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## Expression of serine 194–phosphorylated Fas-associated death domain protein correlates with proliferation in B-cell non–Hodgkin lymphomas

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### Abstract

Fas-associated death domain protein is a key component of the extrinsic apoptotic pathway. In addition, in animal models, Fas-associated death domain protein phosphorylation at serine 194 has been shown to affect cell proliferation, especially in T lymphocytes. The importance of Fas-associated death domain protein phosphorylation at serine 194 for the proliferation of B lymphocytes, however, is uncertain. Here we show in reactive lymph nodes that serine 194 phosphorylated Fas-associated death domain protein is expressed predominantly in the dark (proliferative) zone of germinal centers. In B-cell non–Hodgkin lymphoma cell lines, serine 194 phosphorylated Fas-associated death domain protein levels are substantially higher in highly proliferating cells and lower in serum-starved cells. We also used immunohistochemical analysis to assess Fas-associated death domain protein phosphorylation at serine 194 expression in 122 B-cell non–Hodgkin-type lymphomas. The mean percentage of serine 194 phosphorylated Fas-associated death domain protein positive tumor cells was 81% in Burkitt lymphoma, 41% in diffuse large B-cell lymphoma, 18% in follicular lymphoma, 18% in plasma cell myeloma, 12% in extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue, 11% in mantle cell lymphoma, and 2% in chronic lymphocytic leukemia/small lymphocytic lymphoma ( $P < .0001$ , Kruskal-Wallis test). Furthermore, in chronic lymphocytic leukemia/small lymphocytic lymphoma, serine 194 phosphorylated Fas-associated death domain protein was detected predominantly in proliferation centers. In the entire study group, the percentage of cells positive for serine 194 phosphorylated Fas-associated death domain protein correlated significantly with

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the proliferation index Ki-67 (Spearman  $R = 0.9$ ,  $P < .0001$ ). These data provide evidence that serine 194 phosphorylated Fas-associated death domain protein is involved in the proliferation of normal and neoplastic B cells and has features of a novel proliferation marker.

## Keywords

FADD; Phosphorylation; Proliferation; B cell; Lymphomas

## 1. Introduction

Fas-associated death domain protein (FADD), a molecule of 22 kd, plays a central adaptor role in conveying apoptotic stimuli from death receptors containing a death domain (DD) motif in the extrinsic apoptotic pathway and particularly Fas [1,2]. FADD contains 2 evolutionary highly conserved modules: a carboxy-terminal DD, through which it associates with homologous DDs contained in death receptors, and an amino-terminal death-effector domain (DED), through which it interacts with homologous DEDs contained in the initiator caspases 8/10, and FADD is essential for the nucleation of the death-inducing signaling complex [3,4]. However, a series of experiments with mice bearing a variety of specific *FADD* mutations suggested that FADD is also involved in nonapoptotic processes, including innate immune signaling, hematopoiesis, and cell cycle regulation in lymphocytes [5,6].

FADD can be phosphorylated at serine 194 residue (<sup>ser194</sup>p-FADD), localized at the carboxy-terminal region adjacent to the DD. Regulation of <sup>ser194</sup>p-FADD phosphorylation is critical for nonapoptotic functions of FADD, including cell cycle progression, and for its subcellular localization in the nucleus [7-11]. Although many kinase signaling systems have been implicated in FADD phosphorylation, a recent study demonstrated that casein kinase I $\alpha$  (CKI $\alpha$ ) is responsible for serine 194 phosphorylation of FADD at the G<sub>2</sub>-M transition of the cell cycle [7,12,13].

FADD phosphorylation has been shown to be dysregulated in a variety of epithelial cancers including lung, breast, prostate, and gastric adenocarcinomas [14-17], and <sup>ser194</sup>p-FADD expression has been correlated with prognosis, tumor progression, or response to chemotherapy [14-17]. For example, Chen and colleagues [14] showed that increased expression of <sup>ser194</sup>p-FADD, assessed by immunohistochemistry, correlated positively with adverse prognosis in patients with lung adenocarcinoma. Also, in vitro studies using carcinoma cells have shown that increased <sup>ser194</sup>p-FADD levels augment the antitumor activity of various chemotherapeutic agents [13]. However, the patterns and potential biologic significance of <sup>ser194</sup>p-FADD expression in reactive B cells and B-cell lymphomas are unknown.

In this study, we hypothesized that serine 194 phosphorylation of FADD may play a role in cell proliferation and cell cycle progression in B cells. To address this idea, we assessed <sup>ser194</sup>p-FADD expression in reactive lymphoid tissues and in B-cell non-Hodgkin lymphoma (NHL) cell lines and patient tumor specimens. Our data show that <sup>ser194</sup>p-FADD may represent a novel proliferation marker in normal and neoplastic B cells.

## 2. Materials and methods

### 2.1. Cell lines

Eight B-cell NHL cell lines were assessed in this study including SP-53, Mino, and Jeko-1 (typical mantle cell lymphoma, or MCL), JMP-1 and Z-138 (blastoid variant of MCL), Pfeiffer (diffuse large B-cell lymphoma, or DLBCL), Raji (Burkitt lymphoma, or BL), and REH (B acute lymphoblastic leukemia/lymphoma; B-ALL). All cell lines were maintained under conditions of exponential growth as described previously [18].

For serum deprivation studies, Mino cells were incubated in 20% (control), 10%, or 0.01% fetal bovine serum (FBS) for 24 hours before harvesting. We also reincubated previously serum-starved, synchronized JMP-1 cells with 15% FBS and harvested at various time points.

In another experiment, SP-53 cells were incubated with increasing concentrations of the CKI $\alpha$ -specific inhibitor CKI-7 (Toronto Research Chemicals, Ontario, Canada) up to 250  $\mu$ mol/L for 8 hours and were harvested for analysis of <sup>ser194</sup>p-FADD levels by Western blot analysis.

### 2.2. Western blot analysis

Cells in log-phase growth were collected, washed twice in cold phosphate-buffered saline, and lysed at 4°C in lysis buffer using protease and phosphatase inhibitors as previously described [19]. Western blot analysis of protein extracts was performed as described elsewhere [20]. The antibodies used included rabbit polyclonal antibody specific for <sup>ser194</sup>p-FADD and total FADD (Cell Signaling Technology, Beverly, MA).  $\beta$ -Actin (Sigma, St Louis, MO) was used as control for protein load and integrity.

### 2.3. Bromodeoxyuridine incorporation and immunofluorescence

Mino and Z-138 cells were incubated with bromodeoxyuridine (BrdU) at a final concentration of 10  $\mu$ mol/L in cell culture medium for 1 hour at 37°C. After washing in cold phosphate-buffered saline, cells were fixed with 100  $\mu$ L Cytotfix/Cytoperm buffer for 20 minutes on ice and then treated with DNase for 1 hour at 37°C. After washings, cell suspensions were used for cytospin preparations on coated glass slides. The slides were then incubated with total and <sup>ser194</sup>p-FADD antibodies overnight at 4°C and with fluorescein isothiocyanate-labeled anti-BrdU antibody for 1 hour at room temperature. After appropriate washings, the slides were incubated with a goat antirabbit secondary antibody (Alexa Fluor 594; Invitrogen Corporation, Carlsbad, CA) diluted in 1% bovine serum for 30 minutes. 4,6-Diamidino-2-phenylindole (DAPI) (Invitrogen Corporation) was used as a counterstain.

### 2.4. Tissue samples and immunohistochemical methods

Two reactive lymph nodes, 2 reactive tonsils, and 122 B-cell NHLs were examined including chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL; n = 25); follicular lymphoma (FL; n = 16) including grade 1 (n = 3), grade 2 (n = 11), and grade 3 (n = 2); extranodal marginal zone lymphoma of mucosa-associated lymphoid (MZL) tissue (n

= 5); MCL (n = 15) including 10 typical and 5 blastoid/pleomorphic variants; DLBCL (n = 32); BL (n = 12); and plasma cell myeloma (PCM; n = 17).

Immunohistochemical methods were performed as have been described previously [21]. Full tissue sections (n = 20 tumors) and tissue microarrays (n = 102 tumors) were used. A rabbit polyclonal antibody specific for <sup>ser194</sup>p-FADD (Cell Signaling Technology) was used. Proliferation index was assessed using the MIB1 (Ki-67) antibody (Immunotech, Westbrook, ME). Irrespective of intensity, any staining of tumor cells for <sup>ser194</sup>p-FADD was considered positive. The percentage of positive cells was calculated by counting at least 500 tumor cells.

## 2.5. Statistical analysis

As a continuous variable, the percentage of <sup>ser194</sup>p-FADD-positive tumor cells in different lymphoma types was analyzed using the nonparametric Mann-Whitney and Kruskal-Wallis tests. The Spearman *R* correlation coefficient was used to assess the association between the percentage of <sup>ser194</sup>p-FADD-positive tumor cells and percentage of Ki-67-positive tumor cells (proliferation index) as continuous variables. *P* < .05 was considered significant. All calculations were carried out using the StatView software (Abacus Concepts, Inc, Berkeley, CA).

## 3. Results

### 3.1. <sup>ser194</sup>p-FADD is expressed in the nucleus and is associated with proliferation in B-cell NHL cell lines

Western blot analysis showed <sup>ser194</sup>p-FADD expression in all 8 lymphoma cell lines that included 4 different B-cell NHL lymphoma types, with the levels of phosphorylation being higher in blastoid MCL, DLBCL, BL, and B-ALL cells as compared with typical MCL cells (Fig. 1A, upper panel). The level of FADD phosphorylation inversely correlated with doubling time in these lymphoma cell lines. For example, <sup>ser194</sup>p-FADD was expressed at high levels in Pfeiffer and Raji cells, which have a short doubling time (25-30 hours). By contrast, <sup>ser194</sup>p-FADD expression levels were relatively low in SP-53 and Jeko-1 cells that show a doubling time of 72 and 50 hours, respectively.

To study the subcellular localization of <sup>ser194</sup>p-FADD, immunofluorescence was performed on cytopspins prepared from 3 cell lines, Mino, Z-138, and REH. <sup>ser194</sup>p-FADD was detected primarily in the nucleus of B-cell lymphoma cells with variable staining intensity ranging from faint to strong in individual cells. Higher numbers of strongly positive cells were found in B-ALL (REH) and blastoid MCL (Z-138) than in typical MCL (Mino) (Fig. 1A, lower panel).

We examined the <sup>ser194</sup>p-FADD expression level in the Mino cell line under serum deprivation conditions resulting in growth arrest and decreased total cell numbers (Fig. 1B). Cell counting with trypan blue stain verified that any decreased number of cells was not associated with significant cell death. As shown in Fig. 1B, <sup>ser194</sup>p-FADD expression levels appear to gradually decrease in Mino cells grown in decreasing concentrations of serum (serum starvation), whereas the total FADD levels remained constant. Conversely, JMP-1

cells were first serum starved and then incubated with FBS and collected at various time points. As shown in Fig. 1C, the levels of <sup>ser194</sup>p-FADD expression increased over time in previously synchronized JMP-1. These findings suggest that <sup>ser194</sup>p-FADD expression is associated with cell proliferation.

To investigate the level of FADD phosphorylation in the proliferating fraction of cell lines, BrdU incorporation and double-immunofluorescence studies were performed using Mino and Z-138 cells. As shown in Fig. 1D, a subset of nuclei was positive for both <sup>ser194</sup>p-FADD and BrdU indicating that FADD is highly phosphorylated at serine 194 in the proliferating (BrdU incorporating) cell fraction.

As stated earlier, CKI $\alpha$  has been reported to be responsible for FADD phosphorylation at serine 194 [12,13]. To verify the association of FADD phosphorylation in B-lymphoma cells with CKI $\alpha$  activity, we treated SP-53 cells, growing in 15% FBS, with the CKI $\alpha$ -specific inhibitor CKI-7, and analyzed <sup>ser194</sup>p-FADD levels by Western blot analysis. As shown in Fig. 1E, incubation with increasing concentrations of CKI-7 resulted in gradually decreased <sup>ser194</sup>p-FADD protein levels. This result indicates that CKI $\alpha$  activity is essential for FADD phosphorylation at serine 194 in proliferating B-lymphoma cells and supports the results of others [12,13].

### 3.2. <sup>ser194</sup>p-FADD is highly expressed in the proliferating B-cell compartment in reactive lymphoid tissues

We assessed histologic sections of reactive lymph nodes and tonsils to determine <sup>ser194</sup>p-FADD expression in benign lymphocytes (Fig. 2). <sup>ser194</sup>p-FADD protein was expressed predominantly in the nuclei of germinal center B cells including centroblasts, immunoblasts, and some centrocytes, as well as occasional lymphocytes in the interfollicular areas. Cytoplasmic <sup>ser194</sup>p-FADD expression was identified in lymphocytes in the process of mitosis, in either metaphase or anaphase (Fig. 2).

### 3.3. <sup>ser194</sup>p-FADD is differentially expressed in B-cell NHLs

In B-cell NHLs, <sup>ser194</sup>p-FADD was detected in tumor cell nuclei (Fig. 3A). The number of <sup>ser194</sup>p-FADD expressing cells in different lymphoma types was highly variable, ranging from 0.2% to 95%, and staining intensity was also variable, ranging from weak to strong. The mean (range) percentage of <sup>ser194</sup>p-FADD-positive tumor cells in different B-cell NHL types was 81% (55%-95%) in BL, 41% (14%-79%) in DLBCL, 18% (8%-38%) in FL, 18% (6%-38%) in PCM, 12% (5%-23%) in extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue, 11% (3%-42%) in MCL, and 2% (0.2%-3.5%) in CLL/SLL. <sup>ser194</sup>p-FADD immunostaining highlighted increased positive cells within the proliferation centers of CLL/SLL (Fig. 3B).

As a continuous variable, the percentage of <sup>ser194</sup>p-FADD-positive tumor cells was significantly higher in BL and DLBCL compared with low-grade types of B-cell NHL ( $P < .0001$ , Mann-Whitney test; Fig. 3C). In FL, the small number of grade 3 tumors ( $n = 2$ ) precluded any statistical associations between grade and <sup>ser194</sup>p-FADD staining. In MCL, <sup>ser194</sup>p-FADD expression was significantly higher in tumors with blastoid/

pleomorphic morphology (mean, 20%; range, 13%-42%) compared with tumors with typical features (mean, 6.5%; range, 3%-13%;  $P = .003$ , Mann-Whitney test).

### 3.4. $\text{ser}^{194}\text{p-FADD}$ expression correlates significantly with proliferation index in B-cell NHLs

The percentage of  $\text{ser}^{194}\text{p-FADD}$ -positive cells positively correlated with the proliferation index as assessed by Ki-67 expression for the entire study group, and this association was highly significant (Spearman  $R = 0.9$ ,  $P < .0001$ ; Fig. 4). Of note, in each tumor the percentage of  $\text{ser}^{194}\text{p-FADD}$ -positive tumor cells was almost always lower than the percentage of Ki-67-positive tumor cells (Fig. 4).

## 4. Discussion

This is the first study to provide evidence that FADD phosphorylation at serine 194 is involved in the proliferation of reactive and neoplastic B cells. We further suggest that  $\text{ser}^{194}\text{p-FADD}$  expression has features of a novel proliferation marker in B cells. This suggestion is based on our data showing differential expression patterns of  $\text{ser}^{194}\text{p-FADD}$  in reactive B lymphocytes and various B-NHL types, as well as a highly significant association between  $\text{ser}^{194}\text{p-FADD}$  and the well-established proliferation marker Ki-67.

Previous studies performed in genetically modified mice point to a role for FADD in proliferation of T cells [5,6]. More specifically, T lymphocytes expressing a dominant negative FADD or deficient for FADD showed defective proliferation after T-cell antigen receptor stimulation [22,23]. The assumption that the same holds for B cells after B-cell antigen receptor stimulation, however, was not verified in later studies [24,25]. Nevertheless, B lymphocytes deficient for FADD show defective proliferation after specific innate immune stimuli involving Toll-cell receptor signaling. Furthermore, mice derived from a *RAG1*-negative chimeric background, deficient for FADD in the hematopoietic compartment, lack B lymphocytes [22,25]. Recent in vitro experiments support the hypothesis that involvement of  $\text{ser}^{194}\text{p-FADD}$  in proliferation is not restricted to lymphocytes but applies to a broader spectrum of mammalian cell types including epithelial and other types of mesenchymal cells [9,26].

Our results that showed  $\text{ser}^{194}\text{p-FADD}$  expression predominantly in reactive germinal centers, mostly in cells within the dark (proliferative) zones, recapitulate the pattern of Ki-67 expression and further support the concept that  $\text{ser}^{194}\text{p-FADD}$  is involved in B-cell proliferation. In accord with these findings, the expression levels of  $\text{ser}^{194}\text{p-FADD}$  in B-NHLs were significantly higher in lymphoma types characterized by a higher proliferation index, such as DLBCL and BL, compared with small B-cell lymphomas ( $P < .0001$ , Mann-Whitney test; Fig. 3). In MCL,  $\text{ser}^{194}\text{p-FADD}$  expression levels were significantly higher in blastoid/pleomorphic tumors, known to be characterized by higher proliferation index, than those in typical tumors. In addition, we demonstrated that  $\text{ser}^{194}\text{p-FADD}$  immunostaining identifies the most proliferating component of the lymphoma cell population, such as the proliferation centers in CLL/SLL. In cell lines,  $\text{ser}^{194}\text{p-FADD}$  expression in B-NHL cells after serum starvation and synchronization further supports the hypothesis that  $\text{ser}^{194}\text{p-FADD}$  is involved in proliferation. Recent studies on lung adenocarcinomas that showed



that increased <sup>ser194</sup>p-FADD expression correlated with increased proliferation, tumor progression, and poor prognosis further supports our findings and suggests a broader role for <sup>ser194</sup>p-FADD in cell proliferation [14,27].

Analysis of <sup>ser194</sup>p-FADD expression in lymphomas may have prognostic value. Previous studies have provided evidence that the phosphorylation status of FADD at serine 194 can influence the sensitivity of various solid tumors to chemotherapy [28]. Specifically, increased <sup>ser194</sup>p-FADD levels were reported to enhance the ability of tubulin-disturbing reagents, such as paclitaxel; to induce G<sub>2</sub>/M cell cycle arrest and to synergize with other chemotherapeutic agents, like etoposide, in carcinomas in vitro [13,28]. Although these studies have not been performed in lymphoma cells, it seems possible that <sup>ser194</sup>p-FADD expression may similarly enhance chemotherapeutic sensitivity. Furthermore, the correlation between FADD phosphorylation and proliferation index, the latter having prognostic significance in some NHL types [29], suggests that analysis of <sup>ser194</sup>p-FADD expression may have prognostic value.

Despite the highly significant association between <sup>ser194</sup>p-FADD and Ki-67 expression ( $R = +0.9$ ,  $P < .0001$ ), in individual tumors, the number of <sup>ser194</sup>p-FADD-positive cells was typically smaller than the number of Ki-67-positive cells. This observation suggests that <sup>ser194</sup>p-FADD is expressed in a narrower part of the cell cycle. It has been shown that phosphorylation of FADD at serine residue-194 takes place during the G<sub>2</sub>/M phase of cell cycle, and it is hypothesized that this event modulates a restriction point during the G<sub>1</sub>-S or G<sub>2</sub>-M transition of the cycle, or both, depending perhaps on the cell type [5-7]. Our observation that <sup>ser194</sup>p-FADD is expressed in virtually all cells in mitosis further suggests that <sup>ser194</sup>p-FADD expression is related to G<sub>2</sub>/M phase. It is known that Ki-67 stains all phases of proliferating cells including G<sub>1</sub>, S, G<sub>2</sub>, and M phases. Thus, the lower numbers of <sup>ser194</sup>p-FADD-positive cells compared with Ki-67-positive cells is explained. However, the mechanism of action of <sup>ser194</sup>p-FADD on the cell cycle remains obscure [5,6].

We showed that <sup>ser194</sup>p-FADD is localized predominantly in the nuclei of normal and neoplastic B lymphocytes. Our findings are in agreement with the results from other studies on <sup>ser194</sup>p-FADD localization in normal and neoplastic cells [11,13,14]. Notably, the FADD protein contains nuclear localization and export sequences in the DED and, in some recent studies, a large proportion of total FADD protein was found to be localized in the nucleus [11,30,31]. In addition, the nuclear localization of <sup>ser194</sup>p-FADD is compatible with its role in cell cycle progression. However, <sup>ser194</sup>p-FADD has been previously detected in both the nuclei and the cytoplasm or shown to have altered (cytoplasmic) localization in some cancer cells [17].

Because of the dual role of FADD in apoptosis and cell cycle regulation, disentangling the biologic effects of FADD alterations using in vivo or in vitro systems remains difficult[5]. In many studies in which tumor progression has been shown to correlate with diminished expression of FADD or <sup>ser194</sup>p-FADD, the apoptotic role of FADD is stressed, and FADD is regarded as a tumor suppressor gene [32]. It is still unclear whether nuclear <sup>ser194</sup>p-FADD expression can influence the apoptotic pathways [10]. Some studies have shown that disturbance of FADD phosphorylation at serine residue 194 does not effect the extrinsic

apoptotic pathway, whereas other studies support a negative, or, in special circumstances, a positive effect of <sup>ser194</sup>p-FADD on the apoptotic pathway [8,28,33,34]. It remains possible that nuclear <sup>ser194</sup>p-FADD expression may play a role in coupling the cell cycle and apoptotic pathways [5]. However, although most influences of FADD on the cell cycle seem to be dependent on phosphorylation of serine 194, by contrast, most apoptotic activities of FADD seem to be independent of serine 194 phosphorylation [5,13].

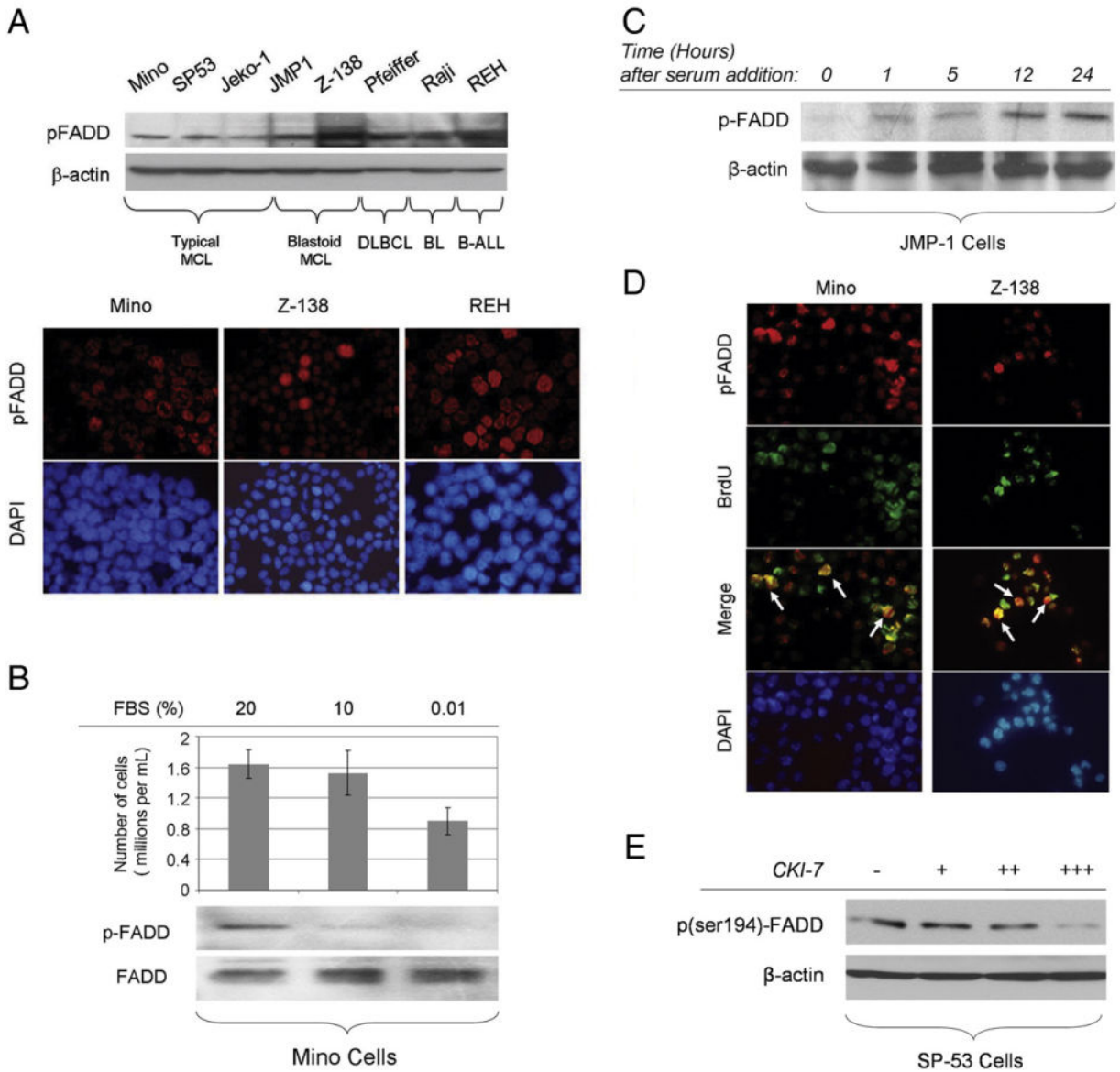
In conclusion, we show that FADD is highly phosphorylated at serine 194 in proliferating reactive and neoplastic B lymphocytes and that <sup>ser194</sup>p-FADD expression may represent a novel marker of cell proliferation in B-NHLs.

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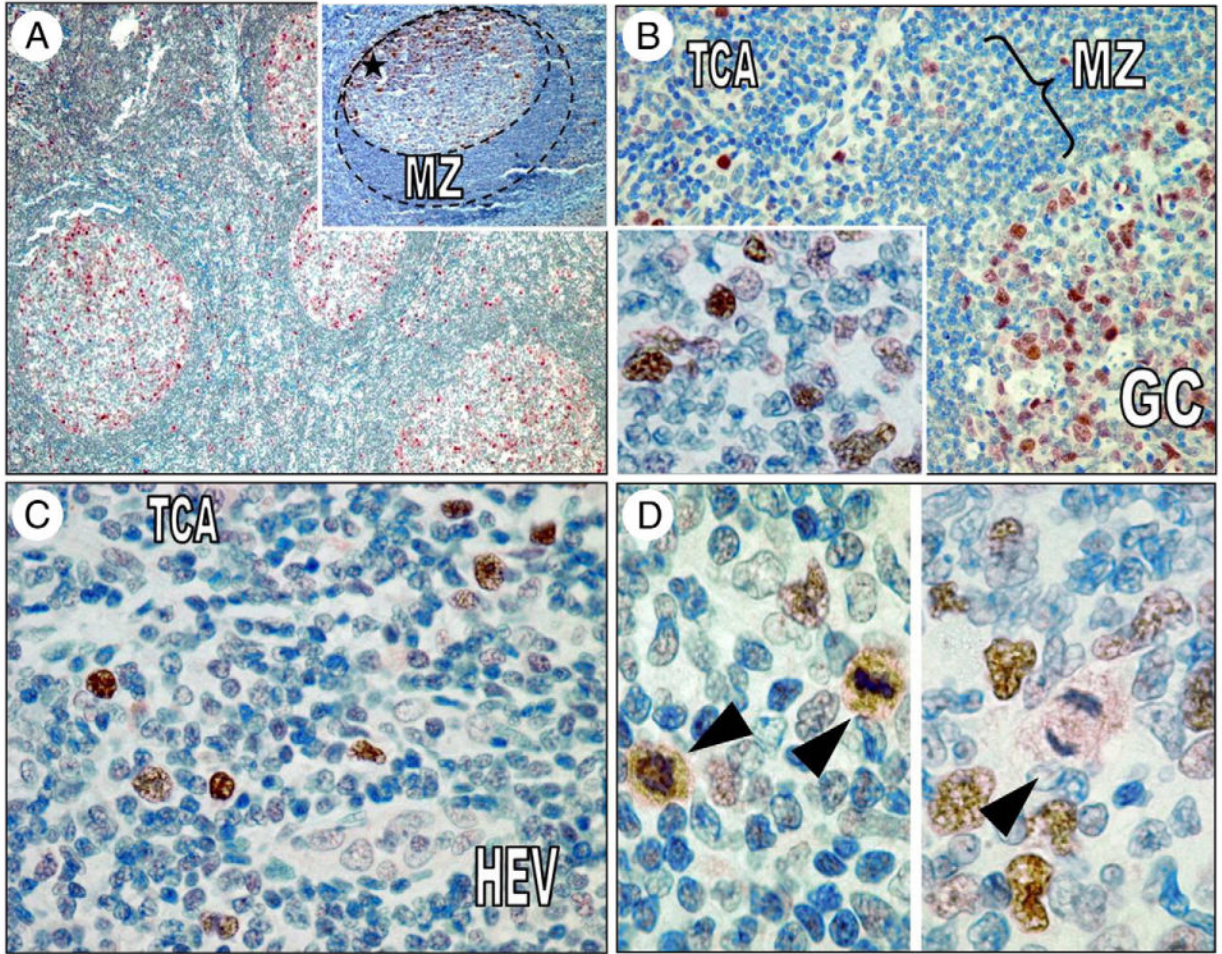
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**Fig. 1.**

A, <sup>ser194</sup>p-FADD is expressed in B-cell NHL cell lines tested at variable levels (upper panel). <sup>ser194</sup>p-FADD protein is detected in the nucleus of Mino, Z-138, and REH cells using immunofluorescence in cytospin preparations (lower panel). B, Mino cells were incubated in 20% (control), 10%, or 0.01% FBS for 24 hours. Western blot analysis shows that the levels of <sup>ser194</sup>p-FADD expression are diminished after 24 hours of serum starvation. The total number of viable cells per volume (in milliliters) is decreased following serum deprivation. C, JMP-1 cells were initially serum starved for cell synchronization, then incubated with FBS, and collected at various time points. The expression levels of <sup>ser194</sup>p-FADD increased gradually over time in these synchronized cells. D, Mino and Z-138 cells were incubated with BrdU for 1 hour at 37°C, and double immunofluorescence was performed using cytospin preparations, antibodies specific for <sup>ser194</sup>p-FADD and BrdU, and DAPI as counterstain. <sup>ser194</sup>p-FADD and BrdU were detected using different secondary

antibodies visible as red and green, respectively. A subset of cells was positive for both <sup>ser194</sup>p-FADD and BrdU as shown with yellow color in merged images, indicating that FADD phosphorylation at serine 194 is more prominent in the BrdU incorporating (proliferating) cell fraction (white arrows). E, Incubation of proliferating SP-53 cells, growing in 15% FBS, with increasing concentrations of the CKI $\alpha$ -specific inhibitor, CKI-7, up to 250  $\mu$ mol/L for 8 hours, resulted in gradual decrease of <sup>ser194</sup>p-FADD levels, as shown by Western blot analysis.

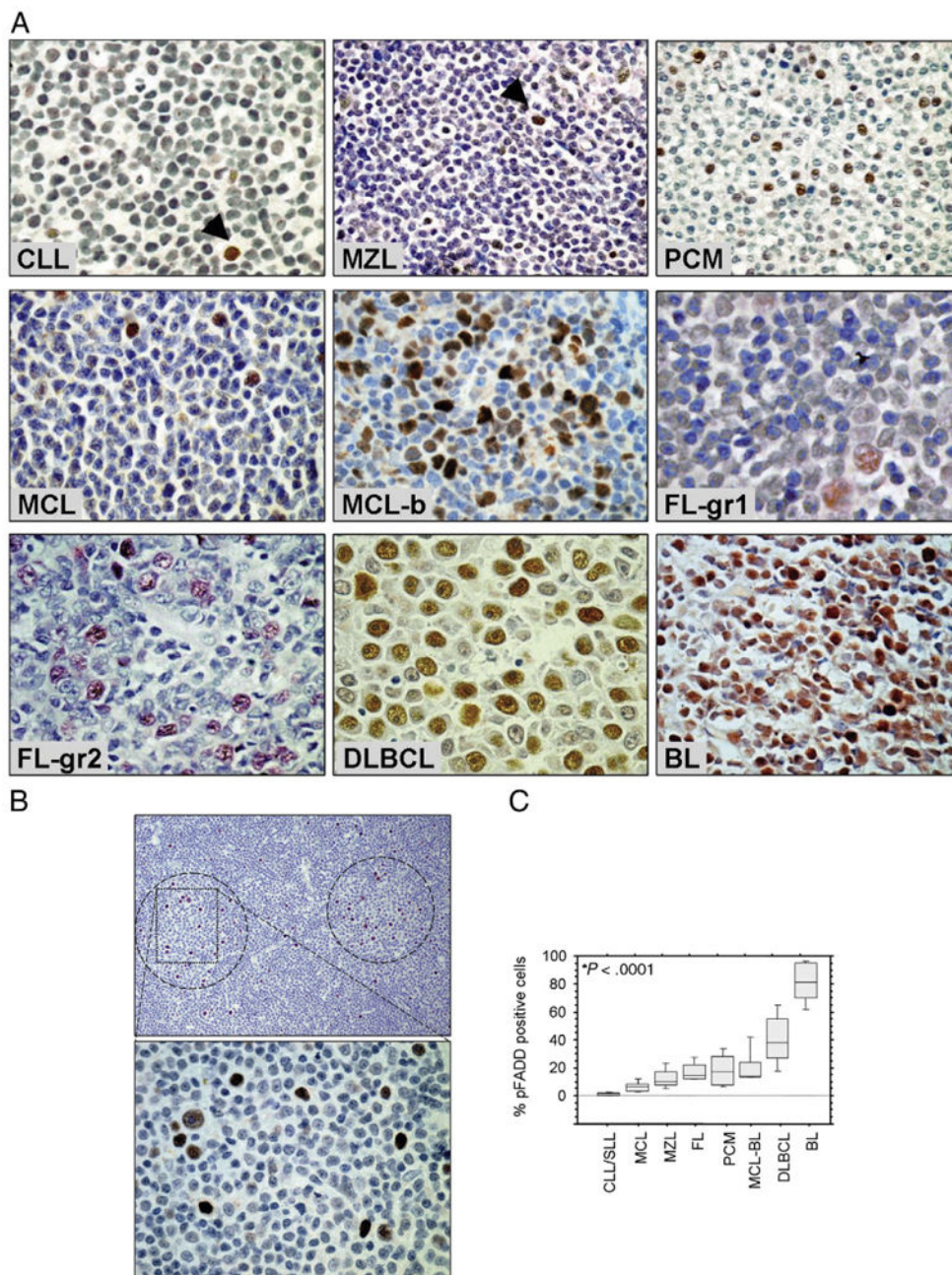




**Fig. 2.**

A,  $\text{ser}^{194}\text{p-FADD}$  is detected predominantly in germinal center B cells in reactive lymph nodes and less frequently in the interfollicular areas. Note the accentuation of expression in the outer limits of germinal centers and the polarization toward the dark zone (inset, asterisk). B,  $\text{ser}^{194}\text{p-FADD}$  protein is expressed in the nuclei of many centroblasts and some centrocytes (inset) in reactive germinal centers at variable intensity. C, In the interfollicular T-areas,  $\text{ser}^{194}\text{p-FADD}$  expression highlights larger lymphocytes, a pattern reminiscent of Ki-67 immunostaining. D, All cells showing cytoplasmic  $\text{ser}^{194}\text{p-FADD}$  expression were in various stages of mitosis (left and right, arrowheads). GC indicates germinal center; MZ, mantle zone; TCA, interfollicular T-cell area; HEV, high endothelial venule.

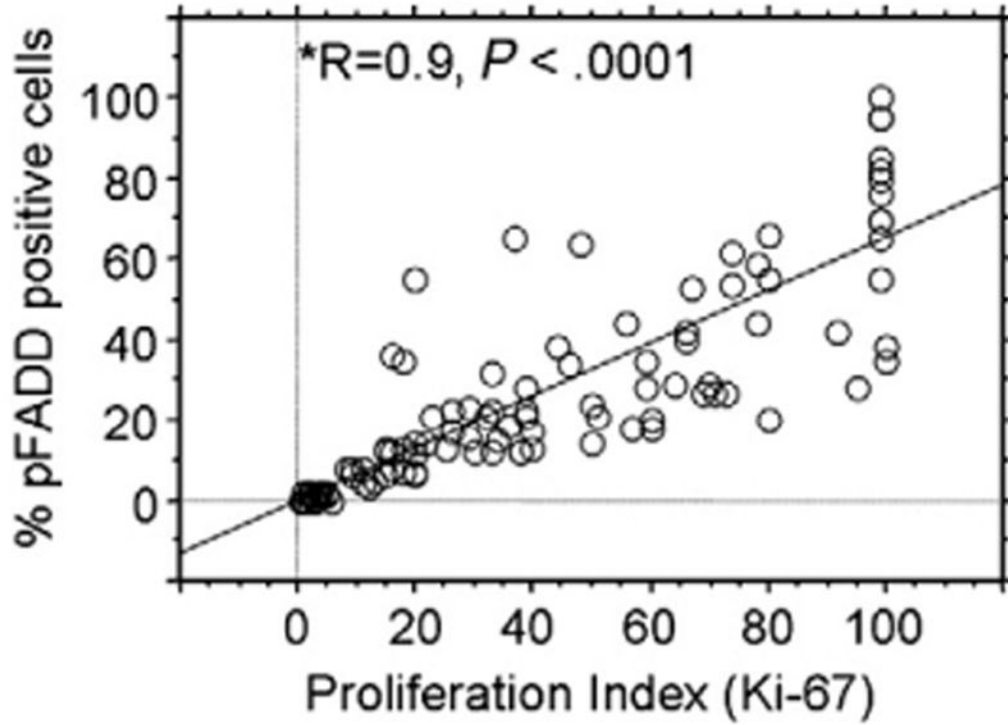




**Fig. 3.**  
 A, Immunohistochemistry showing  $\text{ser}^{194}\text{p-FADD}$  protein expression in tumor cell nuclei from different types of B-cell NHL. The percentage of  $\text{ser}^{194}\text{p-FADD}$ -positive tumor cells is high in highly proliferating lymphoma types such as blastoid/pleomorphic MCL (MCL-b), DLBCL, and BL. Arrowheads indicate single positive cells. B,  $\text{ser}^{194}\text{p-FADD}$  immunostaining identifies proliferation centers (circles) in CLL/SLL (low and high magnifications at the upper and lower panels, respectively). C, Box and whisker plots showing the levels of  $\text{ser}^{194}\text{p-FADD}$  expression (percentage of  $\text{ser}^{194}\text{p-FADD}$ -positive tumor cells) in different types of B-cell NHL (original magnification  $\times 400$ , hematoxylin as

counterstain;  $*P < .0001$ , Kruskal-Wallis test). MZL indicates marginal zone B-cell lymphoma.





**Fig. 4.** In B-cell NHLs, the levels of <sup>ser194</sup>p-FADD expression assessed as the percentage of positive neoplastic nuclei (continuous variable) are significantly associated with tumor proliferation index (Ki-67; \*Spearman  $R = +0.9$ ,  $P < .0001$ ). The bivariate regression plot for the percentage of <sup>ser194</sup>p-FADD-positive and Ki-67-positive tumor cells as continuous variables is shown.