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Cell Cycle Proteins (NFkB, P53, P21, P27) Determination in Patients Samples with Hodgkin's Lymphoma

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Abstract

Background: *p53 tumor suppressor pathway and other cell cycle regulatory proteins has been suggested as a candidate pathogenic mechanism in Hodgkin's lymphoma (HL).*

Aim: *evaluating the frequency of of expression of the cell cycle proteins including the mutant p21Ras ,NFkB, p53 and p27 proteins in the malignant Hodgkin/Reed-Sternberg cells (H/RS cells) of HLsamples and seeking correlation between each protein studied.*

Methods: *A total of 40 archival tissue biopsy samples diagnosed tohave Hodgkin's lymphoma (HL) were included.*

The cell cycle regulatory proteins, NFkB, p53, p27 proteins and the mutant p21Ras oncoprotein were studied using insitu hybridization and immunohistochemistry assays.And study the correlation between p21Ras and each of the NFkB, p53 and p27 proteins.

Results: *The frequency of H/RS cells expression of NFkB, p53, p21 and p27 proteins in HD cases were 17*

(85%), 19 (47%), 29 (72.5) and 9 (22.5%) cases, respectively . The expression was statistically significant [the corresponding Relative Risk (RR) values were 4.52, 5.43, 3.82, and 0.11respectively]. All cell cycle markers gave nuclear pattern of staining except p21Raswhich gave clear cytoplasmic staining. Correlation between the cell cycle marker revealed no correlation betweenexpression of NFkB and any of the other markers, p53, p21 and p27($p>0.05$). p53 exhibited low but insignificant correlation with p21expression ($p>0.05$), and no correlation with p27, while a highlysignificant but inverse correlation was found between p21 and p27($p<0.01$).

Conclussion: *The high expression of the mutant p21Ras and the low expression of p27 both contribute tocell growth and proliferation albeit through different pathways*

Key words: Hodgkin's lymphoma,cell cycle molecules, *in situ* hybridization, immunohistochemistry.

INTRODUCTION

Hodgkin's lymphoma (HL) is a B cell-derived lymphoma characterized by a minority of malignant Hodgkin Reed-Sternberg (HRS) cells that have lost their normal B cell phenotype. Alterations in the cell cycle and apoptosis pathways might contribute to their resistance to apoptosis and sustained cell cycle progression. A key player in both cell cycle arrest and apoptosis is CDKN1A, encoding p21(waf/cip1) (p21). P21 isregulated by p53 and can function as a cell cycle inhibitor when in the nucleus or as an apoptosis inhibitor when localized in the cytoplasm(1). The hallmark of Hodgkin's lymphoma (HL) are mononucleated Hodgkin's cells and multinucleated Reed-Sternberg (HRS) cells, which usually account for only about 1% of cells in the tumor tissue(2). The majority of HRS cells in classical HL are derived from germinal centre B cells that have acquired disadvantageous Ig variable chain gene mutations and escaped from apoptosis(3). Hodgkin and Reed-Sternberg (H/RS) cells are characterized by a profound disturbance of the cell cycle and apoptosis regulation. The constitutive activation of the nuclear factor (NF)-kappaB pathway, which is considered to be involved in the proliferation and survival of H/RS cells. Moreover, substantial evidence that H/RS cells have defective cell cycle and apoptosis regulation has been provided by studies showing that these cells are characterized, in a large proportion of cases, by alterations of the p53, Rb and p27 tumor suppressor pathways, overexpression of cyclins involved in the G1/S and G2/M transition such as cyclins E, D2, D3, A and B1, overexpression of cyclin-dependent kinases such as CDK1, 2 and 6 and overexpression of anti-apoptotic proteins such as c-FLIP, bcl-xl, c-IAP2, X-linked I4P and survivin(4) .Most studies conducted to investigate the pathogenesis of HL focused on the cell cycle checkpoints regulation, namely, the p16INK4a-Rb-E2Fcyclind2/CDK4- cycline/CDK1 pathway which inhibits entry from G0 to G1 phase and progression through G1 restriction point, and the p14ARF/ARF-Mdm2-p53-p21 and p27 pathway,

which controls G1/S transition and S phase progression (5,6).Hence in thisretrospective (archival paraffein embedded tissue)stydy we aimed to assess the Ras protein expression and that of the tumor suppressor proteins p53 and p27 and the nuclear transcriptional factor NFkB in patients with Hodgkinlymphoma.

Materials And Methods

Tissue Samples

A total of 40 tissue samples from Hodgkin's lymphoma cases from archival paraffin-embedded blocks(Retrospective study),were obtained from the Medical City Department of Teaching Laboratories,Division of Histopathology were assayed for detection cell cycle molecules and correlate them with HL. The histopathologic data were approved by specialist in pathology.Biopsy tissues dissected one to two hours after death were obtained from the Institute of Forensic Medicine after signed written consent. Tissue sections from these biopsies were used as negative controls.

Assessment of NFkB factor expression in Tumor Cell by *in*

*Situ*hybridization using *NFkB probe :*Biotin-labeled 371 bp long cDNA probe was purchased from Maxim'sproduct catalog number: SP-10539. (Maxim Biotech Inc. South San Francisco,CA, USA).The assay was performed as recommended by the manufactures sheet.

Evaluation of Tumor Suppressor Gene Expression in Malignant Cells by Immunohistochemistryusing the following monoclonal antibodies which were purchased fromDakoCytomation:

p53: Monoclonal Mouse Anti-Human p53 Protein. Clone Do-7. Code Number.M 7001. Lot 121..*p21*: MonoclonalMouse Anti-Human p21Ras. Code number. M0637. Lot number. 092.

p27: Monoclonal Mouse Anti-Human p27Kip1. Clone SX53G8. Code number. M7203. Lot 038.

Immunohisto chemistry

Paraffins blocks were sectioned at a thickness of 4μm and dried for 16 hours at 56°C before being dewaxed in xylene and rehydrated through a graded ethanol series and washed with phosphate-buffered saline. Antigen retrieval was achieved by heat treatment in a pressure-cooker for 2 minutes in 10 mM citrate buffer (pH 6.5). Before staining the sections, endogenous peroxidase was blocked.

Immunohistochemical staining was performed on these sections using the different monoclonal antibodies ,described forehead. After incubation, immunodetection was performed with light microscope employing diaminobenzidinechromogen as substrate. Sections were counterstained with hematoxylin. The pattern of staining for each Ab was recorded as positive or negative, and high or low expression, taking into account the expression in H/RS cells and different cutoffs for each marker .

Statistical Analysis

The Pearson chi-square and the odds and odds ratio (Relative Risk) wereused to determine significance of relation between the different parameters and markers.

Determination of Correlation Coefficient was made by Pearson correlation to determine degreesof correlation among different markers.

Results

The morphologic features of sections stained for the expression of NFkB in H/RS cells is shown in figure (1).Staining with the tumor suppressor proteins p53 and p27 gave the well known nuclear pattern of staining which was not specific for the malignant cells. With respect to p53 expression, many more H/RS cells gave positive staining than did the surrounding reactive cells. Moreover, the higher staining intensity of H/RS cells enabled adequate discrimination between the positive signals emanating from the two cell types.

This differential staining conformed well to p53 over-expression in the malignant H/RS cells. As for p27, a reverse picture was seen in that most of the positive signals observed emanated from the surrounding reactive cells and only few H/RS cells stained positively for this marker, as depicted in figure (2). This finding indicates that the p27 tumor suppressor gene is actively repressed in Hodgkin's disease. The signal transducer p21Ras showed very clear cytoplasmic staining of H/RS cells of high intensity that obviously distinguished the malignant cells from the fainter staining of reactive and control cells normally encountered in many normal tissues of different origins, these findings are shown in the figure.

Forty cases of HL were tested for the expression of p53, p21 and p27, and20 randomly selected HD cases were tested for the expression of NFkB. Table (1) summarizes the results and reveal the proportion of HL cases exhibiting positive expression of the four cell cycle markers and their relative risk (RR) values. Positive expression of NFkB was found in 17 (85%) out of 20 HL cases tested. Nuclear localization of NFkB specific staining was observed in all HRS cells examined by light microscopy atx40 and x100 magnification (Figuer1), indicating constitutive activation of the factor in these malignant cells. The association of NFkB with HL was statistically very significant [Relative Risk (RR)= 4.52].Nineteen of 40 (47.5%) HL cases showed positive staining for p53 when tested by IHC using p53-specific monoclonal antibodies (Figuer2). This association with HL was found to be very significant (RR= 5.43).The expression of p27 protein, a cyclinD kinase inhibitor (CKI) and the down-stream acting tumor suppressor was remarkably down-regulated; only 9out of 40 (22.5%) cases gave positive results for this protein. No significant association between this marker and HL was found (RR= 0.19).The small-G protein signal-transducing factor p21Ras was positively expressed in 29 of 40 (72.5%) HL cases. The association with HL was found to be statistically significant (RR=3.82).

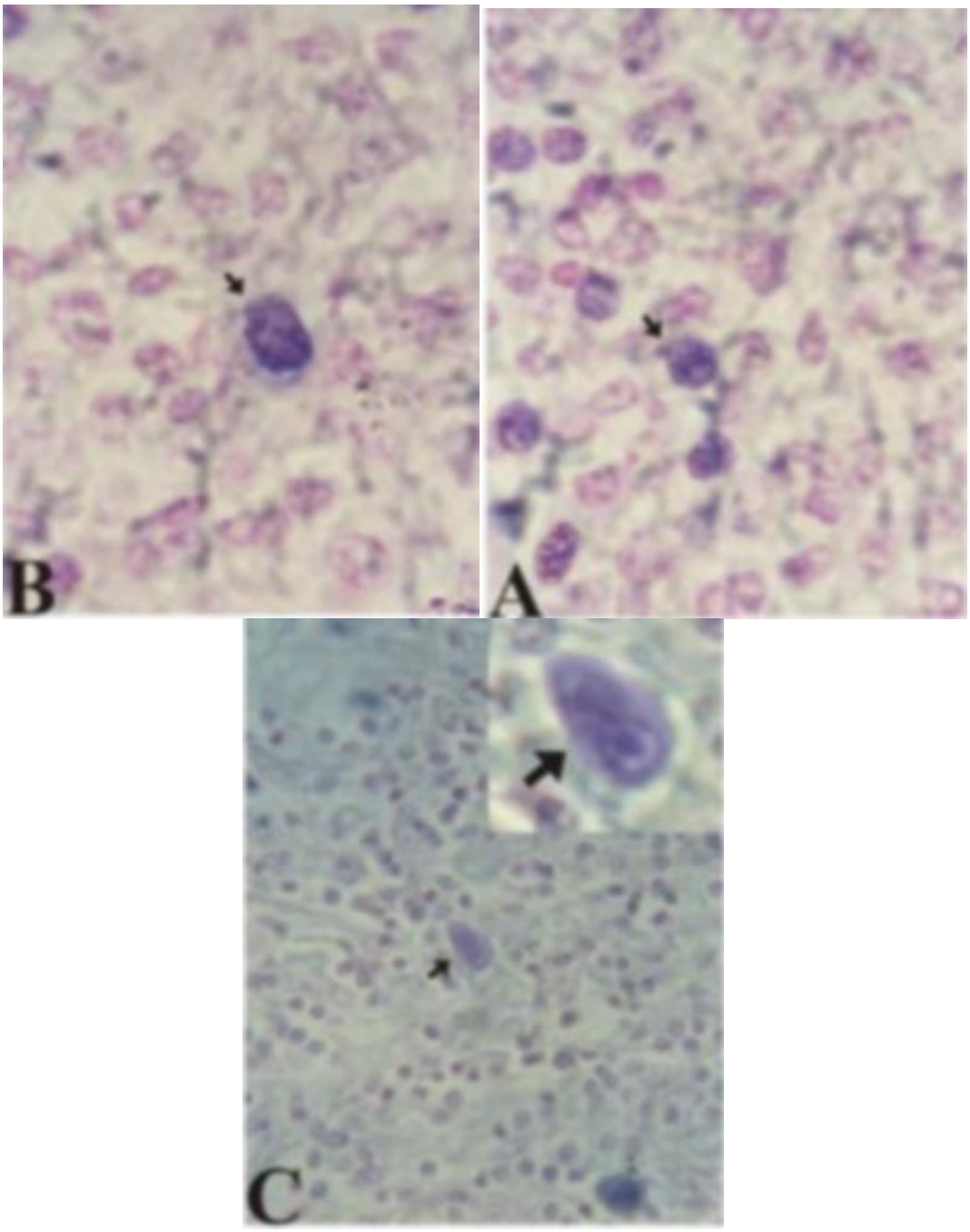
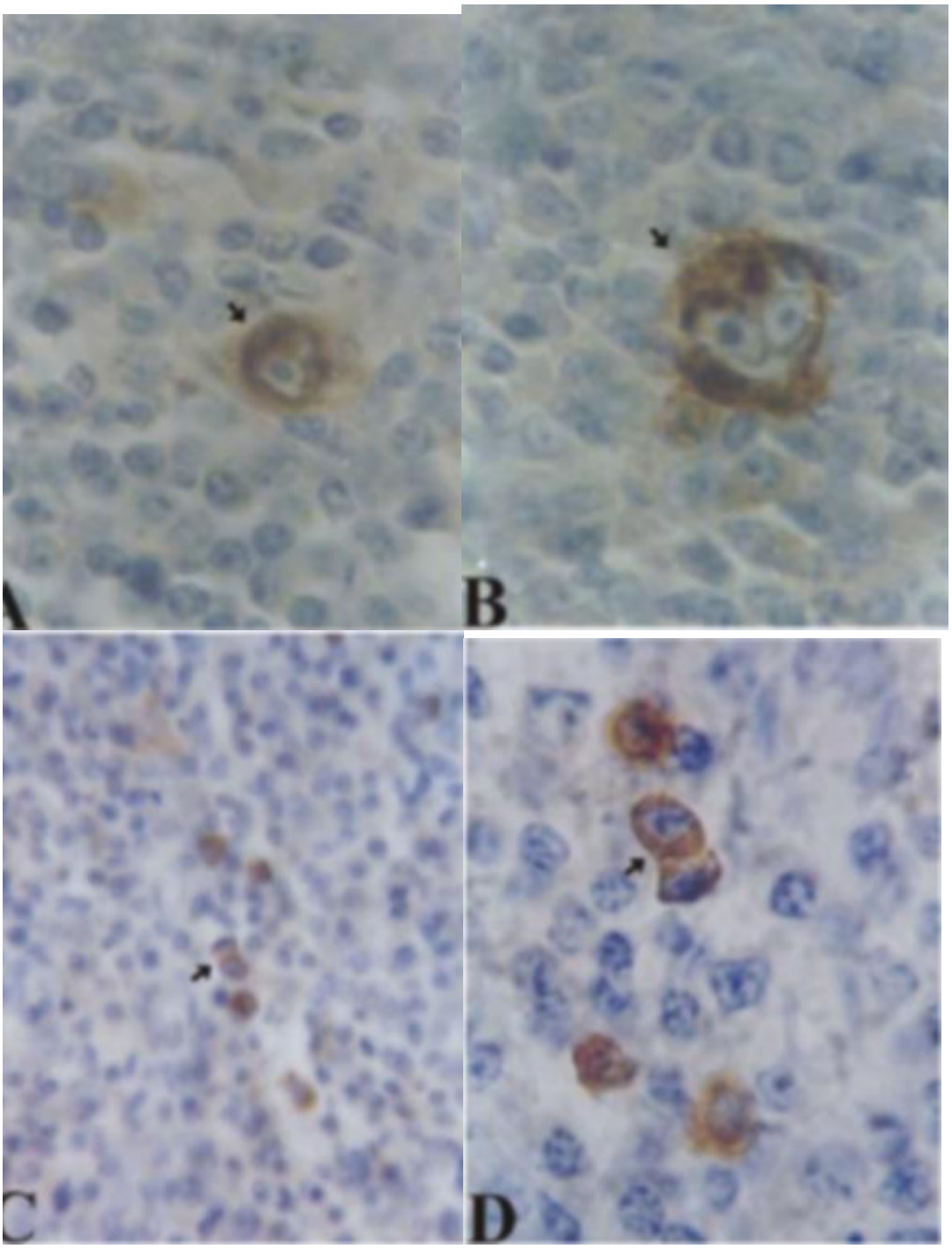


Figure 1. In Situ Hybridization with Nuclear Factor Kappa B (NFkB) -specific probes: A case of Hodgkin's disease shows positive nuclear staining of H/RS cells for NFkB (Arrows). In (A), a positive stained RS cell is seen in the center of the field (Arrow) along with few reactive cells non- specifically stained for NFkB (x40). A similar finding is shown in (B) where a single H/RS cell is shown in the center, but no stained reactive cells are seen(x40). A low-power magnification of a section from the same case shows typical Bi-nucleate RS cell stained positively for NFkB (Blue-purple). At the upper right comer of the same figure is an inset show ing the same RS cell at X100 magnification (Arrow).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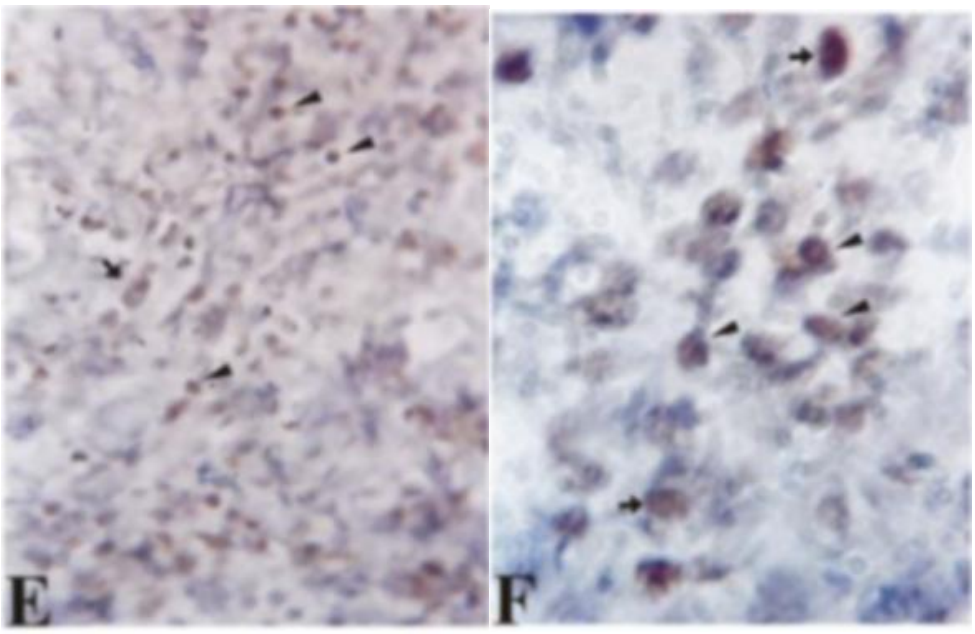

Figure 2.Immunostaining for p21Ras and p27 in HDcases.A to D: Positive cytoplasmic staining of H/RS cells forp2IRas (Arrows) , E and F: Nuclear staining for p27. (x 10) and (x40).

Inter-relationship between the different Cell Cycle Markers in Hodgkin's disease

The relation among the four cell cycle markers, NFkB, p53, p21Ras, and p27, tested in this study were evaluated for the degree of correlation between individual markers. As displayed in Table -2, no correlation between the expression of NFkB and that of any of the other markers, p53, p21Ras, and p27was found, ($p>0.05$). The expression of p53 revealed low correlation with p21Rasexpression which did not reach significance ($p>0.05$), and no correlation with p27 expression. On the other hand, p21Ras expression showed highly significant but inverse correlation with p27 expression ($p<0.01$).

Table 1. Frequency of Expression and Relative Risk of Cell Cycle Markers in Hodgkin's lymphoma samples..

Cell cycle marker(total No. of cases tested)	Frequency of No.(expression)	Relative Risk(RR)
NFkB(20)	17(85)	4.52
P53(40)	19(47)	5.43
$P21^{ras}$(40)	29(72)	3.82
P27(40)	9(22.5)	0.11

Table 2.Correlationamongthedifferentcellcyclemarkers

Markers		NFkB	P53	P21
P53	r	0.082		
	p	>0.05		
P21	r	-0.050	0.153	
	p	>0.05	>0.05	
P27	r	-0.099	-0.057	-0.490
	p	>0.05	>0.05	<0.01

Discussion

There is increasing evidence that multiparameterimmunohistochemical analysis of the complex molecular networks regulating the cell cycle in malignancies provides valuable information for the understanding of the impaired regulation of these networks and permits further insight into oncogenesis (7) This approach was adopted in the present study to analyze the expression of NFkB , p53 ,p21 and p27 in relation with 40 cases of HL.

NFkB. Many previous studies have confirmed unequivocally the constitutiveexpression of the nuclear transcriptional factor NFkB in the malignant H/RScells in most cases evaluated (8). Several mechanisms havebeen suggested to account for the high expression of NFkB in Hodgkin'sdisease(9) . Ligands of TNF family receptors on cells in HL microenvironment contribute to the activation of canonical and non-canonical NF-κB signaling pathways and survival program of HRS cells. Moreover, in HRS cells a number of multiple mutations in negative NF-κB regulators, and also gains and amplifications of positive regulators, cooperate in deregulating these pathways(10).This most explicit explanation for the high expression level in HD isbased on the over-expression and persistent activation of CD40 andCD30 members of the TNFR family in the malignant RS cells, which activateNFkB by initiating signals transduced through the TRAF-TRADD pathways.Here we further confirmed the constitutive expression of activated NFkBby demonstrating nuclear localization of the factor in the malignant RS cells by*in Situ*hybridization using NFkB-specific polynucleotide probe (figures 4.12Ato C). The majority of the RS cells showed the distinct high-intensity staining in positive cells (6).

P53. A tumor suppressor protein that is known to be over-expressed and accumulate in the malignant RS cells of HL. Its expression is activated bycellular DNA damage, hypoxia, irradiation and UV, and mutagenic chemicals in addition to some viral oncoproteins (11). The finding in this study that 47.5% of HD cases exhibited nuclear overexpression of p53 was in

line with the findings in most previous studies although the results varied over a wide range. Garcia and coworkers(2003)(6),mentioned that p53 is frequently expressed but rarely mutated in Hodgkin and Reed-Sternberg (HRS) cells of HL.p53 protein levels are regulated by murine double minute 2 (MDM2) through a well-established autoregulatory feedback loop.(3,12,13).

P21. Perhaps one of the most important findings in this study was the over-expression of the mutant p21Ras protein in HL cases this result is in an agreement with(14). Previous studies have demonstrated evidence that link p21Ras to the activation of NFkB and cyclinD.$p27^{KIP1}$. One of the most interesting observations the results of this study came up with the consistent under-expression of the tumor suppressor protein $p27^{KIP1}$. This result lent further support to the well known inverse relationship between tumor progression and $p27^{KIP1}$ expression. The level of $p27^{KIP1}$ expression in aggressive lymphomas is considered an important prognostic marker. As mentioned above, $p27^{KIP1}$ under-expression supports the notion that the wild-type p53 is transcriptionally inactive. Therefore, the results of our study come in agreement with those found in previous studies, and shows that $p27^{KIP1}$ is actively repressed in HL (15). The finding of the present study that about 22.5% of HL show low expression is in keeping with previous data that down-regulation of p27 expression is very frequent in aggressive lymphoid malignancies (16). In normal lymphoid tissue, $p27^{KIP1}$ is expressed in non-proliferating lymphocytes, whereas proliferating lymphoid cells within germinal centers are often negative(17).

Inter-relationship between the different Cell Cycle Markers in Hodgkin's disease

Low- and high-growth fraction lymphomas are both able to accumulate alterations in cell cycle regulation, most frequently involving tumor suppressor genes such as$p16^{INK4a}$, *p53*, and$p27^{KIP1}$. As a consequence, these tumors behave as highly aggressive lymphomas. The simultaneous inactivation of several of these regulators confers increased aggressivity and proliferative advantage to t*umor cells* (18).Considering the high level constitutive expression of NFkB and p53 shownin previous studies as well as ours, lack of significant correlation between thetwocan be explained as either an attribute of the limited number ofstudied cases or, more likely, that the two factors, acting through differentpathways, were affected to a varying extent by the same pathogenetic process or that each was influenced by a different pathogenetic process in a synergisticmanner that eventually lead to unrestrained cell growth. In the latter case, the up-regulated expression of NFkB is intimately associated with constitutive signaling originating from the TNFR family members including CD30, CD40 (19), while the over-expression and nuclear accumulation of p53 may be related to the deranged function of its negative-feedback regulator Mdm2 (5,20,14). Whether the

two pathogenetic mechanisms are linked together in some way, remains to be determined. On the other hand, the inverse correlation between p21Ras and p27 seemed interesting because the inverse mode of expression of the two in opposite direction (up-regulated p21Ras versus down regulated p27) is conducive to cell cycling(21), despite the fact that they act at different levels of the cell cycle and through different pathways.reduced p27 expression combined with alterations in the status of p53 may have cooperative effects resulting in uncontrolled tumor cell proliferation and aggressive malignancies in experimental mouse models and in humans(22).The p53 pathway regulates apoptosis and cell cycle arrest in G1 phase, depending on the cell type and cell state. P53-dependent G1 arrest is mediated, at least in part, through p53-mediated induction of p21 . The activity and the stability of p53 protein is regulated via interactions with proteins such as mdm2 which allows targeting of p53 to the ubiquitin-mediated proteolytic network. The finding of the present study that about 22.5% of HL show low p27 expression is in keeping with previous data that down-regulation of p27 expression is very frequent in aggressive lymphoid malignancies (22).

Our finding in this study is that 47.5% of HL cases exhibited nuclear overexpressionof p53 was in line with the findings in most previous studies,although the results varied over a wide range. For example, Garcia and coworkers(2003)(6), obtained 15.5% rate of expression. Smolewski*et al* (1998)(23),found 64%p53 reactivity in RS cells.Different studies had claimed that overexpression of p53 protein may indicate prognostic significance for patients(24).The explanation for this aberrant over-expression of p53 has been linkedby (5,25) to a defectiveARF-Mdm2-p53 pathway wherein abnormal Mdm2 (inhibitor of p53) moleculesaccumulate in the nucleoplasm in excess of the amount required to control p53activity. oncogenic-stress-driven senescence depend on p27 and p21(26).It was revealed that low/null p27 expression was significantly correlated with increased p53 expression.(27,28).

Conclusion

Significantly high expression rate of NFkB which was commensurate with H/RS cell proliferative activity and significant expression of p53 was observed which was mostly resulting from nuclear accumulation of transcriptionally inactive protein which indicates an aberrant p53 pathway in H/RS cells .And low expression level of the CDK inhibitor p27 indicating a non-functional upstream activating factors and conforming to the inverse relation between its expression and proliferative activity of the malignant cells. The expression of the mutant p21 Rasoncoprotein in a major proportion of RS cells in , providing evidence for a role of this mutant protein in the pathogenesis of this tumor reminiscent to that established in other mutant Ras–expressing tumors.

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