RESEARCH ARTICLE

Gene Expression Profiling of Non–Hodgkin Lymphomas

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Abstract

Background: Chromosomal translocations are genetic aberrations associated with specific non–Hodgkin lymphoma (NHL) subtypes. This study investigated the differential gene expression profile of Egyptian NHL cases based on a microarray approach. Materials and Methods: The study included tissue samples from 40 NHL patients and 20 normal lymph nodes used as controls. Total RNA was extracted and used for cDNA microarray assays. The quantitative real time polymerase chain reaction was used to identify the aberrantly expressed genes in cancer. Results: Significant associations of 8 up-regulated and 4 down-regulated genes with NHL were observed. Aberrant expression of a new group of genes not reported previously was apparent, including down-regulated NAG14 protein, 3 beta hydroxy-delta 5-c27 steroid oxi-reductase, oxi-glutarate dehydrogenase (lipo-amide), immunoglobulin lambda like polypeptide 3, protein kinase x linked, Hmt1, and caveolin 2 Tetra protein. The up-regulated genes were Rb binding protein 5, DKFZP586J1624 protein, protein kinase inhibitor gamma, zinc finger protein 3, choline ethanolamine phospho-transferase CEPT1, protein phosphatase, and histone deacetylase-3. Conclusions: This study revealed that new differentially expressed genes that may be markers for NHL patients and individuals who are at high risk for cancer development.

Keywords: Non–hodgkin lymphoma - cDNA microarray- up-regulation - down-regulation - markers

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Introduction

Malignant lymphomas are genetically characterized by distinctive chromosomal translocations as the t (14;18) in follicular lymphoma. Traditionally, two main groups of lymphoma have been distinguished: Hodgkin Lymphoma (HL), characterized by large polynuclear cells; and a diverse group of other lymphomas, defined as non-Hodgkin lymphomas (NHL). NHL is the hematologic malignancy with the highest prevalence worldwide (Marcucci and Mele, 2011). Non–Hodgkin lymphomas (NHL) diseases are involving malignant transformation of lymphoid cells. Specific Chromosomal translocations often associated with NHL subtypes (Dyer, 2003; Kuppers, 2005; Ohno, 2006; Bende et al., 2007). NHL-associated translocations result in transcriptional deregulation of proto-oncogene or oncogene (Dyer, 2003; Kuppers, 2005; Ohno, 2006; Bende et al., 2007).

The biological agents associated with NHL are human immunodeficiency virus (HIV) (Killebrew and Shiramizu, 2004) , human T-cell lymphotropic virus 1 (HTLV-1), Hepatitis C virus (HCV), human herpes virus 8 (HHV8) and Epstein Barr virus (EBV) (Lewin et al., 1990; Kanavaros et al., 1995; Gouda et al., 2010). In addition, infection with *Helicobacter pylori* is a risk factor for gastric lymphoma (Alpen et al., 2001).

NHLs are health problem that are increased in incidence (Porcu and Nichols, 1998). NHL incidence rates are higher in developed countries such as those in western Europe, North America, and Australia and lower in South America and Asia, but the rise in incidence has been consistent across countries (Marcucci and Mele, 2011). In Egypt lymphoid malignancies is accounting for 10-12% of all malignancies (Ibrahim et al., 2012; Nasr et al., 2012).

Diffuse large-B-cell lymphoma (DLBCL) is an aggressive malignancy of mature B lymphocytes (Baraniskin et al., 2012; Mey et al., 2012), accounting for roughly 40% of cases of non-Hodgkin's lymphoma and is the most common type in adults (Segal., 2007). DLBCL is one disease have largely failed owing to differential diagnosis (Berget et al., 2012; Tilly et al., 2012). Patients with DLBCL may respond initially to chemotherapy or show a remission (Charbonneau et al., 2012; Guo et al., 2012).

Many genes are involved in NHL-associated translocations regulate the cell cycle, apoptosis, and lymphocyte development, such as *MYC, BCL2, CCND1*,

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and *BCL6* (Kuppers and Dalla-Favera, 2001; Baraniskin et al., 2012). Clinical parameters are accustomed to assess a patient's risk profile but molecular discrepancy within DLBCL is of great important (Alizadeh et al., 2000). Microarray technology is a powerful tool for genomic applications, can profile gene expression on a whole-genome scale.

Materials and Methods

To provide a gene expression for NHL, cDNA microarray is used to characterize the Egyptian NHL patterns. We also profiled the genes in normal samples from normal human tonsil and lymph node.

This study was conducted at Cancer Biology Department, National Cancer Institute, Cairo University. The study included 40 tissue samples from NHL and 20 non cancer lymph nodes from simple hyperplasia, reactive lymph-nodes and inflammatory tonsils (mixed and used as pooled normal). All clinico-pathological features of the studied cancer samples were collected from the medical records. This study was conducted in compliance with the Helsinki Declaration and was approved by the senior staff committee.

In all cases the pathological diagnosis was non-Hodgkin's lymphoma [3 follicular non-Hodgkin's lymphoma, 37 Diffuse large B-cell lymphoma]. Tissues were immediately cut into pieces; one piece was processed for histopathological confirmation. The second portion was immediately snap-frozen and stored in liquid nitrogen for RNA extraction.

RNA extraction and cDNA microarray

Total RNA was isolated using Trizol (Invitrogen, Germany) followed by RNeasy Mini Kit (Qiagen, Germany). RNA quality and quantity were assessed by electrophoresis and optical density respectively (Nanodrop analyzer). Fluorescent cDNA, labelled with the Cy3 dye (Amersham Biosciences, UK), were prepared from each cancer mRNA sample. A normal cDNA, labelled with the Cy5 dye (Amersham Biosciences, UK), was prepared from a pool of mRNAs isolated from pool normal samples. Each Cy3-labelled experimental cDNA probe was combined with the Cy5-labelled normal and the mixture was hybridized to the microarray. Each sample was tested in triplicate on array 15K (Array-I). The fluorescence ratio was quantified for each gene and reflected the relative abundance of the gene in each experimental mRNA sample compared to the normal mRNA pool. After reactions for cDNA synthesis microarray hybridization, washing images were obtained by scanning with Scan Array Express II (Perkin Elmer, USA) and were automatically quantified. The reproducibility of our microarray procedure has been checked and proved to be satisfactory. The repeated hybridization of a same lymphoma sample always showed a good reproducibility with a correlation always above 0.98.

Data analysis

All data were subjected to normalization implemented in the statistical software package R. Hierarchical clustering was obtained with Genesis software using correlation distance and average linkage method.

Real-time PCR analysis

To evaluate genomic gains and amplifications of potential target genes, we performed real-time quantitative polymerase chain reaction (RQ-PCR) using the ABI Prism 7700 Sequence Detector System (Applied Biosystems, USA). Genes *BAG5, BCL2L11, BCLAF1,* and *CASP1* and *8* and *9* were selected for real time analysis (Morton et al., 2009). *BCL2L11* balances the anti-apoptotic influence of *BCL2* and coordinates pro-apoptotic signaling through the intrinsic apoptosis pathway (Khanna et al., 1996; Reed et al., 1996). *BCLAF1* and *BAG5* are both Bcl-2 family members that suppress *BAX* (pro-apoptotic) gene expression, in turn suppressing the *APAF1* gene and inhibiting apoptosis. *CASP9*, the other gene to be replicated, is a pro-apoptotic protease integral to the intrinsic apoptotic pathway, and is responsible for effector caspase activation and apoptosis execution following activation by Apaf-1 bound to cytochrome c released from mitochondria (Allan and Clarke, 2009).

The primers and probes used listed previously (Morton et al., 2009). For controls, β2-microglobulin was used in all cases. Each assay was analyzed by the comparative cycle threshold (CT) method.

Results

All the clinicopathological features of the studied samples were collected from pathology and medical records of patients. The variation in gene expression across 40 NHL and normal samples using 15K cDNA microarrays were showed in Figure 1.

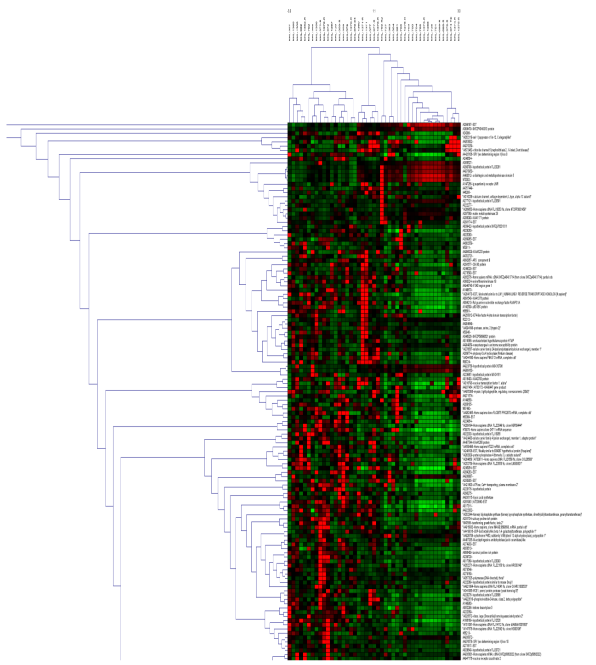

Figure 1. Upregulated Genes. The up-regulated genes were related to cell division, cell adhesion, cytoskeleton, and cell defense and cell metabolism. The down-regulated genes included those associated with cell development, cell cycle, signal transduction, adhesion, cell defense, gene expression and cell metabolism

Table 1. Showed the Most Down-Regulated Genes

UniqueID	Gene name
AA459108	Homo sapiens cDNA: FLJ21421 fis, clone COL04123
AA974394	five-span transmembrane protein M83
AA704749	Homo sapiens cDNA: FLJ22822 fis, clone KAIA3968
AA677361	hypothetical protein
AA703536	HSPC182 protein
AA707083	hypothetical protein dJ473B4
AA465180	phenylalanyl-tRNA synthetase beta-subunit
AA677309	hypothetical protein MGC4614
AA481155	unr-interacting protein
AA458867	hypothetical protein FLJ22301
AA707682	hypothetical protein FLJ11729
AA707332	Homo sapiens cDNA: FLJ21326 fis, clone COL02445
H23117	NAG14 protein
AA707080	3 beta-hydroxy-delta 5-C27-steroid oxidoreductase
AA939100	Homo sapiens mRNA; cDNA DKFZp564O1262 (from clone DKFZp564O1262)
AA677240	polymerase (RNA) II (DNA directed) polypeptide J (13.3kD)
AA481248	CGG triplet repeat binding protein 1
AA706901	purine-rich element binding protein B
AA856769	oxoglutarate dehydrogenase (lipoamide)
AA677326	ornithine carbamoyltransferase
AA677362	Homo sapiens, clone IMAGE:3346451, mRNA, partial cds
AA454650	KIAA0573 protein
AA707081	Homo sapiens cDNA: FLJ23546 fis, clone LNG08361
AA706895	Human DNA sequence from clone RP1-20N2 on chromosome 6q24. Contains the gene for a novel protein similar to yeast and bacterial cytosine deaminase, a possible pseudogene similar to part of Tubulin beta chain, the PEX3 gene for peroxisomal biogenesis
H16820	Homo sapiens chromosome 14 BAC 98L12
AI365523	synovial sarcoma, translocated to X chromosome
H19242	sec61 homolog
H22947	hypothetical protein HDCMC04P
AA706964	RNA helicase-related protein
AI651009	mitogen-activated protein kinase 12
H18423	immunoglobulin lambda-like polypeptide 3
AA465692	KIAA0648 protein
AA609600	solute carrier family 20 (phosphate transporter), member 2
AI536679	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
AA703609	Homo sapiens clone IMAGE:212461, mRNA sequence
AA704323	erbb2-interacting protein ERBIN
H16804	hypothetical protein MGC11335
R51988	Homo sapiens cDNA FLJ13558 fis, clone PLACE1007743
AI014598	DKFZP434H0735 protein
AI337207	transporter similar to yeast MRS2
AA703652	slit (Drosophila) homolog 3
AA452897	Homo sapiens clone IMAGE:23371, mRNA sequence
AA458938	hypothetical protein HDCMC04P
R43648	f-box and leucine-rich repeat protein 6
AI239770	protein kinase, X-linked
AA973768	HMT1 (hnRNP methyltransferase, S. cerevisiae)-like 1

A hierarchical clustering algorithm was used to group genes on the basis of similarity in the pattern with which their expression varied over all samples. The data are shown in a matrix format, with each row representing all the hybridization results for a single cDNA element of the array, and each column representing the measured expression levels for all genes in a single sample. To visualize the results, the expression level of each gene was represented by a color, with red representing expression greater than the mean, green representing expression less than the mean, and the color intensity representing the magnitude of the deviation from the mean.

From the entire set of genes on the microarray, we identified 83 cDNAs significantly expressed in ~80% of samples. To investigate the difference between the NHL patients and normal lymph nodes, we searched for specific aberrant-expressed genes using GoMiner. In all, biological functions were significantly associated with NHL. Interestingly, NHL and normal groups were associated with similar biological functions, although they did not share any common discriminating genes in their signature.

Table 2. Showed the Most Up-Regulated Genes

UniqueID	Gene name
AI016259	Homo sapiens mRNA; cDNA DKFZp434E2118 (from clone DKFZp434E2118); partial cds
AI279103	Homo sapiens cDNA: FLJ22145 fis, clone HEP22070
AI076795	lacrimal proline rich protein
AA458825	mitochondrial translational initiation factor 2
AI290798	hypothetical protein FLJ20281
AA972429	hypothetical protein DKFZp434O1427
AI208335	Homo sapiens cDNA: FLJ21323 fis, clone COL02374
AI126424	E2F-like protein
R99354	uncharacterized hypothalamus protein HT010
AI400612	a disintegrin and metalloproteinase domain 5
AI167373	solute carrier family 12 (potassium/chloride transporters), member 7
AI269079	EST
AI299187	EST
AA983882	DKFZP586J1624 protein
AA973748	RAD54, S. cerevisiae, homolog of, B
AI215853	Homo sapiens clone PP902 unknown mRNA
R19267	kinesin-like 2
AI279479	protein kinase (cAMP-dependent, catalytic) inhibitor gamma
AI202101	Homo sapiens mRNA; cDNA DKFZp761P0615 (from clone DKFZp761P0615)
AI052240	zinc finger protein 3 (A8-51)
AI184893	glutamyl aminopeptidase (aminopeptidase A)
AI269958	BCL2-associated athanogene 3
R27814	choline/ethanolaminephosphotransferase
AA455623	Homo sapiens cDNA: FLJ21205 fis, clone COL00328
AI356480	KIAA0645 gene product
AA936147	Homo sapiens mRNA; cDNA DKFZp434E232 (from clone DKFZp434E232)
AI341917	Ewing sarcoma breakpoint region 1
AI611326	solute carrier family 25, member 13 (citrin)
AI004443	protein phosphatase
AI423435	HCGIV-6 protein
AI244566	EST
AI052298	histone deacetylase 3
AA970166	putative protein similar to nessy (Drosophila)
AA419016	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1
AI283152	hypothetical protein FLJ10583
AI141850	KIAA1161 protein
AI208453	hypothetical protein FLJ10895

In this analysis of 15K cDNA among 40 NHL patients and 20 controls, the overall statistical significance for NHL of the biological pathway(s) by 83 genes (Table 2 and 3). We observed significant associations for 8 up-regulated genes with NHL and 4 genes down-regulated.

cDNA microarray analyses revealed suggestive associations for 37 genes up-regulated with overall, and 46 genes with DLBCL lymphoma but no significant associations with any other follicular lymphoma. The down-regulated genes are BLIMP1, XBP1, NAG14 protein, 3 beta hydroxy-delta 5-c27 steroid oxi-reductase, Oxi-glutarate dehydrogenase (lipoamide), Immunoglobulin lambda like polypeptide 3, Protein kinase x linked, Hmt1 (hnrnp methyltranferase s cervices)- like 1, Caveolin 2 Tetra protein. The up-regulated genes are BCL6, BCL2L, BCL7A, MYC and CCND1, Rb binding protein 5, DKFZP586J1624 protein, Protein kinase inhibitor gamma, Zinc finger protein 3, Choline ethanolamine phospho-transferase CEPT1, Protein phosphatase, Histone deacetylase-3 as in Figure 1.

Validation of DNA microarray results by Real time RT-PCR analysis

By means of quantitative RT-PCR, we further evaluated the expression levels of 8 selected genes, which included 4 up-regulated and 4 down-regulated genes, in NHL cancer tissues, and in global normal from other 20 cases. We found that the expression pattern of 4 genes was significantly high in > 50% (20/40) cases. The levels of mRNA evaluated by real time-PCR were correlated with the microarrays data for each tested gene.

Discussion

Diffuse large B-cell lymphoma (DLB-CL) is an aggressive malignancy of mature B-lymphocytes and the common subtype of non-Hodgkin lymphoma in adults (Bea et al., 2005; Naz et al., 2011). Gene expression profiling provides a quantitative molecular data for human lymphomas disease. The current study from 40 cancerous tissues from NHL showed common genetic variation in cell cycle, apoptosis, and lymphocyte development regulatory genes that may play a role in lymphomagenesis.

A study shows that the target genes differentially expressed in DLB-CL include BCL-6, BLIMP1, and XBP1 (Staudt and Dave, 2005).The current study showed most aberrations genes are: BCL-6, and BLIMP1, and XBP1, MYC and CCND1. Similarly, half of DLB-CL has chromosomal translocations which deregulate expression of BCL-6, MYC, and BCL-2 genes (Wright et al., 2003; Biasoli et al., 2005; Iqbal et al., 2007).

The differentiation of B cells into immunoglobulin-secreting plasma cells is controlled by two transcription factors, B lymphocyte-induced maturation protein 1 (BLIMP1) and X-box-binding protein 1 (XBP1). BLIMP1 is a transcriptional repressor gene that is essential for B cell differentiation. BLIMP1 gene lies on chromosome 6q21-q22.1, a region frequently deleted in B cell lymphomas (Pasqualucci et al., 2006). In the current study BLIMP1 gene was down-regulated in NHL patients. Similar studied reported inactivation in BLIMP1 gene in nearly quarter of activated B cell–like diffuse large cell lymphoma or lack BLIMP1 protein expression, despite the presence of BLIMP1 mRNA. BLIMP1 gene acts as a tumor suppressor gene (Pasqualucci et al., 2006).

XBP1 gene is expressed at a high level in plasma cells and acts downstream of BLIMP1. In the current study XBP1 gene was suppressed in NHL patients compared to pooled normal. Correspondingly, previous report had showed mutations in BLIMP1 gene in B-cell lymphoma. Other study showed that XBP1 and BLIMP1 genes are involved in the pathogenesis in diffuse large B-cell lymphoma (Tate et al., 2009).

The BCL6 gene, a transcription repressor, is the target of multiple chromosomal translocations in NHL (Muramatsu et al., 1996). Translocations in BCL6 gene non-translated region consequently deregulate BCL6 gene expression (Jardin et al., 2007) . In this study we have found upregulation of BCL6 gene expression that was in concordance with others who found that the levels of BCL6 gene expression and protein have been demonstrated to expect the clinical outcome of DLBCL (Lossos et al., 2001). The BCL6 findings from the pooled data set were consistent with our study (Zhang et al., 2005) but do not provide support for two other previous studies of follicular lymphoma (Jardin et al., 2005). Other study examined tumors with a variety of different BCL6 translocations and found no increase in total BCL6 mRNA levels in the NHL specimens harboring BCL6 gene translocation (Lossos et al., 2003). Certainly, some of tumors expressed comparatively low levels of the BCL6 gene . The lymphoma cell lines and majority of NHL tumor specimens expressed BCL6 mRNA predominantly from the rearranged allele that may come under the control of other gene promoters. Conversely, few NHL tumors with BCL6 gene translocations expressed BCL6 mRNA equally in the rearranged and the non-rearranged alleles (Lossos et al., 2003).

In the current study, *MYC* and *CCND1* genes were upregulated in NHL. Both genes play important roles in the cell cycle and/or lymphocyte development. *MYC* and *CCND1* genes have been implicated in lymphomagenesis (Dyer, 2003; Adhikary and Eilers, 2005). There is limited previous research associating lymphoma with common genetic variation in *CCND1*, and no previous research for *MYC*. Because of the importance of *CCND1* and *MYC* in the cell cycle and/or lymphocyte development as well as carcinogenesis.

BCL2L11 is a key pro-apoptotic member of the *BCL2* family that initiates apoptosis in lymphocytes. *BCL2L11* gene is balancing the proliferative and anti-apoptotic effects of *BCL2* (Bouillet et al., 1999). The *BCL2L11* isoforms have varying pro-apoptotic activity (Harada et al., 2004). In the present study upregulation in *BCL2L* gene in NHL samples compared to normal lymph node. Other studies showed BCL2L11 gene with little expression with melanoma progression, renal cell carcinoma, and glioblastoma (Zantl et al., 2007).

BCL7A is participated in chromosomal translocation with *MYC* and *IgH* in a Burkitt lymphoma and B-cell lymphoma cell lines (Zani et al., 1996) . Diminished expression of *BCL7A* has been associated with peripheral T-cell lymphoma (Martinez-Delgado et al., 2004), more aggressive clinical behavior of cutaneous T-cell lymphoma (van Doorn et al., 2005) , and poorer prognosis for DLBCL.

In particular, we found a group of genes that were not reported before, of these the down-regulated genes are NAG14 protein, 3 beta hydroxy-delta 5-c27 steroid oxi-reductase, Oxi-glutarate dehydrogenase (lipo-amide), Immunoglobulin lambda like polypeptide 3, Protein kinase x linked, Hmt1 (hnrnp methyltranferase s cervices)- like 1, Caveolin 2 Tetra protein. The up-regulated genes are Rb binding protein 5, DKFZP586J1624 protein, Protein kinase inhibitor gamma, Zinc finger protein 3, Choline ethanolamine phospho-transferase CEPT1, Protein phosphatase, Histone deacetylase-3. In summary, we found aberration in the expression of specific genes related to Egyptian NHL that may play a role in lymphomagenesis.

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