

Methyltransferase of a cell culture-adapted hepatitis E inhibits the MDA5 receptor signaling pathway

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Hepatitis E virus (HEV) is a causative agent of acute hepatitis and jaundice. The number of human infections is approximated to be over 20 million cases per year. The transmission is mainly via the fecal-oral route and contaminated water and food are considered to be a major source of infection. As a mouse model is not available, a recent development of a cell culture-adapted HEV strain (47832c) is considered as a very important tools for molecular analysis of HEV pathogenesis in cells. Previously, we demonstrated that HEV-encoded methyltransferase (MeT) encoded by the 47832c strain inhibits MDA5- and RIG-I-mediated activation of interferon β (IFN- β) promoter. Here, we report that MeT impairs the phosphorylation and activation of interferon regulatory factor 3 and the p65 subunit of NF- κ B in a dose-dependent manner. In addition, the MeT encoded by the 47832c, but not that of HEV clinical or field isolates (SAR-55, Mex-14, KC-1, and ZJ-1), displays the inhibitory effect. A deeper understanding of MeT-mediated suppression of IFN- β expression would provide basis of the cell culture adaptation of HEV.

Keywords: hepatitis E virus, methyltransferase, interferon

Introduction

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus which is found enveloped in blood and non-enveloped in bile and feces (Denner, 2019). Its genome is known to have 3–4 partially overlapping open reading frames (ORFs): ORF1 encodes 6 non-structural proteins (Koonin *et al.*, 1992; Ropp *et al.*, 2000; Ahola and Karlin, 2015; Kanade *et al.*, 2018; Kang *et al.*, 2018) while ORF2 and ORF3 encode viral capsid protein and an ion channel, respectively (Ding *et al.*, 2017; Kang and Myoung, 2017a; Kang *et al.*, 2018). Recently, ORF4 has been proposed and Nair *et al.* (2016) reported that it is expressed under certain conditions, such as ER stress, whose expression seems to be limited to HEV genotype 1. HEV in-

fections are known to be associated with the most common cause of acute viral hepatitis, accounting for over 20 million cases world-wide (Kang *et al.*, 2018). In healthy subjects, HEV infections are mostly self-limiting and asymptomatic. However, high mortality has been reported if HEV infects pregnant women (Jilani *et al.*, 2007; Navaneethan *et al.*, 2008).

Upon virus infections, type I interferons (IFNs) are rapidly induced, subsequently activating down-stream signaling molecules which leads to anti-viral innate immune responses (Theofilopoulos *et al.*, 2005; Xi *et al.*, 2012; Kang and Myoung, 2017a). The genomes of invading viruses are recognized by pattern recognition receptors (Akira *et al.*, 2006; Fujita *et al.*, 2007; Medzhitov, 2007): toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5). Although both RIG-I and MDA5 recognize double-stranded RNA's (dsRNA's) (Loo *et al.*, 2008; Takeuchi and Akira, 2010), there seem to be subtle differences in the nature, length, and structure of dsRNA's they bind. RIG-I has been reported to bind to relatively short dsRNA's which are blunt-ended with 5'-triphosphates (Kato *et al.*, 2006, 2008). On the contrary, MDA5 does not seem to have strict structural and chemical restrictions, binding to long dsRNA's (Hornung *et al.*, 2006; Pichlmair *et al.*, 2006; Kim and Myoung, 2018; Myoung and Min, 2019). Upon cognate ligand binding, RLR's undergo conformational changes, releasing auto-inhibited caspase activation and recruitment domain (CARD). CARD domains of the RLR's interact with that of mitochondrial antiviral signaling protein (MAVS), which subsequently induces its oligomerization on the outer membrane of mitochondria. Activated MAVS in turn induces activation of down-stream signaling molecules: TANK-binding kinase 1 (TBK1), I κ B kinase ϵ (IKK ϵ), and interferon regulatory factor 3 (IRF3). Before activation, IRF3 resides in the cytoplasm in an auto-inhibited form. Phosphorylation of IRF3 at the C-terminal regulatory domain induces self-dimerization and nuclear translocation of IRF3. Nuclear IRF3 dimers bind to the promoter of IFN- β , leading to transcription of its mRNA.

Previously, we showed that HEV-encoded methyltransferase (MeT) strongly inhibits MDA5-mediated induction of the IFN- β promoter (Myoung and Min, 2019) as well as that of RIG-I (Kang *et al.*, 2018). Here, we demonstrated that MeT efficiently down-regulates the phosphorylation of IRF3 S396. In addition, NF- κ B p65-mediated activation of the NF- κ B promoter was strongly suppressed in a dose-responsive manner, likely due to blockade of phosphorylation at S468 of p65. More importantly, only MeT of HEV genotype 3 (47832c strain) displayed inhibitory activity on MDA5-mediated activation of IFN- β . Taken together, MeT seems to be a novel antagonist of the receptor signaling pathway and delineation

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of its detailed mechanisms will likely pave way to the development of virus-specific therapeutics.

Materials and Methods

Cell culture and reagents

Human embryonic kidney 293T (HEK293T) cells were maintained in Dubecco's modified Eagle's medium (DMEM, Wel-Gene) with supplements (10% fetal bovine serum; Gibco and 1% penicillin/streptomycin; Thermo Fisher Scientific) in a humidifying incubator at 37°C with 5% CO₂. Polyethyleneimine (PEI, Sigma-Aldrich) was used for transfection of DNA at 1:2 ratio (DNA vs PEI). Antibodies were purchased from various manufacturers: anti-FLAG antibody (M2) from Sigma-Aldrich, anti-phospho-IRF3 (S396) and anti-phospho-p65 (S468) from Cell Signaling, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) from Cell Signaling.

DNA constructs and transfection

Construction of expression plasmids of HEV-encoded genes and the host signaling molecules (MDA5, RIG-I, TBK1, IKK ϵ ,

and IRF3) were described elsewhere (Kang *et al.*, 2018; Kim and Myoung, 2018; Myoung and Min, 2019). Sequences of MeT's of 4 reference strains were codon optimized and synthesized by Bionics. Accession numbers of each HEV strain are as follows: Sar-55 (genotype 1, AF444003.1), Mex-14 (genotype 2, KX578717.1), Kernow C-1 (genotype 3, JQ679014.1), and ZJ-1 (genotype 4, JQ993308.1). Synthesized MeT gene fragments were cloned into pCMV10-3XFLAG using *EcoRI* and *XbaI*. For luciferase assay, IFN- β -luc or NF- κ B-luc was co-transfected with plasmids expressing an HEV-encoded gene and a host signaling molecule as indicated. DNA-PEI complex was generated by mixing at 1:2 ratio and incubating at RT for 30 min (Lee *et al.*, 2019a, 2019b, 2019c). DNA-PEI complexes were added dropwise onto cells and cells were incubated for 24 h before harvested for luciferase reporter assay or western blotting.

Luciferase reporter assay

To examine if HEV-encoded MeT regulates the MDA5/RIG-I receptor signaling pathway, luciferase reporter assay was employed as described before (Kang *et al.*, 2018; Kim and Myoung, 2018; Myoung and Min, 2019; Park *et al.*, 2019). Briefly, MeT expressing plasmid was co-transfected into HEK293T cells with IFN- β -Luc/NF- κ B-Luc, β -galactosidase

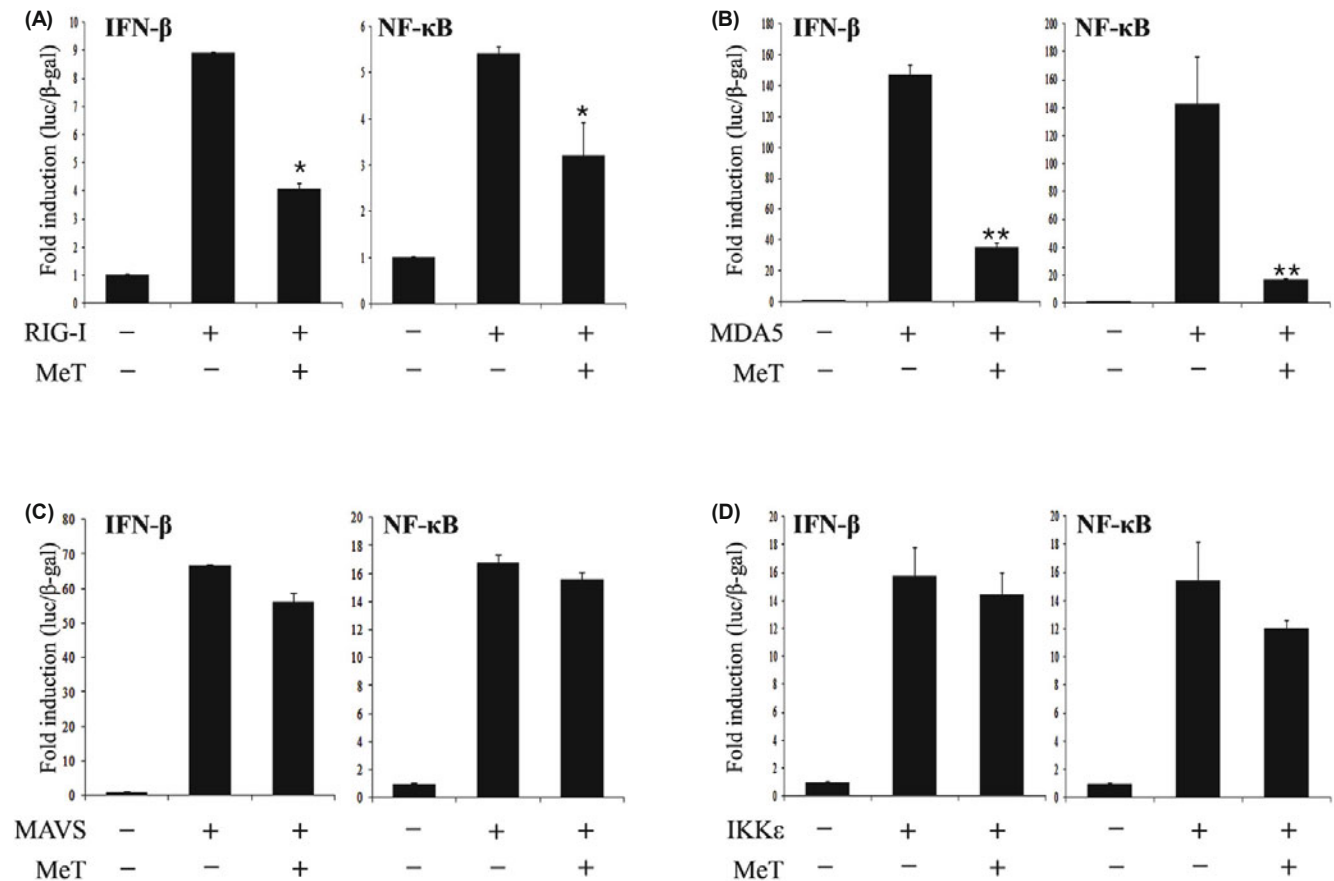
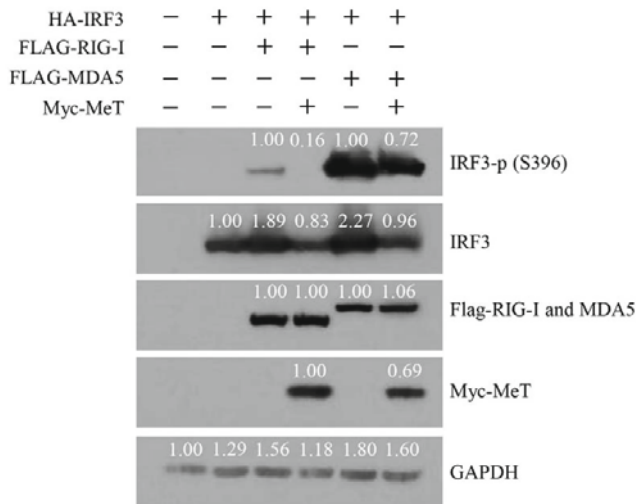


Fig. 1. Methyltransferase inhibits RLR-mediated activation of the IFN- β pathway. HEK293T cells were co-transfected with MeT and RIG-I (A), MDA5 (B), MAVS (C), or IKK ϵ (D). At 24 h post-transfection, cells were lysed and assayed for firefly luciferase activities transcribed from IFN- β -Luc (left panels) or NF- κ B-Luc (right panels). Data presented are mean \pm SD of triplicated samples. * $P < 0.05$; ** $P < 0.01$.



(β -gal) control, and one of signaling molecule gene involved in the pathway as indicated. At 24 h post-transfection, cells were lysed for luciferase assay using Glomax (Promega) following the manufacturer's instructions. Luciferase activities of each experimental group, an indicator of IFN- β promoter induction, were measured and normalized to those of β -gal of each well.

Statistical analysis

Statistical significance was determined by the two-tailed Student's *t*-test. P value less than 0.05 was considered statistically significant. Data shown are one representative from two independent experiments.

Results and Discussion

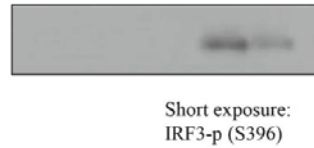
HEV-encoded MeT inhibits RIG-I- and MDA5-mediated induction of the IFN- β and NF- κ B promoters

Previously, we demonstrated that MeT strongly antagonize RIG-I- (Kang *et al.*, 2018) and MDA5- (Myoung and Min, 2019) mediated activation of the IFN- β promoter. To investigate if MeT regulates other signaling molecules of the IFN- β signaling pathway (i.e., MAVS and IKK ϵ) and if NF- κ B activation is also affected, HEK293T cells were co-transfected with MeT and signaling molecules as indicated in Fig. 1. As we previously demonstrated, MeT strongly inhibited RIG-I- and MDA5-mediated activation of the IFN- β promoter (Fig. 1A and B, left panels). Of note, MeT also antagonized induction of NF- κ B promoter as well, which suggests that NF- κ B activation and/or nuclear translocation was inhibited (Fig. 1A and B, right panels). On the other hand, MAVS- and IKK ϵ -mediated activation of the IFN- β signaling pathway was unperturbed, suggesting that MeT-mediated antagonism is largely via inhibition of RLR's.

HEV MeT strongly inhibits RIG-I- and MDA5-mediated phosphorylation of IRF3

RIG-I and MDA5-mediated induction of the IFN- β signal-

Fig. 2. MeT down-regulates RIG-I- and MDA5-mediated phosphorylation of IRF3. HEK293T cells HEK293T cells were co-transfected with Myc-MeT and HA-IRF3, Flag-RIG-I and/or Flag-MDA5 expression plasmids as indicated. At 24 h post-transfection, cells were harvested and analyzed by immunoblotting with anti-p-IRF3 (Ser-396), anti-Flag, anti-Myc, and anti-GAPDH antibodies.



ing pathway ultimately leads to phosphorylation and dimerization of IRF3, a key transcription factor of the pathway. Therefore, we tested if IRF3 phosphorylation is regulated by the expression of HEV MeT. HEK293T cells were co-transfected with MeT and IRF3 together with either RIG-I or MDA5 (Fig. 2). When cells were transfected with IRF3 and RIG-I, IRF3 was efficiently phosphorylated at S396 with total IRF3 levels remaining largely unchanged (Fig. 2, left lanes). How-

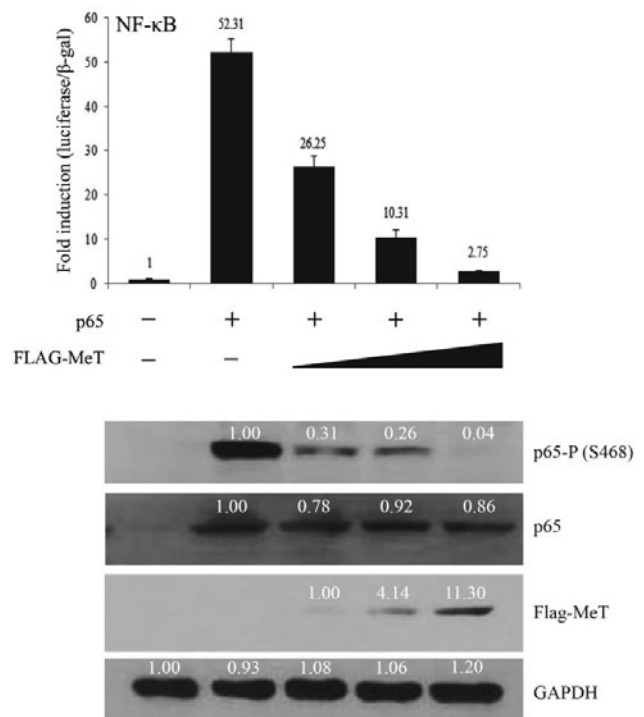


Fig. 3. MeT impairs phosphorylation of the p65 subunit of NF- κ B in a dose-responsive manner. HEK293T cells were co-transfected with FLAG-MeT and the p65 subunit of NF- κ B. At 24 h post-transfection, cells were lysed and assayed for firefly luciferase activity whose expression was driven by the NF- κ B promoter activation.

ever, when MeT was co-expressed, IRF3 phosphorylation was significantly impaired (Fig. 2, middle lanes). The same was true to MDA5-mediated activation of IRF3 (Fig. 2, right lanes).

HEV MeT inhibits MDA5-mediated phosphorylation of the p65 subunit of NF- κ B in a dose responsive manner

As MDA5 strongly inhibits activation of NF- κ B (Fig. 1), phosphorylation of p65 at the S468 residue was probed. An increasing amount (0, 0.5, 1, 2 μ g) of HEV MeT-expressing plasmids were co-transfected with MDA5 and p65 (1 μ g) into HEK293T cells and at 24 h post-transfection, cells were harvested for luciferase assay and western blotting. Of note, p65-mediated activation of the NF- κ B promoter was significantly diminished by MeT co-expression in a dose-dependent manner (Fig. 3, top panels), suggesting that HEV MeT is a true antagonist of NF- κ B. Interestingly, phosphorylation levels of p65, induced by MDA5, were down-regulated by MeT (Fig. 3, bottom panels), suggesting that MeT-mediated inhibition of NF- κ B activation is likely due to impairment of p65 phosphorylation.

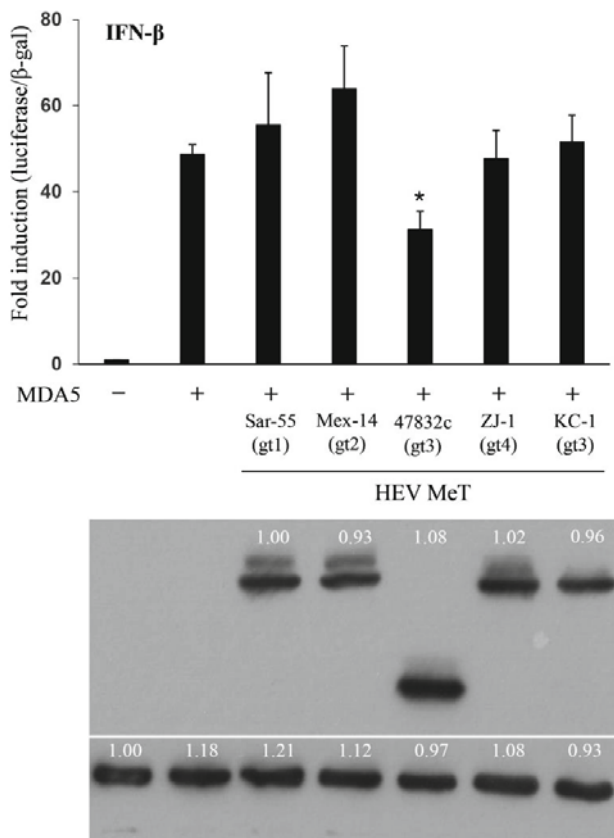


Fig. 4. The MeT of the cell culture adapted HEV strain, but not clinical isolates, displays inhibitory effects on the promoter activity of IFN- β . HEK293T cells were co-transfected with MDA5 and MeT genes from Sar-55 (GT1), Mex-14 (GT2), 47832c (GT3), KC-1 (GT3), and ZJ-1 (GT4), which are all clinical isolates except 47832c. At 24 h post-transfection, cells were harvested for luciferase assay.

MeT of cell culture-adapted 47832c strain displayed inhibitory effects on the IFN- β signaling pathway while MeT's of HEV fields isolates did not

The bottlenecks of studies on HEV and HEV-mediated diseases have been the lack of efficient cell culture system and a small animal model. Recently, Shemmerer *et al.* (2016) reported that a cell culture-adapted HEV strain (HEV 47832c) was successfully isolated and amplified in a subline of A549 (A549/D3). To establish cell culture-adapted HEV 47832c, a clinical isolate of GT3 was passed in A549/D3 over many passages which may have allowed accumulation of mutations for the culture adaptation. To investigate if IFN- β signaling inhibition by HEV 47832c-derived MeT is conserved among those of clinical isolates, MeT's of various HEV strains were cloned and tested (Fig. 4). Interestingly, only MeT of the 47832c strain showed inhibitory activities against activation of the IFN- β signaling while MeT's, derived from other clinical isolates, had little, if any, effect on the pathway.

Discussion

The lack of an efficient cell culture model and a small animal model has greatly hampered in-depth understanding of HEV-mediated pathologies. In this study, we demonstrated that MeT of HEV 47832c strain, a cell culture-adapted HEV strain, displays strong antagonism of MDA5-/RIG-I-mediated receptor signaling pathway.

Previously, we demonstrated that MeT of HEV 47832c strain strongly inhibits MDA5- (Myoung and Min, 2019) or RIG-I- (Kang *et al.*, 2018) mediated induction of the IFN- β signaling pathway. MDA5 and RIG-I are pattern recognition receptors (PRR's) which sense the genomes of invading viral pathogens (Loo *et al.*, 2008; Takeuchi and Akira, 2010; Kang and Myoung, 2017a, 2017b). The notion is consistent with our current findings: MeT strongly antagonized RIG-I- and MDA5-, but not MAVS- or IKK ϵ -, mediated activation of the IFN- β signaling pathway (Fig. 1). In addition, MeT-mediated inhibition of PRR's seems to result in low levels of IRF3 phosphorylation (Fig. 2), leading to low levels of induction of the IFN- β and NF- κ B promoters (Fig. 1). Similarly, MeT inhibited NF- κ B activation (Fig. 1) and phosphorylation of the p65 unit of NF- κ B was suppressed by the expression of MeT in a dose-dependent manner (Fig. 3). These results suggest that MeT efficiently blocks activation of IRF3 and NF- κ B, presumably due to down-regulation of MDA5- and RIG-I-mediated activation of the pathway.

Of note, only MeT of HEV 47832c strain demonstrated inhibitory activity on the MDA5-mediated induction of the IFN- β signaling (Fig. 4) while MeT's, derived from Sar-55, Mex-14, ZJ-1 or Kernow-C1, had little, if any, effect. It is even more interesting because HEV 47832c strain is a cell culture-adapted strain (Shemmerer *et al.*, 2016). A simple hypothesis would be that HEV adaption to cell culture requires effective blockade of the IFN- β signaling. Currently, we are testing this hypothesis by establishing another cell culture adapted HEV strains and by comparing MeT sequences of clinical (original) vs cell culture-adapted HEV strains. Alternatively, cell culture adapted HEV strains have shown host sequence insertions in its genome (Shukla *et al.*, 2011). It

would be very interesting to test if those insertional modifications of the viral genome may be associated with viral antagonisms of IFN- β . Last but not in the least, MeT of HEV 47832c, used in this study, excludes the C-terminal Y domain (so called “iceberg”) while MeT’s other strains encompass it. The Y domain was initially considered an independent viral protein, but now it is considered an integral part of the MeT protein (Ropp *et al.*, 2000; Ahola and Karlin, 2015; Kanade *et al.*, 2018). Studies are currently underway to investigate if MeT with/without the Y domain may have differential functions.

Taken together, we demonstrated that MeT inhibits phosphorylation of IRF3 and NF- κ B, thus blocking efficient induction of the IFN- β promoter. Delineation of detailed molecular mechanisms of MeT-mediated antagonism of the IFN- β pathway may pave way to the development of HEV-specific therapeutics.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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