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Research Article

Analysis of NF-κB Pathway Proteins in Pediatric Hodgkin Lymphoma: Correlations with EBV Status and Clinical Outcome—A Children's Oncology Group Study

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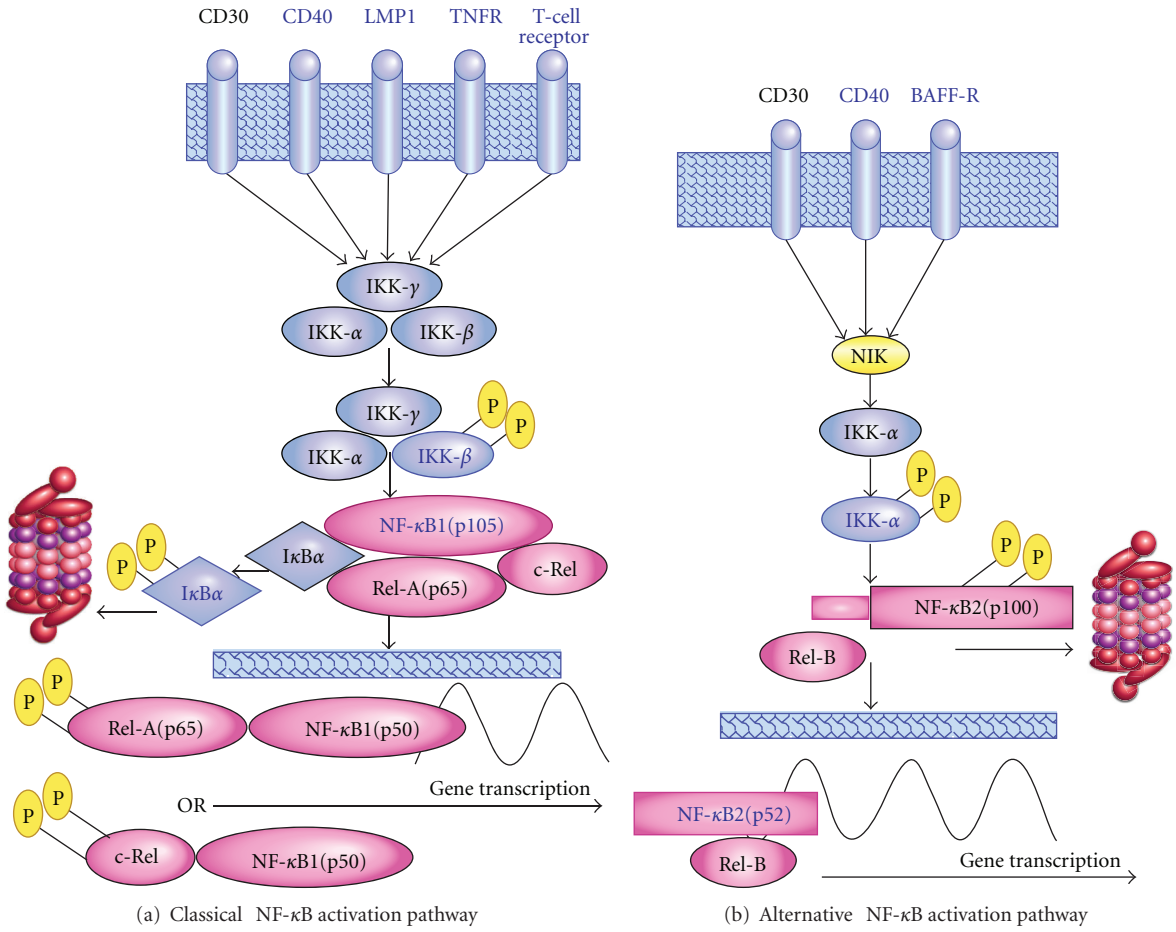
Constitutively active nuclear factor-κB (NF-κB) is integral to the survival of Hodgkin/Reed-Sternberg cells (H/RS) in Hodgkin lymphoma (HL). To investigate NF-κB pathway proteins in pediatric HL, we utilized a tissue microarray compiled from 102 children enrolled in the Children's Oncology Group intermediate-risk clinical trial AHOD0031 (56 male, 78 Caucasian, median age 15 years (range 1–20 years), 85 nodular sclerosing subtype, 23 Epstein-Barr virus (EBV) positive, and 24 refractory/relapsed disease). We examined the intensity, localization, and pathway correlations of NF-κB pathway proteins (Rel-A/p65, Rel-B, c-Rel, NF-κB1, NF-κB2, IκB-α, IKK-α, IKK-β, IKK-γ/NEMO, NIK, and A20), as well as their associations with EBV status and clinical outcome. NF-κB pathway proteins were overexpressed in pediatric HL patients compared to controls. Patients with EBV− tumors, or with rapid early therapy response, had tightly coordinated regulation of NF-κB pathway proteins, whereas patients with EBV+ tumors, or slow early therapy response, had little coordinated NF-κB pathway regulation. High NIK expression was associated with a slow response to therapy and decreased EFS. Elevated Rel-B, NIK, and the NF-κB inhibitor A20 were associated with decreased EFS in multivariate analysis. These studies suggest a pivotal role for the NF-κB pathway in therapy response and patient survival in Hodgkin lymphoma.

1. Introduction

Constitutive activation of NF-κB is one of the hallmarks of H/RS cells in HL [1]. NF-κB is a transcription factor family composed of five members: Rel-A/p65, Rel-B, c-Rel, NK-κB1 (p50/p105), and NF-κB2 (p52/p100) [2]. NF-κB subunits Rel-A and c-rel are sequestered in the cytoplasm by IκB. In the classical pathway (Figure 1(a)), an IκB-kinase (IKK) complex regulates IκB and the release of Rel-A and c-rel into the nucleus. In the alternative pathway, NF-κB is activated by proteasomal cleavage of NF-κB2 (p100) to p52, an NF-κB subunit that binds and activates Rel-B (Figure 1(b)) [3]. Once activated, NF-κB subunits dimerize and translocate into the nucleus, where they activate transcription of NF-κB target genes [4–6]. Both the classical and alternative NF-κB pathways are tightly regulated. In the classical pathway,



(a) Classical NF-κB activation pathway

(b) Alternative NF-κB activation pathway

Figure 1: Classical and alternative NF-κB pathway activation. (a) Activation of the classical NF-κB pathway. Several receptors, including CD30, CD40, and LMP1, induce the classical NF-κB pathway by activating the IKK complex. Phosphorylated IKK-β phosphorylates IκB-α, inducing IκB polyubiquitination and degradation by the 26S proteasome. Following IκB degradation, NF-κB dimerizes with NF-κB1/p50 and translocates into the nucleus where gene transcription is activated. (b) Activation of the alternative NF-κB pathway. The alternative NF-κB pathway is activated by a restricted set of cell-surface receptors, including CD30, CD40, and the BAFF receptor. Alternative NF-κB pathway activation increases the stability of NIK, which phosphorylates and activates IKK-α. IKK-α directly phosphorylates NF-κB2/p100, inducing partial proteolysis of p100 to p52 by the 26S proteasome. The p52 NF-κB subunit dimerizes with Rel-B and translocates into the nucleus, activating gene transcription. Proteins outlined in black were examined in the TMA. Cylindrical structure represents the 26S proteasome.

degradation of IκB-α and IκB-ε is triggered by IKK-β activation. In the alternative pathway, IKK-α is tightly regulated by NF-κB inducing kinase (NIK). IKKs are negatively regulated by A20, an E3 ligase/deubiquitinase, and putative tumor suppressor in HL.

Tissue microarrays (TMA) can be used to uniformly analyze multiple proteins from a large series of biopsy specimens. TMA are particularly informative in the study of HL, in which malignant H/RS cells are surrounded by an extensive inflammatory cell infiltrate. TMA can be used to uniformly assess cellular protein localization and relative protein expression intensity in both H/RS cells and the surrounding microenvironment.

In this study we have evaluated NF-κB pathway protein expression, localization, and coordinate regulation of 102 biopsy specimens from pediatric patients treated with 4 cycles of ABVE-PC (doxorubicin (Adriamycin), bleomycin, vincristine, etoposide, prednisone, and cyclophosphamide), with an interim response assessment following 2 cycles of therapy. We assessed correlations between NF-κB activation and EBV status, early response to therapy, and EFS.

2. Methods

2.1. Treatment Protocol. Diagnostic tissue biopsy samples from accessible lymph nodes were obtained prior to treatment from children and young adults (ages 1–20 years) with HL stages IA or IIA with bulk disease (>6 cm), IB, IIB, IIIA, and IVA, enrolled on the COG intermediate-risk protocol AHOD0031 (clinicaltrials.gov, NCT00025259). Information on stage, bulk disease, and B symptoms (unexplained recurrent fever >38°C, unexplained >10% weight loss, and drenching night sweats) was collected for all patients. A total

Table 1: Demographics of 102 patients with intermediate risk Hodgkin lymphoma treated on the COG-AHOD0031 clinical trial included in TMA analysis.

Demographic	Group	Number
Sex	Male	56
	Female	44
	Unknown	2
Race	Caucasian (including Hispanic)	78
	African-American	10
	Asian	3
	Other	6
	Unknown	5
HL subtype	Nodular sclerosing	85
	Mixed cellularity	8
	Lymphocyte predominant	7
	NOS	2
Response to therapy	Rapid early response (RER)	79
	Slow early response (SER)	20
	Unknown/inevaluable	3
EBV status by LMP1 or EBER	Positive	26
	Negative	76

of 1700 patients were enrolled on this study according to policies of local IRBs. Response assessment was performed by CT after the first 2 cycles of therapy. Patients with ≥60% shrinkage in tumor volume after 2 cycles of therapy were considered to have a rapid early response to therapy (RER) and received additional 2 cycles of ABVE-PC, then randomized to radiation versus no radiation if in complete response (CR) after a total of 4 therapy cycles. Patients with <60% shrinkage in tumor volume after 2 therapy cycles were considered to have a slow early response (SER) and were treated with 2 cycles of DECA (dexamethasone, etoposide, cisplatin, and cytarabine), 2 additional cycles of ABVE-PC, and involved-field radiotherapy. Since FDG-PET was not uniformly available at the time of initiation of the clinical trial, response evaluations were performed based solely on anatomical response by CT.

2.2. Patient Samples for TMA. HL biopsy samples were collected at local institutions using IRB-approved protocols and according to local institutional policies regarding protection of human subjects. Of the 1700 patients enrolled, 336 had paraffin blocks available for analysis. Of those with available material, 105 patients were chosen for the inclusion on the TMA from the first three years of the clinic trial (2003–2006) to allow for the longest clinical outcome followup. Patients chosen for the TMA were representative of those enrolled in the clinical trial with respect to age, sex, race, stage, histology, EBV status, therapy response, and clinical outcome (Table 1, Supplemental Figure 1; see Supplementary Material available online at doi: 10.1155/2012/341629).

Pretreatment biopsy samples were centrally reviewed and subtyped per WHO criteria. Cases were examined by pathologists at the Biopathology Center (BPC, Columbus, OH), and appropriate regions were selected for inclusion in the TMA. The TMA included H/RS-rich regions from 105 patients; 3 patients were later excluded from analysis following quality control assessment (see below).

2.3. Tissue Microarray Construction. A tissue microarrayer device (Beecher Instruments, Silver Spring, MD) was used to construct the TMA. In each case, 0.6 mm diameter cylinders from 4 dense H/RS cell areas were included in the array. Each TMA block initially consisted of 322 spots including 26 control spots from 10 control tissues. Ten control tissues included 3 EBV-infected tonsils, 3 normal tonsils, 3 normal thymuses and 1 normal lymph node. H/RS cell staining intensity was compared to germinal center B cells or immunoblasts when present. For thymus controls, staining intensity was compared to the average intensity across the section.

2.4. Immunohistochemistry (IHC). Tissue microarray slides were deparaffinized with xylene, rehydrated with decreasing concentrations of alcohol, and underwent antigen retrieval using a steam-heat epitope retrieval procedure, for 25 min in 10 mM citrate buffer. Endogenous peroxidase was blocked using 3% H_2O_2/methanol, and slides were incubated using primary antibodies at the corresponding dilutions (Supplemental Table 1), followed by anti-mouse or anti-rabbit secondary horseradish peroxidase conjugate (Biogenex, San Ramon, CA) and liquid diaminobenzidine detection.

2.5. In Situ Hybridization. TMA slides were tested for the presence of EBER transcripts by *in situ* hybridization, and using 4 EBER digoxigenin-labeled probes containing the following sequences 1–4 (BioSource, Invitrogen, Carlsbad, CA) Probe 1: 5′-AGACACCGTCCTCACCACCCGGG-ACTTGTA; Probe 2: 5′-CCCTAGCAAAA-CCTCTAGGG; Probe 3: 5′-GACAACCACAGAC-ACCGTC; Probe 4: 5′AAAACATGCGGACCACCAG.

2.6. Quality Control. TMA slides were immunostained with CD30 antibody and scored for H/RS cell number and CD30 staining intensity. Samples were excluded from analysis if there were <10 H/RS cells/spot, or if a sample spot was missing from >10% of its expected locations on the array across all antibodies. Sample spots were also removed from analysis if H/RS cells had no discernible CD30 staining. Of the total of 322 spots on the array, 14 HL spots were excluded from further analysis. This excluded three HL patients from further analysis, leaving 102 evaluable HL patients. The resulting analysis included 308 spots, representing 102 HL patients and 10 controls.

2.7. IHC Scoring. Protein expression levels were determined by the intensity of IHC staining and were scored as either absent (0), weak (1), or strong (2) as done previously [7]. LMP1 and EBER were scored as either present or absent; to account for differences in processing and fixation among COG institutions and referral sites, both LMP1 and

EBER were scored. NF-κB antibody staining intensity and localization (nucleus and/or cytoplasm) were independently scored by three pathologists (AMS, DLT, and RH/SN). Scores from each pathologist were averaged for each triplicate spot and the three averaged intensity scores for each pathologist were combined to create a final average intensity score (AIS). Cytoplasmic and nuclear protein intensity was scored separately, and samples with discrepancies between pathologists were rescored by consensus review. For bar graphs representing NF-κB subunit intensity in EBV+ versus EBV− tumors, each AIS was placed into one of four intensity bins: 0–0.5 (weak intensity), 0.5–1, 1–1.5, and 1.5–2 (strong intensity).

2.8. Statistical Analysis. NF-κB subunit intensities for HL patients and controls were summarized by box plots and analyzed by the nonparametric Wilcoxon test. Differences between EBV+ and EBV− HL patients, as well as differences between patients with a rapid early response to treatment (RER) and slow early responders (SER), were compared using the nonparametric Wilcoxon test. Among HL patients, correlations between NF-κB subunits were analyzed by the nonparametric Spearman method, with subgroup analyses based on EBV status or early response to therapy (RER versus SER). To control for the overall type I error at 0.05 level when testing the associations using Spearman's method, multiple comparison adjustments were made using the Holm's method [8]. To analyze the association between NF-κB subunits and clinical outcomes, logistic regression models were applied to examine the association between NF-κB subunits and treatment response, and multivariate Cox regression models were carried out to evaluate the associations between NF-κB subunits and EFS, controlling for EBV status, stage, histology, B symptoms, and bulk disease. As these analyses were exploratory in nature, no multiple comparison adjustments were made. The association between EBV status and early response (RER/SER) among patients without A20 expression was tested using the Fishers exact test. The rate of events (progressive disease or relapse) in the TMA cohort was compared to the rate of event in the cohort of AHOD0031 trial using the Chi-squared test.

3. Results

3.1. TMA Sample Demographics and HL Sample Quality Control. The demographics of pediatric HL patients included on the array were similar to those observed in the AHOD0031 clinical trial with respect to age, sex, race, histology, EBV status, and response to therapy (Table 1). A predominance of patients (85%) had nodular sclerosing HL, and most patients were adolescents with a median age of 15 years (range 1–20 years) (Supplemental Figure 1). Lymph node (LN) biopsies were obtained prior to treatment and underwent quality control analysis prior to examination of NF-κB pathway proteins. Two hundred eighty-four of the 296 HL TMA spots (96%) had identifiable H/RS cells. Twenty-six patients (25%) were EBV+, as determined by either LMP1 IHC or EBER *in situ* hybridization. There was 93% agreement in EBV status as determined by marker concordance between LMP-1 IHC and EBER *in situ* hybridization. Using the antibodies chosen (Supplemental Table 1), NF-κB1 had no discernible cytoplasmic staining and NF-κB2 had no discernible nuclear staining.

3.2. NF-κB Pathway Protein Expression in H/RS Cells. Similar to IHC studies performed in adults [9–12], increased nuclear and cytoplasmic expression of three of the five NF-κB subunits (Rel-A/p65, Rel-B, and c-Rel), as well as increased cytoplasmic NF-κB2, was found in H/RS cells when compared to nonneoplastic controls (Figure 2(a)). Increased expression of NF-κB subunits, however, was not universal. The relative protein expression of nuclear NF-κB1, for example, was similar between HL samples and controls (Figure 2(a), right panel).

Pediatric patients also had increased expression of most, but not all, regulatory proteins in the classical and alternative NF-κB pathways (Figure 2(b)). H/RS cells demonstrated increased expression of nuclear phospho-Rel-A, IκB-α (classical pathway), IKK-α and NIK (alternative pathway), cytoplasmic IκB-α, IKK-α, and NIK. However, there was no statistically significant difference in nuclear IKK-β protein expression between HL and nonneoplastic tissues (data not shown). This indicates that, similar to adult HL, both the classical and alternative NF-κB pathways are activated in pediatric HL.

3.3. NF-κB Pathway Protein Regulation in EBV+ versus EBV− Tumors. EBV latent infections are associated with 30–40% of pediatric HL [2]. In this TMA, 26 of the 102 samples were EBV+. Previous work has shown that LMP1, a latent membrane protein expressed in EBV+ H/RS cells, can activate NF-κB subunits and that this activation could be independent of either the classical or alternative NF-κB pathways [13–15].Therefore, it was of interest to determine if NF-κB pathway proteins would be regulated differently in EBV+ HL versus EBV− HL.

In our pediatric TMA, we observed no significant difference in NF-κB subunit intensity or localization between EBV+ and EBV− tumors (Figure 2(c)). Both EBV+ and EBV− HL tumors showed increased expression of most NF-κB subunits. However, as shown in the IHC of two patient samples (Figure 3), there appeared to be very little coordinated activation of NF-κB pathway proteins in EBV+ patients. For example, in an EBV− tumor (Figure 3 (top row)), there were increases in expression of nuclear phospho-Rel-A (patient AIS 1.4), cytoplasmic Rel-B (AIS 1.7), cytoplasmic NIK (AIS 1.7), nuclear c-Rel (AIS 1.7, data not shown), cytoplasmic IκB-α (AIS 1.5, data not shown), and cytoplasmic IKK-β (AIS 1.7) (data not shown). In contrast, EBV+ tumors (Figure 3 (bottom row)) frequently had moderate to strong expression of one protein within the NF-κB pathway, in this case nuclear phospho-Rel-A (patient AIS-1.0), but weak expression of other NF-κB subunits, including nuclear Rel-B (AIS-0.5), cytoplasmic NIK (AIS-0.3), nuclear IκB-α (AIS 0.6, data not shown), and cytoplasmic NF-κB2 (AIS-0.5, data not shown). These

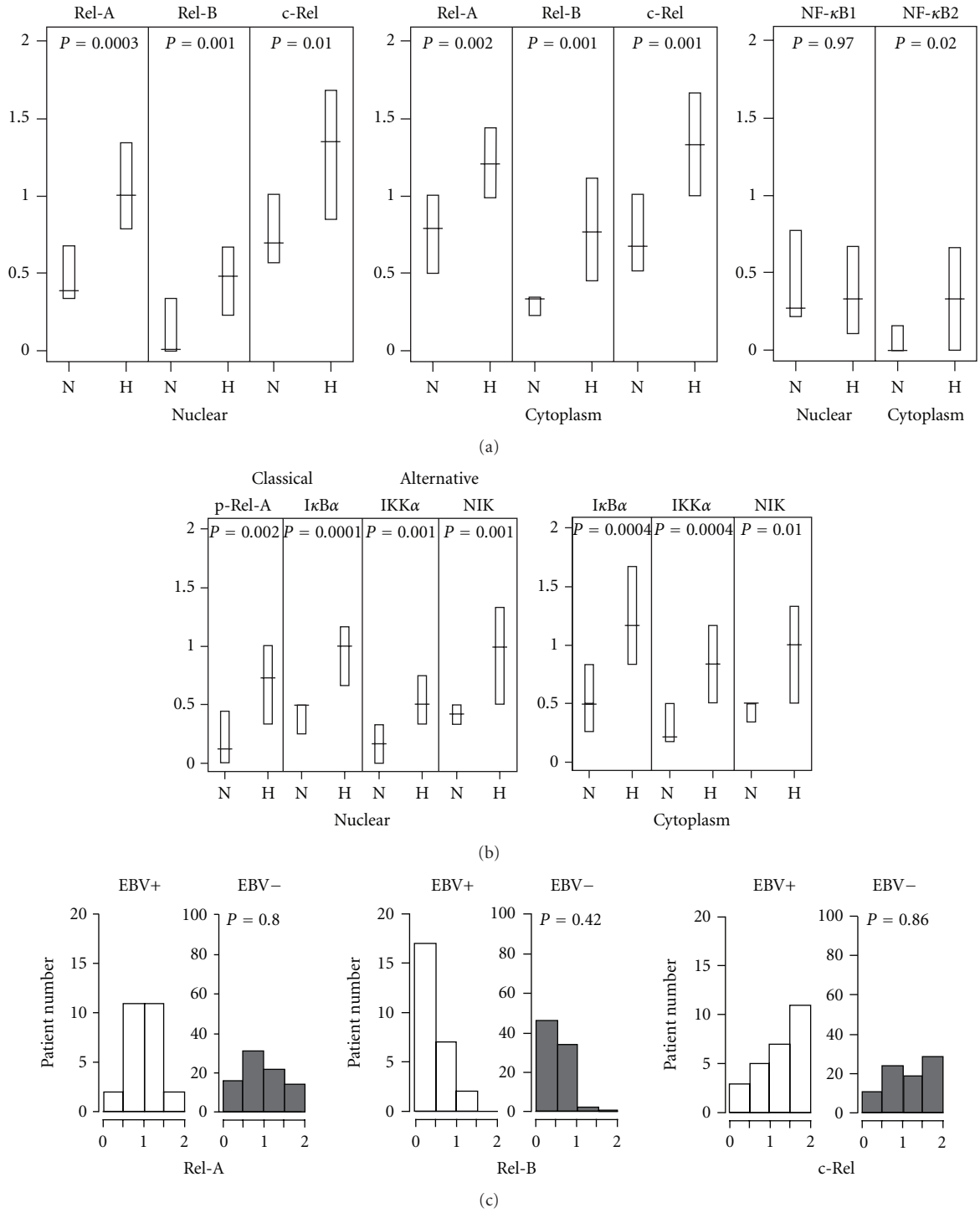


Figure 2: Comparison of NF-κB pathway protein intensities in HL patients with nonneoplastic controls. (a) Nuclear and cytoplasmic NF-κB subunits from 102 HL samples (H) were compared to 10 controls (N). Significance was determined using the Wilcoxon rank-sum test. (b) Nuclear and cytoplasmic NF-κB pathway subunits phospho-Rel-A (p-Rel-A), IκB-α, IKKα, and NIK in HL samples compared to controls. (c) Intensity of nuclear NF-κB subunit expression in EBV+ tumors (white bars) and EBV− tumors (grey bars) for Rel-A, Rel-B, and c-Rel. Patient average intensity scores (AIS) were placed in one of four protein intensity bins: 0–0.5 (very weak intensity), 0.5–1 (weak intensity), 1–1.5 (strong intensity), and 1.5–2 (very strong intensity).

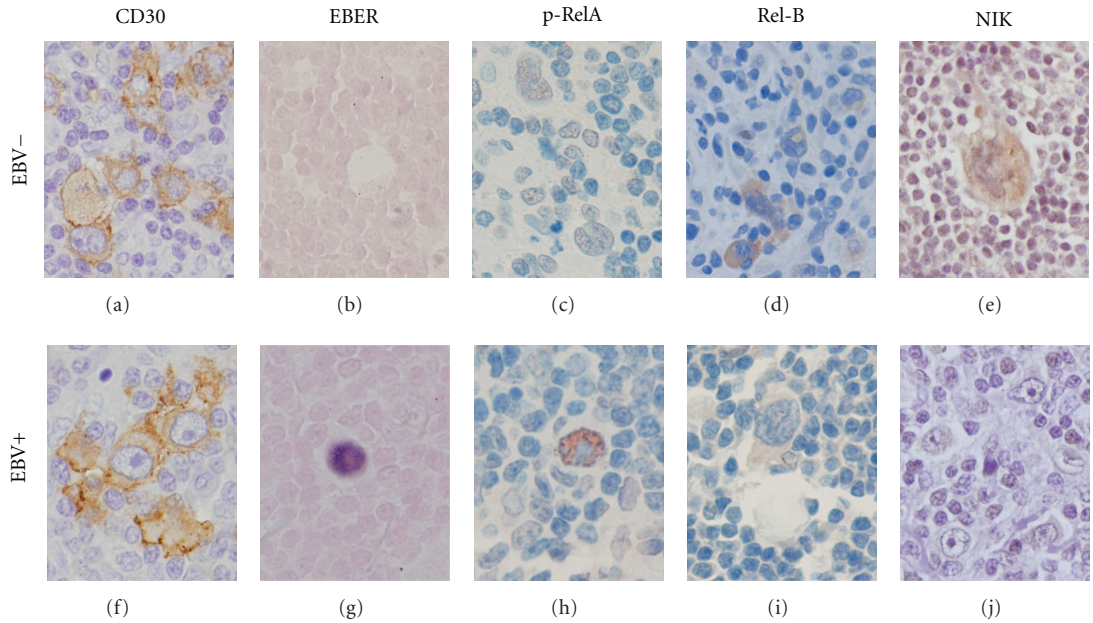


Figure 3: Immunohistochemistry (IHC) and *in situ* hybridization in EBV+ versus EBV− tumors. CD30 (far left panel), EBER *in situ* hybridization, phospho-(P)-Rel-A (center), Rel-B, and NIK (far right panel) in an EBV− patient (top row) and an EBV+ patient (bottom row). Pictures were taken at 400× magnification using an Olympus BX41 microscope. Whole images were corrected for contrast, brightness, and color balance. Details of staining are provided in Supplemental Table 1.

differences in protein intensity expression suggested that there could be differences in NF-κB pathway regulation between the EBV− and EBV+ HL tumors.

To determine the cohesiveness of NF-κB pathway protein regulation between EBV− versus EBV+ HL tumors, we compared NF-κB subunit expression in each HL sample, assessing the degree of coordinate regulation in NF-κB pathway proteins in either the nucleus or the cytoplasm of H/RS cells using Spearman correlation coefficients (Figure 4(A) and Supplemental Figure 3). Proteins were considered to be coordinately regulated if the correlation coefficient between proteins was positive and significant at $P < 0.05$ after correction for multiple comparisons. EBV-negative H/RS cells (left panels) showed many corresponding increases in NF-κB pathway proteins (significant correlation coefficients in white, nonsignificant in grey). Correlation coefficients are detailed in Supplemental Figure 3.

We also examined A20, a negative regulator of both the classical and alternative NF-κB pathways [16] in our pediatric HL population. Of the 102 patients included in the TMA, 11 appeared to express little or no A20 protein (cytoplasmic intensity 0–0.17), including seven of 76 EBV− patients (9%) and four of 26 EBV+ patients (15%). In EBV− HL tumors, we observed tightly coordinated regulation between NF-κB pathway proteins and A20 (Figure 4(A), left panel). For example, cytoplasmic A20 significantly correlated with all cytoplasmic NF-κB pathway proteins except phospho-Rel-A and c-Rel. These data suggest that the NF-κB pathway is tightly regulated in EBV− H/RS cells.

In contrast, EBV+ HL samples demonstrated very little coordinate expression of nuclear or cytoplasmic NF-κB subunits (Figure 4(A), left panel, and Supplemental Figure 4). In total, there were only 3 nuclear and 2 cytoplasmic correlations. Nuclear IKK-γ was inversely correlated with c-Rel ($r = -0.65$, $P = 0.0003$), NIK correlated with IKK-α ($r = 0.63$, $P = 0.0006$), and IKK-β correlated with IKK-γ ($r = 0.63$, $P = 0.0005$). Importantly, there was no evidence that A20 coordinately regulated any NF-κB subunits in EBV+ tumors (Figure 4(A), right panel). These data suggest that EBV+ H/RS tumors have constitutively active NF-κB independent of regulation by the classical NF-κB pathway, the alternative NF-κB pathway, or A20.

3.4. Coordination of NF-κB Pathway Proteins and Treatment Response. Ninety-nine of the 102 patients included in this TMA had a confirmed response assessment after 2 cycles of chemotherapy. NF-κB pathway protein localization and intensity were compared between patients with a rapid early response to therapy (RER, $n = 80$) versus a slow early response to therapy (SER, $n = 19$). For most NF-κB pathway proteins, subcellular localization and intensity of protein expression were similar between RER and SER patients (Supplementary Figure 2). However, there was increased expression of cytoplasmic NIK in SER patients when compared to RER patients ($P = 0.005$, Wilcoxon rank-sum test) (Figure 5(a), Supplemental Figure 2), with 17/19 SER patients having cytoplasmic NIK expression above the mean (i.e., ≥1; range 0–2).

Since NIK was overexpressed in patients with slow early therapy response, we further examined alternative NF-κB pathway regulation in patients with SER. As expected,

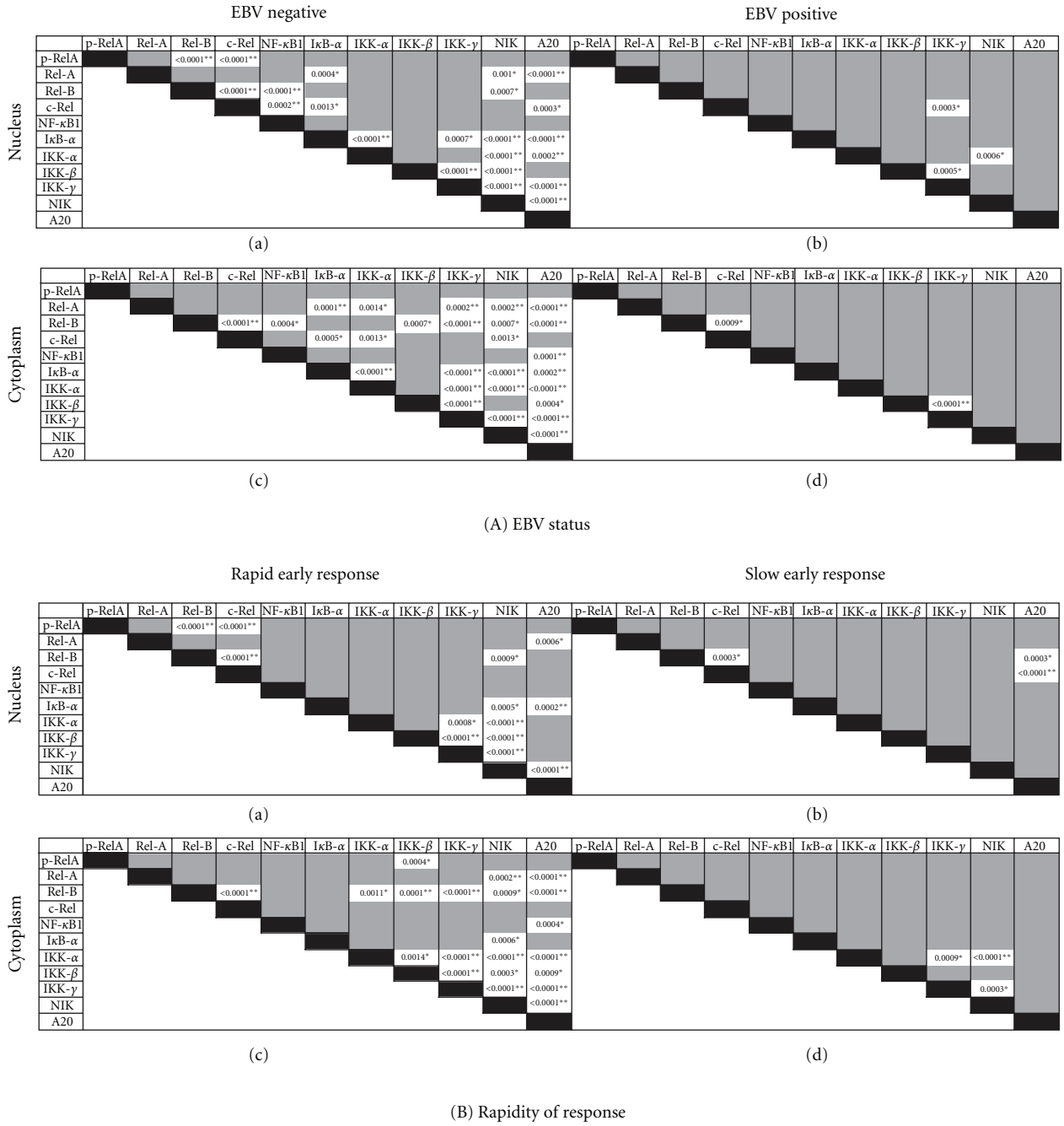


Figure 4: Spearman correlation coefficients between NF-κB pathway proteins stratified by EBV status (A) and rapidity of response to therapy (B) Nuclear (a), (b) and cytoplasmic (c), (d) NF-κB pathway protein correlations were examined in EBV- patients (left panels) and EBV+ patients (right panels). Significant results are shown in white with raw P values; P values after correction for multiple comparisons are noted with asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$). Full data set with Spearman correlation coefficients are shown in Supplemental Figure 3. (B) Nuclear (a), (b) and cytoplasmic (c), (d) NF-κB pathway protein correlations were examined in patients with rapid early response (RER) to ABVE-PC (left panels) versus those with a slow early response (SER) to the same therapy (right panels). Full datasets with Spearman correlation coefficients are shown in Supplemental Figure 4.

increased NIK expression correlated with increased cytoplasmic IKK-α ($r = 0.87$, $P < 0.0001$) (Figure 5(b)). However, there was no significant correlation between NIK and NF-κB2 ($r = 0.12$, $P = 1.0$) or Rel-B ($r = 0.08$ and 0.14, $P = 1.0$). This suggested a potential interruption of alternative NF-κB pathway signaling between IKK-α and NF-κB2 in patients with an SER to therapy (Figures 5(b) and 5(c)). Interestingly, SER patients with increased NIK demonstrated increased expression of nuclear Rel-A (mean expression ≥ 1 in 14/19 SER patients) and c-Rel (mean expression ≥ 1 in 11/19 SER

Table 2: (a) Univariate correlation of nuclear NF-κB pathway proteins and event free survival (EFS). (b) Correlation of NF-κB pathway proteins with EFS (multivariate analysis).

(a)

Protein	Hazard ratio	95% Confidence intervals		Significance
		LL	UL	P value[#]
Phospho-Rel-A	**3.3**	**1.2**	**9.0**	**0.023**
Rel-A	1.3	0.5	3.3	0.57
Rel-B	**3.8**	**1.2**	**11.6**	**0.02**
c-rel	1.3	0.6	2.8	0.57
NF-κB1	1.1	0.5	2.9	0.79
IκB-α	1.1	0.4	3.0	0.83
IKK-α	0.9	0.3	2.9	0.88
IKK-β	1.1	0.4	3.4	0.84
IKK-γ	0.7	0.1	3.4	0.65
NIK	**2.4**	**1.1**	**5.3**	**0.03**
A20	**2.6**	**1.1**	**6.4**	**0.03**

[#]Univariate Cox regression analysis. LL: lower limit; UL: upper limit.

(b)

Protein	Hazard ratio*	LL	UL	P[$]
Nuclear NF-κB pathway proteins				
P-Rel-A	2.9	1.0	8.5	0.05
Rel-B	**5.4**	**1.5**	**19.1**	**0.009**
NIK	**3.1**	**1.2**	**7.9**	**0.015**
A20	**2.8**	**1.1**	**7.2**	**0.03**
Cytoplasmic NF-κB pathway proteins				
Rel-A	**3.9**	**1.1**	**14.0**	**0.038**
Rel-B	2.5	1.0	6.2	0.05
IKK-β	**5.7**	**2.0**	**16.1**	**0.001**

*Controlling for EBV status, histology, stage, bulk disease (>6 m mass), and B symptoms.
[$]Cox regression. 95% CI—LL: lower limit; UL: upper limit.

patients). In contrast, only one SER patient had a nuclear Rel-B intensity ≥ 1. This suggests potential crosstalk between the classical and alternative NF-κB pathways and a lack of tight control of NF-κB pathway proteins in patients with SER.

To further examine NF-κB pathway coordination in RER and SER HL, we examined correlations between NF-κB pathway proteins in individual samples for both the RER versus SER HL subgroups. As expected, we saw several significant associations of NF-κB protein pathway expression in the patients with RER to therapy (Figure 4(b) left panels and Supplemental Figure 4). As expected, the negative regulator A20 significantly correlated with most cytoplasmic NF-κB pathway proteins.

HL patients with SER to therapy, however, demonstrated much less coordinate regulation of NF-κB pathway proteins (Figure 4(b), right panels). A20 showed significant correlations with only two nuclear NF-κB pathway proteins (Rel-B and c-Rel). Cytoplasmic NIK expression, which was significantly increased in SER patients (Figure 5(a)), correlated with increased cytoplasmic expression of IKK-α ($r = 0.86$, $P < 0.0001$) and IKK-γ ($r = 0.73$, $P = 0.019$) (Figure 4(b) and S4), but not with other alternative NF-κB pathway proteins. This suggested that the NF-κB pathway is not as tightly regulated in SER patients as in RER patients. Examining each of the NF-κB pathway proteins and their influence on rapidity of response to therapy (RER/SER) by univariate logistic regression analysis, we noted that increased cytoplasmic NIK reduced the chance of having a RER to therapy (OR = 0.20, 95% CI: 0.07–0.62, $P = 0.005$ (data not shown)), which remained to be statistically significant after controlling for EBV status (data not shown).

3.5. Association of NF-κB Subunit Expression and Event-Free Survival. In this 102 patient cohort, 24 patients had an event defined as either progressive disease or relapse. We determined whether increases in specific NF-κB subunits correlated with clinical outcome, as measured by 3-year EFS, in a univariate analysis (Table 2(a)). Increased nuclear phospho-Rel-A (HR = 3.23, 95% CI: 1.2–9.0, $P = 0.023$), Rel-B (HR = 3.81, 95% CI: 1.21–11.6, $P = 0.021$), NIK (HR 2.4 95% CI: 1.1–5.3, $P = 0.028$), and A20 (HR = 2.6, 95% CI: 1.1–6.4, $P = 0.032$) were all significantly associated with worse EFS (Table 2(a)). Increased cytoplasmic IKK-β also reduced the chance of a prolonged EFS (HR = 3.5, 95% CI: 1.5–7.8, $P = 0.003$) (data not shown). We next determined if any of the NF-κB pathway proteins were associated with EFS in multivariate analyses after controlling for the potential confounding factors of EBV status, tumor histology, stage, bulk disease at presentation, and B symptoms. Nuclear phospho-Rel A, Rel-B, NIK, as well as cytoplasmic IKK-β remained significantly associated with shorter EFS after controlling for these prognostic clinical variables (Table 2(b)).

4. Discussion

We report a comprehensive study of NF-κB pathway activation in pediatric HL patients using TMA. Since all patients whose samples were included on this array were treated with the same chemotherapy for the first 2 cycles, it was possible to correlate NF-κB pathway protein intensity, subcellular localization, and pathway regulation not only with rapidity of treatment response but also, more significantly, with EFS. We show that NF-κB regulation is affected by EBV status and correlates with rapidity of response to therapy and that patients with elevated NIK, a member of the alternative NF-κB pathway, are more likely to respond slowly to therapy and to have worse EFS.

TMA are quite useful in analysis of protein expression in heterogeneous tumors and in HL have the advantage of allowing for the assessment of protein expression intensity and subcellular localization in H/RS cells compared to cells in the microenvironment [17–20]. Previous TMA have favorably correlated with whole tissue section IHC [17, 18] and gene expression arrays [20]. Previous HL TMA

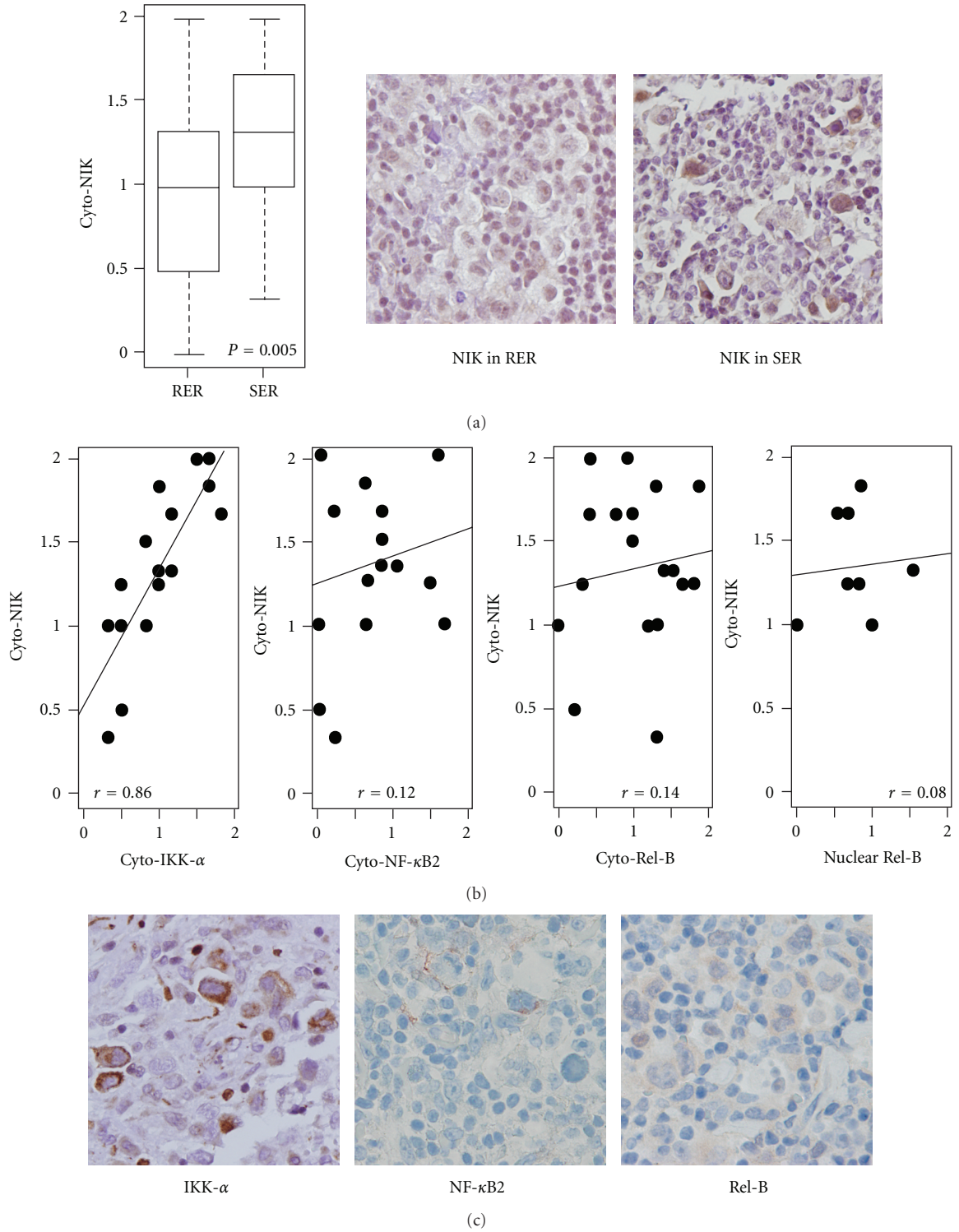


Figure 5: Examination of alternative NF-κB pathway proteins in HL patients with a slow early response (SER) to therapy. (a) NIK expression in SER versus RER. Left panel: mean $\pm$ 2 standard deviations (95% confidence intervals) of NIK levels in RER versus SER. Significance was determined by the Wilcoxon rank-sum test. Middle and right panels: IHC from representative patients comparing NIK expression in an RER patient (middle panel) to an SER patient (right panel). (b) Alternative NF-κB pathway in SER samples: scatter plot and linear regression analysis for cytoplasmic NIK (y-axis) versus cytoplasmic IKK-α (left panel), cytoplasmic NF-κB2 (middle left panel), cytoplasmic Rel-B (middle right panel), and nuclear Rel-B (right panel). (c) Representative IHC from an SER patient samples immunostained with IKK-α (left panel), NF-κB2 (middle panel), and Rel-B (right panel). IHC pictures taken at 400$\times$ magnification using an Olympus BX41 microscope. Whole images were adjusted for contrast and brightness.

studies have examined key regulatory proteins in H/RS cells, including tumor suppressors, cell-cycle checkpoints, and antiapoptosis proteins [17–19]. Two prior TMAs have examined interactions between the NF-κB subunit Rel-A and other cell signaling proteins. Garcia et al. noted a correlation between Rel-A expression and proteins that regulate apoptosis, including TUNL, the retinoblastoma Rb protein, and Stat1 [17]. Rel-A intensity has also been correlated with proteins involved in cell growth and proliferation, including p21, cyclins E and D3, Cdk1, Stat3, and survivin [17, 19].

As previously noted in adult HL [2, 10, 21–23], we noted increased protein expression and nuclear localization of both classical and alternative NF-κB pathway proteins. In H/RS cells, NF-κB proteins are activated by multiple mechanisms. In addition to *REL-A* gene amplification, noted in up to 50% of primary HL samples (reviewed in [2, 24]), 20% of HL cases also have somatic mutations in the genes encoding *IκB-α*, *IκB-ε* [24–27] or *TNFAIP3*, the gene encoding A20 [28, 29]. Recent data in adult HL comparative genomic hybridization showed that >20% of patients had recurrent chromosome gains or losses that impact NF-κB activation, including genomic changes in *REL-A, IKBKB* (IKK-β), *CD40,* and *MAP3K14* (MAP3 kinase) [30]. Nongenomic deregulation can also increase NF-κB activation via self-oligomerization of CD30 [31], NIK overactivation by either CD40, RANK, or BAFF (reviewed in [23]), receptor-independent activation of IKKs [32, 33], and aberrant NF-κB2/p52 by increased proteasomal processing of p100 into p52 [10].

4.1. NF-κB Proteins and EBV Status. Our analysis compared NF-κB pathway protein associations between EBV+ and EBV− tumors. Although this TMA contained fewer EBV+ samples than expected (26 HL samples (25%) versus 40% reported in other developed countries), this is likely due to the preponderance of the nodular sclerosing subtype. In this study we found no difference in either response to treatment (RER versus SER) or EFS between patients with EBV+ versus EBV− HL tumors (RER: 77% versus SER: 82%, $P = 0.57$, Fisher's exact test). Interestingly, EBV+ H/RS tumors had constitutively active NF-κB that appeared to be independent of regulation by the classical NF-κB pathway, the alternative NF-κB pathway, or A20. This activation is likely due to the effects of LMP1 on NF-κB activation. LMP1 has been shown to activate NF-κB through a variety of mechanisms, including simulation of CD40 receptor NF-κB activation [34], as well as TRAF and NIK activation of NF-κB through the LMP1 cytoplasmic tail domains CTAR1 and CTAR2 [35].

Interestingly, although there were no differences in intensity or localization of NF-κB pathway proteins between EBV+ and EBV− tumors (Figure 2(c)), there was a striking lack of coordinate NF-κB pathway regulation in EBV+ tumors (Figure 4(a), right panels). This lack of coordinate regulation in EBV+ tumors could be explained by the presence of EBV proteins in H/RS cells that directly activate NF-κB. For example, the EBV viral protein LMP1 can transactivate NF-κB through multiple mechanisms. In addition to mimicking the active CD40 receptor [36], LMP1 can directly stimulate the TNF receptor-associated death domain protein (TRADD) [37], the PI3 kinase pathway, the JAK-STAT pathway [38], and downregulate CD99 [39].

A20, an E3 ubiquitin ligase/deubiquitinase, inactivates IKK-γ (NEMO) and Traf3, providing a negative feedback loop for both the classical and alternative NF-κB pathways [16]. Inactivating mutations in *TNFAIP3* have been found in up to 40% of adult HL patients with EBV− HL tumors [29]. While our data suggests that fewer pediatric HL patients (10%) have A20 mutations, there are several caveats to this assertion. Although blocked by A20 peptide, it is possible that the A20 antibody could nonspecifically bind to a protein with a similar peptide domain. It is also possible that our antibody is detecting nonfunctional A20 protein. With our sample size, we were unable to detect a significant correlation between lack of A20 expression and EBV status or rapidity of response to therapy. However, increased A20 expression significantly correlated with decreased EFS in both univariate and multivariate analysis.

4.2. NF-κB Pathway Regulation and Therapy Response. Although there were no significant differences in the expression of most NF-κB pathway proteins and the rapidity of response to therapy, NIK expression was significantly higher in SER patients (Figure 5(a)). Previous reports have indicated that increased NIK expression contributes to tumorigenicity in T-cell ALL and H/RS cells [40]. Saitoh et al. demonstrated that increased NIK resulted in cell transformation in rat fibroblasts, an effect blocked by an IκB superrepressor [40]. Although the mechanism of NIK overexpression in HL patients with SER to therapy is unknown, it could be due to alterations in proteins that prevent the proteasomal degradation of NIK, such as decreased E3 ligase cIAP or its adaptor protein Traf3, or an increase of an (as-yet-unidentified) NIK deubiquitinase [3].

In contrast to RER patients, who had significant correlations between cytoplasmic NIK and cytoplasmic IKK-α, IKK-β, IKK-γ, and Rel-B (Figure 4(b), left panels), SER patients showed few significant correlations between NF-κB pathway proteins (Figure 4(b), right panels). The reason for this pathway dissociation in SER patients is unclear but suggests that SER patients may not derive as much clinical benefit from treatment with agents that target NF-κB pathway proteins. Consistent with this hypothesis, Allen et al. demonstrated a limited response of head and neck carcinomas to the proteasome inhibitor bortezomib. In these tumors the classical NF-κB pathway was inhibited but the alternative NF-κB pathway proteins remained unaffected [41].

4.3. NF-κB Pathway Expression and Clinical Outcome. The early response rate for the patients included in the TMA (80% RER) was similar to the overall response rate in the AHOD0031 clinical trial. Outcome for the clinical trial group was excellent; only 248/1712 (14%) patients experienced an event (progressive disease or relapse) following therapy, and the overall 4-year EFS for patients in the AHOD0031 clinical

trial was 85% (Debra L. Friedman, personal communication). In our cohort of 99 evaluable patients, 24 experienced an event, which is slightly higher than the event rate for the entire group ($P = 0.008$, Chi-square test).

In univariate analysis, increases in several NF-κB pathway proteins, including nuclear phospho-Rel-A, Rel-B, A20, and NIK, were significantly associated with worse EFS. In multivariate analysis controlling for prognostic clinical variables, nuclear expression of Rel-B, NIK, and A20 remained significantly associated with worse EFS. Elevated NIK expression was associated with both an increased likelihood of SER and with poor EFS. Within the confines of this retrospective study, these findings suggest that increased expression of several NF-κB pathway proteins, including NIK, is associated with worse EFS independent of clinical presentation or histology.

In summary, this study suggests that NF-κB pathway proteins are overexpressed in pediatric H/RS cells. Some novel insights were provided by the TMA, this pediatric HL cohort. Elevation in the noncanonical pathway protein NIK was associated with both a slow response to therapy and poor EFS. Expression of both classical and alternative NF-κB pathway proteins was coordinately regulated in EBV− tumors and in patients with a rapid early response to therapy. In contrast, there appears to be little coordinate NF-κB pathway regulation in patients with EBV+ tumors or those that have a slow early response to therapy. Five NF-κB proteins, including nuclear Rel-B, NIK, and A20, along with cytoplasmic Rel-A and IKK-β, remained significantly associated with decreased EFS after multivariate analysis. These data suggest that increased expression of certain NF-κB pathway proteins is independently associated with poor clinical outcome.

Authors' Contribution

T. M. Horton designed the research, analyzed the data, and wrote the paper; A. M. Sheehan, D. L. Terrada, R. Hutchison, and S. Narendra performed the research, analyzed the data, and edited the paper; M. F. Wu and H. Liu provided statistical analysis and edited the paper.

Conflict of Interests

The authors report no potential conflict of interests and no competing interests.

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