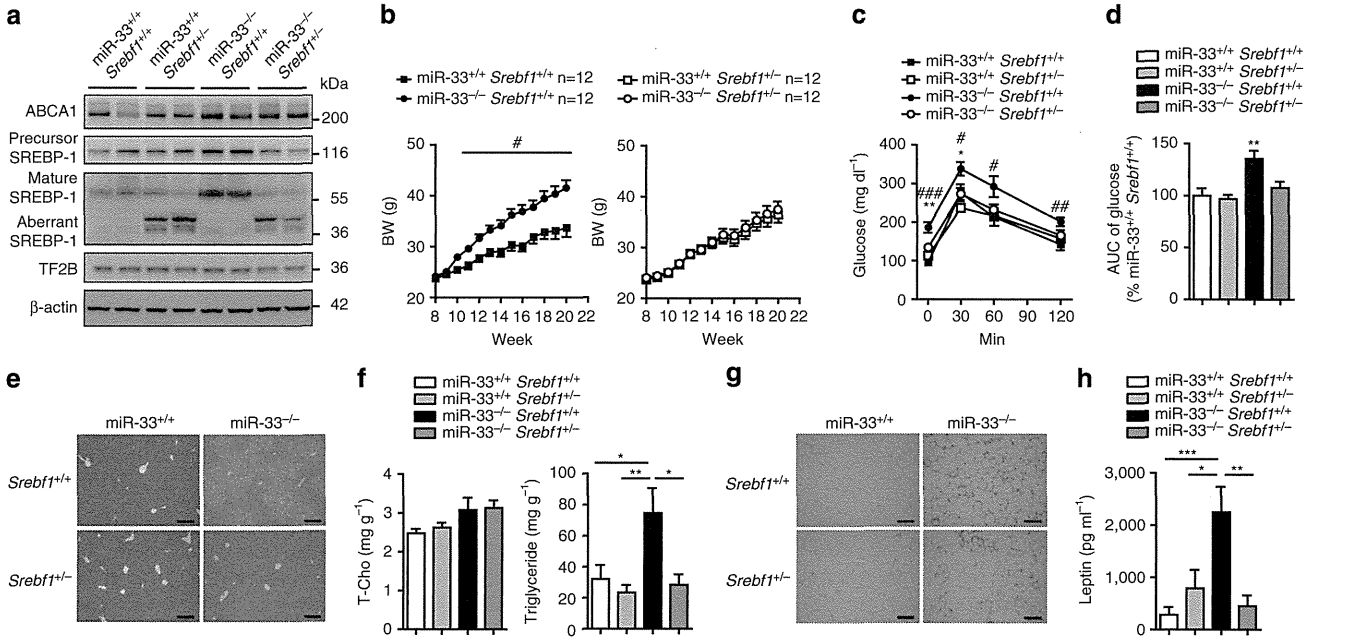

Figure 7 | Reversal of hepatic steatosis by the reduction of SREBP-1 levels. (**a**) Western blotting analysis of SREBP-1 and ABCA1 levels in the livers of miR-33$^{+/+}$*Srebf1*$^{+/+}$, miR-33$^{+/+}$*Srebf1*$^{+/-}$, miR-33$^{-/-}$*Srebf1*$^{+/+}$ and miR-33$^{-/-}$ *Srebf1*$^{+/-}$ mice. Representative western blot images are shown ($n=4$). (**b**) Serial changes in BW levels of miR-33$^{+/+}$*Srebf1*$^{+/+}$, miR-33$^{+/+}$*Srebf1*$^{+/-}$, miR-33$^{-/-}$*Srebf1*$^{+/+}$ and miR-33$^{-/-}$ *Srebf1*$^{+/-}$ mice fed HFD under pair-feeding condition ($n=12$ each). (**c**) Serial changes in glucose levels after intraperitoneal injection of glucose into miR-33$^{+/+}$ *Srebf1*$^{+/+}$, miR-33$^{+/+}$*Srebf1*$^{+/-}$, miR-33$^{-/-}$*Srebf1*$^{+/+}$ and miR-33$^{-/-}$ *Srebf1*$^{+/-}$ mice ($n=11$–12 each). $^{*}P<0.05$ and $^{**}P<0.01$ versus miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ versus miR-33$^{+/+}$*Srebf1*$^{+/+}$ mice in one-way analysis of variance test. (**d**) AUC of glucose levels in glucose tolerance tests in miR-33$^{+/+}$*Srebf1*$^{+/+}$, miR-33$^{+/+}$*Srebf1*$^{+/-}$, miR-33$^{-/-}$*Srebf1*$^{+/+}$ and miR-33$^{-/-}$ *Srebf1*$^{+/-}$ mice ($n=11$–12 each). $^{**}P<0.01$ versus miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice in one-way analysis of variance test. (**e**) Representative microscopic images of the liver of miR-33$^{+/+}$*Srebf1*$^{+/+}$, miR-33$^{+/+}$*Srebf1*$^{+/-}$, miR-33$^{-/-}$*Srebf1*$^{+/+}$ and miR-33$^{-/-}$ *Srebf1*$^{+/-}$ mice fed HFD. Scale bars, 200 μm. (**f**) Cholesterol and triglyceride levels in the liver of miR-33$^{+/+}$*Srebf1*$^{+/+}$, miR-33$^{+/+}$*Srebf1*$^{+/-}$, miR-33$^{-/-}$*Srebf1*$^{+/+}$ and miR-33$^{-/-}$ *Srebf1*$^{+/-}$ mice fed HFD. $^{*}P<0.05$ and $^{**}P<0.01$ in one-way analysis of variance test. (**g**) Representative microscopic images of the adipose tissue of miR-33$^{+/+}$ *Srebf1*$^{+/+}$, miR-33$^{+/+}$*Srebf1*$^{+/-}$, miR-33$^{-/-}$*Srebf1*$^{+/+}$ and miR-33$^{-/-}$ *Srebf1*$^{+/-}$ mice fed HFD. Scale bars, 200 μm. (**h**) Serum leptin levels of miR-33$^{+/+}$*Srebf1*$^{+/+}$, miR-33$^{+/+}$*Srebf1*$^{+/-}$, miR-33$^{-/-}$*Srebf1*$^{+/+}$ and miR-33$^{-/-}$ *Srebf1*$^{+/-}$ mice fed HFD. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ in one-way analysis of variance test. Values are the means ± s.e.m.

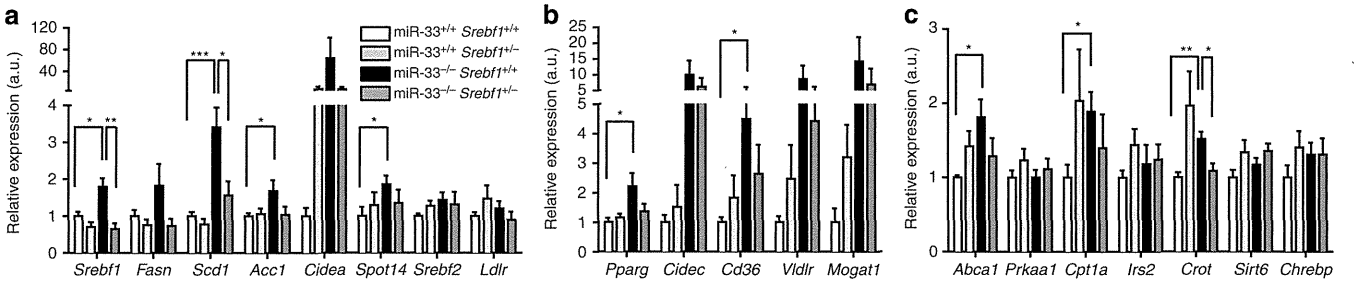

Figure 8 | Relative mRNA expression levels of lipid metabolism-related genes. (**a**) *Srebf*s and lipogenic genes. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ in one-way analysis of variance test ($n=6$–8 each). (**b**) *Pparg* and its downstream genes. $^{*}P<0.05$ in one-way analysis of variance test ($n=6$–8 each). (**c**) Other lipid metabolism-related genes. $^{*}P<0.05$ and $^{**}P<0.01$ in one-way analysis of variance test ($n=6$–8 each). Relative values of lipid metabolism-related genes in the livers of miR-33$^{+/+}$*Srebf1*$^{+/+}$, miR-33$^{+/+}$*Srebf1*$^{+/-}$, miR-33$^{-/-}$*Srebf1*$^{+/+}$ and miR-33$^{-/-}$ *Srebf1*$^{+/-}$ mice fed with HFD. Values are the means ± s.e.m.

(Supplementary Fig. S9a). These results indicate a previously unrecognized relationship between SREBP-1 and SREBP-2 through miR-33 (Supplementary Fig. S9b left).

Until now, there has been little evidence for an interaction between SREBP-1 and SREBP-2. It is known that in cholesterol-rich dietary conditions, SREBP-2 is downregulated at the cleavage level and SREBP-1c is transcriptionally activated through the activation of liver X receptors (LXRs) by the binding of oxysterols[16–19]. However, in sterol-depleted conditions, SREBP-2 is cleaved in the Golgi and the active N-terminal region translocates to the nucleus. Reduction in oxysterol levels leads to the inactivation of liver X receptors, which results in a decrease in SREBP-1c mRNA levels. Recently, it was shown that statin treatment induced hepatic miR-33 expression and at the same time decreased mRNA levels of miR-33 targets, including *Abca1* (ref. 8), *Abcb11* and *Atp8b1* (ref. 20) in mice. Thus, not

only the activation by proteolytic cleavage but also its transcriptional regulation of SREBP-2/miR-33 is important *in vivo*. Our data showed that miR-33 targeted the 3′UTR of *Srebf1* and the upregulation of miR-33 by cholesterol depletion, considerably affecting the reduction of SREBP-1 expression. Therefore, based upon our findings that miR-33 regulates SREBP-1, miR-33 in the intron of *SREBP-2* may amplify reduction in SREBP-1 levels in sterol-depleted conditions (Supplementary Fig. S9b centre). SREBP-1c activates transcription of genes involved in fatty acid and triglyceride synthesis, such as genes encoding acetyl-CoA carboxylase, fatty acid synthase and elovl-6 and stearoyl-CoA desaturase. Therefore, it is possible that in sterol-depleted conditions, acetyl-CoA is preferred as a substrate for cholesterol production and not for fatty acid production, through the upregulation of miR-33. On the other hand, in cholesterol-rich conditions, miR-33 levels decrease[7] and its negative regulation of SREBP-1 may be reduced. Thus, in this situation, acetyl-CoA is preferred as a substrate for fatty acid production (Supplementary Fig. S9b right).

Previously, several different SREBP-1 transgenic (TG) mice have been produced. Liver-specific transgenic mice using a phosphoenolpyruvate carboxykinase (PEPCK) promoter indicated that the livers of the SREBP-1a TG mice (PEPCK-SREBP-1a) were massively enlarged, owing to the accumulation of triglycerides and cholesterol[6]. PEPCK-SREBP-1c TG livers were only slightly enlarged with a moderate increase in triglycerides but not cholesterol. It is interesting that epididymal fat weight was not increased in these mice[6]. However, other liver-specific TG mice lines overexpressing human SREBP-1a and SREBP-1c under the control of the albumin promoter showed a vast accumulation of lipids in the liver and obesity as observed in miR-33$^{-/-}$ mice[21,22]. Therefore, overexpression of SREBP-1 does not show consistent phenotypes and these changes may depend on the different promoters and expression patterns in organs of each transgenic line. Because SREBP-2 and miR-33 are expressed ubiquitously, miR-33$^{-/-}$ mice may have mildly enhanced expression of SREBP-1 throughout the body. Previously developed SREBP-1 TG mice do not resemble miR-33-deficient mice in this context. Thus, obesity and hepatic steatosis developed slowly when fed NC and became prominent when fed HFD. Although SREBP-1c-deficient mice do not show any change in BW, it is possible that some compensatory mechanisms may have occurred in these mice[23]. Because miR-33$^{-/-}$ mice showed a slight but significant increase in food intake when fed HFD, we conducted our crossbreeding experiments in pair-feeding conditions, which may have enabled us to clearly observe the changes. It is interesting that *Ldlr*$^{-/-}$ mice that received anti-miR-33 oligonucleotides once a week for 14 weeks gained weight although the change was not statistically significant[24,25]. Therefore, reduction of miR-33 levels is related to unwanted consequences such as elevated BW. It should also be noted that the expression of *Pparg* and its downstream signalling molecules were enhanced in miR-33$^{-/-}$*Srebf1*$^{+/+}$ mice compared with WT control mice[26–29]. Because these molecules are attenuated in miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice, enhanced expression of *Pparg* may be related to the increase in SREBP-1 levels associated with miR-33 deficiency[30,31].

The phenotype of miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice is not completely the same as that of WT mice, and this may be because of other miR-33 target genes. *Abca1* mRNA expression and serum HDL-C levels still tend to be higher in miR-33$^{-/-}$ *Srebf1*$^{+/-}$ mice than those in miR-33$^{+/+}$ *Srebf1*$^{+/+}$ mice. Moreover, RIP140, another miR-33 target[12,32], promotes the activity of NF-κB and upregulates the expression of genes implicated in inflammation such as TNFα and IL-6 in macrophages. Enhanced expression of RIP140 by miR-33 deficiency may affect the inflammatory conditions in adipose tissue.

It is important to explain why did not compare *Srebf1*$^{+/+}$ with *Srebf1*$^{+/-}$ mice, but only compared *miR-33*$^{-/-}$*Srebf1*$^{+/+}$ and *miR-33*$^{-/-}$*Srebf1*$^{+/-}$ in our experiments. The feedback system of SREBP-2 guarantees appropriate levels of cellular cholesterol. Meanwhile, excess glucose cumulatively activates SREBP-1 and increases triglyceride storage. The latter can be achieved by the fact that *Srebf1* expression is enhanced by SREBP-1 itself and that cleavage of SREBP-1 is less sensitive to sterol-suppression than SREBP-2 (refs 33–36). Therefore, we hypothesize that the differences in the expression of SREBP-1 and its downstream molecules between *Srebf1*$^{+/+}$ and *Srebf1*$^{+/-}$ would be enhanced when SREBP-1 levels are increased by intercrossing with miR-33$^{-/-}$ mice. The *Srebf1* levels are reduced in *Lep*$^{ob/ob}$ × *Srebf1*$^{+/-}$ mice compared with *Lep*$^{ob/ob}$ mice[37]. However, in *Lep*$^{ob/ob}$ mice, the *Srebf1* levels are not considerably enhanced in the liver, but are rather decreased in adipose tissue and the phenotype is different between *Lep*$^{ob/ob}$ and miR-33$^{-/-}$ mice. Because *Srebf1* may be more enhanced in miR-33$^{-/-}$ mice than *Lep*$^{ob/ob}$ mice, intercrossing with *Srebf1*$^{+/-}$ had more effect in miR-33$^{-/-}$ mice than *Lep*$^{ob/ob}$ mice. Similarly, phenotypic difference between *Srebf1*$^{+/+}$ and *Srebf1*$^{+/-}$ mice with WT background may become small because *Srebf1* is not enhanced. Although a threshold may exist for the SREBP-1 levels that distinguishes between the normal condition and lipotoxicity caused by positive energy imbalance, the threshold level of SREBP-1 can only be achieved by feeding the *miR-33*$^{-/-}$ *Srebf1*$^{+/+}$ mice HFD or at an older age. This can also explain the fact that not much difference is observed between the *Srebf1*$^{+/+}$ and *Srebf1*$^{+/-}$ mice at the basal condition. For this reason, one copy of *Srebf1* can rescue the phenotype when miR-33 is absent. Recently, miRNAs have been recognized as therapeutic targets. It seems that it would be efficient to inhibit the function of a small number of miRNAs that have many different targets with similar functions at the same time. However, it is estimated that one miRNA may have hundreds of different target genes, and unpredicted results may be obtained by complete and long-term inhibition of an miRNA. It is also known that the results obtained by antisense oligonucleotide-based medicine are sometimes different from those obtained in miR-deficient mice. For example, the administration of an miR-21 antagomir prevented pressure-overload-induced cardiac hypertrophy and fibrosis in mice[38]; however, miR-21-deficient mice did not show any cardiac differences compared with WT mice under pressure overload[39]. As for miR-33, many target genes have been reported by computer algorithm and *in vitro* experiments such as luciferase-based 3′UTR analysis, only some of which show enhanced expression in miR-33-deficient mice.

A recent report indicated that the inhibition of miR-33a/b in non-human primates increases plasma HDL-C and lowers very-low-density lipoprotein triglycerides[40]. The authors showed a 50% decrease in *SREBF1* mRNA and protein in anti-miR-33-treated monkeys at 12 weeks. They speculated that the decrease in SREBP-1 may result from the derepression of negative regulators of this pathway such as AMPK, which is targeted by miR-33. They actually observed an increase in AMPK (*PRKAA1*) mRNA levels. However, there was no change in AMPKα levels in our experiment, and this point should be clarified in future experiments. It is true that in humans, and not in rodents, there is miR-33b in *SREBF1*. Because miR-33a and miR-33b have the same seed sequence, their targets would be the same. This may explain the differences in AMPKα levels. Further studies may be required to clarify whether there is autoregulation of SREBP-1 by miR-33b. In contrast, the experiment on monkeys

was designed to administer antisense miR-33 for a limited time period. In the present study, we demonstrated that miR-33 deficiency serves to raise SREBP-1, increase fatty acid synthesis and promote fatty acid accumulation in the body. Therefore, long-term therapeutic modulation of miR-33 to cure metabolic diseases requires caution for obesity and related diseases, as miRNAs have potentially many target genes and we cannot detect all of them by computer analysis. Moreover, many genes are affected by these secondary or tertiary target genes. Careful observation of miR-deficient mice enables us to detect overall functions of miRNAs *in vivo*.

In conclusion, these results unravel a previously unrecognized interaction between SREBP-1 and SREBP-2 by the way of miR-33. It will be important to establish the tissue- and time-specific regulation of miR-33 to avoid unexpected side effects.

Methods

Materials. The antibodies used were anti-ABCA1 (NB400-105) (Novus Biologicals, Littleton, CO, USA), anti-GAPDH (14C10; no. 2118S), anti-IRS-2 (no. 4502S), anti-AMPKα (no. 2532), anti-SIRT6 (D8D12; no. 12486) (Cell Signaling Technology, Beverly, MA, USA), anti-β actin (AC-15; A5441, Sigma-Aldrich, St Louis, MO, USA), anti-SREBP-1 (sc-13551, sc-8984), anti-TF2B (sc-225), anti-PPARγ (sc-7273), anti-SCAP (sc-48671) (Santa Cruz Biotechnology, California, USA), antibodies. Anti-rabbit, anti-mouse and anti-goat IgG HRP-linked antibodies were purchased from GE Healthcare (Amersham, UK). *N*-acetyl-leu-leu-norleucinal (Calpain inhibitor; ALLN) and Complete Mini (Protease inhibitor cocktail) were from Roche. Pitavastatin (NK-104) was kindly provided by Kowa (Japan). *PPRE*-luciferase promoter plasmid (*PPRE*-luc) was kindly gifted by Dr Kelly-DP. *FAS*-luciferase promoter plasmid (*FAS*-luc) was obtained from Addgene. *SRE*-luciferase promoter plasmid (*SRE*-luc) was from Dr Yahagi-N. Mouse *Srebf1* was obtained from the FANTOM (functional annotation of the mouse) full-length mouse cDNA clone set, and Mouse *Srebf1* with or without the 3′UTR was cloned into pcDNA3.1.

Cell culture. HepG2 cells were cultured Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Japan) supplemented with 10% fetal bovine serum (FBS). Mouse primary hepatocytes were obtained from male miR-33$^{+/+}$ or miR-33$^{-/-}$ mice at 8–10 weeks of age by the two-step collagenase perfusion method[41]. In brief, hepatocyte suspensions were obtained by passing collagenase type II (Gibco BRL, Life Technologies Inc., Rockville, MD, USA) digested liver through a 70 μm cell strainer, followed by centrifugation to collect the mature hepatocytes. After isolation, hepatocytes were resuspended in DMEM supplemented with 5% FBS, and seeded on collagen type I-coated dishes (Iwaki Asahi Glass Co. Ltd., Japan) at a density of 7×10^4 cells ml^{-1}. After incubation for 24 h, cells were used for experiments. For sterol-depleted experiments, cells were washed twice with DMEM without FBS two times and switched to DMEM containing 5% LPDS (Sigma-Aldrich) with or without pitavastatin (1 μM).

Generation of miR-33$^{-/-}$ *Srebf1*$^{+/-}$ mice. To obtain reduced levels of SREBP-1 in miR-33-deficient mice (miR-33$^{-/-}$ *Srebf1*$^{+/-}$), miR-33$^{-/-}$ mice were mated with *Srebf1*$^{+/-}$ mice, which were a kind gift from Dr Shimano[15]. After being weaned at 4 weeks of age, mice were fed NC containing 4.5% fat until 8 weeks of age, and then switched to HFD (D12451; 45% fat by kcal; Research Diet Inc. New Brunswick, NJ, USA) or kept on NC for the next 12 weeks. All of the experimental protocols were approved by the Ethics Committee for Animal Experiments of Kyoto University. Primers for genotyping were as follows.

WT/KO (miR-33) sense; GGCACTACTTCTGATCCTTC
WT (miR-33) antisense; CAACTACAATGCACCACAGCTG
KO (miR-33) antisense; TTGGGATCCAGAATTCGTGATTAA
WT (*Srebf1*) sense; TGTGTCTGACCTGCAATCCT
WT (*Srebf1*) antisense; AGGCCAACACTAGTAGTCCATTG
KO (*Srebf1*) sense; AGGATCTCCTGTCATCTCACC
KO (*Srebf1*) antisense; GCCAACGCTATGTCCTGATA

Western blotting. Western blotting was performed using standard procedures as described previously[42]. For cell experiments, ALLN (12.5 μg ml^{-1}) was added to the cells 2 h before collection. Samples were lysed in lysis buffer consisting of 100 mM Tris-HCl, pH 7.4, 75 mM NaCl and 1% Triton X-100 (Nacalai Tesque). The lysis buffer was supplemented with complete mini protease inhibitor (Roche), ALLN (25 μg ml^{-1}), 0.5 mM NaF and 10 μM Na_3VO_4 just before use. Nuclear protein was extracted using the CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich) in accordance with the manufacturer's instructions. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Bio-Rad). All samples (20 μg of protein) were suspended in lysis buffer, fractionated using NuPAGE 4–12% Bis-Tris (Invitrogen) gels and transferred to a Protran nitrocellulose transfer membrane (Whatman). The membrane was blocked using 1 × phosphate-buffered saline (PBS) containing 5% non-fat milk for 1 h and incubated with the primary antibody (anti-ABCA1; 1:1,000, anti-ABCG1; 1:1,000, anti-IRS-2; 1:500, anti-AMPKα; 1:1,000, anti-SREBP-1; 1:250 (Supplementary Fig. S10), anti-TF2B; 1:1,000, anti-PPARγ; 1:250, anti-β actin; 1:3,000, anti-GAPDH; 1:3,000, anti-SIRT6; 1:1,000 and anti-SCAP; 1:200) overnight at 4 °C. Following a washing step in PBS-0.05% Tween 20 (0.05% T-PBS), the membrane was incubated with the secondary antibody (anti-rabbit, anti-mouse or anti-goat IgG HRP-linked; 1:2,000) for 1 h at 4 °C. The membrane was then washed in 0.05% T-PBS and detected by ECL Western Blotting Detection Reagent (GE Healthcare), using an LAS-1000 system (Fuji Film). Full-length images on immunoblots are shown in Supplementary Figs S11–S13.

RNA extraction and qRT-PCR. Total RNA was isolated and purified using TriPure Isolation Reagent (Roche), and cDNA was synthesized from 1 μg of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) in accordance with the manufacturer's instructions. For quantitative RT–PCR, specific genes were amplified by 40 cycles using SYBR™ Green PCR Master Mix (Applied Biosystems). Expression was normalized to the housekeeping gene β-actin. Gene-specific primers are listed in Supplementary Table S1.

Quantitative PCR for miRNAs. Total RNA was isolated using TriPure Isolation Reagent (Roche). miR-33 was measured in accordance with the TaqMan MicroRNA assays (Applied Biosystems) protocol, and the products were analysed using a thermal cycler (ABI Prism7900HT sequence detection system). Samples were normalized by U6 snRNA expression.

Measurement of fatty acid synthesis. Following 4 h of fasting, mice were intraperitonially injected with 10 μCi [1-^{14}C]-sodium acetate (PerkinElmer Co., Ltd.). Male mice at 10 weeks of age were sacrificed 30 min after injection and livers were rinsed in ice-cold 1 × PBS. Liver samples were saponified by heating in 3 ml of 30% KOH (w/v) at 70 °C for 15 min, followed by the addition of 3 ml of 95% ethanol (v/v) and continued heating at 70 °C for a further 2 h. Saponified fatty acids were acidified with 3 ml of 9 M H_2SO_4 and extracted with petroleum ether[13,14].

Dual luciferase assays. Full-length PCR fragments of the 3′UTR of *SREBF1* were amplified from human or mouse cDNAs and subcloned downstream of a CMV-driven Firefly luciferase cassette in a pMIR-REPORT vector (Ambion). To create WT or mutant 3′UTR luciferase reporter genes, a fragment of the *Srebf1* 3′UTR as follows was inserted into a pMIR-REPORT vector:

Wild type;

CTTCCAAAACAATCGTGGTATCTTTATTGACTTTTTTTTTTCTGAATG
CAATGACTGTTTTTTTTTTTTTTAAC

Mutant;

CTTCCAAAACAATCGTGGTATCTTTATTGACTTTTTTTTTTCTGA
TACGTATGACTGTTTTTTTTTTTTTTAAC

For *SRE* and *FAS* promoter assay, 293T cells were co-transfected with mouse *Srebf1* with full-length 3′UTR or without 3′UTR, along with expression plasmids for miR-control (negative control), or miR-33. An internal control reporter, *Renilla reniformis* luciferase, driven by the thymidine kinase (TK) promoter (pRL-TK: Promega) was also co-transfected to normalize the transfection efficiency. Luciferase activities were measured using a dual luciferase kit (PicaGene dual kit, Toyo Ink Co.). The relative luciferase activity of each construct (arbitrary unit) was reported as the fold induction.[42]

Lentivirus production and DNA transduction. We produced lentiviral stocks in 293FT cells in accordance with the manufacturer's protocol (Invitrogen). In brief, virus-containing medium was collected 48 h post transfection and filtered through a 0.45-μm filter. One round of lentiviral infection was performed by replacing the medium with virus-containing medium (containing 8 μg of Polybrene per ml), followed by centrifugation at 2,500 rpm for 30 min at 32 °C. Cells were used for analysis 3 days after DNA transduction.

IPGTT and insulin tolerance test. For IPGTT, after overnight fasting, male mice at 20 or 50 weeks of age were injected with 1.5 g kg^{-1} glucose intraperitoneally. For insulin tolerance test, after a 4-h fast, mice were injected intraperitoneally with insulin (0.75 u kg^{-1} and 1.0 u kg^{-1} for NC and HFD, respectively, Humulin R; Eli Lilly Japan KK). Blood was obtained from the orbital vein and glucose levels were measured using a glucose sensor.

Measurement of serum insulin and leptin levels. We quantified serum levels of insulin and leptin in male mice at 20 weeks of age using an ELISA assay kit for mouse insulin and leptin in accordance with the manufacturer's instructions (Shigayagi Co. Ltd, Shibukawa, Japan).

Biochemical analysis of serum. After mice (male at 20 or 50 weeks of age) were fasted for 4–6 h, blood was obtained from the inferior vena cava of anaesthetised mice, and serum was separated by centrifugation at 4 °C and stored at − 80 °C. Biochemical data were measured by standard methods using a Hitachi 7180 Auto Analyzer (Nagahama Life Science Laboratory, Nagahama, Japan).

Measurement of cholesterol and triglyceride in the liver. Lipids in the liver were extracted by the Folch procedure[43]. In brief, lipids were extracted by addition of ice-cold MeOH followed by the addition of ice-cold $CHCl_3$. High purity water was added and the sample kept on ice for an additional 10 min with occasional mixing. The sample was centrifuged for 5 min at 2,000 *g* and the upper (aqueous) phase was removed and reextracted by addition of ice-cold $CHCl_3$:MeOH (2:1, v/v) as above. The upper phase was discarded and both organic phases were combined, dried under nitrogen stream. Lipids were quantified using standard enzymatic colorimetric methods (Sky Light Biotech, Akita, Japan).

Measurement of fat body mass by CT. CT scans were obtained and fat body mass and lean body mass were analysed using a Lathea LTC-100 (Aloka, Tokyo, Japan) under pentobarbital anaesthesia.

DNA microarray analysis. Five liver RNA samples from miR-33$^{+/+}$ or miR-33$^{-/-}$ male mice at 16 weeks of age receiving NC were pooled and analysed using a DNA microarray (3D-Gene Mouse Oligo chip 24 k, Toray, Tokyo, Japan).

Pair feeding. Every other day, male mice received the same amount of food consumed by miR-33$^{+/+}$*Srebf1*$^{+/+}$ mice (WT) on the previous 2 days, from 8 to 20 weeks of age.

Assessment of metabolic rate and activity. Oxygen consumption and activity of male mice at 16 weeks of age were measured with an indirect calorimetric system. In brief, room air was pumped through an acrylic metabolic chamber, and the expired gas was filtered through thin cotton, dried and subjected to gas analysis (model RL-600; Alco System, Tokyo, Japan)[44].

Statistics. Data are presented as means ± s.e.m. Statistical comparisons were performed using unpaired two-tailed Student's *t*-tests or a one-way analysis of variance with the Bonferonni *post hoc* test where appropriate, with a probability value of <0.05 taken to indicate significance.

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Author contributions

T.H., T.N. and K.O. designed the project; T.H., T.N., O.B., Y.K., T.N., M.N., S.U., M.I., M.S. and S.M. performed experiments; T.H., T.N., N.Y., H.S., K.I., H.M., T.M., K.H., N.K., M.Y., T.K., T.K. and K.O. analysed and interpreted data; N.Y. and H.S. contributed materials; and T.H., T.N. and K.O. prepared the manuscript.

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TRIALS

RESEARCH Open Access

The correlation between the number of eligible patients in routine clinical practice and the low recruitment level in clinical trials: a retrospective study using electronic medical records

Eriko Sumi[1*], Satoshi Teramukai[2], Keiichi Yamamoto[3], Motohiko Satoh[4], Kenya Yamanaka[4] and Masayuki Yokode[1]

Abstract

Background: A number of clinical trials have encountered difficulties enrolling a sufficient number of patients upon initiating the trial. Recently, many screening systems that search clinical data warehouses for patients who are eligible for clinical trials have been developed. We aimed to estimate the number of eligible patients using routine electronic medical records (EMRs) and to predict the difficulty of enrolling sufficient patients prior to beginning a trial.

Methods: Investigator-initiated clinical trials that were conducted at Kyoto University Hospital between July 2004 and January 2011 were included in this study. We searched the EMRs for eligible patients and calculated the eligible EMR patient index by dividing the number of eligible patients in the EMRs by the target sample size. Additionally, we divided the trial eligibility criteria into corresponding data elements in the EMRs to evaluate the completeness of mapping clinical manifestation in trial eligibility criteria into structured data elements in the EMRs. We evaluated the correlation between the index and the accrual achievement with Spearman's rank correlation coefficient.

Results: Thirteen of 19 trials did not achieve their original target sample size. Overall, 55% of the trial eligibility criteria were mapped into data elements in EMRs. The accrual achievement demonstrated a significant positive correlation with the eligible EMR patient index ($r = 0.67$, 95% confidence interval (CI), 0.42 to 0.92). The receiver operating characteristic analysis revealed an eligible EMR patient index cut-off value of 1.7, with a sensitivity of 69.2% and a specificity of 100.0%.

Conclusions: Our study suggests that the eligible EMR patient index remains exploratory but could be a useful component of the feasibility study when planning a clinical trial. Establishing a step to check whether there are likely to be a sufficient number of eligible patients enables sponsors and investigators to concentrate their resources and efforts on more achievable trials.

Keywords: Clinical trials, Research patient recruitment, Eligibility determination, Clinical informatics, Accrual

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Background

Clinical trials are essential for gaining and extending knowledge about new therapies, and sufficient patient enrollment in clinical trials is critical to fulfill their scientific objectives. Nevertheless, a number of trials have failed to achieve their target sample size within the original accrual period [1-3]. For such trials, extending the accrual period, modifying the eligibility criteria or, in the worst case scenario, prematurely closing the trial may be necessary. Moreover, many investigators continue to make the same mistakes despite the great advances made in handling clinical trials data using information technology [4].

Many reasons for the low levels of recruitment have been cited, including fewer eligible patients than expected, a smaller percentage of patients agreeing to participate [1,5], time constraints, resource issues, consent interviews and difficulties in identifying the patients [6].

Recently, many screening systems that search electronic medical records (EMRs) or clinical data warehouses derived from EMRs for patients eligible for clinical trials have been developed, and their efficiencies have been evaluated [7-9]. The number of patients who meet the eligibility criteria when medical records are manually reviewed is less (13 to 74%) than the number of potential trial patients identified by an electronic screening system [7,10-15]. Nevertheless, screening systems are promising in that they can provide information on the total eligible patient population at the planning stage of a clinical trial. Estimates of the number of eligible patients enable both the sponsors and the investigators to concentrate their resources and efforts on more achievable and conclusive trials. Moreover, investigators should not put their patients at risk by enrolling them in an inconclusive trial. For more reasonable research programs, we hypothesized that researchers can predict the difficulty of trial patient enrollment by estimating the number of eligible patients using EMR data. We explored how to estimate the number of eligible patients using the EMRs, and using a retrospective design, we tested our hypothesis that the number of eligible patients identified from EMRs correlates with the number of patients actually enrolling in clinical trials.

Methods

Trial data collection

The trials were identified using a departmental database from the Institute for Advancement of Clinical and Translational Science and the University Hospital Medical Information Network Clinical Trial Registry (UMIN-CTR) [16]. The investigator-initiated therapeutic trials that started between July 2004 and January 2011 were included if the following data were available: the trial eligibility criteria, target sample size, number of enrolled patients and accrual period at the Kyoto University Hospital (KUH). The trial eligibility criteria, number of scheduled and enrolled patients and the duration of enrollment were extracted from published papers or from registered information in the UMIN-CTR. For unpublished data, trial protocols and management lists in the departmental database were used after obtaining consent from the relevant principal investigators. The study protocol was approved by the Ethical Committee of the Graduate School of Medicine, Kyoto University (E1175).

Electronic medical records retrieval system

We used an EMR retrieval system that was developed at the Institute for Advancement of Clinical and Translational Science to screen EMRs for patients in KUH [17]. In this system, EMR data, including diagnoses, medications and injections, laboratory tests, radiological or pathological studies, and operative notes, were extracted from the data warehouse to enable the comprehensive and efficient retrieval of patient data.

The replacement of trial eligibility criteria with patient characteristics for a comparison to electronic medical records

We replaced the trial eligibility criteria of the trial protocol with patient characteristics that could be easily compared with EMR data and matched the translated criteria with the data elements in EMRs, referencing the methods of previous studies [10,11,17-21].

After the trial eligibility criteria were collected, three physicians discussed and replaced concepts in the eligibility criteria of each trial with patient characteristics, which were represented by codes, fixed terms or numeric data. Some medical concepts, such as 'severe heart disease', may be interpreted differently depending on the trial and the clinician caring for the patients. The three physicians discussed these concepts and made a general list of how to interpret these concepts as patient characteristics, such as considering a particular concept to be part of a group of diagnoses. The list also included instructions on how to replace the specific medical conditions that do not directly indicate one or more data elements in the EMRs by the data elements in the EMRs related to the conditions. For instance, we replaced the criterion 'patients who do not need intravenous hyperalimentation' with 'no order for high-calorie infusion'. Namely, we aimed to estimate the number of patients who were already receiving care from the trial treatment or from alternative treatments in routine clinical practice rather than estimating the potential number of eligible patients still at the diagnostic stage. Concurrently, we re-categorized the eligibility criteria of the trial protocol into three categories: 'Select', 'Omit' and 'Not applicable'. The 'Select' category indicates that the patient was

included if he or she fulfilled the condition. The 'Omit' category indicates that the patient was excluded if he or she fulfilled the condition but was not excluded if the data were not available or were missing. The 'Not applicable' category indicates that the criterion cannot be searched in the EMRs because of missing or incomplete EMR data; for instance, the data were entered in plain-text freely, were captured as an image or were not entered. Thus, the items in the 'Not applicable' category were neither translated into computable eligibility criteria nor searched for in the EMRs.

Laboratory test requirements were indicated in either the inclusion or exclusion items of the trial eligibility criteria. If the laboratory tests were routinely performed, the requirements of the laboratory tests were categorized as 'Select', and if the test result fulfilled the requirement at least once, the patients were deemed eligible. If the tests were not performed routinely, the requirements of the laboratory tests were categorized as 'Omit'.

The periods of the order or the records to search were critical for estimating the number of patients. We searched for eligible patients using a primary criterion that was recorded during the year for which we wanted to know the number of eligible patients. For the other criteria, such as acute illness or diseases with no prior therapy, we searched eligible patients with other criteria in addition to the primary criterion recorded during the preceding two years for acute illness or diseases with no prior therapy and the previous five years for chronic or recurrent disease.

The degree of concordance with the electronic medical record data

We examined how many and what type of trial eligibility criteria were mapped into the patient characteristics and corresponding data elements of the EMRs to evaluate the completeness of our mapping. We assigned patient characteristics, as mentioned above, to one of the 27 semantic categories defined by Luo *et al.* [22]. One author, a medical doctor, broke up and assigned the eligibility criteria to one of the semantic categories, and another medical doctor validated the results. Then, we counted the number of patient characteristics in the 'Select' or 'Omit' and 'Not applicable' categories.

The number of eligible patients in the electronic medical records

We searched for potentially eligible patients using the EMR retrieval system. Each query in the EMR retrieval system was tested to find errors both in the program and in the search results. A system engineer then examined the number of patients in the 'Select' category, the 'Select' but not the 'Omit' category and the 'Select' and 'Omit' categories to confirm that the first number was equal to the sum of the second and third numbers. After this test, he obtained the estimated number of potentially eligible patients both in the year preceding the start of a trial and in the year in which a trial started using the EMR retrieval system. We did not perform an additional manual review of the medical records after the data extraction.

Statistical analysis

The target sample size, number of patients actually enrolled, scheduled accrual period and actual accrual period were obtained.

The following formula was used to determine the eligible EMR patient index and the accrual achievement:

> The eligible EMR patient index = the number of eligible patients identified by the EMRs per year/the target sample size per year.
> The accrual achievement = the number of enrolled patients per year/the target sample size per year.
> Where, the target sample size per year = the target sample size at KUH/the scheduled accrual period (year).
> The number of enrolled patients per year = the number of patients actually enrolled at KUH/the actual accrual period (year).

We examined the relationship between the eligible EMR patient index and the accrual achievement using Spearman's rank correlation coefficient. The receiver operating characteristic (ROC) curves were used to determine the cut-off value for the eligible EMR patient index that identified the low enrollment trials with accrual achievements <1.0. Furthermore, we examined the consistency between the numbers of searched eligible patients in the year preceding the start of a trial and in the year in which a trial began to evaluate the reliability of the eligible EMR patient index. All statistical analyses were performed using R-2.14.1 and SAS software for Windows.

Results

Trial information

Of the 24 trials screened, 15 trials in the Institute for Advancement of Clinical and Translational Science database met the inclusion criteria, in addition to four trials in the UMIN-CTR (accessed on May 2, 2011). Table 1 indicates the characteristics of the 19 trials. The patient accrual period was extended in seven trials, and six trials recruited 100% or more of their target sample size within the scheduled accrual period.

The replacement of trial eligibility criteria with patient characteristics for comparisons with the electronic medical records

We replaced and matched the trial eligibility criteria for the 19 trials with the data elements in the EMRs. We

Table 1 Characteristics of the trials

Characteristic	Number of trials
Phase	
I/II	7
II	6
IV	1
Not specified	5
Clinical areas	
Cancer	6
Internal medicine	6
Orthopedics	5
Others	2
Participating centers	
KUH only	17
Multicenter study	2
Study start date	
2004 to 2005	3
2006 to 2007	10
2008 to 2009	3
2010	3
Target sample size per year per center[a]	
0 to 9	6
10 to 19	6
20 to 29	2
30 to 39	1
≥40	4
Enrolled patients per year per center	
0 to 9	10
10 to 19	3
20 to 29	1
30 to 39	4
≥40	1

[a]The target sample size was calculated as the total sample size divided by the number of centers in a single trial for which an assigned sample size was not determined. KUH, Kyoto University Hospital.

present an example of this process in a trial titled 'A randomized controlled study of the effectiveness of transcatheter arterial chemoembolization with cisplatin and transcatheter arterial chemoembolization with epirubicin for multiple hepatocellular carcinomas' (Figure 1). The trial candidates were patients who were to receive transcatheter arterial chemoembolization for multiple HCCs at stage 2 to 4a without thrombosis in the portal vein, hepatic vein and the bile duct, although the trial eligibility criteria were only presented as the disease conditions. Therefore, we replaced the disease condition with the related standard treatment for the patients and assessed the radiologic study order in EMRs to narrow down the trial candidates.

The degree of matching with the electronic medical record data

The 318 eligibility criteria from the 19 trials were transformed into 425 patient characteristics. Of the 425 patient characteristics, 408 were related to 18 semantic categories, and 17 were related to a ' no fitting category'. We found that 55% (235 of 425) of the characteristics in the eligibility criteria were matched with data elements in the EMRs. Compared with a previous study by Kopcke *et al.* [21], the degree of matching was similar with respect to both the total and the category (Table 2). The degree of match for each trial ranged from 38% to 75% (median 54%), and the degree was 50% in three trials.

Data retrieval, correlation and receiver operating characteristic analysis

We searched the EMRs for patients who fit the computable criteria characteristics in the 19 trials, counted the number of patients in each trial and calculated the eligible EMR patient index. The accrual achievement demonstrated a significant positive correlation with the eligible EMR patient index ($r = 0.67$, 95% confidence interval (CI), 0.42 to 0.92; Figure 2). The ROC analysis revealed an estimated 1.7 cut-off value for the eligible EMR patient index, with an area under the curve (AUC) of 0.846, a sensitivity of 69.2% and a specificity of 100.0% (Figure 3). None of the nine trials for which the eligible EMR patient index was less than 1.7 achieved their original target sample size within the scheduled accrual period. There were 16 trials in which 50% or more of trial criteria were matched with the data elements in the EMRs. The relationship of the eligible EMR patient index and the accrual achievement in these 16 trials also exhibited a positive correlation ($r = 0.67$, 95% CI, 0.38 to 0.96). The ROC analysis revealed an estimated 1.7 cut-off value for the eligible EMR patient index, with an AUC of 0.867, a sensitivity of 70.0% and a specificity of 100.0%.

The number of identified eligible patients in the year preceding the start of the trial was almost consistent with the number of identified eligible patients in the year in which a trial began (Figure 4). The median ratio of the number of eligible patients in the preceding year to the number in the year a trial began was 1.00 (range, 0.36 to 1.67).

Discussion

We developed a formula to estimate the number of eligible patients using routine EMR data. In half of the tested trials, using the eligible EMR patient index, we

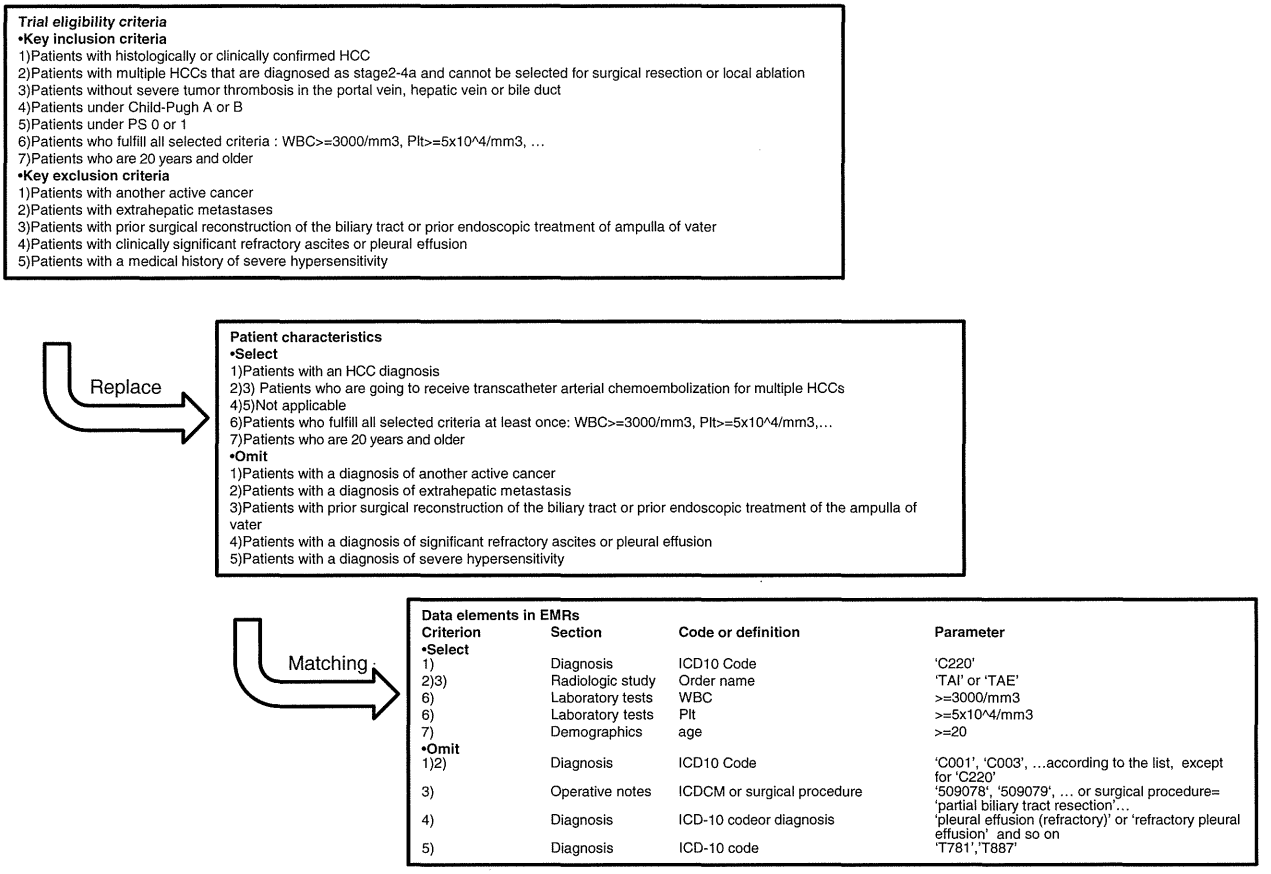

Figure 1 An example of replacement of trial eligibility criteria with patient characteristics for comparisons with the electronic medical records (EMRs). The trial eligibility criteria, replaced patient characteristics and matched data elements of EMRs are presented in the first, second and third columns, respectively. HCC, hepatocellular carcinoma; PS, performance status; TAE, transcatheter arterial embolization; TAI, transcatheter arterial infusion chemotherapy.

were able to predict prior to the start of a trial whether the trial would have a low enrollment because of fewer than expected eligible patients. If researchers are able to accurately predict a shortage of eligible patients, they may modify the eligibility criteria, recruit more participating institutions or abandon the trial to avoid wasting funds and efforts as well as exposing patients to unnecessary risk.

A number of screening methods for EMRs for eligible patients have been developed [7,8,10,11,18-20]. The search method for eligible patients used in our study was based on the patient treatment information rather than the plain text description of the disease in the EMRs. Although we may underestimate the number of potentially eligible patients who were diagnosed with the target disease without standard therapy, we consider those patients to be patients without active disease, patients who are unable to be treated or patients who are unwilling to be treated. Thus, there is little chance to enroll these patients into a clinical trial. When there is no standard therapy for the target disease or the target stage of the disease, one must review the text in the EMRs manually or incorporate an adequate text mining technology to improve the search precision. However, we speculated that the combination of a diagnosis with other information may help refine the estimation [23], and we found that the EMR data and the estimation of the number of patients were accurate enough to predict some of the low enrollment trials.

Approximately one-half of all patient characteristics replaced from the trial eligibility criteria were matched with data elements in EMRs. Considering that the degree of matching in total or by category was not inferior to that achieved in a previous study [21], the included 19 trials are not biased, despite their small number. However, 45% of the patient characteristics were not matched with data elements in the EMRs, which may lead to an overestimation or underestimation of the number of eligible patients in the EMRs. Some trial criteria, such as 'pregnant or lactating', 'measurable disease by RECIST' and 'New York Heart Association class I', were classified as 'Not applicable' and were not considered

Table 2 Degree of translation to the electronic medical record (EMR) data

	Patient characteristics	Data elements in EMRs	Degree of translation[a]	Previous study[b]
Health status	236	141	0.61	0.60
Disease, symptoms and signs	120	80	0.68	0.81
Pregnancy-related activity	12	0	0	0.16
Neoplasm status	24	16	0.67	0.75
Disease stage	10	1	0.10	0.25
Allergy	12	4	0.33	0.17
Organ or tissue status	54	40	0.75	0.74
Life expectancy	4	0	0	0
Treatment or healthcare	45	24	0.55	0.57
Pharmaceutical substance or drug	26	10	0.40	0.35
Therapy or surgery	19	14	0.74	0.74
Device	0	0	NA	0
Diagnostic or lab results	84	47	0.56	0.54
Diagnostic or lab results	84	47	0.56	0.54
Receptor status	0	0	NA	0
Demographics	21	21	1.00	0.85
Age	20	20	1.00	0.95
Special patient characteristic	0	0	NA	0.33
Literacy	0	0	NA	0
Gender	1	1	1.00	1.00
Address	0	0	NA	0
Ethnicity	0	0	NA	0
Ethical consideration	12	0	0	0.08
Consent	8	0	0	0.06
Enrollment in other studies	1	0	0	0
Capacity	2	0	0	0.16
Patient preference	1	0	0	0
Compliance with protocol	0	0	NA	0
Lifestyle choice	10		0.20	0.82
Addictive behavior	5	0	0	0.90
Bedtime	0	0	NA	0
Exercise	0	0	NA	0
Diet	5	2	0.40	0
No fitting category	17	0	0	-
Total	425	235	0.55	0.55

[a]The degree of translation = the number of patient characteristics/the number of data elements in EMRs.
[b]Previous study: the fraction of documentable patient characteristics in previous study [21]. The authors calculated the fraction of patients with any data in at least one corresponding data element for each patient characteristic.

when determining whether the patient should be excluded. In addition, the diagnosis in the EMRs does not necessarily reflect the current condition of the patient. Temporary diagnoses or diagnoses related to the payment of medical insurance are often included in the EMRs and provide false or misleading information that leads to overestimating the number of eligible patients in the EMRs. However, because the exclusion of three trials with low levels of matching did not change the result of the correlation analysis or the ROC analysis, the impact of low levels of matching did not seem to be substantial.

In addition, the eligible EMR patient index is necessary but not sufficient to predict low levels of recruitment. Disappointingly, approximately half (four of ten) of the trials with an eligible EMR patients index greater than 1.7 resulted in low enrollment in this study. This finding

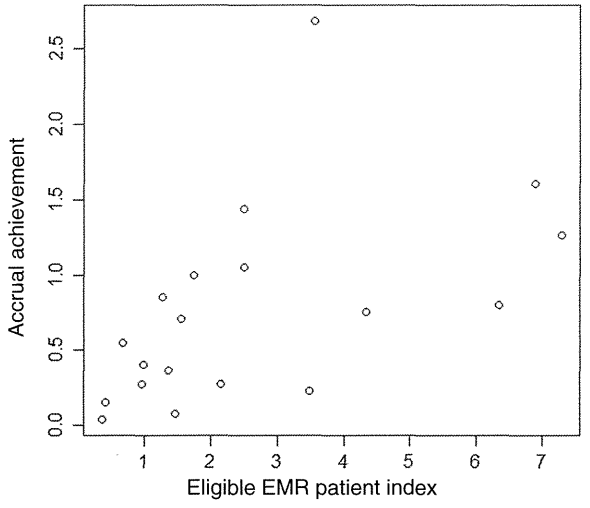

Figure 2 Correlation between the eligible electronic medical record (EMR) patient index and the accrual achievement in 19 trials. The eligible EMR patient index = the number of eligible patients identified from the EMRs per year/target sample size per year. The accrual achievement = the number of enrolled patients per year/target sample size per year.

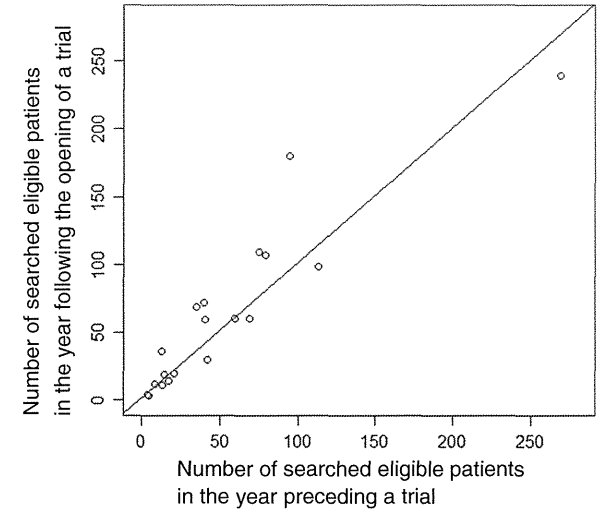

Figure 4 A plot of the number of searched eligible patients both in the year preceding and the year following the opening of a trial.

results from the index not considering the willingness of patients and from the inaccuracy of EMR data. For instance, only 51% of the eligible patients agreed to participate in a cancer trial at one university-based cancer center [24]. Indeed, the consent rates in the trials conducted in our center ranged from 25% to 100% (data not shown). Four times as many patients as the number of eligible patients in EMRs are necessary when the consent rate of the trial is 25%. Therefore, investigators should consider other disincentive factors that would empirically influence a patient's consent, such as foreseeable risks or inconveniences to the patients, and the investigators can then determine whether the trial would in fact achieve its target sample size.

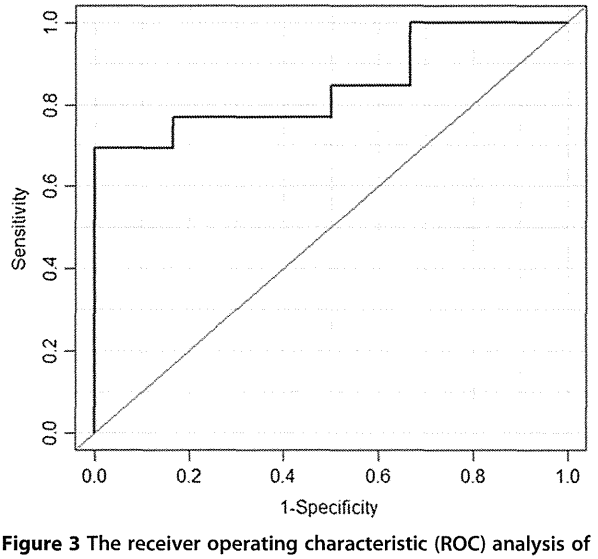

Figure 3 The receiver operating characteristic (ROC) analysis of the eligible electronic medical record (EMR) patient index in 19 trials.

Our method also excluded a manual review of the EMRs. In previous studies, the eligible patients were generally identified by the EMRs in two steps: (1) the patient characteristics were screened and matched with standardized codes (for example, ICD codes) or numeric data; and (2) the medical records were manually reconfirmed by the medical staff [10,11]. Our aim was to obtain the total number of eligible patients instead of examining whether an individual patient was eligible for the trial. Therefore, we did not confirm whether the patients searched by the EMR retrieval system were eligible for the trial by verifying the entirety of their EMR data. As a result, the privacy of patients who had not given consent to participate in the trial is protected, while investigators can still speculate on the feasibility of the trial protocol.

The estimated number of eligible patients during the year of the trial accrual period did not exhibit a substantial increase compared to that of the preceding year. Researchers were unable to enroll more patients than they routinely cared for, regardless of the intensity of the recruitment efforts. The acquisition of new patients may be difficult because of the high degree of development among medical institutions and the guaranteed access by Japanese patients to any institution under the comprehensive medical insurance system. Additionally, most patients in Japan would have already been diagnosed or treated by specialists in a branch of medicine [25].

One limitation of this study was that the process of 'replacement' was not the exact translation of the trial eligibility criteria into data elements in the EMRs and instead depended on physicians' conception or the information presented in the trial protocol; thus, disagreement concerning the replacement or incomplete replacement may occur. Another limitation is that the eligible EMR patient index cannot predict all trials that will result in a failure of accrual (sensitivity, 0.645). The index is designed for a single institution and for a relatively small number of target trials. The cut-off value of the eligible EMR patient index in another institution may be different from ours. To speculate whether there are enough potentially eligible patients at a participating trial site for a multicenter clinical trial, each site must be equipped with an efficient EMR retrieval system. Moreover, this study was exploratory in nature, and prospective studies would be needed to validate the predictive ability of the eligible EMR patient index for future clinical trials.

Conclusions

Our study suggests that in addition to the knowledge of experienced investigators, the health information in EMRs could be a useful component of the feasibility study when planning a clinical trial. Establishing a step to check whether there are likely to be a sufficient number of eligible patients enables sponsors and investigators to concentrate their resources and efforts on more achievable trials.

Abbreviations

AUC: Area under the curve; EMRs: Electronic medical records; KUH: Kyoto University Hospital; ROC: Receiver operating characteristic; UMIN-CTR: University Hospital Medical Information Network Clinical Trial Registry.

Competing interests

All authors declare that they have no competing interests.

Authors' contributions

ES designed the study, replaced the trial eligibility criteria with the EMR data, collected data and wrote the manuscript. KY designed the study, developed the ERS system, wrote computer programs and collected data. ST designed the study and conducted the statistical analysis. MS and KY replaced the trial eligibility criteria with the EMR data. MY is the owner of the ERS systems and supervised the study. All authors read and approved the final manuscript.

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Review

MicroRNAs and Lipoprotein Metabolism

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MicroRNAs (miRNAs; miRs) are small, non-protein-coding RNAs that negatively regulate the gene expression. They bind to specific mRNAs and inhibit translation or promote mRNA degradation. Recently, some miRNAs have been shown to be involved in lipid homoeostasis. In particular, miR-122 and miR-33 have a significant impact on lipid homeostasis and are potential therapeutic targets for treating lipid disorders and/or atherosclerosis. In this review, we describe the current understanding of the function of miRNAs in lipid homeostasis, with a focus on lipoprotein metabolism.

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Key words: MicroRNA, HDL-C, Atherosclerosis

Introduction

MicroRNAs (miRNAs; miRs) are endogenous, small (approximately 20-22 nucleotides in length), non-protein-coding RNAs. miRNAs bind to the 3' untranslated region (UTR) of specific mRNAs according to the complementarity of their sequences and inhibit translation or promote mRNA degradation[1,2]. Initially, miRNAs were discovered in *Caenorhabditis elegans*[3,4] and were later found to be evolutionarily conserved[5,6]. More than 2,500 miRNAs have been identified in humans thus far[7].

miRNAs are usually transcribed as longer primary miRNAs (Pri-miRNAs) by RNA polymerase II (PolII) then processed by the Drosha (RNaseIII)/DGCR8 complex to form pre-mature miRNAs (Pre-miRNAs) in the nucleus. Pre-miRNAs are exported to the cytoplasm through exportin 5 then processed by another ribonuclease enzyme, Dicer, to form mature miRNAs, which are typically 20-22 nucleotides in length. Mature miRNAs are assembled into an RNA-inducing complex (RISC) that inhibits the mRNA expression post-transcriptionally by binding to the 3' UTR of the target mRNAs (**Fig. 1**)[8].

miRNAs have been demonstrated to have many functions in both physiological and pathological states, and some miRNAs have been shown to have a significant impact on lipid homeostasis[8-10]. In particular, the functions of two miRNAs, the liver-specific miR-122 and miR-33, the latter of which is located in the intron of *SREBF2*, have been investigated intensively. In the present review, we summarize the functions of miRNAs in lipid homeostasis, with a focus on lipoprotein metabolism.

miR-122

miR-122 is the most abundantly expressed miRNA in the liver (more than 50,000 copies per cell, 70% of the total miRNA expression in the liver) and was the first miRNA shown to affect lipid metabolism[11-15]. miR-122 inhibition by an antagomir or an antisense oligonucleotide (ASO) results in a significant reduction of the plasma cholesterol levels in mice[11,12]. One report indicated that mice treated with an antagomir exhibit a more than 40% reduction in the plasma cholesterol levels, whereas no effects are observed on the

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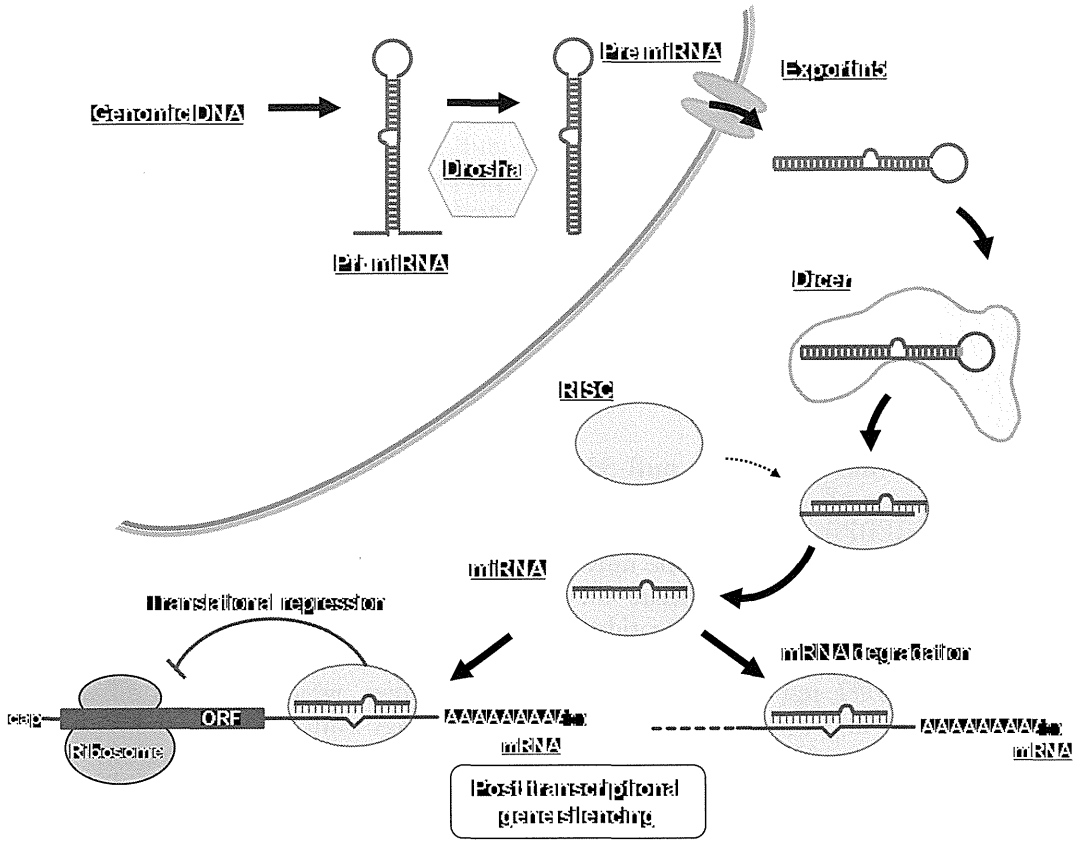

Fig. 1. miRNA biosynthesis pathway.

non-esterified free fatty acid (FFA), triglyceride (TG), bile acid (BA) or glucose levels[11]. This is partly due to a reduction in the expression of 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*), a rate-limiting enzyme of endogenous cholesterol biosynthesis. However, the effects of an antagomir against miR-122 on cholesterol biosynthesis are indirect, and the direct target of miR-122 that impacts the plasma cholesterol levels remains unclear. Another paper reported that mice treated with an ASO showed a 30% reduction in cholesterol, as reflected in both the low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels, with a 40% decrease in the plasma TG levels[12]. The mice exhibited increased hepatic fatty acid oxidation, decreased levels of hepatic fatty acids and cholesterol synthesis and resistance to high-fat diet (HFD)-induced steatosis. The authors explained this phenotype by showing an increased activity of AMP-activated kinase (AMPK) in the mice following miR-122 inhibition. In a study of non-human primates, including African green monkeys, miR-122 inhibition by a locked-nucleic acid-modified oligonucleotide (LNA-antimiR) also resulted in a 30% decrease in plasma cholesterol, predominantly in the LDL fraction, without any apparent liver toxicity[13]. Recently, miR-122 germline and liver-specific knockout mice were generated by two different groups; the mice display a 30% reduction in the total serum cholesterol levels (both HDL and LDL)[14, 15] and exhibit significant inhibition of cholesterol biosynthesis genes, including *Hmgcr*. Moreover, the mice show a reduced expression of microsomal triglyceride transfer protein (*Mttp*), which is essential for the hepatic assembly of very-low-density lipoprotein (VLDL), and demonstrate a significant reduction in the serum TG levels[14]. The mechanism of *Mttp* inhibition due to miR-122 deficiency remains unclear. It is important to note that miR-122-knockout mice display steatohepatitis, fibrosis and hepatocellular carcinoma (HCC), which suggests that miR-122 plays a role not only in lipid metabolism, but also as a tumor suppressor in hepatocytes.

It seems strange that miR-122 ASO-treated mice are resistant, while miR-122-deficient mice developed by two different groups are susceptible, to hepatic steatosis[14, 15]. In a paper by Esau *et al.*, miR-122 ASO treatment reduced the expression of fatty acid oxida-

tion genes, including ACC1 and ACC2[12]. On the other hand, extensive lipid accumulation is observed in miR-122-deficient mice[14, 15]. In the miR-122-deficient mice, a different set of lipogenic genes is altered from that observed in mice treated with miR122-ASO. Therefore, the different inhibitory levels of miR-122 resulted in different gene expression profiles in the liver and may have caused the opposite histology from the viewpoint of steatohepatitis. Further detailed investigations are needed to evaluate the therapeutic approaches for treating lipid disorders using miR-122.

miR-33

Recent studies have indicated that miR-33, which is located in the intron of sterol-regulatory element-binding proteins (SREBPs), controls cholesterol homeostasis[16-20]. There are two miRNAs in the miR-33 family, miR-33a and miR-33b, which share the same seed sequence and differ in only two nucleotides. In humans, miR-33a and miR-33b are encoded in the introns of *SREBF2* and *SREBF1*, respectively[17, 21]. On the other hand, in rodents, miR-33b does not exist, and only miR-33a (designated as miR-33) is present in the intron of *Srebp2*. *SREBF1* encodes SREBP1a and SREBP1c, which primarily regulate lipogenic genes, such as fatty acid synthase (*FASN*), stearoyl-CoA desaturase (*SCD*) and acyl-CoA carboxylase 1 (*ACC1*). *SREBF2* encodes SREBP2, which primarily regulates cholesterol-regulating genes, such as *HMGCR* and low-density lipoprotein receptor (*LDLR*)[22-24]. miR-33a and miR-33b are considered to be co-transcribed and regulate lipid homeostasis with their host genes. Several groups, including ours, have reported that miR-33 targets ATP binding cassette transporter A1 (*Abca1*) *in vivo* using antisense technology or by generating miR-33 knockout mice[16-19]. ABCA1 transports free cholesterol from inside to lipid-poor apolipoprotein A-I (apoA-I) and forms nascent HDL. Therefore, ABCA1 is an essential molecule for HDL biogenesis and reverse cholesterol transport (RCT). Mice treated with LNA antisense oligonucleotides or anti-miR-33 lentivirus exhibit an increased ABCA1 expression in the liver and ABCA1 and ABCG1 expression in macrophages (*Abcg1* is another target of miR-33 in rodents). More importantly, in anti-miR-33-treated mice, the plasma HDL levels are increased 35-50% without affecting other lipoproteins[16-18]. miR-33-knockout mice also show a significant increase in the expression of ABCA1 in the liver and macrophages and a 25% increase in serum HDL[19]. From the viewpoint of reverse cholesterol transport, the beneficial effects of anti-miR-33 therapy include not only increasing the HDL level via the upregulation of ABCA1, but also stimulating bile secretion via the upregulation of ABCB11 and ATP8B1, which are targets of miR-33[25]. Moreover, antisense inhibition of miR-33 results in the regression of atherosclerotic plaque in LDLR-deficient mice by promoting RCT[26]. miR-33 deficiency also reduces the progression of atherosclerosis in apoE-deficient mice (**Fig. 2**)[27]. The cholesterol efflux capacity of the serum in miR-33-knockout mice is higher than that observed in controls, which indicates that the increased level of HDL is functional. Following these studies on atherosclerosis, two different results were reported regarding the effects of the antisense inhibition of miR-33 on the progression of atherosclerosis in LDLR-deficient mice. First, Marquart *et al.* reported that anti-miR-33 therapy does not alter the progression of atherosclerosis in LDLR-deficient mice[28]. On the other hand, Rotllan *et al.* reported that silencing miR-33 inhibits the progression of atherosclerosis in LDLR-deficient mice[29]. The authors discussed the discrepancies between the two studies, which included differences in antisense technology and a considerable difference in diet (1.25% vs. 0.15% cholesterol). It has also been reported that miR-33 regulates fatty acid metabolism by targeting carnitine O-octanoyltransferase (*Crot*), carnitine palmitoyltransferase 1A (*Cpt1a*) and hydroxyacyl-CoA dehydrogenase-3-ketoacyl-CoA thiolase-enoyl-CoA hydratase (a trifunctional protein) β-subunit (*Hadhb*)[20, 30]. In non-human primates (African green monkeys), anti-miR-33 therapy (inhibition of both miR-33a and miR-33b) increases the HDL levels and decreases the VLDL levels without any apparent liver toxicity; the latter effect is not observed in mice[31]. Therefore, further detailed investigations including the study of the miR-33b functions are required to establish whether miR-33 antagonism has the potential to be an efficient therapy in humans.

Other miRNAs

miR-370 directly targets *Cpt1a*, thereby downregulating fatty acid oxidation[32]. miR-370 upregulates the expression of miR-122, which increases the levels of lipogenic genes, such as *SREBP1c*, diacylglycerol acyltransferase-2 (*DGAT2*), *FASN*, and *ACC1*, in cultured HepG2 cells. Like miR-33, miR-758, miR-26 and miR-106b have been shown to target the 3'UTR of *ABCA1* and regulate cholesterol efflux *in vitro*[33-35]. Recently, two independent groups reported that miR-144 also targets the 3'UTR of *ABCA1*, reducing the expression of ABCA1 and cholesterol efflux[36, 37]. miR-144 is upregulated by Farnesoid X Receptor (FXR)

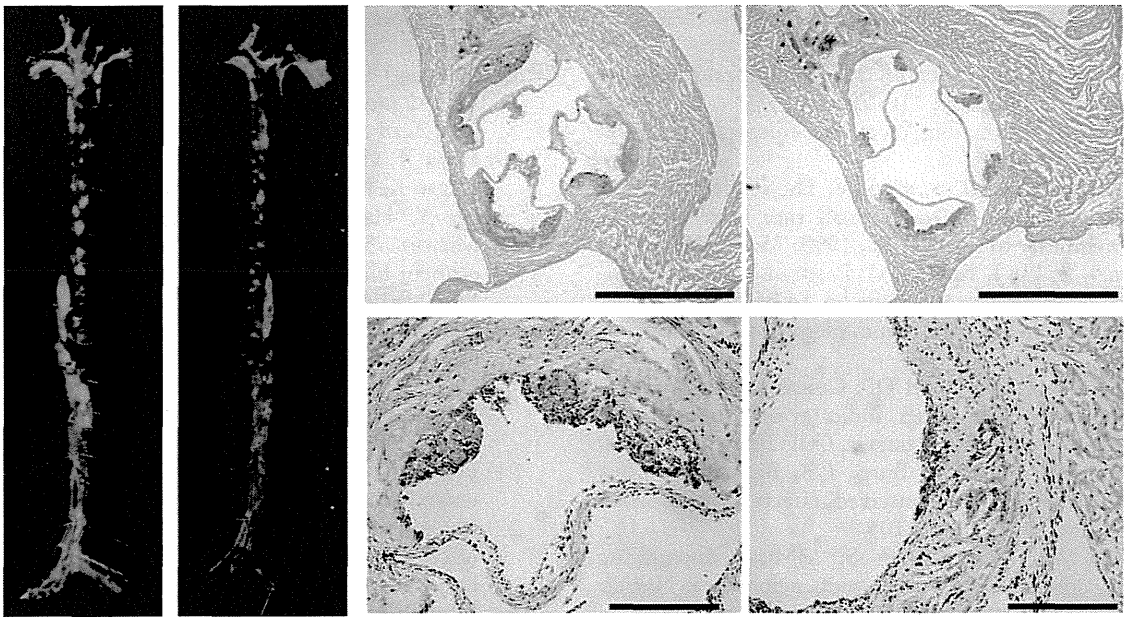

Fig. 2. miR-33 deficiency reduces atherosclerosis (cited from reference[27]).

Left: Representative images of the *en face* analysis of the total aorta in miR-33$^{+/+}$*Apoe*$^{-/-}$ and miR-33$^{-/-}$*Apoe*$^{-/-}$ mice. Right upper: Representative microscopic images of cross-sections of the proximal aorta in miR-33$^{+/+}$*Apoe*$^{-/-}$ and miR-33$^{-/-}$*Apoe*$^{-/-}$ mice. Scale bars: 1 mm.
Right lower: Representative microscopic images of immunohistochemical staining for the macrophage marker CD68 in miR-33$^{+/+}$*Apoe*$^{-/-}$ and miR-33$^{-/-}$*Apoe*$^{-/-}$ mice. Scale bars: 200 μm.

and Liver X nuclear Receptor (LXR). The overexpression of miR-144 reduces the expression of ABCA1 and the plasma HDL levels. On the other hand, silencing miR-144 increases the expression of ABCA1 and the plasma HDL levels in mice. Moreover, miR-33 and miR-144 have an additive effect on the ABCA1 protein expression[37]. Therefore, the inhibition of both miR-33 and miR-144 is potentially a better strategy for increasing the HDL levels.

Very recently, Soh J. *et al.* reported that miR-30c targets the 3'UTR of *Mttp*, leading to reductions in the MTTP activity and apolipoprotein B secretion[38]. Moreover, miR-30c reduces lipid synthesis independently of MTTP. The authors showed that the overexpression of miR-30c reduces the plasma cholesterol and triglyceride levels and improves atherosclerosis, whereas the inhibition of miR-30c increases the plasma cholesterol and triglyceride levels and worsens atherosclerosis in apoE-deficient mice.

Conclusions

miRNAs have been shown to be important regulators of both physiological and pathological states. Recent studies have provided considerable evidence regarding the impact of miRNAs on lipid metabolism, particularly in the preclinical stage. Because one miRNA can have many targets and affect the expression levels of many genes, further investigations are needed to understand the complexity of miRNA biology. miRNA-based therapy may be a promising new approach for the future prevention or treatment of human diseases.

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Disclosures

None.

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