Immunohistochemical mapping of Bcl9 using two antibodies that recognize different epitopes is useful to characterize juvenile development of hepatocellular carcinoma in Myanmar.
Abstract of Dissertation submitted by MYAT THU SOE

Immunohistochemical mapping of Bcl9 using two antibodies that recognize different epitopes is useful to characterize juvenile development of hepatocellular carcinoma in Myanmar

Bcl9 の異なるエピトープを認識する二つの抗体を用いた免疫組織化学的マッピングによるミャンマー国若年性発症肝細胞がんの特徴付け

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Introduction:
Myanmar is a Southeast-Asia country, where the prevalence of HCC is highly reported. Importantly, the juvenile development and poor prognosis of HCC in young generation is common in Myanmar cases, where uptakes of excess iron and a heavy iron-deposition in liver were reported. Previous studies from our laboratories presented that iron overload accelerated the liver cell kinetics abnormally, though the etiopathological mechanisms to induce juvenile development of HCC remain to be elucidated. Bcl9 (B cell lymphoma 9 gene) and Pygopus are core components of β-catenin/TCF complex and indispensable to Wnt/β-catenin signaling. Overexpression of Bcl9 increases cell proliferation, migration, invasion and metastatic potential of tumor cells and is reported in various types of tumors such as colorectal cancer, multiple myeloma and HCC.

Recent studies have demonstrated the nuclear localization of Bcl9 in various tumors including HCC unlike their normal counterparts. This finding seems to be consistent with the function of Bcl9 as a co-activator of β-catenin in the nucleus. However, Bcl9 was also reported to localize in both cytoplasm and nucleus of adrenocortical cells, irrespective of normal and malignant status. In addition, Bcl9 is known to be involved in enamel production and lens development, independently of β-catenin signalling. Consequently, intracellular localization of Bcl9 is currently a controversial issue, mainly because it may form a complex with various molecules at different domains, reflecting a different functional state. Therefore, for a better understanding of Bcl9 function, immunohistochemistry against different epitopes of Bcl9 would be useful. In the present study, we localized Bcl9 in Myanmar HCC immunohistochemically using two different antibodies and analysed the expression profiles of Bcl9 in differentiation grades of Myanmar HCC to evaluate the diagnostic value of Bcl9.

Materials and Methods:
In this study, we examined the expression pattern of Bcl9 immunohistochemically, using two anti-Bcl9 antibodies; one was a conventional polyclonal-antibody (anti-Bcl9ABC) against amino acid no.800-900 of human-Bcl9, while the other (anti-Bcl9BIO) was against amino acid no.50-200, covering Pygopus-binding sites of Bcl9 in paraffin embedded surgically resected liver tissues, which were collected from patients diagnosed with HCC and treated surgically at the Yangon Specialty Hospital, Myanmar. 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). Of the 52 examined samples, 11 were
considered to be normal liver. To evaluate the biological significance of Bcl9 in HCC, we performed immunohistochemical staining and compared the expression profiles with histopathological differentiation grades. For statistical analysis, we used simple IHC-score (0, 1, 2, 3), which was determined by signal intensity because most parts of normal liver parenchyma and HCC nests were stained homogenously.

**Results:**

Bcl9 detected by anti-Bcl9\textsuperscript{BIO} was predominantly localized in the cytoplasm and the expression decreased with the progression of differentiation stage of HCC. Interestingly, localization of Bcl9 seemed to be restricted to certain areas of the cytoplasm especially in advanced HCC. In contrast, the anti-Bcl9\textsuperscript{ABC} signal was detected mainly in the nucleus, but in some cases, a broad or uniform distribution of Bcl9 staining was noted in the cytoplasm of HCC cells. The IHC-score of the normal liver stained with anti-Bcl9\textsuperscript{BIO} was significantly higher than that of any other differentiation stages of HCC (\(P<0.001\)). Moreover, IHC-score by anti-Bcl9\textsuperscript{BIO} tended to demonstrate inverse correlation with poorer differentiation grades of HCC. However, the IHC-score by anti-Bcl9\textsuperscript{ABC} had no statistical significant differences among normal liver and various differentiation stages of HCC (\(P\) value, 0.535).

Finally, we analysed the clinicopathological features according to the IHC-score obtained with two types of anti-Bcl9 antibodies. We divided our HCC patients into two age groups; <40 and \(\geq 40\) years and examined the differences in IHC-score. Very surprisingly, we found IHC-score with anti-Bcl9\textsuperscript{BIO} in age group of <40 years was significantly lower than those of \(\geq 40\) years (\(P\) value, 0.007). In contrast, the IHC-score with anti-Bcl9\textsuperscript{ABC} antibody did not correlate with age (\(P\) value, 0.649). Several other clinicopathological variables, such as sex, HBV and HCV had no impact on IHC-score with anti-Bcl9\textsuperscript{BIO} and anti-Bcl9\textsuperscript{ABC} antibodies.

**Discussion:**

In the present study, we have found that the expression of Bcl9 in HCC detected by anti-Bcl9\textsuperscript{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. 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\textsuperscript{BIO} seems very different from that of conventional anti-Bcl9\textsuperscript{ABC}, even in the cytoplasmic distributions. It was not strange because the epitopes detected by the two antibodies are different and anti-Bcl9\textsuperscript{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 machinery in nuclei, cytoplasmic Bcl9 detected by anti-Bcl9\textsuperscript{BIO} may be regarded as the non-functional state in the Wnt/β-catenin signaling.

One important finding of this study was the significantly lower IHC-score with anti-Bcl9\textsuperscript{BIO} <40 years age group. 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, which covers the epitope of anti-Bcl9\textsuperscript{BIO}, resulting in the depleting the cytoplasmic reservoir of Bcl9. Consequently, this could result in combinatorial 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.

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