様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

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研究課題名（和文）Production of infectious hepatitis E virus (HEV) harboring bioluminescent reporter gene for comprehensive screening of antiviral drugs against HEV and ex vivo evaluation of selected drugs

研究課題名（英文）Production of infectious hepatitis E virus (HEV) harboring bioluminescent reporter gene for comprehensive screening of antiviral drugs against HEV and ex vivo evaluation of selected drugs

研究代表者

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交付決定額（研究期間全体）：（直接経費）　　2,200,000円

研究成果の概要（和文）：本研究では、ルシフェラーゼ遺伝子（nanoKAZ）を有する組換えE型肝炎ウイルス(HEV-nanoKAZ)を開発し、これを利用した薬剤スクリーニングシステムの構築に成功した。このスクリーニング系は、HEVの侵入阻害剤とHEV RNA複製阻害剤を探索することが可能であった。FDA承認薬ライブラリを利用したスクリーニングの結果、4種類の薬剤が同定され、培養細胞において抗HEV効果を有することが確認された。そのうち2剤（azithromycinおよびritonavir）は、培養上清中へのHEV産生および細胞内のORF2蛋白質の発現を強く阻害し、新規抗HEV薬としての可能性が示された。

研究成果の学術的意義や社会的意義
新たに開発したHEV-nanoKAZを利用することにより、これまで同定することのできなかった感染初期過程を阻害する薬剤のスクリーニングが可能となった。今後、さまざまなライブラリを用いたスクリーニングを実施することで、より効果の高い抗HEV候補薬が同定されることが期待される。また、膜に覆われたHEVと膜に覆われていないHEVが利用する感染受容体は同定されておらず、細胞内侵入機構についても不明な点が多く残されている。HEV-nanoKAZシステムは、このようなHEVのライフサイクルの研究へとさらに応用が可能である。

研究成果の概要（英文）：A system consisting of recombinant infectious hepatitis E virus (HEV) harboring a small luciferase gene (nanoKAZ) in ORF1, was developed in this study. It replicated efficiently in cultured cells, was genetically stable, and had morphological characteristics similar to those of the parental virus. Both membrane-associated (eHEV-nanoKAZ) and membrane-unassociated (neHEV-nanoKAZ) particles were infectious. The system was successfully applied in a screening to search for novel anti-HEV drugs. This screening system was able to cover the inhibitor of HEV entry and HEV RNA replication. In the screening, four drugs were identified and confirmed to be effective in cultured cells. Two drugs (azithromycin and ritonavir) strongly inhibited HEV production in culture supernatants, and intracellular expression of ORF2 protein, and may therefore be candidate novel anti-HEV drugs. The results of this study provide evidence supporting the use of this system in more variable HEV studies.

研究分野：ウイルス学

キーワード：hepatitis E virus　bioluminescent　reporter virus　nanoKAZ　hypervariable region　non-enveloped HEV　quasi-enveloped HEV　drug screening

１．研究開始当初の背景

Hepatitis E virus (HEV) is increasingly recognized as the leading cause of acute hepatitis. Although most HEV infection is a self-limiting disease, it could become chronic especially in immunocompromised patients. Currently, there is no specific antiviral drug available against HEV. Off-label drugs such as ribavirin and pegylated interferon α are used for HEV treatment in certain conditions. However, they are associated with severe side-effects and contraindicated in major risk group of pregnant women. On the other hand, ribavirin has increasingly been reported with development of resistance and leads to viral clearance in only 80% of patients treated. Therefore, development of novel and specific antiviral drugs against HEV to provide more options for the treatment is urgently needed.

Our laboratory recently reported a screening method of novel drugs that inhibit HEV replication using the HEV replicon expressing *Gaussia luciferase* (HEV-GLuc). However, this system can only select drugs with inhibitory activity against RNA replication. Here, the applicant sought to identify the potential candidates for novel anti-HEV agents by using the infectious HEV harboring a small bioluminescent reporter gene, nanoKAZ, to comprehensively screen the antiviral drugs against HEV.

２．研究の目的

The purpose of this study was to search for candidates of novel anti-HEV drugs. NanoKAZ reporter system was used to broadly screen the candidate drugs (i.e. Food and Drug Administration [FDA]-approved drug library and small compound library) that are not covered by the HEV-GLuc replicon system. Effectiveness and inhibitory mechanism of selected drugs were analyzed *in vitro*. Identification of the drugs that specifically work in the respective step of HEV life cycle will add the options for combinations of anti-HEV drugs to reduce cell toxicity while improving the effectiveness of the treatment.

３．研究の方法

The outline of research plan is presented in Figure 1, and is briefly described below.

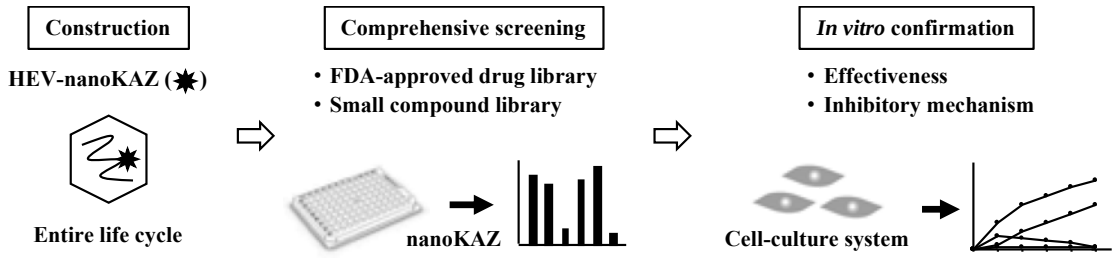

Figure 1. Outline of research plan

(1) Preparation of infectious HEV progenies expressing nanoKAZ for screening assay

The infectious cDNA clone with insertion of nanoKAZ gene in the open reading frame 1 (ORF1) which is involved in viral replication, was constructed. Subsequently, we performed RNA transfection, followed by confirmation of the generation of HEV progenies, the intracellular expression levels of nanoKAZ, intact nanoKAZ insertion (by RT-PCR and sequence analysis), as well as expression of HEV proteins (ORF2 and ORF3) in culture supernatants (by Western blotting) and intracellularly (by immunofluorescence assay, IFA). The morphological characterization of the membrane-associated (eHEV-nanoKAZ) and membrane-unassociated (neHEV-nanoKAZ) particles was confirmed by equilibrium centrifugation in a sucrose density gradient and immune-electron microscopy, while their infectivity was confirmed by detecting the ORF2 protein expression (by IFA) and luciferase activity in the lysates of PLC/PRF/5 cells inoculated with each form of the viral particles. Subsequently, the eHEV-nanoKAZ was subjected to

consecutive passages (passage 1 to 5) to investigate the genetic stability which was confirmed through RT-PCR and sequence analysis. The capability of eHEV-nanoKAZ to replicate in multiple cell lines was confirmed through inoculation into human hepatocarcinoma cell HepG2/C3A (a clonal derivative of HepG2, a human hepatocellular carcinoma cell) and into the non-liver cancer cell lines A549 (human lung adenocarcinoma cell) and Caco-2 (human colorectal adenocarcinoma cell), which have been previously reported to support HEV replication. In addition, it was also inoculated into PXB cells which derived from fresh human hepatocytes isolated from chimeric mice with highly humanized liver (PXB-mouse). The virus growth kinetics was confirmed by quantitation of HEV RNA titer in culture supernatants of the inoculated cells and by measuring the intracellular luciferase activity.

(2) Screening on the FDA-approved drug library and demonstration of anti-HEV activity

Before applying the eHEV-nanoKAZ in drug screening, it was investigated for its sensitivity to known anti-HEV reagents: sucrose (inhibitor of clathrin-mediated endocytosis), ribavirin (inhibitor of HEV RNA replication), genistein (inhibitor of caveola-mediated endocytosis, a negative control for sucrose), and lomibuvir (inhibitor of RNA polymerase NS5B of hepatitis C virus, a negative control for ribavirin). The eHEV-nanoKAZ was then applied in a screening of FDA-approved drug library consisting of 765 drugs. The anti-HEV activity of the four drugs passing the primary and secondary screenings were then confirmed by measuring the luciferase activity in the lysates of the cells inoculated with eHEV-nanoKAZ and treated with each of the drug in various concentrations. Their effect on HEV RNA replication was then investigated by utilizing the HEV-GLuc replicon. The effectiveness of the four hit drugs to inhibit HEV growth was evaluated in cultured cells by using eHEV in either naïve PLC/PRF/5 cells, or in the cells robustly producing HEV in culture supernatants. The HEV RNA titer in culture supernatants was quantitated by real-time RT-PCR, while the expression of ORF2 protein was detected by IFA.

(3) Screening of the small compound library

Other than its application in the screening of FDA-approved drug library, the eHEV-nanoKAZ was applied in the screening of small compound library. The hit compounds will then be subjected to the similar experiments performed above.

４．研究成果

(1) Construction of the recombinant infectious HEV harboring the nanoKAZ gene

We developed a system consisting of recombinant infectious HEV harboring a small luciferase gene (nanoKAZ) in the hypervariable region of the ORF1 (Figure 2). It replicated efficiently in cultured cells, was genetically stable, and had morphological characteristics similar to those of the parental virus. Both membrane-associated (eHEV-nanoKAZ) and membrane-unassociated (neHEV-nanoKAZ) particles were infectious. Other than its ability to efficiently propagate in PLC/PRF/5 cells, the eHEV-nanoKAZ was also able to replicate in a wide variety of cells, ranging from the cancer cell lines HepG2/C3A, A549, and Caco-2 to the normal hepatocyte, PXB-cells, where nanoKAZ is produced specifically, supporting its potential use in a variety of cell culture conditions in future HEV studies.

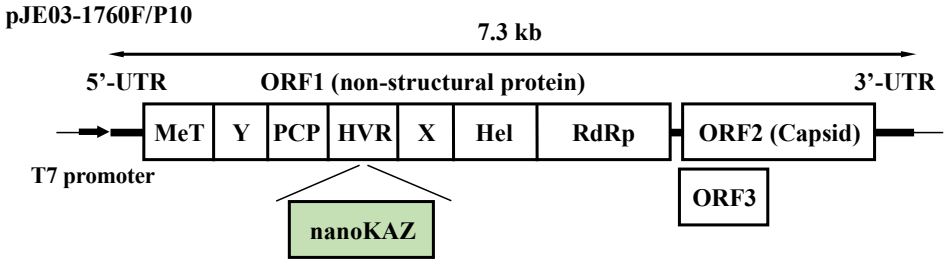

Figure 2. Schematic diagram of the full-length HEV cDNA clone harboring the nanoKAZ gene in the hypervariable region. MeT, methyl transferase; Y, Y domain; PCP, papain-like cysteine protease; HVR, hypervariable region; X, macro domain; Hel, helicase; RdRp, RNA-dependent RNA polymerase.

(2) Application of eHEV-nanoKAZ in drug screening

The eHEV-nanoKAZ system is able to cover the inhibitor of HEV entry (represented by sucrose, an inhibitor of clathrin-dependent endocytosis) and the inhibitor of HEV RNA replication (represented by ribavirin, which inhibits HEV replication *in vitro* by increasing the error rate of viral RdRp) (Figure 3). Solely based on these results, the four hit drugs might inhibit entry step of the HEV life cycle, such as attachment to the cell receptor, internalization, or uncoating.

In order to confirm the utility of HEV-nanoKAZ as functional tool in HEV studies, this system was applied in drug screening. HEV particles circulating in the bloodstream and attaching to hepatocytes in HEV-infected patients are membrane-associated; thus, eHEV-nanoKAZ was applied in the screening. Four drugs were hit in the primary and secondary screenings, namely, gefitinib, chlorpromazine, azithromycin, and ritonavir. Subsequent evaluation confirmed their dose-dependent inhibition of the luciferase activity of eHEV-nanoKAZ-inoculated PLC/PRF/5 cells (Figure 4). On the other hand, these drugs did not affect HEV RNA replication as demonstrated by using the HEV-GLuc replicon system, supporting the notion that they possibly inhibit HEV entry.

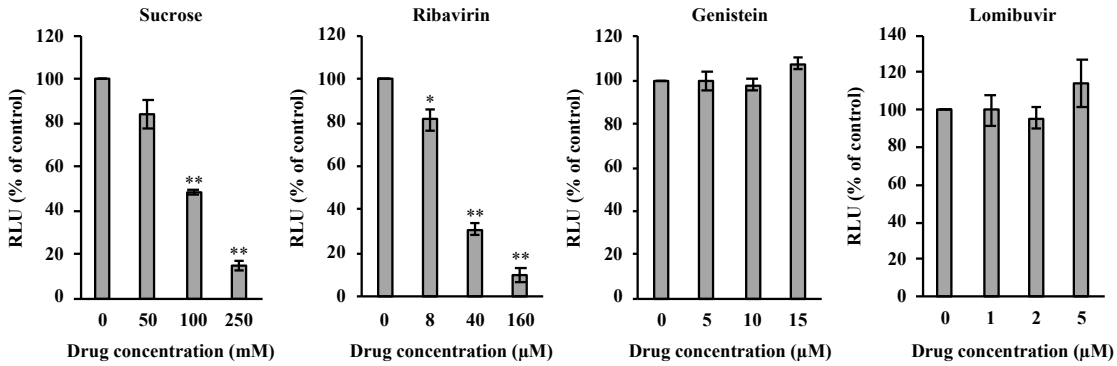

Figure 3. Capability of the eHEV-nanoKAZ to screen the known anti-HEV reagents. The eHEV-nanoKAZ was inoculated to PLC/PRF/5 cells along with drug treatment at various concentrations. Four days after inoculation, the cell lysates were collected and the intracellular luciferase activity was measured. *, $P < 0.05$; **, $P < 0.001$.

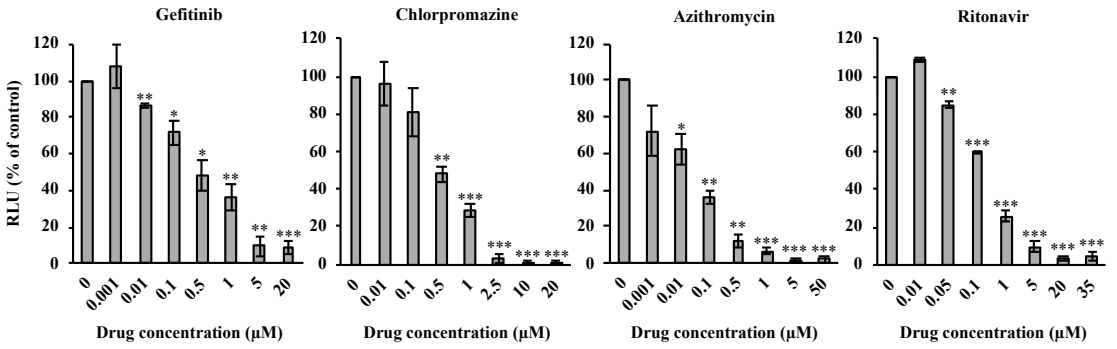

Figure 4. Anti-HEV activity of the hit drugs. The inhibitory activity of gefitinib, chlorpromazine, azithromycin, and ritonavir against HEV was tested at various concentrations. The eHEV-nanoKAZ was inoculated to PLC/PRF/5 cells along with the drug treatment. Cells were lysed four days after inoculation, followed by measurement of luciferase activity. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(3) Evaluation of the effectiveness of the four hit drugs in cultured cells

The effectiveness of the four hit drugs in cultured cells was confirmed in naive and HEV-producing PLC/PRF/5 cells. Two hit drugs (azithromycin and ritonavir) strongly inhibited HEV production in culture supernatants, as well as intracellular expression of ORF2 protein, and may therefore be candidate novel anti-HEV drugs.

(4) Screening of the small compound library using the HEV-nanoKAZ system

In the screening of small compound library by utilizing eHEV-nanoKAZ, several compounds exibited anti-HEV activity. Following this, their inhibition mechanism and their efficacy *in vitro* will be further analyzed.

In conclusion, we developed the HEV-nanoKAZ system as a functional investigation tool with successful application in drug screening assays. The results of this study have emphasized the benefit of using a small-sized luciferase gene in maintaining genetic stability for the successful development of a functional bioluminescent reporter system. The utility of this novel system can be further expanded beyond drug screening to study molecular aspects of the HEV life cycle, taking into consideration the availability of both particle forms (membrane-associated and membrane-unassociated), such as in the investigation of unknown HEV receptors and the elucidation of host factors important for HEV entry. In addition, regarding the major drawbacks associated with the current treatment for HEV infections using the off-label drug ribavirin, the drugs identified in the current study may be potential treatment options for HEV infections.

５．主な発表論文等

〔雑誌論文〕　計3件（うち査読付論文　3件／うち国際共著　1件／うちオープンアクセス　1件）

１．著者名 Primadharsini Putu Prathiwi、Nagashima Shigeo、Okamoto Hiroaki	４．巻 13
２．論文標題 Mechanism of Cross-Species Transmission, Adaptive Evolution and Pathogenesis of Hepatitis E Virus	５．発行年 2021年
３．雑誌名 Viruses	６．最初と最後の頁 909～909
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/v13050909	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

１．著者名 Kobayashi Tominari、Takahashi Masaharu、Ohta Satoshi、Nagashima Shigeo、Primadharsini Putu Prathiwi、Mulyanto、Kunita Satoshi、Murata Kazumoto、Okamoto Hiroaki	４．巻 302
２．論文標題 Production of capsid proteins of rat hepatitis E virus in Escherichia coli and characterization of self-assembled virus-like particles	５．発行年 2021年
３．雑誌名 Virus Research	６．最初と最後の頁 198483～198483
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.virusres.2021.198483	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Primadharsini Putu Prathiwi、Nagashima Shigeo、Nishiyama Takashi、Takahashi Masaharu、Murata Kazumoto、Okamoto Hiroaki	４．巻 96
２．論文標題 Development of Recombinant Infectious Hepatitis E Virus Harboring the nanoKAZ Gene and Its Application in Drug Screening	５．発行年 2022年
３．雑誌名 Journal of Virology	６．最初と最後の頁 e0190621
掲載論文のDOI（デジタルオブジェクト識別子） 10.1128/jvi.01906-21	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

〔学会発表〕　計1件（うち招待講演　0件／うち国際学会　0件）

１．発表者名 Putu Prathiwi Primadharsini, Shigeo Nagashima, Takashi Nishiyama, Masaharu Takahashi, Hiroaki Okamoto, Kazumoto Murata
２．発表標題 Development of recombinant hepatitis E virus harboring nanoKAZ gene and its application to drug screening
３．学会等名 The 68th Annual Meeting of the Japanese Society for Virology
４．発表年 2021年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

自治医科大学医学部 感染・免疫学講座 ウイルス学部門 ホームページ http://www.jichi.ac.jp/virology/

6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考
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研究協力者	高橋　雅春 (Takahashi Masaharu)		
研究協力者	岡本　宏明 (Okamoto Hiroaki)		

7．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

8．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関