Low expression of the DNA repair proteins, Ku70, Ku80, and TDP1, is associated with diffuse large B-cell lymphoma

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Diffuse large B-cell lymphoma (DLBCL) constitutes the largest percent of non-Hodgkin lymphoma (NHL). During B cell development, DNA double strand breaks are processed at immunoglobulin gene segments, which are recombined by mediating the excision, relocation, and re-ligation of these gene segments in a manner that utilizes several of the non-homologous end joining (NHEJ) key enzymes. The NHEJ process is initiated by the sliding of Ku70 and Ku80 over both ends of the broken DNA molecule. The binding of a DNA end with the Ku heterodimer is critical for creation of a scaffold that gives other NHEJ key enzymes binding capability to DNA ends. TDP1 as a broad-spectrum DNA end-processing enzyme, acts at undamaged 3' end to generate 3' phosphates, which prevents error-inducing DNA synthesis during NHEJ. TDP1 is also an essential factor in the DNA repair machinery for TOP1-induced DNA breaks during transcription as well as replication. To investigate the expressions of Ku70/80 and TDP1 in DLBCL, we analyzed the protein levels of Ku70, Ku80, and TDP1 in 25 DLBCL patient samples and 13 normal lymphatic control tissues by using immunohistochemistry. The results showed that very low expression of Ku70 and Ku80 were found in 20% (5/25) and 8% (2/25) of the DLBCL samples, respectively. Moderate expression of Ku70 and Ku80 was detected in 62% and 36% of patient samples, respectively, while all the control lymphatic tissues (13/13) showed high expression of Ku70 and Ku80, which indicated that low expressions of Ku70 and Ku80 were significantly correlated with lymphoma development ($P<0.05$). Furthermore, the results demonstrated that low expression of Ku70 was significantly associated with low expression of Ku80 ($P<0.01$). Moreover, low expression of TDP1 was detected in 84% of patient samples. Statistical analysis results showed that low expression of TDP1 was significantly correlated with lymphoma development ($P<0.01$). In conclusion, this study revealed that DNA repair proteins, Ku70, Ku80, and TDP1, may have negative effects on proliferation and maturation of B lymphocytes and reduced expressions of these proteins are associated with the development of diffuse large B-cell lymphoma.

Keywords: Diffuse large B-cell lymphoma; DNA repair; TDP1; Ku70; Ku80; immunohistochemistry.

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Introduction

Diffuse large B-cell lymphoma (DLBCL) constitutes the largest percent of non-Hodgkin lymphoma (NHL). Patients with DLBCL are curable with combination chemotherapy. However, approximately 50% of patients die due to that disease [1]. Physiological DNA double strand breaks (DSBs) are well-represented in the lymphoid cells of the vertebrate immune system as a way of generating a diversity of antibodies to bind with antigens of invading organisms, which is generated when developed B cells undergo V(D)J recombination that involves assembly of variable (V), diversity (D), and joining (J) segments of the V exon of the immunoglobulin

genes. Adjacent recombination signal sequence (Rss) exists at the 3' end of each V and J segments, and at both ends of each D segment of each gene. Recombination activates genes, RAG-1 and RAG-2 proteins, and then identifies RSS to introduce double-stranded breaks at both strands of DNA at the RSS forming double-stranded breaks (DSB) (Figure 1a). Then, the regular machinery for repairing DSBs by nonhomologous end-joining swings into action [2-4].

After going through V(D)J recombination, B cells subsequently undergo two genetic modifications including somatic hypermutation (SHM) and Class-switch recombination (CSR) to increase the affinity of immunoglobulin. Functional antibody genes are diversified by SHM, which involves the introduction of point mutations into the variable regions of immunoglobulin genes [5]. Hypermutation is triggered by activation-induced (cytidine) deaminase (AID) that is a single strand DNA (ssDNA) deaminase catalyzing conversion reaction of deoxycytidine residues to deoxyuridine on target DNA [6, 7]. CSR, a process that changes the Cμ constant region to another constant region gene (Cγ, Cα, or Cε), involves recombination between switch regions (Figure 1b). These regions are highly repetitive GC-rich sequences of 1–10 kb in length that lie upstream of all immunoglobulin C-region genes except for the δ C-region gene. CSR process involves transcription through mammalian S regions. Researchers found that generation of ssDNA R-loop substrates for the cytidine deaminase action of AID was processed by transcription through mammalian S regions.

In S-region, uracil-DNA deoxyuridine introduced by the AID enzyme is removed by uracil-DNA glycosylase (UNG) [8]. A basic site is arisen from the UNG activity. The apurinic/apyrimidinic endonuclease 1 (APE1) processes the basic site and creates a nick [8-10]. A closely spaced staggered DSB can be formed by similarly generating nick on the opposite strand. In addition, mismatch repair (MMR) machinery could also process mismatch to generate staggered DSB. Accordingly, both UNG deficiency [11] and MMR deficiency result in CSR defects and immunodeficiency [12, 13].

Tyrosyl-DNA phosphodiesterase (TDP1) functions as a general 3’-DNA phosphodiesterase. It acts on the 3’ phospho ∝, β unsaturated aldehyde resulting from β elimination by the base-specific mammalian DNA glycosylases/AP lyases [14]. TDP1 also acts as a broad-spectrum DNA end-processing enzyme [15]. TDP1 is an essential factor in the DNA repair machinery for TOP1-induced DNA breaks during transcription [16] and replication [17]. It can remove covalently bound polypeptides and tyrosyl-phosphates that arise when topoisomerases fail to proceed DNA ligation reaction [17-19].

CSR ends are repaired by non-homologous end joining (NHEJ) which depends on Ku family [20]. The NHEJ process is initiated by the sliding of a protein complex, the Ku70/80 heterodimer, over both ends of the broken DNA molecule. It is currently believed that the association of a DNA end with the Ku heterodimer creates a scaffold for the assembly (attraction) of the other NHEJ key enzymes. The DNA-Ku scaffold attracts the DNA-dependent protein kinase catalytic subunit (DNA-PKCS) to the DSB to form the active protein kinase complex DNA-PK. After capturing both DNA ends together by this kinase, these non-ligatable DNA termini must be processed before final repair of the DSB can take place. If the processing steps are incomplete, there will be defect in the ligation reaction. Several enzymes including nucleases and polymerases have been confirmed that they are able to either remove or fill-in single-stranded, noncompatible overhangs. Finally, ligation reaction of the processed DNA ends is catalyzed by the ligase IV/XRCC4 complex. These reactions are enhanced by XLF/Cernunnos protein [21]. In this study, we evaluated the expression of the DNA break repair proteins, Ku70/80 and TDP1, in DLBCL tissues and non-cancerous lymphoid tissues and tried to find out the relationship between the expression of any of them and the prognosis of DLBCL.

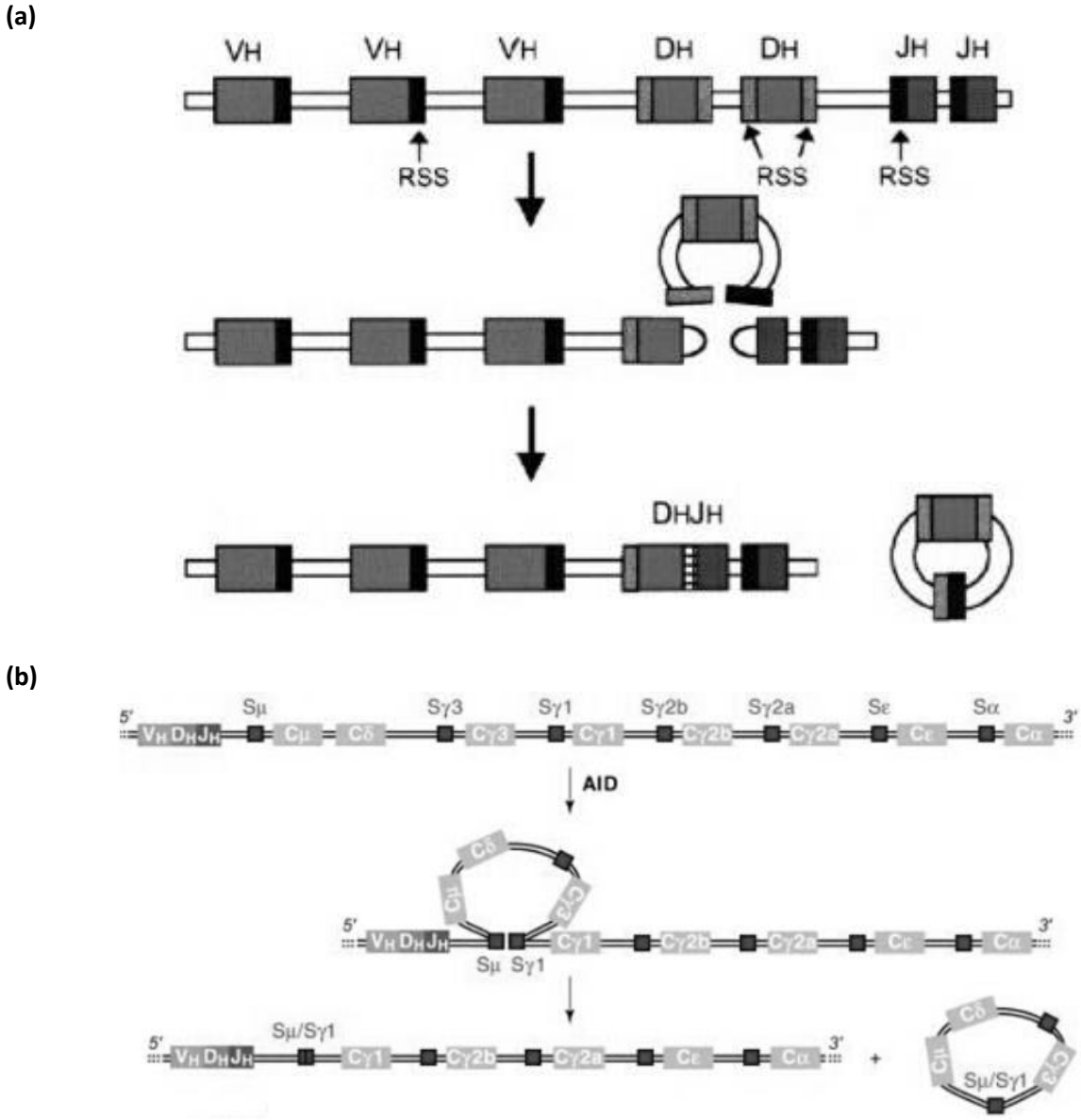

Figure 1. V(D)J recombination and class switching recombination. **(a)** In V(D)J recombination, the RAG1 and RAG2 enzymes cleave the DNA at recombination signal sequences (RSS) creating hairpin-sealed coding ends and blunt signal ends. Artemis, which is recruited and phosphorylated by the Ku/DNA-PK complex, opens the hairpins through its endonuclease activity. The XRCC4/Cernunnos/DNA-Ligase IV complex finally seals coding and signal joins. **(b)** In class switch recombination, DNA breaks are introduced into switch (S) regions after the activity of activation-induced cytidine deaminase (AID). These DNA breaks undergo DNA repair process. Class switch recombination process proceeds for repositioning the constant region genes and deleting the interstitial sequence as an episomal circuit.

Materials and methods

Subjects

Lymph node tissue samples from 25 nodal DLBCL patients (19 females and 6 males with a median age of 55 years old) and 13 normal individuals (8 females and 5 males with a median age of 55 years old) were collected from Oncology Center of Mansoura University, Mansoura, Egypt, and other private labs after obtaining informed consents from all participants according to the approval of the Scientific Ethics Committee of Oncology Center, Mansoura University whose work was undertaken to the provisions of the

Declaration of Helsinki (as revised in Edinburgh 2000). All patients' clinical data were retrieved and validated the diagnosis of DLBCL.

Immunohistochemistry assays of Ku70, Ku80, and TDP1

The immunohistochemistry assays were performed by using Dako reagents and antibodies supplied by Cusabio, Houston, Texas, USA. Briefly, paraffin blocks were cut at 4 µm thick, deparaffinized in xylene, and rehydrated through a graded series of ethanol concentrations. Antigen retrieval was performed in a pressure cooker for 5 mins using Dako Target Retrieval Solution. Endogenous peroxidase activity was blocked by Dako Peroxidase Blocking Reagent for 5 mins. Primary antibodies (Ku70, Ku80, and TDP1) were then applied to the specimen for 1 h at room temperature followed by incubation with labeled polymer-HRP anti rabbit or anti mouse secondary antibody for 30 mins at room temperature. Thorough rinsing with TBST was performed after incubation with each reagent. The slides were visualized using DAB substrate-chromagen and washed with deionized water before counterstaining with haematoxylin. The slides were then dehydrated through a graded series of ethanol concentrations, cleared in xylene, and cover slipped in DPX mounting medium.

The intensity of protein expression of subcellular compartments was detected and grouped as follows: 0 = no staining, +1 = weak staining, +2 = moderate staining, +3 = strong staining. Random fields were selected from representative areas. The demonstration of immunoreactive cells among 100 cells was counted and quantified as percentage. The areas of immunostaining were assessed by calculating the average of the positive cells in five fields. The results were defined as: 0 = less than 5%, 1 = 6-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100%. In addition, the protein expression levels of immunostained cells were also determined as: 0 = negative, +1 = weak, +2 = moderate, +3 = strong. The overall expression levels of the proteins in the section were determined by calculating the integrals of the ''area × intensity'' and were defined as following scales: negative (-): score 0, weak positive (+): score 0-1, moderate positive (++): score 2-4, strong positive (+++): score 5-6.

Statistical analysis

All data were presented as the means or mean percentages ± SD. Software SPSS (version 16.0) (IBM, Ammon, New York, USA) was applied for statistical analysis. The Mann-Whitney U test was used to determine the differences in numerical variables between differently defined groups. Correlations were analyzed using Spearman's rank correlation co-efficient. 95% confidence intervals were estimated for each variable. P value of less than 0.05 had considered statistically significant.

Results

This study aimed to determine the expression of Ku70, Ku80, and TDP1 proteins in lymphoma tissue (DLBCL) comparing to that in normal lymph node tissues (NLT). The protein expression levels were summarized in table 1.

Expression of Ku70, Ku80 in DLBCL and NLT

The overall immunostaining results reflect the low expression of Ku70 protein in the DLBCL tissues with low or very low expression in 20% (5/25) cases, moderate expression in 72% (18/25) cases, and high expression in only 8% (2/25) cases. Importantly, all the normal lymph node tissues showed 100% of high expression of Ku70 (13/13). The results demonstrated that the differential expression levels of Ku70 in DLBCL tissues ranged from low to moderate expressions while all the control lymphatic tissues showed high expression levels. The moderate expression level of Ku70 in DLBCL tissues and the high expression level in normal tissues were shown in figure 2 (A1 and A2). All the immunostaining data was concluded in table 1 and figure 3A.

The immunostaining results of Ku80 also reflected the variations of Ku80 expression levels ranging from low to high expression levels. In

Table 1. Differential immunohistochemical positive rate of Ku70, Ku80, and TDP1 in lymphoma patients and control lymphatic tissues.

Group	Number	TDP1				Ku70				Ku80			
		Non	L	M	H	Non	L	M	H	Non	L	M	H
NLT	13	0	0	13	0	0	0	0	13	0	0	0	13
LT	25	0	21	4	0	0	5	18	2	0	2	9	14

Note: Normal lymph node tissue (NLT). Lymphoma tissue (LT). Expression level low (L), moderate (M), high (H).

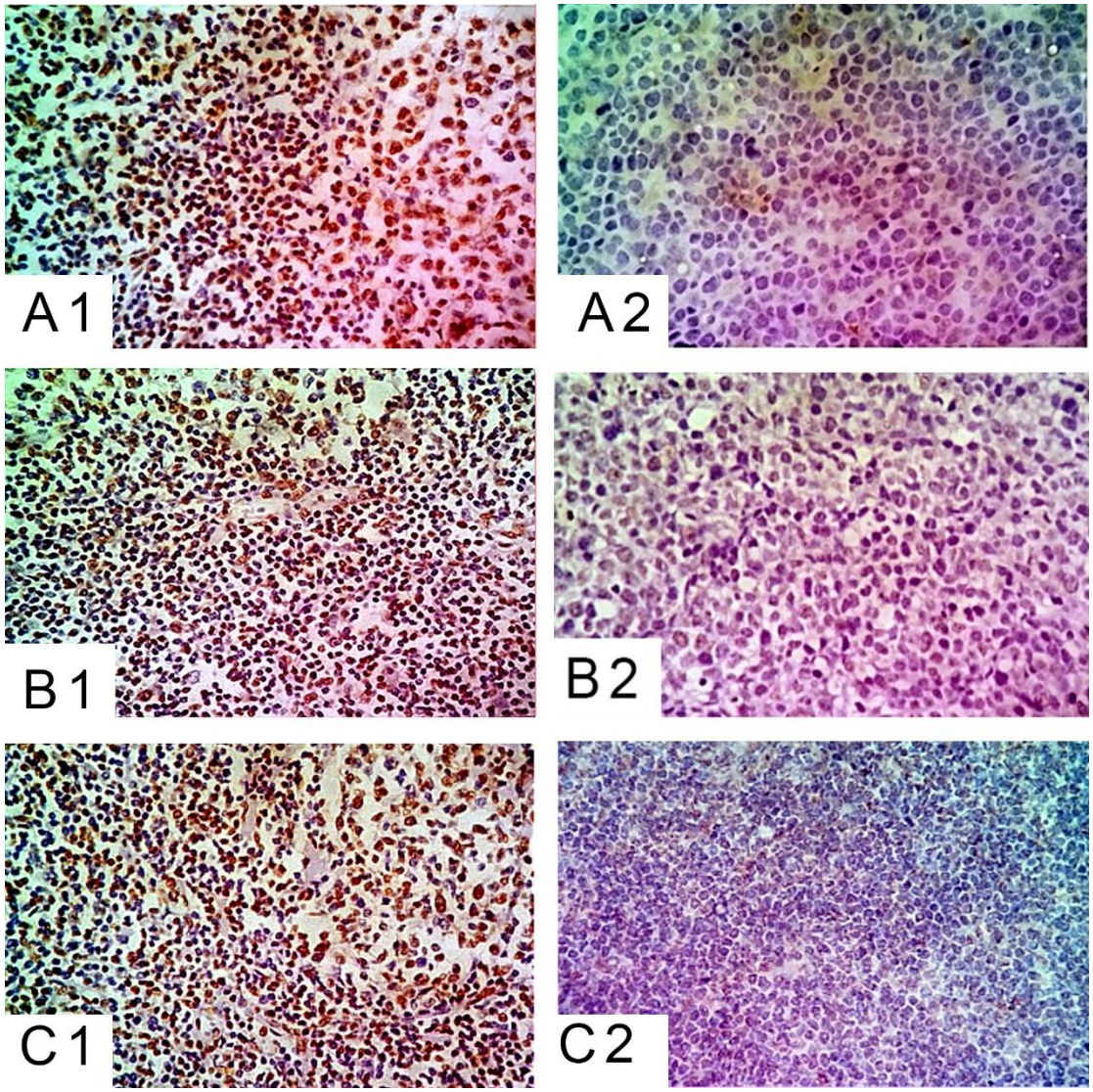

Figure 2. Representative immunohistochemical results of Ku70, Ku80, and TDP1 in lymphoma (DLBCL) and non-lymphoma (NLT) tissues (original magnification: X 400). The NLT groups showed high and/or moderate expression of Ku70, Ku80, and TDP1 while DLBCL groups showed moderate or low expression of Ku70, Ku80, and TDP1. **A1:** Ku70 staining of NLT. **A2:** Ku70 staining of DLBCL. **B1:** Ku80 staining of NLT. **B2:** Ku80 staining of DLBCL. **C1:** TDP1 staining of NLT. **C2:** TDP1 staining of DLBCL.

DLBCL tissues, low and very low expressions of Ku80 were detected in 8% (2/25) cases while moderate expressions of Ku80 were detected in 36% (9/25) cases. The high expression levels of Ku80 were detected in 56% (14/25) of DLBCL tissues. In parallel, all the normal lymph nodes

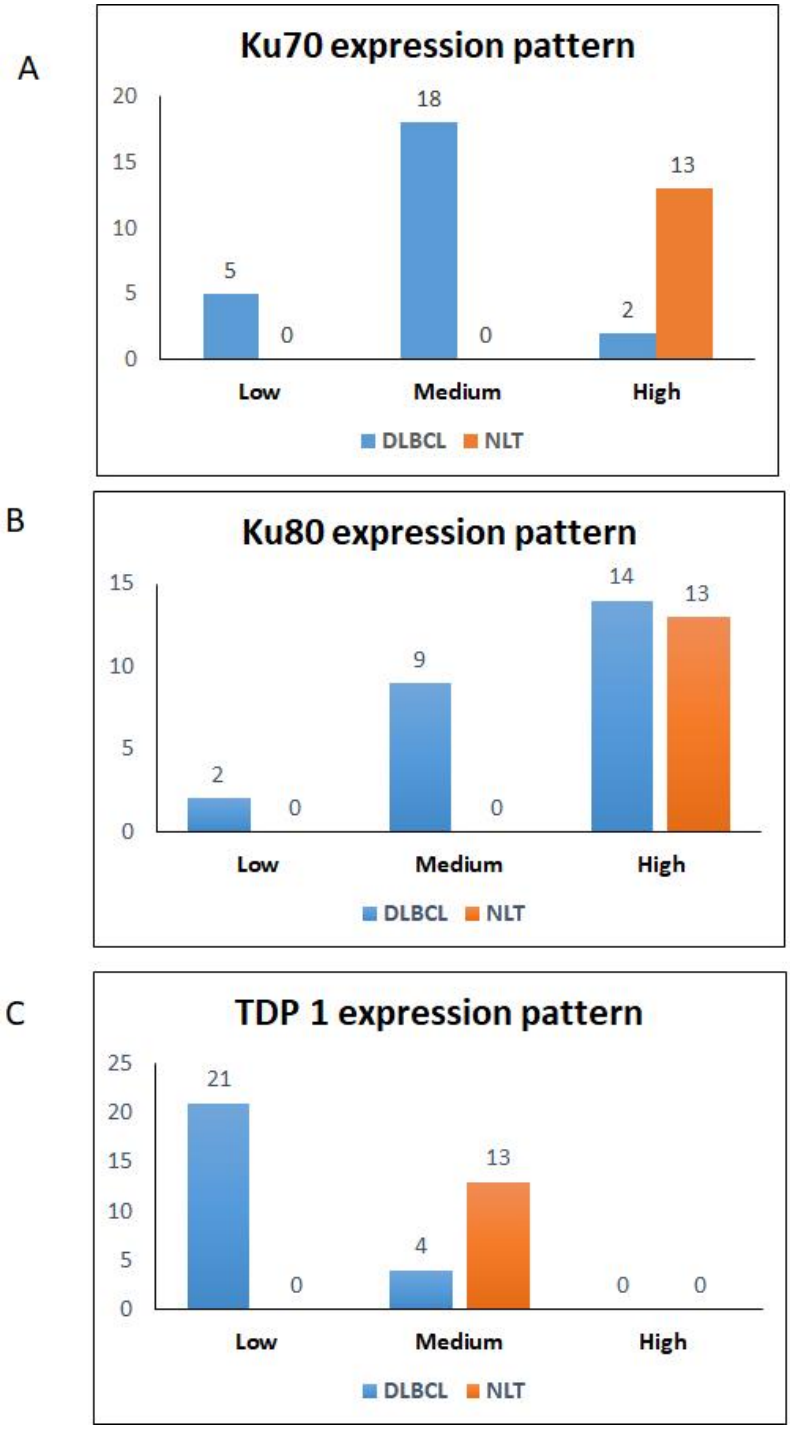

Figure 3. Immunostaining results of Ku70, Ku80, and TDP1.

showed 100% of high expression of Ku80 (13/13). Representative staining images were shown in figure 2 (B1 and B2) while all the immunosatining data of Ku80 in control and DLBCL lymphatic tissues were summarized in figure 3B and table 1.

It is well known that Ku70 and Ku80 are working in heterodimer in the double strand break repair. To determine whether the scoring was reproducible, the 25 sections were scored blindly twice, and a Mann-Whitney test was performed

on the data. There was significant difference between the percentage of cells stained positive for Ku70 and Ku80 proteins in the two data sets (figure 3A and 3B). In-depth statistical analysis demonstrated that down regulation of Ku70 and Ku80 were significantly associated with lymphoma development (table1) ($P<0.05$). There was a significant correlation between the percentage of cells scored positive for both Ku70 and Ku80 ($r=0.57$, $P<0.01$). The resulted data may reflect the importance of both Ku70 and Ku80 in the normal lymph nodes and may indicate that the low expression is associated with carcinogenesis and DLBCL development.

Expression of TDP1 in DLBCL and NLT

Majority of the DLBCL tissues (84%, 21/25) showed very low and low expression of TDP1 while only 16% (4/25) showed moderate expression of the same molecule with none of the tissues showed high expression. Importantly, all the control lymphatic tissues (100%, 13/13) showed moderate expression of TDP1 (figure 2, C1 and C2). All immunostaining data of TDP1 was summarized in figure 3C and table 1. Statistical analysis of the TDP1 staining data showed that low expression of TDP1 was also significantly correlated with lymphoma development ($P<0.01$). These data may indicate the importance of the expression of TDP1 in the normal lymph node tissues and may support the idea that low or very low expression of TDP1 as a repair molecule is associated with DLBCL development.

Discussion

The largest percent of non-Hodgkin lymphoma (NHL) is represented by diffuse large B-cell lymphoma (DLBCL). During B cell development, DNA double strand breaks are processed at immunoglobulin gene segments. The genes which encode immunoglobulins (Ig) are not present in an active form in these lymphocytes. DNA modification process is performed thereafter for generating active Ig genes. During VDJ recombination process, gene segments are recombined by mediating the excision, relocation, and re-ligation in a manner that utilizes several of the NHEJ key enzymes. During these processes, RAG proteins introduce DSBs at gene segments, and then the NHEJ core factors initiate in repairing machinery. Ligation of these segments requires the presence of the NHEJ enzymes ligase IV and XRCC [22, 23]. The inference that CSR ends were repaired by NHEJ was initially supported by *in vivo* and *in vitro* studies which demonstrated that mice deficient in Ku70 or Ku80 severely had defect in CSR process [24].

Several studies have confirmed that the Ku70/80 heterodimer assembly ligation enzyme such as ligase IV/XRCC4 complex and several of the processing enzymes to the synaptic repair complex are, in a manner, similar to the recruitment of DNA-PKCS. One *in vitro* study found that, after interaction with Ku70/80, the ligation activity of ligase IV/XRCC4 increased 20-fold, suggesting that Ku was critical to activate ligase IV/XRCC4 [25]. The sliding of a protein complex of Ku70/80 heterodimer over both ends of the broken DNA molecule is the basic step to initiate the NHEJ process. Several studies showed that the binding of a DNA end with the Ku heterodimer was critical for creation a scaffold that gave other NHEJ key enzymes binding capability to DNA ends. Therefore, Ku80 and Ku70 genetic abnormalities can contribute to cancer susceptibility [26]. Unrepaired DNA can lead to the formation of tumorigenic cells [27]. Previous studies have proved that, in the long evolution of cells from normal to malignant phenotype, accumulation of multiple genetic alterations induce tumor development [28]. Ku70 and Ku80 deficiencies may enhance neoplastic growth, which reflects their roles in tumor suppression [29 ,30]. Ku70/80 and DNA-PK, by their association with DNA repair and recombination, were supposed to be the caretaker genes which belong to class of tumor-suppressor genes that also include ATM, BRCA-1, and BRCA-2 [31]. Some other studies indicated that Ku80 had critical role in the integrity of the genome by a mechanism involving the

suppression of chromosomal rearrangements [34], and loss synergy with p53, which promoted chromosome aberrations including breakage and translocations in fresh cells of Ku80 knock-out mice [32]. This study provided evidence that Ku70, Ku80, and TDP1 were promising biomarkers in non-Hodgkin lymphoma (NHL). The data showed that the incidence of low expressions of Ku70 and Ku80 were 20% and 8%, respectively. Moreover, statistical analysis showed that down expressions of Ku70 and Ku80 were significantly correlated with lymphoma development ($P<0.05$). There was also a good correlation between the numbers of staining positive cells in tumor samples for Ku70 and Ku80 that supported *in vitro* studies of the interdependence of the two heterodimer components. There was a significant correlation between the percentage of cells scored positive for both Ku70 and Ku80 ($r = 0.59$, $P<0.01$).

In recent years, the prognosis of DLBCL patients has significantly improved. However, therapeutic efficacy of some patients is still not ideal. It is also known that the correct diagnosis helps greatly to start with the best treatment regimen. Hence, there is usually a pressing need to best diagnose and start the ideal therapeutic intervention in DLBCL. Tyrosyl-DNA phosphodiesterase (TDP1), an essential factor in the repair machinery of DNA damage resulted from topoisomerase I, has the ability to eliminate covalently bound polypeptides and tyrosyl-phosphates proceed from uncompleted ligation reaction of intermediates. During CSR and SHM, the base-excision-repair (BER) enzyme, uracil- DNA glycosylase (UNG), processes the deoxyuridine which introduced by AID DNA to create a basic site. DSB is created during CSR in S region by processing the basic site. In SHM, the basic site is processed, and then, error-prone DNA polymerases fill in the gap and create mutations. Some reports showed that TDP1 generated DNA strand break with the 3’ phosphate termini from a basic site. Therefore, it can function in a fashion similar to NEIL1 in the APE-independent BER pathway [37]. The importance of TDP1 in DNA repair has been demonstrated by results which indicated that human neurodegenerative disorder, spinocerebellar ataxia with axonal neuropathy (SCAN1), was related to mutation in human TDP1 [33]. In this study, low expression of TDP1 was detected in 84% of DLBCL patient samples. Moreover, statistical analysis showed that low expression of TDP1 was significantly correlated with lymphoma development ($P<0.01$).

Additional report indicated that TDP1 deficiency led to defect in DSB repair [34]. During NHEJ process, a number of end-processing proteins participate in NHEJ. If DNA ends are not processed before the start of ligation enzymes in the ligation reaction, it will result in ligation defect. One mechanism for processing of damaged 3’ termini is TDP1. Recent studies showed that the down-regulated expression levels of Ku70 and Ku80 could be affected by the expression of TDP1 protein when TDP1 was identified as a contaminant in highly purified Ku70/80 from human placenta [35]. TDP1 enhances the activity of protein kinase DNA-PK. Ku70/80 protein forms scaffold with DNA-PK and the effect of TDP1 on DNA-PK may be, in part, due to its interaction with Ku70/80. In addition, poly (ADP-ribose) polymerase 1 (PARP1) has been confirmed involving in NHEJ interacting with TDP1 [36], which may explain in another way how TDP1 can influence NHEJ reaction. The results may indicate that dysregulations of the Ku70/80 heterodimer and TDP1 proteins are associated with DLBCL development. In conclusion, the data of this study represents a pilot study result indicating the negative relationship between major NHEJ proteins and DLBCL development.

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