Research Paper

# RelA NF-κB subunit activation as a therapeutic target in diffuse large B-cell lymphoma

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## ABSTRACT

**It has been well established that nuclear factor kappa-B (NF-κB) activation is important for tumor cell growth and survival. RelA/p65 and p50 are the most common NF-κB subunits and involved in the classical NF-κB pathway. However, the prognostic and biological significance of RelA/p65 is equivocal in the field. In this study, we assessed RelA/p65 nuclear expression by immunohistochemistry in 487 patients with *de novo* diffuse large**

**B-cell lymphoma (DLBCL), and studied the effects of molecular and pharmacological inhibition of NF-κB on cell viability. We found RelA/p65 nuclear expression, without associations with other apparent genetic or phenotypic abnormalities, had unfavorable prognostic impact in patients with stage I/II DLBCL. Gene expressionprofiling analysis suggested immune dysregulation and antiapoptosis may be relevant for the poorer prognosis associated with p65 hyperactivation in germinal center B-cell–like (GCB) DLBCL and in activated B-cell–like (ABC) DLBCL, respectively. We knocked down individual NF-κB subunits in representative DLBCL cells in vitro, and found targeting p65 was more effective than targeting other NF-κB subunits in inhibiting cell growth and survival. In summary, RelA/p65 nuclear overexpression correlates with significant poor survival in early-stage DLBCL patients, and therapeutic targeting RelA/p65 is effective in inhibiting proliferation and survival of DLBCL with NF-κB hyperactivation.**

## INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL), the most common form of aggressive non-Hodgkin lymphoma, accounts for nearly 40% of non-Hodgkin lymphomas [1]. Although most cases of DLBCL are curable with the standard immunochemotherapy regimen, rituximab plus cyclophosphamide, hydroxydaunomycin, vincristine, and prednisone (R-CHOP), 30-40% of patients have drug-resistant disease or recurrence [2]. DLBCL is a highly heterogeneous disease. Based on gene expression profiling (GEP), DLBCL can be classified into two molecular subtypes: germinal center B-cell–like (GCB) and activated B-cell–like (ABC) DLBCL [3]. The ABC subtype of DLBCL typically exhibits constitutive activation of the nuclear factor-kappaB (NF-κB) pathway [4, 5] and patients have inferior clinical outcomes compared with patients with GCB-DLBCL [6, 7]. Recent studies have shown that NF-κB expression is not limited to ABC-DLBCL but also can occur in GCB-DLBCL [8-10].

The NF-κB/Rel family contains five transcription factors: RelA (p65), NF-κB1 (p50; p105), NF-κB2 (p52; p100), RelB, and c-Rel. Only RelA/p65, RelB, and c-Rel had transactivation domains [11]. NF-κB activity is controlled by inhibitors of NF-κB (such as IκBα which inhibits p65/p50 dimers) that keep NF-κB inactive in the cytoplasm. Constitutive activation of NF-κB in ABC-DLBCL is caused by chronic activation of B-cell-receptor (BCR) signaling and elevated IκB kinase (IKK) activities which phosphorylate IκBα. As a result, IκBα is degraded releasing homo- or hetero-dimers of NF-κB to enter the nucleus where NF-κB activates gene transcription [4, 12-14]. *In vivo* the most abundant NF-κB dimers are p50/p65 heterodimers which are ubiquitously expressed in mammalian tissue [11, 15-17], consistent with the highest level of nuclear p50/p65 in DLBCL samples among all NF-κB subunits by our previous studies [10, 18]. Detection of p65/p50 nuclear expression in tumor cells has been considered as homodimers with distinct DNA-binding modes and functions [19-21].

NF-κB activation suppresses apoptosis and promotes tumor cell survival and proliferation, leading to treatment resistance. Different NF-κB subunits had distinct and overlapping functions [22-24]. In addition, transcriptional and functional crosstalk between antiapoptotic NF-κB and proapoptotic p53 (an essential tumor suppressor) plays a critical role in determining the fate of tumor cells [25, 26]. The p65 subunit of NF-κB and p53 counteract each other's function in regulating cell proliferation, metabolism and apoptosis [25, 27-29]. p65 increases MDM2 levels, which decrease the stabilization of p53 and cell death induced by cytotoxic chemotherapy [25]. However, cooperation between p65 and p53 has been also reported [30-33], making interactions between p65/NF-κB and p53 much more complicated. Both p53 and p65 were unexpectedly found necessary for either p53 or NF-κB-directed gene transcription under replicational stress or atypical and classical stimuli for NF-κB. Induced p65 in stimulated cancer cells by pro-inflammatory tumor necrosis factor α (TNF-α) binds to p53 and the p65/p53 complex transcriptionally activates NF-κB target genes (*survivin/BIRC5*, *BCL2*, *BCL-XL*, and *FASL*) [32]. Moreover, p65 and p53 co-regulate induction of proinflammatory genes in monocytes and macrophages [33].

Despite the well-established role of NF-κB signaling in lymphoma pathogenesis and treatment resistance, conflicting results on the prognostic significance of NF-κB and RelA/p65 expression (as a surrogate marker of NF-κB activation) in DLBCL have been reported by previous clinical studies [8, 9, 34-36]. To help clarify the prognostic effect of RelA/p65 nuclear expression, in this study we evaluated nuclear expression of RelA/p65 by immunohistochemistry (IHC) in a large cohort of DLBCL treated with R-CHOP, and studied the prognostic effects and gene expression profiles associated

with p65 nuclear expression. Moreover, we inactivated individual NF-κB subunits in vitro and investigated their differential effects on proliferation and apoptosis of DLBCL cells which highlighted the important therapeutic value of RelA/p65.

## RESULTS

### p65 hyperactivation has significant adverse impact in early-stage DLBCL

p65 expression was evaluable in 487 DLBCL patients, including 287 men and 200 women. GCB/ABC ratio was close to 1 (243 GCB and 239 ABC). The median age of the patients in the study group was 63 years, and 58% of the study cohort had elderly age (≥60). Immunohistochemical results showed that 58% of DLBCL samples had p65 nuclear expression indicative of p65 activation at various levels (Fig. 1A) with a mean level of 14%. p65 nuclear expression was not specific for ABC-DLBCL. In fact, the GCB-DLBCL group had a slightly higher mean level of p65 nuclear expression (16.1%) than the ABC-DLBC group (12.6%) (Fig. 1A). Table 1 showed the clinical and pathological features of the study cohort.

Low levels (10-40%) of p65 nuclear expression did not have significant prognostic impact in DLBCL (Fig. 1B). However, high p65 nuclear expression ($p65^{high}$, ≥50% tumor cells with p65 positive nuclei) correlated with significantly shorter PFS and OS durations in patients with stage I/II DLBCL and in patients with an International Prognostic Index score (IPI) ≤2 (Fig. 1B, Fig. 2A). In contrast, in patients with stage III/IV DLBCL or an IPI >2, p65 expression was not prognostic. $p65^{high}$ patients with stage I/II DLBCL had similar survival rates compared with $p65^{high}$ patients with stage III/IV DLBCL (Fig. 2B).

When analyzed individually in GCB and ABC subtypes, in GCB-DLBCL only, the $p65^{high}$ group frequently had large (≥ 5cm) tumors (65% *vs.* 37%, $P = 0.011$) (Table 1), and significantly decreased PFS ($P = 0.04$, Fig. 2C) and OS ($P = 0.015$) rates than other patients ($p65^{low}$ group, IHC <50%). However, the unfavorable prognostic effect manifested in GCB-DLBCL was limited in stage I/II (Fig. 1C) and minimal in stage III/IV GCB-DLBCL ($P = 0.95$ for PFS and $P = 0.60$ for OS); also, in stage I/II ABC-DLBCL patients, $p65^{high}$ expression also significantly correlated with worse PFS (Fig. 1C).

### p65 nuclear expression correlates with p50 nuclear expression in DLBCL

We found high p65 nuclear expression was significantly associated with $p50^{+}$ and $p50^{high}$ nuclear expression in overall DLBCL, GCB-DLBCL, and ABC-DLBCL (Table 1), suggesting the predominance of p65/p50 dimer activation via the canonical NF-κB pathway [9]. Significant association with c-$Rel^{+}$ nuclear expression was also found in overall DLBCL and GCB-DLBCL (p50/c-Rel is another dimer activated via the canonical pathway [37, 38]). No significant association was observed between $p65^{high}$ and $RelB^{+}$. $p65^{high}$ showed significant association with $p52^{+}$ in overall DLBCL but not in either GCB or ABC subset.

Nuclear expression of p50, p52, and c-Rel did not show further prognostic effects among the $p65^{high}$ patients. We did not observe associations of $p65^{high}$ with any other adverse biomarkers such as *TP53* mutations, *MYC*/*BCL2* translocation, and Myc/Bcl-2 over-expression which may confound the prognostic effects [39-42]. In contrast, in the GCB but not the ABC subgroup, $p65^{high}$ compared with $p65^{low}$ patients less frequently had Bcl-2 overexpression (18% *vs.* 40%, $P = 0.036$).

### p65 hyperactivation has significant adverse impact in patients with wild-type *TP53*

Cases of DLBCL with wild-type *TP53* (*WT-TP53*) had significantly lower levels of *RELA* mRNA ($P = 0.018$, Fig. 1D) and a trend toward lower nuclear p65 levels ($P = 0.11$) than those with mutated *TP53* (*MUT-TP53*), suggesting that wild-type p53 suppressed *RELA* NF-κB expression. Conversely, p65 antagonized p53 function as suggested by survival analysis: in *WT-TP53* DLBCL, patients with $p65^{high}$ expression correlated with significantly decreased PFS ($P = 0.0076$, Fig. 2C) and OS ($P = 0.0082$) rates than patients with $p65^{low}$ tumors, independent of GCB and ABC cell-of-origin. However, when subdivided cohorts by disease stages, we found the prognostic impact was only significant in patients with stage I/II disease ($P < 0.0001$ for PFS, $P = 0.0004$ for OS). Also in *MUT-TP53* patients with stage I/II DLBCL, positive p65 nuclear expression was associated with significant poorer survival; in contrast, opposite trends were observed in *MUT-TP53* patients with stage III/IV DLBCL (Fig. 1E).

### Multivariate survival analysis

Multivariate survival analysis (Cox regression) for high p65 nuclear expression with adjustments for clinical variables confirmed that $p65^{high}$ was an independent adverse prognostic factor in patients with GCB-DLBCL and in patients with *WT-TP53* DLBCL, but not in the overall study group, the ABC-DLBCL subgroup, or the *MUT-TP53* DLBCL subgroup (Table 2).

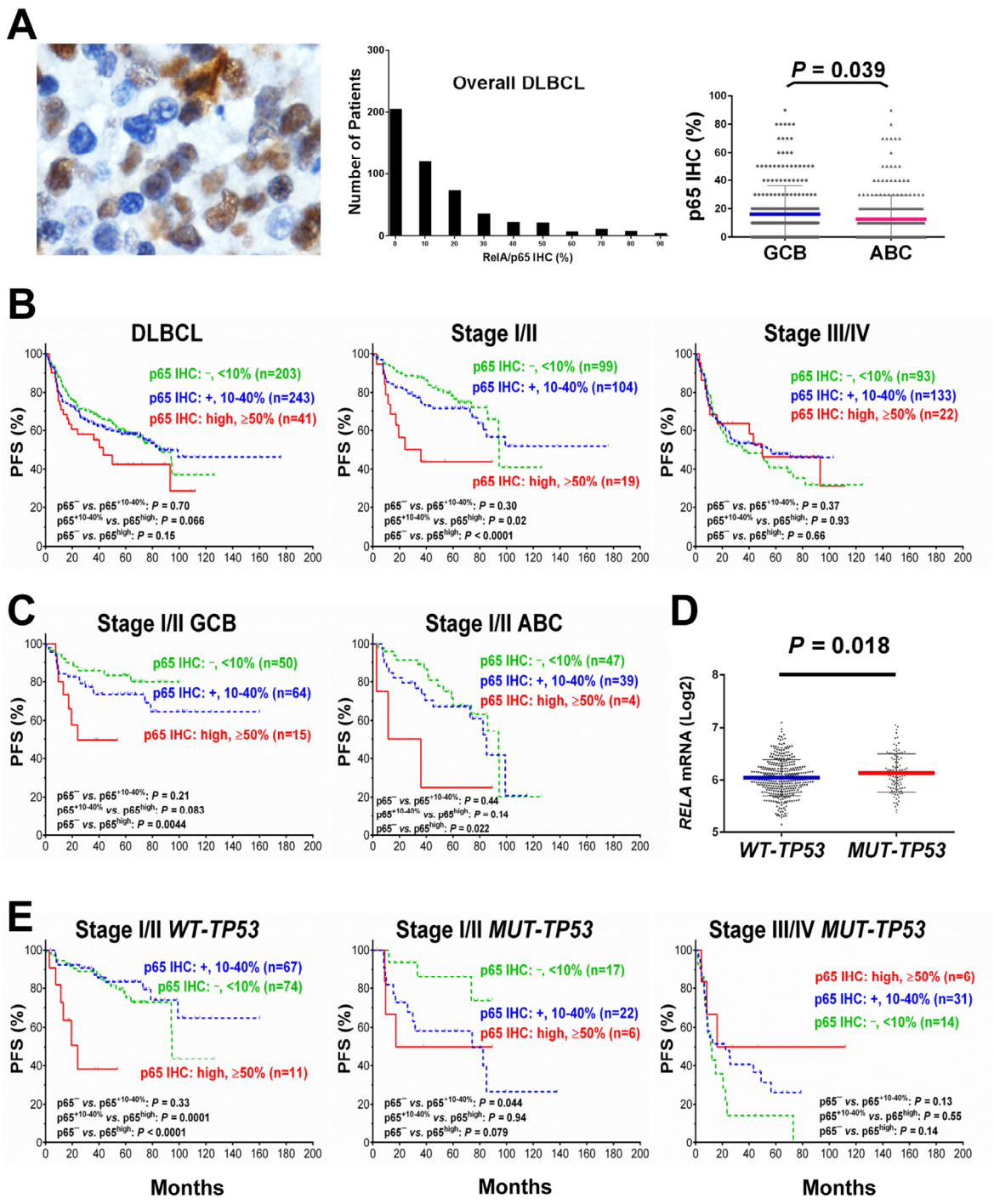


**Figure 1. Nuclear expression of p65 and its effect on progression-free survival (PFS) in diffuse large B-cell lymphoma (DLBCL)** (**A**) Representative immunohistochemical analysis (IHC) and histograms for p65 nuclear expression in DLBCL. The mean expression of nuclear p65 was significantly higher in the germinal center B-cell–like (GCB) subtype than in the activated B-cell–like (ABC) subtype. (**B**) In overall DLBCL, high p65 nuclear expression ($p65^{high}$, ≥50% nuclear expression) was associated with a trend towards worse PFS. In patients with stage I/II DLBCL, $p65^{high}$ correlated with significantly shorter PFS. In patients with stage III/IV DLBCL, $p65^{high}$ did not show signficant prognostic impact. (**C**) $p65^{high}$ correlate with significantly shorter PFS in patients with stage I/II DLBCL independent of GCB/ABC subtypes. (**D**) *TP53* mutation status was significantly associated with higher *RELA* mRNA expression. (**E**) In patients with stage I/II DLBCL, $p65^{high}$ correlate with significantly shorter PFS independent of *TP53* mutation status although more significant in patients with wild-type *TP*53 (*WT-TP53*). In patients with mutated *TP53* (*MUT-TP53*) and stage III/IV DLBCL, $p65^{high}$ was associated with a trend of better PFS.

**Table 1. Clinical characteristics of 487 patients with *de novo* diffuse large B-cell lymphoma (DLBCL).**

| | DLBCL | | | GCB-DLBCL | | | ABC-DLBCL | | | *WT-TP53* | | | *MUT-TP53* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $p65^{high}$ | $p65^{low}$ | *P* | $p65^{high}$ | $p65^{low}$ | *P* | $p65^{high}$ | $p65^{low}$ | *P* | $p65^{high}$ | $p65^{low}$ | *P* | $p65^{high}$ | $p65^{low}$ | *P* |
| Characteristics | N (%) | N (%) | | N (%) | N (%) | | N (%) | N (%) | | N (%) | N (%) | | N (%) | N (%) | |
| **Patients** | 41 | 446 | | 28 | 215 | | 13 | 226 | | 26 | 312 | | 12 | 84 | |
| **Age (years)** | | | | | | | | | | | | | | | |
| <60 | 21 (51) | 183 (41) | 0.21 | 16 (57) | 106 (49) | 0.44 | 5 (39) | 74 (33) | 0.67 | 13 (50) | 124 (40) | 0.31 | 7 (58) | 30 (36) | 0.13 |
| ≥60 | 20 (49) | 263 (59) | | 12 (43) | 109 (51) | | 8 (61) | 152 (67) | | 13 (50) | 188 (60) | | 5 (42) | 54 (64) | |
| **Gender** | | | | | | | | | | | | | | | |
| Female | 8 (20) | 192 (43) | **0.003** | 6 (21) | 95 (44) | **0.022** | 2 (15) | 95 (42) | 0.057 | 6 (30) | 130 (42) | 0.063 | 2 (17) | 38 (45) | 0.06 |
| Male | 33 (80) | 254 (57) | | 22 (79) | 120 (56) | | 11 (85) | 131 (58) | | 20 (70) | 182 (58) | | 10 (83) | 46 (55) | |
| **Stage** | | | | | | | | | | | | | | | |
| I/II | 19 (46) | 203 (47) | 0.90 | 15 (54) | 114 (56) | 0.84 | 4 (31) | 86 (39) | 0.54 | 11 (42) | 141 (48) | 0.60 | 6 (50) | 39 (46) | 0.82 |
| III/IV | 22 (54) | 226 (53) | | 13 (46) | 91 (45) | | 9 (69) | 133 (61) | | 15 (58) | 155 (52) | | 6 (50) | 45 (54) | |
| **B-symptoms** | | | | | | | | | | | | | | | |
| No | 25 (61) | 272 (64) | 0.67 | 18 (64) | 141 (70) | 0.55 | 7 (54) | 127 (59) | 0.73 | 16 (61) | 196 (66) | 0.61 | 7 (58) | 54 (68) | 0.53 |
| Yes | 16 (39) | 151 (36) | | 10 (36) | 61 (30) | | 6 (46) | 89 (41) | | 10 (39) | 99 (34) | | 5 (42) | 26 (33) | |
| **LDH levels** | | | | | | | | | | | | | | | |
| Normal | 14 (34) | 161 (40) | 0.51 | 8 (29) | 86 (44) | 0.12 | 6 (46) | 74 (36) | 0.44 | 10 (39) | 121 (43) | 0.65 | 3 (25) | 28 (36) | 0.46 |
| Elevated | 27 (66) | 247 (60) | | 20 (71) | 109 (56) | | 7 (54) | 134 (64) | | 16 (61) | 160 (57) | | 9 (75) | 50 (64) | |
| **Extranodal sites (n)** | | | | | | | | | | | | | | | |
| 0–1 | 35 (85) | 327 (77) | 0.22 | 23 (82) | 160 (80) | 0.75 | 12 (92) | 163 (74) | 0.15 | 21 (81) | 231 (79) | 0.79 | 11 (92) | 64 (78) | 0.27 |
| ≥2 | 6 (15) | 98 (23) | | 5 (18) | 41 (20) | | 1 (8) | 56 (26) | | 5 (19) | 63 (21) | | 1 (8) | 18 (22) | |
| **Performance status** | | | | | | | | | | | | | | | |
| 0–1 | 34 (87) | 329 (83) | 0.53 | 23 (85) | 158 (86) | 0.87 | 11 (92) | 166 (80) | 0.33 | 22 (85) | 231 (85) | 0.93 | 10 (91) | 69 (90) | 0.89 |
| ≥2 | 5 (13) | 66 (17) | | 4 (15) | 25 (14) | | 1 (8) | 41 (20) | | 4 (15) | 40 (15) | | 1 (9) | 8 (10) | |
| **Size of largest tumor** | | | | | | | | | | | | | | | |
| <5cm | 14 (44) | 192 (58) | 0.11 | 8 (35) | 97 (63) | **0.011** | 6 (67) | 93 (54) | 0.47 | 8 (38) | 146 (62) | **0.035** | 5 (50) | 33 (49) | 0.96 |
| ≥5cm | 18 (56) | 137 (42) | | 15 (65) | 58 (37) | | 3 (33) | 78 (46) | | 13 (62) | 91 (38) | | 5 (50) | 34 (51) | |
| **IPI risk group** | | | | | | | | | | | | | | | |
| 0–2 | 29 (71) | 267 (62) | 0.25 | 21 (75) | 144 (71) | 0.82 | 8 (61) | 118 (54) | 0.78 | 17 (65) | 189 (63) | 1.0 | 10 (83) | 47 (57) | 0.12 |
| 3-5 | 12 (29) | 162 (38) | | 7 (25) | 60 (29) | | 5 (39) | 102 (46) | | 9 (35) | 109 (37) | | 2 (17) | 43 (35) | |
| **Therapy response** | | | | | | | | | | | | | | | |
| CR | 29 (71) | 343 (77) | 0.37 | 17 (61) | 166 (77) | 0.057 | 12 (92) | 172 (76) | 0.18 | 17 (65) | 257 (82) | **0.034** | 9 (75) | 49 (58) | 0.27 |
| Non-CR | 12 (29) | 103 (23) | | 11 (39) | 49 (23) | | 1 (8) | 22 (24) | | 9 (35) | 55 (18) | | 3 (25) | 35 (42) | |
| **Primary origin** | | | | | | | | | | | | | | | |
| Extranodal | 20 (49) | 149 (34) | 0.058 | 14 (50) | 68 (32) | 0.063 | 6 (46) | 79 (36) | 0.44 | 14 (54) | 102 (33) | **0.035** | 4 (33) | 26 (32) | 0.91 |
| Nodal | 21 (51) | 289 (66) | | 14 (50) | 143 (68) | | 7 (54) | 143 (64) | | 12 (46) | 204 (67) | | 8 (67) | 56 (68) | |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cell-of-origin** | | | | | | | | | | | | | | | |
| GCB | 28 (68) | 215 (49) | **0.022** | - | - | - | - | - | - | 18 (69) | 143 (46) | **0.039** | 9 (75) | 49 (58) | 0.35 |
| ABC | 13 (32) | 226 (51) | | - | - | | - | - | | 8 (31) | 165 (54) | | 3 (25) | 35 (42) | |
| **p50 nuclear expression** | | | | | | | | | | | | | | | |
| <20% | 15 (40) | 278 (67) | **0.002** | 12 (46) | 149 (74) | **0.005** | 3 (27) | 129 (60) | **0.05** | 9 (41) | 188 (65) | **0.037** | 6 (50) | 57 (71) | 0.18 |
| ≥20% | 22 (60) | 138 (33) | | 14 (54) | 52 (26) | | 8 (73) | 85 (40) | | 13 (59) | 102 (35) | | 6 (50) | 23 (29) | |
| **p52 nuclear expression** | | | | | | | | | | | | | | | |
| – | 21 (55) | 300 (71) | **0.043** | 14 (54) | 142 (71) | 0.11 | 7 (58) | 157 (72) | 0.33 | 14 (58) | 208 (71) | 0.25 | 5 (46) | 59 (74) | 0.078 |
| + | 17 (45) | 120 (29) | | 12 (46) | 58 (29) | | 5 (42) | 61 (28) | | 10 (42) | 86 (29) | | 6 (54) | 21 (26) | |
| **c-Rel nuclear expression** | | | | | | | | | | | | | | | |
| – | 17 (46) | 297 (72) | **0.002** | 11 (44) | 147 (74) | **0.004** | 6 (50) | 150 (70) | 0.20 | 9 (41) | 207 (73) | **0.003** | 7 (58) | 55 (67) | 0.53 |
| + | 20 (54) | 117 (28) | | 14 (56) | 52 (26) | | 6 (50) | 64 (30) | | 13 (59) | 78 (27) | | 5 (42) | 27 (33) | |
| **Bcl-2 expression** | | | | | | | | | | | | | | | |
| <70% | 27 (66) | 229 (52) | 0.10 | 23 (82) | 127 (60) | **0.036** | 4 (31) | 99 (44) | 0.40 | 17 (65) | 164 (53) | 0.31 | 7 (58) | 40 (48) | 0.55 |
| ≥70% | 14 (34) | 208 (48) | | 5 (18) | 83 (40) | | 9 (69) | 125 (56) | | 9 (35) | 143 (47) | | 5 (42) | 44 (52) | |

Abbreviations: $p65^{high}$, high levels of nuclear p65; $p65^{low}$, low levels of p65 nuclear expression; LDH, lactate dehydrogenase; IPI, international prognostic index; CR, complete remission; PR, partial response; GCB, germinal center B-cell–like; ABC, activated B-cell–like; *WT-TP53*, wild-type *TP53*; *MUT-TP53*, mutated *TP53*. Some of the clinicopathologic data were not available. Percentages are calculated among cases with specific data available. Significant *P* values in bold.

**Table 2. Multivariate analysis of clinicopathologic parameters for survival of patients with diffuse large B-cell lymphoma (DLBCL) treated with R-CHOP.**

| | Overall survival | | | Progression-free survival | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *P* | HR | 95% CI | *P* |
| **DLBCL (*n* = 497)** | | | | | | |
| IPI > 2 | 2.41 | 1.70–3.42 | **< 0.001** | 2.29 | 1.64–3.19 | **< 0.001** |
| Female sex | 1.03 | 0.72–1.49 | 0.86 | 0.99 | 0.70–1.41 | 0.98 |
| Tumor size ≥ 5 | 1.28 | 0.91–1.81 | 0.16 | 1.23 | 0.89–1.71 | 0.21 |
| B-symptoms | 1.35 | 0.94–1.94 | 0.099 | 1.31 | 0.93–1.85 | 0.12 |
| $p65^{high}$ | 1.56 | 0.91–2.68 | 0.11 | 1.44 | 0.85–2.42 | 0.18 |
| **GCB-DLBCL (*n* = 243)** | | | | | | |
| IPI > 2 | 2.47 | 1.40–4.38 | **0.002** | 2.39 | 1.39–4.09 | **0.002** |
| Female sex | 1.00 | 0.55–1.82 | 1.00 | 0.98 | 0.56–1.71 | 0.95 |
| Tumor size ≥ 5 | 1.30 | 0.88–1.91 | 0.19 | 1.40 | 0.82–2.40 | 0.22 |
| B-symptoms | 1.44 | 0.80–2.58 | 0.22 | 1.34 | 0.77–2.33 | 0.31 |
| $p65^{high}$ | 2.30 | 1.14–4.62 | **0.02** | 2.01 | 1.06–3.82 | **0.034** |
| ***WT-TP53* DLBCL (*n* = 338)** | | | | | | |
| IPI > 2 | 2.54 | 1.66–3.88 | **< 0.001** | 2.33 | 1.57–3.46 | **< 0.001** |
| Female sex | 0.98 | 0.63–1.53 | 0.92 | 0.99 | 0.65–1.51 | 0.96 |
| Tumor size ≥ 5 | 1.20 | 0.79–1.84 | 0.39 | 1.09 | 0.73–1.63 | 0.18 |
| B-symptoms | 1.59 | 1.04–2.43 | **0.034** | 1.57 | 1.05–2.33 | **0.028** |
| $p65^{high}$ | 1.91 | 1.04–3.52 | **0.037** | 1.94 | 1.08–3.48 | **0.026** |

Abbreviations: R-CHOP, rituximab with cyclophosphamide, doxorubicin, vincristine, and prednisone; HR, hazard ratio; CI, confidence interval; IPI, International Prognostic Index; $p65^{high}$, high levels of nuclear p65; GCB, germinal center B-cell–like; ABC, activated B-cell–like; WT-TP53, wild-type TP53. *Significant P values in bold.

**GEP analysis suggests different signaling pathways activated in GCB- and ABC-DLBCL**

To gain insight into the molecular mechanisms underlying the prognostic effects of p65 hyperactivation in DLBCL, we compared gene expression profiles of $p65^{high}$ and $p65^{low}$ tumors. $p65^{high}$ patients showed GEP signatures compared with other DLBCL including $p65^{-}$ DLBCL patients (IHC <10%), stronger in GCB-DLBCL than in ABC-DLBCL subset (Fig. 3A, Fig. 4, Tables 3-4).

In line with the unfavorable prognosis of patients with $p65^{high}$ DLBCL, GEP analysis found that *JUN* and *PTPRD* (involved in cell cycle progression) were upregulated (1.43-fold and 1.31-fold respectively) whereas pro-apoptotic *NOXA*/*PMAIP1* and *BTG3* which negatively regulates proliferation and cell cycle progression were downregulated (1.62-fold and 1.45-fold, respectively) in $p65^{high}$ DLBCL compared with $p65^{low}$ DLBCL. *RBMS1* which transactivates *MYC* was upregulated (1.48-fold) in $p65^{high}$ compared with $p65^{low}$ GCB-DLBCL (Table 3). Paradoxically, antiapoptotic

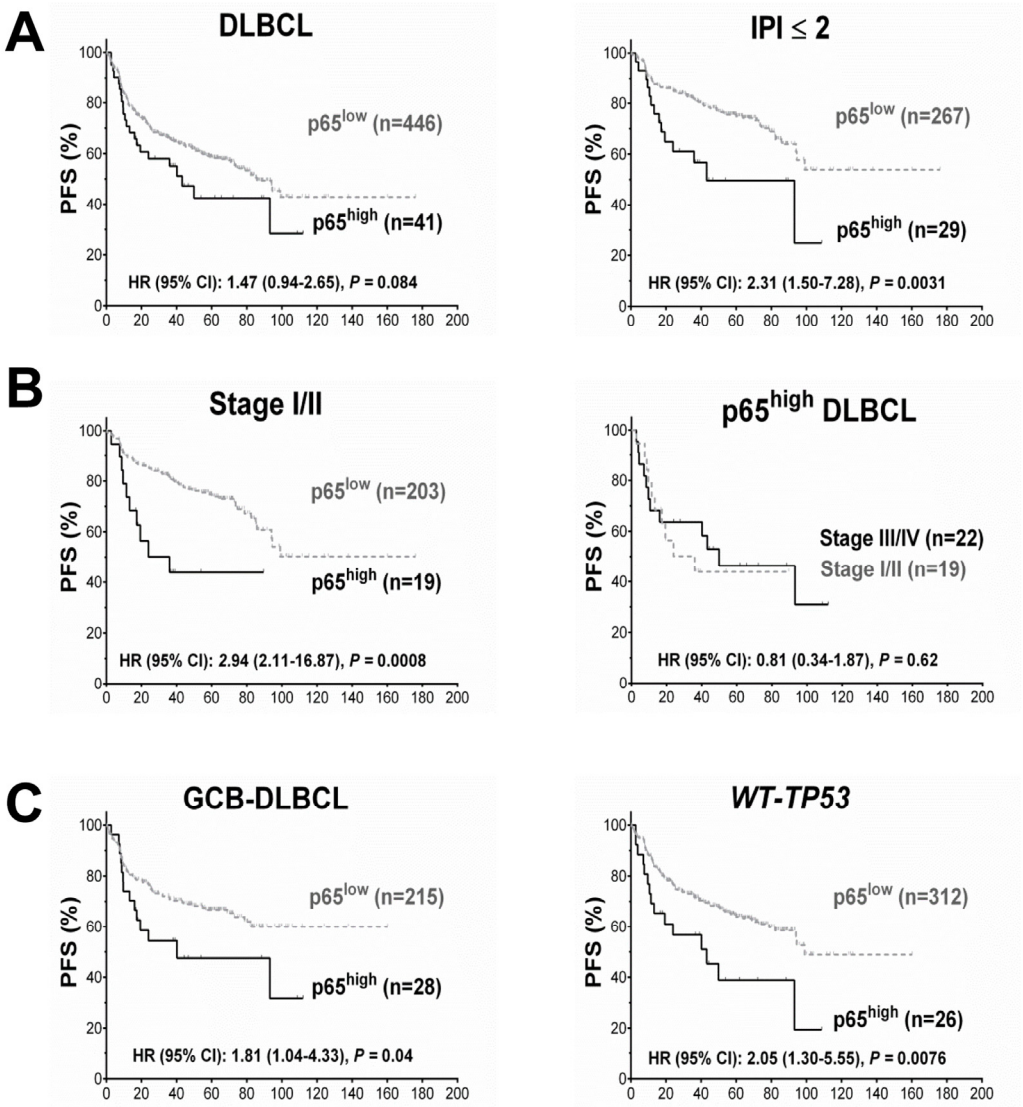


**Figure 2. Prognosis for p65 hyperactivation in diffuse large B-cell lymphoma (DLBCL).** (**A**) In overall DLBCL, high p65 nuclear expression ($p65^{high}$, ≥50% nuclear expression) was associated with unfavorable progression-free survival (PFS). The adverse prognostic impact was significant in patients with an international prognostic index score (IPI) ≤2. (**B**) In patients with stage I/II DLBCL, $p65^{high}$ correlated with significantly poorer PFS. Among $p65^{high}$ DLBCL patients, disease stages did not show further prognostic impact. (**C**) $p65^{high}$ correlated with significantly poorer PFS in patients with GCB-DLBCL and patients with wild-type *TP*53 (*WT-TP53*).

*BIRC6*, *MCM10* (involved in the initiation of eukaryotic genome replication), *CARS* (cysteinyl-tRNA synthetase) and *PA2G4* (involved in growth regulation) were downregulated in p65$^{high}$ patients, and *TENC1* (which inhibits AKT1 signaling) was upregulated in p65$^{high}$ DLBCL.

When analyzed in GCB and ABC subtypes individually, we found such paradoxical association was limited in the GCB subset. *RNF130* involved in apoptosis showed 1.81-fold upregulation in p65$^{high}$ GCB-DLBCL patients. In contrast, in p65$^{high}$ ABC-DLBCL, antiapoptotic *BIRC5* and *BCL2L2* were significantly upregulated whereas pro-apoptotic *NOXA*/*PMAIP1* was significantly downregulated (Fig. 3B-C), in addition to the proliferative signatures (such as upregulation of genes involved in replication, transcription, translation, and metabolism) in ABC-DLBCL (Table 3).

**Table 3. Differentially expressed (canonical activation) genes between p65$^{high}$ *vs.* p65$^{low}$ patients with diffuse large B-cell lymphoma (DLBCL).**

| | p65$^{high}$ vs. p65$^{low}$ | | |
|---|---|---|---|
| Functional categories | In overall DLBCL (FDR <0.30) | In GCB-DLBCL (FDR <0.05) | In ABC-DLBCL (FDR <0.20) |
| Signaling, ion channels | ***TNFRSF1A*** ↑ ***FYN*** ↑ ***LCP2*** ↑ ***PTPRD*** ↑ ***GTPBP2*** ↑ ***PROCR*** ↑ ***TENC1*** ↑ ***ITPR3*** ↑ ***TEK*** ↑ ***CACNA2D1*** ↑ ***AGTRAP*** ↑ *LPAR3* ↓ | ***MT1X*** ↑ ***MT1G*** ↑ ***SERPING1*** ↑ ***PSEN1*** ↑ | ***ARHGEF2*** ↑ ***FGFBP1*** ↑ ***EPHA1*** ↑ ***MS4A3*** ↑ |
| Immune responses, inflammation | | ***CD163*** ↑ ***FCER1G*** ↑ ***CYBB*** ↑ ***GRN*** ↑ ***CD84*** ↑ ***LILRB1*** ↑ | ***DEFB4*** ↑ |
| Cell cycle, DNA metabolism, transcription and translation regulation | ***JUN*** ↑ ***MLLT10*** ↑ ***GATAD2A*** ↑ ***HOXD10*** ↑ ***NFRKB*** ↑ *ZWINT* ↓ *MCM10* ↓ *HMGB1* ↓ *PPP2CA* ↓*UHRF1* ↓ *BTG3* ↓ *ZNF254* ↓ *CARS* ↓ *PA2G4* ↓ *SERBP1* ↓ | ***RBMS1*** ↑ ***ANKRD11*** ↑ ***FAM89B*** ↑ | ***ESRP1*** ↑ ***DPPA4*** ↑ |
| Apoptosis | *PMAIP1* ↓ *BIRC6* ↓ | ***RNF130*** ↑ | |
| Metabolism | ***SULT1A1*** ↑ ***SPTLC2*** ↑ ***SLC25A16*** ↑ ***SLC9A9*** ↑ | ***GLUL*** ↑ ***SERINC1*** ↑ ***CAT*** ↑ ***SLC9A9*** ↑ | ***S100A16*** ↑ ***SLC9A5*** ↑ |
| Transport, trafficking, protein folding, chaperone | ***CPNE8*** ↑ ***DNAJC5*** ↑ ***RHD*** ↑ ***AGFG2*** ↑ | ***SLC8A1*** ↑ ***NPC2*** ↑ ***VAMP5*** ↑ ***DNAJC5*** ↑ | |
| Cell adhesion, cytoskeleton, collagen, extracellular matrix | ***SH3D19*** ↑ ***ITGA6*** ↑ ***MYLK*** ↑ ***COL6A1*** ↑ ***UTRN*** ↑ ***FMOD*** ↑ | ***UTRN*** ↑ | ***CCDC151*** ↑ ***KRT13*** ↑ ***ANTXR2*** ↑ ***COL17A1*** ↑ |
| Degradation, ubiquitination | ***RNASE1*** ↑ | ***SCARB2*** ↑ ***CTSB*** ↑ ***UBA7*** ↑ | ***PSMB1*** ↑ |
| IncRNA genes, unknown function | ***NCRNA00185*** ↑ ***C19orf6*** ↑ ***PLEKHO2*** ↑ ***FAM124A*** ↑ | ***MT1P2*** ↑ ***ZDHHC20*** ↑ ***PLEKHO2*** ↑ | *FAM105B* ↓ ***CG030*** ↑ ***NCRNA00185*** ↑ ***IQCG*** ↑ |

Abbreviations: p65$^{high}$, p65 immunohistochemistry results: ≥50% nuclear expression; p65$^{low}$, p65 immunohistochemistry results: <50% nuclear expression; GCB, germinal center B-cell–like; ABC, activated B-cell–like; FDR, false discovery rate. *Upregulated genes in bold.

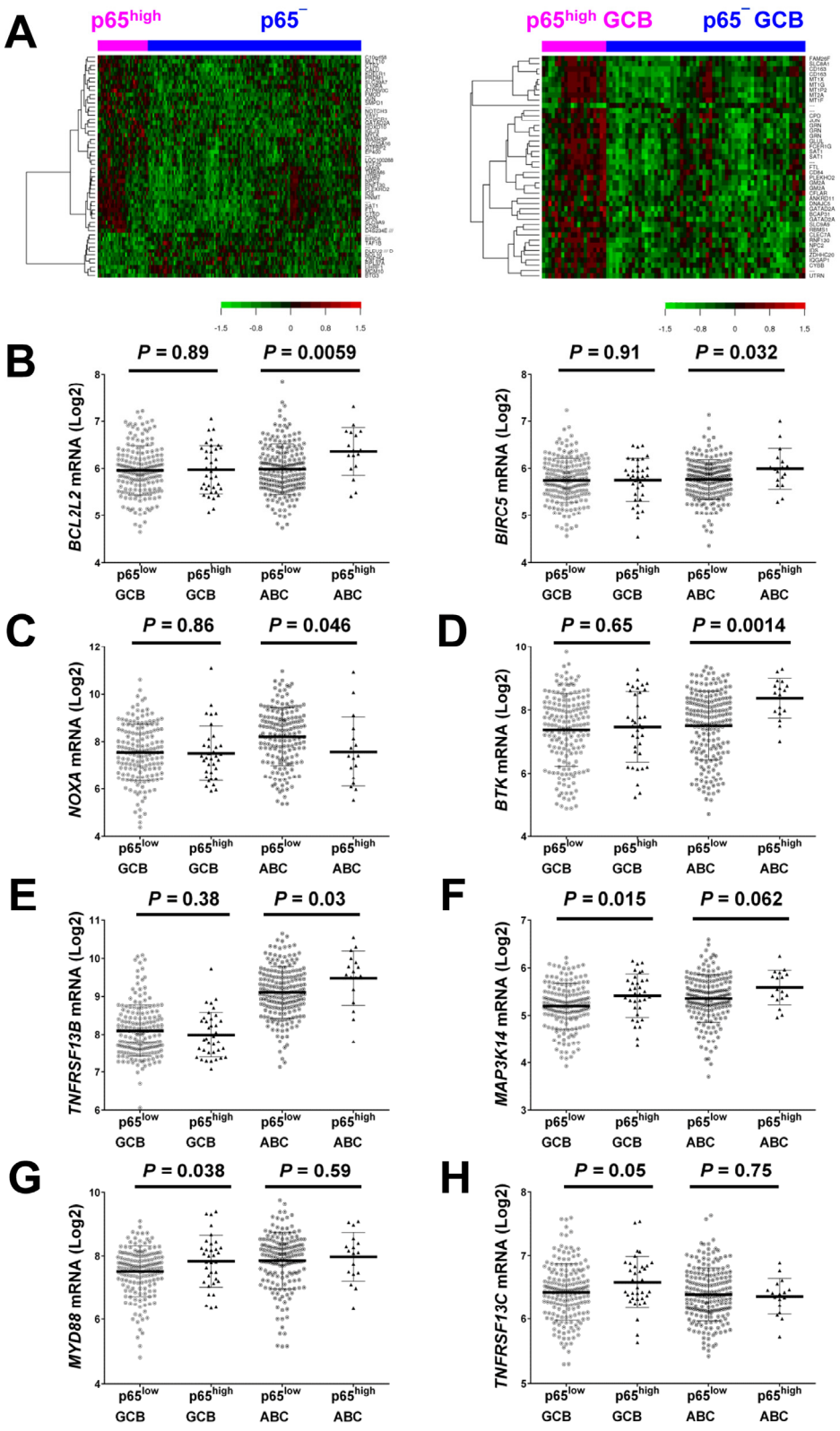


**Figure 3. Gene expression profiling analysis.** (**A**) Heatmaps for comparisons between DLBCL patients with p65$^{high}$ expression (IHC ≥50%) and those without p65 nuclear expression (IHC <10%) in the overall and GCB-DLBCL cohorts (FDR <0.15 and FDR <0.05, respectively). (**B**) *BIRC5/survivin* and *BCL2L2* were significantly upregulated in p65$^{high}$ ABC-DLBCL. (**C**) *NOXA*/*PMAIP1* was significantly downregulated in p65$^{high}$ ABC-DLBCL. (**D**-**E**) *BTK* and *TNFRSF13B* were significantly upregulated in the p65$^{high}$ group in ABC-DLBCL but not in GCB-DLBCL. (**F**-**H**) *MAP3K14/NIK*, *MYD88*, and *TNFRSF13C* were significantly upregulated in the p65$^{high}$ group in GCB-DLBCL but not in ABC-DLBCL.

GEP suggested that in GCB-DLBCL, instead of antiapoptotic mechanisms, dysregulations in immune responses and tumor microenvironment may be relevant for the poor prognosis associated with p65$^{high}$. Such immune signatures included *FCER1G* (Fc fragment of IgE high affinity I receptor for gamma subunit), 2.21-fold upregulation, *CYBB* (critical component of phagocytes generating superoxide), 1.77-fold upregulation, granulin gene *GRN*, 1.63-fold up-regulation, *LILRB1* (a MHC class I receptor resulting in immunosuppression), 1.49-fold upregulation, *CD163* (an antigen exclusively expressed in monocytes and macrophages), 2.46-fold upregulation, and *CD84* (an adhesion molecule involved in regulating receptor-mediated signaling in immune cells), 1.55-fold up-regulation. In the GEP comparison in overall DLBCL, a few immune-related genes were also found up- or down-regulated in p65$^{high}$ DLBCL compared with p65$^{low}$ DLBCL, including upregulation of *LCP2* (lymphocyte cytosolic protein 2, involved in T cell receptor signaling, 1.27-fold) and *TEK* (anti-inflammatory, 1.21-fold), and downregulation of *UHRF1* (an epigenetic regulator promoting proliferation of immunosuppressive Treg cell, 1.48-fold down-regulation) [43] in p65$^{high}$ DLBCL (Table 3).

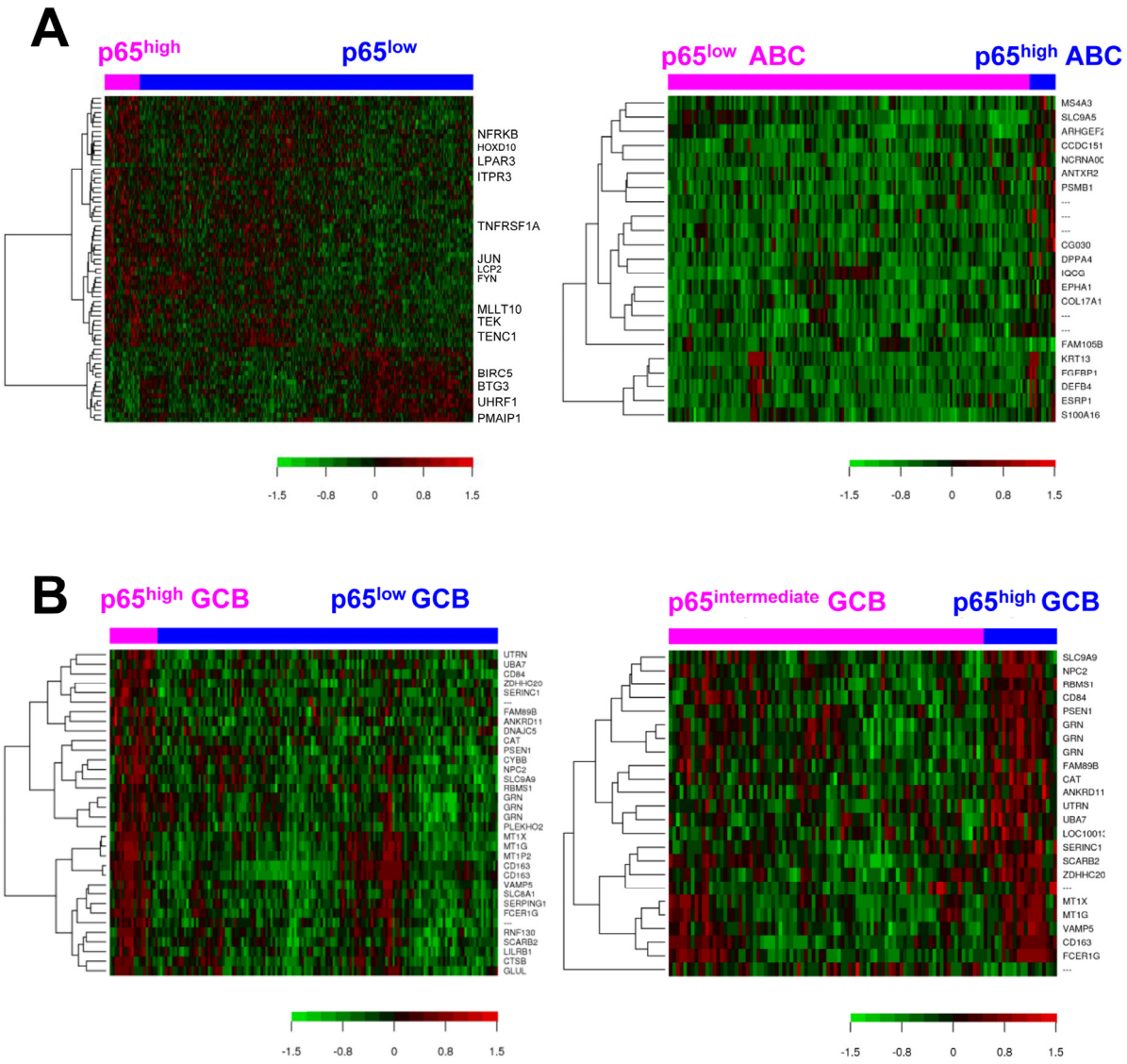


**Figure 4. Gene expression analysis for p65 hyperactivation in diffuse large B-cell lymphoma (DLBCL).** (**A**) Heatmaps for gene differentially expressed between p65$^{high}$ (IHC ≥50%) and p65$^{low}$ (IHC <50%) patients in DLBCL overall and in ABC-DLBCL (false discovery rate <0.30 and <0.20, respectively). (**B**) Heatmaps for genes differentially expressed between p65$^{high}$ (IHC ≥50%) and p65$^{low}$ (IHC <50%) patients and between p65$^{high}$ (IHC ≥50%) and p65$^{intermediate}$ (IHC 10-40%) patients with germinal center B-cell–like DLBCL (false discovery rate <0.05 and <0.20, respectively).