

# The circulating auto-antibodies to p53 protein in the follow-up of lymphoma patients

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**Abstract.** Mutant p53 proteins may be targets of the host immune system - consequently a certain proportion of cancer patients (the percentage varies according to the type of cancer) with tumors that carry p53 missense mutations develop circulating p53 antibodies. The present study was aimed at defining the occurrence of circulating antibodies to p53 protein in patients with various types of non-Hodgkin's lymphomas (NHL). Altogether, the sera of 108 cases with various histological types of NHL and of 20 healthy controls were assessed for the presence of antibodies to p53 protein with an ELISA method. In 73 cases of NHL, also the immuno-histochemical staining for p53 antigen was performed to make a rough estimation of the frequency of mutational events. The development of autoantibodies to p53 protein was observed in approximately 7% of NHL patients (predominately in the more aggressive variants of the disease, but also in one case of small lymphocytic lymphoma). This proportion represents roughly one third of the number of patients assessed (immunohistochemically) to carry a missense p53 mutation in their tumors. The autoantibodies to p53 protein can be used as a tumor marker (early appearance, high specificity) in the follow-up of a subset of NHL patients, but, unfortunately, this subset comprises only approximately 7% of NHL patients.

## Introduction

p53 is a tumor suppressor gene, found on chromosome 17p, the alterations of which are among the most frequent genetic changes detected in human neoplasms. The dramatic effect of such mutations indicates the prominent and central role of wild-type p53 in many critical cell functions. The p53 protein was found to be elevated following DNA damage or other types of cellular stresses and was implicated to operate in the

response to these insults by activating a set of genes whose products facilitate adaptive and protective activities including growth arrest, DNA repair, apoptosis, senescence, differentiation and antiangiogenesis (1-4).

The majority of p53 mutations are missense point mutations giving rise to single amino acid substitutions that abrogate the specific DNA binding activity (5). Concomitantly, the half-life of p53 extends from normal 20 min (wild-type protein) to approximately 48 h (mutant protein) resulting in nuclear accumulation of the mutant protein (6,7). Most of the mutations are clustered in the most highly conserved domains of the gene spanned by four to nine exons (8). Although mutations of the p53 gene are most frequently acquired, they can also be inherited through the Li-Fraumeni syndrome. In these families, one mutant p53 allele is inherited, and the second allele acquires a mutation (9).

p53 can be inactivated not only through mutation, but also at the protein level through complexing with DNA tumor viral oncoproteins, like the SV40 large T antigen, the adenovirus E1B protein, and the human papilloma virus E6 protein (10) or cellular protein MDM-2 (11).

Mutant p53 proteins, as tumor-specific antigens, may be targets of the host immune system indicating that alterations in the expression or properties of p53 associated with tumor development can be detected by screening the sera of cancer patients for anti p53 antibodies (12-18). The formation of autoantibodies to p53 is observed only in patients with p53 missense mutations that express detectable levels of p53 protein in their tumor cells. The p53 specific immune response does not develop in cancer patients bearing stop, splice/stop, splice or frameshift mutations of p53 gene in tumor cells (15).

As the occurrence of autoantibodies to p53 varies among different subtypes of cancers, it also varies among the studies. Namely, the autoantibodies were identified in 2.6% (17) up to 21% (13) of the tested sera of NHL patients. The present study was aimed at determining the frequency of development of circulating antibodies to p53 protein in patients with various types of non-Hodgkin's lymphomas (NHL).

## Materials and methods

**Patients.** In total, 108 cases of NHL (52 males, 56 females; age 19-76 years) were evaluated for the presence of circulating antibodies to p53 protein. The cases were classified according

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Table I. The occurrence of antibodies to p53 in patients with various subtypes of NHL.

| Diagnosis                                                          | No. of cases | No./percentage of immunohistochemically positive cases | No./percentage of cases with autoantibodies to p53 |
|--------------------------------------------------------------------|--------------|--------------------------------------------------------|----------------------------------------------------|
| Lymphoblastic B-cell lymphoma                                      | 3            | 1/33%                                                  | 0                                                  |
| Burkitt's lymphoma                                                 | 3            | 2/67%                                                  | 1/33%                                              |
| Burkitt's like lymphoma                                            | 1            | 0                                                      | 0                                                  |
| T-cell rich diffuse B-cell lymphoma                                | 2            | 0                                                      | 0                                                  |
| Diffuse large B-cell lymphoma                                      | 18           | 7/39%                                                  | 3/17%                                              |
| Follicle center lymphoma grade III                                 | 2            | 1/50%                                                  | 1/50%                                              |
| Follicle center lymphoma grade I and II                            | 11           | 0                                                      | 0                                                  |
| Marginal zone B-cell lymphoma                                      | 3            | 0                                                      | 0                                                  |
| Mantle cell lymphoma                                               | 4            | 0                                                      | 0                                                  |
| Lymphoplasmacytoid lymphoma                                        | 3            | 0                                                      | 0                                                  |
| B-cell chronic lymphocytic leukemia/<br>small lymphocytic lymphoma | 6            | 2/33%                                                  | 1/17%                                              |
| Lymphoblastic T-cell lymphoma                                      | 3            | 0                                                      | 0                                                  |
| Anaplastic large cell lymphoma                                     | 4            | 1/25%                                                  | 0                                                  |
| Anaplastic large cell lymphoma Hodgkin's-like                      | 1            | 0                                                      | 0                                                  |
| Intestinal T-cell lymphoma                                         | 1            | 0                                                      | 0                                                  |
| Angioimmunoblastic T-cell lymphoma                                 | 1            | 0                                                      | 0                                                  |
| Unclassifiable lymphomas                                           | 7            | 1/14%                                                  | 0                                                  |
| Total lymphomas                                                    | 73           | 15/21%                                                 | 6/8%                                               |

to REAL classification (19). In 73 of the cases, the immunohistochemical staining for p53 antigen (applying the commercially available monoclonal antibody DO-7, Dako, Glostrup, Denmark) was also performed to make a rough estimation of the frequency of mutational events, while in other 35 cases, we were unable to obtain additional tissue samples for immunohistochemical analysis. Among the 73 cases, in which the immunohistochemical analysis was also performed, 3 patients had lymphoblastic B-cell lymphoma, 3 Burkitt's lymphoma, 1 Burkitt's-like lymphoma, 2 T-cell rich diffuse B-cell lymphoma, 18 diffuse large B-cell lymphoma, 2 follicle center lymphoma grade III, 11 follicle center lymphoma grade I and II, 3 marginal zone B-cell lymphoma, 4 mantle cell lymphoma, 3 lymphoplasmacytoid lymphoma, 6 B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma, 3 lymphoblastic T-cell lymphoma, 4 anaplastic large cell lymphoma, 1 anaplastic large cell lymphoma Hodgkin's-like, 1 intestinal T-cell lymphoma, 1 angioimmunoblastic T-cell lymphoma, and 7 patients unclassifiable lymphomas. The group of patients, in whom only serological determinations of antibodies to p53 protein were done, consisted of 1 case with Burkitt's-like lymphoma, 13 diffuse large B-cell lymphomas, 1 follicle center lymphoma grade III, 5 follicle center lymphomas grade I and II, 2 marginal zone B-cell lymphomas, 2 mantle cell lymphomas, 3 lymphoplasmacytoid lymphomas, 3 B-cell chronic lymphocytic leukemia/small lymphocytic

lymphomas, 1 lymphoblastic T-cell lymphoma, 2 anaplastic large cell lymphomas, and 2 cases with peripheral T-cell lymphoma, NOS.

In addition, the sera of 20 healthy controls (9 males, 11 females; age 26-43 years) without any signs of malignant disease were assessed for the presence of antibodies to p53 protein by ELISA method.

Finally, one patient (male, age 25 years) with Burkitt's lymphoma, in whom very high levels of autoantibodies to p53 were detected at the time of diagnosis, was followed up for a longer period of time with serial determinations of autoantibodies to p53. In this patient, immunohistochemistry also detected the p53 protein overexpression (indicating an underlying mutation of p53 gene).

**Sample collection.** The blood was collected from the NHL patients at the time of diagnosis and prior to treatment, as well as from healthy controls. The sera were separated from the clot by centrifugation, then aliquoted and stored at -20°C until analysis. The samples were diluted in sample dilution buffer before assaying (so that the measurement results fell inside the area of a standard curve) and the determinations were executed according to manufacturer's instructions.

**Serum determinations.** The determinations of autoantibodies to p53 were performed using the commercially available ELISA kit p53-Autoantikörper ELISA 2. Generation

Table II. The occurrence of antibodies to p53 in patients with various subtypes of NHL and in healthy controls.

| Diagnosis                                                          | No. of cases | No./percentage of cases with autoantibodies to p53 |
|--------------------------------------------------------------------|--------------|----------------------------------------------------|
| Burkitt's-like lymphoma                                            | 1            | 0                                                  |
| Diffuse large B-cell lymphoma                                      | 13           | 2/15%                                              |
| Follicle center lymphoma grade III                                 | 1            | 0                                                  |
| Follicle center lymphoma grade I and II                            | 5            | 0                                                  |
| Marginal zone B-cell lymphoma                                      | 2            | 0                                                  |
| Mantle cell lymphoma                                               | 2            | 0                                                  |
| Lymphoplasmacytoid lymphoma                                        | 3            | 0                                                  |
| B-cell chronic lymphocytic leukemia/<br>small lymphocytic lymphoma | 3            | 0                                                  |
| Lymphoblastic T-cell lymphoma                                      | 1            | 0                                                  |
| Anaplastic large cell lymphoma                                     | 2            | 0                                                  |
| Peripheral T-cell lymphoma NOS                                     | 2            | 0                                                  |
| Total lymphomas                                                    | 35           | 2/6%                                               |
| Healthy controls                                                   | 20           | 1/5%                                               |

(Dianova, Hamburg, Germany). The assay is based on a sandwich technique with the human recombinant p53 protein coated to the microliter well plates which binds the anti-p53 autoantibodies of the serum sample and goat anti-human IgG antibody (as the detection antibody) conjugated to peroxidase. The bound enzymatic activity is determined by addition of a chromogenic substrate and by measuring the resulting colored solution with a spectrophotometer. The concentration of the sample or the standard is directly proportional to the absorbance value measured.

## Results

The results of serological determinations of antibodies to p53 in the group of 73 cases of NHL, in which also the immunohistochemical staining for p53 antigen was performed to make an estimation of the frequency of mutational events, are summarized in Table I.

The antibody response occurred only in the patients that had convincingly positive immunohistochemical determinations of p53 antigen and was distributed predominantly in aggressive forms of NHL. However, the percentages of patients developing the autoimmune response might have been somewhat overestimated, especially in the subtypes with only two or three cases included (i.e., Burkitt's lymphoma and follicle center lymphoma grade III) - due to the small number of cases.

When the sera of 20 healthy controls, plus the sera of another 35 cases with histological diagnosis of NHL in whom we were unable to obtain additional tissue samples for immunohistochemical analysis, were assessed for the presence of circulating antibodies to p53, the results were as indicated in Table II.

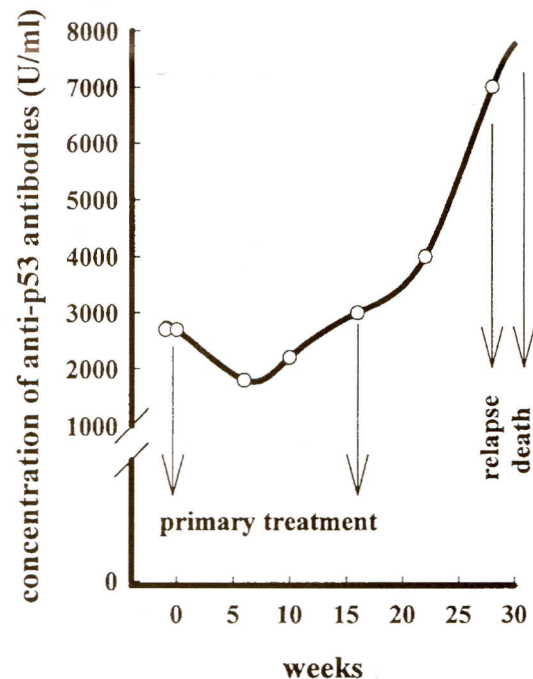


Figure 1. Consecutive serum levels of autoantibodies to p53 protein in a patient with Burkitt's lymphoma reflected the dynamics of the disease. The patient responded well to primary treatment, but experienced a relapse three months later that was resistant to salvage therapy. The relapse had been foretold by the increasing levels of autoantibodies to p53 protein before the primary treatment was completed.

In the group of NHL patients, the overall prevalence of antibodies to p53 protein was similar to the one given for NHL in Table I. Unexpectedly, the antibodies to p53 were

detected also in one case of a healthy control. There were no signs indicating an underlying malignant disease in this person, and autoimmune diseases as a possible explanation for the antibody development were excluded as well.

Finally, one patient in whom very high levels of autoantibodies to p53 were detected at the time of diagnosis, was followed up with serial determinations of autoantibodies to p53. He was diagnosed with Burkitt's lymphoma stage IV (immunohistochemically persuasively positive tumor) and was treated with aggressive chemotherapy that included high dose methotrexate, cyclophosphamide, ifosfamide, vincristine, adriamycin, etoposide, cytosine arabinoside and corticosteroids plus chemotherapeutic central nervous system prophylactic therapy according to BFM protocol. He responded well to primary treatment and there were no clinical signs of residual disease upon completion of the therapy. However, three months later, he was admitted to hospital with a massive relapse. Salvage chemotherapy was applied, but with no effect. The patient died of lymphoma shortly thereafter. The levels of antibodies to p53 were elevated prior to treatment, showing only a minor decrease during the primary treatment and starting to rise before the primary treatment was completed. From there on, the levels increased continuously reflecting the disease progression (Fig. 1).

## Discussion

The frequency of p53 mutations in NHL varies according to the histological type and disease stage. It is high in aggressive lymphomas (Burkitt's and diffuse large B-cell lymphomas) and lower in intermediate and indolent lymphomas. Regardless of the type, the frequency is generally higher in case of relapse or after progression. Most of the mutations are missense mutations, clustered in exons 5-8 of one p53 allele, and are usually associated with the deletion of the other allele through 17p deletion (20).

Although p53 is a cellularly encoded protein, it has been found to be immunogenic and capable of eliciting a p53 specific antibody immune response. About one third of patients (the percentage varies for different types of cancer) with tumors that carry the p53 missense mutations develop circulating p53 antibodies. The antibodies are not seen if there is no p53 accumulation in the tumor cell, and, in case of lung cancer, they can appear before the cancer is detectable. The p53 protein may either be released during tumor cell necrosis, or otherwise it translocates to the surface of the cell, inducing a B-cell response as a result of the breakdown of the immune system tolerance (12-18,21,22).

In contrast to various types of solid tumors (e.g., prostate cancer, breast cancer, colorectal cancer, germ tumors), in lymphomas, there are no appropriate serum tumor markers for an easy assessment of patient's disease status, with lactic dehydrogenase (LDH) and  $\beta$ -2-microglobulin being the best approximations to the definition of a serum lymphoma tumor marker (23). However, in a certain proportion of NHL patients with overexpression of p53 protein in their tumor cells (due either to the mutation of p53 gene or stabilization of gene product by a mechanism other than point mutation), the autoantibodies develop against the p53 protein and can be used for follow-up (13,17).

The specificity of the autoantibodies to p53 protein for the malignant process is reported to be 100% (18), but they are present in malignancy in general, and are not an indicator of the type of malignant disease. The frequency of autoantibody development, on the other hand, is relatively low, approximating only 20% of cancer patients, which is due to the fact that p53 is not mutated in every patient with malignant disease, and that the appearance of antibodies to p53 (protein) is not an obligatory consequence of p53 overexpression in tumor cells (9).

In the present study, the immunohistochemical overexpression of p53 protein was found in 21% of NHL patients, with the highest incidence of p53 immunoreactivity in cases of Burkitt's lymphoma, follicle center lymphoma grade III, and diffuse large B-cell lymphoma. Positive staining was observed also in cases of B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma, lymphoblastic B-cell lymphoma, and anaplastic large cell lymphoma. Bearing in mind all the caveats concerning the discrepancies in the results of immunohistochemistry and determination of the nucleotide sequence, we anyway assume that this percentage gives a rough estimation of the frequency of mutational events.

The development of autoantibodies to p53 protein was observed in approximately 7% of NHL patients - again in the more aggressive variants of the disease, but also in one case of small lymphocytic lymphoma. In the group of patients in which also the p53 immunostaining had been performed the antibody response appeared only in the patients that had convincingly positive immunohistochemical determinations of p53 antigen.

All the determinations were performed at the time of diagnosis, thus confirming the fact that the occurrence of autoantibodies to p53 in the sera of cancer patients is a very early event in the malignant disease (14,24). On the other hand, the specificity of the autoantibodies to p53 protein in our study was not 100% due to unexplainable appearance of the autoantibodies in a healthy control. Up till now, no signs indicating an underlying malignant disease in this person have been observed, and autoimmune diseases as a possible explanation for the antibody development have been excluded as well. In our cases, the sensitivity of autoantibodies to p53 was relatively low, approximating only 7%, which is roughly one third of the number of patients assessed (immunohistochemically) to carry a missense p53 mutation in their tumors.

Finally, when consecutive levels of anti-p53 antibodies were followed in a case of Burkitt's lymphoma, it was found that they correlated well with the disease status, even foretelling the disease progression. Taken together, the autoantibodies to p53 protein can be used as a tumor marker (early appearance, high specificity) in the follow-up of a subset of NHL patients, but, unfortunately, this subset comprises only approximately 7% of NHL patients.

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