学位論文（要約）

Studies on the drug resistance mechanisms

in hepatocellular carcinoma

（肝細胞癌の薬剤耐性機構の解析）

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【Background and Purpose】

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide. With several successful clinical trials, multi-tyrosine kinase inhibitors (TKIs) and immune checkpoint inhibitors (ICIs) are commonly used in clinical practice of unresectable HCC nowadays. TKIs are a class of drugs that inhibit tyrosine kinases activity which implicated in tumorigenesis and progression. ICIs are monoclonal antibodies that release the inhibitory brakes of T cells, resulting in antitumor immune responses. In 2018, a novel TKIs of lenvatinib was approved for unresectable HCC in Japan. Quite recently, the combination therapy of ICIs of atezolizumab which is a programmed death ligand 1 (PD-L1) antibody and bevacizumab which is a VEGF-A antibody have been approval refer to successful phase III clinical trial. Despite there are many effective treatments as lenvatinib and atezolizumab plus bevacizumab available for unresectable HCC, relapse after once responding to systemic therapies still a pressing issue. Regardless of the treatment response, most patients finally get progression in HCC. Thus, it is important to clarify the reasons of progression after once response to lenvatinib as well as atezolizumab plus bevacizumab.

Some previous studies have concentrated on predicting of treatment response threw baseline serum growth factor levels, however the data of sequential changes in growth factors during systemic treatment, especially with respect to progressive disease (PD), have not been completely clarified. In the first chapter of this study, from the aspect of patient serum growth factors, we aimed to comprehend factors with respect to PD and to investigate the sequential changes in serum growth factors during lenvatinib treatment for unresectable HCC. In chapter 2, we aimed to analyze the changes in growth factors during the atezolizumab plus bevacizumab treatment for unresectable HCC to have an insight in mechanism of acquire resistance during atezolizumab plus bevacizumab treatment for HCC. In some basic research regarding lenvatinib resistance mechanisms in HCC cell lines, in the aspect of changes in kinase, cytokine, and microRNA profile, some molecules of great importance have been clarified. However, in the aspect of alteration in gene expression, mechanisms of HCC cells acquire lenvatinib resistance have not been clarified well. Therefore, in Chapter 3, we established lenvatinib resistant cells and analyzed the underlying mechanism regarding resistant to lenvatinib in gene expression level using DNA microarray and pathway analysis, aimed to seize prospective critical genes in lenvatinib resistance cells.

【Methods】

In Chapter 1 and Chapter 2, we detected serum growth factor levels at baseline, best overall response, and PD points by commercial enzyme-linked immunosorbent assays according to the manufacturer's protocols. Studies in Chapter 1 and Chapter 2 are single center retrospective observational studies, in these studies we included unresectable HCC patients who received lenvatinib treatment or atezolizumab plus bevacizumab treatment in Hokkaido University Hospital and excluded patients who were concomitant treated with other anticancer agents or received concomitance chemoembolization treatment. And analyzed the connection between patient treatment response, patient characteristics and the changes in serum growth factors. In Chapter 1 treatment response was evaluated using the modified response evaluation criteria in solid tumors. In Chapter 2 treatment response was evaluated using the response evaluation criteria in solid tumors.

In Chapter 3, we establish lenvatinib resistant cells (Huh7-LenR cells) by continuously exposure human hepatocellular carcinoma cell line of Huh7 cells to lenvatinib. After ensuring that the Huh7-LenR cells was properly resistant to lenvatinib *in vitro* and *in vivo*, we investigated sensitivity of Huh7-LenR cells to other anti-HCC drugs and tried to investigate the key molecule that associated with resistance to lenvatinib by microarray and pathway analysis. Next, we established candidate gene overexpressing HCC cells using lentivirus, and analyzed their resistance to lenvatinib.

【Results】

In Chapter 1, we include 58 HCC patients who treated with lenvatinib, and a total of 8, 24, 18, and 8 patients experienced best overall response of CR, PR, SD, and PD, respectively. Baseline serum ANG-2 levels, relative dose intensity and age were proved associated with lenvatinib treatment response. During the relapse after once responding to lenvatinib, patients can be divided in to four groups according to the changing patterns of serum levels of ANG-2, EGF and HGF. As other prognosis related factors are similar among groups, patient post progression survival was significantly different among four groups.

In Chapter 2, we include 46 HCC patients who treated with atezolizumab plus bevacizumab, and a total of 4, 9, 19, and 14 patients experienced best overall response of CR, PR, SD, and PD, respectively. Baseline serum growth factor levels were similar between patients with or without disease controls and also similar between patients with

or without OR. FGF-19 significantly increased at best overall response point, on the contrary, ANG-2 significantly decreased at best overall response point. VEGF-D, and ANG-2 significantly increased at PD point compared with that at best response point.

In Chapter 3, established Huh7-LenR cells showed resistant to lenvatinib *in vitro* and *in vivo*. Huh7-LenR cells have a lower sensitivity comparing with Huh7 cells under the treatment of various TKIs containing sorafenib, cabozantinib and regorafenib. We focused on candidate gene associates with lenvatinib resistance according to results of microarray analysis and pathway analysis. But individually knockdown or overexpression of candidate gene of GPX8 has no impact on the sensitivity of Huh7 derived cells to lenvatinib.

【Discussion】

In Chapter 1, we found that patients with increased median EGF, ANG-2, and HGF levels between the best overall response and PD points had remarkably shorter PPS than other patient groups. The cause of the remarkably shorter PPS in the patient group with increased median EGF, ANG-2, and HGF than the other groups has not been clarified well, however, refer to several previous studies, simultaneous elevation of these three growth factors (ANG-2, HGF, and EGF) might cause an increase in tumors' malignant potential, deterioration of liver function reserve, and resistance to salvage treatment.

In Chapter 2, in HCC patients who treated with atezolizumab plus bevacizumab, serum VEGF-D level significantly increased at PD point compared with best overall response point. Bevacizumab target VEGF-A, which bind to VEGFR2, While VEGF-C and D could also bind to VEGFR2 and could not be inhibited by bevacizumab. Thus, the increasing serum VEGF-D level might reactivate VEGFR2 mediated signaling, resulted in the acquiring of resistance to atezolizumab plus bevacizumab. Therefore, second line of ramucirumab combination therapy might be effective in patients with high serum VEGF-D levels, since ramucirumab could suppress both VEGF-A and VEGF-D mediated signaling.

In Chapter3, we first established Huh7-LenR cells which have lower sensitivity to different concentrations of lenvatinib. To clarify the underlying mechanism of Huh7-LenR cells resistant to lenvatinib in gene expression levels, we performed microarray and pathway analysis and focused on candidate genes. We found that genes that can reduce oxidative stress may be the key point of resistance in Huh7-LenR cells. Further

studies on other hepatocellular carcinoma cell lines are required.

【Conclusion】

In Chapter 1, our findings demonstrate that the pattern of development of resistance to lenvatinib varies and might determine the prognosis of patients with unresectable HCC. Thus, the evaluation of baseline patient characteristics and the changes in growth factors during lenvatinib could predict treatment response and PPS and can be used to determine subsequent treatments.

In Chapter 2, VEGF-D and ANG-2 significantly increased at PD point compared with best response point. Increased VEGF-D and ANG-2 might be contributed to obtained resistance to atezolizumab plus bevacizumab for unresectable HCC and might be target molecule in the subsequent salvage therapy.

In Chapter 3, we established Huh7-LenR cells which is resistant to lenvatinib treatment and find that gene expression was quite different in Huh7-LenR cells compared with Huh7 cells. Our results showed that GPX8 was a potential candidate that significantly up regulated in Huh7-LenR cells compared with control Huh7 cells. However, individually knockdown and overexpression of GPX8 indicate that GPX8 was not the only key reason of resistance in Huh7-LenR cells. So, in future, we will further focus on other candidates according to results of microarray and pathway analysis.