Full Length Research Paper

Immunohistochemical prognostic indicators of lymphoma tumors

Fawzi Alsheyab, Moh'd Abbas, Yousef Mosameh* and Shawter Ali

Jordan University of Science and Technology, Department of applied Biology, Irbid 22110, Jordan.

Lymphoma, or lymphatic cancer, is a broad term encompassing a variety of cancers of the lymphatic system. This prospective study was initiated to evaluate the expression of two potential biomarkers (p53 and Cyclin D1). Lymphoma tissues were obtained from 50 patients (Royal Medical Services 1990 - 1996), which were diagnosed as Hodgkin's and NonHodgkin's lymphoma. Specimens were reassessed by examining new sections; these sections were analyzed for p53 and Cyclin D1 levels by using immunohisochemisty using formalin/paraffin embedded tissue. Results indicated that the percentages of cases exhibiting staining with p53 and Cyclin D1 were 44 and 72%, respectively. Over-expression of Cyclin D1 was near the range of reported antecedent studies, which confirm the potential oncogenic activity for Cyclin D1 in lymphoma. The expression of Cyclin D1 in both lymphoma in the same level indicates that lymphomas follow similar pathways in Jordanian population.

Key word: Immunohistochemisty, lymphoma tumors, p53, cyclin D1.

INTRODUCTION

Biomarkers play an important role in the diagnosis and prognostic classification of various cancer entities and can moreover be useful in monitoring the patient's clinical course of disease and response to therapy (Montalbán et al., 2004). The p53 gene may block the progression of cell growth cycle and trigger apoptosis in response to DNA damage (Prokocimer and Rotter, 1994; Levine et al., 1991). The mutation of p53 gene causes a loss of tumor-suppressor function, promotes cellular proliferation and inhibits apoptosis (Levine et al., 1991; Xerri et al., 1994; Kastan et al., 1995). The tumor suppressor p53 is essen-tial for cell protection against DNA damage and wide range of cellular stresses including hypoxia, heat shock, exposure to oxidative damage and ontogeny activation (Prokocimer and Rotter, 1994; Levine et al., 1991). The biological activities of p53 are attributed to its ability to arrest the cell cycle at G1 or G2 phase, to induce apoptosis and to maintain genomic stability by modulating DNA repair, replication and recombination (Kastan et al., 1995; Blaszyk et al., 2000). Many of these

effects result from the fact, that p53 acts as transcriptional activator of downstream target genes, such as p21WAF) 1, bax, MDM2 and cyclin G1 (Kastan et al., 1995). Any mutations of p53 tumor suppressor gene result in the loss of its normal function of the guardian of the genome and promote the genetic instability in the cells which may lead to oncogenesis (Xerri et al., 1994; Blaszyk et al., 2000). Although the p53 status has been already investigated in several types of neoplasms, it is still poorly defined in lymphomas.

Cell cycle transitions are governed by the activities of the Cyclin Dependent Kinases (Motokura et al., 1993). The Cyclin Dependent Kinase (CDK) is a heterodimeric complex composed of a catalytic subunit, the CDK and a regulatory subunit called a cyclin (Motokura and Arnold, 1993; Baldin et al., 1993; Hunter and Pines, 1994). The catalytic activity of each CDK is determined by binding of the regulatory cyclin subunit, positive and negative regulatory phosphorylation, and the binding of small polypeptide inhibitors (Baldin et al., 1993; Hunter and Pines, 1994). Each cyclin/CDK complex phosphorylates a unique set of substrates required for continued cell cycle progression. Growth factor stimulation initiates cell cycle progression and is required up to a point in late G1 phase referred to as the restriction point (Motokura et al., 1993;

*Corresponding author. E-mail: ymosameh@hu.edu.jo or ymosameh@yahoo.com. Tel: 00962 777665780.

Xiong et al., 1991; Hunter, 1993; Herber et al., 1994). Beyond the restriction point, cells are committed to a single round of cell division without regard to the presence of mitogenic or anti-mitogenic signals. Progression through the G1 phase requires the activities of both the D-type Cyclins, and cyclin E in combination with their respective CDK subunits 6 with the D-type cyclin dependent kinases being the first to be detected following cell cycle entry (Herber et al., 1994; Sherr, 1996). It is the D-type cyclins (D1, D2 or D3) in combination with their catalytic partner, CDK4 or CDK6 that function as critical integrators of mitogenic signals for the cell cycle machine, making them a natural target during the neoplastic process ((Motokura et al., 1993; Baldin et al., 1993). Of the various cyclin families, over-expression of cyclin D1 is most frequently associated with human malignancy (Motokura et al., 1993; Hunter, 1993; Sherr, 1996). So cyclin D1 acts as a mitogenic sensor for the cell cycle machine and its role as cellular oncogene.

The objective of this study were to characterize the expression of p53, cyclin D1 in lymphoma tumors (HL, NHL) by immunohistochemisty (IHC) and to investigate the significance of these oncoproteins by comparing IHC results in both HL, and NHL, and to assess the prognostic value of expression of these markers.

MATERIALS AND METHODS

Tissue characteristics

The study included 50 patients [31 male (10 HL, 21 NHL) and 19 females (6 HL, 13H)] who were diagnosed with lymphoma, the entire patients underwent endoscopic biopsy between 1990 and 1996. Blocks of tissue were obtained from the archives of the Royal Medical Services. In this series of lymphoma the samples was defined as Hodgkin's and non-Hodgkin's lymphoma.

IHC staining

Sections of formalin-fixed, paraffin-embedded lymphatic tissue were studied by IHC using monoclonal antibodies to p53 (1:50 dilution cloneDO-7) (DAKOCytomation A/S, Denmark), cyclin D1 (BLC-1) (1:50 dilution clone DSC-6) (DAKO Corporation, DAKO Cytomation A/S, Denmark). Both two anti bodies in this cocktail recognize distinct epitopes in the hydrophilic carboxyl region of latent membrane protein (LMP). Immunohistochemical detection of p53 and cyclin D1 was demonstrated by using (DakoCytomation LSAB2 System-HRP) which based on modified labeled avidin-biotin (LAB) technique in which a biotinylated secondary antibody forms a complex with peroxidase-conjugated streptavidin molecule.

5 μm thick sections were cut from the blocks, deparaffinized with xylene and dehydrated through graded concentrations of alcohols. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. The sections were then treated with pressure cocking (Deckoaking Chamber) was used to retrieve the antigen, by target retrieval solution (DakoCytomation S1700), in which the slides placed in containers filled with retrieval solution and heated in an autoclave (121ºC) for 10 min. Retrieval solution is used for both deparaffinizing and unmasking epitopes in formalin-fixed, paraffin-embedded tissues, and also reduces nonspecific background staining. After the tissue sections had been exposed to

Table 1. Expression of cyclin D1 and p53 in both sexes.

Sex	Observed in Cyclin D1	Observed in p53
Male	21/31 (67.7%)	16/31 (51.6%)
Female	15/19 (78.95%)	6/19 (31.6%)
Total	36 /50 (72%)	22/50 (44%)

primary antibodies for 60 min in addition to negative and positive control, they were reacted with 100 μl biotinylated secondary antibody, DakoCytomation (code k0672). Substrate, DakoCytomation liquid DAB Substrate (code k3468) used as a final chromogen, and then sections were counterstained with haematoxylin before mounting.

*Negative control (was done in the same procedure of immunohisochemisty but without the first step: addition for the primary antibody, then counter stained by haematoxylin dye).

Specimen analysis

IHC evaluation was conducted by expert pathologist (Dr. Kanan F., Department of pathology, Jordan University of Science and Technology) who scored and estimated percentage of tumor cells that exhibited nuclear stain and cytoplasmic stain. The percentage of immunoreactive tumor cells for each primary antibody used was evaluated. Tumor cells were considered strongly positive (+2) for p53 (nuclear staining), (+2) for cyclin D1 (nuclear staining and cytoplasmic), if >90% of tumor cells showed stronger expression than surrounding non-neoplastic tissue, intermediately positive (1+) if 10 - 90% were immunoreactive and negative (-) if <10% were immunoreactive. The IHC analysis was performed blind, without the knowledge of the clinical data.

Statistical analysis

Frequencies and correlation between examined markers (Cyclin D1 and p53) expression was determined by using Pearson Chi-square test. Statistical analysis was two sided at significant level of $P \leq 0.05$ and performed by using SPSS version 11 Statistical software.

RESULTS

In the current study the expression of the biomarkers cyclin D1, p53 in HL and NHL samples were done using immunohisochemisty. It was found that the percentages exhibit positive staining for cyclin D1 and p53 of the total samples, as shown in Table 1, were 72% (36/50) and 44% (22/50), respectively.

Immunoreactions to Cyclin D1 was localized in the nuclei and cytoplasm of tumor cell (Figure 1b: A) and all the samples staining was with + 2 for intensity of staining. Regarding the expression of Cyclin D1 to sex, it was 67.7% positive for males and 78.95% positive for female (Figure 2) with no significant differences between the two sexes. The expression of Cyclin D1 in HL and NHL was 58 and 42%, respectively, with no significant differences between the two types (Figure 3).

p53 was expressed with nuclear staining (Figure 1b: B) and the percentage of expression was 44% (22/50)
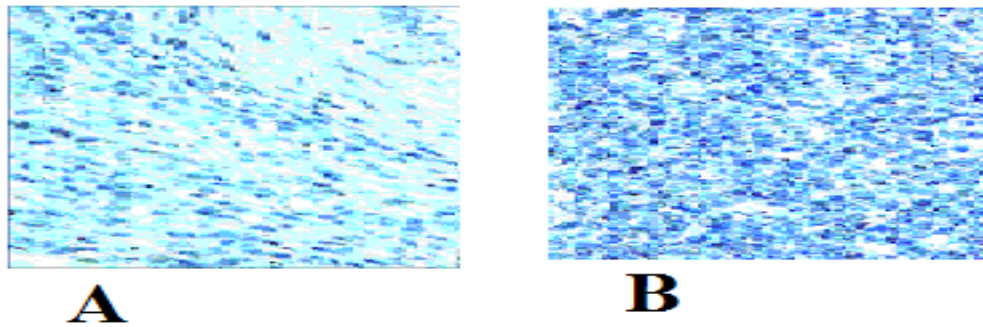
Figure 1a. Immuohistochemical staining for negative control tissues using striptavidin-biotin proxidase method, counter stained with haematoxylin (40 x). A, Cyclin D1 B; and B, p53.
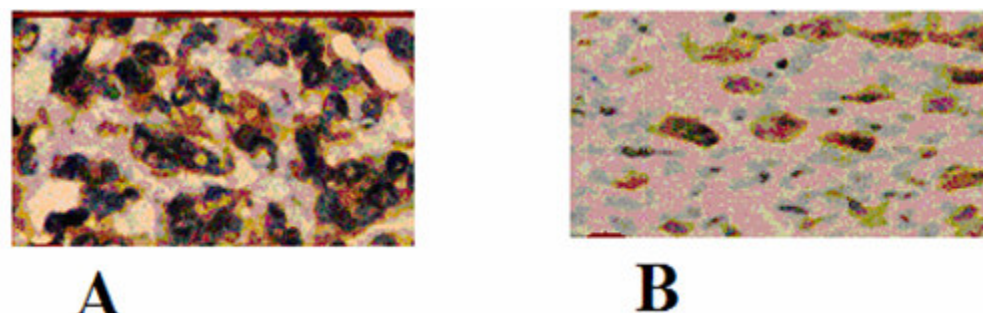

Figure 1b. Immunohistochemical staining for positive cyclin D1 and p53 streptavidin-biotin peroxidase method, counter stained with haematoxylin (40 x). A, cyclin D1; B, p53. Positive cells are brown in lymphoma.
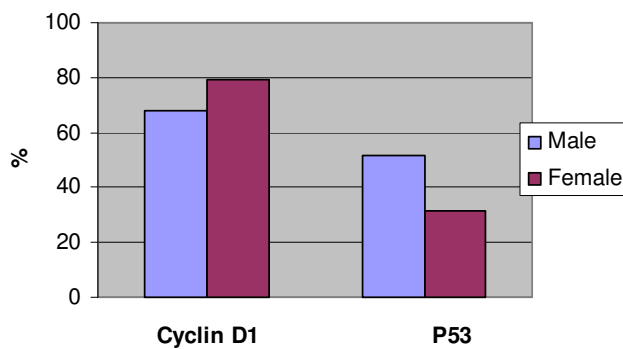

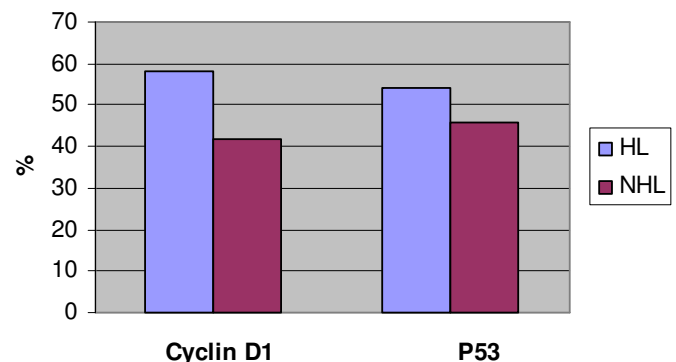**Figure 2.** The expression of Cycline D1 and P53 according to sex.

Figure 3. The expression of Cycline D1 and P53 in HL and NHL.

(Table 1) and the expression according to sex was 51% for males and 31.5% for female's (Figure 2) with no significant differences between the two sexes. The positive stain detected mainly in the nucleus and some in the cytoplasm; positive p53 is mutant type because the primary antibody can recognize an epitope located within amino acids 35 - 45 N-terminuses of wild or mutant types of p53 (Figure 1b: B). The expression of p53 in HL, and NHL was 54, and 46%, respectively, with no significant differences between the two types (Figure 3).

DISCUSSION

Mutant type of p53 can be detected using simple immunohistochemical method, while wild type cannot be detected by standard immunohistochemical methods, because the half-life of the wild type of p53 protein under

physiological conditions is as short as a few min (Xerri et al., 1994). Mutant type of the p53 gene results in the increase of the protein stability and accumulation, making it detectable immunohistochemically (Xerri et al., 1994). It is postulated that there is a strong association between the p53 gene mutations and the detection of p53 reactivity in numerous cells. The p53 alterations are rarely identified in human lymphomas (Xerri et al., 1994; Kastan et al., 1995), except in Burkitt's lymphoma, where the p53 mutations occur in 40% of cases ((Garcia et al., 2003; Smolewski et al., 2000). The frequency of the p53 alterations is related to the tumor grade. In low-grade lymphomas the p53 mutations are rarely reported and are usually associated with tumor progression. In contrast, in highly aggressive lymphomas of B-cell origin, the p53 mutations occur in 30% of cases (Smolewski et al., 2000). However, Nieder et al. (2001) reported that the p53 mutations were present in 10.5% of B-cell non-Hodgkin's lymphomas in general, but at a higher frequency in diffuse large B-cell lymphoma and mantle cell lymphoma. The International Non-Hodgkin's Lymphoma Prognostic Factors Project (1993) analyzed the expression of p53 and p21WAF) 1, a downstream target, useful to screen for the p53 gene mutations and found p53 mutations in 21% of cases. Our results demonstrate that p53 expression is very low (nuclear or cytoplasmic) which is a strong enough to consider the p53 gene mutations or no expression at all. However, no significant difference were observed between the p53 protein expression in Hodgkin's lymphomas and NonHodgkin's lymphoma; mutation of p53 are not restricted to single site so the nuclear stain or cytoplasmic stain cannot be used. Therefore, molecular techniques are required to study the specific mutation within p53 (Nieder et al., 2001). The other finding is that the p53 has no correlation with cyclin D1. Cyclin D1 plays a critical role in the control of cell proli-feration. It is an important target of cellular Ras activity; it is expressed at elevated levels in a number of tumor types (Zhou et al., 1996), particularly in those tumors with activated *ras* genes, and constitutes an Rb kinase which is essential to cell cycle progression. Cyclin D1 expres-sion is low in quiescent cells, but is stimulated following serum addition to high levels at the time the cells pass the restriction point and become committed to enter S phase several hours later. Little information, however, is known about the role of cyclin D1 in controlling the proliferation of continuously cycling cells (Sawa et al., 1998; Weinstat-Saslow et al., 1995). D1 activity is required throughout G1 phase until the beginning of DNA synthesis. In this study, it was observed that cyclin D1 is overexpressed in both Hodgkin's and NonHodgkin's lymphomas by immunohisoc-hemisty method, and positive nuclear and cytoplasmic immunostaining mostly (+2, +2) was observed in 36 samples. Over-expression of Cyclin D1 is high in Jordanian HL and NHL, and further studies are required to determine reasons of cyclin D1 overexpression.

Conclusion

The tumor-suppressor gene p53 plays a key role in damage and repair; it is the most altered gene in human cancer. Lymphomas are one of these tumors and the expression of p53 is low in Jordanian HL and NHL population. Overexpression of Cyclin D1 was detected in lymphomas and the expression was the same in the selected population (HL, and NHL) which may indicate that lymphomas follow similar pathways. This observation suggests that the Cyclin D1 has oncogenic potentiality, resulting from chromosomal translocations or gene amplification.

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