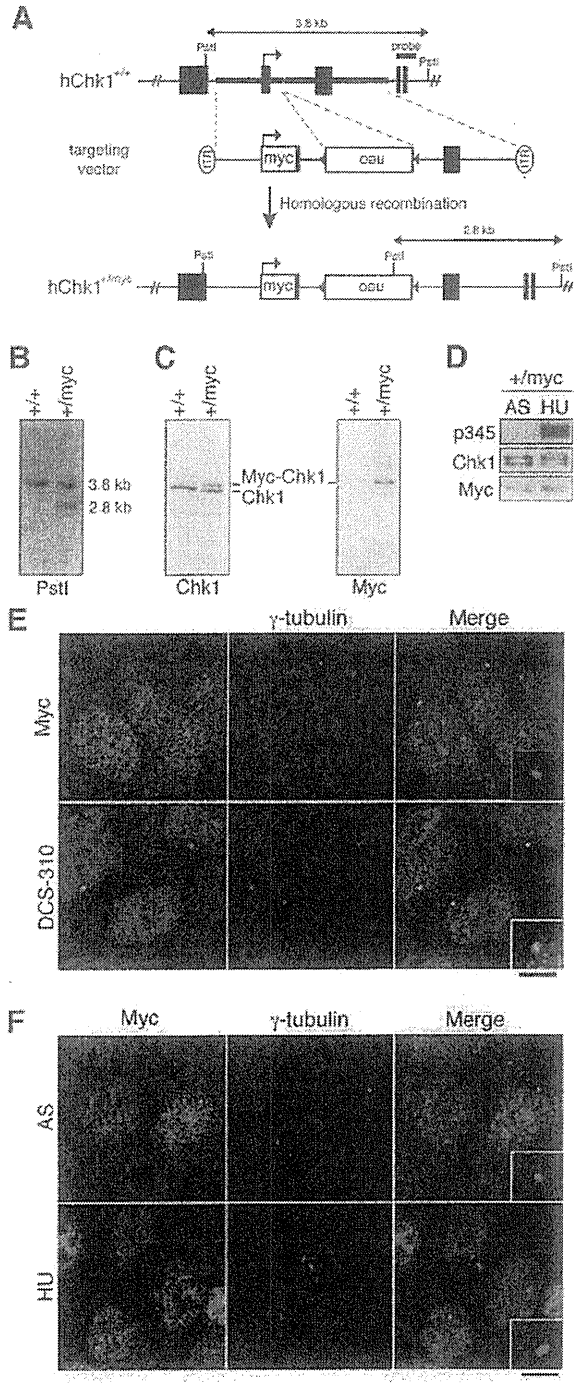

Fig. 2. Chk1 localized mainly in nucleus but not on centrosome. (A) Strategy of Chk1$^{+/myc}$ DLD-1 cell generation. The rAAV targeting vector contains sequences from *CHK1* locus flanking a neomycin resistance (*neo*) marker bounded by *loxP* sites. DNA sequence corresponding to Myc-epitope (amino acid sequence; EQKLISEEDL) was inserted between first ATG and the second codon on exon 2. The diagram indicates the positions of Chk1-derived sequences in the targeting vector and of the labeled hybridization probe relative to *PstI* sites. The predicted restriction map of targeted *CHK1* allele is also shown (hChk1$^{+/myc}$). (B,C) Established heterozygotes (+/myc) were subjected to Southern (B) and western (C) blot analyses. The Southern blot hybridization to *PstI*-digested genomic DNA shows single band of ~3.8 kb in wild-type (+/+) and two bands of ~3.8 kb and ~2.8 kb with similar intensity in heterozygotes (+/myc). Positions of Myc-tagged Chk1 (Myc-Chk1) and wild-type Chk1 (Chk1) are indicated (C). (D) Chk1$^{+/myc}$ DLD-1 cells were treated with 3 mM hydroxyurea (HU) or left untreated (AS) for 16 hours. Then, cells were subjected to western blotting with indicated antibodies. (E,F) Chk1$^{+/myc}$ DLD-1 cells were subjected to immunocytochemistry with anti-Myc or DCS-310, together with anti-γ-tubulin and DAPI. HU treatment was performed as described above. Scale bars: 5 μm.

Myc antibody (Fig. 2E). However, unlike DCS-310, the centrosome was hardly stained by anti-Myc antibody (Fig. 2E). Since Chk1 had been reported to accumulate in the nucleus (Sanchez et al., 1997; Jiang et al., 2003) or on the centrosome (Loffler et al., 2007; Niida et al., 2007) in response to the checkpoint activation, we also stained HU-treated Chk1$^{+/myc}$ cells with anti-Myc antibody. Regardless of the HU treatment, we hardly detected anti-Myc signals on the centrosome (Fig. 2F). However, HU treatment enhanced nuclear Myc signals (Fig. 2F). Since these signals were detected only at low or background levels in parental (Chk1$^{+/+}$) DLD-1 cells (supplementary material Fig. S1), the majority of observed Myc signals in Chk1$^{+/myc}$ cells reflected the staining of Myc-tagged Chk1 but not of endogenous Myc. Thus, these results suggest that Chk1 is localized predominantly in the nucleus but not on the centrosome.

We then searched for DCS-310-reactive centrosomal protein(s) using a protein array system (Goshima et al., 2008). Among 19,900 proteins, we identified 18 proteins (including Chk1) as being DCS-310-reactive (supplementary material Fig. S2 and Table S1). In order to examine which protein(s) localize to the centrosome, we introduced each Flag-tagged protein into HeLa cells. As shown in Fig. 3A,B (also see supplementary material Fig. S3), two independent proteins, bicaudal D homolog 2 (Bicd2) (Splinter et al., 2010) and the non-characterized protein coiled-coil domain containing 151 (Ccdc151), were validated as candidate centrosomal proteins, although Flag-tagged Chk1 was localized predominantly in the nucleus but not on the centrosome. RNA interference (RNAi) experiments showed that depletion of Ccdc151 specifically reduced DCS-310 immunoreactitity on the centrosome (Fig. 3C,D). All these results suggest that centrosomal DCS-310 immunoreactivity reflects the existence of Ccdc151 but not Chk1 on the centrosome.

Nuclear but not centrosomal Chk1 prevents Cdk1 from premature activation

For functional analyses of Chk1, we established Tet-On HeLa cells in which each type of Myc-tagged Chk1 (Myc-Chk1) is expressed in a doxycycline (Dox)-dependent manner. After the addition of 0.3 μg/ml Dox, the expression level of Myc-Chk1 fused to PACT (Myc-Chk1–PACT) was comparable to that of Myc-Chk1 wild-type (WT), Chk1 kinase-inactive mutant (K38M) or of a mutant in which two serine phosphorylation sites of Cdk1 were changed to alanine (S286/301A) (Fig. 4A). Under this condition, Myc-Chk1–PACT localized predominantly on the centrosome, whereas other types of Myc-Chk1 mutant were localized predominantly in the nucleus but not on the centrosome (Fig. 4B). We next evaluated the effect of each protein expression on the G2–M transition and cyclin-B1–Cdk1 activation in the following method (summarized in Fig. 4C). As shown in Fig. 4D, each Tet-On cell line was synchronized at the G1–S boundary by the double-thymidine block

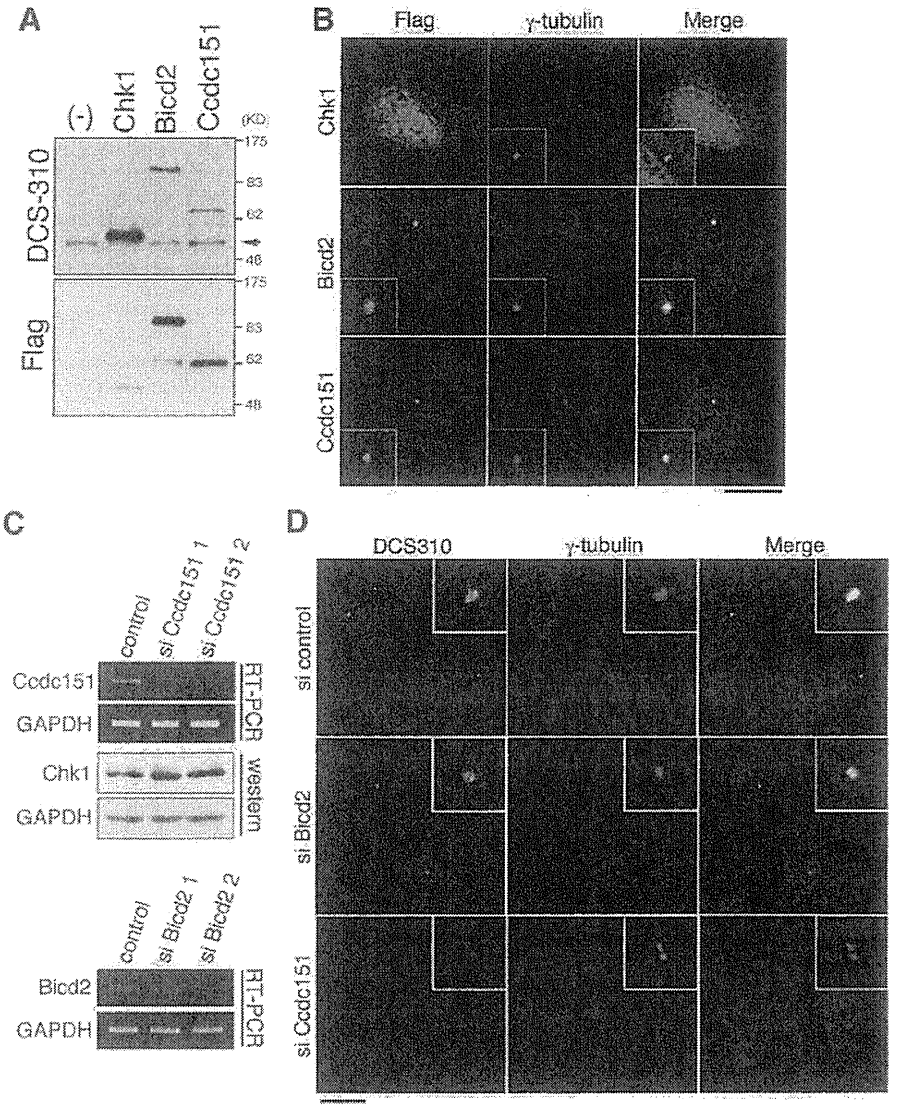

Fig. 3. Centrosomal staining of anti-Chk1 antibody DCS-310 indicates the presence of Ccdc151 but not Chk1 on centrosomes. (**A**,**B**) HeLa cells were transfected with pDEST12.2 (Invitrogen) each carrying a Flag-tagged protein. As a negative control, we used pDEST12.2 carrying only Flag (–). After transfection, cells were subjected to western blotting (A) or immunocytochemistry (B). The arrowhead in A indicates the position of endogenous Chk1. (**C**,**D**) HeLa cells were transfected with siRNA as indicated. At 72 hours after transfection, cells were subjected to RT-PCR, western blotting (C) or immunocytochemistry (D). Since we observed only marginal differences in immunocytochemistry between two sequences targeted to each protein (not depicted), data by using one target sequence are indicated (D). Scale bars: 5 μm.

(DTB) method. At the release of the second thymidine block, we added 0.3 μg/ml Dox to the growing medium for the induction of each Myc-Chk1. From 6–13 hours after the release, cells were collected and then subjected to immunocytochemistry (to evaluate mitotic index; Fig. 4F) or in-vitro H1 kinase assays by using anti-Cyclin B1 immunocomplex (to evaluate the activity of cyclin-B1–Cdk1 complex; Fig. 4G). Without Dox addition (Myc-Chk1 induction), we observed only marginal changes in the timing of mitotic entry among the established cell lines (Fig. 4E). WT expression delayed the timing not only of mitotic entry (Fig. 4F) but also of cyclin-B1–Cdk1 activation (Fig. 4G), compared with the K38M mutant. The timing was more delayed when mutant S286/301A was expressed (Fig. 4F,G), confirming the existence of a positive feedback loop between Cdk1 and Chk1 (Enomoto et al., 2009). However, we observed only marginal effects when Myc-Chk1–PACT was expressed, similar to the experiments with the K38M mutant (Fig. 4F,G). These results suggest that forced immobilization of Chk1 to the centrosome has little impact on the timing of mitotic entry and activation of Cdk1.

These observations appeared to contrast with the previous report that stable induction of GFP-Chk1–PACT inhibited the G2–M transition. We consider that this discrepancy might be partly caused by the difference in the expression level of Chk1–PACT. In the case of higher expression of Myc-Chk1–PACT (by use of 1–2 μg/ml Dox), Myc-Chk1–PACT was not restricted to the centrosome; it also localized to the nucleus (Fig. 4H,I). Under this condition, the expression of Myc-Chk1–PACT also delayed the timing of mitotic entry, like WT expression (Fig. 4J). These results imply one possibility, namely that the localization of mutant Chk1–PACT is highly dependent on its level of expression and that reflects the phenotype.

We also evaluated the effect of the expression of Myc-Chk1 WT with three repeated sequences of NLS at the C-terminus (Myc-Chk1-3×NLS). The expression level of Myc-Chk1-3×NLS was

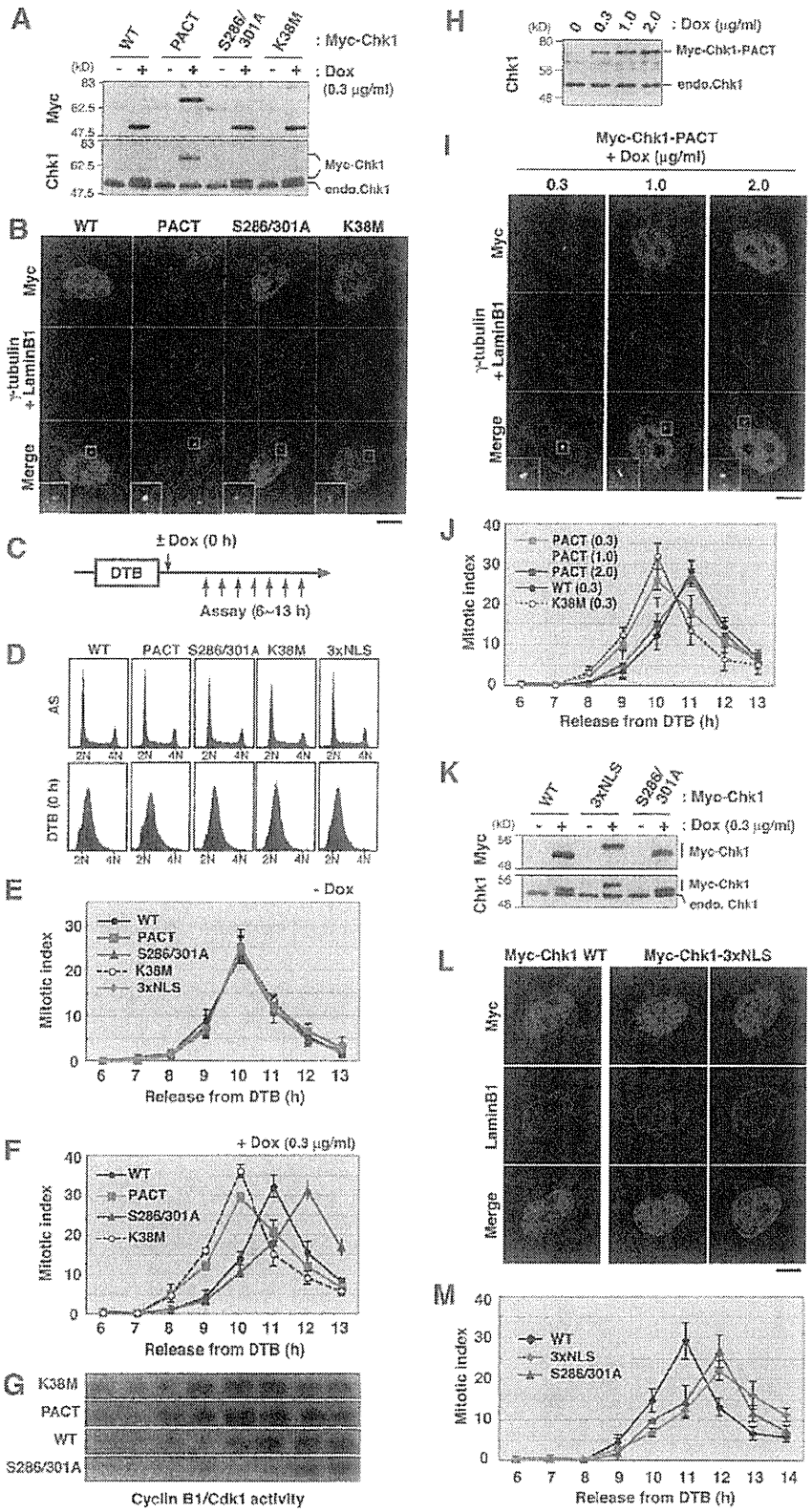

Fig. 4. Nuclear but not centrosomal Chk1 prevents Cdk1 from unscheduled activation. (A–M) Each Tet-ON cell line was incubated with (+) or without (–) doxycycline (Dox) for 16 hours after which the cells were subjected to western blotting (A,H,K) or immunocytochemistry (B,I,L). Positions of endogenous Chk1 (endo. Chk1) or exogenous Myc-Chk1 are indicated (A,H,K). Cells were co-stained with anti-γ-tubulin (B,I) and anti-lamin-B1 (to detect nuclear membrane; B,I,L). To evaluate the effect of each Myc-Chk1 expression on mitotic entry (F,J,M) and Cdk1 activity (G), the following experiments were performed as indicated (C). Each Tet-On cell line was synchronized at the G1–S boundary using the double thymidine block (DTB) method. To confirm the synchrony of each cell line by DTB, some cells were subjected to FACS analyses (Time 0; D). As a control, FACS analyses were also performed using random culture cells (AS; D). Upon release of second thymidine block, Dox was added to the growth medium to induce Myc-Chk1. Between 6 hours and 13 hours after release, cells were collected and subjected to immunocytochemistry (F,J,M) or in vitro H1 kinase assays using anti-Cyclin B1 immunocomplex (G). As a control, the timing of mitotic entry without Myc-Chk1 induction was analyzed (Dox addition; E). Plotted data of mitotic indices at each time point represent mean ± s.e.m. of 200 cells from three independent experiments (E,F,J,M). Cyclin-B1–Cdk1 activity in each sample was visualized through autoradiography of H1 (G). Scale bars: 10 μm.

comparable to that of Myc-Chk1 WT or mutant Chk1 S286/301A (Fig. 4K). Myc-Chk1-3×NLS was localized at higher levels than Myc-Chk1 WT in the nucleus (Fig. 4L). Like that of S286/301A, the expression of Myc-Chk1-3×NLS delayed mitotic entry more than the expression of Myc-Chk1 WT (Fig. 4M). Since the S286/301A mutant was localized predominantly in the nucleus even during the first mitotic phase prophase (Enomoto et al., 2009), all these observations suggest that nuclear Chk1 prevents Cdk1 from unscheduled activation before the G2–M transition.

In this study, we demonstrate that Chk1, when localized in the nucleus but not on the centrosome, inhibits premature Cdk1 activation. Together with our previous study (Enomoto et al., 2009), our findings here strongly support a model according to which the ATR-Chk1 pathway monitors the integrity of genomic DNA in the nucleus before mitosis. In support of this notion, all known regulators of this pathway are localized in the nucleus (see Introduction) and Chk1 accumulates in the nucleus in response to the checkpoint activation (Sanchez et al., 1997; Jiang et al., 2003). Since Chk1 did not translocate to the centrosome even in HU-treated cells (Fig. 2F), Chk1 did not directly inhibit centrosomal cyclin-B1–Cdk1 activation, which was first detected at the G2–M transition (Jackman et al., 2003). Accumulating data has demonstrated a new checkpoint pathway mediated by MAPK (p38) and MAPK-activated protein kinase-2 (MAPKAP kinase-2; MK2) that operates parallel to Chk1 and is activated downstream of ataxia telangiectasia mutated (ATM) and ATR (Bulavin et al., 2001; Manke et al., 2005; Raman et al., 2007; Reinhardt et al., 2007; Reinhardt and Yaffe, 2009). A recent study also revealed that Chk1 controls nuclear events but that the p38–MK2 pathway regulates cytoplasmic events in checkpoint responses (Reinhardt et al., 2010). Interestingly, p38 has been reported to phosphorylate and inhibit Cdc25B (Bulavin et al., 2001), which was proposed to function as a centrosomal Cdc25 (Gabrielli et al., 1996; Karlsson et al., 1999; De Souza et al., 2000; Lindqvist et al., 2005). Therefore, the p38–MK2 pathway is likely to transmit nuclear checkpoint signals generated by Chk1 to other cellular components such as the centrosome.

Our present study documents the importance of spatiotemporally regulated localization of Chk1 in the mitotic entry and paves the way for future studies that evaluate the coordination of centrosomal, cytoplasmic, and nuclear events during the checkpoint response.

Materials and Methods

Generation of the Chk1$^{+/myc}$ DLD-1 and HeLa Tet-On cell lines

The colon adenocarcinoma (DLD-1) cell line was purchased from ATCC (#CCL-221). For the construction of targeting vectors, genomic regions of the *CHK1* locus were amplified from DLD-1 genomic DNA by using primers of 5′ and 3′ homology arms. Then, site-directed mutagenesis (Stratagene, La Jolla, CA) was performed for the addition of a DNA sequence corresponding to Myc epitope (amino acid sequence; EQKLISEEDL). As shown in Fig. 2A, both homology arms and *loxP–neo–loxP* were ligated into the pAAV-MCS vector. Recombinant adeno-associated viruses (rAAVs) were produced according to the manufacturer's protocol (Stratagene). Chk1$^{+/myc}$ DLD-1 cells were established as described previously (Kohli et al., 2004; Rago et al., 2007). Each HeLa Tet-On cell line was generated as reported previously (Ikegami et al., 2008; Enomoto et al., 2009).

Transfection

For the expression of 18 DCS-310-reactive proteins in HeLa cells, pDEST12.2 carrying each Flag-tagged protein was constructed through the homologous recombination between pDEST12.2 and 'FLJ' cDNA clones (Goshima et al., 2008) using Gateway technology (Invitrogen, Carlsbad, CA). Transfection was performed with Lipofectamine™ reagent (Invitrogen) according to the manufacturer's protocol.

All small interfering RNA (siRNA) duplexes were purchased from QIAGEN (Valencia, CA). Target sequences were as follows: Bicd2 siRNAs, 5′-GGAGCUGU-CACACUACAUG-3′ (sequence 1) and 5′-GGUGGACUAUGAGGCUAUC-3′ (sequence 2) (Splinter et al., 2010); Cedc151 siRNAs, 5′-GGAGACUAAGGCACUG-GAA-3′ (sequence 1) and 5′-CAA GGCCUAUCUAAUGGACGA-3′ (sequence 2); and non-silencing siRNA (as a negative control), 5′-UUCUCCGAACGUGUCACGU-3′. Transfection was performed with a mixture of each siRNA (final concentration 25 nM) and Lipofectamine™ RNAiMAX reagent (Invitrogen) according to the transfection procedure.

Antibodies

Antibodies against the following proteins or tags were used in the study: Chk1 (DCS310), γ-tubulin (GTU-88), Flag (M2; Sigma, St Louis, MO); Chk1 phospho-Ser345 (133D3), Myc (9B11; Cell Signaling Technology, Beverly, MA); CyclinB1 (GNS-1; BD Transduction Laboratories, San Diego, CA); rabbit laminB1, GAPDH conjugated to HRP (Abcam, Cambridge, UK).

Immunocytochemistry

Before fixation, cells were grown on glass coverslips. For immunostaining with DCS-310 (except that shown in Fig. 2) or anti-Flag, cells were fixed in –20°C methanol/acetone (1:1) for 7 minutes as described previously (Kramer et al., 2004). In other experiments, cells were treated with 1% (Fig. 2) or 3.7% (Fig. 4) formaldehyde in phosphate-buffered saline (PBS) at room temperature for 10 minutes and then with –20°C methanol for 10 minutes. Cells were incubated with primary antibodies for 1 hour and then with appropriate Alexa-Fluor-conjugated secondary antibodies (Invitrogen) for 30 minutes at room temperature. DNA was also stained with 0.5 μg/ml DAPI for 5 minutes. Each fluorescence image was captured as a single optical section using a Zeiss LSM510 confocal laser-scanning microscope (Carl Zeiss, Thornwood, NY).

In vitro H1 kinase assays using anti-Cyclin B1 immunocomplex

The cyclin-B1–Cdk1 complex was purified as an anti-cyclin-B1 immunocomplex as described previously (Ikegami et al., 2008; Enomoto et al., 2009). This immunocomplex was incubated with histone H1 as described previously (Kasahara et al., 2010). Cyclin-B1–Cdk1 activity in each sample was visualized through the autoradiography of histone H1.

Reverse-transcriptase PCR

Reverse-transcriptase (RT)-PCR was performed using the following primers as described previously (Goto et al., 2006): Cedc151, 5′-CAGGAGAC-CATCAGTCAGCTC-3′ (forward) and 5′-GCAGGTACACGCTGGTAATGT-3′ (reverse); Bicd2, 5′-ACTCGGAGATGAGTGCTTTGA-3′ (forward) and 5′-CACAACGTCCTAAAACCCAGA-3′ (reverse); and GAPDH, 5′-GGCATGGC-CTTCCGTGTTCCT-3′ (forward) and 5′-TCCTTGCTGGGGTGGGTGGTC-3′ (reverse).

FACS analysis

For fluorescence-activated cell sorting (FACS) analysis that shows the DNA content in each group, ~10^6 treated cells were collected by trypsinization, resuspended in buffer solution (CycleTEST™ PLUS kit; Becton-Dickinson, San Diego, CA) and stored at −80°C. Then, we treated cells according to the manufacturer's protocol (CycleTEST™ PLUS kit) and analyzed them using a Becton-Dickinson FacsScan and CellQuest software.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/13/2113/DC1

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Journal of Cell Science

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human reproduction **ORIGINAL ARTICLE** *Reproductive genetics*

SYCP3 mutation may not be associated with recurrent miscarriage caused by aneuploidy

Eita Mizutani [1], Nobuhiro Suzumori [1,*], Yasuhiko Ozaki [1], Kumiko Oseto [1], Chisato Yamada-Namikawa [2], Makoto Nakanishi [2], and Mayumi Sugiura-Ogasawara [1]

[1]Department of Obstetrics & Gynecology, Nagoya City University Graduate School of Medicine, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan [2]Department of Biochemistry II, Nagoya City University Graduate School of Medicine, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

*Correspondence address. Tel: +81-52-853-8241; Fax: +81-52-842-2269; E-mail: og.n.suz@med.nagoya-cu.ac.jp

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BACKGROUND: *SYCP3* mutations have been shown to generate an aberrant synaptonemal complex in a dominant-negative manner and to contribute to abnormal chromosomal behavior that might lead to recurrent miscarriage. We examined whether *SYCP3* mutation is associated with recurrent miscarriage caused by embryonic aneuploidy.

METHODS: The *SYCP3* 657T>C mutation was examined using PCR and sequencing in 101 patients with a history of three or more unexplained recurrent miscarriages and 82 fertile controls with no history of miscarriage. The embryonic karyotype in the aborted conceptus was analyzed.

RESULTS: The 657T>C mutation of *SYCP3* was identified in one patient with a history of six recurrent miscarriages with embryonic euploidy and one fertile woman in the control group. Patients with abnormal and normal chromosome were found to repeat miscarriage with abnormal and normal chromosome, respectively.

CONCLUSIONS: The 657T>C mutation of *SYCP3* may not be associated with recurrent miscarriage caused by aneuploidy. We found no clinical significance of routine examination of the *SYCP3* mutation because only one benign mutation was ascertained in 101 patients.

Key words: SYCP3 / recurrent miscarriage / fetal chromosome / meiosis / polymorphism

Introduction

SYCP3 mutations in women were found to generate an aberrant synaptonemal complex in a dominant-negative manner and to contribute to abnormal chromosomal behavior that might lead to recurrent miscarriage (Bolor *et al.*, 2009). Bolor *et al.* (2009) found *SYCP3* mutations in 2 of 26 (7.7%) patients with recurrent miscarriage. SYCP3 is a DNA-binding protein and a structural component of the synaptonemal complex, which mediates the synapsis or homologous pairing of chromosomes during meiosis of the germ cells. Male mice homozygous for the null mutation of the *Sycp3* gene are sterile as a result of massive apoptotic cell death in the testis during meiotic prophase (Yuan *et al.*, 2000). *Sycp3*-deficient female mice are subfertile with a severely reduced oocyte pool. Although two-thirds of mouse offspring are healthy, one-third is affected by aneuploidy and succumbs during development *in utero* (Yuan *et al.*, 2002). This is consistent with the observations that in humans, a mutation in *SYCP3* was identified in two patients with azoospermia (Miyamoto *et al.*, 2003), and that the lack of *SYCP3* gene expression in human testis may have a negative effect on spermatogenesis and male fertility (Aarabi *et al.*, 2006).

The identifiable causes of recurrent miscarriage may include abnormal chromosomes in either partner (particularly translocations), antiphospholipid antibodies (aPL) and uterine anomalies (Farquharson *et al.*, 1984; Sugiura-Ogasawara *et al.*, 2004, 2010). A currently prevailing hypothesis is that recurrent miscarriage may be a polygenetic disorder associated with both genetic and environmental determinants. Polymorphisms related to thrombophilia, such as Leiden mutation and prothrombin mutation, are known to be associated with recurrent miscarriage, although the mutations are not found in the Asian population (Nelen *et al.*, 1996; Rey *et al.*, 2003; Rai and Regan, 2006; Suzumori and Sugiura-Ogasawara, 2010). However, whether Factor V Leiden and Factor II prothrombin polymorphisms are risk factors for recurrent miscarriage is controversial (Coulam *et al.*, 2006; Goodman *et al.*, 2006).

An abnormal embryonic karyotype causes not only sporadic spontaneous abortion but also recurrent miscarriage because it was found in 51% of recurrent cases (Ogasawara et al., 2000; Carp et al., 2001). Bolor et al. (2009) could not prove an association between the *SYCP3* mutations found in 7.7% of patients and embryonic aneuploidy.

Preimplantation genetic screening (PGS) for aneuploidy has been performed widely; however, there is no evidence of its ability to improve delivery rates (Platteau et al., 2005; The ACOG., 2009; Harper et al., 2010). The *SYCP3* mutation might be a candidate for selection of cases for PGS if an association between the mutation and aneuploidy is established. Also, the 7.7% frequency of *SYCP3* mutation is relatively high because the frequency of translocations, aPL and major uterine anomalies is 4.5% (Sugiura-Ogasawara et al., 2004), 10.7% (Balasch et al.,1990) and 3.2% (Sugiura-Ogasawara et al., 2010), respectively. Here we investigate whether *SYCP3* mutations may be associated with recurrent miscarriage caused by aneuploidy.

Materials and Methods

Patients

All patients underwent a systematic examination, including hysterosalpingography, chromosome analysis for both partners, determination of aPL, including lupus anticoagulant and β2glycoprotein I-dependent anticardiolipin antibodies (Ogasawara et al., 1996), and blood tests for hyperthyroidism, diabetes mellitus and hyperprolactinemia before subsequent pregnancy in Nagoya City University Hospital between 2007 and 2010. A blood sample was taken at the examination and frozen at −70°C before analysis. Patients with identifiable causes of miscarriage, such as translocations, aPL and uterine anomalies, were excluded. The 81 patients for whom a previous or subsequent embryonic (or fetal) karyotype was ascertained at least one time were studied. A further 20 patients for whom the embryonic karyotype was unknown were added.

In Japan, miscarriage is defined as loss within 22 weeks gestation and stillbirth is defined as loss at 22 or more weeks of gestation. Stillbirths after 22 weeks gestation were included in the present study and shown as prior history in Table I.

A total of 101 patients with a history of three or more (3–16) unexplained consecutive first-trimester miscarriages were examined. Subsequent pregnancies were followed up until October 2010. The mean age of participants at examination was 34.4 ± 3.8 years, and the average number of previous miscarriages was 3.8 ± 2.7. Twenty-four patients had a history of live birth and two patients experienced recurrent miscarriage after changing partner, having had a live birth by a previous partner. The mean number of previous live births was 0.27 ± 0.5.

Gestational age was calculated based on basal body temperature charts. Ultrasonography was performed once a week from 4 to 8 weeks of gestation. Dilation and curettage was carried out when miscarriages were diagnosed, and the karyotypes of aborted conceptuses were determined using a standard G-banding technique.

The 82 fertile women with no history of recurrent miscarriage and complications of pregnancy were examined as controls. The fertile controls were recruited in Nagoya City University Hospital and Asamoto Women's Clinic. The mean age of women in the control group was 32.3 ± 6.2 years, and the average number of deliveries was 1.53 ± 0.6.

The study was approved by the Research Ethics Committee of Nagoya City University Graduate School of Medical Sciences. Each patient provided their written consent after full disclosure about the purpose and methods to be employed.

DNA analysis

Genomic DNA was extracted from peripheral blood samples with the Midi Blood DNA Extraction kit (Qiagen, Tokyo, Japan). Oligonucleotide primers were designed to amplify each coding sequence, as well as exon–intron boundaries of the human *SYCP3* gene, encompassing exons 7–9 (GenBank accession number NM_153694). The sense and antisense PCR and sequence primers for *SYCP3* were, respectively, 5′-GATGGCGTG TGCCTATAATCCAAG-3′ and 5′-CGTCTTTATTTAATTGACAGTGT TAG-3′. Additional direct sequence primers were 5′-GTCAT GTTGCTCAGGCTGGTC-3′, 5′-TCTGTGGATTGATAATTATCTACT G-3′, 5′-TCCAATGCTCTGAGAACC-3′ and 5′-TCACCACAGC AAGTTGTG-3′. The coding exons 7–9 and exon–intron boundaries of human *SYCP3* gene were amplified by PCR and sequenced using the Big Dye Terminator v3.1 Cycle Sequencing kit (ABI Prism, Applied Biosystems, Foster City, CA, USA) on a 3100 automated sequencer.

Results

Heterozygous 657T>C mutation in exon 8 of *SYCP3* was ascertained in one of 101 patients who had had six recurrent miscarriage and in one of the 82 fertile controls (Fig. 1). The IVS7-16_19 delACTT previously reported in one patient with recurrent miscarriage or 643delA previously reported in two patients with azoospermia was not found in any patients with recurrent miscarriage or in fertile controls. No other new mutation was found in patients with recurrent miscarriage or controls.

Thirty-two patients experienced miscarriage with a normal embryonic (fetal) chromosome karyotype, and 47 patients presented an abnormal embryonic (fetal) karyotype (Table I). Two patients had miscarriages with both normal and abnormal karyotype.

Patient No. 77 with the 657T>C mutation was 31 years old and experienced a total of six miscarriages and no live birth. Available fetal karyotypes were shown as 46, XX and 46, XY. The control with the 657T>C mutation had a history of one live birth and no miscarriage.

Nine patients (No. 39–47) had repeated miscarriage with an abnormal karyotype, and seven patients (No. 75–81) had repeated miscarriage with a normal karyotype. Only 2 out of 18 (11.1%) patients had experienced miscarriages with both abnormal and normal embryonic karyotypes.

Discussion

In the present study, we found a heterozygous 657T>C mutation in exon 8 of the *SYCP3* gene in one patient and one fertile control. We could not find the IVS7-16_19 delACTT reported in one patient with recurrent miscarriage or 643delA reported in two patients with azoospermia in any patients with recurrent miscarriage or in fertile controls (Miyamoto et al., 2003; Bolor et al., 2009). No other new mutation was found in patients with recurrent miscarriage or controls.

Bolor et al. (2009) reported that 7.7% (2 of 26) patients with unexplained recurrent miscarriage were found to carry independent heterozygous nucleotide alterations, IVS7-16_19delACTT and 657T>C in *SYCP3*, neither of which was present among a control group of 150 fertile women. They also reported that analysis of transcripts from minigenes harboring each of these two mutations revealed that both mutations affected normal splicing, possibly resulting in the

Table I Previous miscarriage, live birth and subsequent pregnancy outcome with karyotype analysis ($n = 101$ patients).

Pt.	Prior history			Age (year)	Subsequent pregnancy			Karyotype	Cumulative live birth
	No. of P.M.	No of L.V.	Karyotype of previous miscarriage		Outcome		Karyotype of miscarriage		
1	2	2		37	f		47,XX,+16	A	y
2	3	0	#3 47,XY,+16	34	s	s		A	y
3	4	1		39	f		47,XX,+22	A	y
4	2	1		37	f	s	47,XX,+21	A	y
5	2	0		38	f	s	48,XX,+8,+22	A	y
6	3	0		28	f	s	47,XY,+16	A	y
7	3	0	#3 69,XXY	34	s			A	y
8	4	1	#3 47,XY,−13,+i(13)(p10),+i(13)(q10)[11]/46,XY,−13,+i(13)(q10)[19]	33	no			A	y
9	4	1	#2 47,XY,+18 stillbirth 32w	32	s			A	y
10	2	0		31	f	s	47,XX,+22	A	y
11	3	0	#3 45,X	42	s			A	y
12	4	1	#4 48,XY,+10,+13[12]/47,XY,+13[8]	35	no			A	y
13	3	0	#3 47,XX,+12	40	s			A	y
14	4	0	#4 46,XY,5cenh+,add(8)(p23)[7]/46,XY,5cenh+[13]	32	s			A	y
15	4	1	#3 47,XY,+7	38	s			A	y
16	4	1	#4 47,XY,+3	34	no			A	y
17	3	1	#3 47,XY,+16	36	s			A	y
18	4	0	46,XX,del(6)(q27)[12]/46,XX,add(6)(q27)[3]/46,XX,add(6)(q27)[2]/	37	s		46,XX,der(6)t(1;6)(q11;q27)[2]/46,XX,der(6)t(6;9)(q27;q12)[1]	A	y
19	2	0		33	f	s	48,XX,+15,+20	A	y
20	3	0	#3 45,XY, −21	30	f	s	ND*	A	y
21	3	1	#3 45,X	32	no			A	y
22	2	0	#2 47,XX,+22	32	f	s		A	y
23	3	1		40	f	s	48,XX,+14,+15	A	y
24	3	0	#3 47,XY,+16	31	s			A	y

Continued

Table I *Continued*

Pt.	Prior history			Age (year)	Subsequent pregnancy			Karyotype	Cumulative live birth
	No. of P.M.	No of L.V.	Karyotype of previous miscarriage		Outcome		Karyotype of miscarriage		
25	4	0	#3 47,+22	39	s			A	y
26	2	0		37	f		47,XY,?	A	n
27	3	0		34	f		47,XY,+16	A	n
28	2	0		39	f		47,XX,+16	A	n
29	2	0		32	f		47,XY,+16	A	n
30	5	0	#5 46,X,+16	27	f		Bio Misc	A	n
31	3	0		28	f		47,XX,+13	A	n
32	5	0		46	f		47,XX,+22	A	n
33	3	0	#3 47,XX,add(2)(q37),+20	42	no			a	n
34	4	0	#1 stillbirth 16w; #4 47,XY,+15	39	no			a	n
35	3	0		41	f		47,XX,+16	a	n
36	4	0		43	f		48,XY,+16,+21	a	n
37	4	0		38	f		46,X,+3	a	n
38	6	0	#1 stillbirth 33w; #4 45,X	37	no			a	n
39	7	0	#5 47,XX,+16; #7 45,X	30	s			aa	y
40	6	0	#6 47,XY,+16	32	f	s	47,XX,+13	aa	y
41	2	1	47,XY,+21***	30	f		47,XX,+5	aa	y
42	3	0	#3 47,XX,+16	29	f	s	47,XX,+3	aa	y
43	6	1	#6 47,XX,+16	41	f		47,XX,+12	aa	y
44	2	0	#2 46,XY,add(8)(p23)	33	f		47,XY,+16	aa	n
45	4	0	#2 45,X; #4 47,XX,+idic(8)(q?21.2)	35	no			aa	n
46	2	0		30	f	f	47,XX,+15;45,X	aa	n
47	2	0	#2 47,XX,+15	35	f		45,X	aa	n
48	14	2	#12 47,XX,+16 #14 46,XY, 2 children with previous husband	45	no			an	y
49	4	1	#3 47,XY,+16;#4 46,XX	30	f		46,XY	ann	y
50	4	0	#4 46,XY	35	s	s		n	y
51	4	0	#1 Stillbirth 28w; #4 46,XX	28	s		On-going preg.EDC06/27/11	n	y
52	4	0	#3 Stillbirth 18w	35	f	s	46,XY stillbirth 33w	n	y
53	3	0	#3 46,XY	33	s			n	y
54	5	1	#5 46,XY	36	s			n	y
55	4	1	#3 46,XY	36	no			n	y

56	3	0	#3 46,XX	30	s				n	y	
57	3	0	#3 46,XX	32	s				n	y	
58	2	0		34	f	s		46,XX	n	y	
59	3	1	#3 46,XX	35	no				n	y	
60	3	1	#3 46,XX	33	s				n	y	
61	4	0	#4 stillbirth 15w	37	f	s		46,XX stillbirth 13w	n	y	
62	3	0	#3 46,XX	33	s				n	y	
63	3	1	#3 46,XY	37	s				n	y	
64	3	0		26	f			46,XX	n	n	
65	3	0		34	f			Normal Karyotype	n	n	
66	5	0	46,XY	40	no				n	n	
67	2	0		32	f			46,XX	n	n	
68	3	0	#3 46,XY	35	f			NT**	n	n	
69	2	0		39	f			46,XX	n	n	
70	3	0	#3 46,XX	28	no				n	n	
71	2	0		36	f			46,XX	n	n	
72	2	0		30	f			46,XY	n	n	
73	3	0	#3 46,XX	34	no				n	n	
74	2	0		31	f			46,XY	n	n	
75	3	0	#2 46,XX; #3 46,XY	31	s				nn	y	
76	2	0	#2 46,XY	22	f	s		46,XY; On-going preg.EDC 05/27/11	nn	y	
77	5	0	#5 46,XY	31	f			46,XY	nn	n	
78	5	0	#3 46,XX; #4 46,XY; #5 46,XY	36	f	f	s	46,XX,t(11;19)(q21;q13.1) [4]/46,XX[26], Bio Misc	nnnn	y	
79	6	0	#3#,4#,6 46,XX,inv(9); #5 46,XY,inv(9)	31	s			On-going preg.EDC 02/23/11	nnnn	y	
80	9	0	#5 46,XX;#6 46,XX;#8 46,XY	38	f			46,XX	nnnn	n	
81	13	0	#4 46,XY; 46,XX; 46,XX; 46,XX	33	f	f	f	46,XX; 46,XX; 46,XX	Nnnnnnn	n	
82	3	0		31	s					y	
83	3	0		39	s					y	
84	3	0		26	s					y	
85	3	0		38	s					y	
86	23	0		33	s					y	
87	3	1		35	f			Bio Misc		y	
88	3	0		28	s					y	
89	3	0	#2 stillbirth 15w	30	s					y	
90	3	1		35	no					y	

Continued

Table I *Continued*

Pt.	Prior history			Age (year)	Subsequent pregnancy		Karyotype	Cumulative live birth
	No. of P.M.	No of L.V.	Karyotype of previous miscarriage		Outcome	Karyotype of miscarriage		
91	3	2	2 children with previous husband	34	s			y
92	3	1		38	s	On-going preg.EDC03/24/11		y
93	3	0		36	s			y
94	5	0		34	f	ND*		n
95	2	0		28	f	Bio Misc		n
96	4	0		41	no			n
97	4	0		42	no			n
98	3	0		33	no			n
99	4	0		31	no			n
100	3	0	#1 stillbirth 18w; #3 stillbirth 23w	32	no			n
101	4	0		37	f	Bio Misc		n
	3.782178	0.267327		34.347				
	2.681805	0.507762		4.4033				

*ND, not detected; **NT, not tested; ***Live birth, He is 8 years old; Pt., patient; P.M., previous miscarriage; L.V., Live Birth; Bio Misc, biochemical miscarriage, decreasing hCGs < 1500 mIU/ml; age, age at examination karyotype; a, aneuploidy; n, normal karyotype (euploidy) from Pt. 1–81, karyotype were knowed in prolr history or subsequent pregnancy; 'Outcome' reflect the conclusion of subsequent pregnancy; 's' means success in live birth delivery; 'f' means failure, miscarriage.

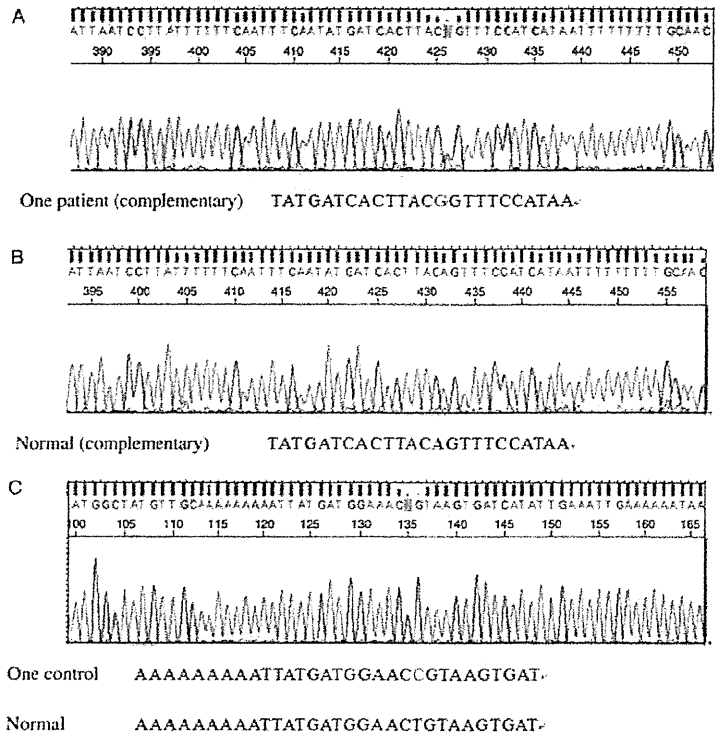

Figure 1 Nucleotide sequence results for the *SYCP3* gene in women with recurrent miscarriage and a control. (**A**) Heterozygous 657T>C mutation in exon 8 of the *SYCP3* gene of one patient with a history of six recurrent miscarriages. (**B**) Normal genotype in one patient with recurrent miscarriage. (**C**) Heterozygous 657T>C mutation in exon 8 of the *SYCP3* gene in one control with a history of one live birth and no miscarriages. From (A–C) sequences are all of the same region, and (A) and (B) sequences are complementary.

production of proteins that were mutated at the C-terminus. However, the effects of the *SYCP3* mutations on non-disjunction or the function of the synaptonemal complex have not been clear in mammals so far.

Our patient with 657T>C had repeated miscarriages with euploidy. It may be that the 657T>C mutation is a polymorphism without the specific function ascertained in the Bolor *et al.* (2009) study in humans. Further studies with larger numbers and a wide range of cases are needed to define whether the *SYCP3* mutations can be a cause of recurrent miscarriage.

Sycp3-deficient mice show complete meiotic arrest leading to male infertility (Yuan *et al.*, 2000). Miyamoto *et al.* (2003) identified in two azoospermia patients a 1 bp deletion of the *SYCP3* gene (643delA) that results in a premature stop codon and truncation of the C-terminal, coiled-coil-forming region of the SYCP3 protein. The mutant protein showed greatly reduced interaction with the wild-type protein *in vitro* (Miyamoto *et al.*, 2003). Reynolds *et al.* (2007) suggested that azoospermia associated with a decrease in the *DAZ* gene function in humans might, in part, be the consequence of failure at synapsis caused by reduced levels of the SYCP protein. However, no female patient with the 643delA mutation of SYCP3 has been reported. The infertile women might have the mutation because embryos with trisomy or monosomy except 45,X are frequently seen by PGD.

Our data showed that among the normal fetal karyotypes, XX and XY were found at a similar frequency, indicating that fetal rather than maternal karyotypes were obtained. In this study, about 90% of patients with abnormal and normal embryonic (fetal) karyotype tended to have repeat miscarriages with abnormal and normal karyotypes, respectively. The results suggest that unexplained recurrent miscarriage should be grouped as two types: one is miscarriage caused by abnormal embryonic karyotype and the other is 'real' unexplained recurrent miscarriage.

Moreover, the prognosis of a successful pregnancy for patients with an abnormal embryonic karyotype was better than for patients with a normal embryonic karyotype (Ogasawara *et al.*, 2000). No therapeutic approach to improve the rate of live birth could be found at this time (Kaandorp *et al.*, 2010). Thus, the gene associated with unexplained recurrent miscarriage with normal embryonic karyotype is more important (Suzumori and Sugiura-Ogasawara, 2010).

The results of our study suggest no clinical significance of routine screening for the presence of the *SYCP3* mutation in women with recurrent miscarriage because we detected only one benign mutation in 101 such patients. Future studies in mammalian animal models are likely to accelerate our understanding of the molecular mechanisms involved in recurrent miscarriage and will provide additional candidate genes to be screened in recurrent miscarriage patients and embryos with genetic factors.

Authors' roles

N.S., Y.O., M.N. and M.S.-O. were involved in conception and design; E.M., K.O. and C.Y-N. conducted data analysis; E.M., N.S. and M.S.-O. were involved in drafting the article. All authors agreed final approval of the version to be published.

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Forum Minireview

Novel Findings for the Development of Drug Therapy for Various Liver Diseases: Genetic Variation in *IL-28B* Is Associated With Response to the Therapy for Chronic Hepatitis C

Masaya Sugiyama[1,3,4], Yasuhito Tanaka[2,*], Makoto Nakanishi[1], and Masashi Mizokami[3]

[1]*Department of Biochemistry and Cell Biology,* [2]*Department of Virology & Liver Unit,*
Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho, Mizuho-ku, Nagoya 467-0001, Japan
[3]*The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine,*
1-7-1 Kohnodai, Ichikawa 272-8516, Japan
[4]*JSPS Research Fellow*

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Abstract. Hepatitis C infection is a global health problem. Spontaneous viral clearance was observed in approximately 30% of individuals with acute infection. In the therapy using a combination of pegylated interferon-α and ribavirin, approximately 50% of chronic hepatitis C patients infected with high viremia of hepatitis C virus infection (HCV) genotype 1 reached a sustained viral response. These findings were strongly expected to reflect variations of the host genome. To reveal genetic effects against viral clearance or treatment response, four independent groups applied a genome-wide association study (GWAS) to HCV infection. These groups almost simultaneously reported a strong association of interleukin (IL)-28B polymorphisms with viral clearance or final decision of HCV therapy. The discovered single nucleotide polymorphisms (SNPs) also revealed the enigma that the viral clearance rate was dependent on ethnic type. The significant SNPs are useful for prediction prior to treatment because of the strong association with clinical outcome. In addition, the unexpected results revealed by GWAS could promote the development of a novel drug related to IL-28B. Herein, we present current understanding in regard to the relationship between host variations and clinical outcome of hepatitis C.

Keywords: hepatitis C virus, genome-wide association study, interleukin-28, interferon-λ, single nucleotide polymorphism, liver disease

1. Introduction

Chronic infection with hepatitis C virus (HCV) presents a significant health problem worldwide with approximately 3% of the world population, that is, more than 170 million people. Only 20% – 30% of HCV-infected individuals recover spontaneously. The remaining 70% – 80% going on to develop chronic infection have a significant risk for progressive liver fibrosis and subsequent liver cirrhosis (LC) and hepatocellular carcinomas (HCC) (1). Successful treatment of chronic hepatitis C would reduce the morbidity and mortality of patients because around 8% of patients progressing to LC will develop HCC annually (2).

Spontaneous clearance following acute infection occurs in some cases for reasons that remain unclear, and previous studies report that 50% – 85% of patients progress to chronicity. The relationship between race and spontaneous viral clearance following acute infection have been reported (3 – 6). These characteristics based on ethnic types would suggest the effect of a host genetic factor on HCV infection.

*Corresponding author. ytanaka@med.nagoya-cu.ac.jp
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2. HCV treatment and the response

The current standard of care for the HCV infections comprises pegylated interferon (PEG-IFN)-α 2a or 2b plus ribavirin (RBV). Successful treatment, termed "sustained virological response (SVR)", was defined by an HCV RNA negative after 6 months of completing therapy, whereas a transient viral response (TVR) was defined as a reappearance of HCV RNA in serum after treatment was discontinued in a patient who had undetectable HCV RNA during the therapy or on completion of the therapy (Fig. 1). A non-viral response (NVR) was defined as cases with detectable viremia after and during treatment. The standard therapy is effective in only 42% – 52% of patients with HCV genotype 1 in the US and Europe (7 – 9). A significant difference in response to PEG-IFN&RBV therapy between ethnicities were reported: the SVR achievement of African Americans was only approximately 20% – 28% compared to 40% – 52% in Caucasian patients with genotype 1 infection (10 – 12) and 57% vs. 82% for genotype 2/3 (13). The current therapies are limited by expensive, ineffectiveness in part of the patients, and numerous potentially severe side effects, which cause dose reduction and/or premature termination of treatment. Additionally, premature withdrawal from IFN-based therapy (14) was necessary for 10% – 14% of the patients, leading to failure of the HCV therapy.

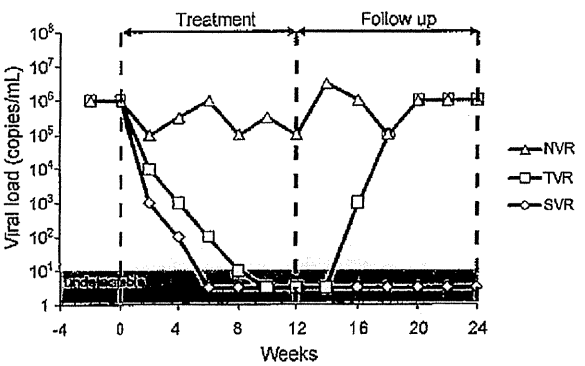

Fig. 1. Representative changes of HCV viral load in patients treated with PEG-IFN&RBV combination therapy. The response type under the therapy of PEG-IFN&RBV is divided into three groups: SVR, TVR, or NVR. SVR is defined as successful treatment, which is HCV RNA negative after 6 months of completing therapy. TVR is defined as a transiently negative for HCV RNA during treatment. However, after the end of therapy, HCV RNA of the patients reappears with impaired liver function in TVR. NVR is defined as a constitutive high viremia during and after treatment. SVR, sustained viral response; TVR, transient viral response; NVR, non-viral response

3. Viral factors associated with HCV therapy

To prevent treatment failure in such patients, we must identify the predictive factors leading to treatment failure as well as production of severe side effects in the clinic. Previous studies have reported that viral titer, mutations, or gene expression levels of innate immunity could be prediction factors for NVR using clinical specimens of chronic hepatitis C patients. Several viral factors such as genotype 1 (HCV-1), high baseline viral load, viral kinetics during treatment, and amino acid pattern in the IFN sensitivity–determining region have been found to be significantly associated with the outcome by a number of independent studies (15 – 17). Accumulated data have provided strong evidence that approximately 20% of patients with HCV genotype 1 have NVR to PEG-IFN&RBV. The reliable prediction for NVR would allow avoidance of side effects and reduce the cost of treatment in the 20% of patients with HCV-1 before starting the treatment.

4. Host factors associated with response to PEG-IFN&RBV therapy

Several host factors related to viral clearance have been reported based on clinical features or laboratory data, for example, gender, age <40 years, low HCV RNA level prior to treatment, lack of liver cirrhosis, and HCV genotypes 2/3 (18, 19). As for host genetic factor, candidate gene approaches have been adopted to identify host factors related to clinical outcomes, single nucleotides polymorphisms (SNPs), copy number variation (CNV), or insertion/deletion of genes. The approach could latently find weak associations and show significant differences because only one or a limited number of SNPs or gene loci are detected in candidate genes. The focused approach, however, contains the restraint to detect crucial factors. In detail, the selection of candidate regions for genetic study depends on the researcher's knowledge or the present data of the gene pathway.

In contrast, a recent genome-wide association study (GWAS) approach using high-throughput genotyping technology usually for SNPs, ranging from 300,000 to 900,000 SNPs in each sample, is able to detect strong association factors affecting disease susceptibility and drug response without any a-priori hypotheses on causative SNPs apart from the hypotheses (20, 21). On the basis of the GWAS, four independent groups assessed the role of genetic variation on response to PEG-IFN&RBV combination therapy for chronic hepatitis C patients, and the data was reported in a short-term (21 – 24). In all cases, the conclusive finding was that polymorphisms in or near the *IL-28B* gene strongly de-

termined the outcome of HCV therapy.

5. Study design of four studies for GWAS

Ge et al. and Suppiah et al. studied genetic variants associated with SVR to PEG-IFN&RBV therapy in individuals infected with HCV genotype 1 (21, 22). The former examined genetic factors associated with treatment response in patients from the IDEAL trial (Individualized Dosing Efficacy vs. flat dosing to Assess optimaL pegylated interferon therapy) (25), a large randomized controlled trial involving Caucasian, American-African, and Hispanic individuals in North America (n = 1137) (Table 1). The latter study group analyzed Caucasians consisting of 293 Australian individuals (Northern European ancestry) with HCV genotype 1 and also validates an independent replication cohort consisting of 555 Europeans from the UK, Germany, Italy, and Australia. These two study groups mainly investigated GWAS in Caucasians and analyzed host factors associated with SVR.

Tanaka et al. studied host factors associated with the response to PEG-IFN&RBV treatment in 142 Japanese patients with chronic hepatitis C of HCV genotype 1 for GWAS and prepared an independent replication cohort of 172 Japanese (Table 1) (24). In this study, patients were divided into three groups, SVR, TVR, or NVR. NVR vs. virological responder (VR) consisting of SVR and TVR was used for the predication of NVR factors. The data set of SVR vs. non-SVR (TVR and NVR) was constructed to discover the host factor related to SVR (Fig. 1).

Rauch et al. investigated 465 Caucasians infected with HCV genotypes 1, 2, 3, or 4 to reveal genetic variations associated with response to the combination therapy (23). A case control study was designed to detect genetic variations related to SVR in European individuals. Three study groups, except Suppiah et al., selected patients receiving at least 80% of the recommended treatment dose to emphasize genetic associations.

6. Identification of strongly significant SNPs associated with PEG-IFN&RBV therapy

Ge et al. identified a genetic polymorphism (rs12979860) near the *IL-28B* gene on chromosome 19, also known as IFN-λ3 (Fig. 2). Individuals with the CC genotype showed the association with an approximately two-fold change in response to PEG-IFN&RBV treatment compared with those with the TT genotype, both among patients of European ancestry ($P = 1.06 \times 10^{-25}$) and African-Americans ($P = 2.06 \times 10^{-3}$). An important finding in the study is the strong correlation between being a carrier of this SNP and SVR rates in diverse ethnic groups, which is significantly more frequent in European-Americans and Asian populations than in African-Americans. Approximately 23% – 55% of Africans (<40% of African-Americans) carry advantageous C-allele frequency of rs12979860, compared with approximately 53% – 85% of Europeans (<70% of European-

Table 1. Four GWAS groups studying host factor related to the response to HCV therapy

Study (Ref. No.)	Ge et al. (22)	Suppiah et al. (21)	Tanaka et al. (24)	Rauch et al. (23)
Region	Northern America	Northern Europe, Australia	Japan	Switzerland
Ancestry	Caucasian/ African/ Hispanic	Caucasian	Japanese	Caucasian
GWAS size	871/ 191/ 75	293	142	465
Replication	No replication	555	172	No replication
Case/ control	SVR vs. non-SVR	SVR vs. non-SVR	SVR vs. non-SVR SVR&TVR vs. NVR	SVR vs. non-SVR
Adherence	Over 80% adherent to PEG-IFN&RBV during the first 12 weeks of therapy	Not controlled	Over 80% adherent to PEG-IFN&RBV during the first 12 weeks of therapy	Over 80% adherent to PEG-IFN&RBV during the first 12 weeks of therapy
HCV genotype	1	1	1	1,2,3,4
Significant SNPs	rs12979860	rs8099917	rs8099917	rs8099917
P value	1.37×10^{-8}	9.25×10^{-7}	1.18×10^{-7}	3.10×10^{-8}
OR (95% CI)	3.1 (2.1 – 4.7)	1.98 (1.57 – 2.52)	12.1 (6.5 – 22.4)*	5.19 (2.9 – 9.3)
Platform	Illumina 610-quad	IlluminaCNV370-quad	Affymetrix SNP 6.0	Illumina Human 1M-duo, Human Hap550, Human610W-quad

*The combined value in the study in comparison with SVR vs. non-SVR.

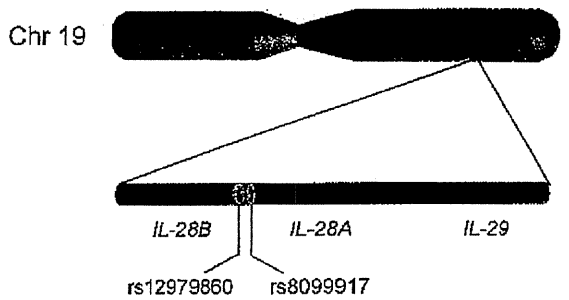

Fig. 2. *IL-28B* gene and SNPs location in chromosome 19. Four independent GWAS discovered SNPs strongly associated with the response to PEG-IFN&RBV therapy around *IL-28B* in chromosome 19. Ge et al. (22) reported rs12979860 as a strongly associated SNP, whereas Suppiah et al. (21), Tanaka et al. (24), and Rauch et al. (23) detected a statistical difference on rs8099917. These 2 SNPs are in strong linkage disequilibrium because these loci are very near to each other. The interferon lambda family consists of *IL-29* (IFN-λ1), *IL-28A* (IFN-λ2), and *IL-28B* (IFN-λ3), which are induced by type I IFN, or bacterial and viral infection.

Americans) and approximately 90% of Chinese and Japanese. Ge et al. showed that the SVR rates across different population groups displayed a striking concordance with the C-allele frequency. This SNP explained about half of the difference in response rates between African-Americans and Europeans.

Suppiah et al. and Tanaka et al. revealed the strong association of particular haplotypes of SNPs around *IL-28B* in the population of PEG-IFN&RBV therapy. The most significant SNPs in both study groups was rs8099917 (8 kb upstream of *IL-28B*) associated with SVR in European and Japanese patients (Fig. 2). Suppiah et al. also identified the association of rs8099917 in European ancestry with HCV genotype 1 based on the determination of SVR factors (combined $P = 9.25 \times 10^{-9}$, OR = 1.98, 95% CI = 1.57 – 2.52) (21). Homozygotes for the risk allele (rs8099917 G-allele) showed 2-fold higher risk of treatment failure than that of major allele homozygotes. In the gene expression assay, the minor allele of rs8099917 tended to suppress mRNA levels of *IL-28A/B*.

Tanaka et al. identified several SNPs significantly associated with NVR to PEG-IFN&RBV therapy in the GWAS and the replication study. All significant SNPs were located near the *IL-28B* locus on chromosome 19. The SNPs, rs12980275 or rs8099917, validated in an independent replication cohort showed the strongest association (combined $P = 2.84 \times 10^{-27}$ and 2.68×10^{-32}; OR = 17.7, 95% CI = 10.0 – 31.3; OR = 27.1, 95% CI = 14.6 – 50.3, respectively) (24). Interestingly, the minor alleles of the SNPs were accumulated in NVR (minor allele frequency of NVR = 74.3% for rs12980275 and 75.0% for rs8099917). Multivariate analyses containing genetic and clinical factors revealed that rs8099917 was the strongest predictor for response to therapy ($P = 0.0001$, OR = 37.68, 95% CI = 16.71 – 83.85).

The fourth GWAS was published on the response to HCV therapy, Rauch et al. studied patients infected with HCV genotype 1, 2, 3, or 4 (23). Rauch et al. also identified several SNPs around the *IL-28B* gene on chromosome 19 (Fig. 2). The strongest association with treatment failure was found with rs8099917 ($P = 3.11 \times 10^{-8}$, OR = 5.19). Interestingly, rs8099917 did not associate with the response to PEG-IFN&RBV therapy in genotype 2 or 3 patients. The contribution of host factors to genotype 2 or 3 clearance would be low because HCV genotype 2 or 3 is likely to be eliminated by the standard therapy compared with genotype 1. In individuals infected with HCV genotypes 1 and 4, the SVR rate of the patients harboring the minor allele was 28%, whereas that of the major allele homozygotes reached 63%. However, patients infected with genotypes 2 or 3 showed high viral response rate, approximately 80%, without statistical significance between the patients and the control.

7. The influence of genetic background on the statistical analysis

For the prediction of SVR, OR of the Japanese population was much higher than that of the other populations (Table 1). Individuals harboring the risk allele of rs8099917 or rs12979860 was approximately 10% in Asia, whereas the risk allele frequency was generally over 20% in European Caucasians. Moreover, individuals with the risk allele were the major population in individuals with African ancestry. The differences of allele frequency might explain, in part, the observed discrepancy in the response rate of viral clearance and the statistical power between racial groups.

Tanaka et al. extracted the data of TVR patients to analyze the genetic background. The minor allele frequency (MAF) of the strongly associated SNPs (rs8099917, located in the intergenic region between *IL-28A* and *IL-28B*) in TVR was similar to that of the SVR population (Fig. 3) (24). The statistical analysis for SVR prediction (SVR vs. non-SVR) using the SNPs showed lower statistical power (OR = 12.1) than that of NVR prediction (NVR vs. SVR plus TVR, OR = 27.1), indicating that the significant SNPs are strongly associated with the outcome of NVR. In other words, TVR patients share similar genetic background with SVR patients, and they would achieve SVR by prolonged therapy or PEG-IFN&RBV plus protease inhibitor.

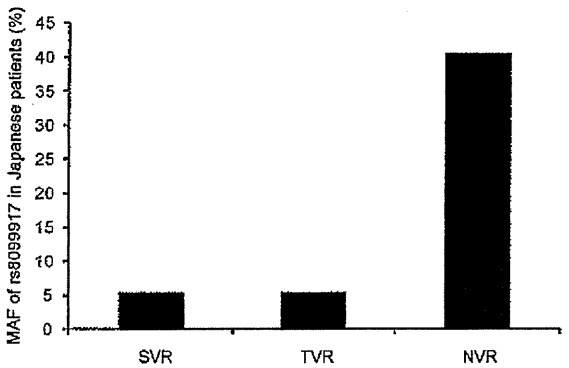

Fig. 3. Minor allele frequencies (MAF) of rs8099917 in each response type of chronic hepatitis C patients reported by Tanaka et al. (24). In the Asian population, the MAF of rs8099917 is approximately 10% according to Thomas et al. (20) and the dbSNPs international database. The MAF of chronic hepatitis C patients under a combination therapy of PEG-IFN&RBV revealed the deviation from that of the general population. The minor allele of rs8099917 accumulated in the population of NVR (approximately 40%), whereas those of SVR and TVR were occurred at lower frequency than those of the general population (approximately 5%).

8. SNPs associated with spontaneous clearance of HCV

Two study groups searched for common SNPs related to spontaneous elimination of HCV using a Caucasian cohort. Thomas et al. performed a candidate gene study on the rs12979860 SNP reported by Ge et al. to determine whether the SNP was also associated with spontaneous clearance of HCV infection (20). This study included 388 individuals with spontaneous HCV clearance and 620 with persistent HCV infection in a cohort consisting of HCV and HIV/HCV co-infected patients. The strong association of rs12979860 with spontaneous recovery was found in European and African American individuals (OR = 2.6, 95% CI = 1.9 – 3.8; OR = 3.1, 95% CI = 1.7 – 5.8, respectively) (Table 2). The association was also independent of co-infection with HIV, type of HCV transmission, and history of HBV infection.

Rauch et al. revealed the host factor associated with spontaneous clearance of HCV based on GWAS technology mounting more than 500K SNPs (23). The case-control study was designed for 347 individuals with spontaneous HCV clearance, 567 individuals with chronic hepatitis C, and 448 individuals with HCV/HIV co-infection. The significant SNP was also rs8099917 (combined $P = 6.07 \times 10^{-9}$, OR = 2.31, 95% CI = 1.74 – 3.04). The effect of HIV co-infection was also similar to that of HCV mono-infection ($P = 8.25 \times 10^{-5}$, OR = 2.16, 95% CI = 1.47 – 3.18; $P = 1.96 \times 10^{-5}$, OR = 2.49, 95% CI = 1.64 – 3.79, respectively) compared to Thomas et al. Note that rs8099917 was in strong linkage disequilibrium with rs12979860 in European and Asian individuals (26). These reports described by Thomas et al. and Rauch et al. seem to lead crucially identical results.

9. The characteristics of *IL-28B* and the IFN-λ family

IL-28B, referred to as IFN-λ3, belongs to the IFN-λ family, which consists of *IL-29*/IFN-λ1, *IL-28A*/IFN-λ2, and *IL-28B*. The *IL-28B* gene has been recently discovered and classified into type III IFN, a member of the class II cytokine family. This class II family includes type I, II, and III IFN and the *IL-10* family (*IL-10*, *IL-19*, *IL-20*, *IL-22*, *IL-24*, *IL-26*, *IL-28*, and *IL-29*). Peripheral blood mononuclear cells (PBMCs) and dendritic cells are main sources of IFN-λ (27, 28), which is induced by IFN-α, viral infection, and/or stimulations of toll-like receptors. IFN-λ behave as a interferon stimulated gene (ISG) of IFN-α, which is expressed at low levels by a broad variety of human cells, similar to IFN-α (29).

The signal pathway of IFN-λ is initiated through a membrane receptor distinct from that of type I IFN. The receptor is composed of heterodimer molecules consisting of an IL-28RA/IFN-λR1 subunit and IL-10R2 subunit (27, 28). The IL-10R2 subunit is expressed broadly and shared by IL-10, IL-22, IL-26, and IFN-λ. Compared with the IFN-α /-β receptor, which is ubiquitously expressed, the IL-28RA receptor has a more restricted distribution. The signal transduction of IFN-λ receptor is mediated via Jak1 and Tyk2, which can induce the phos-

Table 2. Summary of associated SNPs regarding spontaneous clearance of HCV

Study (Ref. No.)	Thomas et al. (20)		Rauch et al. (23)		
SNPs	rs12979860		rs8099917		
Population	European	African	HCV mono-infection	HCV/HIV co-infection	Combined
P value	1.0×10^{-7}	1.0×10^{-4}	1.96×10^{-5}	8.25×10^{-5}	6.07×10^{-9}
OR	2.6	3.1	2.49	2.16	2.31
95% CI	1.85 – 3.84	1.75 – 5.88	1.64 – 3.79	1.47 – 3.18	1.74 – 3.04

phorylation of STAT1 and STAT2 molecules and is followed by the expression of ISG (30).

10. The antiviral effect of IFN-λ against HCV in basic studies or clinical trials

Antiviral effects of IFN-λs against HCV have been reported before the discovery of the association with the response to HCV therapy. The treatment of IFN-α, or IFN-λ1 inhibited HCV replication at similar levels at low concentrations (31). The combination treatment of IFN-α and *IL-29/28A* enhanced the antiviral effect against HCV replicon synergistically (32). In microarray analysis on ISG induction of IFN-α/β or IFN-λ1, IFN-λ1 showed a unique pattern of ISG expression compared to that of IFN-α/β (31). For example, a total of 19 genes, which were not detected in the IFN-α–treated cells, were specifically up-regulated by IFN-λ1 at the late phase of treatment, indicating the signal pathway downstream of IL-28R1 could differ from that of IFN-α and possess a important biological function, although the pattern of signal transduction currently thought to be similar to that of IFN-α R1/2 (33). Further studies are needed to elucidate the biological consequences of these differences.

As described above, HCV replication is inhibited by the antiviral effects of IFN-λ. IFN-λ might have potential as a therapeutic agent against chronic hepatitis C in patients. A pegylated IFN-λ1 has already been tried against chronic hepatitis C in phase 1B trials (34). Interestingly, sufficient antiviral effects were observed but not severe side effects. The expression pattern of the IFN-λ receptor is restricted in specific organs. The high expression of the receptor was observed in the pancreas, liver, prostate, or thyroid, whereas the central nerve system (the bone marrow or the brain) showed the low expression (27, 28). These results could explain the avoidance of severe toxicity induced by IFN-α/β.

11. Conclusions

The recent discovery revealed by GWAS technology provides the unexpected role of *IL-28B* in HCV infection. The findings could be strong evidence to enhance the development of a novel therapeutic strategy and basic studies on IFN-λs. The SNPs around the *IL-28B* gene could improve the diagnostics for the prediction of spontaneous clearance and the response to anti-HCV treatment. However, approximately 20% – 30% of the total homozygotes with the risk alleles in Caucasians and 20% of heterozygotes/homozygotes with risk alleles in the Japanese population achieved a SVR and vice versa (21 – 24), indicating that the response to a combination therapy is not inevitably restricted because of genetic factors. To improve the prediction rate, especially, host epigenetic, rare SNPs, mutations, or viral factors are eligible candidates to consider when trying to establish an adequate tailor-made therapy. Although the strongly associated SNPs may have a big impact on the type of therapy and outcome, this is the first step in the tailor-made therapy for HCV infection. Further functional studies of IFN-λs and the significant SNPs should be investigated to improve the positive predictive value using the point mutation analysis of the targeted polymorphisms (35). For applying a practical tailor-made therapy, it is also necessary to reveal the cause of exceptional cases that do not follow the *IL-28B* genotyping.

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