

CORRELATION BETWEEN PRO- AND ANTI-APOPTOTIC GENE EXPRESSION AND TELOMERASE ACTIVITY IN B- CELL LYMPHOMAS

CRISTINA DANIELA GOIA^a, ANCA ROXANA LUPU^b, CORALIA BLEOTU^a, CONSTANTA ANTIPA^a, LUMINIȚA VELT^b, ELENA POPA^b, GABRIELA CONSTANTINESCU^b, MIHAELA CLOSCA^b and GABRIELA ANTON^a

a. "Stefan S. Nicolau" Institute of Virology, 285, Mihai Bravu Ave., 030304, Bucharest, Romania

b. Coltea Hospital, Hematology Clinic, 1, I.C. Bratianu Blv., Bucharest, Romania

cdgoia@yahoo.com

Received December 15, 2008

The growth of lymphoid cells is regulated by a delicate balance between molecules controlling cell survival and cell death. Although some studies examined telomerase activity and Bcl-2, Mcl1 and p53 gene expression in non-Hodgkin's lymphomas (NHL), side-by-side analysis of these genes in different types of NHL is still missing. Therefore, blood specimens representing 60 cases of different NHL types were obtained from the Hematology Clinic, Coltea Hospital. Total RNA was isolated and reverse-transcribed in order to evaluate Bcl-2, Mcl1 and p53 mRNA expression and telomerase activity. The results showed a high expression of hTERT mRNA in the majority of NHL cases (85%) but also different expression levels of the anti-apoptotic genes. On the other hand, p53 mRNA expression was negative in over 55% of the cases, the negative correlation between Bcl-2 and p53 mRNA expression suggesting that the former was negatively regulated by the latter. The results suggest that pro- and anti- apoptotic genes Bcl-2, Mcl1 and p53 take part in the regulation of apoptosis in NHL and contribute to a different extent in progression to malignancy. The molecular mechanisms controlling cell proliferation and death in non-Hodgkin's lymphoma are complex, probably involving many genes, including those taken under study.

Key words: Lymphoma; Apoptosis; Telomerase; Bcl-2, p53.

INTRODUCTION

Non-Hodgkin B cell lymphomas (NHL) are among the few malignancies whose incidence and mortality increased in Europe and North America over the last few decades. In most developed European countries, NHL mortality rose up to the mid 1990s, and started to level off or decline in the following decade. Overall, in the European Union, mortality from NHL declined from 4.3/100,000 to 4.1 in men and from 2.7 to 2.5 in women between the late 1990s and the early 2000s. It is estimated that approximately 66000 patients will be diagnosed with and almost 20000 will die of non-Hodgkin lymphoma in 2008 all over the world¹. The rates are, however, still increasing in Eastern Europe. In Romania over 1300 new cases are

diagnosed every year and about 600 patients die of NHL.

Alterations in gene expression controlling apoptosis have been observed in most of B- cell lymphomas². The integrated molecular mechanism that controls apoptosis and cell cycle progression (the existence of regulatory molecules that interface between apoptosis and cell cycle progression) has been implicated and widely investigated³. Among these molecules, the B-cell lymphoma-leukemia 2 (Bcl2) protein family represents one of the major groups of apoptosis regulatory proteins. At least 15 of Bcl2 family members have been identified in mammalian cells and, despite their similar structures, Bcl2 family members can either facilitate cell survival (BCL-xL, MCL1) or promote cell death (BAX, BAK, BCL-xS).

MCL-1 (myeloid cell leukemia 1) is a member of the BCL-2 family that has a very short protein half-life. Since its identification in 1993, a great number of studies have suggested that MCL-1 plays an important role in various cell survival pathways⁴.

However, only recently the molecular mechanism by which MCL-1 antagonizes apoptosis started to be elucidated. MCL-1 is rapidly degraded in response to cell death signals and is immediately re-induced by survival stimuli. These results indicate that MCL-1 plays an apical role in many cell death and survival regulatory programs⁵.

The Bcl-2 gene was first discovered in follicular lymphoma that bear a t(14;18)(q32;q21) translocation, which results in the over-expression of BCL-2 protein in germinal center cells. Over-expression of BCL-2 contributes to oncogenesis by blocking apoptosis thereby promoting cell survival; interestingly this over-expression has been shown to upregulate telomerase activity *in vitro*⁶. Telomerase is a ribonucleoprotein polymerase that maintains telomere ends by addition of the telomere repeat TTAGGG. The enzyme consists of a protein component with reverse transcriptase activity (encoded by this gene) and a RNA component which serves as a template for the telomere repeat. Telomerase plays a role in cell cycle, as it is normally repressed in postnatal somatic cells resulting in progressive shortening of telomeres. Deregulation of telomerase expression in somatic cells may be

involved in oncogenesis⁷. For instance, telomerase activity has been reported to have an adverse prognostic significance in several malignancies including neuroblastoma, gastric cancer and breast cancer⁸. Induction of hTERT (telomerase reverse transcriptase) expression is essential for telomerase activation during cellular immortalization and tumor progression. *In vitro* studies have shown that p53, a tumor suppressor gene downregulates the transcription of hTERT in malignant cells. Several studies demonstrated that p53, a pro-apoptotic gene, is frequently inactivated during oncogenesis and is associated with poor prognosis⁹.

The aim of the present study was to investigate the relationship between hTERT expression and p53, Bcl-2 and Mcl1 gene expression in 60 samples from patients with NHL.

MATERIALS AND METHODS

Study group

60 patients (31–78 years old, 45% women, 55% men) diagnosed with different NHL subtypes in the Hematology Clinic of Coltea Hospital were included in the study group. NHL phenotype distribution is shown in Figure 1. A group of 20 healthy donors were included in the control group.

Total RNA isolation

Total RNA was isolated from cells separated from 2 ml peripheral blood samples (collected on EDTA from lymphoma patients and healthy donors) using High pure RNA isolation kit (Qiagen) according to the manufacturer's protocol. The quality of isolated RNA was assessed by determining the concentration and purity using Nanodrop spectrophotometer.

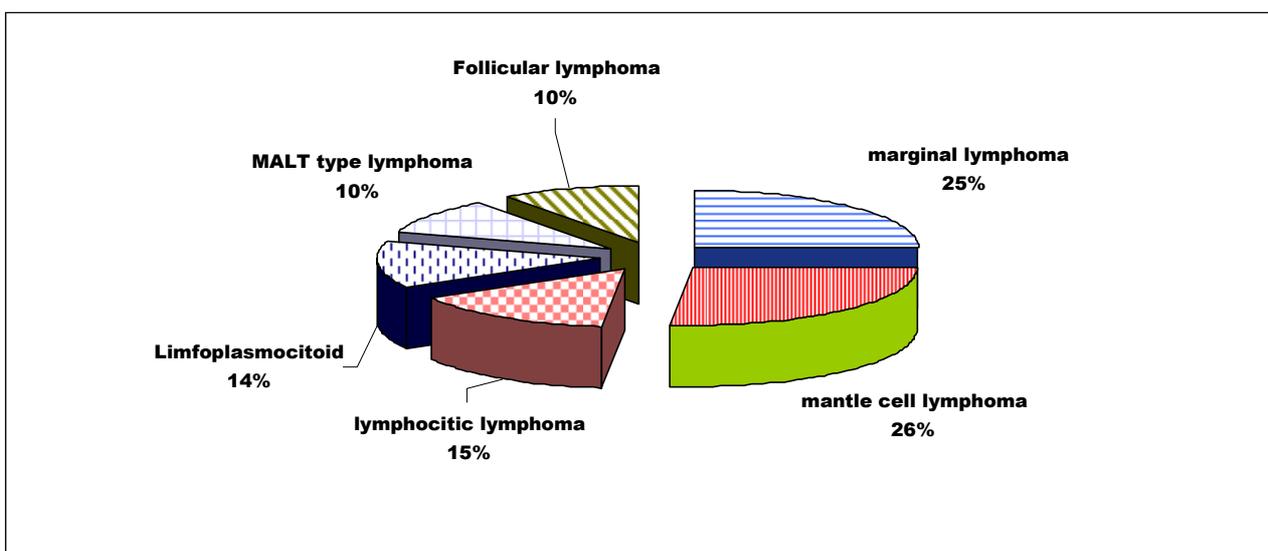


Fig. 1. Distribution of the different types of non-Hodgkin lymphomas in the group of patients investigated.

Reverse-transcription

Total isolated RNA was reverse-transcribed with Promega reagents. Briefly, 2,5 µg of RNA, 1µl oligodT and 1µl dNTPs were mixed and incubated 5 minutes at 65°C. After cooling on ice, 4 µl RT buffer, 2 µl DTT and 1µl RNase inhibitor were added to the mix. After 2 minutes incubation at 37°C, 1 µl of MuMLV reverse transcriptase was added and the incubation proceeded for 60 minutes at 37°C and 15 minutes at 70°C.

RT-PCR

In order to determine cDNA quality, a RT-PCR assay for GAPDH *house-keeping* gene was performed on all samples.

RT-PCR assays for Bcl-2, Mcl1, hTERT and p53 were performed by using *in house* designed primers and synthesised by Invitrogen. The primers and amplicon size are shown in Table 1 and PCR conditions are presented in Table 2. Reaction mix consisted in 2 µl cDNA, PCR buffer 5×, 1,5 mM MgCl₂, 400 pmols of each primer, 200 µM of each nucleotide and 2 units of Taq polymerase (Promega reagents). The results obtained were analyzed after agarose gel electrophoresis (2%) and ethidium bromide staining, using UVP BioDoc-It analysis program (Biometra).

Table 1

Primers used for RT-PCR and the correspondent product size

Genes	Primers	Amplicon size
GAPDH	F: 5'- ACCACAGTCCATGCCATCAC-3' R: 5'- TCCACTACCCTGATGCTGTA-3'	450 bp
Bcl-2	F: 5'- CGGCCTCTGTTTGATTTCTC-3' R: 5'- AGCCTGCAGCTTTGTTTCAT-3'	350 bp
Mcl1	F: 5'- GCACTTACTGTAAGGCTATC-3' R: 5'- TCGCTGGGTAAGTTCGGG-3'	504 bp
hTERT	F: 5'- CGGAAGAGTGTCTGGAGCAA-3' R: 5'- GGATGAAGCGGAGTCTGGA- 3'	145 bp
p53	F: 5'- CCACCATCCACTACAACACTAC-3' R: 5'-CCTTCTGTCATGAACATGAG-3'	507 bp

Table 2

RT-PCR conditions

Genes	Initial Denaturation	Denaturation	Annealing	Elongation	Final elongation	Number of cycles
GAPDH	95°C/5 min	95°C/60 sec.	56°C/90sec	72°C/60sec.	72°C/5 min	35
Bcl-2	95°C/5 min	95°C/60 sec.	60°C/60sec	72°C/60sec.	72°C/5 min	35
Mcl1	95°C/5 min	94°C/30 sec	55°C/60sec	72°C/30sec	72°C/5 min	40
hTERT	95°C/5 min	95°C/40 sec	60°C/50sec	72°C/50sec	72°C/5 min	35
p53	94°C/5 min	94°C/60 sec	50°C/2 min	72°C/2 min	72°C/15min	40

RESULTS AND DISCUSSIONS

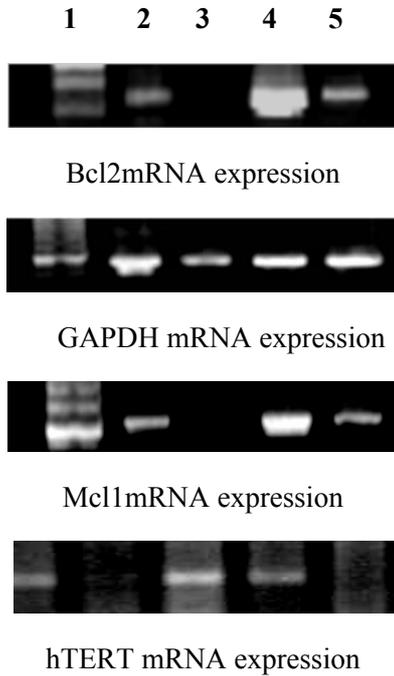


Fig. 2. hTERT, Mcl1, Bcl-2 mRNA expression in 4 representative NHL samples as determined using RT-PCR. GAPDH mRNA expression was also included as a control for cDNA quality. Lane 1: DNA ladder; lanes 2-3: mRNA expression in samples from mantle cell lymphoma patients; lanes 4-5: mRNA expression in samples from marginal zone lymphoma patients.

Table 3

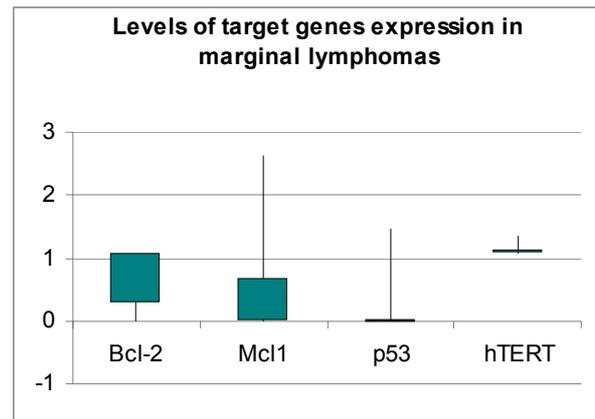
Expression levels of Bcl-2 family members, hTERT and p53 mRNA (absence, hipoeexpression, hiperexpression) compared with GAPDH mRNA expression in NHL cases studied.

Levels of expression	Bcl-2 (n=60)	Mcl1 (n=60)	p53 (n=60)	hTERT (n=60)
-	50% (30)	25% (15)	56,6% (34)	0%
Hipoexp.	25% (15)	15% (9)	33,3% (20)	15% (9)
Hiperexp.	25% (15)	60% (36)	10% (6)	85% (51)

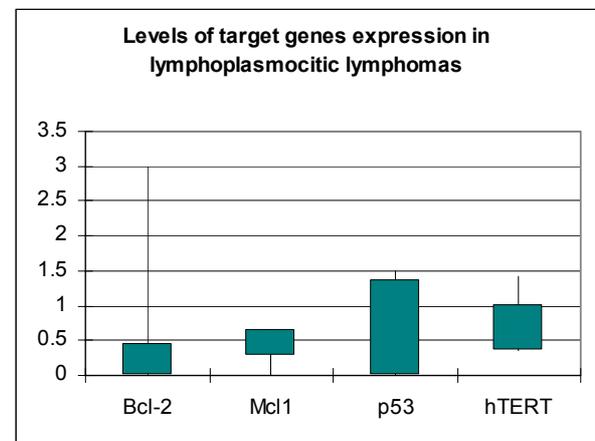
In the management of patients with lymphoma, it would clearly be useful to have information which would form the basis of reliable prognostic criteria. There have been a variety of classifications of NHL all of which claim to have prognostic value. The definition of broad categories of disease within NHL has certainly

been useful but in individual patients it can be unreliable. In this context, researchers focus on establishing novel biomarkers to better characterize different disease entities within NHL. Such candidate biomarkers are represented by pro- and anti-apoptotic genes like those studied here. A possible correlation of the expression levels of these genes with telomerase activity could offer a better knowledge of molecular mechanisms involved in different NHL types.

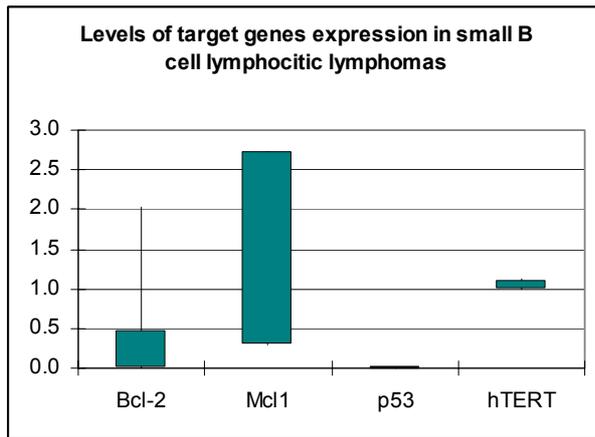
In Figure 3 (A to F), the levels of target genes mRNA expression in different lymphoma types are shown. According to this data, there is a high Mcl1 mRNA expression in small B cell lymphocitic lymphomas, but, on the other hand, p53 expression is almost absent. In some types of lymphomas (follicular and lymphocitic lymphomas) there is a low expression of p53 suggesting a poor prognosis for these patients. Telomerase activity is elevated in virtually all cases which also indicated an unfavourable disease outcome.



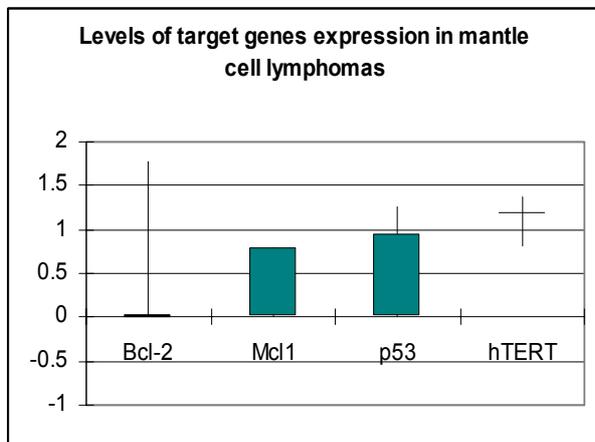
A



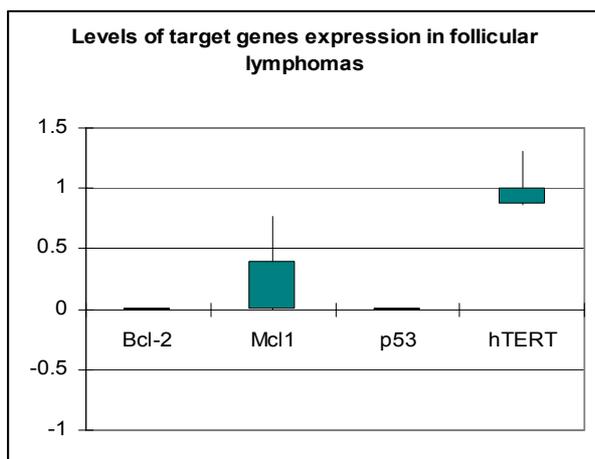
B



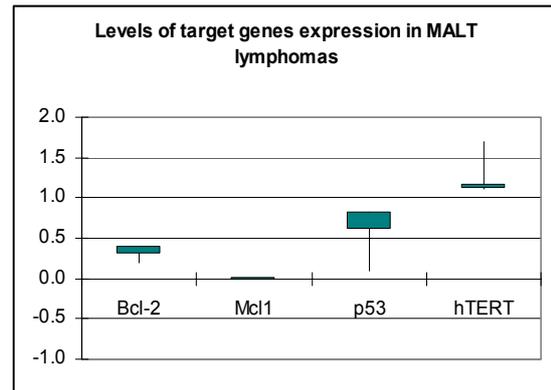
C



D



E



F

Fig. 3. Distribution of GAPDH-relative gene expression of Bcl-2, Mcl1, p53, hTERT mRNA in patients with: A. marginal lymphomas, B. lymphoplasmocytic lymphomas, C. small B cell lymphocytic lymphomas, D. mantle cell lymphomas, E. follicular lymphomas, F. MALT lymphomas.

The overall results of RT-PCR for the selected genes are shown in table 3. hTERT mRNA expression is present in 85% of the total cases. Our findings support the hypothesis of other researchers^{10,11,12} that telomerase functions in mature B-cell neoplasms by preferentially stabilizing short telomeres, thereby preventing the activation of cellular crisis in malignant cells with chromosomal telomere loss. Telomerase is controlled *in vivo* along with the cell cycle and is not constitutively active in B-NHL but our data provide evidence that telomerase may represent a candidate gene in B-NHL and can possibly have a prognosis value (could indicate a poor outcome in patients with hTERT mRNA expression).

The Bcl-2 gene product is an anti-apoptotic molecule that modulates the mitochondrial release of cytochrome *c*, and the interaction of apoptosis activating factors with caspase 9 and Bax (Bcl-2 associated \times protein). p53 is a tumor suppressor gene that maintains genomic stability either by inducing cell cycle arrest or apoptosis. In our study group there is a lack of Bcl-2 mRNA expression in half of the cases supporting the known data according to which altered Bcl-2 and p53 gene expression is involved in lymphomagenesis. On the other hand, our data suggest that the Mcl1 high expression level (60%) could be considered a prognostic factor in NHL.

CONCLUSIONS

Enhanced telomerase activity combined with deregulation of the factors responsible for cell survival and proliferation may contribute to the development and progression of lymphomas. The molecular mechanisms involved in the control of cell proliferation and death in non Hodgkin lymphoma are very complex, probably involving a wide range of genes, including p53, Bcl2 and Mcl1. On the other hand, cellular immortalization may be associated with an increased resistance to apoptosis and an increase for telomerase activity. Our preliminary data suggest that high hTERT mRNA expression may be related to poor prognosis and shorter survival in NHL. In conclusion, further investigation in a larger group of patients is needed in order to assess the clinical relevance of telomerase activity and genes involved in apoptosis.

REFERENCES

1. Ries, L.A.G., Melbert, D., Krapcho, M., Stinchcomb, D.G., Howlader, N., Horner, M.J., Mariotto, A., Miller, B.A., Feuer, E.J., Altekruse, S.F., Lewis, D.R., Clegg L., Eisner, M.P., Reichman, M., Edwards, B.K., *SEER Cancer Statistics Review, 1975–2005*, National CancerInstitute.Bethesda,MD,<http://seer.cancer.gov/csr/1975-2005/>, based on November 2007 SEER data submission, posted to the SEER web site, **2008**.
2. Pagnano, K.B., Silva, M.D., Vassallo, J., Aranha, F.J., Saad, S.T., *Apoptosis-regulating proteins and prognosis in diffuse large B cell non-Hodgkin's lymphomas*, *Acta Haematol*, **2002**, 107:29–34.
3. MacNamara, B., Wang, W., Chen, Z., *Telomerase activity in relation to pro- and anti-apoptotic protein expression in high grade non-Hodgkin's lymphomas*, *Haematologica*, **2001**, 86: 386–393.
4. Leoncini, L., Del Vecchio, M.T., Megha, T., *Correlations between apoptotic and proliferative indices in malignant non-Hodgkin's lymphomas*. *Am J Pathol*, **1993**, 142:755–63.
5. Soini, Y., Raunio, H., Paakko, P., *High-grade malignant non-Hodgkin's lymphomas differ from low-grade lymphomas in the extent of apoptosis and their expression of bcl-2, mcl-1, BAX and p53*, *Tumour Biol*, **1998**, 19:176–85.
6. Ely, S.A., Chadburn, A., Dayton, C.M., Cesarman, E., Knowles, D.M., *Telomerase activity in B-cell non-Hodgkin lymphoma*. *Cancer*, **2000**, 89: 445–452.
7. Shay, J.W., Zou, Y., Hiyama, E., Wright, W.E., *Telomerase and cancer*, *Hum Mol Genet.*, **2001**, 10: 677–685.
8. Collins, K., Mitchell, J.R., *Telomerase in the human organism*, *Oncogene*, **2002**, 21:564–579.
9. Xu, D., Wang, Q., Gruber, A., *Downregulation of telomerase reverse transcriptase mRNA expression by wild type p53 in human tumor cells*, *Oncogene*, **2000**, 19:5123–33.
10. Du, M., Singh Husseuin, A., Isaacson, P.G., *Positive correlation between apoptotic and proliferative indices in gastrointestinal lymphomas of mucosa-associated lymphoid tissue (MALT)*, *J Pathol*, **1996**, 178: 379–384.
11. Charalambous, G.K., Gomatos, I.P., Konstadoulakis, M.M., *Protein expression of bax, bcl-2, and p53 in patients with non-Hodgkin's gastric lymphoma: prognostic significance*, *World J Surg*, **2000**, 24: 608–614.
12. Xu, W., Sheng, R., Sheng, Z., Xu, T., *The expression and clinical significance of proliferative antigen Ki-67 and apoptosis-antagonizing antigen Bcl-2 in non-Hodgkin's lymphoma*, *Zhonghua Nei Ke Za Zhi*, **2001**, 40: 452–465.