Cell Cycle 9:3, 557-563; February 1, 2010; © 2010 Landes Bioscience

Yin Yang 1 overexpression in diffuse large B-cell lymphoma is associated with B-cell transformation and tumor progression

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Key words: non-Hodgkin lymphoma, Yin Yang 1, Bcl-6, Pax-5

Yin Yang 1 (YY1), a multifunctional transcription factor, has been shown to be involved in the pathogenesis of several cancer types. However, its role in hematological malignancies has not yet been fully investigated. In the present study, using computational methods, we showed that YY1 transcript levels were significantly increased in the high-grade lymphomas, including Burkitt's lymphoma and diffuse large B-cell lymphoma (DLBCL), compared with those of both low-grade lymphomas and normal B-cells. The significant increase in gene expression resulted in a significant increase also at protein level in three NHL cell lines. The association of YY1 expression with some clinical-pathological features in DLBCL showed a positive correlation between a high level of YY1 mRNA and high levels of BCL-6 protein. Moreover, by analyzing the large series of DLBCL in the Hummel dataset, we identified the transcription factor PAX-5 among the top 50 genes positively correlated with YY1. These findings are also supported by the biological network analysis in which the top network, with the highest score, associated with YY1 expression levels in DLBCL is cellular movement, hematological system development and function, and immune response. Overall these data suggest that YY1 is involved in B cells transformation which gives rise to high-grade lymphomas through a dysregulation in the normal development of B cells affecting cell cycle and cellular motility.

Introduction

Non-Hodgkin lymphomas (NHL) are a heterogeneous group of lymphoproliferative malignancies with variable patterns of behavior and responses to therapy. Due to the combination of rituximab with chemotherapy, currently, a significant percentage of patients with NHL may be successfully cured. However, a fraction of these patients relapse after treatment due to the development of chemoresistance.[1] Thus, the further characterization of mechanisms of lymphomagenesis may be useful for the identification of new therapeutic targets.

Yin Yang 1 (YY1), a multifunctional transcription factor, has been shown to be involved in the development and/or progression of several cancer types. By gene expression analysis of publicly available datasets from 9 different oncotypes for a total of 552 cancer samples and 276 normal samples, we recently observed that, in several tumor types, YY1 transcript levels were higher when compared to those of the normal counterparts.[2] However, YY1 involvement in hematopoietic malignancies, such as NHL, remains poorly defined.

YY1 was found to inhibit FAS expression and render cells resistant to FAS ligand-mediated apoptosis.[3] YY1 overexpression may promote p53 degradation in an MDM-2 dependent manner or may inhibit its transcriptional activity by sequestering the p53 coactivator p300 promoting a more malignant phenotype.[4] High levels of YY1 inhibit the expression of p53 target genes after DNA damage[5] and downregulation of YY1 expression has been shown to restore the cellular sensitivity to p53-mediated apoptosis in response to DNA damage.[6] The numerous lines of evidences suggesting that YY1 overexpression may promote p53 degradation or inhibit its transcriptional activity may provide insight into the role of YY1 in lymphomagenesis.

Computational approaches, not only may help in the identification of new gene lists (signatures) suitable for tumor subgrouping on the basis of their aggressiveness and outcome[7] but may also contribute to the evaluation of the role of a specific gene in the genesis, progression and resistance to therapy. Here, we exploited computational methods with the aim of evaluating the expression of YY1 in NHL histotypes compared to normal B-cells and focusing on its potential role in lymphoma development and progression.

Results

Expression of YY1 in B cell lymphomas. The analysis of YY1 expression levels in association with B cell transformation was

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Submitted: 10/05/09; Revised: 10/29/09; Accepted: 11/06/09
Previously published online: www.landesbioscience.com/journals/cc/article/10554

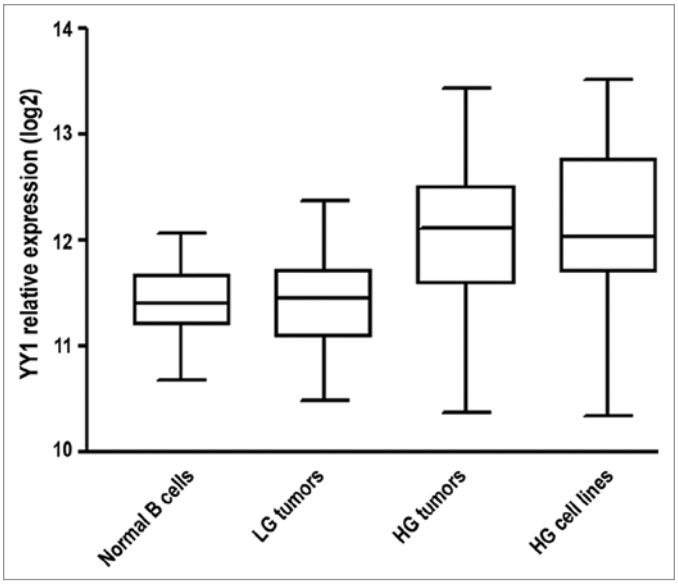

Figure 1. Association of YY1 gene expression levels and B cell transformation. The analysis of YY1 expression levels was performed using a dataset of microarray data obtained on 196 samples out of the 336 present in the original dataset (Basso et al.[8]). Normal B cells as well as LG B cell malignancies (Follicular Lymphoma, Mantle cell Lymphoma, B cell chronic lymphocytic leukemia, Hairy cell Leukemia) presented a significantly lower YY1 expression as compared to HG tumors and HG cell lines ($p < 0.0001$ by one-way ANOVA and by Dunnett' post test $p < 0.0001$ versus B cells). Results are reported as boxed quartiles (median, 25th, and 75th percentile) and whiskers (minimum and maximum).

performed using a dataset of microarray data from human lymphoma specimens (Basso et al. PMID: 15778709). YY1 transcript levels were found significantly increased in the 86 HG tumor biopsies and in the 21 HG tumor cell lines with respect to 25 samples of normal B cells and to 64 LG tumor biopsies (**Fig. 1**). When the HG tumors were analyzed separately the YY1 overexpression was found to be significantly associated only to BL and DLBCL but not to primary effusions.

Validation of gene expression was performed by in vitro experiments. In particular, we evaluated if the significant increase in gene expression resulted in a significant increase also at protein level. YY1 expression was analyzed in NHL cell lines (Raji, Daudi and Ramos) by western blot (WB). In **Figure 2A** a representative experiment of the three performed is presented. The expression of YY1, as evaluated by densitometric analysis of the bands, was significantly higher in all NHL cell lines compared to normal B-cells ($p < 0.001$).

To confirm that WB was a valid approach to further understand the role of YY1 in cancer, YY1 protein level was evaluated in numerous cell lines from solid tumors where a different pattern of expression between normal and tumor tissue was experimentally or computationally observed.[2] In absence of certified cell lines from various normal tissues, two preparations of normal fibroblasts were used for comparison. As shown in the representative experiment reported in **Figure 2B** high YY1 protein expression was observed in the majority of cells lines (A274, A375, CAKI-1, CAKI-2, HT1080, MCF-7, HT-29, HS913T, SK-LMS1, SW626), while levels comparable to normal control were observed in 3 cell lines (A-172, Hela, MG63).

Functional analysis of YY1 gene expression. To shed light on the role of YY1 in DLBCL development, we used a dataset of publicly available microarray gene expression data of DLBCL that was obtained using a very informative platform.[9] YY1 expression was initially analyzed for its possible association with the available clinical-pathological parameters. Among these parameters, the positive expression of BCL-6 protein in the tumors was significantly associated with higher levels of YY1 gene transcription (**Fig. 3**).

The global gene expression was used to evaluate the Pearson correlation between expression of each one of the genes present on the platform and YY1 gene expression. The analysis yielded 372 genes correlated at $\alpha = 0.001$ significance level (254 positively correlated and 118 negatively correlated). The top genes and their expression on the entire dataset are shown in the heat map (**Fig. 4**) and the list of the genes is reported in the **Supplementary Table 1**. The heat map indicates that among the genes most positively correlated with YY1 are present other transcription factors already associated, such as E2F3 ($r = 0.35$; $p < 0.0001$). Interestingly, the heat map also shows that the mRNA expression level of YY1 is positively correlated with that of PAX-5 ($r = 0.40$; $p < 0.0001$) already known to be involved in the lymphoma development (**Fig. 4**).

Biological network analysis using the proprietary ingenuity pathway analysis software showed 29 biological networks significantly associated to YY1. In **Table 1** are reported the networks with the most significant scores linking YY1 to regulation of cellular motility, development of hematological system and cell cycle. Numerous genes coordinatively modulated with YY1 in 3 of the 6 most significant networks are related to cellular movement; furthermore molecules involved in the carbohydrate metabolism and the small molecule biochemistry are present in 2 of the 6 networks. In **Figure 5** is shown the network potentially involving YY1 in the regulation of cellular movement, hematological system development and function and immune response in DLBCL. For further details of each network see **Supplementary Table 2**.

Discussion

Eighteen years after the discovery of YY1, a large number of YY1 regulated genes has been described. Several different, sometimes contradictors, mechanisms have been implicated as modes determining YY1-mediated transcriptional activation or repression (reviewed in ref. 13). The putative role of YY1 in tumorigenesis is supported by its known interaction in the cell cycle regulation[4] and, accordingly, many studies were conducted to evaluate the involvement of YY1 in several solid tumor types.[14-19]

In this study we have provided evidence that YY1 is expressed at significantly higher levels in high-grade lymphomas, including Burkitt's Lymphoma and DLBCL, when compared to normal B cells. DLBCL is the most frequent non-Hodgkin's lymphoma subtype, accounting for 30% to 35% of all lymphoma cases.[20] It is characterized by an aggressive behavior due to its typically rapid growth and limited survival in the absence of effective treatment.

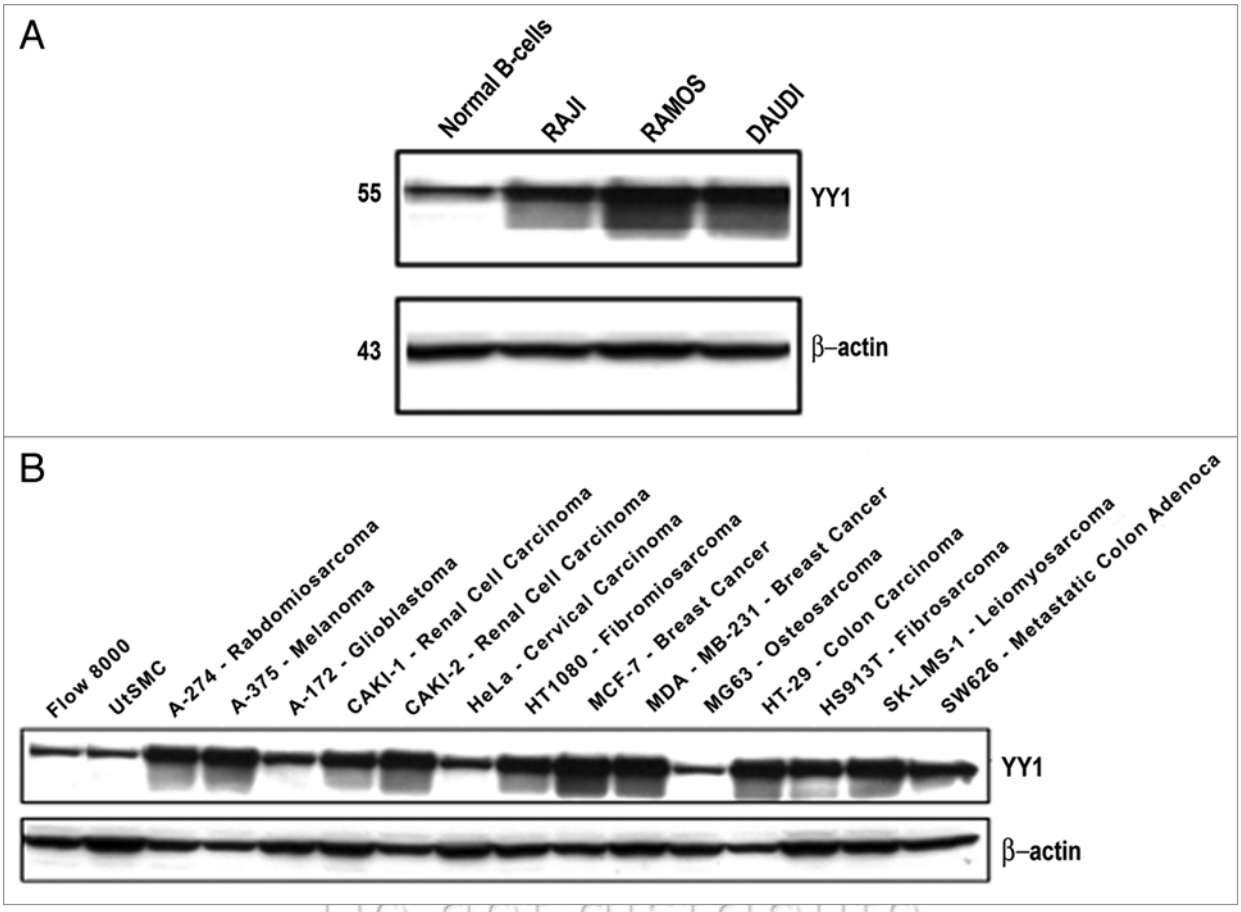

Figure 2. YY1 protein expression in tumor cell lines and normal cells as assessed by western blot analysis. (A) NHL cell lines were compared to normal B cells. (B) Solid tumor cell lines of various origin (see material and methods for details) were compared to normal fibroblasts. A representative experiment of the three performed is reported. Actin is used as protein loading control.

Currently, DLBCL may be cured in a significant percentage of patients, depending on the initial characteristics of the tumor and the host. Despite these advances, a considerable portion of DLBCL patients does not achieve long-term remission using current therapeutic approaches.[21]

Recently, in the attempt to identify "indicator genes" predictive of outcome in NHL, YY1 has been shown to have a statistically significant higher expression in the neoplastic nodes compared with reactive nodes, as assessed by real-time PCR.[22]

Here we documented that YY1 was detected at increased levels by genomic RNA analysis in a very large panel of DLBCL cases and demonstrated that the increased gene expression resulted in overexpression at protein level as demonstrated by WB. Since, several reports of YY1 expression have been generated in solid tumors using WB, we analyzed YY1 protein levels by WB also in numerous solid tumor cell lines to confirm that our validation approach was correct. The obtained results are essentially in line with literature data, in fact YY1 protein overexpression in cell lines was already reported in breast cancer for MCF-7 cells and in osteosarcoma for SAOS-2 cells.[19] Overall cell lines, as also documented by the comparison of microarray data and functional assays in ovary carcinoma[16] could represent a good model system to further evaluate the significance of YY1 expression

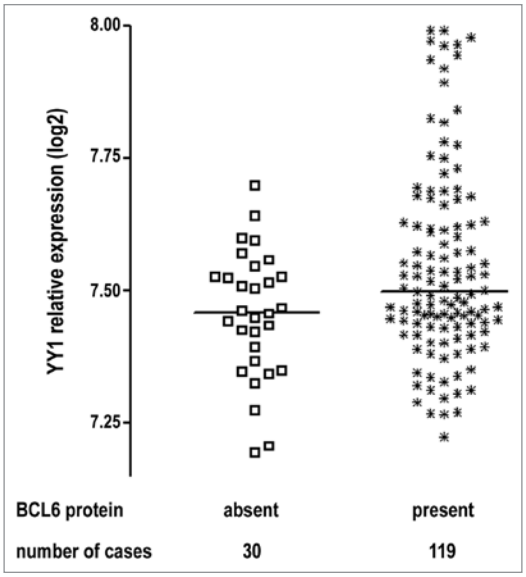

Figure 3. YY1 gene expression levels in DLBCL samples expressing or not BCL6 protein. The analysis was performed using data from Hummel dataset[9] and the difference was statistically evaluated by the Mann Whitney assay (p = 0.0380).

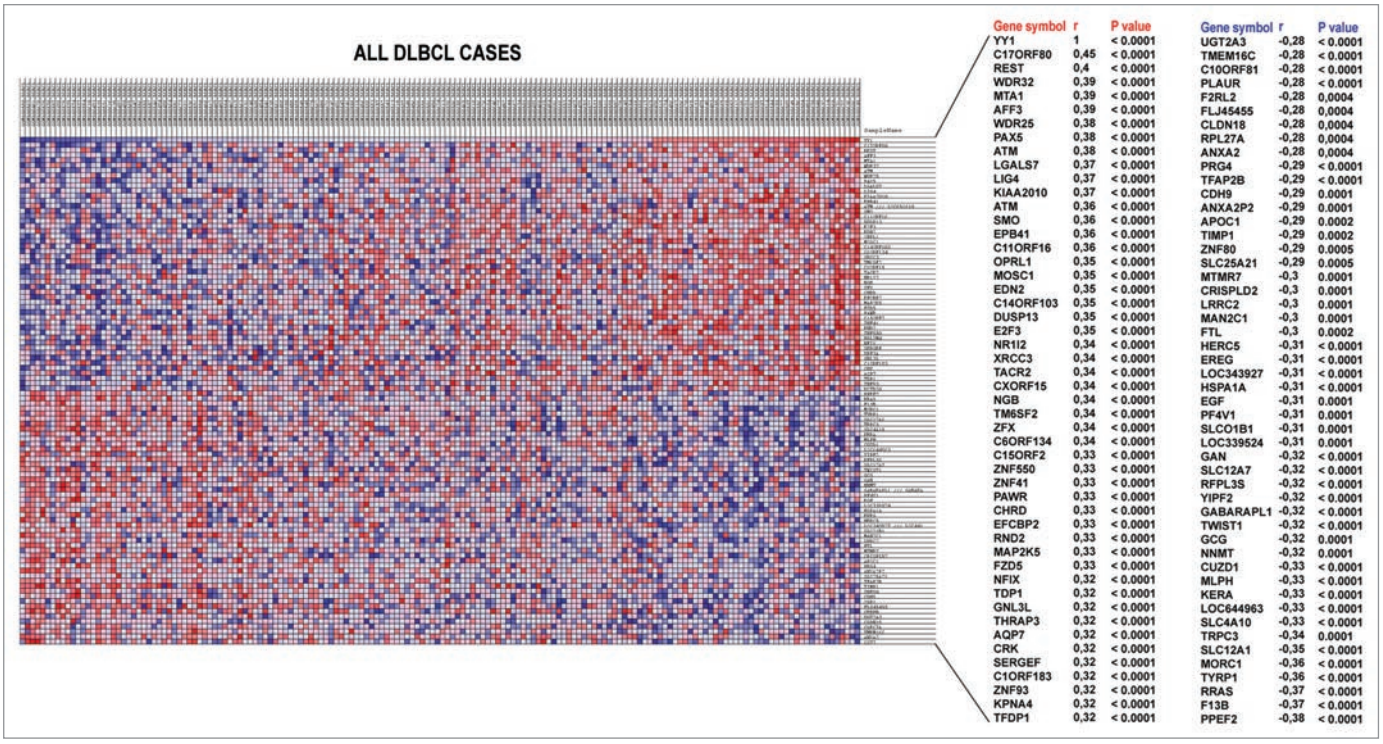

Figure 4. Heat map of YY1 correlated genes. The top 50 genes positively (red fill) and negatively (blue fill) correlated with YY1 by Pearson correlation analysis.

dysregulation in NHL; accordingly analyses of functional aspects are ongoing in different cellular models.

The association of YY1 expression with some clinical-pathological features in DLBCL, reported in Hummel et al.[9] showed a positive correlation between a high level of YY1 mRNA and high levels of BCL-6 protein. Likewise a positive correlation was documented between low level of YY1 mRNA and the absence of BCL-6 protein in lymphoma cells. It was already shown that BCL-6 is overexpressed in the same high-grade lymphoma cell lines[23,24] in which we observed the overexpression of YY1. However, the association between the two molecules is probably not direct. In fact, when we analyzed on the same case material all the genes that were significantly co-regulated with the YY1 mRNA expression, BCL-6 was not significantly associated with mRNA levels. *BCL-6* is a proto-oncogene encoding a nuclear transcriptional repressor, with pivotal roles in germinal center formation and regulation of lymphocyte function, differentiation and survival. The BCL-6 protein, expressed in 70% of DLBCL, is a zinc finger transcriptional repressor which, in normal cells, is expressed selectively by germinal center (GC) B cells, but not by immature B-cell precursors or differentiated plasma cells.[23] It has been also shown that expression of BCL-6 is an absolute requirement for GC formation and function.[25] Mutations in BCL-6 are regarded as a marker of B-cell transit through the GC because, in normal lymphoid tissues, they occur in approximately 30% to 50% of GC and memory B cells, whereas these are absent in pre-GC, virgin B cells.[26] On this basis, BCL-6 mutations have been proposed as a genetic marker useful for defining the histogenesis of B-cell lymphoproliferations displaying a GC or a post-GC phenotype.[27] BCL-6 mutations were suggested to result from a molecular mechanism similar to somatic hypermutation of Ig genes.[28] Furthermore, the prognostic value of BCL-6 mutation in lymphoid malignancy has been recently demonstrated (reviewed in ref. 29). Therefore, the potential indirect association between YY1 and BCL-6 deserves further functional analysis. If this relationship between YY1 and BCL-6 will be confirmed and functionally defined, it may have an important value in terms of therapeutic approaches.

By analyzing the large series of DLBCL in the Hummel dataset, we identified the transcription factors E2F3 and PAX-5 among the top 50 genes positively correlated with YY1. Similarly, Matsumara et al.[16] showed the association of YY1 with E2F3 in ovarian cancer. This finding further supports the power of our results obtained in DLBCL since the authors used computational approaches similar to those applied in the present study. Moreover, the authors report that such association is consistent with the involvement of YY1 in the cell cycle.[16] For the first time, here we document the association of YY1 with PAX-5 in DLBCL. Several studies have demonstrated that PAX-5 plays an important role in the control of B-cell proliferation and differentiation. It was shown that its overexpression results in proliferation of splenic B cells and spontaneous proliferation of B-lymphoma cell lines was observed after treatment with antisense oligonucleotides.[30] Overexpression of PAX-5 was already observed in high-grade lymphoma cell lines.[24] Importantly, PAX-5 itself downregulates the p53 expression[31] and YY1 overexpression promotes p53 degradation.[4] Moreover, it was reported that PAX-5 mutations are associated with lymphoma development in both immunocompromised

Table 1. List of the top networks as defined by the IPA software

ID	Networks	Score	Focus genes	Genes of networks
1	**Cellular Movement,** Hematological System Development and Function, Immune Response	41	25	ADCY, ↑**CARD10**, ↓**CCL3**, ↓**CEBPB**, ↑**CHKA**, ↓**CST7**, ↑**ERCC4**, ↓**EREG**, Galpha, ↑**GLPIR**, ↑**GNAI3**, ↓**GNAI5**, ↑**HIPK3**, ↑**HMGAI**, ↑**HTRIA**, IFNBeta, ↑**IFNAI7**, Igm, IL12, ↓**IL17A**, ↑**ILIRL2**, Interferonalpha, LDL, ↑**LGALS7**, ↓**MEFV**, NFκB, ↓**OLRI**, ↑**PI3**, ↑**RAD52**, Rock, ↓**SERPINA1**, ↑**SNIPI**, STAT5a/b, ↓**TNFAIP6**, ↑**UMOD**
2	Cell morphology, Cancer, **Cellular Movement**	38	24	Actin, ALP, Alpha actin, **ANXA2,** Ap1, Calmodulin, ↑**CASP2,** Caspase, ↑**CRYBB2,** ↓**CTSB,** ↑**CXORF15,** ↑**DLAT,** ↓**EGF,** ↑**EPB41,** F Actin, ↓**GP5,** Hdac, ↓**HSD11B1,** ↓**HSPB1,** IL1, ↑**ITPKA,** ↑**KCNQ2,** ↓**LPA,** ↑**MOAP1,** ↓**PPEF2,** ↑**REPS2,** ↑**RIPK1,** ↓**RPL27A**, ↑**SMAD6,** TGF beta, Timp, ↓**TIMP1,** ↓**TIMP2,** ↑**TIMP3,** ↓**TWIST1**
3	Cell Cycle, Gene Expression, *Carbohydrate Metabolism*	36	23	Akt, ↓**APOC1**, ↑**ASH1L**, ↑**BOK**, ↑**CCNE2 (includes EG:9134)**, Ck2, ↑**COL13A1**, ↑**CSNK2A1**, ↑**CUL4A**, Cyclin A, Cyclin E, E2f, ↑**E2F3**, ↓**GADD45G**, Histone h3, Hsp90, Insulin, ↑**LUC7L2**, ↓**MSTN**, N-cor, ↑**NFIX**, ↑**NR1I2**, Proteasome, ↑**PTCH2**, ↑**REST**, RNA polymerase II, ↑**SETD1A**, ↓**SLCO1B1**, ↑**SMO**, ↑**SORBS2**, ↑**SP1**, ↑**TFDP1**, ↑**THRAP3**, VitaminD3-VDR-RXR, ↑**YY1**
4	Organismal Functions, *Carbohydrate Metabolism, Small Molecule Biochemistry*	32	20	↓**AGXT,** ↑**AKT2**, Alcohol group acceptor phosphotransferase, AMPK, ↑**ATM,** ↑**CACNG2**, Calpain, Cbp/p300, Creb, ↑**EDN2**, ERK1/2, ↑**FRS2,** Galphai, ↓**GCG,** ↑**GIP,** ↑**GRM1,** ↑**GTF2I**, ITPR, ↑**MAPK1,** ↑**MAPK8IP2,** ↓**MLPH,** ↑**NCAPD3,** ↓**NFAT5,** NFAT complex, ↓**NPHP1,** ↑**NPHP4,** p70 S6k, PDGF, PKA, PP2A, ↑**PRPF4B,** ↑**SCNN1B,** STAT, ↓**TRPC3,** ↓**TYRP1**
5	**Cellular Movement,** Reproductive System Development and Function, Cell-to-cell Signaling and Interaction	22	16	CD3, ↑**CRK,** ERK, FGFR, ↑**FXN,** ↑**GFER**, IgE, ↓**IL9**, Integrin, ↓**ITGA5,** ↑**ITGA9 (includes EG:3680),** JNK, ↑**MAP2K5,** MAP2K1/2, MEK, ↑**MGAT3**, MMP, Nfat, ↓**PLAUR,** RAC, RAF, RAP1, RAS, RAS homolog, ↓**RRAS,** RSK, RTK, ↓**SLC12A7**, SOS, ↑**TNFSF8**, VEGF
6	Drug Metabolism, Lipid Metabolism, *Small Molecule Biochemistry*	22	16	↑**ABCE1,** AKAP12, ALDH1A1, ↓**API5S2,** ARNT2, ↑**CALML3**, CD52, COTL1, CXCR5, ↓**GAN,** ↑**GLRX5,** ↑**GMCL1,** HPRT1, MGMT, ↑**MSH3,** MYH7, ↑**NPAS1**, NUDC, ↓**OGN,** ↑**PDLIM4,** retinoic acid, RPL29, RPL23A, RPS19, ↑**STIM1 (includes EG:6786),** ↑**STK16,** ↑**TACR2,** TERF1, TLE1, ↓**TMSB10,** ↑**TNRC4**, UBA1, VHL

The score is based on a p-value, which estimates the likelihood that the Network Eligible Molecules that are part of a network are found therein by random chance alone; Mathematically, the score is the negative exponent of this p-value; Functions present in more than 1 network are identified either in bold or in italics or italics underlined; Focus genes significantly correlated with YY1 are in bold; ↑positive correlation, ↓negative correlation.

and immunocompetent patients.[32-34] Consequently, we can speculate that PAX-5 and YY1 may collaborate in the control of B-cell proliferation and that their deregulated expression may contribute to abnormal proliferation and thus to lymphomagenesis. These observations are also supported by previous studies where YY1 is involved in controlling multiple stages of early B-cell development, especially the pro-B-to-pre-B-cell transition[35] and by the biological network analysis, described herein, in which 29 biological networks are significantly associated with YY1. As shown in **Figure 5**, the top network associated with YY1 expression levels in DLBCL with the highest score is cellular movement, hematological system development and function, immune response.

Taken together, these data suggest that YY1 is involved in B cells transformation which gives rise to high-grade lymphomas through a dysregulation in the normal development of B cells affecting cell cycle and cellular motility. Molecular investigations are warranted to further support our computational functional analyses. If this is, in fact the case, the regulation of YY1 expression in NHL may be envisaged as a potential new therapeutic intervention.

Methods

Computational analyses. Two independent datasets were used.

(a) Basso K, et al. (2005).[8] The samples were hybridized using Affymetrix HG-U95Av2. The analysis of YY1 expression levels in association with B cell transformation was performed on 196 samples out of the 336 present in the original dataset. The selected dataset includes: 21 high grade (HG) cell lines [10 Burkitt lymphoma (BL), 3 Type III BL and 8 diffuse large B cell lymphoma (DLBCL)], 86 HG tumors (17 BL, 60 DLBCL and 9 primary effusion lymphomas), 64 low grade (LG) tumors (34 B cell chronic lymphocytic leukemia, 16 hairy cell leukemia, 6 follicular lymphoma and 8 mantle cell lymphoma) and 25 normal B cells subpopulations (5 preparations each from: cord blood B cells, naïve B cells, memory B cells, centroblasts, centrocytes). Scaled gene-expression values were obtained using RMA method with R software from Bioconductor.

(b) Hummel et al. (2006).[9] The samples were hybridized on Affymetrix U133A GeneChips. This dataset consists of 220 B-cell lymphomas, including 18 aggressive B-NHL, 28 atypical Burkitt Lymphomas (BL), 9 BL and 165 DLBCL. Probe level

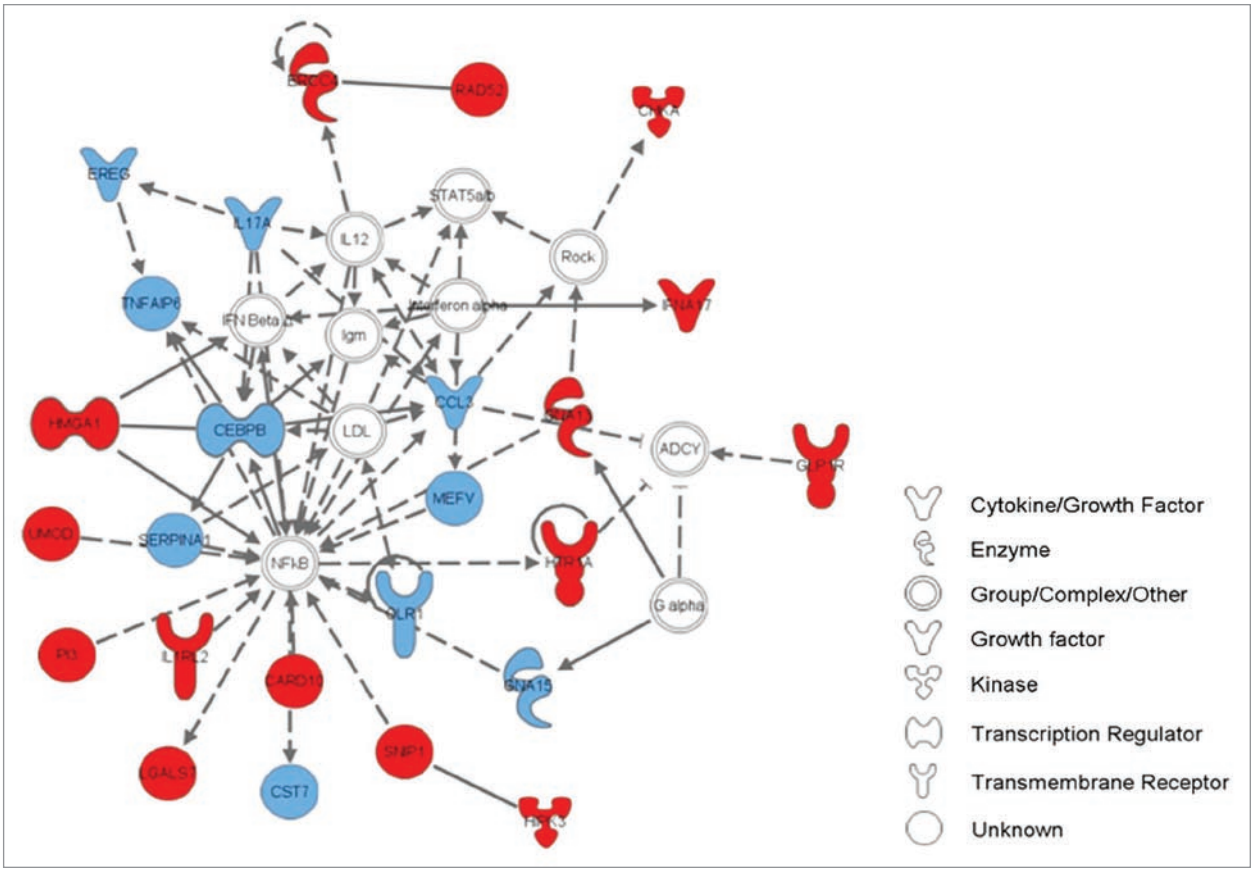

Figure 5. YY1 association with the network "Cellular Movement, Hematological System Development and Function, Immune Response" by Ingenuity pathway analysis. The analysis was performed using data from Hummel dataset.[9] Ingenuity pathway analysis predicts that 15 of the 35 genes involved in this network are significantly correlated with YY1 (red fill); while, 10 genes are inversely correlated (blue fill) with YY1. For the list of genes and details see Table 1 and Supplementary Table 2.

normalization was done using the calibration and variance stabilization method.[10] Probe-set summarization was performed using the median polish method on the normalized data.[11]

Cell lines. The Ramos, Raji and Daudi cell lines (human non-Hodgkin's B cell lymphoma) were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Invitrogen Corp., Carlsbad, CA), 50 IU/mL penicillin, and 50 μg/mL streptomycin (all from Cellgro, Herndon, VA). Blood samples from healthy donors were separated on a Ficoll gradient and the mononucleate cells (PBMC) collected were cultured in RPMI 1640, (1%) L-glutamine, (1%) penicillin, streptomycin and stimulated with pokeweed mitogen (PWM-GIBCO) 20 Mg/ml. Cells were maintained at 37°C and 5% CO_2 for incubation. The following solid tumor cell lines A375 (melanoma), A172 (glioblastoma), CAKI-1 and CAKI-2 (renal cell carcinoma), HeLa (Cervical carcinoma), HT1080 and Hs913T (fibrosarcoma), A274 (Rabdomyosarcoma), MG63 (osteosarcoma), SK-LMS1 (Leiomyosarcoma), MCF-7 (breast carcinoma), HT-29 (colon carcinoma), SW626 (adenocarcinoma), and normal fibroblast preparations (FLOW 8000 and UtSMC) were also obtained from the ATCC and cultured as indicated in the datasheets.

Western blot analysis. Cell lysates were prepared using radioimmunoprecipitation assay buffer (Assay Designs, Inc., Ann Arbor, MI) supplemented with one tablet of protease inhibitor mixture (Roche, Indianapolis, IN). Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Total protein lysates (20–30 μg) were subjected to electrophoresis in 10% SDS-PAGE, and the resolved proteins were transferred onto nitrocellulose membranes (Amersham, Arlington Heights, IL) as described previously.[12] The polyclonal anti-β-actin Ab was purchased from Chemicon International; the monoclonal anti-YY1, was obtained from Santa Cruz Biotechnology. Gel images were acquired with a Duoscan T1200 scanner (AGFA) and densitometric analysis of the bands was done using the Image-Pro Plus 4.1 program.

Statistical analysis. The non parametric Mann-Whitney and one-way ANOVA tests were used to compare two or three or more groups, respectively. A p-value <0.05 was considered significant. Pearson correlation coefficients were computed for each gene with YY1 gene expression and the p value were computed using the function cor.test of the software package R. A α = 0.001 was considered as significance level. Statistical analyses and graphics were generated using R version 2.7.2 (2008-08-25) and Graphpad Prism version 3.02.

Network and pathway analysis. Analysis of gene interactions was performed using Ingenuity Pathways Analysis (IPA) tools version 3.0 (www.ingenuity.com, www.ingenuity.com). Gene symbols of each probe set correlated with YY1 were used as input to generate biological networks based on a curated list of molecular interactions in IPA. For each of these networks a score was assigned: it represents the likelihood that the Network Eligible Molecules that are part of a network are found therein by random chance alone. Mathematically, the score is the negative exponent of this p-value calculation.

Acknowledgements

The authors thank Dr. Maria Luisa Sensi for her excellent contribution. This work was partially supported by the Italian Association for Cancer Research-AIRC (S.C.) and by the Italian League against Cancer-LILT (M.L.).

Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/CastellanoCC9-3-Sup.pdf

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