p53 protein expression in non-Hodgkin's lymphoma. Comparative study with the wild type p53 induced proteins mdm2 and p21/waf1

M Tzardi, Ch Kouvidou, I Panayiotides, K Stefanaki, D Rontogianni, E Zois, K Koutsoubi, G Eliopoulos, G Delides, P Kanavaros

Abstract

Aims—To investigate the pattern of expression of p53 protein and two wild type p53 induced proteins (mdm2 and p21/waf1) as an indirect way of assessing p53 gene status in non-Hodgkin's lymphoma.

Methods—Formalin fixed, paraffin wax embedded tissue from 87 cases of nodal non-Hodgkin's lymphoma, comprising 52 high grade and 35 low grade tumours, was stained by immunohistochemistry for p53, mdm2 and p21/waf1 proteins.

Results—p53, mdm2 and waf1/p21 proteins were expressed in 36/52, 21/52 and 31/52 high grade and 3/35, 21/35 and 3/35 low grade non-Hodgkin's lymphomas, respectively. Parallel p53/mdm2 protein expression was found in 23 cases (21 high grade and two low grade). These 23 cases were also positive for p21/waf1 protein expression. Discordant p53 positive/mdm2 negative protein expression was found in 16 cases (15 high grade and one low grade). Eleven (10 high grade and one low grade) of these 16 cases were p21/waf1 positive and the remaining five high grade non-Hodgkin's lymphomas were p21/waf1 negative. Mdm2 and p21/waf1 proteins were not expressed in the absence of p53 protein expression.

Conclusions—p53, mdm2 and waf1/p21 protein expression is more frequently associated with aggressive histotypes of non-Hodgkin's lymphoma. Parallel expression of p53, mdm2 and p21 proteins may represent non-Hodgkin's lymphomas with a wild type p53 gene as mdm2 and p21 proteins can be induced by the wild type gene. In these cases p53 protein expression may result from stabilisation via complex formation with the mdm2 protein. This could be important in the pathogenesis of these cases as mdm2 may deregulate the p53 dependent growth suppressive pathway. Discordant p53 positive/mdm2 negative/p21 negative protein expression may represent non-Hodgkin's lymphoma with p53 gene mutations unable to activate expression of mdm2 and p21 proteins. Non-Hodgkin's lymphoma with p53 positive/mdm2 negative/p21 positive protein expression may have either wild type p53 with deregulated mdm2 gene expres-

sion or mutated p53 gene with p53 independent p21 expression.

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p53 gene mutations are the most frequent genetic alterations found in human tumours.¹⁻⁴ p53 protein expression either secondary to p53 gene mutation or because of adhesion to other cellular or viral proteins can be detected by immunohistochemistry.^{4,5} An indirect way to analyse p53 gene status is by the investigation of protein expression induced by wild type p53. One of these is the mdm2 protein, which forms a complex with p53 thereby inhibiting p53 function.⁶⁻⁹ Wild type p53 induces mdm2 transcription via a p53 responsive element in the first intron of the mdm2 gene.^{10,11} p53 and mdm2 proteins interact with each other, forming a self-regulating feedback loop.^{11,12} p21/waf1 protein is a wild type p53 inducible protein which inactivates the cyclin/cyclin dependent kinase (CDK) complexes, blocking cell cycle progression at the G₁ to S interface.¹³⁻¹⁶ There is evidence that p53, after DNA 'damage', increases the expression of p21 protein, which binds to cyclin/CDK complexes resulting in inhibition of retinoblastoma (Rb) protein phosphorylation.¹³⁻¹⁶ In addition, p21/waf1 protein blocks the action of proliferating cell nuclear antigen (PCNA), impeding DNA replication.¹⁷ In tumour cells that have either lost p53 protein or contain an altered form of p53, p21 concentrations are dramatically reduced or the protein is absent.¹³⁻¹⁵ This could result in abnormal control of DNA replication or loss of coordination between DNA replication and the cell cycle.¹⁷ Both could lead to genomic instability which is directly related to oncogenesis.

Previous investigations on non-Hodgkin's lymphomas, showed that p53 protein expression is a frequent finding in the high grade variant.^{4,5,18,19} p53 protein expression in non-Hodgkin's lymphoma is sometimes secondary to p53 gene mutations, but in many cases is independent of mutation.^{4,5,18-20} In addition, mdm2 protein has been detected in a proportion of high grade non-Hodgkin's lymphomas, but less frequently than p53 protein.²⁰ Prompted by these data, we investigated, using

Department of Pathology, University Hospital of Heraklion, Heraklion
M Tzardi
Ch Kouvidou
I Panayiotides
K Stefanaki
K Koutsoubi
G Delides
P Kanavaros

Department of Pathology, Evangelismos Hospital of Athens, Athens
D Rontogianni

Department of Pathology, Venizelion Hospital of Heraklion, Heraklion
E Zois

Department of Haematology, University Hospital of Heraklion, Heraklion
G Eliopoulos

Correspondence to:
Dr P Kanavaros, University of Crete, Faculty of Medicine, 1393 Heraklion, Crete, Greece.

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immunohistochemistry, the expression of p53, mdm2 and p21/waf1 in a series of 87 cases of nodal non-Hodgkin's lymphoma, to obtain indirect information about the status of p53 gene in these tumours.

Methods

All 87 cases of nodal non-Hodgkin's lymphoma were retrieved from the archives of the Departments of Pathology, University Hospital and Venizelion Hospital of Heraklion, Greece. These were classified according to the updated Kiel classification (1988) into 52 high grade (47 B non-Hodgkin's lymphomas and five T non-Hodgkin's lymphomas) and 35 low grade (32 B non-Hodgkin's lymphomas and three T non-Hodgkin's lymphomas) tumours. The material comprised 18 follicular centroblastic/centrocytic B non-Hodgkin's lymphomas, three centrocytic (mantle cell) B non-Hodgkin's lymphomas, nine immunocytomas, two lymphocytic B non-Hodgkin's lymphomas, three angioimmunoblastic lymphadenopathy T non-Hodgkin's lymphomas, four immunoblastic B non-Hodgkin's lymphomas, 39 centroblastic B non-Hodgkin's lymphomas, two Burkitt-type B non-Hodgkin's lymphomas, three pleomorphic medium and large cell

Table 1 Expression of p53, mdm2 and p21/waf1 proteins in non-Hodgkin's lymphoma. Results are expressed as number positive/total number

Non-Hodgkin's lymphoma	p53	mdm2	p21/waf1
High grade	36/52	21/52	31/52
Low grade	3/35	2/35	3/35
Total	39/87	23/87	34/87

T non-Hodgkin's lymphomas, two anaplastic large cell B non-Hodgkin's lymphomas, and two anaplastic large cell T non-Hodgkin's lymphomas. Four reactive lymph nodes were also included as controls.

IMMUNOHISTOCHEMISTRY

Immunostaining was performed on formalin fixed, paraffin wax embedded tissue sections using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method.²¹ Monoclonal antibodies directed against mdm2 (Oncogene Sciences), p53 (D07: Dako, Glostrup, Denmark) and p21/waf1 (Oncogene Sciences) were applied at a dilution of 1 in 100 for three hours. The bridging rabbit antimouse (Z 259) and APAAP complexes (D 314) were obtained from Dako. All sections were heated in citrate buffer in a microwave oven prior to incubation with all antibodies, as described previously.²² Positive control slides were included in all tests and consisted of paraffin wax sections from a patient with Hodgkin's disease known to be positive for p53, mdm2 and p21/waf1 proteins. Negative control slides were prepared by omitting the primary antibody. In all cases the quality of antigenic preservation was tested by immunostaining for PCNA (Oncogene Sciences).

Results

IMMUNOHISTOCHEMISTRY

The results are summarised in table 1.

p53 PROTEIN EXPRESSION

p53 nuclear staining was found in 39/87 cases of non-Hodgkin's lymphoma. In correlating p53 expression with histological type, positive staining was observed in three of 35 low grade and in 36/52 high grade non-Hodgkin's lymphomas. p53 positive cells represented 1-5%, 5-25%, 25-50%, and >50% of lymphoid cells in 28, five, four, and two cases, respectively (fig 1). The three low grade non-Hodgkin's lymphomas exhibited p53 nuclear staining in 1-5% of tumour cells. Rare p53 positive cells were found in germinal centres and in interfollicular areas in all reactive lymph nodes.

MDM2 PROTEIN EXPRESSION

Mdm2 nuclear staining was found in 23/87 cases of non-Hodgkin's lymphoma (21/52 high grade and two of 35 low grade). Mdm2 positive cells represented 1-5% of the lymphoid cells in all but four high grade non-Hodgkin's lymphomas with 5-25% positive cells and one high grade non-Hodgkin's lymphoma with 25-50% positive cells (fig 2). Rare mdm2 positive cells were found in germi-

Figure 1 p53 protein expression in a high grade non-Hodgkin's lymphoma.

Figure 2 Mdm2 protein expression in a high grade non-Hodgkin's lymphoma.

Figure 3 p21/waf1 protein expression in a high grade non-Hodgkin's lymphoma.

nal centres and in interfollicular areas in all reactive lymph nodes.

p21/WAF1 PROTEIN EXPRESSION
p21 nuclear staining was found in 34/87 cases of non-Hodgkin's lymphoma (31/52 high grade and three of 35 low grade). p21 positive cells represented 1–5% of the lymphoid cells in all but six high grade non-Hodgkin's lymphomas with 5–25% positive tumour cells (fig 3). Rare p21 positive cells were found in germinal centres and in interfollicular areas in all reactive lymph nodes.

RELATIONS BETWEEN EXPRESSION OF p53, MDM2 AND p21
Parallel p53/mdm2 expression was found in 23/87 cases of non-Hodgkin's lymphoma (21 high grade and two low grade) (table 2). All 23 cases were also p21/waf1 positive. Discordant p53 positive/mdm2 negative expression was found in 16/87 cases (15 high grade and one low grade). Eleven of these 16 cases (10 high grade and one low grade) were p21/waf1 positive and the remaining five high grade non-Hodgkin's lymphomas were p21/waf1 negative. Mdm2 and p21/waf1 proteins were not expressed in the absence of p53 protein expression.

Discussion
p53 protein expression can be a consequence of p53 mutation, which stabilises the protein, or it could depend on other factors, such as association with other viral or cellular proteins.^{1 4 5 18–20} An indirect way of assessing p53 gene status is by the investigation of the expression of mdm2 and p21/waf1 proteins. Wild type p53 is able to upregulate waf1 gene transcription, resulting in an enhanced expression of p21 protein.^{13 14} This protein mediates

cell cycle arrest by inhibiting the formation of cyclin/CDK complexes.^{13–16} Wild type p53 also induces transcription of the mdm2 gene.^{10 11} This gene, localised at 12q13–14, when amplified or overexpressed, or both, may increase the capacity of the cell to undergo tumorigenesis. The mdm2 gene has been found to be amplified in human sarcomas²³ and gliomas,²⁴ and is overexpressed in breast cancer, Hodgkin's disease, acute leukaemias, chronic lymphocytic leukaemias, and B cell lymphomas.^{25–28} It encodes a nuclear protein, which can bind p53, inhibiting p53 transcriptional control by concealing its transactivational domain.⁸

Prompted by these data we investigated p53, mdm2 and p21/waf1 protein expression in non-Hodgkin's lymphoma. We found the expression of these three proteins was more frequently associated with high grade non-Hodgkin's lymphoma. These proteins were rarely found in the low grade variant. Our results for p53 and mdm2 are in keeping with previous findings^{4 5 18 20 29–31} and indicate that p53, mdm2 and p21/waf1 protein expression is associated with aggressive histological types of non-Hodgkin's lymphoma.

Two patterns of p53/mdm2 protein expression were found in non-Hodgkin's lymphoma. (1) Simultaneous p53/mdm2 protein expression in 23 cases of non-Hodgkin's lymphoma (21 high grade and two low grade). All of these cases were also p21/waf1 positive. There is evidence that mdm2 protein is present mainly in cases with wild type p53.^{24–28} In addition, p21/waf1 protein expression is induced by the wild type p53 gene.¹³ Thus, the detection of mdm2 and p21/waf1 proteins in these cases of non-Hodgkin's lymphoma suggests that the p53 gene is not mutated and that mdm2 expression could be one of the factors leading to stabilisation of the p53 protein. This observation is supported by previous findings that of 17 cases of non-Hodgkin's lymphoma with mdm2 gene overexpression, 14 had a wild type p53 gene.²⁷ This could be of interest with regard to the pathogenesis of the p53 positive/mdm2 positive cases. As mdm2 can inhibit p53 mediated transactivation,^{7–9} it is possible that mdm2 expression may be involved in the pathogenesis of some, predominantly high grade non-Hodgkin's lymphomas, by deregulating the p53 dependent growth suppressive pathway. Previous studies have investigated the molecular mechanisms of mdm2 gene deregulation in lymphoid neoplasms.^{26 27 30} By Southern blotting, in only one case of follicular B non-Hodgkin's lymphoma³⁰ of a total of 162 cases of non-Hodgkin's lymphoma and lymphoid leukemias tested was mdm2 gene amplification detected.^{26 27 31} This suggests that mechanisms other than gene amplification, possibly mdm2 gene overexpression^{26 27} or enhanced translation,³² may play a role in deregulating mdm2 gene expression in non-Hodgkin's lymphomas and lymphoid leukemias.

(2) p53 protein expression without mdm2 protein expression in 16 cases of non-Hodgkin's lymphoma (15 high grade and one

Table 2 Patterns of p53, mdm2 and p21/waf1 protein expression

Pattern of expression	Number of cases	Histotype
p53+/mdm2+/p21+	23	21 High grade, 2 low grade
p53+/mdm2+/p21–	0	
p53+/mdm2–/p21+	11	10 High grade, 1 low grade
p53+/mdm2–/p21–	5	All high grade

low grade). This pattern suggests deregulation of the self-regulating feedback loop which is formed by the interaction between mdm2 and p53 proteins.^{11,12} Five of these 16 cases were p21 negative high grade non-Hodgkin's lymphomas. In these cases, it is possible that mutations rendering the p53 gene incapable of activating the mdm2 and p21/waf1 genes might have occurred. In support of these findings, some high grade non-Hodgkin's lymphomas with p53 positive/mdm2 negative protein expression harboured p53 gene mutations^{4,20} and p53 gene mutations were found in 45% of high grade non-Hodgkin's lymphomas.^{3-5,18,19,30}

In this respect, it is worth noting that some of these mutations were found in exons 4 or 10, outside the classic 'hot spot' regions (exons 5 to 8).⁵ The remaining 11 cases (10 high grade and one low grade) had a p53 positive/mdm2 negative/p21 positive pattern of expression. It is possible that, in these cases, mdm2 gene deregulation might have occurred in the absence of p53 gene mutation. Alternatively, if the p53 gene is mutated in these cases then p21/waf1 protein expression might have occurred through a p53 independent pathway, as in vitro p21 expression can be triggered by differentiation inducing agents in p53 negative haemopoietic model systems.³³ However, data from non-Hodgkin's lymphoma suggest that, at least in these tumours, high concentrations of p21 are associated with wild type p53 gene expression.³⁴ It is of interest that expression of mdm2 and p21/waf1 proteins was not found in the absence of p53 protein expression in non-Hodgkin's lymphoma. This is in agreement with the finding that a lack of p53 protein expression prevents mdm2 and p21/waf1 transactivation.^{6,7,10,13}

With regard to the p53 negative/mdm2 negative/p21 negative non-Hodgkin's lymphomas, it is possible that p53 gene is in the wild type configuration or that the p53 gene is mutated but inactivated because of nonsense or frame-shift mutations.^{4,20} Moreover, the possibility that some non-Hodgkin's lymphomas with p53 missense point mutations do not exhibit p53 immunostaining may also be considered in view of previous findings in these tumours.⁵ Finally, it is of interest to compare the different patterns of p53/mdm2/p21 protein expression in non-Hodgkin's lymphoma with the constant p53 positive/mdm2 positive/p21 positive pattern of expression in Reed-Sternberg cells in all cases of Hodgkin's disease.³⁴ Sanchez-Beato *et al* postulate that the p53 gene in Hodgkin's disease is a tumour suppressor gene and it has been hypothesised that p53 gene mutation could be secondary phenomena in Hodgkin's disease, leading to transformation to a more aggressive variant or anaplastic large cell lymphoma. Taken together, the above data suggest that immunohistochemical studies on p53, mdm2 and p21/waf1 protein expression may provide indirect information about the status of the p53 gene in human lymphomas and are a useful adjunct to molecular biology studies.

In summary, our study shows that different patterns of concordant or discordant p53/

mdm2/p21 protein expression occur in non-Hodgkin's lymphoma, suggesting that derangement of the growth suppressive pathway involving these proteins may play a role in the pathogenesis of a proportion of non-Hodgkin's lymphomas. Further studies at the DNA and RNA level are required to gain more insight into this issue.

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