coefficient (R^2) value for the standard curves for all genes tested was 0.994 $\pm$ 0.02 (mean $\pm$ SD). The initial QRT-PCR confirmations were performed using TaqMan probes on 15 genes that were selected according to the following criteria: at least 3-fold increased or decreased gene expression in at least three of five cases with a minimal average difference value of one times the background value ($\sim$500) in either the low- or the high-grade samples. Because of the inclusion of genes with low average difference values, we expected a high percentage of false-positive microarray findings and confirmed 5 of 15 genes tested. Those genes were ABL2 (ARG), NEK2, HOXA1, and TPRC (PRCC) with increased expression, and PDCD1 with decreased expression; all had average difference values several-fold above the background value.

To improve the signal/noise ratio, a repeat data analysis was performed, increasing the average difference value requirements as described above to five times the background value (2500). The RT-PCR confirmation rate for genes selected according to these criteria was 78%. Because of different program algorithms, Genespring-generated fold changes were usually higher than the Affymetrix calculations and the RT-PCR confirmation results. To compare these sets of data, we made a cutoff of at least 2-fold changes for Affymetrix software and QRT-PCR data. Figure 2 shows a good correlation of microarray and RT-PCR results for a representative gene, PDCD1. Table 5 lists all confirmed genes and shows the RT-PCR data for all seven matched samples, including two additional matched pairs that were used only for PCR analysis. In addition, the mean fold change comparing five unrelated FL with five de novo DLBCL is shown. Genes with a significance of $P < 0.05$ using a two-tailed Student's t test are indicated by asterisk. These results demonstrate a heterogenous pattern. Some genes are differentially expressed only in paired samples of FL/transformed DLBCL, whereas others are also found to be differentially expressed comparing unrelated cases of FL and de novo DLBCL.

Extended Confirmation of Microarray Data with QRT-PCR Data in an Independent Case Series

For further confirmation, we selected an unrelated series consisting of 5 normal lymph nodes, 15 FL (3 FL grade 1, 6 FL grade 2, 6 FL grade 3), 12 DLBCL that transformed from a known low-grade lymphoma (5 from FL, 7 from other lymphomas including small lymphocytic lymphoma [SLL] and MALT), 24 de novo DLBCL, and lymphoma cell lines for comparison. Figure 3A shows the expression data for ABL2. The higher expression of ABL2 in de novo DLBCL compared with FL is statistically significant ($p = 0.0038$). In addition, ABL2 expression in FL grade 3 is higher than in grades 1 or 2, and the set of DLBCL that transformed from low-grade lymphomas contains a group of cases that have increased ABL2 expression when compared with FL grade 1. Figure 3B shows increasing NEK2 expression levels when comparing FL

Table 2. Classification of differentially expressed genes in matched samples of transformed B-cell lymphomas before and after transformation to a high-grade NHL

Cell/function	GB No.		Gene expression	$P < 0.05$
T cells				
Down	X60992	CD6		+
	M37271	CD7		+
	U93049	FYB	Fyn binding proteins	+
	U26174	GZMK	Granzyme K	+
	D49394	HTR3A	Serotonin receptor 3A,	
	X76223	MAL	T lymphocyte maturation-associated protein	+
	X63380	MEF2B	MADS box transcription enhancer factor	
	D30755	NAF1	Nef-associated factor	+
	L20971	PDE4B	Phosphodiesterase 4B	+
	U25956	SELPLG	P-selectin glycoprotein ligand	+
	U60800	SEMA4D	CD100	
	X59871	TCF7	Transcription factor 7	+
	M12886	TRB	T cell receptor beta locus	+
Dendritic and stromal cells				
Up	D56495	REGL	Matrix protein	
Down	M26004	CR2	Complement component R2, CD21	
	M92934	CTGF	Connective tissue growth factor	
	X53331	MGP	Matrix Gla protein	
	U77180	SCYA19	MIP-3b, exodus-3	+
Transcription factors				
Up	U52682	IRF4	MUM1	
	X79439	NOTCH3	Receptor	
	M93650	PAX6	Transcription factor	
Down	U15460	B-ATF	Basic leucine zipper transcription factor	
	X62535	DGKA	Diacylglycerol kinase, alpha	+
	M91196	ICSBP1	Interferon consensus sequence binding protein 1	
	D45906	LIMK2	Lim domain kinase 2	+
	U27655	RSG3	Regulator of G-protein signaling 3	
	M97936	STAT1	Signal transducer and activator of transcription 1	
	S73591	VDUP1	Induced by VitD3	+
Evading apoptosis				
Down	X59350	CD22	BL-CAM	
	M35878	IGFBP3	Insulin-like growth factor binding protein 3	+
	M33552	LSP1	Lymphocyte-specific protein	+
	U64863	PDCD-1	Programmed cell death 1	+
	M98539	PGD2S	Prostaglandin D_2 synthase	+
	M63928	TNFRSF7	CD27	+
Tissue invasion and metastasis				
Up	D83542	CDH15	Cadherin 15, M cadherin	
	M32304	TIMP	Tissue inhibitor of metalloproteinases	
Down	X69819	ICAM3	Intercellular adhesion molecule 3	+
	J05070	MMP9	Matrix metalloproteinase 9	+
Proliferation and metabolism				
Up	D13118	ATP5G1	ATP synthase	
	X00351	ACTB	Actin, beta	
	D13627	CCT8	Chaperonin-containing TCP1, subunit 8	
	L27943	CDA	Cytidine deaminase	
	U05340	CDC20	p55	
	Y08374	CHI3L1	Cartilage glycoprotein-39	
	M64108	Col14A1	Udulin1	+
	U50743	FXYD2	Na,K-ATPase gamma	
	X01677	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	
	M90299	GCK	Glucokinase	
	J02621	HMG14	High-mobility group protein 14	

Strict selection criteria were used. Genes with uniform changes resulting in a significance of $p < 0.05$ are indicated. GB, GenBank accession number.

Table 2. *Continued*

Cell/function	GB No.		Gene expression	$P < 0.05$
Proliferation and metabolism				
	X06985	HMOX1	Hemeoxygenase	
	M34458	LMNB	LaminB1	
	M21904	MDU1	CD98 heavy chain	
	D49489	P5	Protein disulfide isomerase-related	
	Z48501	PABPC	Poly(A)-binding protein, cytoplasmic	
	X94453	PYCS	Pyrroline-5-carboxylate synthetase	+
	M81758	SCN4A	Na-channel	
	Y10032	SGK	Serum glucocorticoid regulated kinase	
	J02683	SLC25A	Solute carrier family 25	
	M34338	SRM	Spermidine synthase	
	D21261	TAGLN2	Transgelin 2, actin crosslinking protein	
	V00599	TUBB	Tubulin, beta	
	U73379	UBCH10	Ubiquitin carrier protein E2-C	
Down	U37408	CTBP1	C-terminal binding protein 1	
	X04412	GSN	Gelsolin, actin-depolymerizing factor	
	D45248	PSME2	Proteasome activator subunit 2	
	X72889	SMARCA	HBRM	
Miscellaneous				
Up	A28102	GABARα3	Receptor	+
	L32831	GPR3	G protein-coupled receptor 3	
	M32053	H19	Oncofetal RNA	
	L41919	HIC1	Hypermethylated in cancer 1	
	U31342	NUCB1	Nucleobindin	
	U49278	UBE2V	Ubiquitin-conjugating enzyme E2 variant	
Down	X57522	ABCB2	ATP-binding cassette subfamily B member 2	
	M34041	ADRA2B	Adrenergic, alpha 2B receptor	
	X05409	ALDH2	Aldehyde dehydrogenase, mitochondrial	
	J03600	Alox 5	Arachidonate 5-lipoxygenase	
	M63262	ALOX5AP	5-lipoxygenase activating protein	+
	X68149	BLR1	Burkitt lymphoma receptor 1, CXCR5	
	X78992	BRF2	Butyrate response factor 2	
	U97502	BT3.3	Butyrophilin	
	U90552	BTN3A1	Butyrophilin, subfamily 3, member A1	
	M59815	C4A	Complement component C4A	+
	J03507	C7	Complement component 7	+
	L35594	ENPP2	ATX, autotaxin	+
	M62831	ETR101	Immediate early protein	
	Y12670	HSOBRGRP	Leptin receptor gene-related protein	
	U19247	IFNGR α	Interferon gamma receptor alpha chain	+
	L27560	IGFBP5	Insulin-like growth factor binding protein 5	
	L00022	IGHE	Immunoglobulin heavy epsilon chain	
	V00563	IGHM	Immunoglobulin heavy constant mu	
	X58529	IGHM	Immunoglobulin heavy constant mu	
	X57206	ITPKB	Inositol 1,4,5-triphosphate 3 kinase B	
	U01062	ITPR3	Inositol 1,4,5-triphosphate receptor	
	M87860	LGALS2	Human S-lac lectin L-14-II	+
	U89922	LTB	Lymphotoxin beta, TNFSF3	+
	U05572	MAN2B1	Mannosidase	
	U51010	NNMT	Nicotinamide-methyl transferase	
	U08021	NNMT	Nicotinamide-methyl transferase	
	X97267	PTPRCAP	LPAP, protein tyrosise phosphatase	
	Z14982	PSMB8	Proteasome subunit, beta type 8	
	U08815	SF3A3	Splicing factor 3a, subunit 3	+
	U00968	SREBF1	Sterol regulatory element binding factor 1	
	X82240	TCL1	T-cell leukemia/lymphoma	
	L40357	TRIP 7	Thyroid hormone interactor receptor 7	
	M63379	TRPM-2	Clusterin	+

Strict selection criteria were used. Genes with uniform changes resulting in a significance of $p < 0.05$ are indicated. GB, GenBank accession number.

Table 3. Correlation of immunohistochemistry and microarray gene expression data of serial samples of transformed B-cell lymphomas at the time of initial diagnosis and time of transformation

NHL patient	Gene	Immunohistochemistry		Microarray gene expression
		Low grade	High grade	Low- to high-grade comparison
No. 1	VEGF	+	++	4-fold up
	c-MYC	+	++	14-fold up
	CD20	+	0	11-fold down
	CD22	+	0	6-fold down
	BCL2	++	+	2-fold down
	IRF4	−	++	8.5-fold up
	Ki67	ND	++	4.3-fold up
No. 3	IRF4	−	+	11.6-fold up
	BCL2	++	++	Expressed
	CD20	+	+	Expressed
No. 4	IRF4	−	−	5.9-fold down
	BCL2	+	+	Expressed
	CD20	+	+	Expressed
No. 5	IRF4	−	−	3.4-fold up
	BCL2	+	+	Expressed
	CD20	+	+	Expressed

The microarray results correlated well with protein expression data obtained at the time of either initial diagnosis or transformation. Because of limited amounts of available tissue, no material for further immunohistochemistry was available for case nos. 2, 6, and 7. VEGF, vascular endothelial growth factor; c-MYC, MYC proto-oncogene; CD20, B-lymphocyte surface antigen B1; CD22, B-cell membrane protein; BCL2, B-cell CLL/Lymphoma 2; Ki67, Proliferation marker; 0 = negative, ± = faint, + = positive, ++ = highly positive.

grades 1, 2, and 3 with transformed or de novo DLBCL. The higher expression of NEK2 in de novo DLBCL compared with FL is statistically significant ($p = 0.0477$). In addition, all DLBCL cell lines express NEK2 at high levels with only marginal expression in the follicular dendritic cell line HK. Figure 3C demonstrates statistically significant decreased expression levels of PDCD1 in transformed DLBCL ($p = 0.0048$) and de novo DLBCL ($p = 0.0029$) when compared with FL. All DLBCL cell lines expressed only minimal levels of PDCD1. The mantle cell lymphoma cell lines JEKO1 and NCEB expressed more PDCD1 than the DLBCL lines but at minimal levels when compared with the patient lymphoma samples. Figure 3D shows the expression of VDUP1. Although the expression levels were not different comparing FL with de novo DLBCL, a difference between FL and transformed DLBCL was observed that just missed the level of statistical significance ($p = 0.0708$). Again, the DLBCL cell lines showed only very low VDUP1 expression.

Immunohistochemical Confirmation of Microarray Data in an Independent Case Series

For further confirmation, we selected an unrelated series of 50 NHL including 16 FL grades 1 to 3, 13 DLBCL that transformed from low-grade lymphomas (6 from FL, 7 from other low grade lymphomas), and 21 de novo DLBCL. Frozen sections were stained with antibody specific for interferon regulatory factor 4/multiple myeloma oncogene 1 (IRF4/MUM1) (Table 6). Only 6% of FL (one individual) expressed IRF4/MUM. This case was a grade 3 FL with 2+ staining in 70% of cells. In marked contrast, 81% of de novo DLBCL (17 of 21) expressed IRF4/MUM1, as did 69% of DLBCL that transformed from preexisting low-grade lymphomas (9 of 13). Within this latter group, IRF4/MUM1 was expressed in 83% of DLBCL that transformed from FL (5 of 6) and in 57% of DLBCL (4 of 7) that transformed from other low-grade lymphomas (MALT, SLL). In most cases, staining was of 2 to 3+ intensity in more than 20% of neoplastic cells. Representative staining for IRF4/MUM1 is illustrated in Figure 3.

Discussion

The process of transformation of low-grade to high-grade NHL is not well characterized, and new approaches to elucidate the underlying mechanisms are needed. We were able to obtain seven matched samples of NHL from the same patients before and after transformation. All but one of the cases were FL grade I that transformed to a high-grade NHL. One case was a marginal zone lymphoma that transformed to a DLBCL. With gene expression profiling of these matched samples and QRT-PCR in an extended series of lymphomas, we identified and validated highly differentially expressed genes, generating new hypotheses of the underlying transforming events.

The transformation of FL is a heterogenous process. Uniform gene expression changes seen in all transformations are therefore not expected. To identify changes that might occur in a subgroup, we selected genes that were altered in at least three of five paired samples for which microarray data were available. The genes were selected according to very strict criteria to keep the validation rate using QRT-PCR as high as possible. Those genes were then grouped according to their known function. Studies analyzing the core

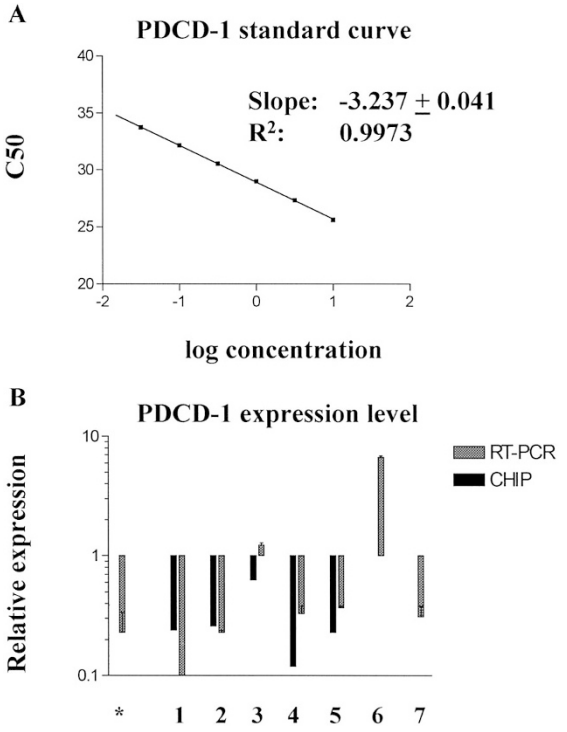

Figure 2.

Quantitative real-time PCR (QRT-PCR) of PDCD1. This figure shows the standard curve and the correlation of PCR and microarray data of PDCD1 as a representative sample. A, Standard curve: the standard curves were generated by serial dilutions of 100 ng/μl cDNA samples in five ÷10-fold dilution steps and used for regression analyses. B, Correlation of microarray data and QRT-PCR. The comparison of five different FL compared with five unrelated de novo diffuse large B-cell lymphoma (DLBCL) is indicated by the asterisk. Cases 1 to 5 are the paired samples of transformed B-cell lymphoma for which microarray data are available. Numbers 6 to 7 are additional paired samples of transformed B-cell lymphoma for which microarray data were not available because of limited amounts of RNA.

meiotic transcriptome in budding yeast have demonstrated that only 10% of specifically regulated genes during meiosis were in fact essential for that process (Kaback and Feldberg, 1985; Primig et al, 2000). Extrapolation of those findings led us to assume that the percentage of functionally essential genes among the group of differentially expressed genes in FL transformation might be rather low. We attempted to increase the odds of finding functionally relevant candidate genes by concentrating on genes that were not related to contaminating T or dendritic cells and whose known functions implied a possible pathogenic role. We found genes with decreased expression that reflect the presence of contaminating germinal center type T cells and follicular dendritic cells in the low-grade lymphoma samples compared with the DLBCL. In addition, a cluster of genes associated with cell growth and metabolism reflected the different growth rates between low- and high-grade lymphomas. Most of these genes were thought to be of less interest as they appeared unrelated to the transforming events. But we are aware that "contaminating" stromal cells may have a contributing role in the growth of lymphoma cells, such as producing proteins that remodel the extracellular matrix (Alizadeh et al, 2000).

For validation, we compared protein expression data obtained routinely at times of diagnosis and progression with the microarray gene expression data and found a very good correlation. Our confirmation rate of selected genes using QRT-PCR (78%) compares favorably with a published confirmation rate of 71% (Rajeevan et al, 2001). Several genes that we found to be increased have already been published as markers for transformed/aggressive lymphoma, providing independent validation of our data and our search strategy. These genes include nucleobindin (Kubota et al, 1998), IRF4 (Falini et al, 2000; Iida et al, 1997; Tsuboi et al, 2000), and tissue inhibitor of metalloproteinases (Kossakowska et al, 1993, 1999). Nucleobindin has DNA-binding ability (Miura et al, 1992) and may play a role in G protein-regulated and $Ca2^{+}$-regulated signal transduction (Lin et al, 2000). IRF4, also known as MUM1, is involved in the chromosomal translocation t(6;14) that occurs in multiple myeloma, and it has been found to have oncogenic activity in vitro (Iida et al, 1997). Expression of tissue inhibitor of metalloproteinases is associated with a poor clinical outcome in NHL (Kossakowska et al, 1993, 1999). In the case of IRF4/MUM1, we confirmed expression of the protein by immunohistochemistry in an unrelated series of 50 lymphomas. Only 1 of 16 FL expressed IRF4/MUM1 (a case of FL grade 3), whereas 69% of transformed DLBCL and 81% of de novo DLBCL prominently expressed the protein.

While this manuscript was under review, Lossos et al (2002) published their series of FL that transformed to DLBCL. Neither we nor Lossos et al found uniformly differentially expressed genes throughout all paired patient samples. In their series, consistent changes were observed only in up to 4 of 12 paired cases. Instead, a marked heterogeneity was observed, with consistent expression changes occurring only in a subset of transformed DLBCL. These findings are consistent with a multistep transformation process involving many alternative pathways in transformed DLBCL. Some genes are differentially expressed only in paired samples of FL/transformed DLBCL, whereas others are also found to be differentially expressed comparing unrelated cases of FL and de novo DLBCL.

Figure 4 lists selected, confirmed, differentially expressed genes in transformed FL in the context of their likely functional contribution to the transformation step. Genes that provide growth stimulation, potentially decrease apoptotic cell death, or lessen cell adhesion are shown. New hypothetical pathogenic mechanisms of FL transformation are represented by the following selected candidate genes that we found to be differentially expressed in transformed lymphomas.

ARG, also known as *ABL2*, is an ABL-related gene with a structure similar to the Abelson gene (ABL) (Kruh et al, 1986). It is highly related to ABL at its SH3, SH2, and kinase domains but is only 30% similar at the C-terminal level (Kruh et al, 1990). The function of ARG is not well understood. However, in a recently reported t(1;12)(q25,p13) translocation in a patient with acute leukemia, ARG was fused to the TEL gene

Table 4a. Primers and probes used for quantitative RT-PCR

Gene	5′ Primer	3′ Primer	Probe (5′ – FAM – 3′ TAMRA)
ABL2	AGCATGCTGATGGGTTCTCT	AGGTTCCTCTGTGCAAAGCT	CTCCTGCCCAGCAAGAGGCG
HOXA1	AGTACGGCTACCTGGGTCAA	CGCGTCAGGTACTTGTTGAA	AACGCGGTGCGCACCAACTT
NEK2	GAAGAGGGCGACAATTAGGA	CTCGCTCTCGCTCCTGTAAC	CGCAGGATTCCAGCCCTGTATTGA
PDCD-1	GCTAAACTGGTACCGCATGAG	CAGTTGTGTGACACGGAAGC	ACGGACAAGCTGGCCGCCTT
TPRC	ATGCCGCTGGTGCTTATTAT	ATCGATGAAGGAGGCATCTG	ATCCTGCACAGGACCCGGCC

Primer probes were designed using the PRIMER3 software (http://www.genome.wi.mit.edu/cgi-bin/primer3_www.cgi) using sequence data from the NCBI database. Shown are the primers and probes of confirmed genes.

Table 4b. Primers and melting points (MP) for quantitative SYBRGreen RT-PCR

Gene	5′ Primer	3′ Primer	MP
B-ATF	AGCGAAGACCTGGAGAAACA	TTCAGCACCGACGTGAAGTA	83.5
CD22	TGACCTGGAAAGCTTCATCC	GGAACCTTCCCATCCTTTGT	80.5
CD27	CATCACTGCCAATGCTGAGT	GGGTTTGGAAGAGGATCACA	85.5
CDC20	AAGACCTGCCGTTACATTCC	TTCCCAGAACTCCAATCCAC	83
CR2	ATCCTAAGAGGCCGAATGGT	CCTTCAAGGTGAAGCCAAAC	79.5
ETR101	GAAGTGCAGAAAGAGGCACA	TGCGGGAGTGATACATCTTC	83.5
ICAM3	GTGTTTGAAGGAAGGCTCCA	TGTATTTGCCTCGTGAGCTG	85
IGFBP3	TGGTAGTTGTGCAGCATCGT	ATCAGGAAGGACCAGAGCAA	79
IGHM	CACCAAGTCCACCAAGTTGA	GGTGTGGGTTTTCACAGCTT	86
ICSBP1	CCAGGACTGATTTGGGAGAA	AATGGAGGCATCCACTTCCT	82
IRF4	AGAAGAGCATCTTCCGCATC	CCTTTAAACAGTGCCCAAGC	87.5
ITPK3	AGGCCATTCGAACCACTCTA	TTGGCCTGTTCCTTCTTGTC	83
NBK	TGTATGAGCAGCTCCTGGAA	AGTCATGCCAAGAACCTCCA	80
NOTCH3	CAGCCTCATGGCAGAATAGA	TGCAGCTTCTCCAACACATC	78.5
NUCB1	GTGACCAGAAGGAGGTGGAC	GGTCCGAGATCACAGATGCT	84
PDCD-1	TGCAGCTTCTCCAACACATC	CATGCGGTACCAGTTTAGCA	79.5
RGP3	CAGTGTTCCAAGCCTTCCTT	CTTGGATGCCATCTTGGACT	83.5
TCF-1	GCAACCTGAAGACACAAGCA	GCAATGACCTTGGCTCTCAT	84
TRPM2	GGTCTGGCACCCAAAGATAA	GACAAGCCTTGCTCTGGAAA	80
VDUP1	AGCCTTCGGGTTCAGAAGAT	TTGGATCCAGGAACGCTAAC	83.5

Primer probes were designed using the PRIMER3 software (http://www.genome.wi.mit.edu/cgi-bin/primer3_www.cgi) using sequence data from the NCBI database. Shown are the primers and probes of confirmed genes. MP in degrees Celsius.

(Cazzaniga et al, 1999; Iijima et al, 2000). This fusion probably increased the tyrosine kinase activity of ARG. In vitro studies showed that the ARG kinase can be inhibited by STI571 (Okuda et al, 2001). The gene expression level of ARG was increased in our transformed FL samples. We speculate that the ARG gene might be involved in activating translocations in transformed FL.

NEK2 is a serine/threonine-protein kinase that is involved in mitotic regulation. It has been found to be a core component of the centrosome and, upon overexpression, NEK2 can stimulate centrosome splitting (Fry et al, 1998). During development, this protein accumulates throughout S phase and shows maximal levels in late G2. This expression pattern is highly reminiscent of that of A and B cyclins (Fry et al, 1995). NEK2 is a member of the NIMA-related kinase family. NIMA was first identified in the filamentous fungus *Aspergillus nidulans* as a protein whose kinase activity is essential for mitotic entry (Fry et al, 1995). Temperature-sensitive *nimA* mutants arrest in G2 at the restrictive temperature, whereas overexpression of NIMA leads to a rapid entry into mitosis from any point in the cell cycle (Fry, 2002). The first report that NEK2 expression is increased in certain tumor cells has come from microarray studies of Ewing tumor-derived cell lines (Wai et al, 2002). Here, we report that NEK2 is overexpressed in transformed and de novo DLBCL. To our knowledge, this is the first report of high levels of NEK2 in primary human tumors.

We have found that programmed cell death 1 (PDCD1) is decreased in transformed and de novo DLBCL. It is an Ig superfamily member, containing an immunoreceptor inhibitory motif in the cytoplasmic tail and is thought to be involved in the negative regulation of B-cell proliferation and differentiation (Freeman et al, 2000). PDCD1 is activated in lymphatic cell lines undergoing programmed cell death (Ishida et al, 1992), suggesting a role of this protein in apoptosis. When PDCD1 is disrupted, mice develop a lupus-like disease (Nishimura et al, 1999) or an autoimmune dilated cardiomyopathy (Nishimura et al, 2001). We have found this gene to be very poorly expressed in MCL (Hofmann et al, 2001), and we propose that the low expression of PDCD1 may contribute to the accumulation of abnormal B cells in NHL.

Table 5. Genes with QRT-PCR confirmed differential expression in matched samples of transformed B-cell lymphomas before and after transformation to a high-grade NHL

Matched pairs of low-grade and high-grade NHL from the same patient				Comparison of unrelated NHL		
				Relative expression ± SD		
Gene	GB No.	Description	Confirmed cases in seven paired samples	FL ($n = 5$)	DLBCL ($n = 5$)	p value
Increased expression						
ABL2	M35296	ARG, Abelson-related gene	3 of 7	1.00 ± 0.15	0.81 ± 0.60	0.5465
CDC20	U05340	p55CDC	4 of 7	1.00 ± 0.46	1.03 ± 0.61	0.9358
HOXA1	U37431	Homeobox A1	6 of 7	1.00 ± 0.48	1.17 ± 0.39	0.5499
IRF4	U52682	Interferon regulatory factor 4	4 of 7	1.00 ± 0.37	1.54 ± 1.20	0.3113
NBK	X89986	BCL2-interacting killer	4 of 7	1.00 ± 0.44	3.97 ± 3.82	0.1227
NEK2	Z29066	NIMA-related kinase 2	4 of 7	1.00 ± 0.56	2.18 ± 0.87	0.0421*
NOTCH3	X79439	Notch (*Drosophila*) homolog 3	5 of 7	1.00 ± 0.38	7.06 ± 12.84	0.2419
NUCB1	U31342	Nucleobindin	3 of 7	1.00 ± 0.22	0.59 ± 0.22	0.0186*
TPRC	X99720	Papillary renal carcinoma associated	5 of 7	1.00 ± 0.33	0.73 ± 0.24	0.1774
Decreased expression						
B-ATF	U15460	Basic leucin zipper transcription factor	3 of 7	1.00 ± 0.22	1.32 ± 1.05	0.5259
CD22	X59350	B-cell membrane glycoprotein	3 of 7	1.00 ± 0.41	0.20 ± 0.22	0.0060*
CD27	M63928	TNF receptor superfamily member 7	5 of 7	1.00 ± 0.20	0.48 ± 0.43	0.0345*
CR2	S62696	3d/Epstein-Barr virus receptor 2	5 of 7	1.00 ± 0.48	0.35 ± 0.29	0.0347*
ETR101	M62831	Immediate early protein	4 of 7	1.00 ± 0.58	0.83 ± 0.38	0.5899
ICAM3	X69819	Intercellular adhesion molecule 3	3 of 7	1.00 ± 0.25	0.67 ± 0.26	0.0727
ICSBP1	M91196	IFN consensus sequence binding protein	4 of 7	1.00 ± 0.41	0.51 ± 0.41	0.0876
IGFBP3	M35878	Insulin-like growth factor binding protein	4 of 7	1.00 ± 0.38	0.21 ± 0.12	0.0028*
IGHM	U24685	Immunoglobulin heavy constant mu	4 of 7	1.00 ± 1.09	0.94 ± 1.48	0.9440
ITPK3B	X57206	Inositol 1,4,5-triphosphate 3-kinase B	4 of 7	1.00 ± 0.39	0.43 ± 0.29	0.0310*
PDCD-1	U64863	Programmed cell death 1	5 of 7	1.00 ± 0.39	0.23 ± 0.11	0.0027*
RGP3	U27655	Regulator of G-protein signalling 3	4 of 7	1.00 ± 0.44	0.68 ± 0.17	0.1852
TCF-1	M57732	Hepatic nuclear factor 1	5 of 7	1.00 ± 0.46	0.12 ± 0.10	0.0030*
TCL1	X82240	T-cell leukemia/lymphoma 1A	3 of 7	1.00 ± 0.82	0.02 ± 0.03	0.0280*
TPRM-2	M63379	Clusterin	5 of 7	1.00 ± 0.29	0.08 ± 0.09	0.0001*
VDUP1	S73591	Up-regulated by 1,25-dihydroxyvit. D-3	5 of 7	1.00 ± 0.44	0.15 ± 0.14	0.0025*

A total of seven matched samples of transformed B-cell lymphomas before and after transformation to a high-grade NHL was available. Six cases were FL and one a marginal zone lymphoma that transformed to a high-grade NHL. Five matched sample pairs had enough RNA available for gene expression profiling, but all seven sample pairs were used for validation studies using quantitative RT-PCR. Shown are all confirmed genes that were selected according to strict criteria (see Table 2), as well as earlier confirmed genes that were selected according to lesser restrictions but nevertheless confirmed per RT-PCR. These genes are ABL2 (ARG), NEK2, HOXA1, and TPRC (PRCC), and PDCD1. In addition, the mean fold change comparing five unrelated FL with 5 DLBCL is shown. Genes with uniform changes resulting in a significance of $p < 0.05$ are indicated by asterisk. GB, GenBank accession number.

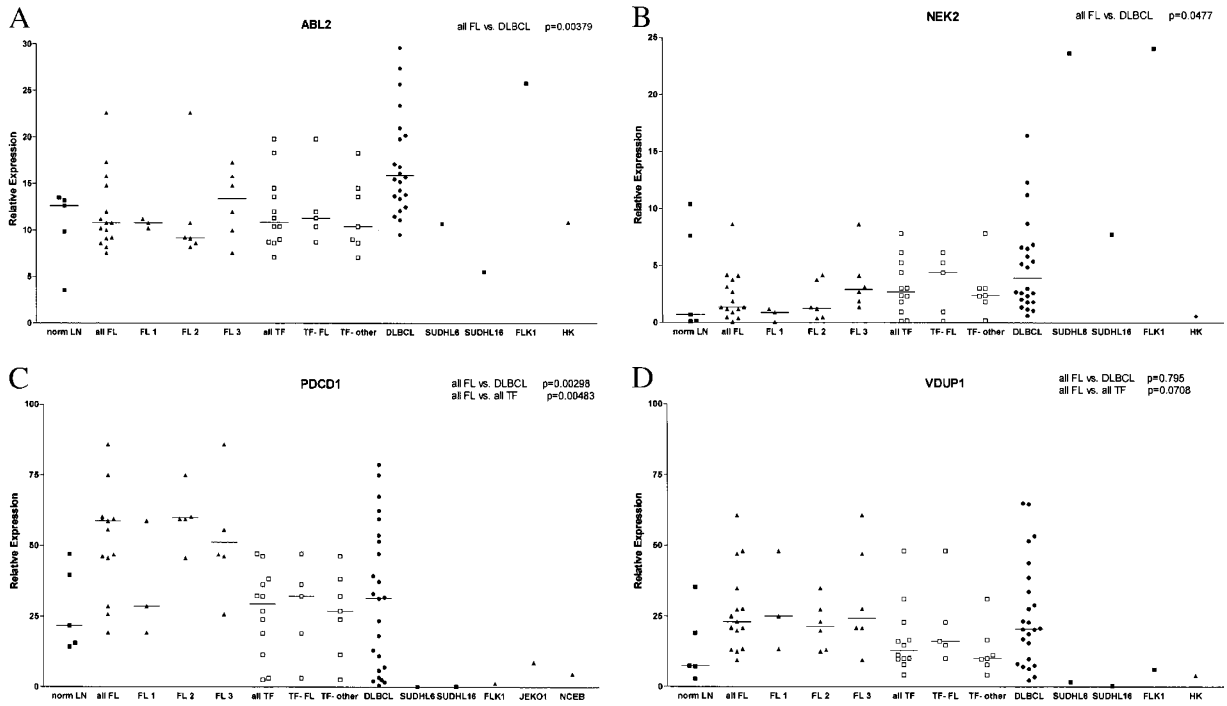

Figure 3.

QRT-PCR in an unrelated series of lymphomas including 5 normal lymph nodes, 15 FL (3 FL grade 1, 6 FL grade 2, and 6 FL grade 3), 12 DLBCL that transformed from a known low-grade lymphoma (5 from FL, 7 from other lymphomas including SLL and MALT), 24 de novo DLBCL, and lymphoma cell lines for comparison were selected. Shown are the expression data for (A) ABL2, (B) NEK2, (C) PDCD1, and (D) VDUP1. The *p* values comparing different lymphoma subtypes are shown.

Table 6. Confirmation of microarray data with immunohistochemistry for IRF4/MUM1 in an independent case series

Histology	n	IRF4/MUM1 positive n	%
FL			
Grade I	3	0	0
Grade II	6	0	0
Grade III	7	1	14
Total	16	1	6
DLBCL (transformed)			
Prior FL	6	5	83
Prior other low-grade lymphoma	7	4	57
Total	13	9	69
DLBCL (de novo)	21	17	81

Confirmation of the microarray data used immunohistochemistry in an independent case series of 50 NHL cases, including 16 FL grades 1–3, 13 DLBCL that transformed from a low-grade lymphoma (6 from FL, 7 from other low-grade lymphomas), and 21 de novo DLBCL. Frozen sections were stained with an antibody against IRF4/MUM1.

We found VDUP1 expression to be decreased in transformed DLBCL and in a subset of de novo DLBCL. Vitamin D_3 up-regulated protein 1 (VDUP1)/thioredoxin-binding protein-2 (TBP-2) has been previously isolated and characterized as a gene up-regulated by 1,25-dihydroxyvitamin D3 in HL-60 cells (Chen and DeLuca, 1994). It has also been shown that VDUP1 is a cytoplasmic protein that binds to and inhibits active (reduced) thioredoxin (TRX), with amino acids 155 to 225 required for binding (Junn et al, 2000). VDUP1 has been implicated as a redox-sensitive tumor suppressor. By inhibiting the function of TRX, VDUP1 plays a role in cell proliferation and oxidative stress by influencing the redox state of the cell (Nishiyama et al, 1999). TRX has been shown to modulate DNA-binding activity of several transcription factors, such as TFIIIC, BZLF1, nuclear factor-κB, Ref-1, and p53, while inducing AP-1 through de novo transcription of c-jun and c-fos (Toledano and Leonard, 1991). Although VDUP1 is suppressed in HTLV-I transformed T-cell lines, overexpression of VDUP1 suppresses their cell growth, indicating its possible involvement in tumorigenesis (Masutani and Yodoi, 2002). In addition, it has been shown that the histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates VDUP1, and down-regulates TRX in prostate and bladder cancer cell lines (Butler et al, 2002). Further, VDUP1 mRNA levels were found to be reduced in human breast and colon tumor tissue compared with matched samples of normal tissues (Butler et al, 2002). To our knowledge, this is the first report describing differential VDUP1 expression in FL and DLBCL.

In summary, we have identified and validated differentially expressed genes in transformed FL, including already known markers of high-grade NHL, but also new candidate genes including ABL2, NEK2, PDCD1, and VDUP1. The transformation of FL to DLBCL is a heterogenous process, and confirmed gene alterations in a subset of investigated cases might be

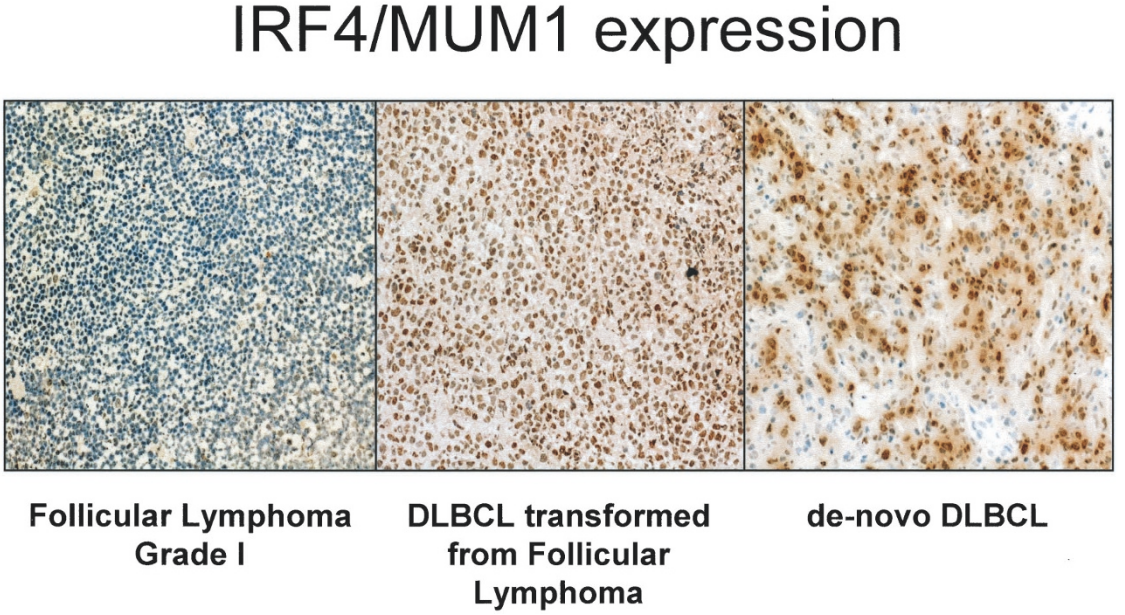

Figure 4.
Representative, confirmatory immunohistochemistry for interferon regulatory factor 4/multiple myeloma oncogene 1 (IRF4/MUM1) was performed in an unrelated series of 50 lymphomas, including FL, transformed B-cell lymphomas, and de novo DLBCLs. Shown is a characteristic pattern of staining in a grade 1 FL, de novo and transformed DLBCL.

significant, generating new hypotheses about deregulated pathways (Figure 5).

Materials and Methods

Patients

Seven pairs of snap-frozen lymph nodes, derived from patients who transformed from low- to high-grade B-cell lymphoma were obtained largely from the central lymphoma repository of the Southwest Oncology Group lymphoma collection. The lymphomas were classified according to the World Health Organization classification scheme (Jaffe et al, 2001). Five cases (nos. 2, 3, 4, 6, and 7) were FL grade I that transformed to DLBCL; one FL grade I transformed to a Burkitt-like lymphoma (no. 1), and one marginal zone lymphoma transformed to a DLBCL (no. 5). The successive transformation of the original low-grade lymphoma clone was evidenced by identical light chain restrictions of the low- and high-grade lymphomas in five of the seven matched patient samples where data were available. Additional unrelated cases of five FL and five de novo DLBCL were used for QRT-PCR studies in each of the studied candidate genes. For further confirmation, a larger series of 50 unrelated cases including 16 FL grades 1 to 3, 13 DLBCL that transformed from low-grade lymphomas (6 transformed from FL, 7 from other low-grade lymphomas), and 21 de novo DLBCL were selected for immunohistochemistry studies. Five normal lymph nodes, 15 FL (3 grade 1, 6 grade 2, 6 grade 3), 12 DLBCL that transformed from a known low-grade lymphoma (5 from FL, 7 from other lymphomas including SLL, and MALT), 24 de novo DLBCL, and lymphoma cell lines were used for QRT-PCR confirmation studies. The SUDHL-6, SUDHL-16, and FLK1 cell lines are derived from patients with DLBCL harboring the t(14;18) translocation. HK is a follicular dendritic cell line; JEKO1 and NCEB are mantle cell lymphoma cell lines.

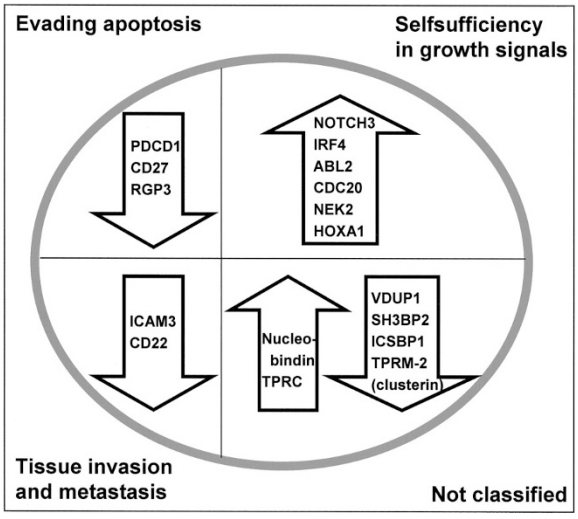

Figure 5.
Confirmed differentially expressed genes in transformed B-cell lymphoma. The change of gene expression is indicated by up/down arrows. The candidate genes are classified according to the likely functional consequences of their changed expression for lymphoma progression.

RNA Extraction

Frozen sections (30 serial 20-μm sections) were collected for RNA extraction. The areas selected were uniformly either low- or high-grade NHL, without visi-

ble significant contamination with other tissues in hematoxylin and eosin (HE) stains and were microdissected when necessary. Total RNA was extracted using STAT-60 (Tell-Test, Inc., Friendswood, Texas), followed by a purification step using the RNeasy cleanup kit (QIAGEN, Inc., Valencia, California), according to the manufacturer's protocol. The RNA quality was confirmed on an agarose gel.

Oligonucleotide Microarrays

The HuGeneFL Array (Affymetrix Inc., Santa Clara, California) provides gene expression data for 5600 full-length human sequences. Each set of matched samples was studied with two microarrays. The preparation and microarray processing was performed per standard Affymetrix protocol as previously published (Hofmann et al, 2001). The raw data image was analyzed using the GeneChip Analysis Suite (Affymetrix Inc.), and the data for each microarray were normalized by global scaling to a target value of 2500. The average background noise was in the range of 500. For quality control purposes, we evaluated the percentage of "present" calls per chip; the cutoff was set at 20%. Using the housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase, we evaluated the ratio of average difference values of the 3' versus 5' mRNA expression; sufficient RNA integrity was assumed if that ratio was $\leq$2.0.

Data Analysis

We performed a supervised data analysis with matched paired samples of transformed low-grade lymphoma. For five of the paired samples, enough RNA was available to perform microarray analyses (Samples 1 to 5). Because of a limited case series and the high likelihood of nonuniform transforming events, we chose to search for highly differentially expressed genes in at least three of our five cases. In addition, we selected genes with sufficiently high average difference values to increase our confirmation rate with secondary techniques. The raw data values were required to be 5-fold higher than the background level of 500, and the fold changes were at least 3-fold. We used the GeneChip analysis suite for pairwise comparisons and the Genespring software (Silicon Genetics, Inc., Redwood City, California) for clustering, statistical analysis, and visualization. The value of statistical analyses was limited because of the low case numbers and resulted in statistical significance in only those situations in which the changes were uniform within the whole study set. The p values for the microarray data of differentially expressed genes were generated for the five paired samples using a paired t test, with a significance level α at 0.05. However, highly differentially expressed genes in a subset of cases might still be meaningful and were therefore included in further confirmation studies. For hierarchical clustering of genes and patient samples, we used the Pearson correlation algorithm.

QRT-PCR

After DNase treatment with RQ1 DNAseI (Promega, Madison, Wisconsin), 2 μg of total RNA was reverse transcribed using Superscript II (Life Technologies, Grand Island, New York) according to the manufacturer's protocol in a total volume of 50 μl. Quantitative PCR was performed using either TaqMan probes or SYBRGreen for the detection of fluorescence during amplification. The initial RT-PCR confirmations were performed using TaqMan probes. All PCR primers (Life Technologies) and TaqMan probes (Applied Biosystems, Foster City, California) were designed using the PRIMER3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and sequence data from the NCBI database (Table 4, a and b). The primer Tm was 58° C to 60° C. The TaqMan probes were labeled with the reporter dye 6-carboxyfluorescein in the 5'end and the quencher dye 6-carboxy-tetramethyl-rhodamine in the 3'end. For TaqMan probes, each amplification reaction contained 5 μl of a 1:10 dilution of the original cDNA, 12.5 μl of the Universal TaqMan 2$\times$ PCR mastermix (Applied Biosystems), and specific primers in 300 nM and the probe in 100 nM concentration in a final volume of 25 μl. All reactions were performed in triplicates in an iCycler iQ system (BioRad, Hercules, California), and the thermal cycling conditions were 2 minutes at 50° C, 10 minutes at 95° C, followed by 45 cycles of 95° C for 15 seconds and 60° C for 1 minute. Normalization was performed using the expression of 18S rDNA as an internal control. β-actin is located on chromosome 7, which is often aberrant in lymphomas (Johansson et al, 1995). It was shown to be up-regulated in the majority of our transformed lymphoma samples and was therefore unsuitable for normalization purposes.

For economy, we used SYBRGreen detection for selected genes. A 25-μl RT-PCR reaction consisted of 2.5 μl of 10$\times$ HotStarTaq PCR buffer, 10 mM of each dNTP, 10 pmol of each primer, 0.25 μl of SYBRGreen (Molecular Probes, Eugene, Oregon) in a 1:600 dilution in 1% dimethyl sulfoxide, 0.625 U of HotStarTaq (Qiagen, Valencia, CA), and 50 ng cDNA, or 1 pg of cDNA when measuring 18S. In initial experiments, gene fragments were amplified using PCR conditions as follows: 95° C for 15 minutes, followed by 45 cycles of 95° C for 15 seconds, 60° C for 15 seconds, 72° C for 30 seconds. Melting temperatures of these fragments were determined according to the manufacturer's protocol. The thermal cycling conditions for SYBRGreen RT-PCR were 95° C for 15 minutes, followed by 45 cycles of 95° C for 15 seconds, 60° C for 15 seconds, 72° C for 30 seconds, and the specific melting temperature for 20 seconds. The standard curves were generated by serial dilutions of 100 ng/μl sample cDNA in five $\div$10-fold dilution steps and used for regression analyses. SYBRGreen QRT-PCR gave identical results to the TaqMan RT-PCR as tested on a limited set of genes (data not shown). The variance of the triplicate measurements was $<$5% for both TaqMan and SYBRGreen RT-PCR. When correlating fold changes, the Genespring generated values were usually higher than the GeneChip calculations and the

RT-PCR confirmation results. The cutoff was set to at least 2-fold changes when correlating microarray data (GeneChip software) and QRT-PCR data. The *p* values for the QRT-PCR data of the unrelated NHL series were generated using a two-tailed Student's *t* test, assuming equal variance, with a significance level α at 0.05.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Grogan et al, 1995). Briefly, we used snap-frozen sections fixed in 4° C 100% acetone and applied the relevant antibodies using standard immunoperoxidase techniques in an automated immunostainer "VMS 320" (Ventana Medical Systems, Tucson, Arizona) or a Dako autostainer (Carpenteria, California).

Immunohistochemistry staining was graded by two observers according to the intensity of the stain (1 to 3+) as well as the percentage of positive cells. To be considered IRF4/MUM1 positive, we required at least 1+ staining intensity in >10% of cells.

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