www.impactjournals.com/oncotarget/ Oncotarget, 2017, Vol. 8, (No. 8), pp: 13782-13791

Research Paper

# Clinical impact of serum survivin positivity and tissue expression of EBV-encoded RNA in diffuse large B-cell lymphoma patients treated with rituximab–CHOP

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**Keywords:** *survivin, epstein-barr virus, diffuse large B-cell lymphoma*

***Received:*** *May 14, 2016* ***Accepted:*** *January 03, 2017* ***Published:*** *January 13, 2017*

## ABSTRACT

**Survivin is an inhibitor of apoptosis and is upregulated by Epstein–Barr virus (EBV) latent genes. Given the frequent association of EBV with lymphoid malignancies, survivin is expected to have prognostic value in diffuse large B-cell lymphoma (DLBCL). Thus, we measured the pretreatment serum level of survivin in DLBCL patients and analyzed its association with survival outcome and EBV status, as represented by EBV-encoded RNA (EBER) in DLBCL. Pretreatment serum survivin level was measured in patients registered in a prospective cohort study (*n* = 210), and serum survivin-positivity was defined as any detectable level of survivin. EBV status was determined using EBER *in situ* hybridization, and EBER-positivity was defined as 20% of examined cells showing nuclear positivity. Mean serum survivin level was higher in patients with relapsed or refractory disease than with responsive disease (59.89 pg/mL versus 17.34 pg/mL, *P* = 0.041). Serum survivin-positive patients had worse overall and progression-free survival (*P* = 0.023 and 0.022, respectively). Serum survivin positivity was associated with unfavorable characteristics including stage. In patients with non-germinal center B-cell type DLBCL, serum survivin-positive patients also had significantly worse survival than serum survivin-negative patients (*P* < 0.001). EBER-positivity was found in 6.7% (14/210) of patients, and EBER-positive patients had worse survival (*P* < 0.05). Patients having concomitant positivity for serum survivin and EBER expression (2.8%, 6/210) showed extremely poor prognosis. In the present era of rituximab in DLBCL, DLBCL with serum survivin positivity showed adverse clinical features and followed worse clinical course, especially in non-GCB subtype DLBCL. EBER-positivity was still associated with worse outcomes in DLBCL.**

## INTRODUCTION

Treatment outcomes for diffuse large B-cell lymphoma (DLBCL) have been improved with the addition of rituximab to cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP) chemotherapy [1]. However, up to one-third of patients still develop relapsed or refractory disease after treatment and die because of disease progression [2]. DLBCL is a widely heterogeneous disease with diverse clinical

courses and a variety of molecular aberrations [3]. DLBCL subtypes at higher risk of relapsed or refractory disease include activated B-cell (ABC) subtype DLBCL and DLBCL harboring MYC rearrangement [4, 5]. Epstein–Barr virus (EBV)-positive DLBCL of the elderly has been consistently shown to have poor treatment outcomes in Asian countries [6–8]. Of note, EBV-positive DLBCL of the elderly is more common in Asian populations and is characterized by a higher frequency of the ABC-like immunophenotype and increased activity of the NF-kB and JAK–STAT pathways [7, 9]. In these DLBCL subtypes with a higher risk of relapsed or refractory disease, novel prognostic markers and treatment approaches are expected.

Survivin is a unique member of the inhibitor of apoptosis family and is one of the transcriptional targets of STAT3 and p53 proteins [10–13]. Survivin is overexpressed in diverse cancers such as non-small cell lung cancers, colorectal cancers, and lymphomas [14–17]. Previous lymphoma studies have reported that survivin overexpression, as detected by immunohistochemistry, is associated with poor clinical outcomes of DLBCL, especially ABC subtype DLBCL [17–19]. Interestingly, in terms of the diverse mechanisms of survivin upregulation, preclinical data have shown that survivin can be upregulated by EBV latent genes and is considered to contribute chemoresistance and poor clinical outcomes in EBV-associated malignancies such as EBV-positive gastric cancer and EBV-positive DLBCL [20, 21].

Despite the use of immunohistochemistry in previous studies, survivin protein concentration has not been investigated as a prognostic marker of DLBCL. Therefore, we measured the level of survivin using easily available pretreatment serum samples from DLBCL patients and analyzed the clinical impact of serum survivin level and its possible association with EBV status in DLBCL patients.

## RESULTS

### Characteristics of patients

Survivin was detected (range 1.7–2795.8 pg/mL) in the serum of 26 patients of the 210 patients (12.4%, 26/210). Receiver-operating characteristic (ROC) curve analysis could not find the optimal cut-off value of serum survivin due to low sensitivity and specificity. Patients were dichotomized into survivin-positive and -negative groups according to the presence of survivin in serum. The comparison of patients' characteristics at diagnosis showed that serum survivin positivity was closely associated with poor performance status (ECOG 2–4, $P < 0.001$), more advanced stage (stage III–IV, $P < 0.001$), two or more sites of extranodal involvement ($P < 0.001$), bone marrow involvement ($P < 0.001$), and higher International Prognostic Index (IPI) risk groups (high–intermediate/high, $P < 0.001$). EBER expression was observed in only 14 patients, and the frequency of EBER positivity was significantly higher in serum survivin-positive patients (19.2%, 5/26) than in serum survivin-negative patients (4.9%, 9/184) ($P = 0.018$). Among the 198 patients whose immunophenotype was available, 91 (46.0%) and 107 (54.0%) were classified with the GCB and non-GCB histologic subtypes, respectively. However, the cell of origin did not differ significantly according to serum survivin positivity (Table 1).

### Association of serum survivin positivity and EBER expression with survival outcome

The mean serum survivin level was significantly higher in patients with, than in those without, relapsed or refractory disease (59.89 pg/mL versus 17.34 pg/mL, $P = 0.041$) (Figure 1). The percentage of serum survivin-positive patients was higher in patents with relapsed or refractory disease than in those without it showing borderline significance (18.8% versus 9.2%, $P = 0.072$). The overall 3-year OS and PFS rates of patients were 74.0% and 68.2%, respectively, for a median follow-up duration of 42.2 months (range, 0.3–83.4). Patients with serum survivin positivity showed significantly worse OS and (median OS, both not reached, $P = 0.023$) and PFS (median PFS, 21.1 months versus not reached, $P = 0.022$) compared with patients with serum survivin negativity (Figure 2A and 2B). EBER-positive patients also showed substantially worse OS and (median OS, 14.7 months versus not reached, $P = 0.007$) and PFS (median PFS, 6.9 months versus not reached, $P < 0.001$) compared with EBER-negative patients (Figure 2C and 2D).

### Prognostic value of serum survivin positivity in non-GCB and EBER-positive patients

We performed subgroup analysis according to the cell of origin and EBER expression status. Among 91 DLBCL patients with the GCB subtype, there were no significant differences in OS and PFS between serum survivin-positive and -negative groups (Figure 3A and 3B). However, in the 107 patients with the non-GCB subtype, serum survivin positivity was significantly associated with worse OS (median OS, 6.9 months versus not reached, $P < 0.001$) and PFS (median PFS, 6.9 months versus not reached, $P < 0.001$) compared with patients with serum survivin negativity (Figure 3C and 3D). Although the number of patients was relatively small, the association of serum survivin positivity with survival outcome was analyzed in terms of EBER expression. Serum survivin-positive patients showed a consistent trend of worse survival outcomes irrespective of EBER status (Figure 4A–4D). Of note, the subgroup with concomitant EBER positivity and serum survivin positivity (2.8%, 6/210) showed extremely poor prognosis: median OS and PFS were boths 2.2 months (Figure 4C and 4D).

**Table 1: Baseline characteristic of patients**

| | All patients (*n* = 210) | Serum survivin (–) (*n* = 184) | Serum survivin (+) (*n* = 26) | *P*-value |
|---|---|---|---|---|
| **Age, no. (%)** | | | | 0.204 |
| 60 y or less | 123 (58.6) | 111 (60.3) | 12 (46.2) | |
| Older than 60 y | 87 (41.4) | 73 (39.7) | 14 (53.8) | |
| **Sex, no. (%)** | | | | 0.999 |
| Male | 128 (61.0) | 112 (60.9) | 16 (61.5) | |
| Female | 82 (39.0) | 72 (39.1) | 10 (38.5) | |
| **Performance status, no. (%)** | | | | **< 0.001** |
| ECOG 0–1 | 173 (82.4) | 159 (86.4) | 14 (53.8) | |
| ECOG 2–4 | 37 (17.6) | 25 (13.6) | 12 (46.2) | |
| **Ann Arbor stage, no. (%)** | | | | **< 0.001** |
| Limited, I–II | 103 (49.0) | 102 (55.4) | 1 (3.8) | |
| Advanced, III–IV | 107 (51.0) | 82 (44.6) | 25 (96.2) | |
| **No. of ENI, no. (%)** | | | | **< 0.001** |
| 0 or 1 | 136 (64.8) | 129 (70.1) | 7 (26.9) | |
| 2 or more | 74 (35.2) | 55 (29.9) | 19 (73.1) | |
| **LDH no. (%)** | | | | 0.834 |
| ULN or below | 113 (53.8) | 98 (53.3) | 15 (57.7) | |
| Over ULN | 97 (46.2) | 86 (46.7) | 11 (42.3) | |
| **IPI risk group, no. (%)** | | | | **< 0.001** |
| Low/Low intermediate | 130 (61.9) | 126 (68.5) | 4 (15.4) | |
| High intermediate/High | 80 (38.1) | 58 (31.5) | 22 (84.6) | |
| **B symptom, no. (%)** | | | | **0.003** |
| Negative | 157 (74.8) | 144 (78.3) | 13 (50.0) | |
| Positive | 53 (25.2) | 40 (21.7) | 13 (50.0) | |
| **Bone marrow involvement** | | | | **< 0.001** |
| Negative | 191 (91.0) | 177 (96.2) | 14 (53.8) | |
| Positive | 19 (9.0) | 7 (3.8) | 12 (46.2) | |
| **Bulky disease, no. (%)** | | | | 0.701 |
| No | 193 (91.9) | 168 (91.3) | 25 (96.2) | |
| Yes | 17 (8.1) | 16 (8.7) | 1 (3.8) | |
| **Response to front-lineTx** | | | | 0.543 |
| CR or PR | 181 (86.2) | 157 (86.2) | 24 (92.3) | |
| SD or PD | 29 (13.8) | 27 (13.8) | 2 (7.7) | |
| **EBER status, no. (%)** | | | | **0.018** |
| Negative | 196 (93.3) | 175 (95.1) | 21 (80.8) | |
| Positive | 14 (6.7) | 9 (4.9) | 5 (19.2) | |
| **Cell of origin, no. (%)** | | | | 0.528 |
| GCB subtype | 91 (46.0) | 78 (45.1) | 13 (52.0) | |
| Non-GCB subtype | 107 (54.0) | 95 (54.9) | 12 (48.0) | |

### Prognostic factor analyses

In the univariate analysis, the following clinical factors were associated with worse OS: age older than 60 years ($P < 0.001$), poor performance status (ECOG 2–4, $P < 0.001$), advanced stage (stage III–IV, $P = 0.003$), two or more sites of extranodal involvement ($P = 0.002$), bone marrow involvement ($P = 0.025$), non-GCB histological subtype ($P = 0.007$), serum survivin positivity ($P = 0.023$), and EBER positivity ($P = 0.007$). Multivariate analysis showed that EBER positivity retained its significantly poor prognostic impact for worse OS [hazard ratio (HR) 2.5; 95% confidence interval (CI), 1.1–5.6; $P = 0.025$]. Other independent prognostic factors for worse OS were age older than 60 years (HR 2.4; 95% CI, 1.4–4.1; $P = 0.001$) and poor performance status (ECOG 2–4) (HR 2.8; 95% CI, 1.5–5.1; $P = 0.001$). The multivariate analyses of PFS and OS are summarized in Table 2. Serum survivin positivity lost its independent predictive power for poor prognosis in the multivariate analysis because of strong multicollinearity between survivin positivity and baseline clinical parameters, as shown in Table 1. However, in a multivariate analysis of the 107 DLBCL patients with the non-GCB subtype, serum survivin positivity showed a trend toward an association with worse OS (HR 2.3; 95% CI, 0.9–5.6; $P = 0.067$).

## DISCUSSION

Survivin is a unique member of the inhibitor of apoptosis family and plays roles in both cell survival and cell mitosis in cancer [10, 22]. Previous lymphoma studies have shown that survivin overexpression is associated with poor survival outcomes [17, 19]. However, there are some pitfalls in the interpretation of previous data. The first is the scarcity of data for uniformly R-CHOP-treated DLBCL populations. Many of the previous studies included DLBCL patients who were treated with mainly a CHOP regimen, except for the notable recent data published by Liu et al. about the International Diffuse Large B-cell Lymphoma Rituximab-CHOP Consortium Program [18]. The second pitfall is the absence of uniform criteria for defining survivin expression positivity using immunohistochemistry. Some reports used a value greater than 5% as the survivin-positive cut-off, whereas others have used

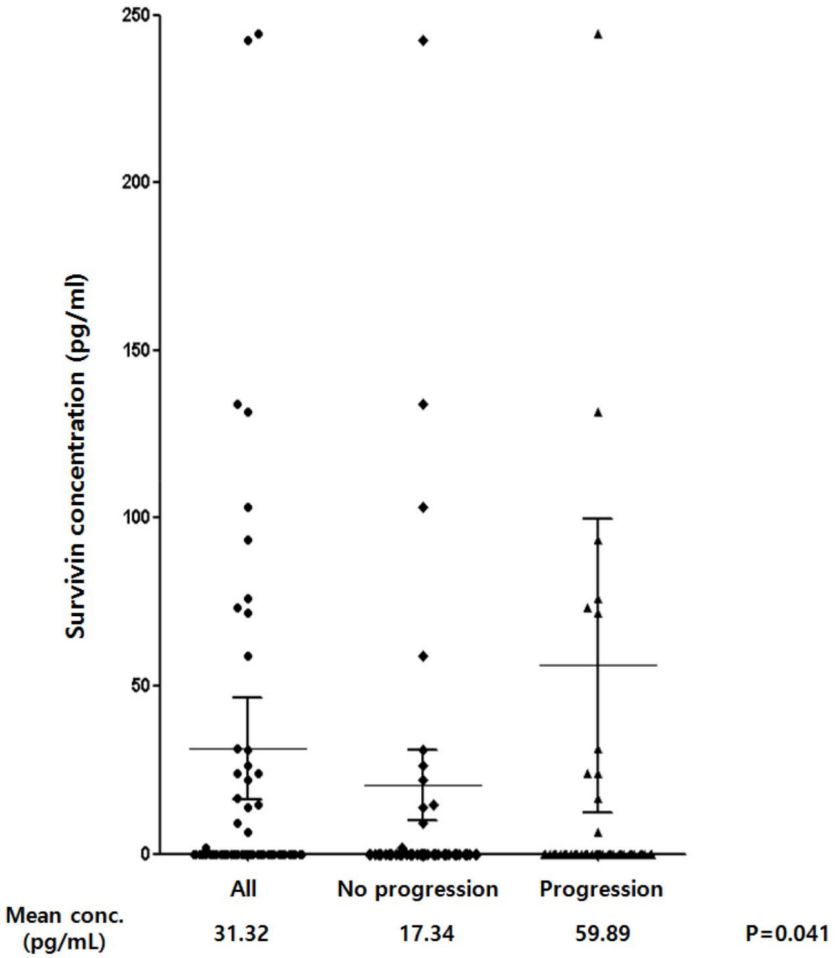


**Figure 1: Serum levels of survivin in all patients ($n = 210$), patients without relapsed or refractory disease ($n = 146$), patients with relapsed or refractory disease ($n = 64$).**

**Table 2: Multivariate analysis of survival**

| | PFS | | | OS | | |
|---|---|---|---|---|---|---|
| | HR (95% CI) | | *P* | HR (95% CI) | | *P* |
| **EBER status** | | | **0.001** | | | **0.025** |
| Negative | 1 | | | 1 | | |
| Positive | 3.4 | (1.7–7.0) | | 2.5 | (1.1–5.6) | |
| **Age** | | | **0.003** | | | **0.001** |
| 60 y or less | 1 | | | 1 | | |
| Older than 60 y | 2.1 | (1.3–3.4) | | 2.4 | (1.4–4.1) | |
| **Sex** | | | 0.500 | | | 0.925 |
| Female | 1 | | | 1 | | |
| Male | 0.8 | (0.5–1.4) | | 1.0 | (0.6–1.7) | |
| **ECOG performance** | | | **0.007** | | | **0.001** |
| 0–1 | 1 | | | 1 | | |
| 2–4 | 2.2 | (1.2–3.9) | | 2.8 | (1.5–5.1) | |
| **No. of ENI** | | | **0.013** | | | 0.150 |
| 0 or 1 | 1 | | | 1 | | |
| 2 or more | 2.1 | (0.7–2.6) | | 1.6 | (0.9–3.0) | |
| **Stage** | | | 0.369 | | | 0.552 |
| Limited, I–II | 1 | | | 1 | | |
| Advanced, III–IV | 1.3 | (0.7–2.6) | | 1.3 | (0.6–2.5) | |

cut-offs of 10%, 25%, 30%, or 45% [17–19, 23, 24]. For localizing survivin immunostaining, some reports have used cytoplasmic survivin positivity, whereas others have used nuclear positivity, mixed-type positivity, or an immunoreactivity scoring system [17–19, 23, 24]. Thus, these previous studies have reported highly variable percentages of survivin-positive DLBCL in the range of 39.3% to 84.9% [19]. Given these pitfalls, we performed this study using survivin protein concentration in easily available serum samples from DLBCL patients who were uniformly treated with R-CHOP chemotherapy.

This study revealed that serum survivin positivity was more prevalent in patients with poor performance status, more advanced stage, two or more sites of extranodal involvement, bone marrow involvement, and higher IPI risk. Liu et al. recently reported similar results that survivin expression was associated with a higher IPI risk score, higher number of extranodal disease, and higher Ki-67 index [18]. Meta-analysis also showed that positive survivin expression was associated with inferior OS and there was a significant association between survivin expression and advanced clinical stage (III and IV), higher IPI score (3–5), elevated serum LDH, presence of bone marrow involvement [19]. However, the biological mechanisms underlying release of survivin from the cell to peripheral blood are not completely understood. The distinct adverse clinical features shown in patients with serum survivin positivity suggest that serum survivin might indicate high tumor burden.

This study showed that serum survivin positivity was significantly associated with inferior survival outcomes in DLBCL patients who were uniformly treated with R-CHOP chemotherapy. Notably, subgroup analysis according to histological subtype suggested that serum survivin positivity had a more pronounced prognostic impact in patients with the non-GCB subtype DLBCL. These results are similar to those reported recently by Liu et al. [18]. Considering the unmet need for additional effective treatment for ABC subtype DLBCL, these results suggests that survivin might be a useful prognostic marker and a therapeutic target in ABC subtype DLBCL.

We also investigated possible associations between serum survivin positivity and EBER status based on preclinical evidence that survivin can be upregulated by EBV latent genes and can contribute to poor clinical outcomes of EBV-associated malignancies [21]. The percentage of EBER positive patients was significantly higher in the serum survivin-positive group than in the serum survivin-negative group (19.2% versus 4.2%). This study also reaffirmed that EBER positivity had a poor prognostic impact on DLBCL in the rituximab era. Patients with concomitant EBER positivity and serum survivin positivity showed extremely poor prognosis, with median OS and PFS both of 2.2 months.

However, this study have several limitations. To have a stronger evidence of survivin as a prognostic marker or a therapeutic target, further validation researches are needed to investigate the correlation between positive

serum survivin and survivin expression in the tumor tissue. The large number of serum survivin-negative patients can skew the difference of serum survivin level between patients with or without relapsed or refractory disease. Total number of EBV-positive DLBCL patients with concurrent positive serum survivin was small. So, we should cautiously interpretate the results and the results need to be confirmed on a larger cohort. Loss of independent prognostic power of serum survivin positivity in the whole population limit the universal application of serum survivin as a prognostic marker in DLBCL. However, in the subgroup analysis of non-GCB subtype, we found a more pronounced prognostic impact of serum survivin (Figure 3) and strong trend with borderline statistical significance (HR 2.3 *P*-value = 0.067) in the multivariate analysis. This suggests serum survivin is still noteworthy for further investigation as a prognostic marker especially in non-GCB subtype DLBCL patients.

A selective survivin suppressant, YM155, demonstrates potent antitumor activities in a wide variety of cell lines and xenograft models, including lymphomas [25–27]. The first phase I clinical trial of YM155 has been published and has reported that YM155 produced an objective response in three of five patients with non-Hodgkin lymphoma [28]. In a phase II clinical trial, single-agent YM155 was well tolerated but demonstrated minimal activity in refractory DLBCL with a response rate of 2.4% [29]. However, preclinical data have demonstrated promising synergistic effects of YM155 when combined with rituximab, rituximab plus bendamustine, or a STAT3 inhibitor [30–32]. Further clinical trials using combination regimens with YM155 in the treatment of lymphomas are expected.

In summary, DLBCL patients with serum survivin positivity showed distinct adverse clinical features and followed a significantly worse clinical course, especially in those with non-GCB subtype DLBCL. In this rituximab era, EBER positivity remains a predictor of poor prognosis for patients with DLBCL. Our findings also suggest that further studies are needed to examine the feasibility of

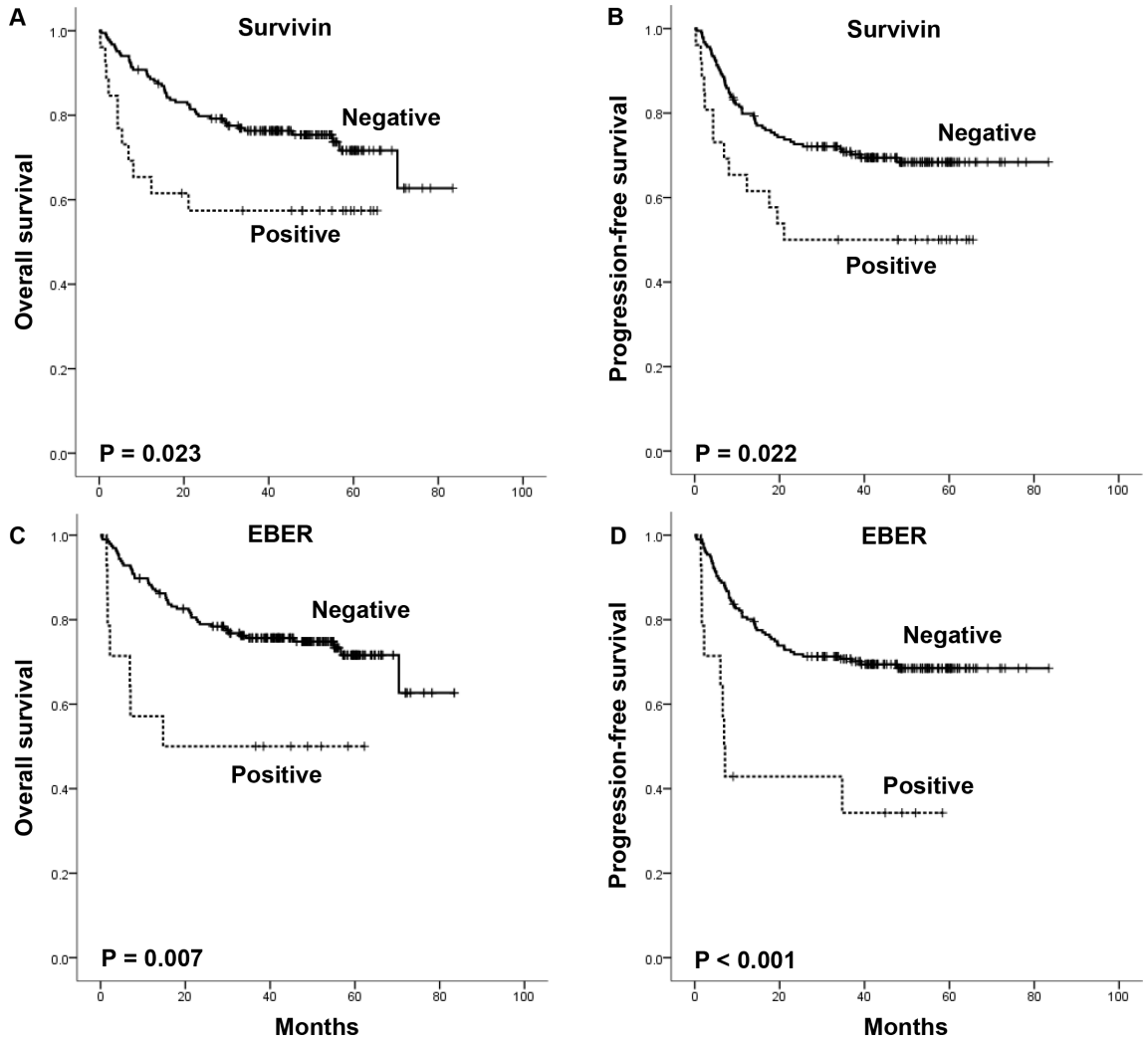


**Figure 2: Overall survival and progression-free survival of the patients according to serum survivin positivity and EBER status.**

using survivin as a therapeutic target in DLBCL patients with the subtypes having higher risk of relapsed or refractory disease, such as ABC subtype DLBCL and EBV-positive DLBCL of the elderly.

## MATERIALS AND METHODS

### Patients

This study analyzed samples from DLBCL patients enrolled in our prospective cohort study between September 2008 and December 2011 (NCT#00822731). Using the following inclusion criteria, we selected 210 patients. (1) Patients should be pathologically confirmed as having DLBCL according to the World Health Organization (WHO) classification. (2) Patients should have chemotherapy-naïve, newly diagnosed DLBCL and receive R-CHOP chemotherapy as their first treatment. (3) Patients should have available pretreatment serum samples collected at diagnosis for measurement of serum survivin level. (4) Patients should have an adequate amount and quality of paraffin-embedded biopsy specimens or unstained slides for EBV-encoded RNA (EBER) *in situ* hybridization (ISH). The clinical data including disease and survival status were updated in September 2015, and the protocol was approved by the Samsung Medical Center Institutional Review Board. Written informed consent was obtained from the patients for enrollment in the prospective cohort study and the use of samples for research.

### Pathology review

The pathology of the DLBCL cases was confirmed by an expert hematopathologist (Y.H.K.) using the WHO classification. To determine the cell of origin of DLBCL, immunohistochemical staining was performed in formalin-fixed paraffin-embedded specimens using a panel of monoclonal antibodies against CD10 (Dakopatts, Copenhagen, Denmark), BCL-6 (Dakopatts), and MUM-1 (Dakopatts). Stained slides were reviewed, and the cell

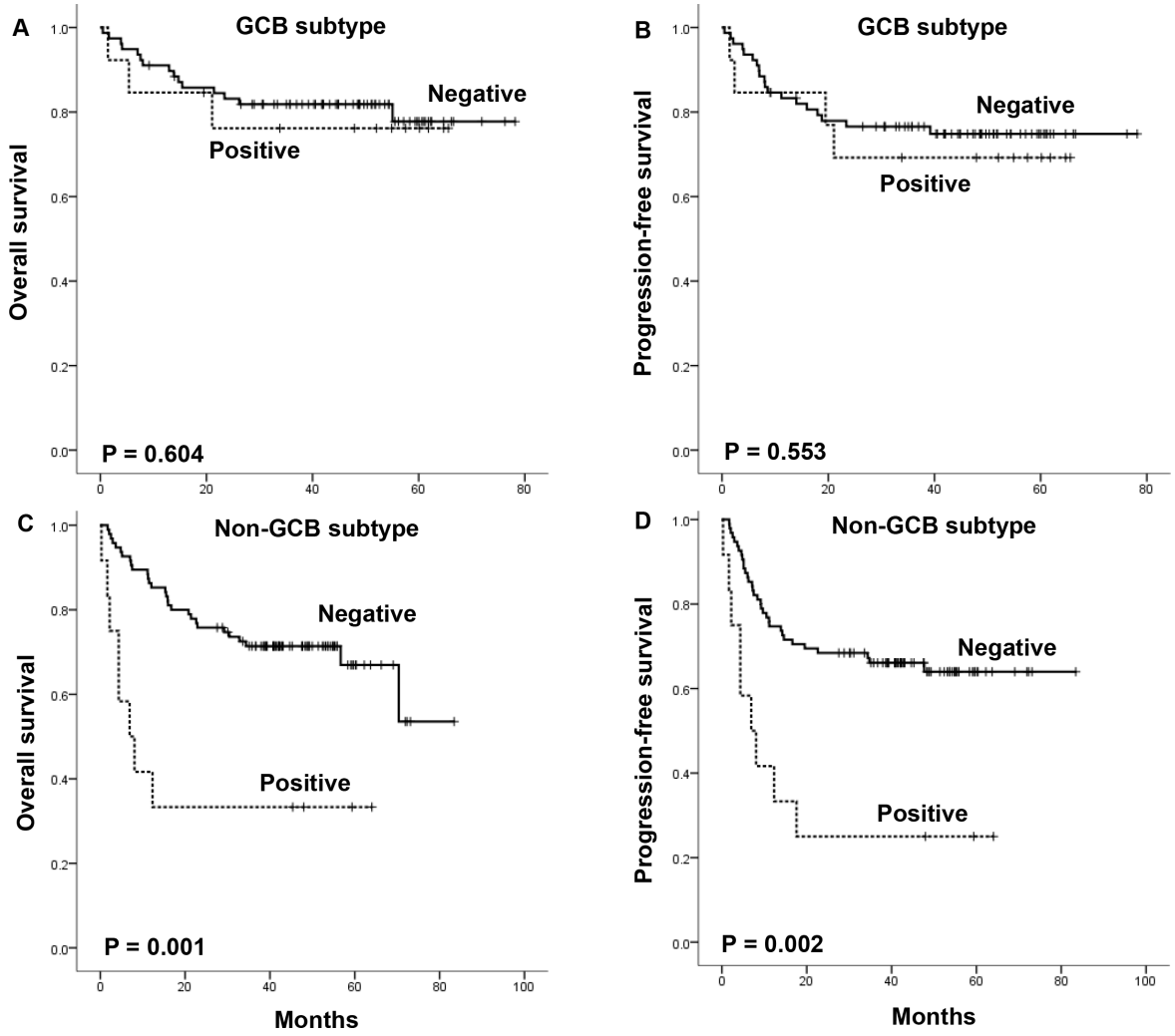


**Figure 3: The impact of serum survivin positivity on overall survival and progression-free survival according to cell of origin of diffuse large B-cell lymphoma.**

of origin was determined by expert hematopathologists (M.H. and Y.H.K.) according to the results of immunohistochemistry. Thus, patients were classified as having the germinal center B-cell-like (GCB) or non-GCB subtype based on the Hans algorithm, as proposed previously [33]. EBER was detected using ISH and an EBV ISH kit (Leica Microsystems, Bannockburn, IL, USA). We used EBV-negative lymphoid tissues and the hybridization mixture without EBV oligonucleotides as negative controls. A positive reaction was defined as more than 20% of examined cells showing nuclear positivity, as applied in our previous series [6, 7, 34].

### Serum survivin assay with archived serum samples

Serum survivin concentration was measured in archived frozen samples of the aforementioned prospective cohort study. Archived serum sample aliquots had been stored at –80°C and were thawed before use in the cytokine assay. The concentration of survivin, an antiapoptosis protein, was measured in serum using the Procarta cytokine profiling kit (Panomics, San Diego, CA, USA), and all measurements were performed in duplicate according to the manufacturer's instructions.

### Statistical analysis

Intergroup comparisons were performed using Fisher's exact test for categorical variables. Progression-free survival (PFS) was calculated from the date of diagnosis to the first day of disease progression, relapse, or death from any cause. Overall survival (OS) was calculated from the date of diagnosis to death. PFS and OS were censored on the last date of follow-up. Survival curves were estimated by Kaplan–Meier method, and the survival distributions were compared using the log-rank test. Multivariate analysis was performed using Cox regression analysis. *P*-values less than 0.05 were considered to be significant, and two-sided tests were used in all calculations. Statistical analyses were performed using the software package IBM PASW version 18.0 (SPSS Inc., Chicago, IL, USA).

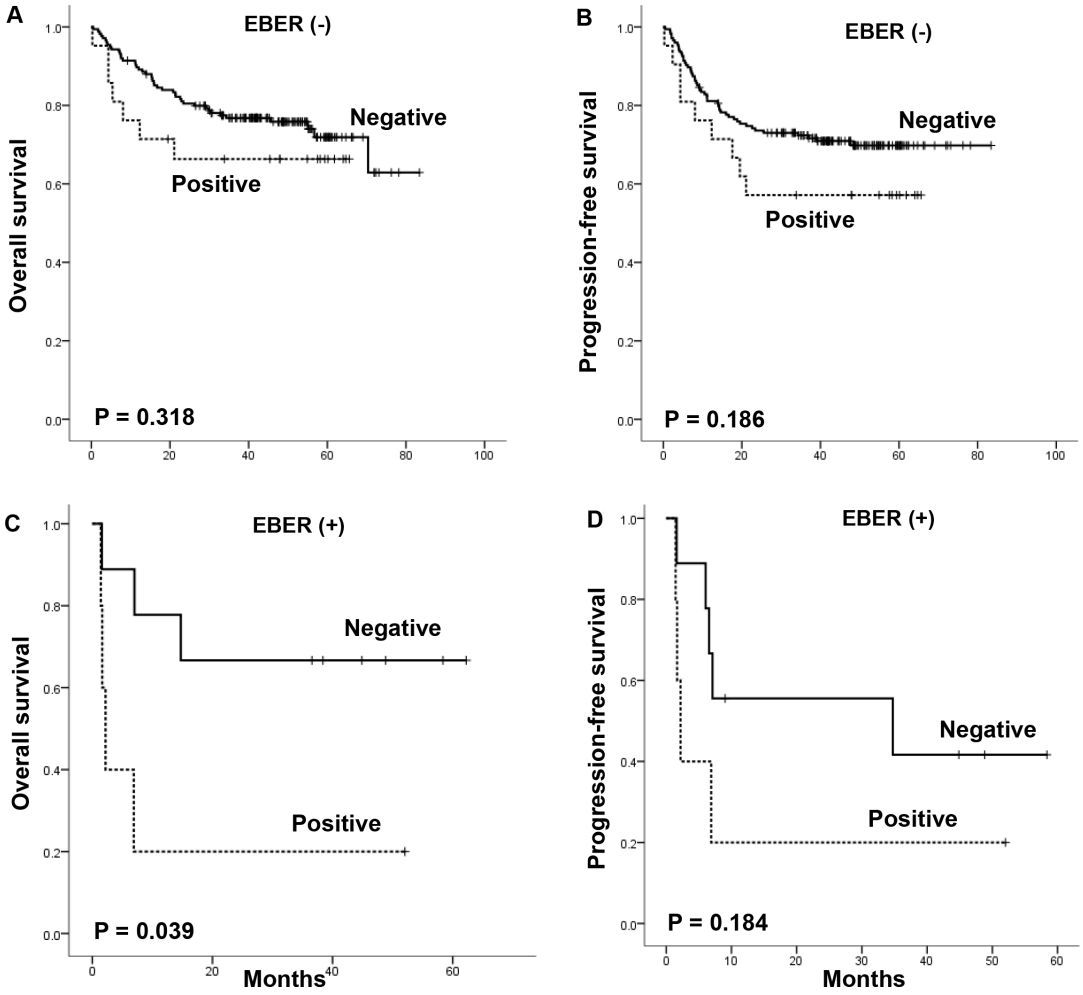


**Figure 4: The impact of serum survivin positivity on overall survival and progression-free survival according to EBER status.**

## ACKNOWLEDGMENTS

We specially express out appreciation to Won Seog Kim for sharing his ideas with us during the course of this research.

## CONFLICTS OF INTEREST

There is no conflicts of interest that all authors should disclose.

## FUNDING

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2014R1A2A1A11049853).

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