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 HL samples and seeking correlation between each protein studied.

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

The cell cycle regulatory proteins, NFkB, p53, p27 proteins and the mutant p21Ras oncoprotein were studied using in situ 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 0.11 respectively]. All cell cycle markers gave nuclear pattern of staining except p21Ras which gave clear cytoplasmic staining. Correlation between the cell cycle marker revealed no correlation between expression of NFκB and any of the other markers, p53, p21 and p27 (p > 0.05), p53 exhibited low but insignificant correlation with p21 expression (p > 0.05), and no correlation with p27, while a highly significant but inverse correlation was found between p21 and p27 (p < 0.01).

**Conclusion:** The high expression of the mutant p21Ras and the low expression of p27 both contribute to cell growth and proliferation albeit through different pathways.

**Key words:** Hodgkin’s lymphoma, cell cycle molecules, *in situ* hybridization, immunohistochemistry.
which controls G1/S transition and S phase progression (5,6). Hence in this retrospective (archival paraffin embedded tissue) study we aimed to assess the Ras protein expression and that of the tumor suppressor proteins p53 and p27 and the nuclear transcriptional factor NFκB in patients with Hodgkin lymphoma.

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 NFκB factor expression in Tumor Cell by in Situ hybridization using NFκB probe:
Biotin-labeled 371 bp long cDNA probe was purchased from Maxim’s product 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 Immunohistochemistry using the following monoclonal antibodies which were purchased from DakoCytomation:

Immunohistochemistry

Paraffins blocks were sectioned at a thickness of 4 μm 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 beforehand. After incubation, immunodetection was performed with light microscope employing diaminobenzidine chromogen 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) were used to determine significance of relation between the different parameters and markers.

Determination of Correlation Coefficient was made by Pearson correlation to determine degrees of 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, and 20 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 at x40 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 9 out 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 corner of the same figure is an inset showing the same RS cell at X100 magnification (Arrow).
Figure 2. Immunostaining for p21Ras and p27 in HD cases. A to D: Positive cytoplasmic staining of H/RS cells for p21Ras (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 p27 was found, (p>0.05). The expression of p53 revealed low correlation with p21Ras expression 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.

<table>
<thead>
<tr>
<th>Cell cycle marker (total No. of cases tested)</th>
<th>Frequency of expression</th>
<th>Relative Risk (RR)</th>
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<tbody>
<tr>
<td>NFkB (20)</td>
<td>17 (85)</td>
<td>4.52</td>
</tr>
<tr>
<td>P53 (40)</td>
<td>19 (47)</td>
<td>5.43</td>
</tr>
<tr>
<td>P21ras (40)</td>
<td>29 (72)</td>
<td>3.82</td>
</tr>
<tr>
<td>P27 (40)</td>
<td>9 (22.5)</td>
<td>0.11</td>
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Discussion

There is increasing evidence that multiparameter immunohistochemical 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 NFκB, p53, p21 and p27 in relation with 40 cases of HL.

NFκB. Many previous studies have confirmed unequivocally the constitutive expression of the nuclear transcriptional factor NFκB in the malignant H/RScells in most cases evaluated (8). Several mechanisms have been suggested to account for the high expression of NFκB in Hodgkin's disease (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 is based on the over-expression and persistent activation of CD40 and CD30 members of the TNFR family in the malignant RS cells, which activate NFκB by initiating signals transduced through the TRAF-TRADD pathways. Here we further confirmed the constitutive expression of activated NFκB by demonstrating nuclear localization of the factor in the malignant RS cells by in situ hybridization using NFκB-specific polynucleotide probe (figures 4.12A to 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 by cellular 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 Table 2. Correlation among the different cell cycle markers

<table>
<thead>
<tr>
<th>Markers</th>
<th>NFκB</th>
<th>P53</th>
<th>P21</th>
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<tr>
<td>P53</td>
<td>r</td>
<td>0.082</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>P21</td>
<td>r</td>
<td>-0.050</td>
<td>0.153</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>P27</td>
<td>r</td>
<td>-0.099</td>
<td>-0.057</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
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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.p27KIP1. One of the most interesting observations the results of this study came up with the consistent under-expression of the tumor suppressor protein p27KIP1. This result lent further support to the well known inverse relationship between tumor progression and p27KIP1 expression. The level of p27KIP1 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 p27KIP1 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, p27KIP1 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 p16INK4a, p53, and p27KIP1. 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 tumor cells (18). Considering the high level constitutive expression of NFkB and p53 shown in previous studies as well as ours, lack of significant correlation between the two can be explained as either an attribute of the limited number of studied cases or, more likely, that the two factors, acting through different pathways, were affected to a varying extent by the same pathogenetic process or that each was influenced by a different pathogenetic process in a synergistic manner 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 overexpression of 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. Smolewskiet 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 linked by (5,25) to a defective ARF-Mdm2-p53 pathway wherein abnormal Mdm2 (inhibitor of p53) molecules accumulate in the nucleoplasm in excess of the amount required to control p53 activity. 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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