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Regular Article

Immunohistochemical Mapping of Bcl9 Using Two Antibodies that Recognize Different Epitopes Is Useful to Characterize Juvenile Development of Hepatocellular Carcinoma in Myanmar

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B-cell lymphoma 9 (Bcl9) is the core component of Wnt/β-catenin signaling and overexpressed in nuclei of various tumors, including hepatocellular carcinoma (HCC). However, the extent of Bcl9 expression relative to HCC differentiation stage and its functional aspects are poorly understood. In this study, we examined the expression pattern of Bcl9 immunohistochemically, using two anti-Bcl9 antibodies; one was a conventional polyclonal-antibody (anti-Bcl9^{ABC}) against amino acid no.800–900 of human-Bcl9, while the other (anti-Bcl9^{BIO}) was against amino acid no.50–200, covering Pygopus-binding sites of Bcl9. Immunohistochemistry using anti-Bcl9^{BIO} demonstrated distinctive staining in the cytoplasm, while the anti-Bcl9^{ABC} signal was detected in both cytoplasm and nuclei of HCC cells, reflecting different states of Bcl9 function because Pygopus-binding to Bcl9 is essential to exert its function together with β-catenin in nucleus. Quantitative analysis revealed a significantly higher immunohistochemical-score by anti-Bcl9^{BIO} in normal liver comparing various differentiation grades of HCC ($P < 0.004$), whereas no significant difference was noted with anti-Bcl9^{ABC}. Interestingly, immunohistochemical-score of anti-Bcl9^{BIO} in patients aged < 40 years was significantly lower than that of ≥ 40 years group ($P < 0.01$). The results indicated that anti-Bcl9^{BIO} detected cytoplasmic Bcl9, which does not bind to Pygopus suggesting it could be a useful indicator for development of HCC in young Myanmar patients.

Key words: Bcl9, Pygopus, β-catenin, juvenile, hepatocellular carcinoma

I. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third leading cause of cancer-related mortality in the Asia-Pacific region [14, 31]. Liver cancer rates are the highest in East and Southeast Asia and Northern and Western Africa [27]. In Myanmar, a Southeast Asia country, where the prevalence of HCC is highly reported and more importantly, the juvenile development and poor prognosis of HCC in the young generation is common, where a heavy deposition and uptakes of excess iron was reported [22]. Subsequent studies from our laboratories presented that iron overload accelerated the liver cells kinetics abnormally [2], though the etiopathological mechanisms to induce juvenile development of HCC remain to be elucidated.

Abnormal upregulation of the canonical Wnt/β-catenin

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signaling pathway was found to play a role in the development of certain types of cancers, such as gastric, colorectal and breast cancer including HCC. β-catenin is a key effector and plays a fundamental role in this pathway [10, 20]. In the absence of Wnt ligands, β-catenin is phosphorylated and degraded by a destruction complex which consists of adenomatous polyposis coli (APC), Axin, glycogen synthase kinase-3β (GSK3β) and casein kinase 1α [9, 15]. However, the presence of the ligand disrupts the destruction, allowing the unphosphorylated β-catenin to be translocated into the nucleus. Then, nuclear β-catenin binds to the TCF transcription factor and activates the expression of various genes such as c-Myc and Cyclin D1 which are needed to be involved in cell proliferation, cell migration and cell survival [11, 26].

Bcl9 (*B cell lymphoma 9* gene) and Pygopus are core components of β-catenin/TCF complex and indispensable to Wnt/β-catenin signaling. The domain structure of Bcl9 includes five homology domains (HD1 to HD5) where HD1 to HD3 are highly conserved between *Drosophila*, zebrafish and mammals [16], whereas HD4 and HD5 are highly conserved among mice, humans and vertebrates [1]. Bcl9 is translocated to nucleus by Pygopus and functions as an adaptor protein between Pygopus and β-catenin [16]. Overexpression of Bcl9 increases cell proliferation, migration, invasion and metastatic potential of tumor cells and was reported in various types of tumors such as colorectal cancer, multiple myeloma and HCC [5, 21].

Recent studies demonstrated the nuclear localization of Bcl9 in various tumors, such as multiple myeloma, breast cancer, colon carcinoma and HCC cells unlike their normal cell counterparts. This finding seems to be consistent with the function of Bcl9 as a co-activator of β-catenin in the nucleus. [18, 24]. However, Bcl9 was also reported to localize in both cytoplasm and nucleus, irrespective of the normal and malignant status of adrenocortical cells [6]. In addition, Bcl9 is known to be involved in enamel production and lens development, independently of β-catenin signalling [7, 8]. Consequently, the intracellular localization of Bcl9 is currently a controversial issue, mainly because it may form different complexes [25], reflecting a different functional state. Therefore, for a better understanding of Bcl9 function, we need to analyse the different epitopes by immunohistochemistry.

In the present study, we mapped the localization of Bcl9 in Myanmar HCC immunohistochemically using two different antibodies. The anti-Bcl9^{BIO} antibody recognizes the Pygopus-binding domain of Bcl9, an epitope essential for the nuclear localization of Bcl9 and could be masked by the binding of Pygopus. The other one was anti-Bcl9^{ABC}, which can react with the different epitopes of Bcl9 and had been widely used in the previous studies [12, 30]. Finally, we analysed the expression profiles of Bcl9 in differentiation grades of Myanmar HCC and clinicopathological parameters to evaluate the diagnostic value of Bcl9.

II. Materials and Methods

Chemicals and antibodies

3-Aminopropyltriethoxysilane (APS), bovine serum albumin (BSA, essentially fatty acid- and globulin-free), 30% Brij® L23 solution, normal goat IgG and rabbit IgG were purchased from Sigma-Aldrich (St. Louis, MO, USA). Normal mouse IgG was from Dako (Glostrup, Denmark). Mouse monoclonal anti-human Bcl9 (BMR00368; 2.0 μg/ml) was a gift from Bio Matrix Research Inc. (Nagareyama, Chiba, Japan) and rabbit polyclonal anti-human Bcl9 (ab37305; 5.0 μg/ml) was purchased from Abcam (Cambridge, MA, USA) [12, 30]. Mouse monoclonal anti-β-catenin (CTNNB1, UM500015; 1.33 μg/ml) was purchased from OriGene (Rockville, MD, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (AP308P; 10.0 μg/ml) and HRP-conjugated goat anti-rabbit IgG (AP307P; 10.0 μg/ml) antibodies were from Millipore (Temecula, CA, USA). 3,3'-Diaminobenzidine-4HCl (DAB) was from Dojin Chemical Co. (Kumamoto, Japan). Permount was purchased from Thermo Fisher Scientific (Hudson, NH, USA). All other reagents used in this study were from Wako Pure Chemicals (Osaka, Japan).

Clinical samples

Surgically resected liver tissues were collected from patients diagnosed with HCC, who were treated surgically at the Yangon Specialty Hospital (YSH), Myanmar and fixed with 10% formalin and embedded in paraffin using standard procedures. Normal liver samples, resected away from the tumor lesions were also collected and processed. A total of 52 liver specimens were collected (Age; 20–82 years, mean ± SD; 53.2 ± 9.1, 31 (60%) male and 21 (40%) female). Data on hepatitis B virus (HBV) and hepatitis C virus (HCV) were obtained by the rapid diagnostic test from Standard Diagnostics (SD, Inc., Abbott Laboratories, Chicago, IL, USA) for HBV (SD Bioline HBsAg) and HCV (SD Bioline HCV) (Tables 1 and 2).

Ethical approval for this study was obtained from the Ethics Review Committee of the Department of Medical Research, Yangon (Ethics/DMR/2018/059) and all liver specimens were collected after obtaining written informed consent of the patients in accordance with the Declaration of Helsinki.

Histopathological examination

Liver specimens were cut into 5 μm thickness serial sections and mounted on APS-coated slides. Using hematoxylin and eosin (H&E) stained slides, histopathological examination was conducted by two pathologists, blinded to the patients' basic and clinical data at Pathology Research Division, Department of Medical Research (DMR), Yangon using WHO classification [4]. The results were later confirmed by a Japanese pathologist who was also blinded to the clinical background. Of the 52 examined samples, 11 (21%) were considered normal liver and 41 (79%)

Table 1. *Clinicohistopathological features of the patients (sorted by histological classification)*

Patient number	Age	Sex	Histological classification	HBV	HCV	Anti-Bcl9^{BIO}	Anti-Bcl9^{ABC}	β-catenin
1	46	F	Normal	−	+	++	−	++
2	47	M	Normal	+	−	+++	+++	+++
3	49	F	Normal	NA	NA	++	+++	+++
4	50	M	Normal	NA	NA	+++	+++	+++
5	50	F	Normal	+	−	+++	+	++
6	52	M	Normal	NA	NA	++	+	++
7	54	F	Normal	−	−	+++	+++	+++
8	55	F	Normal	+	−	+++	+++	+++
9	58	F	Normal	−	−	++	++	+++
10	58	M	Normal	−	+	+	+++	++
11	62	M	Normal	NA	NA	++	−	−
12	37	F	Well	−	+	+	++	+
13	41	F	Well	+	−	+	−	−
14	46	M	Well	−	+	−	++	+++
15	48	M	Well	+	−	++	++	+++
16	48	F	Well	+	−	++	+	−
17	51	M	Well	+	−	+	+	−
18	51	M	Well	NA	NA	++	++	+
19	55	F	Well	+	−	++	+++	+++
20	58	F	Well	−	−	+	+	+
21	64	M	Well	−	+	+	+	+++
22	64	M	Well	+	−	++	+++	+++
23	65	F	Well	+	−	−	+	−
24	71	M	Well	−	−	+	−	++
25	20	F	Moderate	+	−	−	++	+++
26	26	M	Moderate	+	−	−	++	++
27	39	M	Moderate	+	−	+	++	++
28	40	M	Moderate	+	−	−	+	+
29	47	M	Moderate	−	+	++	++	+
30	48	F	Moderate	−	+	+	−	+
31	53	F	Moderate	−	+	−	++	++
32	53	F	Moderate	NA	NA	++	+	−
33	56	M	Moderate	+	−	+	+++	+++
34	62	F	Moderate	+	−	+	++	++
35	63	F	Moderate	−	+	−	+	−
36	64	M	Moderate	−	+	−	−	+++
37	76	M	Moderate	−	−	−	++	−
38	77	F	Moderate	NA	NA	++	+	+
39	82	M	Moderate	NA	NA	+	++	++
40	37	M	Poor	−	+	−	+++	+
41	38	M	Poor	+	−	−	−	+
42	42	M	Poor	+	−	−	+++	++
43	43	M	Poor	+	+	+	++	−
44	50	M	Poor	NA	NA	−	+	−
45	51	M	Poor	+	+	−	−	+
46	51	M	Poor	+	NA	−	+	+
47	58	M	Poor	−	+	−	++	+++
48	59	F	Poor	+	−	++	++	++
49	62	F	Poor	+	−	−	++	+++
50	62	M	Poor	−	+	++	++	+
51	64	M	Poor	+	−	+	++	+
52	65	M	Poor	+	−	+	++	−

M, male patient; F, female patient; HBV, hepatitis B virus; HCV, hepatitis C virus; normal, normal liver; well, well differentiate HCC; moderate, moderately differentiated HCC; poor, poorly differentiated HCC; NA, not applicable; −, no staining (negative in HBV and HCV); +, weak staining (positive in HBV and HCV); ++, moderate staining; +++, strong staining.

Table 2. *Clinicohistopathological features of the patients (sorted by age)*

Patient number	Age	Sex	Histological classification	HBV	HCV	Anti-Bcl9^{BIO}	Anti-Bcl9^{ABC}	β-catenin
1	20	F	Moderate	+	−	−	++	+++
2	26	M	Moderate	+	−	−	++	++
3	37	F	Well	−	+	+	++	+
4	37	M	Poor	−	+	−	+++	+
5	38	M	Poor	+	−	−	−	+
6	39	M	Moderate	+	−	+	++	++
7	40	M	Moderate	+	−	−	+	+
8	41	F	Well	+	−	+	−	−
9	42	M	Poor	+	−	−	+++	++
10	43	M	Poor	+	+	+	++	−
11	46	F	Normal	−	+	++	−	++
12	46	M	Well	−	+	−	++	+++
13	47	M	Normal	+	−	+++	+++	+++
14	47	M	Moderate	−	+	++	++	+
15	48	M	Well	+	−	++	++	+++
16	48	F	Well	+	−	++	+	−
17	48	F	Moderate	−	+	+	−	+
18	49	F	Normal	NA	NA	++	+++	+++
19	50	M	Normal	NA	NA	+++	+++	+++
20	50	F	Normal	+	−	+++	+	++
21	50	M	Poor	NA	NA	−	+	−
22	51	M	Well	+	−	+	+	−
23	51	M	Well	NA	NA	++	++	+
24	51	M	Poor	+	+	−	−	+
25	51	M	Poor	+	NA	−	+	+
26	52	M	Normal	NA	NA	++	+	++
27	53	F	Moderate	−	+	−	++	++
28	53	F	Moderate	NA	NA	++	+	−
29	54	F	Normal	−	−	+++	+++	+++
30	55	F	Normal	+	−	+++	+++	+++
31	55	F	Well	+	−	++	+++	+++
32	56	M	Moderate	+	−	+	+++	+++
33	58	F	Normal	−	−	++	++	+++
34	58	M	Normal	−	+	+	+++	++
35	58	F	Well	−	−	+	+	+
36	58	M	Poor	−	+	−	++	+++
37	59	F	Poor	+	−	++	++	++
38	62	M	Normal	NA	NA	++	−	−
39	62	F	Moderate	+	−	+	++	++
40	62	F	Poor	+	−	−	++	+++
41	62	M	Poor	−	+	++	++	+
42	63	F	Moderate	−	+	−	+	−
43	64	M	Well	−	+	+	+	+++
44	64	M	Well	+	−	++	+++	+++
45	64	M	Moderate	−	+	−	−	+++
46	64	M	Poor	+	−	+	++	+
47	65	F	Well	+	−	−	+	−
48	65	M	Poor	+	−	+	++	−
49	71	M	Well	−	−	+	−	++
50	76	M	Moderate	−	−	−	++	−
51	77	F	Moderate	NA	NA	++	+	+
52	82	M	Moderate	NA	NA	+	++	++

M, male patient; F, female patient; HBV, hepatitis B virus; HCV, hepatitis C virus; normal, normal liver; well, well differentiate HCC; moderate, moderately differentiated HCC; poor, poorly differentiated HCC; NA, Not applicable; −, no staining (negative in HBV and HCV); +, weak staining (positive in HBV and HCV); ++, moderate staining; +++, strong staining.

diagnosed as HCC. With regard to the histopathological grade, 13 cases were graded as well differentiated, 15 cases as moderately and 13 cases as poorly differentiated HCC.

Immunohistochemistry

Enzyme immunohistochemistry (IHC) was performed on paraffin sections of HCC as described previously [3, 17, 23]. All procedures were performed at room temperature. Briefly, the paraffin sections were deparaffinized with toluene and autoclaved in 10 mM citrate buffer (pH 6.0, 120°C for 15 min). Endogenous peroxidase was inactivated with 0.3% H_2O_2 in methanol for 15 min. Then, the sections were preincubated with normal goat IgG (500 μg/ml) in 1% BSA in PBS for 1 h and then reacted with the primary antibody dissolved in 1% BSA in PBS for overnight. After washing three times with 0.075% Brij 35 in PBS for 10 min each, the sections were reacted with HRP-conjugated secondary antibody for 1 hr. Then, the sections were washed again with 0.075% Brij 35 in PBS and the sites of HRP were visualized with DAB and H_2O_2 with nickel and cobalt ions. In each experimental run, the adjacent sections were reacted with normal mouse or rabbit IgG in the place of specific primary antibody and served as negative control. Finally, the sections were dehydrated and mounted.

Quantitative analysis

The stained slides were judged as positive or negative based on comparison with the negative control results. The staining intensity was graded as 0, 1, 2 and 3 representing negative, weak, moderate and strong staining, respectively, and used as immunohistochemical-score (IHC-score) for statistical analysis.

Statistical analysis

All data were expressed as mean ± SEM. The Wilcoxon-Mann-Whitney test was used for assessment of the statistical significance between age and sex groups. One-way ANOVA followed by Tukey's range test was used for comparison of statistical significance between histopathological classifications. P value < 0.05 denoted the presence of a statistically significant difference. All analyses were performed with KaleidaGraph 4 (Hulinks Inc., Tokyo, Japan).

III. Results

Immunohistochemical characterization of anti-Bcl9^{BIO} and anti-Bcl9^{ABC} antibodies

To discriminate the states of Bcl9, we used two antibodies that recognize different portions of Bcl9 as epitopes. One is a monoclonal anti-Bcl9 antibody from Bio Matrix Research Inc. (anti-Bcl9^{BIO}), which was raised against the recombinant protein, no.50–200 amino acid (aa) residues of human-Bcl9 because the residue covers the major part of Pygopus-binding domain of Bcl9 (HD1, aa no.177–204) [24], it was expected that the association of Pygopus with HD1 might disturb the binding of anti-Bcl9^{BIO} to Bcl9. The other was rabbit polyclonal anti-Bcl9 antibody purchased from Abcam (anti-Bcl9^{ABC}), which was raised against synthetic peptide carrying aa no.800–900 of human-Bcl9, which is located near the N-terminus of HD4 (aa no.997–1048) of Bcl9. The latter had been used for immunohistochemical examination of tumors, including HCC, in previous studies reported by other groups [12, 30] (Fig. 1A).

As shown in Fig. 1B, immunohistochemical staining using anti-Bcl9^{BIO} revealed the signals for Bcl9 mainly in the cytoplasm but not in the nuclei of well differentiated HCC cells. In comparison, immunostaining with anti-Bcl9^{ABC} detected the signals in either the cytoplasm or the nuclei, or both in adjacent serial sections that were used for anti-Bcl9^{BIO}. When we examined the expression of β-catenin, the signals was limited to the plasma membrane and no convincing co-localization with Bcl9. In addition, tissues stained with normal IgG in place of the primary antibody presented no staining.

Expression profile of Bcl9 detected by two antibodies in various types of histopathological grades of Myanmar HCC

Although overexpression of Bcl9 in the cytoplasm and nuclei was reported in HCC with the use of anti-Bcl9^{ABC} [18, 30], our understanding of the functional status has been limited. To evaluate the biological significance of Bcl9 in HCC, we performed immunohistochemical staining using the two types of anti-Bcl9 antibodies in Myanmar HCC specimens and compared the expression profiles according to the histopathological differentiation grades. As shown in Fig. 2, Bcl9 detected by anti-Bcl9^{BIO} was distinctively localized in the cytoplasm and the expression decreased with the progression of differentiation stage of HCC (Fig. 2A). Interestingly, localization of Bcl9 seemed to be restricted to certain areas of the cytoplasm especially in advanced HCC. On the other hand, the anti-Bcl9^{ABC} signal was detected mainly in the nucleus, but in some cases, a broad or uniform distribution of Bcl9 staining was noted in cytoplasm of HCC cells (Fig. 2A). For statistical analysis, we used simple IHC-score (0, 1, 2, 3), which was determined by the signal intensity because most parts of the normal liver parenchyma and HCC nests were stained homogenously. The IHC-score of the normal liver stained with anti-Bcl9^{BIO} was significantly higher than that of any other differentiation stages of HCC (P < 0.01, Fig. 2B). Moreover, IHC-score by anti-Bcl9^{BIO} tended to demonstrate inverse correlation with poorer differentiation grades of HCC. In contrast, the IHC-score by anti-Bcl9^{ABC} had no statistical significant differences among normal liver and various differentiation stages of HCC (P value, 0.535; Fig. 2B).

Possible correlation between various clinicopathological parameters and Bcl9 expression

Finally, we analysed the clinicopathological features according to the IHC-score obtained with two types of anti-

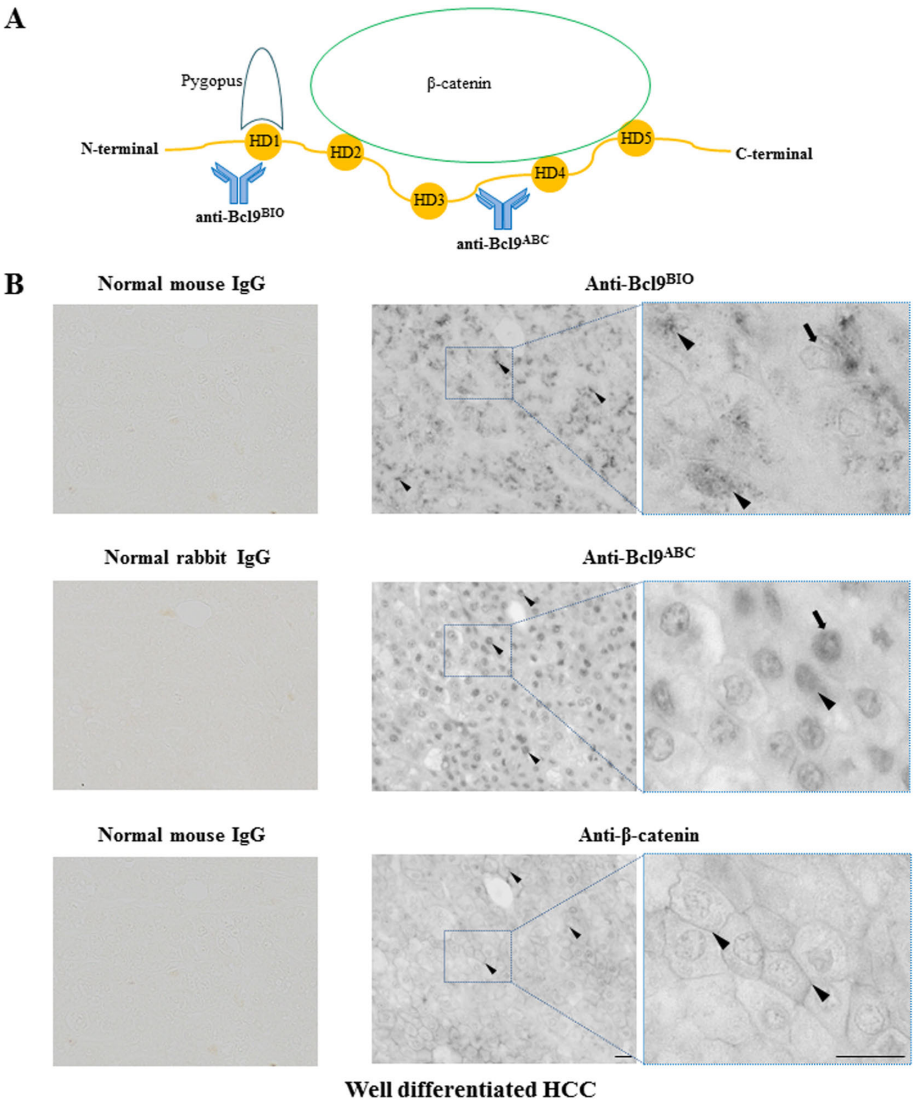

Fig. 1. Characterization of anti-Bcl9^{BIO} and anti-Bcl9^{ABC} antibodies. **A:** Schematic representation of the functional domain of Bcl9 and its interaction with Pygopus and β-catenin. Bcl9 contains 1420 amino acid (aa) residues and has five homology domains (HD1; aa no.177–204, HD2; 349–371, HD3; 467–490, HD4; 997–1048 and HD5; 1223–1254). The HD1 domain of Bcl9 recognizes the nuclear protein, Pygopus, while HD2 domain facilitates the interaction with β-catenin. The HD4 and HD5 regions of Bcl9 act as transactivation domains and synergize with β-catenin. Anti-Bcl9^{BIO} (BMR00368) was raised against aa no.50–200 residues of the Bcl9 while anti-Bcl9^{ABC} (ab37305) was raised against aa no.800–900 residues. **B:** Immunohistochemical localization of Bcl9 by both antibodies and β-catenin in serial sections of a well differentiated HCC. The same concentration of normal mouse or rabbit IgG was used instead of the specific antibody. Left panel; negative controls. Middle panel; immunohistochemical results with the indicated antibody. Right panel; higher magnification of the images of the dotted-square in the middle panel. Arrowheads; typical positive cells to each epitope of anti-Bcl9^{BIO} and anti-Bcl9^{ABC}. Arrows; the same cell which is positive to both epitopes of anti-Bcl9^{BIO} and anti-Bcl9^{ABC}. Bar = 50 μm.

Bcl9 antibodies. Based on the recent study that identified Asians older than 40 years of age to be at highest risk of HCC among other populations [19], we divided our HCC patients into two age groups; < 40 and ≥ 40 years and examined the differences in IHC-score. Very surprisingly, we found a significant difference in IHC-score obtained with anti-Bcl9^{BIO} between the two age groups (*P* value, 0.007, Fig. 3A). When the age group was divided into < 45 and ≥ 45 years, significance was still identified (*P* value, 0.017), although no more significant difference (*P* value, 0.572) was noted when 50 years was used as the cut-off value. In contrast, the IHC-score with anti-Bcl9^{ABC} antibody did not correlate with age (*P* value, 0.649, 0.631 and 0.844, for 40, 45 and 50 years cut-off values, respectively, Fig. 3A). Several other clinicopathological variables, such as sex, HBV and HCV had no impact on IHC-score with anti-Bcl9^{BIO} and anti-Bcl9^{ABC} antibodies (Fig. 3B–D).

IV. Discussion

In the present study, we have found that the expression

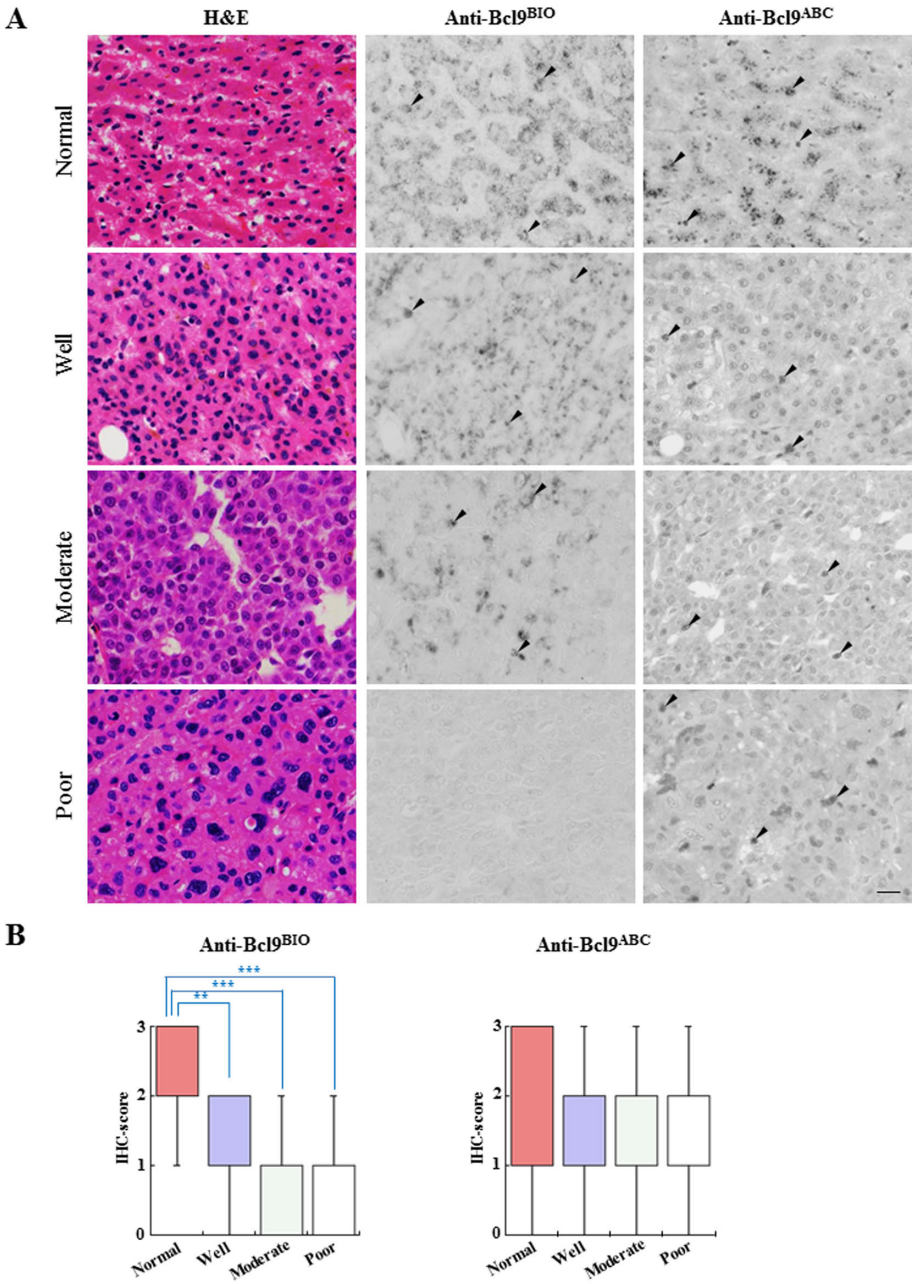

Fig. 2. Immunohistochemical localization of Bcl9 detected by anti-Bcl9^{BIO} and anti-Bcl9^{ABC} in various histopathological grades of Myanmar HCC. **A:** H&E staining (left panel) and immunohistochemical localization of Bcl9 detected by anti-Bcl9^{BIO} (middle panel) and anti-Bcl9^{ABC} (right panel) in serial sections of normal liver, well, moderately and poorly differentiated HCC. Arrowheads; typical positive cells to each epitope of anti-Bcl9^{BIO} and anti-Bcl9^{ABC}. Bar = 100 μm. **B:** Quantitative analysis of IHC-score in normal liver and various histopathological grades of HCC: IHC-score obtained by anti-Bcl9^{BIO} (left graph, normal; 2.4 ± 0.2, well; 1.2 ± 0.2, moderate; 0.7 ± 0.2, poor; 0.5 ± 0.2) and IHC-score with anti-Bcl9^{ABC} (right graph, normal; 2.0 ± 0.4, well; 1.5 ± 0.3, moderate; 1.5 ± 0.2, poor; 1.8 ± 0.3). Data are mean ± SEM. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

of Bcl9 in HCC detected by anti-Bcl9^{BIO} was limited to the cytoplasm and tended to display inverse correlation with progression of differentiation grades of Myanmar HCC. Moreover, the expression level was significantly lower in younger patients less than 40 years than their older counterparts. The localization of Bcl9 detected with both antibodies in the same cells revealed the specificity of each antibody, while the expression pattern of Bcl9 detected by anti-Bcl9^{BIO} seems very different from that of conventional anti-Bcl9^{ABC}, even in the cytoplasmic distributions (Fig. 2). It was not strange because the epitopes detected by the two antibodies are different and anti-Bcl9^{BIO} is considered to react with the HD1 domain unoccupied with Pygopus, which promotes the translocation of Bcl9 from the cytoplasm to the nucleus. Considering that Bcl9 is involved in transcription regulation as a component of the β-catenin

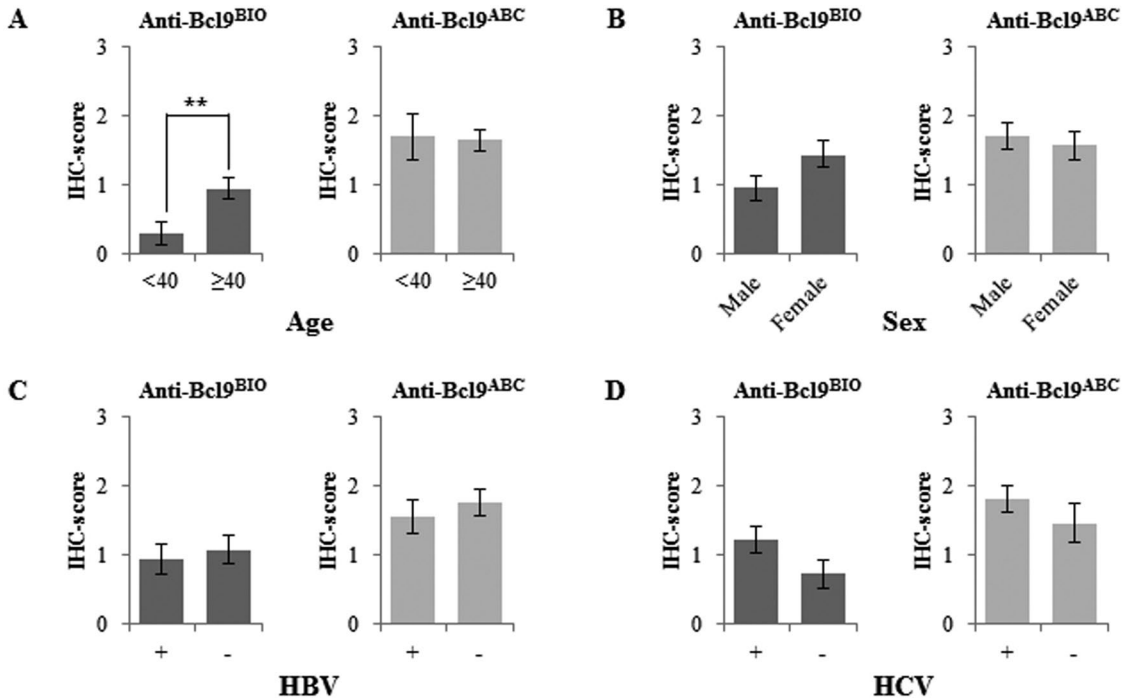

Fig. 3. Relationships between clinicopathological parameters and IHC-score of sections stained with anti-Bcl9^{BIO} and anti-Bcl9^{ABC} antibodies. **A:** Age, **B:** Sex, **C:** HBV infection and **D:** HCV infection. Data are mean ± SEM. IHC-score, immunohistochemical-score; HBV, hepatitis B virus; HCV, hepatitis C virus; +, positive; -, negative; $^{**}P \leq 0.01$.

machinery in nuclei, cytoplasmic Bcl9 detected by anti-Bcl9^{BIO} may be regarded as the non-functional state in the Wnt/β-catenin signaling.

Using anti-Bcl9^{ABC}, Hyeon *et al.* demonstrated previously that Bcl9 expression correlated significantly with HCC in younger age, higher Edmondson grade, more microvascular invasion and increased intrahepatic metastasis [12]. However, the expression of Bcl9 detected by anti-Bcl9^{ABC} in normal liver found in our study had a discrepancy from the previous reports where Bcl9 was not detected in normal liver [12, 30]. The difference might be due to the use of non-cancerous parts as normal liver in our study. Besides, almost 90% of our HCC specimens were positive to either HBV or HCV infection, suggesting that the difference could be also due to regenerating changes that could affect the expression of Bcl9.

Furthermore, statistical analysis of the expression profiles of Bcl9 detected by anti-Bcl9^{ABC} as well as β-catenin in our study failed to display any significant difference between normal liver parts and various differentiation stages of HCC. In this regard, previous study reported that β-catenin was localized in the nucleus in 35.3% of HCC cases [13], whereas β-catenin was found mostly in the cytoplasm and plasma membrane of HCC cells in the present study and nuclear localization was detected only in 2 cases out of 41 cases. These discrepancies were probably due to the difference in pre-existing etiological factors in the developmental properties of Myanmar HCC, especially environmental inorganic toxic substances, such as iron, arsenic which have been reported to affect the pathogenesis of HCC [22, 28, 29].

One important finding of this study was the significantly lower IHC-score with anti-Bcl9^{BIO} < 40 years age group (Fig. 3A). This finding suggests that in the younger HCC cases including juvenile HCC, almost all Bcl9 would be activated and translocated into the nucleus by binding to Pygopus, depleting the cytoplasmic reservoir of Bcl9. Consequently, this could result in combinational imbalance of β-catenin machinery, leading to the promotion of the juvenile HCC development in Myanmar patients. Although the cytoplasmic expression of Bcl9 seems to be a new hepatological parameter, further studies with human and animal model specimens are needed to draw a solid conclusion about the role of Bcl9 in the juvenile development of HCC.

V. Conflicts of Interest

The authors declare that there are no conflicts of interest.

VI. Acknowledgments

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VII. References

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