Level of PAX5 in differential diagnosis of non-Hodgkin's lymphoma

Brij Bharti, Sachin Shukla, Ratnakar Tripathi, Suman Mishra, Mohan Kumar[1], Manoj Pandey[2] & Rajnikant Mishra

Departments of Zoology, [1]Pathology, [2]Surgical Oncology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

Background & objectives: The PAX5, a paired box transcription factor and B-cell activator protein (BSAP), activates B-cell commitment genes and represses non-B-cell lineage genes. About 14 transcript variants of *PAX5* have been observed in human. Any alteration in its expression pattern leads to lymphogenesis or associated diseases and carcinogenesis in non-lymphoid tissues. Its mechanisms of function in pathophysiology of non-Hodgkin's lymphoma (NHL) are unclear. This study was intended to explore influence of PAX5 in cascade of NHL pathogenesis and diagnosis.

Methods: Samples of 65 patients were evaluated by immunohistochemical staining for cellular localization of PAX5, CD19, CD3, cABL, p53, Ras and Raf and by TUNEL assay, RNA-isolation and reverse transcriptase (RT)-PCR, Western blot analysis, and lactate dehydrogenase (LDH) specific staining.

Results: B-cell type NHL patients were positive for PAX5, p53, Ras, CD19, Raf and CD3. All of them showed TUNEL-positive cells. The differential expression pattern of PAX5, CD19, p53, CD3, ZAP70, HIF1α, Ras, Raf and MAPK (mitogen-activated protein kinase) at the levels of transcripts and proteins was observed. The LDH assay showed modulation of LDH4 and LDH5 isoforms in the lymph nodes of NHL patients.

Interpretation & conclusions: The histological observations suggested that the patients represent diverse cases of NHL like mature B-cell type, mature T-cell type and high grade diffuse B-cell type NHL. The findings indicate that patients with NHL may also be analyzed for status of PAX5, CD19 and ZAP70, and their transcriptional and post-translational variants for the differential diagnosis of NHL and therapy.

The PAX5, a member of the paired box transcription factor, is expressed within the haematopoietic system and brain. The *PAX5* and its transcript variants have been promising markers of B-cell lineage, haematological neoplasms and various types of cancers including bladder carcinomas, neuroendocrine tumours and astrocytomas[1-4]. The presence of PAX5, CD19 and CD20 indicates B-cell lineage or mature lymphomas[2,5,6] whereas that of CD3 suggests T-cell lymphoma[7]. Other than PAX5, several molecular markers (CD markers) have been suggested for differential diagnosis of non-Hodgkin's lymphoma (NHL)[8,9].

In recent years, advances in genomic and proteomic technologies have provided molecular insights into B- and T-cell non-Hodgkin's lymphomas (NHLs) for their precise classification, risk-stratification, and target-antigens. However, lack of identified novel targets for the distinct NHL subtypes in patients, and dysregulation of intracellular oncogenic processes during lymphomagenesis[10] make the treatment challenging. The spleen and lymph nodes are common peripheral sites of lymphoma but it has also been found in extralymphatic organs like stomach, intestine, and skin. Although morphologic and microscopic differences of lymph nodes facilitate assessment of prognostically and therapeutically relevant features[11], but classification of haematolymphoid neoplasm requires help of differential diagnostic procedures[12]. Reports suggest that the PAX5, and ZAP70 (zeta chain associated protein kinase 70), a cytoplasmic tyrosine kinase positively regulate CD19. The ZAP70 is positively regulated by the CD3 that indicates an association between ZAP70 and PAX5. The ZAP70 activates several downstream cascades but the relationship to Ras-Raf mediated MAPK (mitogen-activated protein kinase) cascade is unknown in lymphoma. It is also not clear if PAX5 is influenced by Ras/Raf/MAPKs. It is presumed that there is PAX5-mediated alternate pathway that regulates progression of lymphoma because PAX5 has been found upregulated during treatment by inhibitors of tyrosine kinases[13]. The present study describes molecular and immunohistological evaluations of patients showing diverse cases of NHL and suggests importance of PAX5, CD19 and ZAP70 in differential diagnosis of NHL.

Material & Methods

This study was conducted in the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India, during February 2009 to January 2012. The study protocol was approved by the institutional ethics committee.

Immunohistochemical analysis and TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) assay: Majority of the biopsy samples were obtained by excision of cervical lymph nodes whereas some of them were obtained from auxiliary lymph node excision. Biopsy samples of 65 patients with confirmed NHL were collected. Samples were fixed with 4 per cent formaldehyde overnight and then embedded in paraffin wax. Serial (5 μm thick) sections were de-paraffinized and re-hydrated in alcohol downgrade series. The sections were fully hydrated for 30 min and stained with haematoxylin followed by differentiation in acid water. The sections were dehydrated in upgrade alcohol serries and stained with eosin for histological evaluation. For immunohistochemical analysis, sections were rehydrated by alcohol downgrade series and then in running water. Antigen retrieval was done in 0.1 per cent trypsin for 10 min and blocked with 1 per cent bovine serum albumin (BSA) followed by treatment of primary antibody at 1:200 dilutions for 4-6 h or overnight at 4°C. The sections were washed with phosphate buffered saline (PBS) and incubated with horseradish peroxidase (HRP) conjugated secondary antibody as per protocol of super sensitive polymer-HRP IHC detection system (BioGenex, USA). In this study primary antibodies anti-cABL, anti-CD19, anti-CD3, anti-Ras, anti-Raf (Abcam, UK), anti-PAX5, and anti-p53 (Santacruz Biotech, USA) were used.

TUNEL assay was performed for the detection of apoptotic bodies in NHL biopsy sections, using Apo-Brdu-IHC™ *In Situ* DNA Fragmentation Assay Kit (BioVision, Inc, USA).

Analysis of expression pattern of markers by reverse transcriptase polymerase chain reaction (RT-PCR): Total RNA was extracted from biopsy samples of NHL patients by PureLink RNA Mini kit (Life technologies, USA) using manufacturer's protocol. One microgram of total RNA was reverse transcribed in 20 μl reaction mixture using oligo-dT primer according to the protocol for first strand cDNA synthesis kit (Fermentas, USA). The resulting cDNA was used as a template for PCR. All reactions were done in triplicate. The expression levels of *PAX5, ZAP70, CD19, Ras, Raf, MAPK, p53* and *HIF-1-alpha* (hypoxia inducible factor -1α subunit) were studied with the gene specific sets of primers and normalized against β-*actin* internal control gene (Table I)[14-17]. The gene specific primers used here were self designed and validated.

Protein expression pattern by Western blotting: The extract of biopsy samples was prepared in Tris-Cl 0.1M, *p*H 7.2, containing protease inhibitor. 50 μg protein was resolved on 10 per cent denaturing poly-acrylamide gel using standard method[18]. The polypeptide pattern was immobilized on polyvinylidene difluoride (PVDF) membrane, and probed with primary antibodies, anti-CD19, anti-CD3 (Abcam), anti-PAX5, and anti-p53 (Santacrutz Biotech., USA) after blocking with 5 per cent non-fat milk in PBS. Detection was done with

Primer name	Primer sequence	Amplicon size (bp) and target
Table I. Forward and reverse primers (5′-3′) to amplify genes in RT-PCR experiments. All primers are self designed and validated		
PAX5 F[15]	5'-AATGACACCGTGCCTAGCGT-3'	381
R	5'-GGTGGTGAAGATGTCTGAGT-3'	(824-1204)
CD19 F[14]	5'-GGAGAGTCTGACCACCATGCCACC-3'	1827
R	5'-AAGGGGACTGGAAGTGTC -3'	(47-1918)
Zap-70 F[16]	5'- TCTCCAAAGCACTGGGTG-3'	524
R	5'-AGCTGTGTGTGGAGACAACCAAG -3'	(1306-1829)
Raf F[14]	5'-TCCAGGAGACCAAATTCCAG-3'	610
R	5'-GTGCAAGCATTGATGTCCTC-3'	(796-1405)
Ras F[14]	5'-TGTGGATGAGTACGACC-3'	337
R	5'-ACGAATCCCGTAACTC-3'	(281-617)
p53 F[14]	5'- AGAGACCGCCGTACAGAAGA-3'	285
R	5'-GCATGGGCATCCTTTAACTC-3'	(685-911)
MAPK F[14]	5'-TCTCCCGCACAAAAATAAGG-3'	213
R	5'-TCGTCCAACTCCATGTCAAA-3'	(1038-1250)
HIF1A F[14]	5'-CAGTGTCTAGCCAGGCCTTGAC-3'	813
R	5'-AGTGAAGCACCTTCCACGTTGC-3'	(178-990)
β-Actin F[17]	5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3'	661
R	5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'	(552-1212)

F, forward; R, reverse

Superscript numerals denote reference numbers

PAX5, paired box gene 5; *CD19*, cluster of differentiation 19; *ZAP70*, zeta chain of T cell receptor associated protein kinase 70; *RAF*, rapidly accelerated fibrosarcoma kinase; *RAS*, rat sarcoma GTPase family; *p53*, Tumor protein 53; *MAPK*, mitogen-activated protein kinase; *HIF1A,* hypoxia-inducible factor 1-alpha; *β-Actin*, beta actin

electrochemiluminescence (ECL)-system (supersignal west pico chemiluminescent substrate, Thermo Scientific, USA) with HRP-conjugated secondary antibody. β-actin (Sigma - Aldrich, Inc., USA) was used as the reference protein for constitutive expression.

Lactate dehydrogenase (LDH) specific staining: Native polyacrylamide gel (8.0%) electrophoresis was performed to analyze LDH isoforms and enzyme activity of lymph nodes from NHL patients. Gels were stained for LDH specific activity as described earlier[14].

Results

Of the 65 patients, 43 (66%) were diagnosed as NHL and classified into different types of NHL according to the WHO classification[19]. Of the 43 NHL patients, 20 (46.5%) showed T-cell type NHL and 23 patients (53.5%) showed B-cell type NHL. Of the 23 B-cell type NHL, eight patients showed high grade large cell B-cell type NHL, seven showed mature B-cell type NHL, five showed intermediate B-cell type NHL, and one patient showed follicular type B-cell NHL (Table II). The case histories of patients, their diagnosis and immunophenotypic analysis of

their biopsy samples showed variable penetrance and expressivity of NHL.

One patient having intestinal lymphoma but no tumour cells in lymph nodes showed preserved architecture with germinal centre (Fig. 1A-B). Patient P1 presented with diffuse superficial and deep lymphadenopathy showed effacement of the architecture by the monotonous population of atypical lymphoid cells with opened up nuclei (Fig. 1 C-D). Lymph node from lymphoma patient P2 showed loose clusters of cells and necrosis in whole tissue (Fig. 1 E-F). The lymph nodes of patients showed immunoreactive cells for PAX5 (77%), CABL (66%), CD19 (77%), CD3 (77%), Raf (88%), Ras (100%), and p53 (100%) (Fig. 2A-C).

To observe the effect of progression of NHL on the apoptosis, TUNEL assay was performed with the sections of biopsy samples of NHL patients. Figure 3 shows a representative micrograph of TUNEL positive cells.

Levels of transcripts of PAX5, CD19, ZAP70, p53, HIF1A, MAPK, Ras and Raf in NHL patients: The levels

Table II. A brief summary of analysis of NHL (non-Hodgkin's lymphoma) patients (n=43), origin of biopsy sample, histological and diagnostic findings and immunophenotypes based on the presence or absence of various molecular markers

Site and specimen	Histology and diagnosis	Immunophenotypes	Pateints showing particular immunophenotype (% of 43 NHL patients)
Cervical lymph nodes excision	Mature B-cell type NHL (small monomorphic cells)	PAX5+/ CD3-/ CD19+/ p53+/ cABL+/ Ras+	4
	Mature B-cell type NHL (large pleomorphic cells)	PAX5-/ CD3-/ CD19-/ p53+/ cABL-/ Ras+	16.2
	High grade diffuse B-cell NHL	PAX5+/ CD3+/ CD19+/ p53+/ cABL+/ Ras+	18.6
	Intermediate type B-cell NHL (Small round cells with prominent nucleoli and scanty cytoplasm)	PAX5+/ CD3+/ CD19+/ p53+/ cABL+/ Ras+	11.6
Auxiliary lymph nodes excision	T-cell type NHL (Monomorphic small cells)	PAX5-/ CD3+/ CD19-/ p53+/ cABL+/ Ras+	46.5

of transcripts of *PAX5* and associated markers *CD19, ZAP70, p53, HIF1A, MAPK, Ras* and *Raf* were found altered in NHL patients (Fig. 4A). The *PAX5* expression could only be observed in patient P2, whereas *MAPK* and *Raf* were detected only in patients P2 and P3. The other candidates (*CD19, ZAP70, p53, Ras* and *HIF1A*) showed variable expression in different patients. *CD19* and *ZAP70* were relatively higher in P2 and P3 than in P1 and P4 patients. The *p53* and *HIF1A* showed higher expression in patient P2 than patients P1, P3 and P4 (Fig. 4A). The expression patterns of *PAX5* (25%) and associated markers *CD19* (100%), *ZAP70* (100%), *p53* (75%), *HIF1A* (100%), *MAPK* (50%), *Ras* (75%) and

Raf (50%) were variable. The Western blot analysis of patient samples showed presence of PAX5 (66%), CD3 (33%), CD19 (66%), and all patients showed presence of p53 (Fig. 4B).

Modulation of LDH isoforms: The results of enzyme-specific staining of LDH of NHL patients showed modulation of LDH isoforms (Fig. 5). Patient P1 showed three isoforms of LDH *i.e.*, LDH4, LDH3, and LDH2 while the patient 2 (P2) showed four isoforms of LDH including LDH5, LDH4, LDH3, and LDH2. The reactive or control lymph node showed only three isoforms of LDH including LDH3,
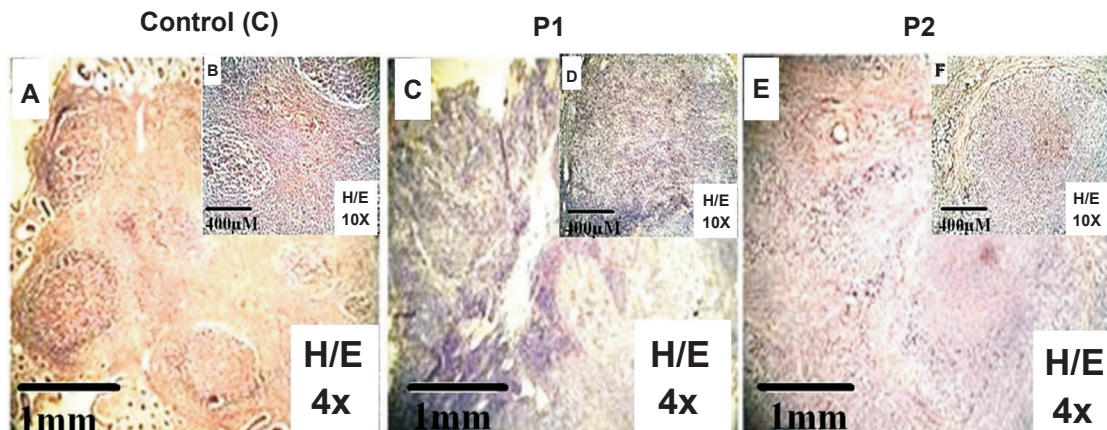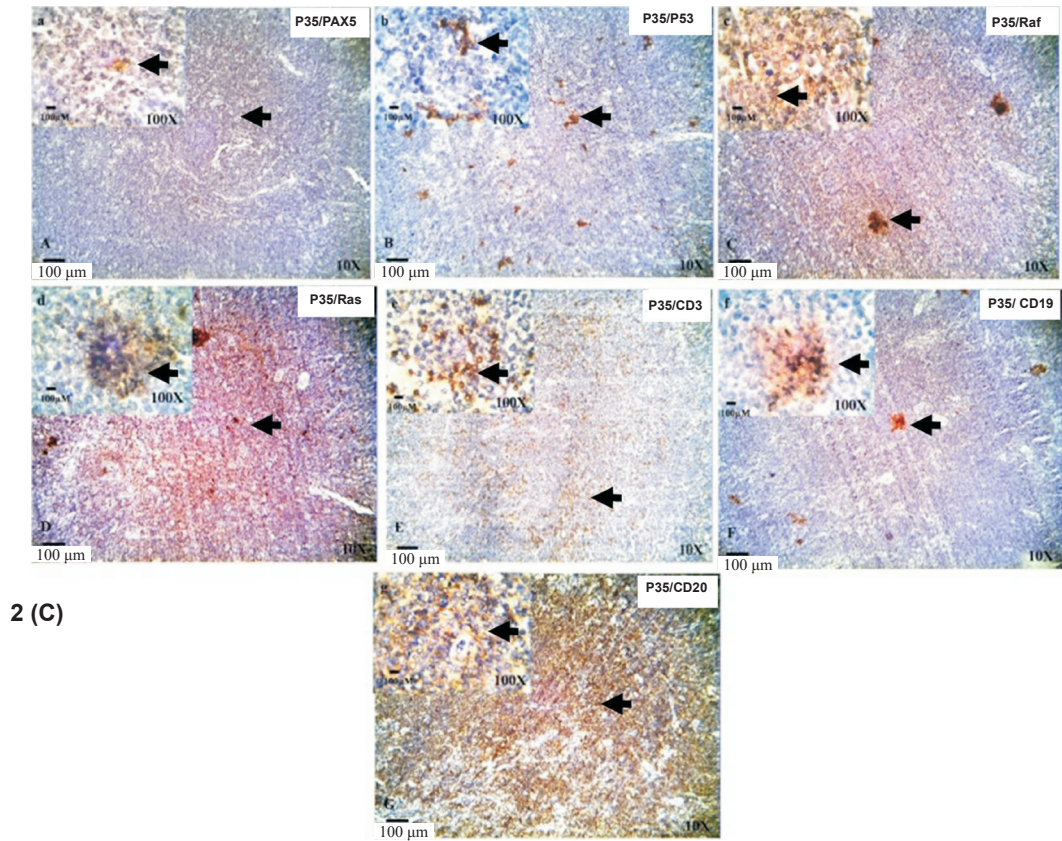

Fig. 1. Histochemical analysis of reactive lymph node from a patient having intestinal lymphomna but no tumour cells in lymph nodes. Reative lymph nodes show prominent strands of monocytoid B-cells, surrounding a reacting germinal centre just beneath the epithelial cells of intestine at 4x **(A)** and at 10x **(B)**. Lymphoma patient P1 shows dense, highly cellular sheets and nest of monotonous cells at 4x **(C)** and 10x D); and patient P2 shows loose clusters of cells and necrosis in whole tissue at 4x **(E)** and at 10x **(F)**.

2 (A)

2 (B)
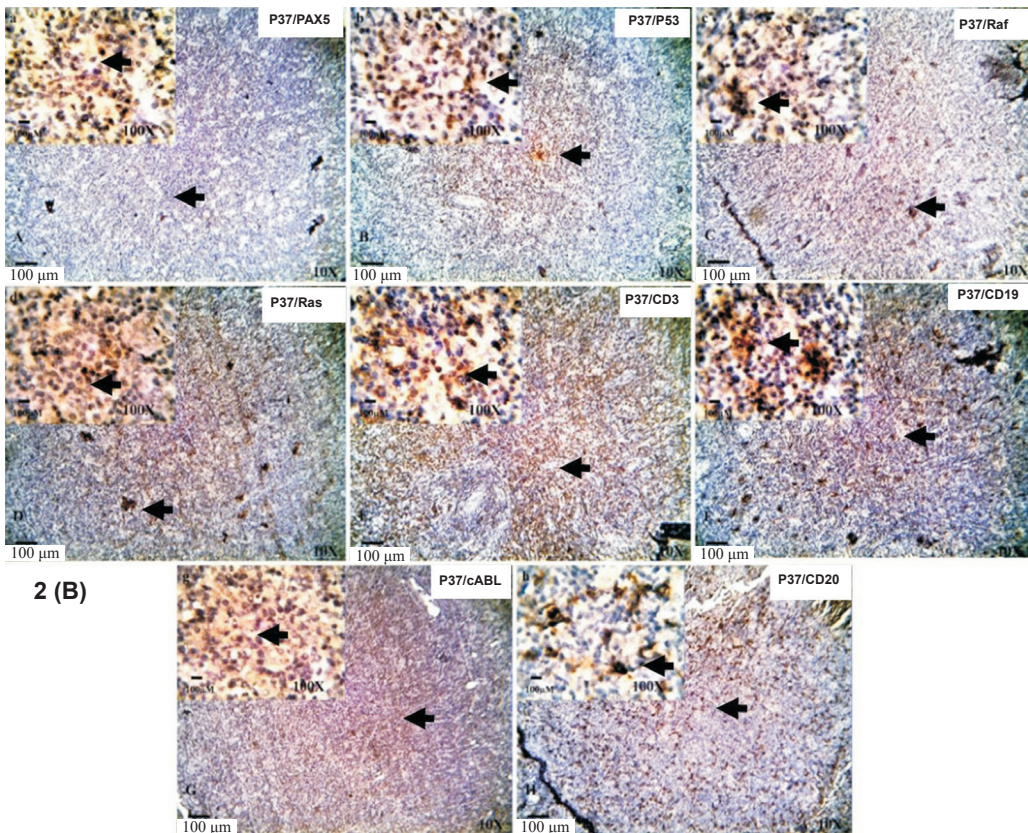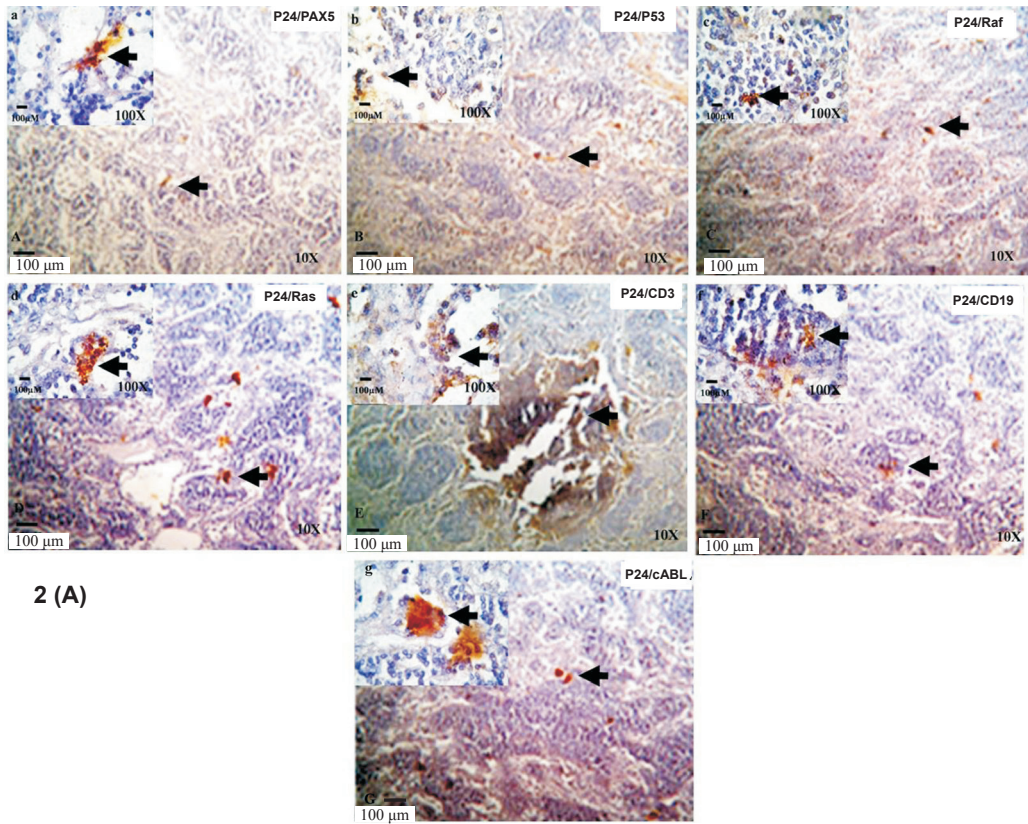
Fig. 2. Immunohistochemical analysis of lymph node sections of non-Hodgkin's lymphoma (NHL) patients **(2A-C)**. The PAX5, CD3, CD19, CD20, cABL, Ras, Raf and p53 immunoreactive cells are observed in NHL patients. Inset (higher magnification, 100X) images enable easier identification of cells expressing specific markers. Arrows indicate immuno reactivity to specific markers.
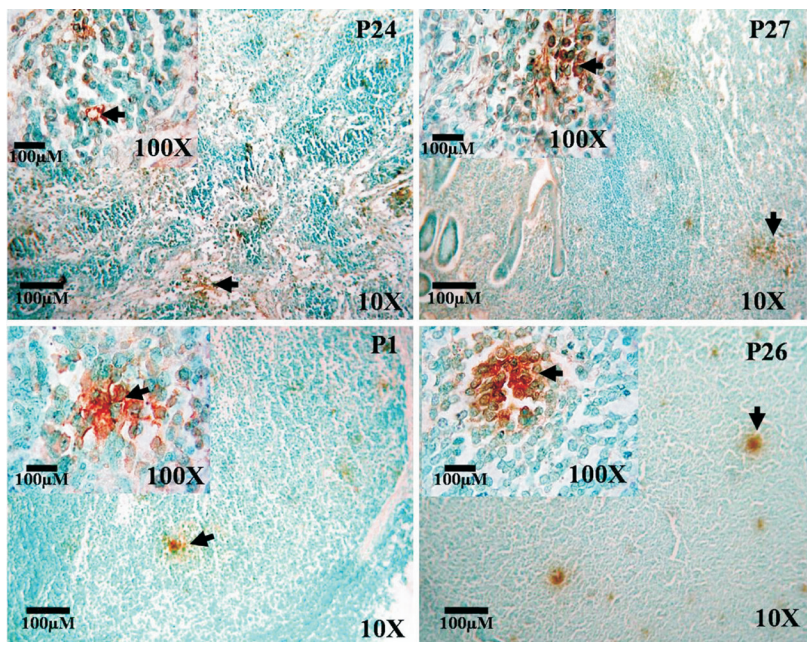
Fig. 3. TUNEL-positive cells in different non-Hodgkin's lymphoma (NHL) sections. The figures in inset are shown in higher magnification (100X) images enabling easier identification of cells. P1, P24, P26 and P27 are patients' numbers. Arrows show area of apoptotic cells.
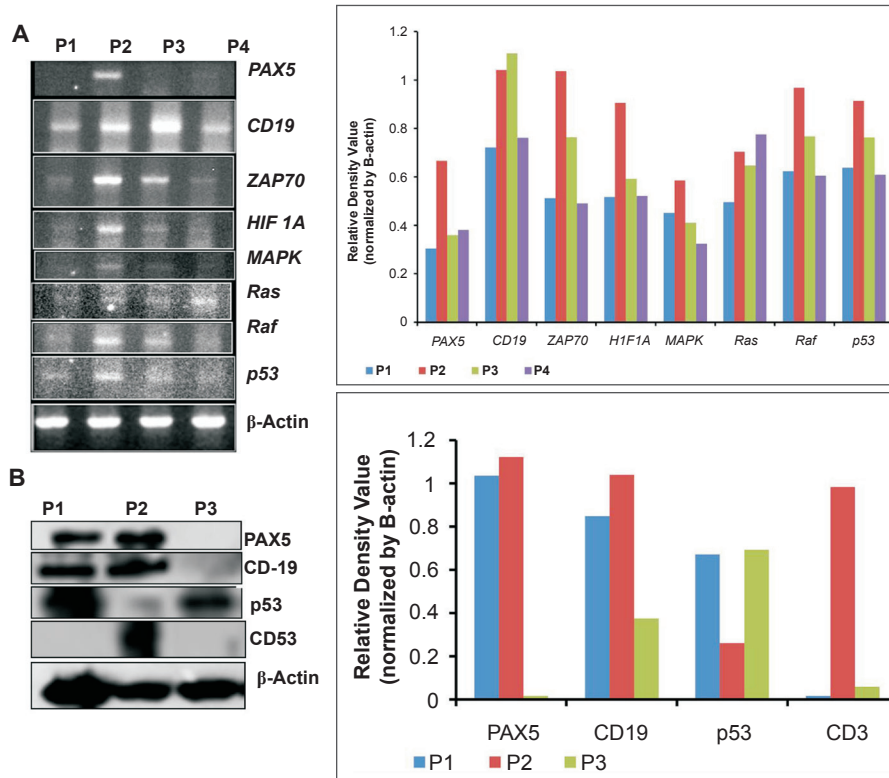
Fig. 4. Modulation of expression of different candidate genes at transcript **(A)** and protein **(B)** levels in NHL patients. **A.** RT-PCR analysis shows expression of different candidate genes (*PAX5, CD19, ZAP70, HIF1A, MAPK, Ras, Raf* and *p53*), and their quantitative analysis **B**. Western blot analysis shows modulation of candidate proteins (PAX5, CD19, CD3 and p53) and their quantitative analysis in different NHL patients. P1-P4 represent NHL patients.
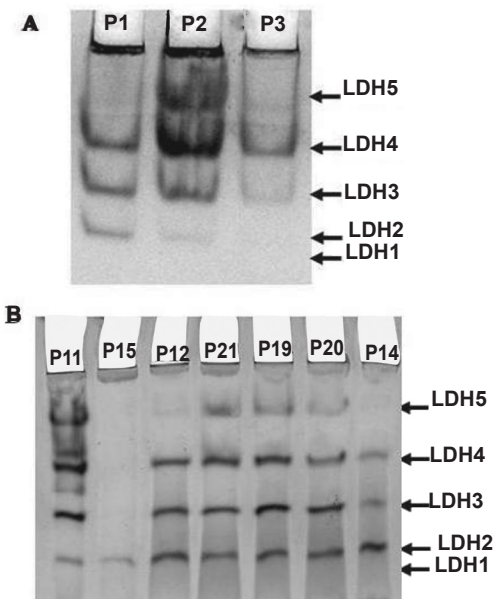
Fig. 5 A. Modulation of LDH isoforms (LDH2, LDH3, LDH4 and LDH5) were observed by enzyme specific staining of serum of representative NHL patients (P1, P2 and P3). **B.** Shows similar analysis of seven more NHL patients. Arrows show LDH isoforms.

LDH4, and LDH5 at detectable level (Fig. 5A). The activities of LDH isoforms LDH3, LDH4 and LDH5 were higher in P2 while that of LDH2 was higher in P1. While some of them showed all the five LDH isoforms (LDH1, LDH2, LDH3, LDH4 and LDH5), LDH2 and LDH3 were absent in P15. LDH4 was absent in P12, P14 and P15. LDH5 was absent in P12, P14 and P19 (Fig. 5B).

Discussion

The NHL covers about 90 per cent of diagnosed lymphomas that affect lymph nodes, spleen, bone marrow and other organs of immune system[19]. Distinction between histologically similar tumours is often critical as therapeutic options often differ[20]. The expression of PAX5, and CD19 was detectable in a few cases by both immunohistochemistry and Western blot analysis. Since PAX5 also regulates expression of p53 in haematopoietic cells, it was interesting to observe presence of p53 in lymph nodes of lymphoma bearing patients. It is known that overexpression of wild type p53 induces cells to express cytoplasmic

immunoglobulin heavy chain and a B-cell specific antigen[21]. Co-expression of both T- and B-cell markers was in agreement with expression patterns of neoplastic T-cells showing positive signals for CD3, CD4, CD5, CD8, CD45, and CD20 and negative signals for other B-cell markers such as CD79a and PAX5[22]. Some specific type of B-cell lymphomas show negative staining for CD20[23] while some T-cell lymphomas show positive staining for CD20[24]. It is suggested that although CD markers are specific to particular cell types but in neoplastic conditions their expression becomes non-specific[7]. The expression of B- and T-cell markers may not be aberrant but indicative of an antigenic phenotype present on small population of cells.

The expression of PAX5, CD19 and CD3 in these cases recapitulated the conditional *Pax5* deletion in mice that allowed mature B-cell from peripheral lymphoid organs to dedifferentiate *in vivo* back to uncommitted progenitors in the bone marrow, which rescued T lymphopoiesis in the thymus of T cell deficient mice[25]. The *Pax5* and its isoforms are well known for normal B-cell development and differential modulation of *Pax5* is suggested in case of malignancies[3,26]. The expression of PAX5 in immunohistochemistry and Western blot suggested its promising role in B-cell specific NHL. However, the co-expression of PAX5, CD19, p53, Ras and Raf indicates involvement of Ras-Raf mediated pathway influenced by PAX5 (Fig. 6).

The LDH activity was elevated because glucose was used as main energy source through glycolysis. Kimura *et al*[27] have reported a relationship between expression of CD19 and level of LDH in B-cell lymphoma that may be associated with energy transduction and redox-sensitive factor, PAX5. The modulation of activity of LDH isoforms in lymphoma may be due to induced hypoxic condition. The elevated activity of LDH5, LDH4 and LDH3 could be due to differences in the rate of synthesis, degradation or excretion of the enzymes in lymphoma. A tumour-induced cachexia and metabolic inefficiency is also presumed[28,29].

In conclusion, the analysis of cell types and immunoreactivity showed various types of cells including lymphocytes and medullar sinuses. The variable immunoreactivity of markers in patients indicated mixed type of lymphoma. The expression levels of *PAX5, CD19, CD3, p53, Ras, Raf, ZAP70, HIFIA,* and *MAPK* indicated involvement of Ras-Raf mediated pathway influenced by PAX5. The modulation of activity of LDH isoforms (LDH2) may be due to
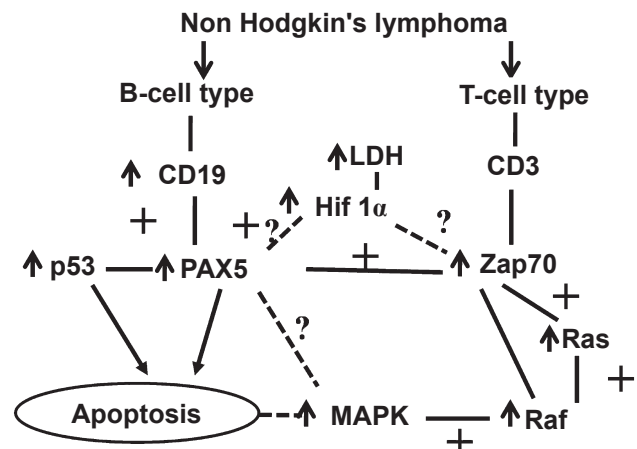
Fig. 6. A model showing association of PAX5, CD19, CD3, p53, Ras, Raf, MAPK and Zap70 in B-cell and T-cell type non-Hodgkin's lymphoma. The (+) sign indicates the positive relationship and (?) indicates presumed relationship. Non-Hodgkin's lymphoma affects B- and T-cell types by modulating their specific markers. In B-cell CD19, PAX5 and p53 expression upregulated whereas in T-cell CD3 may have a role in the upregulation of ZAP70, Ras, Raf and MAPK. PAX5 positively regulates expression of ZAP70 and ZAP70 might be a regulatory point of non-Hodgkin's lymphoma by Ras-Raf dependent pathway. Increased p53 and PAX5 levels induce apototosis which is also supported by upregulation of HIF1A and LDH. PAX5, paired box gene 5; CD19, cluster of differentiation 19; ZAP70, zeta chain of T cell receptor associated protein kinase 70; RAF, rapidly accelerated fibrosarcoma kinase; RAS, rat sarcoma GTPase family; p53, tumour protein 53; MAPK, mitogen-activated protein kinase; HIF1A, Hypoxia-inducible factor 1-alpha; LDH, lactate dehydrogenase.

induced hypoxic condition. Almost all patients were p53 positive but variable TUNEL positivity indicated p53 independent apoptosis. The patients with NHL may be tested for molecular markers and differential diagnosis with PAX5, CD19 and ZAP70 to decide appropriate therapy of NHL.

***Conflicts of Interest*:** None.

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Reprint requests: Dr Rajnikant Mishra, Biochemistry & Molecular Biology Laboratory, Department of Zoology,
Banaras Hindu University, Varanasi 221 005, Uttar Pradesh, India
e-mail: rmishraa@bhu.ac.in