Impact of K-ras Mutation and C-myc Overexpression on the Prognosis of Diffuse Large B-Cell Lymphomas

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ABSTRACT

Diffuse large B-cell lymphoma(DLBCL) is the prototype of aggressive lymphomas. The abnormalities such as translocation, deletion, duplication are stated to affect the progress and the prognosis of the disease. This study investigates the presence and impact on the prognosis of K-ras mutation and C-myc overexpression in patients with DLBCL. Twenty eight patients diagnosed with DLBCL and 29 healthy controls, whose diagnostic lymph node biopsy was reactive, were enrolled in this study. Paraffin blocks were analyzed for K-ras mutations and C-myc expressions. The effects of C-myc and K-ras on overall and disease-free survival were evaluated. Of the 28 patients, three (10.7%) K-ras mutations were detected. K-ras mutation (+) cases have an average survival time of 28 months, while those without the mutation were 49 months. The average duration of disease-free survival was 17.6 months in patients with K-ras mutation, while it has been identified as 45 months in patients without mutations (p= 0.072). C-myc overexpression in 17 patients(61%) was detected. Median overall survival was 33 months in male patients, while it was 65 months in female patients (p= 0.006). The median disease-free survival time was 23 months in male patients, although 64 months in female patients (p= 0.003). Complete remission rate was 30% in C-myc overexpression group, while 69% in remaining group with the cut-off 2.48 (p= 0.02). The presence of K-ras and C-myc overexpression in patients with DLBCL does not affect overall and disease-free survival. C-myc overexpression harms complete remission. Gender was found to be influential on the overall and disease-free survival; meanwhile, the female gender was a better prognosis.

Keywords: Lymphoma, K-ras, C-myc

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid neoplasia in adults.[1] Many cancers are known to occur as a result of proto-oncogene activation or mutation. The most common oncogenes in human cancers are members of the Ras family.[2] Myc protein expression is seen in the majority of DLBCL cases. In some studies, it has been observed that overexpression is associated with poor prognosis.[3] Nearly 70% of cancers in humans have high levels of Myc expression. Members of the Myc gene family have malignant potential. Chromosomal translocations and amplifications activate the Myc genes, and they transform into oncogenes.[4] The C-myc gene is a transcription factor found in the cell nucleus, which encodes the protein C-myc.[5] It has also been shown that increased Myc expression stimulates apoptosis.[6,7]

K-ras is a protein encoded by the K-ras proto-oncogene and is involved in normal cell proliferation signal transduction. A mutated K-ras becomes a potent oncogene. K-ras has been shown to stabilize the Myc protein. Thus, Myc and Ras stimulate tumor development by various mechanisms.[8,9]

We aimed to investigate whether K-ras mutation and C-myc overexpression affect the prognosis in DLBCLs. It is crucial to identify risk factors and define genetic changes to manage better subgroups that fail with standard treatments in the future and determine targeted therapies.

PATIENTS AND METHODS

The study was conducted at Faculty of Medicine, Department of Internal Diseases, Division of Hematology. The study protocol was reviewed and accepted by the Ethics Committee of University Senate (08.12.2010-2010/16). The University Research Projects unit accepted it with the project number TSU-11-3675 and covered all financial expenses (genetic and pathology tests). The medical records of patients admitted to the hematology outpatient clinic between April 2004 and September 2013, who were diagnosed with DLBCL and had been followed up, were retrospectively reviewed. Thirty-one patients whose data were sufficient were included in the study. Three patients with insufficient lymph node samples were excluded from the study. As the control group, 29 individuals who were not diagnosed with malignancy, who underwent diagnostic lymph node excision, and reported as reactive or benign lymph nodes were included.

In this study, formalin-fixed paraffin-embedded (Formalin Fixed Paraffin Embedded, FFPE) DLBCL tissues and reactive lymph node tissue samples belonging to the control group were used as material. Patient data were obtained from Faculty of Medicine's file archive. The following parameters were recorded, and IPI (international prognostic index) values at the time of diagnosis were calculated: age, gender, stage of the disease, splenomegaly, bone marrow involvement, EKOG performance score, chemotherapy received, number of cycles, chemotherapy response, whether receiving radiotherapy, whether there is an autologous bone marrow transplant.

The patients were staged according to the Cotswold staging system.

The total and disease-free survival time of the patients were determined. Surviving patients were evaluated at intervals of 3-6 months.

Three sections were taken from the blocks into two separate Eppendorf tubes. K-ras mutation and C-myc expression were analyzed in the sections in the Medical Genetics Department.

K-Ras Mutation Analysis

DNA isolation was performed with a tissue DNA isolation kit from sections taken from paraffin-blocked lymph nodes of patients and control group. Then, codon 12, 13, and 61 mutations were studied with the K-ras kit from DNA samples. The method's principle is based on the immobilization of products amplified in a polymerase chain reaction (PCR) with Streptavidin Sepharose High-Performance beads using primers targeting codon 12, 13, and 61. Single-stranded DNA is prepared from these samples, and appropriate sequence primers are attached to the DNA. Samples are then analyzed with Pyromark Q24 MDx (QIAGEN,971460).

C-Myc Gene Expression Analysis

From paraffin blocks obtained from lymph nodes of patients and control group, 3-5 sections were taken with Shadan type microtone into Eppendorf tubes of 4 μm thickness. RNA was isolated from paraffin sections using the Qiagen miRNeasy FFPE kit. The obtained RNAs were measured in a spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies, USA) at a wavelength of 260 nm, and microgram (μg) values per microliter (μL) were determined. A transcriptor high fidelity cDNA synthesis kit (ROCHE) was used for cDNA synthesis. The RNAs obtained were converted into cDNA with the transcriptor high fidelity cDNA synthesis kit (ROCHE) in RT-PCR with the catalog number 05081955001. The primer sequences specific to the cDNAs of the genes investigated are given below. Amplifications in 20 μL total reaction volume; cDNA was performed using mRNA specific primers, UPL probe, Light Cycler TaqMan

Table 1. Mean age and gender ratios of patients and control group

		Patient (n=28)	Control (n=29)	p
Age, mean ± SD		58.2857 ± 13.75408	59.8621 ± 9.31099	0.61*
Gender	Female n (%)	15 (53.6%)	14 (48.3%)	0,79**
	Male n (%)	13 (46.4%)	15 (51.7%)	

* Student T-test, ** Chi-square Test

Master mix (Roche, Germany), and distilled water. To normalize C-myc gene expression levels, the housekeeping gene Beta-Actin mRNA expression level was taken as a reference. The experiment was repeated twice for each concentration. The samples' target gene concentrations and reference gene concentrations were made by Relative Quantification, and the 2-Cq method was applied with the Efficiency=2 method.

Beta Actin gene primers: GGCCAGGTCATCACCATT

C-myc gene primers: TGGCCGAAATGAAAGAGAAG

Statistical Analysis

Normal distribution was evaluated using the Shapiro-Wilk test. The independent two-sample T-test and Mann-Whitney U tests were used for quantitative variables; the Pearson chi-square method and Fisher's exact chi-square test were used for qualitative variables. ROC analysis was used to evaluate the unfavorable prognostic effect of C-myc, and the area under the curve was calculated with a 95% confidence level. The Youden index was also used to determine the optimum cut-off value for C-myc, and sensitivity and specificity values for this cut-off value were calculated. The Kaplan-Meier method was used to evaluate C-myc and K-ras effect on total and disease-free survival, and comparisons were made with the Log-rank test. Data analysis was evaluated using IBM SPSS Statistics 20.0 (IBM Inc, Chicago, ILL, USA). $p< 0.05$ was accepted as a significance level.

RESULTS

The characteristic of control group and patients were shown in Table 1.

Complete response was observed in the 13, partial response was observed in nine of them. While two of the patients remained stable, four people were unresponsive to treatment.

The patients' survival was compared with the gender. While 80% of female patients were alive, this rate was only 23% in male patients (p= 0.007).

The average follow-up period of the patients enrolled in this study was 37.75 months; the mean overall survival was 50 months. The average overall survival of male patients was 33.4 months, while of female patients was 65.9 months (p= 0.006).

In terms of disease-free survival, the mean disease-free survival time of the patients was 46 months. The average disease-free survival time of male patients was 23.2 months, while female patients were 64.7 months (p= 0.003), (Table 2).

Table 2. Gender-survival rates at the end of follow-up

	n	Alive, n (%)	p	Survival Mean (months ± SD)	p	Disease-free survival Mean (months±SD)	p
Male	13	3 (23.1%)	**0.007**	33.4 ± 6.83	**0.006**	23.2 ± 6.32	**0.003**
Female	15	12 (80.0%)		65.9 ± 6.83		64.7 ± 7.48	
All	28	15 (53.5%)		50.1 ± 5.80		46.2 ± 6.66	

Table 3. Overall and disease-free survival times with K-ras mutation and C-myc overexpression

			Mean Overall Survival (months)	p	Mean Disease-free Survival (months)	p
K- ras	+	3 (%10.7)	28.0	0.093	17.667	0.072
	-	25 (%89.3)	49.8		45.076	
C-myc	< 1.95	11 (%39)	49.6	0.740	42.3	0.920
	> 1.95	17 (%61)	47.4		45.9	

K-ras mutation was detected in 3 (10.7%) of 28 patients. No mutation was found in the control group. At the end of the study, all three patients with K-ras died, while 12(48%) of 25 patients without K-ras died. All point mutations were at the 12th codon.

While the mean survival time was 28 months for those with K-ras mutation, it was 49.8 months for those with no mutation (p= 0.093), (Table 3).

While the mean disease-free survival time was 17.6 months in patients with K-ras mutation, it was 45.1 months in those without the mutation (p= 0.072), (Table 3.

C-myc expression was detected in both the patient group and control group. There was a significant difference between the expression values of the two groups (p= 0.001). The optimum cut-off value was 1.95. Above this value was accepted as overexpression.

C-myc overexpression was detected in 17 (61%) of the patients. The average survival of these patients was 47.3 months. The average survival of the remaining 11 patients was 49.6 months (p> 0.5) (Table 3). Disease-free survival time was 45.9 months in patients with C-myc overexpression; and 42.3 months in patients without overexpression (p> 0.05), (Table 3).

When C-myc overexpressing patients were compared with those who did not, according to the status of having complete remission after first-line chemotherapy, complete remission was detected in 46% of the overexpression group and 53% in the other group (p> 0.05). However, when we decreased the sensitivity and increased the specificity instead of taking 1.95, which is the cut-off value, and accepted the cut-off value of 2.48, which is the optimum value, a significant change was found in the full remission rates. The complete remission rate was 30% in the C-myc overexpressing group and 69% in the other group (p= 0.02).

There were only two patients with both K-ras mutations and C-myc expression above the cut-off value. These two patients' average life expectancy was 32 months; the remaining patients' average life expectancy was 50 months (p> 0.05).

According to their IPI scores, the patients were divided into two groups: low (IPI= 0, 1, 2) and high (IPI= 3, 4, 5) risk groups. The overall survival of 22 patients in the low IPI group was 51 months on average; the high IPI group average was 42 months (p> 0.05).

The average disease-free survival of the two groups divided according to low and high IPI values was 48 months and 35 months, respectively (p> 0.05).

The patients were divided into two groups according to the age of 60, which is the cut-off value in IPI scoring. Fifteen were under 60; 13 were over 60 years-old. The average survival time of patients under 60 years-old was 54 months; above 60 was 44 months (p> 0.05). Disease-free survival rates were also similar (p> 0.05).

DISCUSSION

Diffuse large B-cell lymphoma is a heterogeneous subgroup, which makes diagnosis and treatment processes difficult, and requires different treatment approaches. It can be seen in all age groups and both genders, including children, and is more common in men than in women.[10] Tomita et al.,[11] evaluating prognostic factors in patients with DLBCL, mentioned the negative effect of male gender on overall survival. In our study, both total and disease-free survival times were significantly lower

in men with a diagnosis of DLBCL compared to women (p= 0.006). It raises the question of whether we should prefer more aggressive treatments if performance conditions are appropriate.

In most of the studies conducted, the FISH method examines C-myc expression levels in tissues; then, pathologists state the expression rates as percentages. There is no standard value yet for the limit of overexpression. While some studies consider the patient group's median value to be the limit, other studies accept staining over 50-60% as overexpression, but these results are subjective.

In our study, the real-time PCR method was used, which is much more sensitive than FISH and prevents human errors. The optimal cut-off value, which demonstrates the statistical difference between the patient and control groups, was accepted, and its effect on prognosis was investigated. Also, the analyzes were repeated with different cut-off values by decreasing the sensitivity and increasing the specificity.

Although Dave et al.[12] stated that C-myc expression is characteristic of burkitt lymphoma, Schrader et al.[13] reported that it may also be in DLBCL subgroups and has prognostic significance. Schrader's study found that a high C-myc index increased premature death rates 3.4 times.

In the study of Magić et al.,[14] significant C-myc gene overexpression was detected by the PCR method in approximately one-third (29%) of B-cell lymphomas.

Green et al.,[15] found Myc overexpression in 35 (17%) of 205 DLBCL patients. Also, it has been mentioned that the localization of the Myc protein in the cell nucleus has a very high specificity and sensitivity in predicting Myc gene changes.

In our study, C-myc overexpression was detected in 61% of the patients. The higher C-myc positivity rate than the other studies was attributed to the RT-PCR technique's different sensitivity using the FISH method. We thought that performing analyses with simultaneous FISH and PCR techniques in future studies will reveal this difference more clearly.

In the study conducted by Klapper et al.[16], Myc changes were observed in 14 (7.9%) of 177 DLBCL patients whose tissues were evaluated by the FISH method. While the survival of Myc (+) patients was significantly lower than the Myc (-) group (p= 0.047), no significant difference was found between disease-free survival (p= 0.062).

In the study conducted by Savage et al. in patients receiving R-CHOP treatment, Myc changes were detected in 12 (8.8%) of 135 DLBCL patients. When Myc-associated DLBCL was compared with all other DLBCL types, both five-year disease-free survival (31% and 66%, p= 0.006) and overall survival (33% and 72%, p= 0.016) were significantly lower in the Myc (+) group. The adverse effects of IPI score, AB cell phenotype, extra-nodal, and bone marrow involvement on disease-free survival were determined in their analysis. When CNS relapse is considered the endpoint, Myc changes are predictive for CNS relapse in patients treated with R-CHOP.[17] Additionally Malkan et al. mentioned that extensive relapses may alter the outcomes; in the cases who reached second remission in DLBCL, disease-free survival is lower and stem cell transplantation may be a good treatment option.[18]

Akasaka et al.[19] studied C-myc translocation by inverse PCR method; while the 2-year survival rate was 37.2% in the C-myc (+) DLBCL group (n= 12), they found it 62.6% in the C-myc (-) DLBCL group. The C-myc (+) group showed a significantly poor prognosis (p= 0.04). However, there was no significant difference between the five-year survivals.

In the study conducted by Hummel et al.[20] in 2006, DLBCL patients carrying Myc translocations showed a more aggressive clinical presentation. The five-year survival rate was lower than patients without translocation.

As seen in our study, Myc protein synthesis occurs in the average population, but these levels are significantly lower than the patient group (p= 0.001). New studies are needed to standardize and determine the critical limit values.

In the Nitsu study, total and disease-free survival of patients with DLBCL and burkitt lyphoma with 8q24 translocation were compared; no significant difference was found. It has been suggested that B-cell lymphomas with 8q24 translocation may be

a group with a poor prognosis independent of these two diseases.[21] The rate of having complete remission in patients with DLBCL was 67% in the 8q24 translocation (+) group and was 83%, significantly lower than the 8q24 translocation (-) group.

Myc overexpression is seen in 5-40% of Mantle Cell Lymphomas (MHL). In the study conducted by Oberley et al.[22], it was found that high Myc expression alone was associated with poor prognosis and KI67 proliferation index in MHL. It has also been suggested that keeping the cut-off values high will increase the predictive value. When the cut-off value was increased in our study, the C-myc overexpressing group's rate to enter into complete remission was significantly lower (p= 0.02).

Magić et al.[14] showed that while K-ras point mutation was detected in two (6.45%) of 31 B-cell NHL cases, H-ras point mutation was detected in only one case (3.23%). No mutation was found in other patients and healthy controls. In our study, K-ras mutation was detected in three (10.7%) of 28 DBBHL patients, while no mutation was found in the control group. The effect of K-ras presence on total and disease-free survival was not found statistically significant. However, the numerical difference was quite significant. It is thought that conducting studies with larger patient groups will contribute to the literature.

The study by Tran et al.[8] demonstrates that future targeted therapies could result in tumor regression. It was found that Myc is a more potent oncogene than K-ras in lymphomas. When Myc and K-ras were inactivated together, complete regression was found in both lymphomas and lung cancer. It was mentioned that targeting a single oncogene in treatment has not been successful in every case in cancer treatment. The results found are that simultaneous Myc and mutant K-ras inactivation will be useful in tumor regression.[8]

This study has a few limitations. This is a single-center study performed in a small number of patients diagnosed at a tertiary center and whose biopsies are eligible for the study. Our results must be complemented by future global multicenter studies to achieve stronger results.

As a result, the presence of K-ras and C-myc overexpression in patients with DLBCL does not affect overall and disease-free survival. C-myc overexpression is a negative factor in terms of complete remission. Gender was found to be influential on the overall and disease-free survival; meanwhile, the female gender was a better prognosis.

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