Virology 462-463 (2014) 42–48

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

The flavonoid apigenin inhibits hepatitis C virus replication by decreasing mature microRNA122 levels

Chikako Shibata [a], Motoko Ohno [a], Motoyuki Otsuka [a,b,*], Takahiro Kishikawa [a], Kaku Goto [c], Ryosuke Muroyama [c], Naoya Kato [c], Takeshi Yoshikawa [a], Akemi Takata [a], Kazuhiko Koike [a]

[a] Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, 5-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
[b] Japan Science and Technology Agency, PRESTO, Kawaguchi-shi, Saitama 332-0012, Japan
[c] Unit of Disease Control Genome Medicine, Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

ARTICLE INFO

Article history:
Received 5 May 2014
Accepted 13 May 2014
Available online 14 June 2014

Keywords:
HCV
MicroRNA
Replicon
Polyphenol

ABSTRACT

Despite recent progress in the development of direct-acting antivirals against hepatitis C virus (HCV), chronic HCV infection remains an important health burden worldwide. MicroRNA122 (miR122), a liver-specific microRNA (miRNA), positively regulates HCV replication, and systemic application of antisense oligonucleotides against miR122 led to the long-lasting suppression of HCV viremia in human clinical trials. Here, we report that apigenin, a flavonoid and an inhibitor of maturation of a subset of miRNAs, inhibits HCV replication in vitro. Apigenin decreased the expression levels of mature miR122 without significantly affecting cell growth. Because supplementation of synthesized miR122 oligonucleotides or overexpression of constitutively active TRBP blocked these effects, the inhibitory effects of apigenin on HCV replication seemed to be dependent on the reduction of mature miR122 expression levels through inhibition of TRBP phosphorylation. Thus, apigenin intake, either through regular diet or supplements, may decrease HCV replication in chronically infected patients.

Introduction

Hepatitis C virus (HCV) constitutes a significant health problem worldwide, with an estimated 130–170 million people chronically infected (Scheel and Rice, 2013). Chronic HCV infection leads to severe liver diseases, including advanced liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). A recent HCV therapy consisting of a triple combination of pegylated interferon α (peg IFN-α), ribavirin, and protease inhibitors increased cure rates (Jacobson et al., 2011; Poordad et al., 2011; Bacon et al., 2011; Sherman et al., 2011). However, substantial side effects, resistance, and drug-drug interactions are concerns with this therapy. Although an IFN-free regimen with direct-acting antivirals (DAAs) is beginning to reach patients and increase cure rates (Manns and von Hahn, 2013; Deuffic-Burban et al., 2014), several issues remain, including treatment failure, resistant clones, and economic burden.

MicroRNA122 (miR122) is a highly abundant microRNA (miRNA) expressed in the liver and essential for the stability and propagation of HCV RNA (Jopling, 2012; Pfeffer and Baumert, 2010; Jopling et al., 2005). It binds to two closely spaced target sites in the highly conserved 5′-untranslated region (5′-UTR) of the HCV genome. These sites are conserved across all HCV genotypes and subtypes (Li et al., 2011). This positive regulation through 5′-UTR sites is a unique process, as compared to the usual function of miRNAs in the repression of gene expression via 3′-UTRs in target mRNAs. Although the precise mechanisms by which miR122 positively regulates HCV replication through its binding to the 5′-UTR of the HCV genome are not yet fully elucidated (Jopling, 2012), it was demonstrated that LNA-based anti-miR122 oligonucleotides led to the long-lasting suppression of HCV viremia and improvement of HCV-induced liver pathology in chimpanzees (Lanford et al., 2010). Based on experimental results, human clinical trials using miravirsen, an LNA-modified DNA phosphorothioate antisense oligonucleotide against miR122, have been conducted, and in Phase 2a studies miravirsen resulted in a dose-dependent reduction in HCV levels, without major adverse events and with no escape mutations in the miR122 binding sites of the HCV genome (Janssen et al., 2013). The miR122 binding sites are conserved across all HCV genotypes and subtypes, and miR122 could represent a host target for antiviral therapy.

We previously demonstrated that the flavonoid apigenin (4′,5,7-trihydroxyflavone) has inhibitory effects on the maturation of a subset of miRNAs and on subsequent miRNA function (Ohno et al., 2013). These effects were mediated by the inhibition of TRBP

* Corresponding author at: Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, 5-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel.: +81 3 3815 5411x37966; fax: +81 3 3814 0021.
E-mail address: otsukamo-tky@umin.ac.jp (M. Otsuka).

http://dx.doi.org/10.1016/j.virol.2014.05.024

phosphorylation through inhibition of ERK activation (Ohno et al., 2013). We reported that the administration of apigenin to mice improved glucose intolerance induced by overexpression of miR103 in the liver, likely through suppression of mature miR103 expression. Moreover, we found that miR122 was also affected by apigenin (Ohno et al., 2013).

We hypothesized that apigenin may exert inhibitory effects on HCV replication by decreasing mature miR122 expression levels. In this study, we assessed the effects of apigenin on HCV replication and the possible molecular mechanisms by using an in vitro HCV replicon reporter system. Based on our findings, we propose potential novel management methods for chronic HCV infection and possibly other pathological states mediated by miR122.

Results

Apigenin inhibits the biogenesis of miR122

We recently showed that apigenin inhibits the biogenesis of a subset of miRNAs (Ohno et al., 2013). Because previous screening of the comprehensive miRNA expression changes revealed that miR122 was one of the miRNAs affected by apigenin (NCBI Gene Expression Omnibus (GEO) accession number: GSE46526) (Ohno et al., 2013), we here measured mature miR122 levels in Huh7 cells by quantitative RT-PCR, after apigenin treatment for 5 days. While the expression levels of let-7g, an unrelated miRNA, were not affected, apigenin significantly reduced mature miR122 expression levels in a dose-dependent manner (Fig. 1a). Northern blotting confirmed the reduced expression levels of miR122 after apigenin treatment (Fig. 1b). By contrast, expression of miR122 precursor, but not let-7g precursor, was increased after apigenin treatment (Fig. 1c), as we described previously (Ohno et al., 2013). Consistent with the reduced levels, miR122 function was inhibited by apigenin, as determined by the increased reporter values from a transiently transfected reporter construct with two miR122 target sites in tandem in its 3′-UTR (Fig. 1d). The effect was miR122 function-specific because no effects were observed with use of a reporter construct with mutations in the miR122 target sites (Fig. 1d). Apigenin had no significant effects on Huh7 cell growth at a final concentration of up to 5 μM (50 μM apigenin slightly reduced the cell number) (Fig. 1e). These results suggest that up to 5 μM of apigenin inhibits the expression levels of mature miR122 in Huh7 cells without affecting cell viability.

Apigenin inhibits HCV replication

Because miR122 positively regulates HCV replication (Jopling et al., 2005), we hypothesized that apigenin might inhibit HCV replication by decreasing the expression levels of mature miR122. To test this hypothesis, we used Huh7 cells harboring an HCV replicon reporter construct (HCV-Feo), referred to as Huh7-Feo cells (Yokota et al., 2003). These cells continuously carry a replicon expressing a chimeric protein consisting of firefly luciferase and neomycin phosphotransferase under the HCV 5′ IRES and can be used to monitor intracellular HCV replication by measuring luciferase activity (Fig. 2a) (Yokota et al., 2003). Treatment of Huh7-Feo cells with apigenin (final concentration 5 or 50 μM) for 5 days significantly reduced HCV-Feo replication (Fig. 2b). The pattern of luciferase values closely matched the pattern of luciferase protein expression levels (Fig. 2c). The decrease in replication observed with 5 μM apigenin treatment was only slightly less than that obtained following the transfection of anti-miR122 oligonucleotides at 10 pM. Although 50 μM apigenin may have adverse effects on cell viability, as described above, these results suggest that 5 μM apigenin can significantly inhibit HCV replication without affecting cell viability, possibly through downregulation of mature miR122 expression levels.

Apigenin inhibits HCV replication through downregulation of miR122 levels

To further investigate the mechanisms of the observed negative effects of apigenin on HCV replication, we applied synthesized mature miRNAs to Huh7-Feo cells to determine whether miRNA supplementation could antagonize the effects of apigenin. Overexpression of miR122 after the transfection of synthesized miR122, compared with the expression levels of miR122 in cells with no treatment or with let-7g transfection, was confirmed by Northern blotting (Fig. 2d). As expected, overexpression of miR122 efficiently antagonized the negative effects of apigenin on HCV replication, while supplementation of an unrelated miRNA, let-7g, had no effect on HCV replication (Fig. 2e). These results suggest that apigenin inhibits HCV replication by downregulating mature miR122 expression.

Phosphorylation mimic TRBP blocks the effects of apigenin

Because we previously demonstrated that apigenin inhibits the maturation of a subset of miRNAs by inhibiting the phosphorylation of TRBP, which contributes to the maturation of a subset of miRNAs by binding to Dicer (Paroo et al., 2009). We constructed a flag-tagged TRBP-expressing construct and a phosphorylation mimics in which serine was substituted with aspartic acid (TRBP (SD)) (Paroo et al., 2009). The expression levels of the wild-type TRBP and TRBP(SD) constructs were comparable (Fig. 3a). After stably expressing these constructs in Huh7-Feo cells by lentiviral transduction, we determined the expression levels of miR122. While overexpression of wild-type TRBP slightly enhanced the expression levels of miR122, apigenin significantly reduced its expression levels (Fig. 3b). However, in TRBP(SD)-expressing cells, miR122 expression levels were significantly increased and were not affected by apigenin treatment (Fig. 3b), probably due to the phosphorylation mimic TRBP(SD) being constitutively active. Consistent with the changes in miR122 expression levels, replication of the HCV replicon, as determined by luciferase values, was inhibited by apigenin in cells expressing wild-type TRBP but not in cells expressing TRBP(SD), which showed a slight increase in replication (Fig. 3c). These results suggest that apigenin inhibits HCV replication through the inhibition of mature miR122 expression levels, probably by modulating TRBP phosphorylation, consistent with our previous report (Ohno et al., 2013).

Discussion

In this study, we demonstrate that apigenin inhibits HCV replication by decreasing the expression levels of mature miR122, possibly through inhibition of the phosphorylation of TRBP, an important factor for the maturation of a subset of miRNAs (Paroo et al., 2009).

Our study revealed that apigenin inhibits HCV replication. A liver-specific miRNA, miR122, has been reported to be linked with pleiotropic physiological functions (Jopling, 2012; Otsuka et al., 2014), such as liver development, cholesterol metabolism, iron metabolism, and fatty acid metabolism (Takata et al., 2013a). A particularly intriguing function of miR122 is its role in promoting HCV replication (Jopling et al., 2005). The success of miravirsen, an LNA-modified DNA phosphorothioate antisense oligonucleotide against miR122, against HCV in a Phase 2a study (Janssen et al., 2013) shows its promise as a novel anti-HCV drug and as the first miRNA-targeting therapy to be trialed. While miravirsen hybridizes

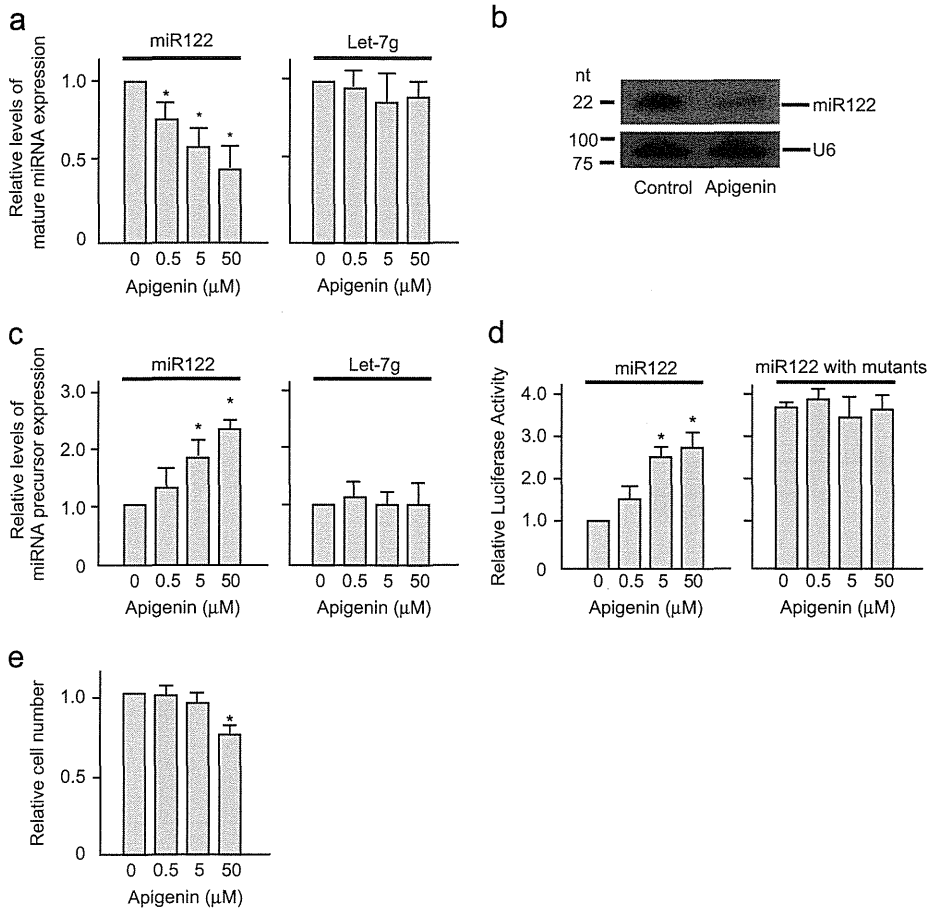

Fig. 1. Apigenin decreases mature miR122 expression levels. (a) The expression levels of mature miRNAs in Huh7 cells were determined by quantitative RT-PCR. Cells were treated with apigenin at the indicated doses for 5 days. Expression levels of mature miR122 and let-7g were determined. Relative expression levels are indicated as the means ± s.d. of three independent experiments. * $p < 0.05$. (b) The expression levels of mature miR122 were determined by Northern blotting. Huh7 cells were treated with 5 μM apigenin for 5 days. Expression levels of mature miR122 were determined by Northern blotting. U6 levels were determined as a loading control by reprobing the same membrane. Representative results from three independent experiments are shown. (c) The expression levels of miRNA precursors in Huh7 cells were determined by quantitative RT-PCR. Cells were treated with apigenin at the indicated doses for 5 days. Expression levels of miR122 and let-7g precursors were determined. Relative expression levels are indicated as the means ± s.d. of three independent experiments. * $p < 0.05$. (d) Endogenous miR122 function was determined by reporter assay. Huh7 cells were transiently transfected with reporter constructs containing miR122 binding sites or mutants. Cells were treated with apigenin at the indicated doses for 36 h. Data represent the mean ± s.d. of three independent experiments. * $p < 0.05$. (e) The number of cells was counted after treatment with apigenin at the indicated doses for 5 days. Experiments were performed in duplicate in a single test and the data represent the means ± s.d. of three independent tests. * $p < 0.05$.

to the 5′ region of mature miR122, resulting in sequestration and inhibition of miR122 (Janssen et al., 2013), it also binds to the stem-loop structure of pri- and pre-miR-122 and inhibits both Dicer- and Drosha-mediated processing of miR122 precursors (Gebert et al., 2014). Therefore, the importance of miR122 in HCV replication appears to depend on its expression level as well as its binding capacity. Because apigenin reduced the expression levels of mature miR122, and supplementation of synthesized mature miR122 blocked the effects of apigenin on HCV replication, the inhibitory effects of apigenin on HCV replication seem to be dependent on reduced levels of mature miR122. However, other potential molecular mechanisms for the effects of apigenin on HCV replication may exist.

Apigenin decreased the expression levels of a subset of mature miRNAs, including miR122, similar to the case of miR103, as we observed previously using microarray analyses (GEO accession number: GSE46526) (Ohno et al., 2013). We reported that the decreased maturation of miR103 was due to decreased phosphorylation of TRBP resulting from inhibition of ERK activities (Paroo et al., 2009). Because overexpression of the TRBP(SD) increased the levels of mature miR122, it appears that the reduced maturation of miR122 was also dependent on the activity of TRBP, which was inhibited by apigenin (Ohno et al., 2013). However, mature miRNA levels might be regulated not only by their synthesis but also, potentially, by their degradation, although this has not yet been established definitively (Jopling, 2012), and the effects of apigenin on the levels of mature miRNAs may be more diverse than expected. These effects should be explored in future studies involving the identification of the specific molecular target with which apigenin directly interacts and the development of an apigenin synthesis method, as has been achieved for resveratrol, another pleiotropic polyphenol (Snyder et al., 2011).

miR122 levels are frequently reduced in HCC compared with background liver tissues (Hou et al., 2011; Kutay et al., 2006; Gramantieri et al., 2007; Tsai et al., 2009), and lower miR122 expression levels in HCC tissues are correlated with a poor prognosis (Kojima et al., 2011). Because mice lacking miR122 in the liver showed spontaneous inflammation and liver tumors (Hsu et al., 2012; Tsai et al., 2012), miR122 may function as a tumor suppressor. However, to date no detectable liver toxicity has been reported with antisense oligonucleotide inhibition of miR122 in mice, primates, or humans (Lanford et al., 2010; Janssen et al., 2013; Elmén et al., 2008; Krützfeldt et al., 2005). We reported previously that transgenic mice expressing an antisense oligonucleotide specific for

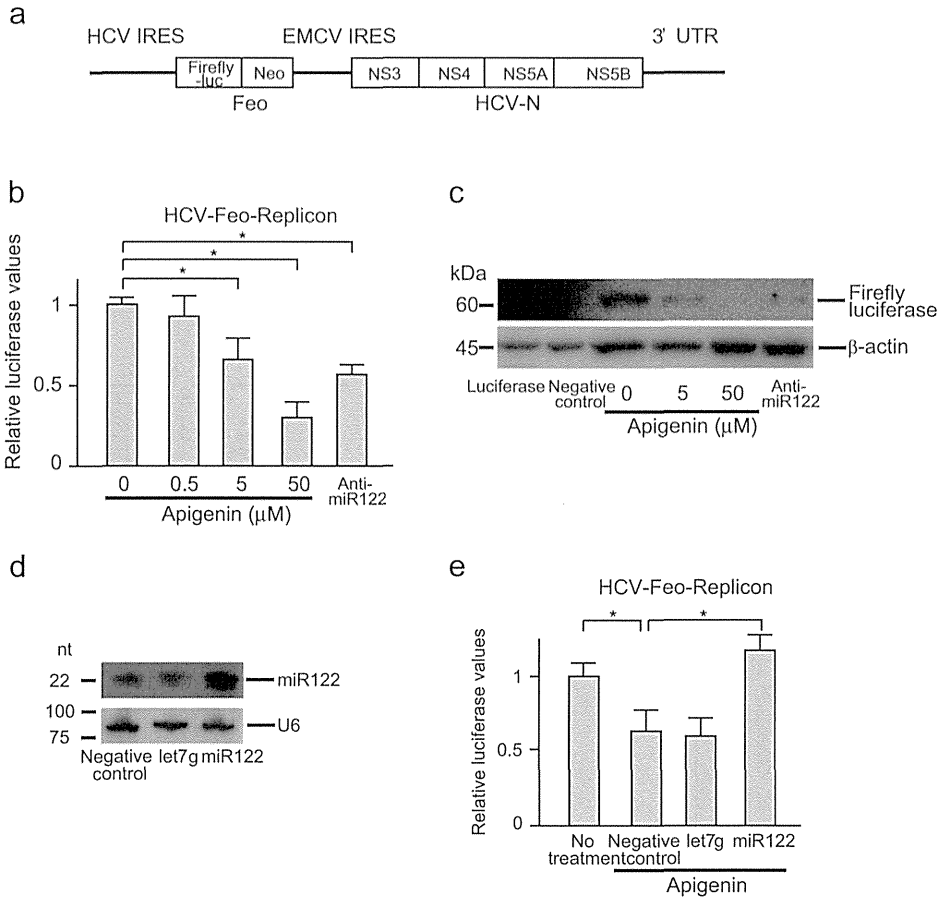

Fig. 2. Apigenin inhibits HCV replication in vitro by modulating miR122 levels. (a) Structure of the HCV replicon reporter. IRES, internal ribosomal entry site; firefly-luc, firefly luciferase gene; neo, neomycin resistance gene. NS3, NS4, NS5A, NS5B, and HCV are nonstructural proteins. Fusion genes consisting of the firefly luciferase gene and neomycin resistance gene are referred to as Feo. Huh7-Feo cells harbor an HCV replicon reporter construct (HCV-Feo) and expresses a chimeric protein consisting of firefly luciferase and neomycin phosphotransferase under the HCV 5′ IRES. (b) Apigenin inhibited replication of the HCV replicon reporter construct. Huh7-Feo cells were treated with apigenin for 5 days and the luciferase values were determined. Synthesized anti-miR122 oligonucleotides were transfected as a reference. Data represent the means ± s. d. of three independent experiments. * $p < 0.05$. (c) Apigenin inhibits the expression of luciferase protein under the HCV 5′ IRES of the reporter construct. Huh7-Feo cells were treated as described in (b) and luciferase protein was quantified by Western blotting. 293T cell lysates obtained after transient transfection with luciferase-expressing plasmid or control plasmid were used as controls. Representative results from three independent experiments are shown. (d) and (e) Synthesized miR122 blocks the inhibitory effects of apigenin on HCV replication. Synthesized mature miR122 and let-7g were transfected into Huh7-Feo cells. (d) Cells were harvested 48 h after transfection and mature miR122 levels were determined by Northern blotting. U6 levels were used as a loading control. Representative results from three independent experiments are shown. Synthesized miR122 overexpression blocked the inhibitory effects of apigenin on HCV replication (e) Huh7-Feo cells were treated or not treated with 5 μM apigenin for 5 days. Synthesized miRNAs were transfected 48 h before measurement. Luciferase values for HCV-Feo were determined by reporter assay. Data represent the means ± s.d. of the absolute luciferase values of three independent experiments. * $p < 0.05$.

miR122 showed no spontaneous pathological features (Kojima et al., 2011). In addition, mice treated with apigenin for 2 weeks suffered no detectable harmful events in our previous studies (Ohno et al., 2013). Therefore, while several miRNAs other than miR103 and miR122 are affected by apigenin (Ohno et al., 2013), apigenin treatment in vivo seems to be a safe and convenient method of reducing the expression levels of a subset of miRNAs. One of the reasons for this is that the effects of apigenin are relatively mild; it reduces but does not completely abolish target miRNA expression levels. The effects of apigenin as well as its long-term safety need to be determined in a small animal model.

The appropriate dose of apigenin in this in vitro study was 5 μM. The fasting plasma concentration of flavonoids, including apigenin, is proportional to their intake. Intake of 100 mg flavonoids results in a plasma concentration of ~410 nM (Cao et al., 2010). Apigenin is abundant in parsley, celery, and other herbs, according to the USDA Database for the Flavonoid Content of Selected Foods (Release 3.1). For example, fresh parsley contains 215.46 mg apigenin per 100 g edible portion, dried parsley contains 4303.50 mg, and celery seeds contain 78.65 mg. The plasma concentration of apigenin theoretically reaches 1.7 μM, assuming one eats 10 g of dried parsley per day. Thus, although it is not impossible to reach a plasma apigenin concentration of 5 μM through normal dietary intake, apigenin supplementation may be required to obtain an appropriate dose. However, the apigenin concentration in the liver may be higher than the plasma due to direct blood flow from the intestine, which absorbs nutrients, and eating foods rich in apigenin may be sufficient to reach the appropriate liver concentration. The concentration of apigenin in liver tissues should thus be determined in future studies.

In HCV therapy, treatment has become more effective with the advent of DAAs (Scheel and Rice, 2013; Manns and von Hahn, 2013). Thus, it is uncertain the extent to which our finding, that apigenin may inhibit HCV replication, contributes to patient care in the DAA era. However, worldwide access to drugs and the implementation of economical therapy are major challenges (Scheel and Rice, 2013; Manns and von Hahn, 2013), in addition to cases of non-responders and patients with clones resistant to DAAs. Our findings may provide novel insights into HCV management. The combination of apigenin with other agents, including

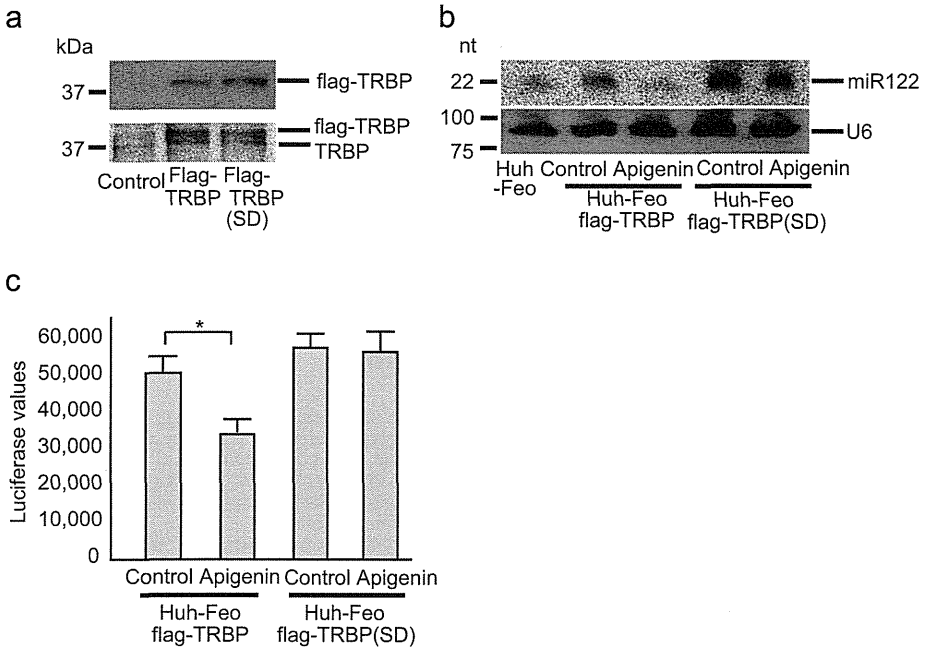

Fig. 3. Phosphorylation mimic TRBP blocks the inhibitory effects of apigenin on HCV replication. (a) Confirmation of flag-tagged TRBP-expressing construct expression. The expression of flag-tagged TRBP or phosphorylation mimic TRBP (TRBP(SD)) was confirmed by Western blotting using anti-flag and anti-TRBP antibodies. 293T cells were transiently transfected with constructs expressing the indicated proteins. Representative results from three independent experiments are shown. (b) TRBP (SD) blocked the inhibitory effects of apigenin on mature miR122 expression. Huh7-Feo cells were transduced with TRBP- or TRBP(SD)-expressing lentiviruses and selected. The expression levels of mature miR122 in Huh7-Feo cells, Huh7-Feo cells with TRBP expression, and Huh7-Feo cells with TRBP(SD) expression were determined by Northern blotting after 5 days of 5 μM apigenin treatment. U6 levels were used as a loading control. Representative results from three independent experiments are shown. (c) TRBP(SD) blocked the inhibitory effects of apigenin on HCV replication. Huh7-Feo cells with TRBP or TRBP(SD) expression were treated with 5 μM apigenin for 5 days and then subjected to luciferase assay. Data represent the means ± s.d. of the absolute luciferase values of three independent experiments. * $p < 0.05$.

conventional interferon, to eliminate HCV remains important. In addition, reducing miR122 expression may benefit patients with high cholesterol levels, because targeting miR122 with antisense oligonucleotides in vivo, including miravirsen in humans, decreases elevated cholesterol levels (Janssen et al., 2013). The effects of taking apigenin or eating foods rich in apigenin are worth considering.

Materials and methods

Cell culture

Huh7 and 293T cells were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). Huh7-Feo cells that harbor an HCV replicon reporter construct (HCV-Feo) were kindly provided by Yokota et al. (2003). All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Reagents

Apigenin was purchased from Wako Chemicals (Osaka, Japan) and was dissolved in dimethyl sulfoxide (DMSO). Apigenin was added to the cell culture media at a 1:1000 dilution to reach the final working concentration. An equal volume of DMSO was used as a negative control.

Antibodies

Anti-luciferase antibody (#PM016) was purchased from MBL (Nagoya, Japan). Anti-β-actin antibody (#A5441), anti-flag M2 antibody (#F3165), and anti-TRBP antibody (SAB4200111) were purchased from Sigma (St. Louis, MO).

Western blotting, transfection, and dual luciferase assays

Western blotting, transfection, and dual luciferase assays were performed as we previously described (Kojima et al., 2011).

Quantitative RT-PCR analysis of miRNA expression

To determine miR122 and let-7g expression levels, cDNA was first synthesized from RNA, and quantitative PCR was then performed using the Mir-X miRNA First-Strand Synthesis and SYBR qRT-PCR Kit (Clontech, Mountain View, CA). miRNA precursor expression levels were determined according to a previous report (Suzuki et al., 2009) using the reported primers. Relative expression values were calculated by the CT-based calibrated standard curve method. These calculated values were then normalized to the expression of U6 snRNA. The reverse primer was provided in the kit.

Plasmids, viral production, and transduction

The firefly luciferase-based reporter carrying miR122-responsive elements in its 3′-UTR, which was used to examine miR122 function, and the internal control Renilla luciferase-based plasmids have been described previously (Kojima et al., 2011). A reporter with mutations in the miR122-responsive elements was constructed by inserting annealed oligonucleotides containing mutant miR122 binding sites downstream of the luciferase gene of pGL4. The oligonucleotide sequence used was CAA ACA CCA TTG TCA CAG CAG T. An HCV replicon expressing a chimeric protein consisting of firefly luciferase and neomycin phosphotransferase under the HCV 5′ IRES was used to monitor intracellular HCV replication levels (Yokota et al., 2003). To construct plasmids expressing wild-type TRBP or phosphorylation mimic TRBP (TRBP(SD)), flag-tagged TRBP cDNAs, amplified by PCR using plasmids containing cDNAs provided

by Paroo et al. (2009) as templates, were cloned into the pLVSIN vector (Takara, Shiga, Japan) at the *Not*1 site by a standard infusion method (Clontech).

Lentiviral production and transduction

Lentiviral particles carrying the flag-tagged TRBP-expressing constructs were produced using a pPACKH1 lentivector packaging plasmid mix according to the manufacturers' recommendations (System Biosciences). Huh7-Feo cells were transduced with lentiviruses using polybrene (EMD Millipore, Billerica, MA, USA), and were then selected with puromycin (6 μg/ml).

Northern blotting of miRNAs

Northern blotting of miRNAs was performed as described previously (Takata et al., 2013b). Briefly, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 10 mg of RNA were resolved in denaturing 15% polyacrylamide gels containing 7 M urea in 1 × TBE and then transferred to a Hybond N+ membrane (GE Healthcare) in 0.25 × TBE. Membranes were UV-crosslinked and prehybridized in hybridization buffer. Hybridization was performed overnight at 42 °C in ULTRAhyb-Oligo Buffer (Ambion) containing a biotinylated probe specific for miR122 (CAA ACA CCA TTG TCA CAC TCC A) that had been heated to 95 °C for 2 min. Membranes were washed at 42 °C in 2 × SSC containing 0.1% SDS, and the bound probe was visualized using a BrightStar BioDetect Kit (Ambion). Blots were stripped by boiling in a solution containing 0.1% SDS and 5 mM EDTA for 10 min prior to rehybridization with a U6 probe (CAC GAA TTT GCG TGT CAT CCT T).

Cell counting

Relative cell proliferation was assessed using Cell Counting Kit-8 (Dojindo Laboratories), as we previously described (Kojima et al., 2011).

Synthesized miRNAs and transfection

Synthesized anti-miR122 oligonucleotides, mature miRNA122, and let7g mimics were purchased from Sigma and were transiently transfected at a final concentration of 10 pM using RNAi Max Reagent according to the manufacturer's instructions (Sigma).

Statistical analysis

When the variances were equal, statistically significant differences between groups were identified using Student's *t*-test. When the variances were unequal, Welch's *t*-test was used instead. *P* values <0.05 were considered to indicate statistical significance in in vitro experiments.

Author contributions

C.S. and M.Otsuka planned the research and wrote the manuscript. C.S., M.Ohno, T.K., T.Y. and A.T. performed the majority of the experiments. K.G., R.M., and N.K. provided materials and supported some parts of experiments. C.S., M.Ohno and M.Otsuka analyzed the data. K.K. supervised the entire project.

Acknowledgments

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (# 25293076, #25860520, and #24390183) (to M.O., T.Y., K.K.), by Health Sciences Research Grants of The Ministry of Health, Labour and Welfare of Japan (to K.K.), by a grant from the Mishima Kaiun Memorial Foundation (to M. Ohno), and by grants from the Honjo International Scholarship Foundation and the Japanese Society of Gastroenterology (to M.O.).

References

Bacon, B.R., Gordon, S.C., Lawitz, E., Marcellin, P., Vierling, J.M., Zeuzem, S., et al., 2011. Boceprevir for previously treated chronic HCV genotype 1 infection. New Engl. J. Med. 364, 1207–1217.

Cao, J., Zhang, Y., Chen, W., Zhao, X., 2010. The relationship between fasting plasma concentrations of selected flavonoids and their ordinary dietary intake. Br. J. Nutr. 103, 249–255.

Deuffic-Burban, S., Schwarzinger, M., Obach, D., Mallet, V., Pol, S., Pageaux, G.P., et al., 2014. Should we await IFN-free regimens to treat HCV genotype 1 treatment-naive patients? A cost-effectiveness analysis (ANRS 12188). J. Hepatol, in press.

Elmén, J., Lindow, M., Schütz, S., Lawrence, M., Petri, A., Obad, S., et al., 2008. LNA-mediated microRNA silencing in non-human primates. Nature 452, 896–899.

Gebert, L.F., Rebhan, M.A., Crivelli, S.E., Denzler, R., Stoffel, M., Hall, J., 2014. Miravirsen (SPC3649) can inhibit the biogenesis of miR-122. Nucleic Acids Res. 42, 609–621.

Gramantieri, L., Ferracin, M., Fornari, F., Veronese, A., Sabbioni, S., Liu, C.G., et al., 2007. Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. Cancer Res. 67, 6092–6099.

Hou, J., Lin, L., Zhou, W., Wang, Z., Ding, G., Dong, Q., et al., 2011. Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as therapeutic target for hepatocellular carcinoma. Cancer Cell 19, 232–243.

Hsu, S.H., Wang, B., Kota, J., Yu, J., Costinean, S., Kutay, H., et al., 2012. Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. J. Clin. Investig. 122, 2871–2883.

Jacobson, I.M., McHutchison, J.G., Dusheiko, G., Di Bisceglie, A.M., Reddy, K.R., Bzowej, N.H., et al., 2011. Telaprevir for previously untreated chronic hepatitis C virus infection. New Engl. J. Med. 364, 2405–2416.

Janssen, H.L., Reesink, H.W., Lawitz, E.J., Zeuzem, S., Rodriguez-Torres, M., Patel, K., et al., 2013. Treatment of HCV infection by targeting MicroRNA. New Engl. J. Med 368, 1685–1694.

Jopling, C., 2012. Liver-specific microRNA-122: biogenesis and function. RNA Biol. 9, 137–142.

Jopling, C.L., Yi, M., Lancaster, A.M., Lemon, S.M., Sarnow, P., 2005. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science 309, 1577–1581.

Kojima, K., Takata, A., Vadnais, C., Otsuka, M., Yoshikawa, T., Akanuma, M., et al., 2011. MicroRNA122 is a key regulator of α-fetoprotein expression and influences the aggressiveness of hepatocellular carcinoma. Nat. Commun. 2, 338.

Krützfeldt, J., Rajewsky, N., Braich, R., Rajeev, K.G., Tuschl, T., Manoharan, M., et al., 2005. Silencing of microRNAs in vivo with 'antagomirs'. Nature 438, 685–689.

Kutay, H., Bai, S., Datta, J., Motiwala, T., Pogribny, I., Frankel, W., et al., 2006. Downregulation of miR-122 in the rodent and human hepatocellular carcinomas. J. Cell. Biochem. 99, 671–678.

Lanford, R.E., Hildebrandt-Eriksen, E.S., Petri, A., Persson, R., Lindow, M., Munk, M. E., et al., 2010. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 327, 198–201.

Li, Y.P., Gottwein, J.M., Scheel, T.K., Jensen, T.B., Bukh, J., 2011. MicroRNA-122 antagonism against hepatitis C virus genotypes 1-6 and reduced efficacy by host RNA insertion or mutations in the HCV 5′ UTR. Proc. Natl. Acad. Sci. U.S.A. 108, 4991–4996.

Manns, M.P., von Hahn, T., 2013. Novel therapies for hepatitis C—one pill fits all? Nat. Rev. Drug Discov. 12, 595–610.

Ohno, M., Shibata, C., Kishikawa, T., Yoshikawa, T., Takata, A., Kojima, K., et al., 2013. The flavonoid apigenin improves glucose tolerance through inhibition of microRNA maturation in miRNA103 transgenic mice. Sci. Rep. 3, 2553.

Otsuka, M., Kishikawa, T., Yoshikawa, T., Ohno, M., Takata, A., Shibata, C., et al., 2014. The role of microRNAs in hepatocarcinogenesis: current knowledge and future prospects. J. Gastroenterol. 49, 173–184.

Paroo, Z., Ye, X., Chen, S., Liu, Q., 2009. Phosphorylation of the human microRNA-generating complex mediates MAPK/Erk signaling. Cell 139, 112–122.

Pfeffer, S., Baumert, T.F., 2010. Impact of microRNAs for pathogenesis and treatment of hepatitis C virus infection. Gastroenterol. Clin. Biol. 34, 431–435.

Poordad, F., McCone, J., Bacon, B.R., Bruno, S., Manns, M.P., Sulkowski, M.S., et al., 2011. Boceprevir for untreated chronic HCV genotype 1 infection. New Engl. J. Med. 364, 1195–1206.

Scheel, T.K., Rice, C.M., 2013. Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. Nat. Med. 19, 837–849.

Sherman, K.E., Flamm, S.L., Afdhal, N.H., Nelson, D.R., Sulkowski, M.S., Everson, G.T., et al., 2011. Response-guided telaprevir combination treatment for hepatitis C virus infection. New Engl. J. Med. 365, 1014–1024.

Snyder, S.A., Gollner, A., Chiriac, M.I., 2011. Regioselective reactions for programmable resveratrol oligomer synthesis. Nature 474, 461–466.

Suzuki, H.I., Yamagata, K., Sugimoto, K., Iwamoto, T., Kato, S., Miyazono, K., 2009. Modulation of microRNA processing by p53. Nature 460, 529–533.

Takata, A., Otsuka, M., Yoshikawa, T., Kishikawa, T., Ohno, M., Koike, K., 2013a. MicroRNAs and liver function. Minerva Gastroenterol. Dietol. 59, 187–203.

Takata, A., Otsuka, M., Yoshikawa, T., Kishikawa, T., Hikiba, Y., Obi, S., et al., 2013b. MicroRNA-140 acts as a liver tumor suppressor by controlling NF-κB activity by directly targeting DNA methyltransferase 1 (Dnmt1) expression. Hepatology 57, 162–170.

Tsai, W.C., Hsu, P.W., Lai, T.C., Chau, GY, Lin, CW, Chen, CM, et al., 2009. MicroRNA-122, a tumor suppressor microRNA that regulates intrahepatic metastasis of hepatocellular carcinoma. Hepatology 49, 1571–1582.

Tsai, W.C., Hsu, S.D., Hsu, C.S., Lai, T.C., Chen, S.J., Shen, R., et al., 2012. MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. J. Clin. Investig. 122, 2884–2897.

Yokota, T., Sakamoto, N., Enomoto, N., Tanabe, Y., Miyagishi, M., Maekawa, S., et al., 2003. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep. 4, 602–608.

J Gastroenterol (2015) 50:261–272
DOI 10.1007/s00535-014-1000-9

The Japanese Society of Gastroenterology

REVIEW

MICA SNPs and the NKG2D system in virus-induced HCC

Kaku Goto · Naoya Kato

Received: 23 March 2014 / Accepted: 17 September 2014 / Published online: 1 October 2014

Abstract Hepatocellular carcinoma (HCC) is one of the most frequent causes of cancer-related death globally. Above well-known risk factors for HCC development ranging from various toxins to diseases such as diabetes mellitus, chronic infection with hepatitis B virus and hepatitis C virus (HCV) poses the most serious threat, constituting the cause in more than 80 % of cases. In addition to the viral genes intensively investigated, the pathophysiological importance of host genetic factors has also been greatly and increasingly appreciated. Genome-wide association studies (GWAS) comprehensively search the host genome at the single-nucleotide level, and have successfully identified the genomic region associated with a whole variety of diseases. With respect to HCC, there have been reports from several groups on single nucleotide polymorphisms (SNPs) associated with hepatocarcinogenesis, among which was our GWAS discovering MHC class I polypeptide-related sequence A (MICA) as a susceptibility gene for HCV-induced HCC. MICA is a natural killer (NK) group 2D (NKG2D) ligand, whose interaction with NKG2D triggers NK cell-mediated cytotoxicity toward the target cells, and is a key molecule in tumor immune surveillance as its expression is induced on stressed cells such as transformed tumor cells for the detection by NK cells. In this review, the latest understanding of the MICA–NKG2D system in viral HCC, particularly focused on its antitumor properties and the involvement of MICA SNPs, is summarized, followed by a discussion of targets for state-of-the-art cancer immunotherapy with personalized medicine in view.

Keywords MHC class I polypeptide-related sequence A · Single nucleotide polymorphism · Hepatitis C virus · Hepatitis B virus · Hepatocellular carcinoma · Natural killer

Abbreviations

ADAM	A disintegrin and metalloprotease
ATM	Ataxia–telangiectasia mutated
CHB	Chronic hepatitis B
CHC	Chronic hepatitis C
DAP10	DNAX-activating protein of 10 kDa
GWAS	Genome-wide association study
HBV	Hepatitis B virus
HBx	Hepatitis B virus encoded X protein
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDAC	Histone deacetylase
IFN	Interferon
Met	Methionine
MIC	MHC class I chain
MICA	MHC class I polypeptide-related sequence A
MICA-129	Amino acid 129 of MHC class I polypeptide-related sequence A
miRNA	MicroRNA
mMICA	Membrane-bound MHC class I polypeptide-related sequence A
MMP	Matrix metalloproteinase
NKG2D	Natural killer group 2D
NKG2DL	Natural killer group 2D ligand
sMICA	Soluble MHC class I polypeptide-related sequence A

K. Goto · N. Kato (✉)
Division of Advanced Genome Medicine, The Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan
e-mail: kato-2im@ims.u-tokyo.ac.jp

K. Goto
Japan Society for the Promotion of Science, Tokyo 102-8472, Japan

SNP	Single nucleotide polymorphism
TGF-β	Transforming growth factor β
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent causes of cancer-related death, with 700,000 individuals dying annually [1]. Beside a wide variety of well-known risk factors for HCC development represented by aflatoxin B_1, heavy alcohol intake, nonalcoholic fatty liver disease, diabetes, and hemochromatosis [2], chronic infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) poses the greatest threat, constituting the cause in around 80 % of cases [3] and resulting in 250,000 new cases of HCC and an estimated 500,000–600,000 deaths per year [4, 5]. While the pathogenic effects of viral genes have been investigated intensively [6], the pathophysiological importance of host genetic factors has also been greatly and increasingly recognized [7], though the analyzed genes and genomic regions were limited, as we previously reported [8]. Recent whole-genome and whole-exome sequencing analyses in addition to genome-wide association studies (GWAS) are innovative methods that comprehensively explore the host genome, and have successfully identified the genomic regions associated with various diseases [9, 10]. With respect to HCC, some groups performed whole-genome/whole-exome sequencing and discovered novel alterations of cancer-related genes and pathways [11], and others found the single nucleotide polymorphisms (SNPs) responsible in GWAS [12], among which was our study identifying MHC class I polypeptide-related sequence A (MICA) as a susceptibility gene for HCV-induced HCC [13]. MICA is a natural killer (NK) group 2D (NKG2D) ligand (NKG2DL), directly triggering NK cell-mediated cytokine release and cytotoxicity toward the target cells [14], even in the presence of inhibitory signals under certain conditions [15], via NKG2D, which also works as a costimulatory receptor in $CD8^+$ T cells [16], and is a key molecule in tumor immune surveillance as the expression is induced on stressed cells such as transformed tumor cells for the detection by NK cells [16, 17]. In the Japanese population, we identified a SNP, rs2596542, in the 5′ flanking region of MICA strongly associated with HCV-induced HCC in a GWAS and further demonstrated the association of the SNP with progression from chronic hepatitis C (CHC) to HCC. The risk allele of rs2596542 was also found to correlate with lower soluble MICA (sMICA) protein level in HCV-induced HCC, and therefore in the report we discussed the possibility that individuals who carry the rs2596542 A allele would express low levels of membrane-bound MICA (mMICA) in response to HCV infection, leading to poor or no activation of immune cells, including NK cells, and would progress from CHC to HCC. The data suggested the significance of tumor immune surveillance via MICA in HCC development. Here we briefly summarize the knowledge of the MICA–NKG2D system activating NK cells, which are highly accumulated in the liver, representing 30–50 % of all hepatic lymphocytes [18] and increasing to even 90 % in patients with hepatic malignancy [19], with their anti-HCC actions impaired in chronic infection with HBV and HCV, and simultaneously consider effects of MICA variations such as SNPs in each case. Finally, targets and modalities of cancer immunotherapy for the prevention of HCC exploiting NK cells via MICA will be discussed with personalized medicine in view.

The MICA–NKG2D system and NK cell activation

NK cells are major effector lymphocytes known to act as initial defense against viral infection and malignant transformation [20]. Activated NK cells directly eliminate the target cells through several mechanisms, such as releasing perforin and granzyme, causing apoptosis by caspase-dependent and caspase-independent pathways, death-receptor-mediated apoptosis via Fas ligand or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), secreting effector molecules including interferon (IFN)-γ and antibody-dependent cellular cytotoxicity through CD16, concomitantly exerting indirect immune effects including IFN-γ-driven induction of cytotoxic T lymphocytes from $CD8^+$ T cells and differentiation of $CD4^+$ T cells to a T_h1 response, regulation of antibody production by B cells, and provision of tumor antigen for and regulatory activation of dendritic cells [21, 22]. For the selective reactivity, NK cell activation is licensed by MHC class I ligand through self-recognizing inhibitory receptors [23] and controlled by activating receptors comprising four main families of molecules, including NKG2D, the greatest one in natural killing [24], and a family of inhibitory receptors via engagement of the corresponding ligands, whose list keeps growing with newly identified members [25]. MICA is one of the MHC class I chain (MIC)-related genes composing the MIC family in NKG2DL molecules [26], whose expression is absent in general on the surface of healthy cells but is augmented by cellular stresses including viral infection and malignant transformation [27], reportedly through pathways and factors encompassing ataxia-telangiectasia mutated (ATM) or ATM- and Rad3-related signaling, transcription factors Sp1 and Sp3,

heat shock factor 1, and E2F family transcription factors, while being affected also by epigenetic regulation [28]. After they have subsequently gone through posttranscriptional steps typified by microRNA (miRNA)- and proteasome-mediated processing, the ligand proteins are finally presented on the membrane surface. Once MICA is engaged by NKG2D on NK cells, DNAX-activating protein of 10 kDa (DAP10) becomes phosphorylated, accompanied by the recruitment of phosphatidylinositol 3-kinase (PI3K) or a growth factor-receptor-bound protein 2 (GRB2)–Vab1 complex for full NK activation [29], thus displaying a critical role of MICA in NK cell-mediated immunity.

MICA is a transmembrane protein consisting of three extracellular domains [17], and is reported to produce sMICA through alternative splicing, proteolytic shedding, or exosome secretion [30, 31]. Sheddases of MICA have been well investigated, in particular, and matrix metalloproteases [32, 33] and a disintegrin and metalloprotease (ADAM) proteases were shown to shed MICA [30, 34–36]. Shedding is expected to decrease the amount of cell surface MICA [37], and sMICA downregulates NKG2D as exemplified by facilitated endocytosis and degradation of NKG2D on tumor-infiltrating T cells [31], resulting in decreased NK cell cytotoxicity as suggested in many studies. In cancer patients, elevated serum level of sMICA was detected [14, 37], and sMICA-containing serum from pancreatic carcinoma patients inhibited NK cell and δγ T cell-mediated cytotoxicity, which was rescued by neutralization of sMICA [38]. The data endorsed the notion that MICA shedding and sMICA production operate as a mechanism of tumor cells escaping NKG2D-mediated immune surveillance.

From a genetic perspective, the highly polymorphic feature of MICA [39] is of interest because MICA was demonstrated to participate in well-known immune diseases such as rheumatoid arthritis [40], Behçet's disease [41], type I diabetes [42], and celiac disease [43], adding to other disorders, including liver fibrosis in schistosomiasis [44], with which MICA polymorphism was associated. For instance, among MICA variants such as those with a variable number of tandem repeats constituted of four, five, six, seven, eight, nine, or ten repeats of GCT as A4, A5, A6, A7, A8, A9, and A10, respectively, a well-recognized transmembrane polymorphism MICA-A5.1, which causes a frameshift mutation for a premature stop codon, leading to a truncated MICA protein without a cytoplasmic tail [41, 45], is noteworthy. In HCC patients compared with healthy controls, MICA-A5.1 polymorphism was increased, and the individuals with the polymorphism had increased sMICA levels [46]. The shorter MICA protein encoded by MICA-A5.1 was discussed to be easily detached from the carcinoma cell membrane, and indeed correlation of MICA-A5.1 with higher sMICA levels in other types of cancer or inflammatory diseases has been reported [47, 48]. SNPs in the MICA gene were also found to be associated with susceptibility to chronic inflammatory diseases, among which rs1051792 is well studied and corresponds to a methionine (Met)/valine (Val) polymorphism at amino acid 129 of MICA (MICA-129), with the Met allele (MICA-129Met) as a strong binder to NKG2D [49]. The SNP was reportedly implicated in several major disorders, exemplified by inflammatory bowel disease [50], type 1 diabetes [51], and chronic graft-versus-host disease [52], beside other MICA SNPs involved in rheumatoid arthritis [53] and HCV-induced HCC in our GWAS [13], underscoring the immunopathological impact of genetic variance even on carcinogenesis.

Virus-induced HCC

HCV is reported to chronically infect 130 million to 170 million people globally [54], and accounts for 60 % of HCC's causative agents in the USA, Europe, Egypt, and Japan [3]. About half of the people chronically infected with HCV develop chronic liver disease (CLD), with 5–20 % progressing to cirrhosis within 5–20 years, 1–2 % of which develop HCC each year [55]. The complex pathogenic effects of persistent HCV replication as a risk factor for HCC [56] have been ascribed to the activation of cellular oncogenes and/or the inactivation of tumor suppressor genes [57] and derangement of multiple signaling pathways [58–60], exemplified by the core protein and nonstructural proteins NS3 and NS5A [6]. Also, during the progression from the CLD through fibrosis to cirrhosis, accompanied inflammation, oxidative stress, localized hypoxia, epithelial-to-mesenchymal transition, and angiogenesis are all eventually contributory to HCCs [3], the vast majority of which develop in a cirrhotic liver [61]. Furthermore, miRNAs were recently exhibited to play carcinogenic roles [62] in HCV-induced HCC, interfering with multiple target genes and pathways associated with cancer [63]. Beside these factors, alteration of immunity well illustrated by the reduced tumor surveillance function of NK cells in addition to a weakened impact of $CD8^+$ T cells is crucially involved in HCC development [64]. In chronic HCV infection, suppression of NK cell activation due to impaired interleukin (IL)-15 production [65] and decreased expression of activation-associated receptors [66] was observed, and especially intrahepatic NK cells demonstrated reduced cytotoxicity and TRAIL expression [67]. In cirrhosis, decreased NK cell function was suggested to be a possible factor related to HCC development [68], in keeping with regulatory effects of NK cells on intrahepatic fibrosis via their cytotoxicity toward hepatic stellate cells indicated in CHC [69]. In patients with HCC,

Table 1 Association of rs2596542 with hepatitis B virus (*HBV*)- or hepatitis C virus (*HCV*)-induced hepatocellular carcinoma (*HCC*)

Risk allele	Control	Patient background	OR	*P*	Reference
A	HCV negative	HCV, Japanese	1.39	4.21×10^{-13}	Kumar et al. [13]
A	CHC	HCV, Japanese	1.36	3.13×10^{-8}	Kumar et al. [13]
G[a]	No HCC	HCV, European			Lange et al. [81]
G	Healthy	HBV, Japanese	1.19	0.029	Kumar et al. [94]
A	Non-HCC	HBV, Vietnamese	1.5	0.01	Tong et al. [95]
A	LC	HBV, Vietnamese	1.6	0.009	Tong et al. [95]

CHC chronic hepatitis C, *LC* liver cirrhosis, *OR* odds ratio

[a] Susceptibility genotypes are indicated in the original study by Lange et al. [81].

tumors progress despite the presence of NK cell response [70], suggesting immunosuppressive mechanisms encompassing immunosuppressive cytokines, regulatory T cells, and myeloid-derived suppressor cells [64], which were found to suppress the cytokine production as well as the cytotoxic capacity of NK cells [71]. Furthermore, the levels of highly cytotoxic NK subsets of $CD56^{dim}$ were dramatically reduced peripherally and in tumor regions, and these subsets exhibited reduced levels of IFN-γ production and cytotoxicity [72]. Dysregulation of the NKG2D system was observed to be responsible for diminished tumor-specific NK cell responses. For instance, NKG2D was downmodulated on NK cells via transforming growth factor β (TGF-β)-induced by IL-10, which was produced from monocytes stimulated by NS5A [73]. Moreover, NKG2DLs were also targeted by tumors for their evading NKG2D-mediated anticancer immunity in HCC [74], and in particular proper expression and function of MICA have been found to be perturbed. Cell surface MICA expression was downregulated by NS3/N4A in HCV-infected cells [75], and NS2 and NS5B were responsible for an HCV-associated decrease in MICA expression [76]. On the other hand, the levels of sMICA were elevated in patients with advanced HCC, associated with impaired activation of NK cells [77]. ADAM9 was subsequently shown to be responsible for the shedding of MICA [34] with another critical sheddase, ADAM10 [35], both of which were overexpressed in HCC, decreasing its NK cell sensitivity via sMICA production.

Genetic variance of MICA was shown to affect HCC development. The amount of MICA-A5.1 polymorphism, giving rise to a truncated protein, was increased in HCC patients, with higher serum levels of sMICA [46], and the MICA-A4 frequency was increased in end-stage liver disease and HCC [78], in agreement with the same trend as in our GWAS though not statistically significant [13]. We also identified the SNP rs2596542 (Table 1) in the 5′ flanking region of MICA strongly associated with HCV-induced HCC in the GWAS and sMICA levels, and suggested the rs2596542 A allele would express low levels of mMICA in response to HCV infection [13]. A subsequent study investigated the effects of rs2596542 and candidate SNPs, showing strong linkage disequilibrium with it, on the binding of nuclear proteins to the genomic segment including rs2596542. The analyses led to the discovery of rs2596538 located 2.8 kb upstream of the MICA gene, showing the binding to transcription factor Sp1 at its protective G allele, which was also associated with higher sMICA level among 246 HCV-induced HCC patients, and the direct influence of a SNP on MICA transcription [79]. The data support the pathophysiological effects of SNPs on gene regulation and provide backing for the notion that MICA genetic variation could be a prognostic biomarker for CHC patients though the effects of specific genetic traits in susceptibility to HCC may vary from one population to another [80] as was seen in the protective impact of the rs2596542 A allele on HCC development in the European population [81], whereas the SNP was not correlated with HCC recurrence after hepatectomy [82].

Around two billion people are infected with HBV, and more than 350 million worldwide have become long-term carriers, with 60 % of HCC being associated with HBV in Africa and Asia [3]. Whereas chronic HBV infection and the consequential liver injury are, as was the case with HCV-induced HCC, acknowledged risk factors for HCC development [56], which is reportedly due to the perturbation of signaling pathways and genes prominently by HBV-encoded X protein (HBx) [83] and integration of the viral DNA sequences into the human genome, again disruption of tumor immune surveillance was conspicuous in NK cell hypofunction. In chronic hepatitis B (CHB) patients, lower activity of NK cells was observed [66, 84], and the capacity of IFN-γ production by NK cells was impaired [85]. Murine chronic HBsAg carriers also exhibited decreased number, declined activation, and attenuated cytotoxicity of hepatic NK cells [86], whose cytotoxicity was also reported to control hepatic stellate cells in HBV-related liver disease [87]. Reduced level of NKG2D/DAP10 was correlated with high levels of $TGF\text{-}\beta_1$, a well-studied downregulator of NKG2D, in HBV-

infected patients in the immunotolerant phase [88], increasing the risk of HCC. NKG2DLs were also down-regulated by TGF-β [89], and more specifically, HBV-mediated disruption of MICA as an NKG2DL has been found. In HepG2.2.15 cells, the expression of mMICA was strongly diminished [90], and RNA interference-mediated inhibition of HBV induced MICA upregulation, conferring the cells with more susceptibility to lysis by NK cells [91]. In HBsAg-overexpressing HCC cells, suppression of the MICA expression via induction of miRNAs targeting the 3′ untranslated region of MICA bringing about reduced sensitivity to NK cells was identified [92], in accordance with a report of the significant role of miRNAs in MICA expression regulation [93]. On the other hand, we also reported higher sMICA level to be associated with poorer prognosis and vascular invasion in HCC patients infected with HBV, showing a significantly shorter survival [94], concurrent with an increase of sMICA levels in HBV-infected patients [95] and correlation of a high sMICA level with tumor size, poor prognosis, and low NKG2D expression on $CD56^{+}$ NK cells among patients with advanced HCC [96]. Intriguingly, HBx was associated with an enhanced expression of membrane type 1 matrix metalloproteinase (MMP), MMP2, and MMP3 [97–99] and was identified to both transactivate MMP9 and suppress tissue inhibitor of metalloproteinase (TIMP) 1 and TIMP 3 [100, 101], presumably promoting shedding of mMICA.

A number of studies demonstrated substantial effects of MICA polymorphisms in HBV infection over the course of HCC development. Among MICA microsatellite variants, the genotype A6/A9 was contributory to an increased risk of HCC compared with liver cirrhosis, and genotype A5/A6 was observed more frequently in HCC than in CHB, though the allele A5.1 did not turn out to be related to HBV infection and the induced HCC in a Vietnamese population [95], inferring crucial roles of MICA variances. The group also demonstrated the risk of HBV-induced HCC was increased by the allele MICA-129Met, a strong binder to NKG2D [49], similarly to a polymorphism, MICA*015, with high binding affinity for NKG2D associated with HBV persistence [102], and suggested the strong binding could have effects similar to those of elevated sMICA levels and reduced NKG2D expression. As for the SNP rs2596542 (Table 1), we also demonstrated the G allele, the protective allele expressing a higher level of mMICA in HCV-induced HCC in a previous study [13], to increase the risk of HCC in HBV infection, associated with elevated sMICA levels in Japanese patients [94]. It was speculated that the HBx-driven increase in expression of MMPs leading to both sMICA level elevation and mMICA level reduction could explain the HBV-infection-characteristic response of the SNP. In a Vietnamese cohort, the A allele of rs2596542 was associated with increased progression from cirrhosis to HCC in HBV infection but not from CHB to cirrhosis or from other early stages of HBV infection, at least indicating the significance of the SNP in the liver disease progression irrespective of viral factors [95]. Another study investigating rs2596542 in HBV infection was conducted in Han Chinese and did not find significant differences of the allele frequency between CHB and HCC patients, although the haplotype of the rs2596542 G allele with the A allele of rs9275572, a reported risk allele in HCV-induced HCC [13], exhibited a protective effect on the occurrence of HBV-induced HCC, which indicated that the rs2596542 G allele strengthened the protective impact of the rs9275572 A allele [103]. In a Saudi Arabian population, whereas the C allele of rs2596542 was more frequently distributed in HBV-infected patients than in healthy controls, the T allele frequency was increased in patients categorized as being active carriers, as having cirrhosis, or as having cirrhosis-induced HCC in comparison with inactive carriers,owing to which the SNP's disease-determining activity and the existence of additional factors for disease progression in HBV infection [104]. All these results again depict various impacts of specific genetic traits in susceptibility for HCC among diverse populations [80], with the possibility that a locus could exert opposite effects in different ethnic groups owing to distinct genetic backgrounds and environmental pressures [103]. Especially, different mechanisms of HCC development presumed to operate over the course of HBV infection from those in HCV infection are well perceived as observed in the cases of rs2596542 and rs9275572 above, which further complicates the genetic understanding of virally induced HCC. Although a coherent explanation for the roles of rs2596542 and related SNPs in *MICA* necessitates further studies in diverse populations at larger scales and functional analyses, the critical involvement of MICA SNP(s) in HBV-mediated liver diseases leading to HCC has thus been increasingly recognized.

HCC immunotherapy

For the prevention of HCC occurrence in HBV and HCV infections, the essential option is antiviral treatment [105], which constitutes administration of antiviral drugs against HBV infection [106], with maintained viral response by a nucleoside/nucleotide analog reducing the risk of HCC even in cirrhotic patients [107], and against HCV infection [108], with a lower rate of HCC in patients achieving a sustained virologic response (SVR) even in the presence of cirrhosis [109]. Still, the anti-HCC efficacy of viral replication inhibition is limited in HBV infection for several reasons such as drug resistance [110] and covalently closed

circular DNA in the nucleus, a persistent source of recurrence [111], and unfortunately HCC does emerge as was observed in a Greek study reporting no effect of complete viral suppression on the HCC incidence in patients with cirrhosis [112]. Likewise, HCV is well known to acquire resistance to antivirals owing to the high error rate of replication [113], and also multiple cases of HCC development in treatment responders even after they had achieved a SVR were documented [114]. Generally, such latency of HCC development has led to its diagnosis at an advanced stage, where sorafenib is the only agent approved for HCC treatment, but has limited efficacy [115], therefore requiring novel and better efficacious HCC therapies. Remarkably, cancer immunotherapy was selected by *Science* as the breakthrough of the year in 2013 thanks to its entirely new way of treating cancer via the immune system rather than targeting the tumor itself and successful results across tumor types [116]. Hence, in addition to classic immunological strategies against carcinogenic viruses represented by vaccination against HBV [117] and IFN-α treatment in HBV infection [118] or HCV infection [119], boosting antitumor immune cells for HCC treatment irrespective of viral factors is of an increasing interest. A variety of immune-based strategies involving cytokines, cell-mediated vaccines, monoclonal antibodies, and immune adjuvants have been in trials in HCC [120]. Liver NK cells from a deceased donor liver can be used advantageously for the treatment of liver transplantation because of HCC [121]. The MICA–NKG2D system is also a preferred target of immunotherapeutic intervention [122]. For example, IL-12 found to improve NK cell cytotoxicity through upregulation of NKG2D expression [123] and inhibited liver carcinogenesis in animal models [124, 125]. In turn, induction of MICA expression is another alternative for stimulation of NK cell activity. Histone deacetylase (HDAC) inhibitors were demonstrated to enhance the expression of NKG2DLs on cancer cells, promoting NKG2D-mediated cytolysis [126]. Valproic acid, a well-known antiepileptic drug with HDAC inhibitory activity, increased MICA transcription in and NK-mediated lysis of HCC cells [127]. Additional HDAC inhibitors such as panobinostat, belinostat, and resminostat in the presence or absence of sorafenib have been tested in phase I/II studies in advanced HCC [128]. Furthermore, bortezomib, an anticancer agent with proteasome-inhibitory activity, was identified to elevate mMICA expression in hepatoma cells at low doses, resulting in the stimulation of cytotoxicity and IFN-γ production of co-cultured NK cells in a tumor-specific manner [129]. The effects of the drug with preferable tumor selectivity in NK cell activation on HCC have started to be evaluated in clinical trials [130].

Although we have focused our attention here on antitumor properties of the MICA–NKG2D system, pathogenic aspects of the NKG2D-driven immune responses [131] also need to be considered, especially in therapeutic upmodulation of NK cell cytotoxic activity, as NK cells were also found to contribute to liver injury during chronic hepatitis viral infection [132], in concordance with a report that the pathogenesis of HCC is mediated by immunity [133], leading to inflammation and regeneration. In chronic HBV infection, liver injury was correlated with hypercytolytic NK cells in immune-activated patients [134], and liver damage was due to activated and TRAIL-expressing NK cells lacking target specificity during hepatic flares in e antigen CHB patients [135]. In CHC, polarization of NK cells toward cytotoxicity was suggested to contribute to liver injury [136], and activated NK cells were also identified to cause aberrant DNA methylation via reactive oxygen species production in patients and a mouse model of chronic HBV or HCV infection [137]. Specifically, NKG2DLs were responsible for NK cell-mediated hepatocyte injury via NKG2D recognition on a background of HBV [138]. Indeed, upregulation of MICA protein level followed by hepatic NK cell activation and hepatocellular damage was detected in nonalcoholic steatohepatitis patients [139], progressing to HCC [140]. Hence, for safe management of the MICA–NKG2D signaling, it is indispensable to avert adverse and immunopathological side effects, including autoimmune reactions. Moreover, tumor selectivity of immunomodulatory drugs enhancing MICA expression is of great benefit as well; these drugs include bortezomib, which increases mMICA expression in hepatoma cells but not in nonmalignant primary human hepatocytes [129]. From a virological perspective, different pathophysiological effects between HBV and HCV on MICA expression and modification are to be taken into consideration as mentioned for the HBV-characteristic enhancement of sMICA production [94]. Additionally, the status of sMICA production should be examined in MICA expression-targeted therapy to guarantee mMICA expression, otherwise functionally dominant negative effects of sMICA on NKG2D [37] could be exacerbated by the treatment. Accordingly, MICA sheddases were presented as interventional targets, and in HCC the chemotherapeutic agents sorafenib and epirubicin were discovered to possess inhibitory activities against ADAM9 [34] and ADAM10 [35], respectively. Further insights into the immunological roles of sMICA in HCC development are evidently needed for the development of better therapeutic schemes.

As reviewed in previous sections, all these immunopathological processes discussed could be potentially affected by MICA variations such as SNPs, causally influencing therapeutic directions together with other factors. For instance, in those CHC patients with the rs2596542 A allele failing to induce MICA expression in response to HCV [13], drugs to increase MICA expression

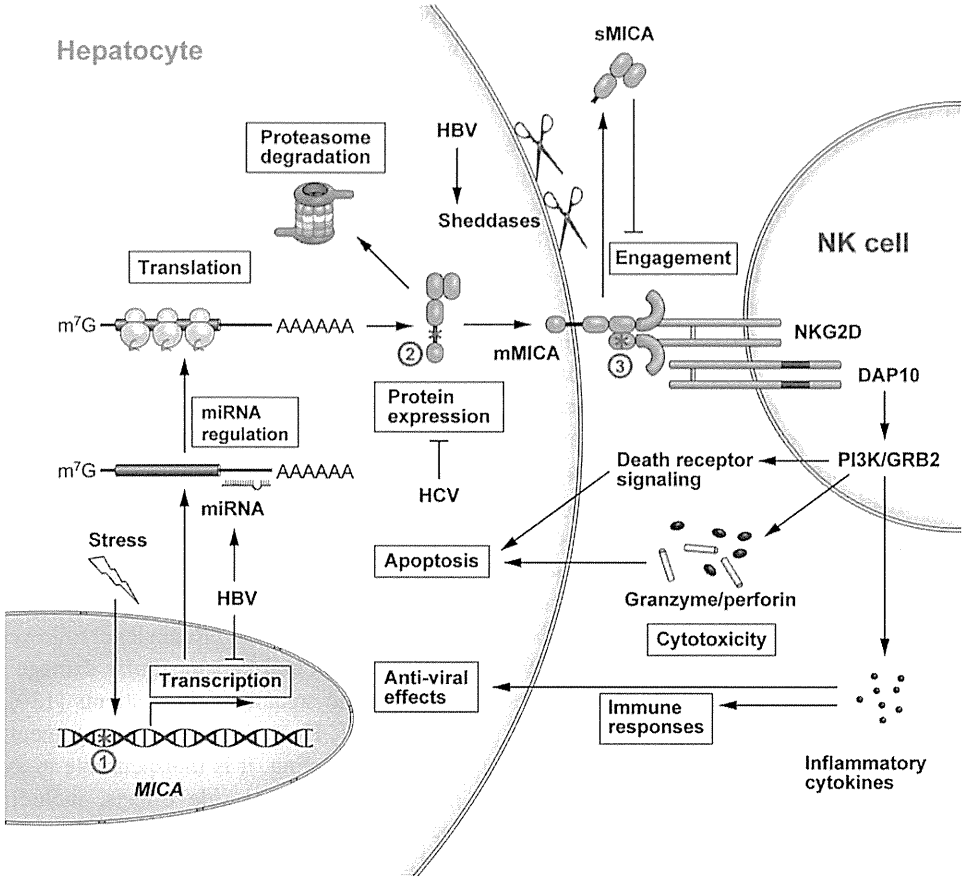

Fig. 1 Current molecular genetic understanding of the MHC class I polypeptide-related sequence A (*MICA*)–natural killer group 2D (*NKG2D*) signaling in viral hepatocellular carcinoma. Briefly, the expression of MICA is initiated by stress-induced transcription of the messenger RNA (mRNA), being subsequently regulated by microRNAs (*miRNA*) and translated into the protein. This is followed by its presentation on the cell surface while the protein is under the control of proteasome-dependent degradation. Membrane-bound MICA (*mMICA*) is engaged by NKG2D on natural killer (*NK*) cells, which is disturbed by soluble MICA (*sMICA*) generated by sheddases, and their activation is triggered through phosphatidylinositol 3-kinase (*PI3K*)/growth factor receptor-bound protein 2 (*GRB2*) mediators. Consequently, those NK cells exert cytotoxic effects via perforin/granzyme secretion and death receptor signaling, and release inflammatory cytokines such as interferon-γ, which brings about antiviral effects and further immune responses against infection and cancer. On the other hand, viruses are known to counteract the anticarcinogenic action of the MICA–NKG2D system by targeting MICA biogenesis. Hepatitis C virus (*HCV*) reportedly disrupts the expression of MICA protein, and hepatitis B virus (*HBV*) deters MICA mRNA transcription and upmodulated miRNAs to that, and promotes sheddases. The important single nucleotide polymorphisms (SNPs) and other polymorphisms in the MICA gene discussed in this review are graphically highlighted: *1* rs2596542 upstream of the MICA transcription start site, *2* the polymorphisms with a variable number of tandem repeats, and *3* MICA-129Met. *DAP10* DNAX-activating protein of 10 kDa

may be of benefit, though additional factors, including ethnic background, should have an influence [81], whereas MICA sheddase inhibition could be rather effective in HBV-induced HCC patients with the rs2596542 G allele exhibiting converse impacts of the SNP on carcinogenesis [94]. In another ethnic group where the rs2596542 A allele is associated with HBV-induced HCC, administration of both MICA expression inducers and sheddase inhibitors would appear to be reasonable [95]. These examples are based on the current understanding of the SNP's genetic involvement in MICA transcription [79], and better specific pharmacological regulation of MICA expression at the messenger RNA level could be realized if further detailed mechanistic analyses of the transcriptional elements and modes of SNP involvement were conducted [141]. Additionally, biological effects of MICA SNPs correlated with HCC, and their functional association with other SNPs represented by MICA sheddases would provide useful guidance for prescription as individuals with unique combinations of SNPs are assumed to possess unique properties of MICA biogenesis [142]. On the other hand, rs2596542 and related MICA SNPs were proposed to be possible prognostic biomarkers owing to their strong association with HCC as shown in HBV infection [94] and CHC [79], as well as sMICA [96]. Further investigation is needed to distinguish the stage of CHC progress the rs2596542 A

allele affects, and multiple genetic variants would be more useful than a single SNP especially for HCC risk prediction [143, 144]. Expectedly, SNPs constitute a part of the determinants for personalized immunochemotherapy for HCC [145], with causes, disease stages, ethnicities, and drug properties adding to other risk factors synchronously in the calculation as discussed herein.

Conclusion

In recent years, the therapeutic development of safe and effective antiviral drugs suppressing replication of HBV or HCV was substantially fostered [146], eventually leaving the issue of HCC development in the aftermath of viral activities. For patients in need of regular HCC surveillance irrespective of favorable responses to antiviral therapies achieving an undetectable level of viral replication [111, 147] as well as those with HCC, immunotherapeutic intervention through NKG2D signaling might become an attractive option, as argued herein [25, 115]. Notably, the genetic identification of MICA in HCV-induced HCC by the GWAS has resulted in more attention being drawn to the roles of the MICA–NKG2D system and NKG2D-expressing immune cells such as NK cells in virus-induced HCC (Fig. 1). The information on MICA SNPs not only reveals to us the novel immunobiological properties of the ligand in the MICA–NKG2D system, but also gives us a balanced direction for immunomodulatory modalities [148] together with other factors showing prognostic values in each case, resulting in our coming a step closer to personalized immunotherapy.

Acknowledgments N.K. is supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare, Japan. K.G. is a recipient of Research Fellowships of the Japan Society for the Promotion of Science (JSPS) for Young Scientists. The authors appreciate critical reading of the manuscript and comments by Dr. Masahisa Jinushi, Center for Infection-Associated Cancer, Institute for Genetic Medicine, Hokkaido University, Hokkaido, Japan.

Conflict of interest The authors disclose no conflict of interest.

References

1. Yang JD, Roberts LR. Hepatocellular carcinoma: a global view. Nat Rev Gastroenterol Hepatol. 2010;7:448–58.
2. Aravalli RN, Steer CJ, Cressman EN. Molecular mechanisms of hepatocellular carcinoma. Hepatology. 2008;48:2047–63.
3. Arzumanyan A, Reis HM, Feitelson MA. Pathogenic mechanisms in HBV- and HCV-associated hepatocellular carcinoma. Nat Rev Cancer. 2013;13:123–35.
4. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. 2005;55:74–108.
5. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology. 2007;132:2557–76.
6. Shlomai A, de Jong YP, Rice CM. Virus associated malignancies: the role of viral hepatitis in hepatocellular carcinoma. Semin Cancer Biol. 2014;26:78–88.
7. Miki D, Ochi H, Hayes CN, Aikata H, Chayama K. Hepatocellular carcinoma: towards personalized medicine. Cancer Sci. 2012;103:846–50.
8. Kato N, Ji G, Wang Y, Baba M, Hoshida Y, Otsuka M, et al. Large-scale search of single nucleotide polymorphisms for hepatocellular carcinoma susceptibility genes in patients with hepatitis C. Hepatology. 2005;42:846–53.
9. Manolio TA. Bringing genome-wide association findings into clinical use. Nat Rev Genet. 2013;14:549–58.
10. Han ZG. Functional genomic studies: insights into the pathogenesis of liver cancer. Annu Rev Genomics Hum Genet. 2012;13:171–205.
11. Nishida N, Kudo M. Recent advancements in comprehensive genetic analyses for human hepatocellular carcinoma. Oncology. 2013;84(Suppl 1):93–7.
12. Chaiteerakij R, Addissie BD, Roberts LR. Update on biomarkers of hepatocellular carcinoma. Clin Gastroenterol Hepatol. 2013. doi:10.1016/j.cgh.2013.10.038.
13. Kumar V, Kato N, Urabe Y, Takahashi A, Muroyama R, Hosono N, et al. Genome-wide association study identifies a susceptibility locus for HCV-induced hepatocellular carcinoma. Nat Genet. 2011;43:455–8.
14. Kahraman A, Fingas CD, Syn WK, Gerken G, Canbay A. Role of stress-induced NKG2D ligands in liver diseases. Liver Int. 2012;32:370–82.
15. Watzl C. The NKG2D receptor and its ligands-recognition beyond the "missing self"? Microbes Infect. 2003;5:31–7.
16. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science. 1999;285:727–9.
17. Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, Spies T. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc Natl Acad Sci U S A. 1996;93:12445–50.
18. Bjorkstrom NK, Kekalainen E, Mjosberg J. Tissue-specific effector functions of innate lymphoid cells. Immunology. 2013;139:416–27.
19. Castello G, Scala S, Palmieri G, Curley SA, Izzo F. HCV-related hepatocellular carcinoma: from chronic inflammation to cancer. Clin Immunol. 2010;134:237–50.
20. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol. 2008;9:503–10.
21. Miletic A, Krmpotic A, Jonjic S. The evolutionary arms race between NK cells and viruses: who gets the short end of the stick? Eur J Immunol. 2013;43:867–77.
22. Cheng M, Chen Y, Xiao W, Sun R, Tian Z. NK cell-based immunotherapy for malignant diseases. Cell Mol Immunol. 2013;10:230–52.
23. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature. 1986;319:675–8.
24. Marcus A, Gowen BG, Thompson TW, Iannello A, Ardolino M, Deng W, et al. Recognition of tumors by the innate immune system and natural killer cells. Adv Immunol. 2014;122:91–128.
25. Shabani Z, Bagheri M, Zare-Bidaki M, Hassanshahi G, Arababadi MK, Mohammadi Nejad M. NK cells in hepatitis B virus infection: a potent target for immunotherapy. Arch Virol. 2014;159:1555–65.
26. Kasahara M, Yoshida S. Immunogenetics of the NKG2D ligand gene family. Immunogenetics. 2012;64:855–67.

27. Chan CJ, Smyth MJ, Martinet L. Molecular mechanisms of natural killer cell activation in response to cellular stress. Cell Death Differ. 2013;21:5–14.
28. Lopez-Soto A, Huergo-Zapico L, Acebes-Huerta A, Villa-Alvarez M, Gonzalez S. NKG2D signaling in cancer immunosurveillance. Int J Cancer. 2014;. doi:10.1002/ijc.28775.
29. Nausch N, Cerwenka A. NKG2D ligands in tumor immunity. Oncogene. 2008;27:5944–58.
30. Salih HR, Rammensee HG, Steinle A. Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. J Immunol. 2002;169:4098–102.
31. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature. 2002;419:734–8.
32. Sun D, Wang X, Zhang H, Deng L, Zhang Y. MMP9 mediates MICA shedding in human osteosarcomas. Cell Biol Int. 2011;35:569–74.
33. Liu G, Atteridge CL, Wang X, Lundgren AD, Wu JD. The membrane type matrix metalloproteinase MMP14 mediates constitutive shedding of MHC class I chain-related molecule A independent of a disintegrin and metalloproteinases. J Immunol. 2010;184:3346–50.
34. Kohga K, Takehara T, Tatsumi T, Ishida H, Miyagi T, Hosui A, et al. Sorafenib inhibits the shedding of major histocompatibility complex class I-related chain A on hepatocellular carcinoma cells by down-regulating a disintegrin and metalloproteinase 9. Hepatology. 2010;51:1264–73.
35. Kohga K, Takehara T, Tatsumi T, Miyagi T, Ishida H, Ohkawa K, et al. Anticancer chemotherapy inhibits MHC class I-related chain a ectodomain shedding by downregulating ADAM10 expression in hepatocellular carcinoma. Cancer Res. 2009;69:8050–7.
36. Waldhauer I, Goehlsdorf D, Gieseke F, Weinschenk T, Wittenbrink M, Ludwig A, et al. Tumor-associated MICA is shed by ADAM proteases. Cancer Res. 2008;68:6368–76.
37. Chitadze G, Bhat J, Lettau M, Janssen O, Kabelitz D. Generation of soluble NKG2D ligands: proteolytic cleavage, exosome secretion and functional implications. Scand J Immunol. 2013;78:120–9.
38. Marten A, von Lilienfeld-Toal M, Buchler MW, Schmidt J. Soluble MIC is elevated in the serum of patients with pancreatic carcinoma diminishing gammadelta T cell cytotoxicity. Int J Cancer. 2006;119:2359–65.
39. Bahram S, Bresnahan M, Geraghty DE, Spies T. A second lineage of mammalian major histocompatibility complex class I genes. Proc Natl Acad Sci U S A. 1994;91:6259–63.
40. Mok JW, Lee YJ, Kim JY, Lee EB, Song YW, Park MH, et al. Association of MICA polymorphism with rheumatoid arthritis patients in Koreans. Hum Immunol. 2003;64:1190–4.
41. Mizuki N, Ota M, Kimura M, Ohno S, Ando H, Katsuyama Y, et al. Triplet repeat polymorphism in the transmembrane region of the MICA gene: a strong association of six GCT repetitions with Behçet disease. Proc Natl Acad Sci U S A. 1997;94:1298–303.
42. Park Y, Lee H, Sanjeevi CB, Eisenbarth GS. MICA polymorphism is associated with type 1 diabetes in the Korean population. Diabetes Care. 2001;24:33–8.
43. Tinto N, Ciacci C, Calcagno G, Gennarelli D, Spampanato A, Farinaro E, et al. Increased prevalence of celiac disease without gastrointestinal symptoms in adults MICA 5.1 homozygous subjects from the Campania area. Dig Liver Dis. 2008;40:248–52.
44. Gong Z, Luo QZ, Lin L, Su YP, Peng HB, Du K, et al. Association of MICA gene polymorphisms with liver fibrosis in schistosomiasis patients in the Dongting Lake region. Braz J Med Biol Res. 2012;45:222–9.
45. Tonnerre P, Gerard N, Chatelais M, Poli C, Allard S, Cury S, et al. MICA variant promotes allosensitization after kidney transplantation. J Am Soc Nephrol. 2013;24:954–66.
46. Jiang X, Zou Y, Huo Z, Yu P. Association of major histocompatibility complex class I chain-related gene A microsatellite polymorphism and hepatocellular carcinoma in South China Han population. Tissue Antigens. 2011;78:143–7.
47. Tamaki S, Kawakami M, Yamanaka Y, Shimomura H, Imai Y, Ishida J, et al. Relationship between soluble MICA and the MICA A5.1 homozygous genotype in patients with oral squamous cell carcinoma. Clin Immunol. 2009;130:331–7.
48. Lu M, Xia B, Ge L, Li Y, Zhao J, Chen F. Role of major histocompatibility complex class I-related molecules A*A5.1 allele in ulcerative colitis in Chinese patients. Immunology. 2009;128:e230–6.
49. Steinle A, Li P, Morris DL, Groh V, Lanier LL, Strong RK, et al. Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse RAE-1 protein family. Immunogenetics. 2001;53:279–87.
50. Lopez-Hernandez R, Valdes M, Lucas D, Campillo JA, Martinez-Garcia P, Salama H, et al. Association analysis of MICA gene polymorphism and MICA-129 dimorphism with inflammatory bowel disease susceptibility in a Spanish population. Hum Immunol. 2010;71:512–4.
51. Raache R, Belanteur K, Amroun H, Benyahia A, Heniche A, Azzouz M, et al. Association of major histocompatibility complex class 1 chain-related gene a dimorphism with type 1 diabetes and latent autoimmune diabetes in adults in the Algerian population. Clin Vaccine Immunol. 2012;19:557–61.
52. Boukouaci W, Busson M, de Peffault Latour R, Rocha V, Suberbielle C, Bengoufa D. MICA-129 genotype, soluble MICA, and anti-MICA antibodies as biomarkers of chronic graft-versus-host disease. Blood. 2009;114:5216–24.
53. Kirsten H, Petit-Teixeira E, Scholz M, Hasenclever D, Hantmann H, Heider D, et al. Association of MICA with rheumatoid arthritis independent of known HLA-DRB1 risk alleles in a family-based and a case control study. Arthritis Res Ther. 2009;11:R60.
54. Thomas DL. Global control of hepatitis C: where challenge meets opportunity. Nat Med. 2013;19:850–8.
55. Strader DB, Wright T, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C. Hepatology. 2004;39:1147–71.
56. Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology. 2004;127:S35–50.
57. Jeong SW, Jang JY, Chung RT. Hepatitis C virus and hepatocarcinogenesis. Clin Mol Hepatol. 2012;18:347–56.
58. Goto K, Lin W, Zhang L, Jilg N, Shao RX, Schaefer EA, et al. The AMPK-related kinase SNARK regulates hepatitis C virus replication and pathogenesis through enhancement of TGF-β signaling. J Hepatol. 2013;59:942–8.
59. Tsai WL, Chung RT. Viral hepatocarcinogenesis. Oncogene. 2010;29:2309–24.
60. Kato N, Yoshida H, Ono-Nita SK, Kato J, Goto T, Otsuka M, et al. Activation of intracellular signaling by hepatitis B and C viruses: C-viral core is the most potent signal inducer. Hepatology. 2000;32:405–12.
61. Caldwell S, Park SH. The epidemiology of hepatocellular cancer: from the perspectives of public health problem to tumor biology. J Gastroenterol. 2009;44(Suppl 19):96–101.
62. Otsuka M, Kishikawa T, Yoshikawa T, Ohno M, Takata A, Shibata C. The role of microRNAs in hepatocarcinogenesis: current knowledge and future prospects. J Gastroenterol. 2013;49(2):173–84.
63. Negrini M, Gramantieri L, Sabbioni S, Croce CM. microRNA involvement in hepatocellular carcinoma. Anticancer Agents Med Chem. 2011;11:500–21.

64. Schmidt N, Neumann-Haefelin C, Thimme R. Cellular immune responses to hepatocellular carcinoma: lessons for immunotherapy. Dig Dis. 2012;30:483–91.
65. Jinushi M, Takehara T, Tatsumi T, Kanto T, Groh V, Spies T, et al. Autocrine/paracrine IL-15 that is required for type I IFN-mediated dendritic cell expression of MHC class I-related chain A and B is impaired in hepatitis C virus infection. J Immunol. 2003;171:5423–9.
66. Lunemann S, Malone DF, Hengst J, Port K, Grabowski J, Deterding K, et al. Compromised function of natural killer cells in acute and chronic viral hepatitis. J Infect Dis. 2014;209:1362–73.
67. Varchetta S, Mele D, Mantovani S, Oliviero B, Cremonesi E, Ludovisi S, et al. Impaired intrahepatic natural killer cell cytotoxic function in chronic hepatitis C virus infection. Hepatology. 2012;56:841–9.
68. Friedman SL. Mechanisms of hepatic fibrogenesis. Gastroenterology. 2008;134:1655–69.
69. Glassner A, Eisenhardt M, Kramer B, Korner C, Coenen M, Sauerbruch T, et al. NK cells from HCV-infected patients effectively induce apoptosis of activated primary human hepatic stellate cells in a TRAIL-, FasL- and NKG2D-dependent manner. Lab Invest. 2012;92:967–77.
70. Korangy F, Hochst B, Manns MP, Greten TF. Immune responses in hepatocellular carcinoma. Dig Dis. 2010;28:150–4.
71. Hoechst B, Voigtlaender T, Ormandy L, Gamrekelashvili J, Zhao F, Wedemeyer H, et al. Myeloid derived suppressor cells inhibit natural killer cells in patients with hepatocellular carcinoma via the NKp30 receptor. Hepatology. 2009;50:799–807.
72. Cai L, Zhang Z, Zhou L, Wang H, Fu J, Zhang S, et al. Functional impairment in circulating and intrahepatic NK cells and relative mechanism in hepatocellular carcinoma patients. Clin Immunol. 2008;129:428–37.
73. Sene D, Levasseur F, Abel M, Lambert M, Camous X, Hernandez C, et al. Hepatitis C virus (HCV) evades NKG2D-dependent NK cell responses through NS5A-mediated imbalance of inflammatory cytokines. PLoS Pathog. 2010;6:e1001184.
74. Kamimura H, Yamagiwa S, Tsuchiya A, Takamura M, Matsuda Y, Ohkoshi S, et al. Reduced NKG2D ligand expression in hepatocellular carcinoma correlates with early recurrence. J Hepatol. 2012;56:381–8.
75. Wen C, He X, Ma H, Hou N, Wei C, Song T, et al. Hepatitis C virus infection downregulates the ligands of the activating receptor NKG2D. Cell Mol Immunol. 2008;5:475–8.
76. Kim H, Bose SK, Meyer K, Ray R. Hepatitis C virus impairs natural killer cell mediated augmentation of complement synthesis. J Virol. 2014;88:2564–71.
77. Jinushi M, Takehara T, Tatsumi T, Hiramatsu N, Sakamori R, Yamaguchi S, et al. Impairment of natural killer cell and dendritic cell functions by the soluble form of MHC class I-related chain A in advanced human hepatocellular carcinomas. J Hepatol. 2005;43:1013–20.
78. Lopez-Vazquez A, Rodrigo L, Mina-Blanco A, Martinez-Borra J, Fuentes D, Rodriguez M, et al. Extended human leukocyte antigen haplotype EH18.1 influences progression to hepatocellular carcinoma in patients with hepatitis C virus infection. J Infect Dis. 2004;189:957–63.
79. Lo PH, Urabe Y, Kumar V, Tanikawa C, Koike K, Kato N, et al. Identification of a functional variant in the MICA promoter which regulates MICA expression and increases HCV-related hepatocellular carcinoma risk. PLoS One. 2013;8:e61279.
80. Ngamruengphong S, Patel T. Molecular evolution of genetic susceptibility to hepatocellular carcinoma. Dig Dis Sci. 2014;59:986–91.
81. Lange CM, Bibert S, Dufour JF, Cellerai C, Cerny A, Heim MH, et al. Comparative genetic analyses point to HCP5 as susceptibility locus for HCV-associated hepatocellular carcinoma. J Hepatol. 2013;59:504–9.
82. Motomura T, Ono Y, Shirabe K, Fukuhara T, Konishi H, Mano Y, et al. Neither MICA nor DEPDC5 genetic polymorphisms correlate with hepatocellular carcinoma recurrence following hepatectomy. HPB Surg. 2012;2012:185496.
83. Feitelson MA, Bonamassa B, Arzumanyan A. The roles of hepatitis B virus-encoded X protein in virus replication and the pathogenesis of chronic liver disease. Expert Opin Ther Targets. 2014;18:293–306.
84. Li Y, Wang JJ, Gao S, Liu Q, Bai J, Zhao XQ, et al. Decreased peripheral natural killer cells activity in the immune activated stage of chronic hepatitis B. PLoS One. 2014;9:e86927.
85. Peppa D, Micco L, Javaid A, Kennedy PT, Schurich A, Dunn C, et al. Blockade of immunosuppressive cytokines restores NK cell antiviral function in chronic hepatitis B virus infection. PLoS Pathog. 2010;6:e1001227.
86. Chen Y, Wei H, Sun R, Tian Z. Impaired function of hepatic natural killer cells from murine chronic HBsAg carriers. Int Immunopharmacol. 2005;5:1839–52.
87. Rehermann B. Pathogenesis of chronic viral hepatitis: differential roles of T cells and NK cells. Nat Med. 2013;19:859–68.
88. Sun C, Fu B, Gao Y, Liao X, Sun R, Tian Z, et al. TGF-beta1 down-regulation of NKG2D/DAP10 and 2B4/SAP expression on human NK cells contributes to HBV persistence. PLoS Pathog. 2012;8:e1002594.
89. Zwirner NW, Fuertes MB, Girart MV, Domaica CI, Rossi LE. Cytokine-driven regulation of NK cell functions in tumor immunity: role of the MICA-NKG2D system. Cytokine Growth Factor Rev. 2007;18:159–70.
90. Chen Y, Cheng M, Tian Z, Hepatitis B. virus down-regulates expressions of MHC class I molecules on hepatoplastoma cell line. Cell Mol Immunol. 2006;3:373–8.
91. Tang KF, Chen M, Xie J, Song GB, Shi YS, Liu Q, et al. Inhibition of hepatitis B virus replication by small interference RNA induces expression of MICA in HepG2.2.15 cells. Med Microbiol Immunol. 2009;198:27–32.
92. Wu J, Zhang XJ, Shi KQ, Chen YP, Ren YF, Song YJ, et al. Hepatitis B surface antigen inhibits MICA and MICB expression via induction of cellular miRNAs in hepatocellular carcinoma cells. Carcinogenesis. 2013;35:155–63.
93. Kishikawa T, Otsuka M, Yoshikawa T, Ohno M, Takata A, Shibata C, et al. Regulation of the expression of the liver cancer susceptibility gene MICA by microRNAs. Sci Rep. 2013;3:2739.
94. Kumar V, Yi Lo PH, Sawai H, Kato N, Takahashi A, Deng Z. Soluble MICA and a MICA variation as possible prognostic biomarkers for HBV-induced hepatocellular carcinoma. PLoS One. 2012;7:e44743.
95. Tong HV, Toan NL, Song LH, Bock CT, Kremsner PG, Velavan TP. Hepatitis B virus-induced hepatocellular carcinoma: functional roles of MICA variants. J Viral Hepat. 2013;20:687–98.
96. Li JJ, Pan K, Gu MF, Chen MS, Zhao JJ, Wang H, et al. Prognostic value of soluble MICA levels in the serum of patients with advanced hepatocellular carcinoma. Chin J Cancer. 2013;32:141–8.
97. Ou DP, Tao YM, Tang FQ, Yang LY. The hepatitis B virus X protein promotes hepatocellular carcinoma metastasis by upregulation of matrix metalloproteinases. Int J Cancer. 2007;120:1208–14.
98. Yu FL, Liu HJ, Lee JW, Liao MH, Shih WL. Hepatitis B virus X protein promotes cell migration by inducing matrix metalloproteinase-3. J Hepatol. 2005;42:520–7.

99. Lara-Pezzi E, Gomez-Gaviro MV, Galvez BG, Mira E, Iniguez MA, Fresno M, et al. The hepatitis B virus X protein promotes tumor cell invasion by inducing membrane-type matrix metalloproteinase-1 and cyclooxygenase-2 expression. J Clin Invest. 2002;110:1831–8.
100. Kim JR, Kim CH. Association of a high activity of matrix metalloproteinase-9 to low levels of tissue inhibitors of metalloproteinase-1 and -3 in human hepatitis B-viral hepatoma cells. Int J Biochem Cell Biol. 2004;36:2293–306.
101. Chung TW, Lee YC, Kim CH. Hepatitis B viral HBx induces matrix metalloproteinase-9 gene expression through activation of ERK and PI-3 K/AKT pathways: involvement of invasive potential. FASEB J. 2004;18:1123–5.
102. Karacki PS, Gao X, Thio CL, Thomas DL, Goedert JJ, Vlahov D, et al. MICA and recovery from hepatitis C virus and hepatitis B virus infections. Genes Immun. 2004;5:261–6.
103. Chen K, Shi W, Xin Z, Wang H, Zhu X, Wu X, et al. Replication of genome wide association studies on hepatocellular carcinoma susceptibility Loci in a chinese population. PLoS One. 2013;8:e77315.
104. Al-Qahtani AA, Al-Anazi M, Abdo AA, Sanai FM, Al-Hamoudi W, Alswat KA, et al. Genetic variation at -1878 (rs2596542) in MICA gene region is associated with chronic hepatitis B virus infection in Saudi Arabian patients. Exp Mol Pathol. 2013;95:255–8.
105. Guan YS, He Q. Role of antiviral therapy in the management of hepatocellular carcinoma. Anticancer Drugs. 2013;24:337–43.
106. Fletcher SP. Delaney WEt. New therapeutic targets and drugs for the treatment of chronic hepatitis B. Semin Liver Dis. 2013;33:130–7.
107. Kurokawa M, Hiramatsu N, Oze T, Yakushijin T, Miyazaki M, Hosui A, et al. Long-term effect of lamivudine treatment on the incidence of hepatocellular carcinoma in patients with hepatitis B virus infection. J Gastroenterol. 2012;47:577–85.
108. Manns MP, von Hahn T. Novel therapies for hepatitis C—one pill fits all? Nat Rev Drug Discov. 2013;12:595–610.
109. Ogawa E, Furusyo N, Kajiwara E, Takahashi K, Nomura H, Maruyama T, et al. Efficacy of pegylated interferon alpha-2b and ribavirin treatment on the risk of hepatocellular carcinoma in patients with chronic hepatitis C: a prospective, multicenter study. J Hepatol. 2013;58:495–501.
110. Chotiyaputta W, Lok AS. Hepatitis B virus variants. Nat Rev Gastroenterol Hepatol. 2009;6:453–62.
111. Abu-Amara M, Feld JJ. Does antiviral therapy for chronic hepatitis B reduce the risk of hepatocellular carcinoma? Semin Liver Dis. 2013;33:157–66.
112. Papatheodoridis GV, Manolakopoulos S, Touloumi G, Vourli G, Raptopoulou-Gigi M, Vafiadis-Zoumbouli I, et al. Virological suppression does not prevent the development of hepatocellular carcinoma in HBeAg-negative chronic hepatitis B patients with cirrhosis receiving oral antiviral(s) starting with lamivudine monotherapy: results of the nationwide HEPNET. Greece cohort study. Gut. 2011;60:1109–16.
113. Salvatierra K, Fareleski S, Forcada A, Lopez-Labrador FX. Hepatitis C virus resistance to new specifically-targeted antiviral therapy: a public health perspective. World J Virol. 2013;2:6–15.
114. Morgan RL, Baack B, Smith BD, Yartel A, Pitasi M, Falck-Ytter Y. Eradication of hepatitis C virus infection and the development of hepatocellular carcinoma: a meta-analysis of observational studies. Ann Intern Med. 2013;158:329–37.
115. Miamen AG, Dong H, Roberts LR. Immunotherapeutic approaches to hepatocellular carcinoma treatment. Liver Cancer. 2012;1:226–37.
116. Couzin-Frankel J. Breakthrough of the year 2013. Cancer immunother. Sci. 2013;342:1432–3.
117. Lavanchy D. Viral hepatitis: global goals for vaccination. J Clin Virol. 2012;55:296–302.
118. Gerlich WH. Medical virology of hepatitis B: how it began and where we are now. Virol J. 2013;10:239.
119. Wendt A, Adhoute X, Castellani P, Oules V, Ansaldi C, Benali S, et al. Chronic hepatitis C: future treatment. Clin Pharmacol. 2014;6:1–17.
120. Gao Q, Shi Y, Wang X, Zhou J, Qiu S, Fan J. Translational medicine in hepatocellular carcinoma. Front Med. 2012;6:122–33.
121. Ohira M, Nishida S, Tryphonopoulos P, Tekin A, Selvaggi G, Moon J, et al. Clinical-scale isolation of interleukin-2-stimulated liver natural killer cells for treatment of liver transplantation with hepatocellular carcinoma. Cell Transplant. 2012;21:1397–406.
122. Spear P, Wu MR, Sentman ML, Sentman CL. NKG2D ligands as therapeutic targets. Cancer Immun. 2013;13:8.
123. Zhang C, Zhang J, Niu J, Zhou Z, Tian Z. Interleukin-12 improves cytotoxicity of natural killer cells via upregulated expression of NKG2D. Hum Immunol. 2008;69:490–500.
124. Xia X, Li X, Feng G, Zheng C, Liang H, Zhou G. Intra-arterial interleukin-12 gene delivery combined with chemoembolization: anti-tumor effect in a rabbit hepatocellular carcinoma (HCC) model. Acta Radiol. 2013;54:684–9.
125. Chang CJ, Chen YH, Huang KW, Cheng HW, Chan SF, Tai KF, et al. Combined GM-CSF and IL-12 gene therapy synergistically suppresses the growth of orthotopic liver tumors. Hepatology. 2007;45:746–54.
126. Ullrich E, Koch J, Cerwenka A, Steinle A. New prospects on the NKG2D/NKG2DL system for oncology. Oncoimmunology. 2013;2:e26097.
127. Armeanu S, Bitzer M, Lauer UM, Venturelli S, Pathil A, Krusch M, et al. Natural killer cell-mediated lysis of hepatoma cells via specific induction of NKG2D ligands by the histone deacetylase inhibitor sodium valproate. Cancer Res. 2005;65:6321–9.
128. Worns MA. Systemic therapy and synergies by combination. Dig Dis. 2013;31:104–11.
129. Armeanu S, Krusch M, Baltz KM, Weiss TS, Smirnow I, Steinle A, et al. Direct and natural killer cell-mediated antitumor effects of low-dose bortezomib in hepatocellular carcinoma. Clin Cancer Res. 2008;14:3520–8.
130. Kim GP, Mahoney MR, Szydlo D, Mok TS, Marshke R, Holen K, et al. An international, multicenter phase II trial of bortezomib in patients with hepatocellular carcinoma. Invest New Drugs. 2012;30:387–94.
131. Van Belle TL, von Herrath MG. The role of the activating receptor NKG2D in autoimmunity. Mol Immunol. 2009;47:8–11.
132. Chen Y, Wei H, Sun R, Dong Z, Zhang J, Tian Z. Increased susceptibility to liver injury in hepatitis B virus transgenic mice involves NKG2D-ligand interaction and natural killer cells. Hepatology. 2007;46:706–15.
133. Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol. 2005;5:215–29.
134. Zhang Z, Zhang S, Zou Z, Shi J, Zhao J, Fan R, et al. Hypercytolytic activity of hepatic natural killer cells correlates with liver injury in chronic hepatitis B patients. Hepatology. 2011;53:73–85.
135. Dunn C, Brunetto M, Reynolds G, Christophides T, Kennedy PT, Lampertico P, et al. Cytokines induced during chronic hepatitis B virus infection promote a pathway for NK cell-mediated liver damage. J Exp Med. 2007;204:667–80.
136. Ahlenstiel G, Titerence RH, Koh C, Edlich B, Feld JJ, Rotman Y, et al. Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon-alfa-dependent manner. Gastroenterology. 2010;138(325–35):e1–2.

137. Okamoto Y, Shinjo K, Shimizu Y, Sano T, Yamao K, Gao W, et al. Hepatitis virus infection affects DNA methylation in mice with humanized livers. Gastroenterology. 2014;146:562–72.
138. Zou Y, Chen T, Han M, Wang H, Yan W, Song G, et al. Increased killing of liver NK cells by Fas/Fas ligand and NKG2D/NKG2D ligand contributes to hepatocyte necrosis in virus-induced liver failure. J Immunol. 2010;184:466–75.
139. Kahraman A, Schlattjan M, Kocabayoglu P, Yildiz-Meziletoglu S, Schlensak M, Fingas CD, et al. Major histocompatibility complex class I-related chains A and B (MIC A/B): a novel role in nonalcoholic steatohepatitis. Hepatology. 2010;51:92–102.
140. Rosmorduc O, Fartoux L. HCC and NASH: how strong is the clinical demonstration? Clin Res Hepatol Gastroenterol. 2012;36:202–8.
141. Cox ST, Madrigal JA, Saudemont A. Diversity and characterization of polymorphic 5′ promoter haplotypes of MICA and MICB genes. Tissue Antigens. 2014. doi:10.1111/tan.12400.
142. Raulet DH, Gasser S, Gowen BG, Deng W, Jung H. Regulation of ligands for the NKG2D activating receptor. Annu Rev Immunol. 2013;31:413–41.
143. Chaiteerakij R, Addissie BD, Roberts LR. Update on biomarkers of hepatocellular carcinoma. Clin Gastroenterol Hepatol. 2013. doi:10.1016/j.cgh.2013.10.038.
144. Hoshida Y, Fuchs BC, Tanabe KK. Genomic risk of hepatitis C-related hepatocellular carcinoma. J Hepatol. 2012;56:729–30.
145. Della Corte C, Aghemo A, Colombo M. Individualized hepatocellular carcinoma risk: the challenges for designing successful chemoprevention strategies. World J Gastroenterol. 2013;19:1359–71.
146. Delaney WEt. Molecular virology of chronic hepatitis B and C: parallels, contrasts and impact on drug development and treatment outcome. Antiviral Res. 2013;99:34–48.
147. Liver EAftSot. EASL clinical practice guidelines: management of hepatitis C virus infection. J Hepatol. 2014;60:392–420.
148. Gao B. Natural killer group 2 member D, its ligands, and liver disease: good or bad? Hepatology. 2010;51:8–11.

PLOS | ONE

RESEARCH ARTICLE

The Characteristic Changes in Hepatitis B Virus X Region for Hepatocellular Carcinoma: A Comprehensive Analysis Based on Global Data

Wenwen Li[1], Kaku Goto[1], Yasuo Matsubara[1], Sayaka Ito[1], Ryosuke Muroyama[1], Qiang Li[2], Naoya Kato[1]*

1 Division of advanced genome medicine, Advanced clinical research center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan, **2** Affiliated Infectious Disease Hospital of Shandong University, Jinan, Shandong, China

* kato-2im@ims.u-tokyo.ac.jp

Citation: Li W, Goto K, Matsubara Y, Ito S, Muroyama R, Li Q, et al. (2015) The Characteristic Changes in Hepatitis B Virus X Region for Hepatocellular Carcinoma: A Comprehensive Analysis Based on Global Data. PLoS ONE 10(5): e0125555. doi:10.1371/journal.pone.0125555

Academic Editor: Tatsuo Kanda, Chiba University, Graduate School of Medicine, JAPAN

Received: December 31, 2014

Accepted: March 25, 2015

Published: May 5, 2015

Data Availability Statement: All relevant data are within the paper.

Funding: The study was supported by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Objectives

Mutations in hepatitis B virus (HBV) X region (HBx) play important roles in hepatocarcinogenesis while the results remain controversial. We sought to clarify potential hepatocellular carcinoma (HCC)-characteristic mutations in HBx from HBV genotype C-infected patients and the distribution of those mutations in different disease phases and genotypes.

Methods

HBx sequences downloaded from an online global HBV database were screened and then classified into Non-HCC or HCC group by diagnosis information. Patients' data of patient age, gender, country or area, and viral genotype were also extracted. Logistic regression was performed to evaluate the effects of mutations on HCC risk.

Results

1) Full length HBx sequences (HCC: 161; Non-HCC: 954) originated from 1115 human sera across 29 countries/areas were extracted from the downloaded 5956 HBx sequences. Genotype C occupied 40.6% of Non-HCC (387/954) and 89.4% of HCC (144/161). 2) Sixteen nucleotide positions showed significantly different distributions between genotype C HCC and Non-HCC groups. 3) Logistic regression showed that mutations A1383C (OR: 2.32, 95% CI: 1.34-4.01), R1479C/T (OR: 1.96, 95% CI: 1.05-3.64; OR: 5.15, 95% CI: 2.53-10.48), C1485T (OR: 2.40, 95% CI: 1.41-4.08), C1631T (OR: 4.09, 95% CI: 1.41-11.85), C1653T (OR: 2.58, 95% CI: 1.59-4.19), G1719T (OR: 2.11, 95% CI: 1.19-3.73), and T1800C (OR: 23.59, 95% CI: 2.25-247.65) were independent risk factors for genotype C HBV-related HCC, presenting different trends among individual disease phases. 4) Several