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研究課題名（和文）Elucidation of host factors and the associated pathways responsible for cellular permissiveness to hepatitis E virus replication and identification of the potential inhibitors

研究課題名（英文）Elucidation of host factors and the associated pathways responsible for cellular permissiveness to hepatitis E virus replication and identification of the potential inhibitors

研究代表者

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交付決定額（研究期間全体）：（直接経費）　　3,500,000円

研究成果の概要（和文）：これまでに、PLC/PRF/5細胞のクローニングを行い、E型肝炎ウイルス（HEV）に対して増殖効率の異なるクローン化細胞の樹立に成功している。本研究では、高許容性細胞と低許容性細胞のRNAマイクロアレイによる発現遺伝子の比較解析に基づき、HEV感染培養系を用いて、高発現または低発現する遺伝子のHEV増殖効率への影響を解析した。その結果、HEVの複製に対する細胞の許容性に関与する遺伝子を複数同定することに成功した。また、同定された遺伝子と関連が認められる化合物ライブラリのスクリーニングにより、野生型HEVに対して阻害活性を有する化合物を同定することができた。

研究成果の学術的意義や社会的意義
新たに同定されたHEV感染の許容性に関与する宿主因子は、新規抗HEV薬開発の標的と成り得ることが示唆された。今後、さまざまな関連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、HEVの複製機構に関しては未だ不明な点が多く残されている。今回同定された宿主因子は、HEVの感染や複製の分子機構を理解する上でも重要な知見である。

研究成果の概要（英文）：Subclones of a single PLC/PRF/5 cell line demonstrated up to 10,000-folds difference in the permissiveness to hepatitis E virus (HEV) replication. Based on the results of RNA microarray analysis of highly permissive and poorly permissive subclones, several genes were silenced by using small interfering RNA (siRNA) followed by screening using eHEV-nanoKAZ and evaluation in cell culture. Among them, possible genes involved in cellular permissiveness to HEV were identified. One hit gene was used as the target for screening on a small compound library by three distinct HEV reporter systems. Among the hit compounds, one with strongest inhibition on luciferase activity was further evaluated in cultured cells. It exhibited moderate inhibition on HEV growth in an evaluation in cell culture system using a wild-type HEV-3 strain. The results of this study will enhance the insights on HEV molecular aspects and will be useful for the development of novel and specific anti-HEV drug.

研究分野：ウイルス学

キーワード：Hepatitis E virus　Host cellular factor　Anti-HEV drug　Microarray　Cell culture　Replication efficiency　Reporter virus　Drug screening

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１．研究開始当初の背景

Hepatitis E virus (HEV) is increasingly recognized as the leading cause of acute hepatitis. In immunocompromised patients, HEV can cause chronic hepatitis. Fulminant and chronic cases require anti-HEV drugs. The off-label drug ribavirin is the current mainstay therapy. However, major drawbacks such as significant side effect of dose-dependent anemia has restricted its clinical application, thus, novel and specific anti-HEV drugs are urgently needed.

Successful viral replication relies on cellular host factors and targeting proviral host factors for antiviral development is appealing due to the lower likelihood of resistance development. However, the host factors associated with permissiveness to HEV infection have not been fully elucidated. Subclones of PLC/PRF/5 cells have variable permissiveness to HEV replication even when inoculated with the same virus, suggesting that aside from viral factor itself, cellular factors might be involved in determining host susceptibility to HEV replication, and therefore, can be the target for development of specific anti-HEV drug.

２．研究の目的

The purpose of this study is to identify the cellular factors which play a role to determine the host permissiveness to HEV replication and to identify small compounds which can inhibit the cellular factor(s) through the specific pathways as the candidate for the development of novel and specific anti-HEV drug.

３．研究の方法

1) Gene expression profiling by using the result of RNA microarray analysis

Analysis of RNA microarray data from highly permissive and poorly permissive PLC/PRF/5 subclones led to the selection of 15 upregulated genes and 15 downregulated genes. Fifteen selected upregulated genes were silenced by small interference RNA (siRNA)-mediated gene knockdown. The siRNA was transfected to the highly permissive PLC/PRF/5 subclone, followed by inoculation with HEV-nanoKAZ (a recombinant HEV harboring small luciferase gene in the non-structural protein, ORF1)[1] to screen the upregulated genes. The intracellular luciferase activity was then measured.

Similarly, selected fifteen downregulated genes were silenced by siRNA-mediated gene knockdown. The siRNA was transfected to poorly permissive PLC/PRF/5 subclone followed by inoculation with HEV-nanoKAZ to screen the downregulated genes. The intracellular luciferase activity was then measured.

(2) Evaluation of the effect of hit genes on HEV replication efficiency in cell culture

The effect of hit upregulated genes and downregulated genes on HEV replication efficiency was confirmed in cell culture. For the hit upregulated genes, siRNA was transfected to the highly permissive PLC/PRF/5 subclone, followed by inoculation with wild-type HEV-3 strain. On the other hand, the hit downregulated genes were overexpressed by transfection of recombinant plasmid of each gene to the highly susceptible PLC/PRF/5 subclone, followed by inoculation with wild-type HEV-3 strain. Additional siRNA transfection or plasmid transfection was performed during monitoring of virus growth kinetics in cell culture. The monitoring was performed for 10 days by quantitation of HEV RNA in culture supernatants of inoculated cells using real-time RT-PCR. The knockdown efficiency or the expression of target protein during the monitoring was confirmed by Western blotting.

(3) Screening on ubiquitination small compound library

One of the candidate host genes is associated with ubiquitination, therefore, was used as the target for a small compound library to be screened. Screening was performed by using three HEV reporter systems recently established in our lab, each covering specific step in HEV life cycle: HEV-GLuc replicon[2] (covering middle step), HEV-nanoKAZ[1] (covering early and middle steps), or HEV-HiBiT/ΔORF2s[3] (covering middle and late steps) in the presence of small compounds, and the luciferase activity was measured to identify compound with anti-HEV activity.

(4) Evaluation of hit compound in cell culture

The compound with anti-HEV activity hit in the screening was evaluated further in cell culture using wild-type HEV-3. HEV growth kinetics was monitored for 12 days by quantitation of HEV RNA in the culture supernatants using real time RT-PCR.

４．研究成果

(1) Gene expression profiling and evaluation of the effect of hit genes on HEV replication efficiency in cell culture

Subclones derived from a single PLC/PRF/5 cell line exhibited a remarkable difference in HEV replication efficiency, with variances of up to 10,000-fold. Direct comparison on the permissiveness level of the highly susceptible and poorly susceptible subclones in supporting the HEV replication by using lower inoculum titer further confirmed the difference. Analysis of RNA microarray data from highly permissive and poorly permissive PLC/PRF/5 subclones led to the selection of 15 upregulated genes and 15 downregulated genes.

Fifteen selected upregulated genes were silenced by siRNA-mediated gene knockdown. The silenced genes were then screened by using eHEV-nanoKAZ in highly susceptible subclone. Silencing any of six genes resulted in decreased intracellular luciferase activity (Figure 1, left). To confirm the effect of the six genes in HEV replication efficiency, wild-type HEV-3 strain was inoculated to the highly susceptible PLC/PRF/5 subclone with each of the six upregulated genes being silenced. The growth kinetic in cell culture was monitored for 10 days. Among the six genes, silencing any of four genes led to decreased HEV replication efficiency in cell culture (Figure 1, right). The knockdown efficiency was maintained during monitoring period as confirmed through Western blotting.

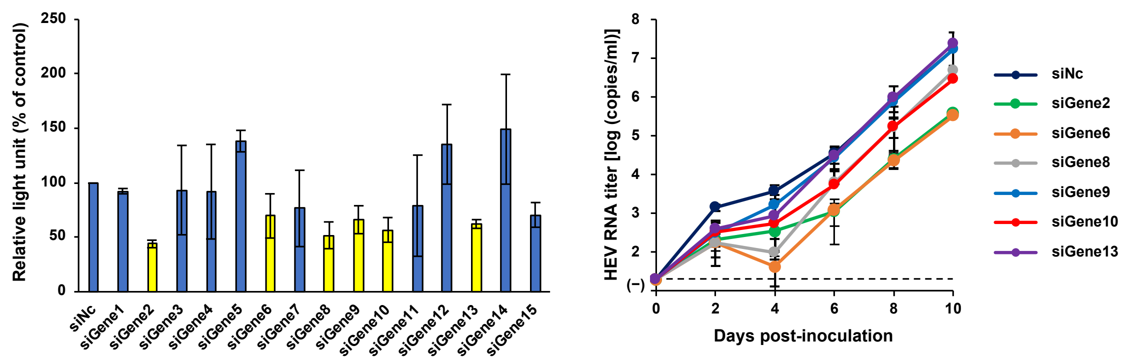

Figure 1. Gene expression profiling for upregulated genes in highly susceptible PLC/PRF/5 subclone by using siRNA-mediated gene knockdown. (Left) Screening on the upregulated genes by HEV-nanoKAZ. (Right) Examination of the effect of selected upregulated genes on HEV replication efficiency by using wild-type HEV-3 strain in cell culture.

On the other hand, the selected fifteen downregulated genes were silenced and then screened by using eHEV-nanoKAZ in poorly susceptible PLC/PRF/5 subclone. Silencing any of the four downregulated genes in the poorly permissive PLC/PRF/5 subclone led to a slight increase in HEV replication efficiency (Figure 2, left). Overexpression of the selected downregulated genes in the highly susceptible PLC/PRF/5 subclone resulted in decreased HEV replication efficiency in cell culture (Figure 2, right). The expression of each target protein was maintained during the monitoring period as confirmed through Western blotting.

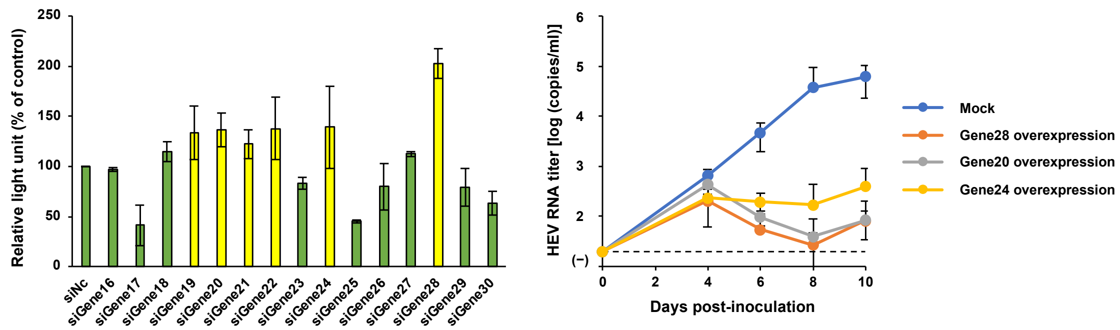

Figure 2. Gene expression profiling for downregulated genes in poorly permissive PLC/PRF/5 subclone by using siRNA-mediated gene knockdown. (Left) Screening on the downregulated genes by HEV-nanoKAZ. (Right) Examination of the effect of selected downregulated genes on HEV replication efficiency by using wild-type HEV-3 strain in cell culture and gene overexpression.

(2) Screening on ubiquitination small compound library with three distinct HEV reporter systems

One hit gene was upregulated in the PLC/PRF/5 subclone with highest susceptibility to HEV replication, and introduction of siRNA decreased HEV replication efficiency. It encodes ubiquitin ligase. Since ubiquitin was suggested to play a role in HEV life cycle, a screening on a ubiquitination compound library was performed by using three HEV reporter systems to search for candidate compound with activity against HEV: HEV-GLuc replicon (covering HEV RNA replication)[2], HEV-nanoKAZ (covering HEV entry and RNA replication)[1], and HEV-HiBiT (covering HEV particle formation and release)[3].

(3) Evaluation of hit compound in cell culture

Among the hit compounds, one with strongest inhibition on luciferase activity was further evaluated in cultured cells by using the wild-type HEV-3 strain. By 12 days post-inoculation, the highest dose (10 μM) already decreased the HEV RNA titer in culture supernatants of the infected cells to 1,000 lower than that of untreated control cells.

In summary, several genes potentially contributing to cellular susceptibility to HEV replication were identified. In addition, a small compound with anti-HEV activity targeting one of the selected candidate genes exhibited moderate inhibition on HEV growth in an evaluation in cell culture system using a wild-type HEV-3 strain. The results of this study will enhance the insights on HEV molecular aspects and will be useful for the development of novel and specific anti-HEV drug.

＜引用文献＞

1.Primadharsini, P. P.; Nagashima, S.; Nishiyama, T.; Takahashi, M.; Murata, K.; Okamoto, H. Development of recombinant infectious hepatitis E virus harboring the nanoKAZ gene and its application in drug screening. J Virol. 2022, 96(6):e0190621.

2.Nishiyama, T.; Kobayashi, T.; Jirintai, S.; Kii, I.; Nagashima, S.; Primadharsini, P. P.; Nishizawa, T.; Okamoto, H. Screening of novel drugs for inhibiting hepatitis E virus replication. J Virol. Methods 2019, 270, 1-11.

3.Nagashima, S. P., P.P.; Nishiyama, T.; Takahashi, M.; Murata, K.; Okamoto, H. Development of a HiBiT-tagged reporter hepatitis E virus and its utility as an antiviral drug screening platform. J Virol. 2023, 97(9):e0050823.

５．主な発表論文等

〔雑誌論文〕　計7件（うち査読付論文　7件／うち国際共著　0件／うちオープンアクセス　6件）

１．著者名	４．巻
Primadharsini Putu Prathiwi、Nagashima Shigeo、Takahashi Masaharu、Murata Kazumoto、Okamoto Hiroaki	14
２．論文標題	５．発行年
Ritonavir Blocks Hepatitis E Virus Internalization and Clears Hepatitis E Virus In Vitro with Ribavirin	2022年
３．雑誌名	６．最初と最後の頁
Viruses	2440～2440
掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
10.3390/v14112440	有
オープンアクセス	国際共著
オープンアクセスとしている（また、その予定である）	-

１．著者名	４．巻
Primadharsini Putu Prathiwi、Nagashima Shigeo、Tanaka Toshinori、Jirintai Suljid、Takahashi Masaharu、Murata Kazumoto、Okamoto Hiroaki	15
２．論文標題	５．発行年
Development and Characterization of Efficient Cell Culture Systems for Genotype 1 Hepatitis E Virus and Its Infectious cDNA Clone	2023年
３．雑誌名	６．最初と最後の頁
Viruses	845～845
掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
10.3390/v15040845	有
オープンアクセス	国際共著
オープンアクセスとしている（また、その予定である）	-

１．著者名	４．巻
Takahashi Masaharu、Kunita Satoshi、Nishizawa Tsutomu、Ohnishi Hiroshi、Primadharsini Putu Prathiwi、Nagashima Shigeo、Murata Kazumoto、Okamoto Hiroaki	15
２．論文標題	５．発行年
Infection Dynamics and Genomic Mutations of Hepatitis E Virus in Naturally Infected Pigs on a Farrow-to-Finish Farm in Japan: A Survey from 2012 to 2021	2023年
３．雑誌名	６．最初と最後の頁
Viruses	1516～1516
掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
10.3390/v15071516	有
オープンアクセス	国際共著
オープンアクセスとしている（また、その予定である）	-

１．著者名	４．巻
Nagashima Shigeo、Primadharsini Putu Prathiwi、Nishiyama Takashi、Takahashi Masaharu、Murata Kazumoto、Okamoto Hiroaki	97
２．論文標題	５．発行年
Development of a HiBiT-tagged reporter hepatitis E virus and its utility as an antiviral drug screening platform	2023年
３．雑誌名	６．最初と最後の頁
Journal of Virology	e0050823
掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
10.1128/jvi.00508-23	有
オープンアクセス	国際共著
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	-

1．著者名 Primadharsini Putu Prathiwi、Nagashima Shigeo、Nishiyama Takashi、Okamoto Hiroaki	4．巻 15
2．論文標題 Three Distinct Reporter Systems of Hepatitis E Virus and Their Utility as Drug Screening Platforms	5．発行年 2023年
3．雑誌名 Viruses	6．最初と最後の頁 1989～1989
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/v15101989	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 Primadharsini Putu Prathiwi、Takahashi Masaharu、Nishizawa Tsutomu、Sato Yukihiro、Nagashima Shigeo、Murata Kazumoto、Okamoto Hiroaki	4．巻 16
2．論文標題 The Full-Genome Analysis and Generation of an Infectious cDNA Clone of a Genotype 6 Hepatitis E Virus Variant Obtained from a Japanese Wild Boar: In Vitro Cultivation in Human Cell Lines	5．発行年 2024年
3．雑誌名 Viruses	6．最初と最後の頁 842～842
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/v16060842	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 Kanda Tatsuo、Li Tian-Cheng、Takahashi Masaharu、Nagashima Shigeo、Primadharsini Putu Prathiwi、Kunita Satoshi、Okamoto Hiroaki, et al.	4．巻 －
2．論文標題 Recent advances in hepatitis E virus research and the Japanese clinical practice guidelines for hepatitis E virus infection	5．発行年 2024年
3．雑誌名 Hepatology Research	6．最初と最後の頁 1-30
掲載論文のDOI（デジタルオブジェクト識別子） 10.1111/hepr.14062	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 －

〔学会発表〕　計2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, Masaharu Takahashi, Kazumoto Murata, Hiroaki Okamoto
2．発表標題 Analysis of ritonavir inhibition target in hepatitis E virus life cycle and the efficacy of its combination with ribavirin in cultured cells
3．学会等名 The 69th Annual Meeting of the Japanese Society for Virology
4．発表年 2022年

１．発表者名 Putu Prathiwi Primadharsini, Shigeo Nagashima, Masaharu Takahashi, Kazumoto Murata, Hiroaki Okamoto
２．発表標題 Analysis of host factors underlying cellular susceptibility to hepatitis E virus replication
３．学会等名 The 70th Annual Meeting of the Japanese Society for Virology
４．発表年 2023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 https://www.jichi.ac.jp/virology/

６．研究組織

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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