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財団法人　日中医学協会　御中

貴財団より助成金を受領して行った研究テーマについて報告いたします。

添付資料：**研究報告書**

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1．助成金額：600,000円

2．研究テーマ

薬剤耐性ウイルス出現における自然免疫効果分子APOBECの役割

3．成果の概要

B型肝炎病態をモデル化した実験系においてAPOBECファミリーの発現プロファイリングを行い、インターフェロンでAPOBEC3GとF, TGFβでAIDの発現誘導を見いだした。さらにAPOBEC3GとAIDの強制発現でラミブジン耐性変異の源泉となりうる遺伝子変異がおこる事を見いだした。

4．研究業績

（1）学会における発表　　無・(有)（学会名・演題）

・日本生化学会　北陸支部第28回大会（2010年5月29日　福井）
　B型肝炎ウイルス高頻度突然変異における塩基除去修復因子UNGの作用"
　喜多村晃一、Zhe Wang、Sajeda Chowdhury、Guoxin Liang、村松正道
・第14回国際免疫学会議（2010年8月22日～27日　神戸）
　"Base excision repair by uracil DNA glycosylase counteracts interferon-induced hypermutation on hepatitis B virus DNA"
　Kouichi Kitamura, Zhe Wang, Sajeda Chowdhury, Guoxin Liang, Yoshiko Mitsuya, Miyuki Simadu, Miki Koura, Taketomo Ozaki, and Masamichi Muramatsu

−日中医学協会助成事業−

薬剤耐性ウイルス出現における自然免疫効果分子APOBECの役割

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Background:

In HBV story, lamivudine strongly suppresses HBV replication, so that prognosis of chronic hepatitis due to HBV is much improved. However, long-term cohort studies revealed surprisingly rapid emergence of lamivudine resistant HBVs in the chronic HBV infected patients. Point mutation in drug target gene is responsible for such drug resistance. It is proposed that such point mutations are generated by error of DNA polymerases during virus replication.

On the other hand, numerous cytokines are involved in immune responses, as well as antiviral activity, viral clearance, apoptosis, and fibrogenesis. In HBV study, it has been known that IFN and TGF-β1 can inhibite HBV replication in vitro experiment. In clinic treatment, Moreover, IFNs are quite classical approach to cure the HBV infected patient. It is also known that both IFNs and TGF-β1 can induce some APOBECs expression. Since APOBECs can mutate HBV genomic DNA, it is plausible that APOBECs upregulated by cytokine stimulation during hepatitis generate a drug resistant clone. To evaluate the possibility, first of all, expression profiling has been performed to decide which APOBECs can be a generater of drug resistant virus.

Results:

Fig1, IFN-α-induced expression of APOBEC genes in HepG2 cells. Expression of APOBEC cytidine deaminases and adenosine deaminase acting on RNA (ADAR). cDNA pools were synthesizes from human hepatoma cells(HepG2 cells), which had been cultivated for 9 hours with and without IFN-α, and were analysed by Reverse Transcription-PCR for the expression of AOPBEC3s and ADAR1. β-actin cDNA was amplified as a loading control. The PCR products were analysed on 2% agrose gel and then stained with SYBR Green .

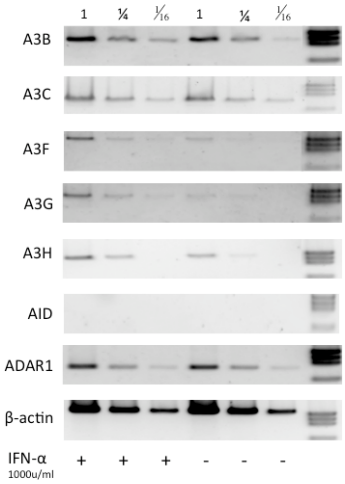


Fig2, IFN γ-induced expression of APOBEC genes in HepG2 cells. Expression of APOBEC cytidine deaminases and adenosine deaminase acting on RNA (ADAR). cDNA pools were synthesizes from human hepatoma cells(HepG2 cells), which had been cultivated for 48 hours with and without IFN-γ, and were analysed by Reverse Transcription-PCR for the expression of AOPBEC3s and ADAR1. β-actin cDNA was amplified as a loading control. The PCR products were analysed on 2% agrose gel and then stained with SYBR Green .

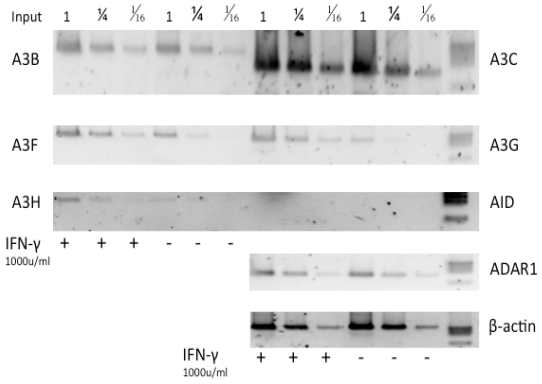


Fig3, TGF-β1-induced expression of APOBEC genes in HepG2 cells. . HepG2 cells total RNA was isolated before(0 hr), 12hr and 15hr after stimulation with human TGF-β1(10ng/ml).APOBEC gene expression were assessed by RT-PCR. The PCR products were analysed on 2% agrose gel and then stained with Ethidium bromide.

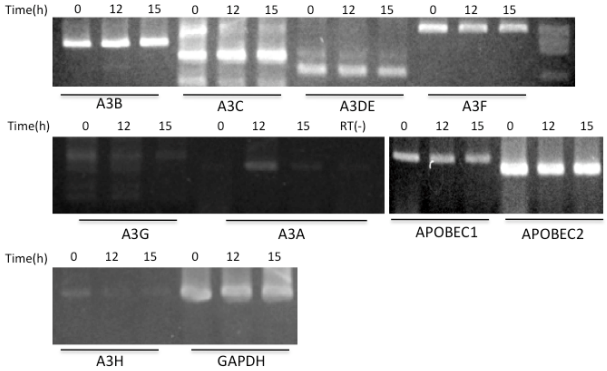


Fig4, TGF-β1-induced expression of APOBEC genes in Huh7 cells. Fig.4. HuHh7 cells total RNA was isolated before and 48hr after stimulation with human TGF-β1(10ng/ml). APOBEC genes expression were assessed by RT-PCR. The PCR products were analysed on 2% agrose gel and then stained with Ethidium bromide.

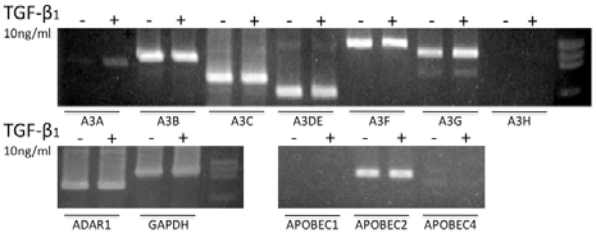
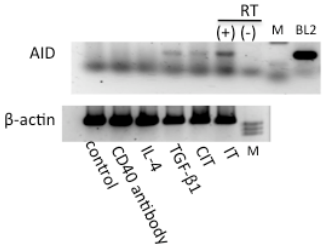


Fig5, AID expression by CIT stimulation 2 days in Huh7 cells. Induction of AID mRNA expression in Huh7 cells. cells were either unstimulated or stimulated for 2 days with anti-CD40(10ug/ml), IL-4(150ng/ml), TGF-β1(10ng/ml) and antiCD40+IL-4+TGF-β1. AID gene expression was assessed by RT-PCR. The PCR products were analysed on 2% agrose gel and then stained with SYBR Green . Here, BL2 serves as postive control.



Conclusion:

In our semi-quantitative PCR experiment, we find that a: IFN-α not only could robustly induce APOBEC3G expression but also modest induce APOBEC3F and APOBEC3H expression in HepG2 cell line, however, no significant induction in APOBEC3A、3B、3C、ADAR1. b: IFN-γ has the twin feature in APOBEC family genes expression in HepG2 cell line. c: TGF-β could remarkably up-regulate APOBEC3A expression in both HepG2 and Huh7 cell line. d: in Huh7 cells, we found not TGF-β1 but also CIT(antiCD40+IL-4+TGF-β1) or IT(IL-4+TGF-β1) could induce AID transcript expression. But anti-CD40 or IL-4 alone did not exhibit any apparent induction. This synergic effect is first time proposed by us in hepatoma cell.

Summary:

Hepatocellular carcinoma (HCC) is one of the most prevalent of human malignancies, and individuals who are chronic

Hepatitis B virus(HBV) carries have a greater than 100 fold increased relative risk of developing HCC. Lamivudine treatment has been very useful in reducing HBV DNA leveles in patient sera and shows very limited side effects(8).It was reported that drug resistant HBV appears in high frequency(2), with the incidence of resistance after 1 year of treatment ranging from 17% to 32%(3,9). Moreover, 65% of patients who have been treated with lamivudine for 5 years appear drug resistant (1). Most of HBV researchers accept that cytokines, like IFNs, IL-4 and TGF-β1, are participated in HBV infection process. During HBV infection period, our immune system releases these factors to defend ourself. Cytidine deaminase is a member of a much larger family of tissuse-restricted deaminases that exhibit RNA editing and/or DNA mutating activity. In human, APOBEC family which includes activation-induced deaminase(AID)、APOBEC1、 APOBEC2、 APOBEC3A~H and APOBEC4 is a dominant cytidine deaminase. Many study already shown that some of APOBECs could inhibit viral replication, like HIV、HBV as well HPV, in vitro and vivo.

In our detection system, Semi-quantitative RT-PCR confirmed induction of APOBEC3G and F expression with both IFNα and IFNg stimulations in our cell lines. Consistent with previous studies from other research groups, APOBEC3G is the major responder of interferon stimulation among APOBEC3 families in our cell lines(4-7). Besides, we further proposed that another APOBEC predominant member, namely AID, was induced by CIT(antiCD40+IL-4+TGF-β1) in Huh7 cells. We also observed that TGF-β1 had robust suppression effect in HBV replication in vitro experiment (data not shown). These results might reflect that potential mediator between cytokines and viral is deaminase. Finally we assume that point mutation in HBV drug resistant might be resulted from deaminase reaction by APOBECs, especially G(Guanine) to A(Adenine) and C(Cytosine) to T(Thymine), during HBV replication process.

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Materials and methods

IFNα(recombinant IFNα-2b) and IFNγ(recombinant IFNg-1a) were purchased from Schering-Plough and Shionogi, respectively. Huamn TGF-β1, (R&B. Systems, Minneapolis, MN). SYBR Green was purchased from Invitrigene.

Cell culture

Human hepatoma HuH-7 and HepG2 cells were cultivated at 37°C in Dulbecco's modified Eagle medium containing 10% fetal calf serum(Wako, Osaka, Japan).

Semi quantitative RT-PCR

RNA was prepared from cultivated human liver cells using TRIsure according to the manufacturer's instruction. Five micrograms total RNA from hepatoma cells was reverse transcribed using a commercially available cDNA synthesis kit(SuperScript® III Reverse Transcriptase, invitrogen, CA) and oligo-dT primer. Conventional polymerase chain reaction (PCR) amplification of target cDNAs was performed.

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