an RNA probe carrying wild-type or mutant (p.E415G) sequence. Coomassie blue staining of the RNA affinity-purified proteins showed a ~30 kDa protein in the wild-type RNA probe but not in the p.E415G probe (Fig. 3b, open arrowhead). In addition, we noticed *de novo* binding of a ~55 kDa protein in the p.E415G probe (Fig. 3b, closed arrowhead). Mass spectrometry analysis of the excised bands disclosed that the molecule bound to the wild-type RNA probe was SRSF1, and that to the p.E415G RNA probe was hnRNP H. We confirmed the identity of bound proteins by immunoblotting with specific antibodies against SRSF1 (32–4500, Invitrogen) and hnRNP H (A300–511A, Bethyl Laboratories) (Fig. 3c). The immunoblotting indeed demonstrated that SRSF1 binds only to the wild-type probe, whereas hnRNP H binds only to the p.E415G probe.

SRSF1 enhances and hnRNP H silences inclusion of exon 16. We next examined the effects of SRSF1 and hnRNP H on splicing of exon 16 by siRNA-mediated downregulation of SRSF1 and hnRNP H in HeLa cells. SRSF1 and hnRNP H were efficiently downregulated in HeLa cells (Fig. 3d). Downregulation of SRSF1 induced exclusion of exon 16 in the wild-type minigene (Fig. 3e, lane 3), whereas downregulation of hnRNP H induced inclusion of exon 16 in the p.E415G minigene (Fig. 3e, lane 6), indicating that SRSF1 functions as a splicing enhancer for the wild-type minigene and hnRNP H functions as a splicing silencer for the p.E415G minigene. We observed similar splicing alterations with a second set of siRNAs targeting different sites of each mRNA in HeLa cells (Fig. S1a and b).

We next confirmed that SRSF1 and hnRNP H indeed work on the identified *cis*-element and not on the other sites. To this end, we tethered SRSF1 or hnRNP H to the target using the bacteriophage MS2 coat protein. We made an effector construct expressing MS2-tagged SRSF1 and hnRNP H proteins (MS2-SRSF1 and MS2-H, respectively), and the target minigene construct (pCI-*COLQ*-MS2), in which the MS2-binding site was substituted for the native target site. As expected, tethering of SRSF1 to the target induced inclusion of exon 16 (Fig. 3f, lane 4), whereas tethering of hnRNP H caused skipping of exon 16 (Fig. 3f, lane 6). Lack of the splicing modulating effects of SRSF1 and hnRNP H without the MS2-tag indicates that neither SRSF1 nor hnRNP H works at the other sites (Fig. 3f, lanes 3 and 5). We also confirmed that MS2 alone or MS2-fused hnRNP L (hnRNP L-MS2) had no effect on splicing of pCI-*COLQ*-MS2 (Fig. 3f, lanes 2 and 7). Thus, SRSF1 and hnRNP H exert splicing-enhancing and splicing-silencing activities exclusively on the identified target, respectively.

Molecular basis of binding of SRSF1 versus hnRNP H and splicing regulation. Previous reports suggest that SRSF1 binds to GA-rich sequence[12,23,24] and hnRNP H binds to poly(G) sequence[25–27]. The wild-type *COLQ* exon 16 harbors a motif of GGAGGA, and the patient's mutation (p.E415G) convert the GGAGG motif to GGGGG. We identified that SRSF1 binds to wild-type RNA probe, whereas hnRNP H binds to the p.E415G RNA probe. The GGAGG motif indeed matches to the functional SELEX consensus of SRSF1 [(G/C)(A/G)(G/C)A(G/C)GA][28], as well as to *in vivo* binding motifs 2 and 3 identified by CLIP-seq (cross-linked immunoprecipitation and deep sequencing)[12]. On the other hand, GGGGG motif also matches to the previously identified *in vitro* and *in vivo* binding motif of hnRNP H[25–27]. Therefore, a single nucleotide substitution switches binding of the splicing-enhancing SRSF1 to the splicing-suppressing hnRNP H, and causes aberrant splicing in patient's muscle.

We further dissected the molecular basis of splicing regulation by SRSF1 and hnRNP H. Analysis of artificial mutations showed that any mutations affecting the core GGAGG motif affected inclusion of exon 16 (Fig. 2). We therefore examined binding of SRSF1 and hnRNP H to the mutant RNA probes. RNA affinity purification followed by immunoblotting revealed that SRSF1 showed a strong binding affinity for GGAGG, and weak binding affinities for GGTGG and GGCGG (Fig. 4a,b). However, we did not detect binding of SRSF1 to GGGGG. In contrast, hnRNP H showed a strong binding affinity for GGGGG, whereas it could not bind to other motifs. The results of the binding assay were consistent with the splicing analysis of minigenes harboring these motifs (Fig. 2). To further confirm that SRSF1 and hnRNP H indeed regulate splicing of each motif, we overexpressed SRSF1 or hnRNP H in HeLa cells along with a minigene harboring each motif. As expected, SRSF1 exerted an additive effect on exon inclusion for minigenes harboring GGAGG (Fig. 4c), GGCGG (Fig. 4e) and GGTGG (Fig. 4f), to which SRSF1 was able to bind (Fig. 4b). On the contrary, SRSF1 had no effect on a minigene harboring GGGGG (Fig. 4d), to which SRSF1 could not bind. Similarly, overexpression of hnRNP H had no effect on GGAGG (Fig. 4c), GGCGG (Fig. 4e) and GGTGG (Fig. 4f), to which hnRNP H could not bind. In contrast, hnRNP H caused complete skipping of exon 16 harboring GGGGG (Fig. 4d), to which hnRNP H was able to bind (Fig. 4b). Therefore, binding of SRSF1 and hnRNP H to each motif (Fig. 4b) exerted the expected effects on splicing of exon 16 (Fig. 4c–f).

We next asked if there is any binding competition between hnRNP H and SRSF1 for the GGAGG or GGGGG motif, because the G nucleotide in the middle of the motif is an acceptable binding site for SRSF1[12]. For this purpose, we examined splicing of exon 16 of the wild-type (GGAGG) and p.E415G (GGGGG) minigenes after knocking down SRSF1 and/or hnRNP H. For the wild-type minigene, knockdown of SRSF1 alone and knockdown of both SRSF1 and hnRNP H showed similar degrees of exon skipping (Fig. 4g), indicating that hnRNP H had no effect on splicing of exon 16 even in the absence of SRSF1. Similarly, for the p.E415G minigene, SRSF1 had no effect on splicing of exon 16 even in the absence of hnRNP H (Fig. 4h). Taken together, lack of binding of SRSF1 to GGGGG and lack of binding of hnRNP H to GGAGG were not due to competition between SRSF1 and hnRNP H.

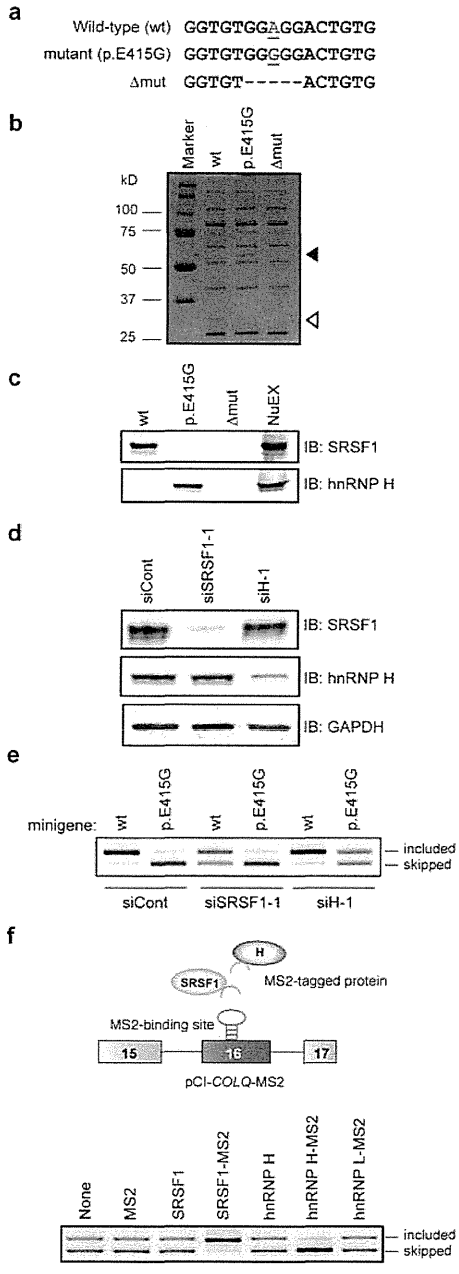

Figure 3. p.E415G compromises binding of a splicing-enhancing SRSF1 and gains binding of a splicing-suppressing hnRNP H. (**a**) Biotinylated RNA probes. (**b**) Coomassie blue staining of RNA affinity-purified products of HeLa nuclear extract. A ~30-kDa protein (open arrowhead) is detected only with the wild-type (wt) probe but not with the p.E415G or deletion mutant (Δmut) probe. In contrast, a ~55-kDa protein (closed arrowhead) is detected only with the p.E415G mutant. (**c**) Immunoblots (IB) of RNA affinity-purified proteins probed with indicated splicing *trans*-factors. The wild-type exon 16 (wt) binds to SRSF1and the p.E415G mutation disrupts its binding. The mutation gains *de novo* binding to hnRNP H. NuEX indicates 5% of the nuclear extract used in the assay. (**d**) Immunoblotting (IB) of HeLa cells treated with siRNA against control (siCont), SRSF1 (siSRSF1-1), and hnRNP H (siH-1) showing efficiency of siSRSF1-1 and siH-1. (**e**) RT-PCR of wild-type (wt) and p.E415G *COLQ* minigenes in HeLa cells treated with indicated siRNAs. A representative gel image of three independent experiments is shown. (**f**) Schematic presentation of a reporter minigene (pCI-*COLQ*-MS2) and *trans*-acting effectors. SRSF1 and hnRNP H (ovals) are fused to the artificial MS2 coat protein (an inverted U shape). MS2 coat protein-binding hairpin RNA is substituted for the splicing regulatory site of exon 16 to directly tether MS2 coat protein-fused SRSF1 and hnRNP H. RT-PCR of pCI-*COLQ*-MS2 minigenes in HeLa cells that are co-transfected with the indicated effectors. A representative gel image of three independent experiments is shown.

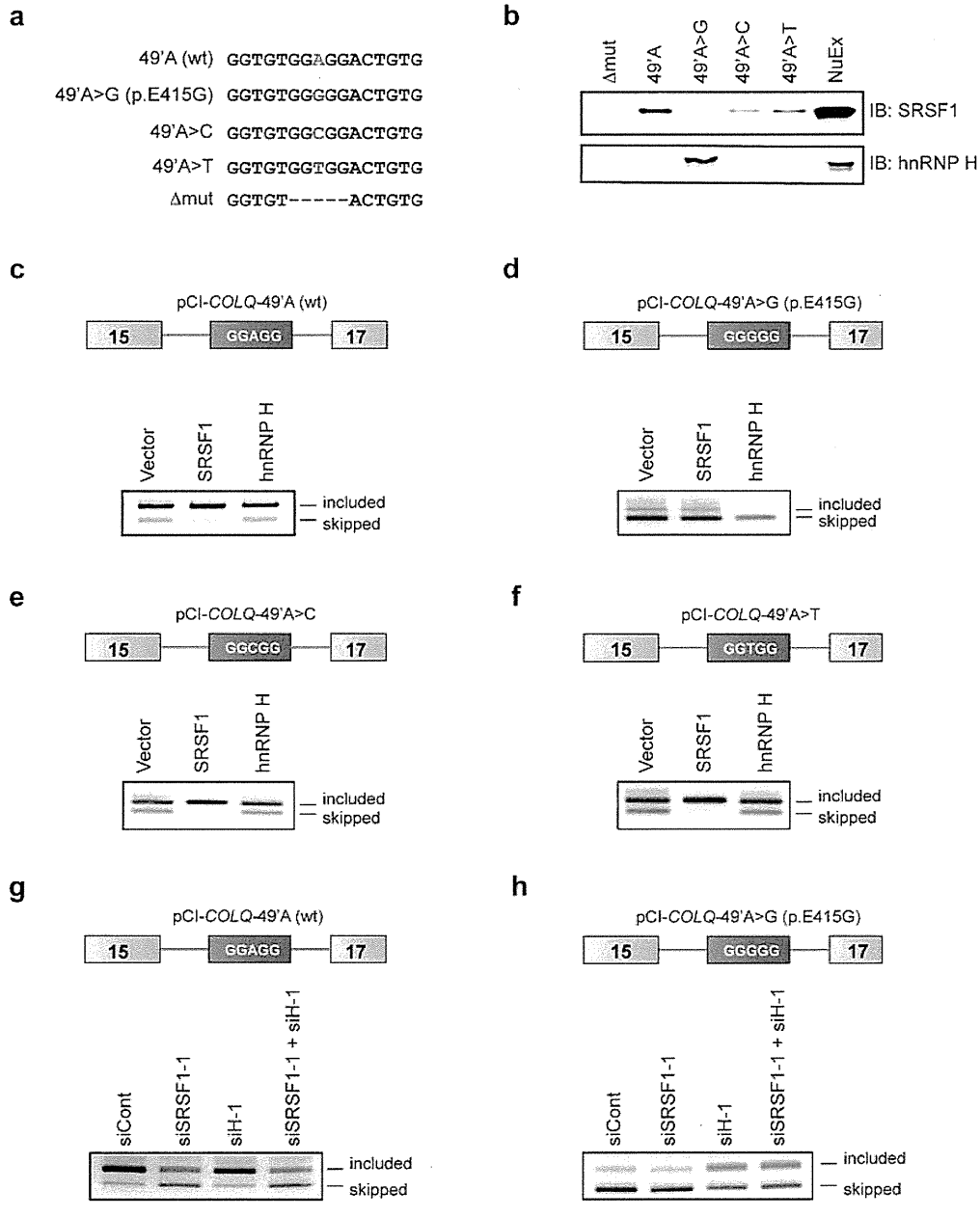

Figure 4. Molecular basis of binding and functional regulation of SRSF1 and hnRNP H. (**a**) Sequences of RNA probes carrying wild-type nucleotide (A), the patient's mutation (G), and artificial mutations (C, T, and Δmut) in *COLQ* exon 16. (**b**) Immunoblots (IB) of RNA affinity-purified proteins bound to each RNA probe. NuEX indicates 5% of the nuclear extract used in the assay. (**c**–**h**) RT-PCR of pCI-*COLQ* minigenes harboring the indicated motifs that are co-transfected with the indicated *trans*-factors (**c**–**f**) or siRNAs (**g**,**h**) in HeLa cells. Representative gel images of two independent experiments are shown.

p.E415G impairs splicing of the downstream intron by disrupting SRSF1-induced recruitment of U1 snRNP 70K to the downstream 5′ splice site. We next asked if the p.E415G mutation compromises removal of the upstream or downstream intron. We constructed two sets of minigenes, both of which carried either wild-type (wt) or mutant (p.E415G) sequence. The structure of upstream set was "exon 15-intron 15-exon 16" (E15E16-wt and E15E16-p.E415G), and that of downstream set was "exon 16-intron 16-exon 17" (E16E17-wt and E16E17-p.E415G). We examined the splicing efficiency of these minigenes in HeLa cells. We found that both E15E16-wt and E15E16-p.E415G were spliced to a similar extent, suggesting that p.E415G has no effect on splicing of the upstream intron (Fig. 5a). In contrast,

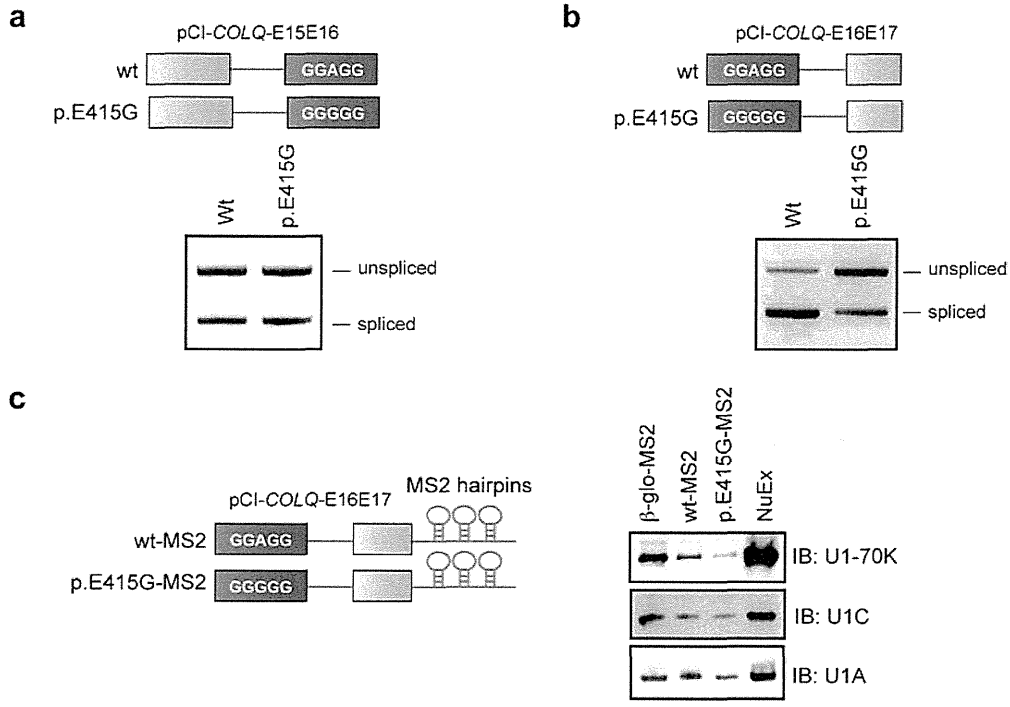

Figure 5. p.E415G compromises recognition of the downstream 5′ splice site by U1-70K. (**a**,**b**) RT-PCR of E15E16 (wt and p.E415G) and E16E17 (wt and p.E415G) minigenes in HeLa cells. (**c**) Schematic structures of MS2-attached wild-type (wt) and p.E415G substrates used for isolation of early spliceosome complex (left). Immunoblotting (IB) of purified spliceosome complex assembled on the indicated substrates (right). β-glo-MS2 is a control construct carrying MS2-attached human β-globin exon 1-intron 1-exon 2[35]. A representative gel image of three independent experiments is shown. Signal intensities of U1-70K bound to wt-MS2 are on average 2.19-fold (SD = 1.21, $n = 3$, $p < 0.05$ by Student's *t*-test) higher than those bound to p.E415G-MS2.

splicing of E16E17-p.E415G was inefficient compared to E16E17-wt (Fig. 5b), indicating that p.E415G has an inhibitory effect on splicing of the downstream intron.

We next monitored binding of the associated factors (U1 snRNPs) to the downstream 5′ splice site of the wild-type and p.E415G pre-mRNA substrates. We isolated the early spliceosome complex using the MS2-attached wild-type and p.E415G RNA substrates, and analyzed the associated factors by immunoblotting. We found that E16E17-wt-MS2 and β-globin-MS2 efficiently associated with U1 snRNP 70K (U1-70K) (Fig. 5c, lanes 1 and 2). In contrast, association of U1-70K with E16E17-p.E415G-MS2 was less efficient (Fig. 5c, lane 3). There were no appreciable differences in association of U1 snRNP C (U1C) and U1 snRNP A (U1A) between E16E17-wt-MS2 and E16E17-p.E415G-MS2. These observations along with compromised binding of SRSF1 due to p.E415G suggests that SRSF1 likely promotes recruitment of U1 snRNP 70K to the downstream 5′ splice site to achieve efficient inclusion of exon 16.

Global effects of the GGGGG and GGAGG motifs in the human and mouse genomes on pre-mRNA splicing. We next looked into the global significance of the SRSF1-binding GGAGG and hnRNP H-binding GGGGG motifs in the human and mouse genomes. We quantified splicing efficiencies of exons carrying either GGAGG or GGGGG by calculating the percent-spliced-in's (PSI's) of RNA-seq data of the human and mouse brains. In both human and mouse, PSI's of exons carrying GGGGG were significantly lower than those with GGAGG (Fig. 6, Table S3). We also observed a similar tendency in exons flanked by GGGGG- and GGAGG-bearing introns (Fig. S2, Table S3). Thus, a part of human and mouse exons exploit the SRSF1-binding GGAGG and hnRNP H-binding GGGGG motifs to regulate alternative splicing events. It was also interesting to note that human PSI's were lower than mouse PSI's, which supports the notion that humans have evolved by acquiring alternative splicing events.

Discussion

The plasticity and complexity of splicing code enable finely tuned splicing regulation in humans and expand the transcriptome and proteome diversities. However, the increasing splicing complexity predisposes to aberration of splicing regulation that can affect cellular physiology and lead to splicing diseases[29]. Among the numerous splicing codes, ESEs constitute an important class of splicing *cis*-elements

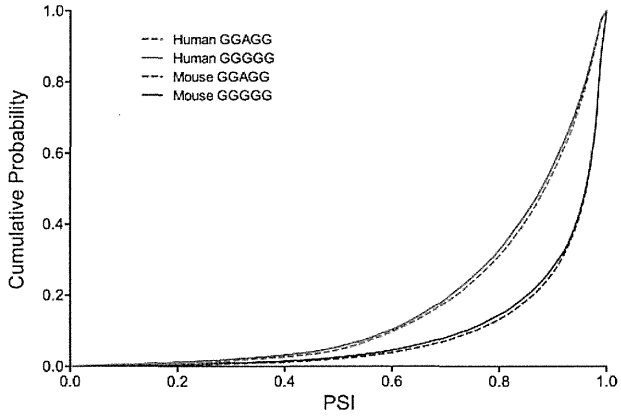

Figure 6. Cumulative distribution function (CDF) plot of PSI's of exons carrying GGGGG or GGAGG that are expressed in the human and mouse brains. PSI is a ratio of inclusion of each exon in the RNA-seq data. CDF is a fraction of exons with PSI's of less than or equal to a specific value. PSI's of exons with GGGGG are shifted to the left compared to those with GGAGG in both human and mouse, indicating that GGGGG-carrying exons are predisposed to be skipped than GGAGG-carrying exons. $P = 0.00003$ between human GGGGG and human GGAGG; $P = 0.00016$ between mouse GGGGG and mouse GGAGG by Student's *t*-test. Mean and SD are indicated in Table S3.

that were acquired in the course of evolution. However, ESEs ironically became vulnerable targets of disease-causing mutations. More than 16–20% of the missense mutations of the human mismatch-repair genes hMSH2 and hMLH1 are predicted to disrupt ESEs and affect splicing of the mutant exons[30]. Therefore, molecular dissection of the effects of ESE-disrupting mutations on RNA splicing is essential to understand disease pathomechanisms and to develop rational therapy to correct splicing errors.

We here investigate the underlying mechanisms of a pathogenic splicing mutation in *COLQ* identified in a patient with endplate AChE deficiency, which causes aberrant skipping of a constitutive exon 16[21]. We demonstrate that the mutation disrupts an ESE and *de novo* generates an ESS to induce exclusive exon skipping. We show that the ESE in *COLQ* exon 16 harbors a high affinity-binding motif for SRSF1. Introduction of artificial mutations in the middle of the core GGAGG motif reveals that SRSF1 preferentially binds to 'A' but not to 'G', 'C', or 'T'. Although binding of SRSF1 to the patient's 'G' mutation is the weakest among the three mutant nucleotides, the difference is marginal. Nevertheless, the 'G' mutation markedly causes exon skipping, whereas the effects of artificial 'C' and 'T' mutations on splicing are moderate. Exclusive binding of hnRNP H to the 'G' mutation is likely to have an additional splicing silencing effect and to account for marked skipping of exon 16. We demonstrate that lack of SRSF1 and gain of hnRNP H is the underlying cause of exclusive skipping of exon 16 in the patient's muscle.

Demonstration of aberrant splicing of *COLQ* not only uncovers the splicing maladies at the patient's endplates, but also allows us to understand SRSF1-mediated splicing regulation under physiological conditions. To further characterize this regulation in cellular context, we examined splicing of endogenous *COLQ* exon 16 in immortalized human myogenic cells (KD3)[31–34] by manipulating the expression levels of SRSF1 and hnRNP H. As expected, overexpression of SRSF1 (Fig. S1c, lane 3) induces inclusion of exon 16 (Fig. S1d, lane 2), whereas overexpression of hnRNP H (Fig. S1c, lane 5) has no effect on splicing of exon 16 (Fig. S1d, lane 3). We also examined the relative expression levels of SRSF1 and hnRNP H in both HeLa and KD3 cells. We found that SRSF1 and hnRNP H are similarly expressed at RNA and protein levels in both KD3 and HeLa cells (Fig. S1e and f). In minigene analysis, we proved that hnRNP H had no effect on splicing of wild-type exon even when SRSF1 is depleted (Fig. 4g). These observations suggest that loss of SRSF1-binding motif (GGAGG) and acquisition of hnRNP H-binding motif (GGGGG) are critical determinants of aberrant skipping *COLQ* exon 16 at the patient's endplates. The patient may somehow overexpress hnRNP H at the endplates, which may have exacerbated aberrant splicing of *COLQ* exon 16.

Point mutation-mediated transition of splicing *trans*-factors and splicing antagonism between two *trans*-factors is also evident in other genes expressed at the NMJ. We reported a point mutation in *CHRNA1* encoding the acetylcholine receptor (AChR) α subunit in a patient with CMS, which switches binding of a splicing-suppressing RNA-binding protein hnRNP L to a splicing-enhancing RNA-binding protein hnRNP LL[35]. The switch induces inclusion of a non-functional exon P3A into *CHRNA1* transcript, which subsequently nullifies expression of AChR on the cell surface and leads to endplate AChR deficiency. In contrast to hnRNPs L and LL in *CHRNA1*, SRSF1 and hnRNP H do not compete for an identical binding site in *COLQ*. Similar antagonistic splicing regulation is also observed in *SMN1* and *SMN2* pre-mRNAs. *SMN1* and *SMN2* are closely related paralogs with only a single nucleotide substitution (C in *SMN1* and T in *SMN2*) at the 6th nucleotide of exon 7. SRSF1 induces inclusion of exon 7

in *SMN1*[36,37]. The C-to-T substitution in *SMN2* gains binding of a splicing-suppressing hnRNP A1[38,39]. Additionally, the C-to-T substitution in *SMN2* may[36,37] or may not[38,39] attenuates binding of SRSF1. Thus, antagonistic splicing regulation by binding of antagonistic splicing *trans*-factors to similar motifs can occur in both physiological and pathological conditions.

Dissection of mechanistic basis of splicing of *COLQ* exon 16 reveals that p.E415G disrupts splicing of the downstream intron, but has no effect on splicing of the upstream intron. Analysis of the purified spliceosome complex reveals that p.E415G inhibits the association of U1 snRNPs in early spliceosome complex by disrupting the recruitment of U1-70K in the downstream 5′ splice site. This suggests that SRSF1 probably enhances recognition of the downstream 5′ splice site of *COLQ* exon 16 by U1 snRNP 70K. Previous studies have repeatedly demonstrated that SRSF1 promotes early spliceosome assembly by interacting with U1-70K[40–43]. The RNA recognition domains (RRM) of SRSF1 promotes the bridging of the RRM of U1-70K to pre-mRNA[40–43], thereby allowing multiple binary interactions including RNA-protein, protein-protein, and RNA-RNA, which are essential for the stability of spliceosomal E complex. Therefore, partial dissociation of U1-70K from spliceosome E complex formed on the p.E415G pre-mRNA substrate is likely due to loss of SRSF1-binding. However, we cannot exclude the possibility that hnRNP H exerts an additional inhibitory effect on recruitment of U1 snRNPs to the 5′ splice site.

Having characterized the antagonistic splicing regulation mediated by SRSF1 and hnRNP H that bind to GGAGG and GGGGG, respectively, we next examined the global antagonistic splicing regulation of the GGAGG and GGGGG motifs in the human and mouse genomes. Analysis of RNA-seq data of the human and mouse brains reveals that exons carrying GGGGG have a higher ratio of exon skipping compared to those carrying GGAGG in both human and mouse. Therefore, the hnRNP H-binding GGGGG motif and the SRSF1-binding GGAGG motif are likely to regulate alternative splicing events in some exons. Thus, a single nucleotide substitution that occurred in the course of evolution potentially increases the proteome diversity by activating or suppressing alternative splicing, and the GGGGG and GGAGG motifs are likely to be a representative pair used in alternative regulation of splicing events.

Materials and Methods

Patient. The current study was in accord with and approved by the Institutional Review Boards of the Mayo Clinic and Nagoya University Graduate School of Medicine. The patient gave written informed consent to participate in the study. The studies were performed in accordance with the relevant guidelines.

Cell culture and transfection. HeLa cells were cultured in the Dulbecco's minimum essential medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich). HeLa cells were transfected using FuGENE 6 (Roche) according to manufacturer's instructions, unless otherwise indicated. Immortalized human myogenic cells (KD3) were grown and transfected as described previously[31].

Construction of *COLQ* minigene for splicing analysis. To construct a *COLQ* minigene, a 5-kb fragment spanning the 5′ end of exon 15 to the stop codon in exon 17 (Fig. 1c) was amplified by PCR using human genomic DNA isolated from HeLa cells. The forward primer carried an XhoI restriction site followed by the Kozak consensus sequence, 5′-CCACCATG-3′, at the 5′ end. The Kozak sequence was introduced to retain the normal open reading frame of *COLQ*. The reverse primer harbored a NotI restriction site at the 5′ end. The PCR amplicon was cloned into a cytomegalovirus-based expression vector pCI (Promega). The patient's mutation and artificial mutations were engineered into the pCI minigene using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

RT-PCR for splicing analysis. Total RNA was extracted 40 h after transfection using Trizol (Invitrogen), followed by DNase I treatment. cDNA was synthesized with an oligo-dT primer using ReverTra Ace (Toyobo). PCR amplifications were performed by GoTaq (Promega), using primer pairs 5′-CAGCTGACCCCTTTCTACCC-3′ and 5′-AGCGGCAGGGCGTGGAGT-3′.

RNA affinity purification assay. Biotinylated RNAs were synthesized with the RiboMAX System (Promega) using a PCR-amplified fragment. The PCR-amplicon was generated by annealing two primers followed by overlap extension PCR[44]. Forward primer carried the T7 promoter sequence at the 5′ end. In each 20 μl reaction, 2 μg DNA template was transcribed by T7 RNA polymerase in the presence of 7.5 mM UTP, 7.5 mM ATP, 7.5 mM GTP, 4.5 mM CTP and 3.0 mM Biotin-14-CTP (Invitrogen).

The RNA affinity purification method was slightly modified from the previously reported protocols[35]. Biotinylated RNAs (0.75 nmol) and HeLa nuclear extract (40 μl) (CilBiotech) were mixed in a binding buffer [20 mM HEPES pH 7.8, 150 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.05% Triton X, 1× Protease Inhibitor Cocktail (Active Motif)]. A binding reaction of 500 μl was incubated at 30 °C for 2 h with gentle agitation. In parallel, 50 μl streptavidin-conjugated beads (Streptavidin-sepharose, GE Healthcare) were blocked with a 1:1 mixture of 1 ml binding buffer containing yeast tRNA (0.1 mg/100 μl of beads) and 1 ml PBS containing 4% BSA at 4 °C with rotation for 1 h. After blocking, the beads were washed twice in 1 ml binding buffer and mixed with the binding reaction for 2 h at 4 °C with gentle rotation. After washing the beads four times with 1 ml binding buffer, RNA-bound proteins were harvested

in SDS loading buffer by boiling at 95 °C for 5 min. The purified proteins were fractionated on a 10% SDS-polyacrylamide gel and stained with Coomassie blue or resolved by immunoblotting.

Mass spectrometry. The band of interest was excised from the Coomassie blue-stained gel and processed for in-gel digestion by Trypsin Gold (Promega) according to the manufacturer's protocols. Nanoelectrospray tandem mass analysis was executed using an LCQ Advantage Mass Spectrometry System (Thermo Finnigan) combined with a Paradigm MS4 HPLC System (Michrom BioResources) equipped with a Magic C18AQ column of 0.1 mm in diameter and 50 mm in length (Michrom BioResources). Reversed-phase chromatography was performed with a linear gradient (0 min, 5% B; 45 min, 100% B) of solvent A (2% acetonitrile with 0.1% formic acid) and solvent B (90% acetonitrile with 0.1% formic acid) at an estimated flow rate of 1 μl/min. Ionization was performed with an ADVANCE Captive Spray Source (Michrom BioResources) with a capillary voltage at 1.7 kV and temperature of 150 °C. A precursor ion scan was carried out using a 400–2000 mass to charge ratio (m/z) prior to MS/MS analysis. Multiple MS/MS spectra were resolved by the Mascot program version 2.4.1 (Matrix Science).

siRNA-mediated knocking down and splicing analysis of minigenes. We synthesized siRNA for human SRSF1 with the sequence of 5′-CCAAGGACAUUGAGGACGUTT-3′ (Sigma Genosys). We previously synthesized siRNA for human hnRNP H[26]. To rule out the possible off-target effects, a second set of siRNAs were similarly synthesized: 5′-GGAAAGAAGAUAUGACCUATT-3′ for human SRSF1 and 5′-GGAAGAAAUUGUUCAGUUC-3′ for human hnRNP H. The control siRNA was AllStar Negative Control siRNA (1027281) by Qiagen.

Cells were plated 24 h before transfection in a six-well culture plate (1.5×10^5 cells/well). The transfection reagent included each siRNA duplex at a final concentration of 30 nM in the Opti-MEM medium, 1 μl Lipofectamine 2000 (Invitrogen), and 500 ng of the minigene in 100 μl DMEM. Three days after incubation at 37 °C, the cells were harvested and were subjected to immunoblotting analysis. Total RNA was also isolated from the harvested cells and RT-PCR was performed for splicing analysis.

cDNA overexpression and minigene splicing. To construct an expression vector of human SRSF1, we first amplified human SRSF1 cDNA by RT-PCR using total RNA from human skeletal muscle (Clontech). We then cloned the cDNA amplicon into pCDNA3.1D/V5-His TOPO vector (Invitrogen) according to manufacturer's instructions to obtain pcDNA-SRSF1. The construction of human hnRNP H cDNA in pCDNA3.1D/V5-His TOPO vector (pcDNA-hnRNP H) was previously described[26]. Prior to transfection, cells were plated 24 h in a six-well culture plate (1.5×10^5 cells/well) and transfected with 1 μg of the expression construct, 500 ng of the minigene, and 3.5 μl of FuGENE 6 (Roche) in 100 μl Opti-MEM medium according to the manufacturer's instructions. Three days after incubation at 37 °C, the cells were harvested and were subjected to immunoblotting analysis. For splicing analysis, total RNA was also isolated from the harvested cells and RT-PCR was performed.

Harvesting cells for immunoblotting. Cells were washed twice in PBS and harvested in PBS with 1 × Protease Inhibitor Cocktail. After centrifugation at 2,000 × *g* for 5 min, the pellets were suspended in buffer A (10 mM HEPES-NaOH pH 7.8, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.1% Nonidet P-40, 1 × Protease Inhibitor Cocktail) and kept for 30 min on ice. Following sonication, samples were centrifuged at 20,000 × *g* for 5 min. The supernatants were collected as total cell lysate.

MS2-mediated artificial tethering of *trans*-factor. Artificial tethering was performed by co-transfection of a reporter minigene and an effector construct as previously described[35]. We introduced the MS2-binding site (5′-ACATGAGGATCACCCATGT-3′)[35] in the minigene by replacing the native target using the QuikChange Site-Directed Mutagenesis Kit, so that effector molecule can bind to the artificially inserted target site in the reporter minigene. To construct pcDNA-SRSF1-MS2, an insert encoding MS2 was isolated from pcDNA-hnRNP L-MS2[35] using XhoI and XbaI restriction enzymes, purified, and cloned into the respective sites of pcDNA-SRSF1. We previously made pcDNA-hnRNP H-MS2 expressing hnRNP H-MS2[26] and pcDNA-MS2 expressing MS2 alone[35].

Splice site function and early spliceosomal complex assays. We constructed E15E16 (wt and p.E415G) minigenes spanning *COL9* exon 15 to 16 and E16E17 (wt and p.E415G) minigenes spanning *COL9* exon 16 to 17 in pcDNA3.1D/V5-His-TOPO vector (Invitrogen). Amplicons were generated by PCR using pCI-*COL9* (wt or p.E415G), and cloned into pcDNA3.1D/V5-His-TOPO vector.

We introduced three copies MS2-coat protein binding hairpin sequences at the 3′ end of E16E17 (wt and E415G) constructs using the megaprimer method[45]. At first, we PCR-amplified a fragment harboring three copies MS2-coat protein binding hairpin sequences from pSP64-MS2 vector[35] with the primers carrying complementary sequences to E16E17 minigene where the MS2-sequences is being inserted. The PCR amplicon was used as a megaprimer for the QuikChange site-directed mutagenesis system. These vectors were used as templates to generate MS2-attached RNA substrates of E16E17 (wt and p.E415G) for isolation of early spliceosomal complex. As control, we used MS2-attached human β-globin exon 1-intron 1-exon 2 construct (pSP64-HβΔ6-MS2) as previously described[35].

MS2-affinity isolation of early spliceosomal complex. One pmol of the RNA probe (β-globin-MS2, E16E17-wt-MS2, or E16E17-p.E415G-MS2) was incubated with a 20-fold molar excess of MS2-MBP fusion protein[46] before mixing it with HeLa nuclear extract. Fifty μl of HeLa nuclear extract was pre-treated with 10 μl (bead volume) of amylose resin (New England Biolabs) overnight at 4 °C. The pre-treated HeLa nuclear extract was incubated at 37 °C for 30 min with a mixture of the RNA probe and the MS2-MBP fusion protein at final concentrations of 60 mM KCl and 25% HeLa nuclear extract. Then amylose resin beads (20 μl) was added in the mixture and incubated at 4 °C for 30 min with gentle rotation. After washing the resin four times with wash a buffer (20 mM HEPES pH 8.0, 150 mM KCl, and 0.05% Triton X-100), bound proteins were eluted with 10 mM maltose solution and subjected to SDS-PAGE followed by immunoblot analyses.

Analysis of the GGGGG and GGAGG motifs in the human and mouse genomes. To understand the effects of the SRSF1-binding GGAGG motif and hnRNP H-binding GGGGG motif on pre-mRNA splicing in the human and mouse genomes, we analyzed RNA-seq of the brains of human (Illumina BodyMap 2.0 at http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-513/) and mouse[47]. RNA-seq of human and mouse brains had 63,966,169 and 93,246,802 paired-end reads, respectively. RNA-seq fastq files were mapped to the human genome hg19/GRCh37 or the mouse genome mm9 using TopHat version 2.0.12[48]. The mapping efficiency was 87.0% and 78.5%, respectively. The mapped reads were analyzed at the transcript level with Cufflinks version 2.2.1[49]. Among 217,852 and 206,107 exons annotated in Ensembl release 65, 114,971 (52.8%) and 128,785 (62.5%) exons were expressed in the human and mouse brains, respectively. The numbers of GGGGG- and GGAGG-bearing exons among the expressed exons are shown in Supplementary Table S1. The copy numbers of GGGGG- and GGAGG-motifs within an exon are shown in Supplementary Table S2. The percent-spliced-in (PSI) values of the expressed exons carrying GGGGG or GGAGG were calculated using MISO version 0.5.2[50]. We compared PSI values of the motif-bearing and motif-lacking exons. Cumulative distribution functions were plotted with Prism 6.0f (GraphPad software).

Antibodies. Antibodies used in this study were anti-SRSF1 (32–4500, Invitrogen), anti-U1-snRNP 70K (U1-70K) (H111, kindly provided by Akila Mayeda, Division of Gene Expression Mechanism, Fujita Health University), anti-U1 snRNP C (U1C) (4H12, Sigma-Aldrich), anti-U1 snRNP A (U1A) (PA5-27474, Thermo Fisher Scientific Pierce), anti-His-tag (D293-1, Medical & Biological Laboratories) and anti-GAPDH (Sigma-Aldrich).

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Author Contributions

K.O. and A.G.E. conceived the project. M.A.R. and A.M. designed experiments; M.A.R. performed most of the experiments with the help of Y.A., F.N., M.N., K.B.A. and J.T. performed *in silico* analyses; M.A.R., A.G.E. and K.O. wrote the paper.

Additional Information

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TRIALS

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TRIALS

STUDY PROTOCOL Open Access

Adding smartphone-based cognitive-behavior therapy to pharmacotherapy for major depression (FLATT project): study protocol for a randomized controlled trial

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Abstract

Background: Major depression is one of the most debilitating diseases in terms of quality of life. Less than half of patients suffering from depression can achieve remission after adequate antidepressant treatment. Another promising treatment option is cognitive-behavior therapy (CBT). However, the need for experienced therapists and substantive dedicated time prevent CBT from being widely disseminated.

In the present study, we aim to examine the effectiveness of switching antidepressants and starting a smartphone-based CBT program at the same time, in comparison to switching antidepressants only, among patients still suffering from depression after adequate antidepressant treatment.

Methods/design: A multi-center randomized trial is currently being conducted since September 2014. The smartphone-based CBT program, named the "Kokoro-App," for major depression has been developed and its feasibility has been confirmed in a previous open study. The program consists of an introduction, 6 sessions and an epilogue, and is expected to be completed within 9 weeks by patients. In the present trial, 164 patients with DSM-5 major depressive disorder and still suffering from depressive symptoms after adequate antidepressant treatment for more than 4 weeks will be allocated to the Kokoro-App plus switching antidepressant group or the switching antidepressant alone group. The participants allocated to the latter group will receive full components of the Kokoro-App after 9 weeks.

The primary outcome is the change in the total score on the Patient Health Questionnaire through the 9 weeks of the program, as assessed at week 0, 1, 5 and 9 via telephone by blinded raters. The secondary outcomes include the change in the total score of the Beck Depression Inventory-II, change in side effects as assessed by the Frequency, Intensity and Burden of Side Effects Rating, and treatment satisfaction.

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Discussion: An effective and reachable intervention may not only lead to healthier mental status among depressed patients, but also to reduced social burden from this illness. This paper outlines the background and methods of a trial that evaluates the possible additive value of a smartphone-based CBT program for treatment-resistant depression.

Trial registration: UMIN-CTR: UMIN000013693 (registered on 1 June 2014)

Keywords: Behavior therapy, Cognitive therapy, Computer-assisted therapy, Depression, Randomized controlled trials

Background

Major depression is the second leading cause of deterioration of quality of life in humankind [1]. The economic loss due to depression is estimated at approximately 2 trillion yen per year in Japan [2]. Approximately 30,000 people a year die due to suicide in Japan. Half of these suicide victims are estimated to suffer from depression immediately before committing suicide [3].

The first-line treatment for depression in clinical settings is pharmacotherapy, especially antidepressant treatment; however, less than 50 % of patients receiving acute-phase antidepressant treatment for 2 to 4 months can achieve remission [4]. Other effective treatment options for depression include cognitive-behavior therapy (CBT); this has been shown to be as efficacious as pharmacotherapy [5] and to be more efficacious when combined with pharmacotherapy than pharmacotherapy alone [6]. CBT can, therefore, be a viable treatment option not only for patients preferring CBT to pharmacotherapy but also for patients still suffering from depression after an adequate trial of antidepressant treatment.

However, patients willing to receive CBT can rarely do so even in developed countries, because a typical course of CBT consists of sixteen 1-hour face-to-face sessions led by an experienced therapist. On the other hand, CBT delivered via the Internet or computers has been recently provided in Western countries, including Australia, the United Kingdom, the Netherlands and Sweden. The efficacy of computer-based or Internet-based CBT has been examined in previous systematic reviews. A systematic review and meta-analysis, combining results from 6 randomized controlled trials (RCTs) with 645 participants, estimated an effect size of computer-based CBT to be 0.78 (95 % confidence interval (CI): 0.59 to 0.63) in comparison with treatment as usual (TAU) or waiting-list controls [7]. Another systematic review combining results from 16 RCTs with 2807 participants showed that computer-based CBT led to a greater proportion of dropouts but to better efficacy at an effect size of 0.48 (0.33 to 0.63) in the short-term follow up than those in control conditions, whilst to neither superiority nor inferiority in the long term [8].

In the wake of recent developments in information and communication technology (ICT), CBT delivered via smartphones can be a better treatment option for depression than a computer-based one in terms of accessibility and portability. A clinical trial involving 52 participants in the community has shown that smartphone-based CBT was not inferior to computer-based CBT at 3-month follow up [9].

Given the vast number of patients with depression and the still very limited accessibility of effective CBT for them, it will be very meaningful and helpful to develop a CBT program taking advantage of this rapidly evolving ICT. In the present study, we aim to examine the effectiveness of adding a smartphone-based CBT program to switching antidepressants in comparison to that of switching antidepressants alone, for patients still suffering from depression after adequate antidepressant treatment. Among antidepressants, a systematic review and multiple-treatment meta-analysis evaluating the comparative efficacy of 12 newer antidepressants has suggested that escitalopram and sertraline are the most favorable in terms of efficacy and acceptability [10]. We aim to examine the effectiveness of switching from the previous antidepressant to escitalopram or sertraline and starting a smartphone-based CBT program at the same time, in comparison to switching to escitalopram or sertraline only, among patients still suffering from depression after adequate antidepressant treatment. We hypothesized that adding a smartphone-based CBT program to switching antidepressants could lead to greater improvement in depression symptoms among patients with treatment-refractory depression than switching antidepressants alone.

Methods/design

Trial design

A multi-center, parallel-arm, rater-blinded RCT has been planned. Participants who still suffer from full or residual major depressive disorder after adequate antidepressant treatment will be randomly allocated to either of the two intervention arms: 1) switching antidepressants to escitalopram or sertraline plus smartphone-based CBT program the "Kokoro-App" ("kokoro" means "mind" or "heart" in Japanese) consisting of 8 sections, or 2) switching antidepressants to escitalopram or sertraline only for 8 weeks after 1 week of introductory lead-in. The primary outcome is the slope of the Patient Health Questionnaire-9 (PHQ-9) through 0 to 9 weeks. The secondary outcomes include the slope of the Beck Depression Inventory (BDI-II) through 0

to 9 weeks; treatment satisfaction at 9 weeks; continuation of antidepressant pharmacotherapy up to 9 weeks; and the slope of the Frequency, Intensity and Burden of Side Effects Rating (FIBSER) through 0 to 9 weeks.

Interventions

Smartphone-based CBT program: "Kokoro-App"

The smartphone-based CBT program the "Kokoro-App" for iPhones and iPads (Apple Inc., Cupertino, CA, USA) has been developed, based on an empirically supported CBT manual [11–13], and pilot tested for feasibility and acceptability. It consists of eight sections, including one introductory section, two sessions on self-monitoring, two sessions on behavioral activation, two sessions on cognitive restructuring, and one epilogue.

The Kokoro-App can be completed within 7 weeks at the fastest. The main program consists of dialogues between characters who explain the principles and skills of CBT (Fig. 1). Homework needs to be completed by the participant between sessions. The participant can proceed to the next session 1 week after they start the previous one and after they finish the homework. One session needs approximately 30 minutes to complete. The central trial office sends an Email to each participant to encourage him/her to complete the session and the homework once every week during the program.

The participants and their attending physicians can check the entries made by the participants into the program by looking at the website (Kokoro-App Web). They can thus discuss the contents uploaded to the Kokoro-App Web during their consultations.

Security of the data exchanged through the Internet has been certificated by Secure Sockets Layer (SSL). An identification number and a password are required to enter the program or website when: 1) a participant logs in to the program at week 0; 2) a participant logs in to the Kokoro-App Web; and 3) the attending physician logs in to the Kokoro-App Web.

Antidepressant pharmacotherapy

In the present study, antidepressants that the participants had been taking before entry to the study will be switched to escitalopram or sertraline. A systematic review and a multiple-treatment meta-analysis have suggested that escitalopram and sertraline are the most favorable in terms of the efficacy and acceptability among 12 newer antidepressants [10]. The attending physician will start escitalopram or sertraline at entry (week 0) and aim to stop antidepressants other than these two by week 5 and to prescribe either 5–10 mg/day of escitalopram or 25–100 mg/day of sertraline at week 5.

Fig. 1 Screen shots from the Kokoro-App on an iPhone

Concomitant interventions

From week 0 through week 9, mood stabilizers (e.g., lithium, valproic acid, carbamazepine and lamotrigine), antipsychotics, electroconvulsive therapy, repetitive transcranial magnetic stimulation, individual CBT, and individual interpersonal therapy will not be allowed. Two antidepressant drugs may be prescribed until week 5 while the drug previously prescribed before entry is tapered and discontinued. After week 5, either escitalopram or sertraline should be prescribed as antidepressant monotherapy.

Any psychotherapy that is not specifically designed for depression, anxiolytics and hypnotics can be prescribed, and dosage of these can be changed between week 0 and week 9. Group psychotherapy during this period is allowed, but may not be changed or started.

The period after the assessment at week 9 is a follow-up phase, and any treatment is allowed at the attending physician's discretion as TAU.

Participants

The inclusion criteria for the participants are:

1) Men or women aged between 25 and 59 years upon entry into the study. We will limit participants to those aged 25 or older because patients younger than this age have been reported to be susceptible to increased suicidality after taking antidepressants in comparison with those taking placebo [14];
2) Primary diagnosis as major depressive disorder without psychotic features, according to the *Diagnostic and Statistical Manual of Mental Disorders, version 5* (DSM-5). This is confirmed by the treating psychiatrist through the semi-structured interview using the Primary Care Evaluation of Mental Disorders (PRIME-MD) [15]. It is not necessary that the patient satisfies the full criteria for a current major depressive episode upon entry. As long as the patient does not experience remission lasting for 2 months or more, the patient is considered to be in the same major depressive episode. Comorbid secondary diagnosis of anxiety disorders is allowed;
3) Not having taken either escitalopram or sertraline for the current episode;
4) Being antidepressant-resistant, defined as scoring 10 or more on the BDI-II at entry after taking one or more kinds of antidepressants at an adequate dosage for 4 or more weeks (corresponding to Stage I, II or III according to the criteria by Thase and Rush [16]), and it is judged by the attending physician that the patients should be switched to escitalopram or sertraline;
5) Taking only one kind of antidepressant at entry, and not taking any antipsychotics or mood stabilizers. Concomitant use of anxiolytics or hypnotics is allowed;
6) Willing to do the Kokoro-App program, and being judged suitable for the program by the attending physician;
7) Being used to smartphones, which is confirmed by the following conditions: a) the patient uses an iPhone, iPad (Apple Inc., Cupertino, CA, USA), Android smartphone or an equivalent in daily life; b) has an Email address for daily use; and c) has a mobile phone number for daily use;
8) Being an outpatient at entry, and having no plan to be hospitalized more than 1 week for any reason within 4 months;
9) Having no plan to transfer to a different hospital within 4 months;
10) Being able to respond to assessments about symptoms and side effects via telephone; and
11) Being able to understand and sign a written informed consent.

The exclusion criteria include:

1) Having taken any of the following interventions for the current episode: a) monoamine oxidase inhibitors (corresponding to Stage IV according to the criteria of Thase and Rush [16]); b) electroconvulsive therapy or repetitive transcranial magnetic stimulation (corresponding to Stage V [16]); c) both escitalopram and sertraline; or d) face-to-face individual CBT or face-to-face interpersonal psychotherapy;
2) Any of the following comorbid illnesses: a) past history of schizophrenia, schizoaffective disorder, or bipolar and related disorders according to DSM-5; or b) current diagnosis of neurocognitive disorders, feeding and eating disorders, substance-related and addictive disorders or borderline personality disorder;
3) Imminent risk of suicide as judged by the treating physician;
4) Physical illnesses possibly interfering with pharmacotherapy by escitalopram or sertraline, including: a) a possible prolonged QT syndrome, as judged through an interview with the attending physician; b) prolonged heart-rate corrected QT interval (QTc) in the electrocardiogram within 1 month (male: QTc > 450 ms, female: QTc > 470 ms [17]); c) taking medication known to prolong the QT interval and being judged unsuitable to take escitalopram or sertraline by the attending physician; d) severe or extremely unstable cardiovascular disease, such as current or past severe bradycardia, congestive heart failure or hypokalemia; e) severe or extremely unstable hepatic, renal, respiratory, blood,

or endocrine function, or central nervous system disease or head injury; f) terminal stage of physical illnesses; g) currently taking pimozide; or h) history of hypersensitivity or allergy to escitalopram or sertraline;
5) Being currently pregnant or breastfeeding;
6) Currently participating in another clinical intervention study;
7) Family members living with the researchers of the present study; or
8) Being unable to understand the written Japanese language.

Trial sites

A participating trial site must fulfill the following eligibility criteria:

1) Have a department of psychiatry or of psychosomatic medicine;
2) The principal trial physician and all the participating trial physicians at the site have understood the study protocol; and
3) The site is within mobile phone range.

On the other hand, a trial site will be ineligible if it satisfies one or more of the following conditions:

1) The principal trial physician withdraws consent for participating in the study;
2) No participant is registered for 6 months; or
3) The steering committee of the present trial judges the site to be inappropriate to recruit participants.

Initial trial sites include Nagoya City University Hospital in Aichi, Waseda Clinic in Gifu, Kochi University Hospital and Atago Hospital in Kochi, Japan.

Assessment measures

Screening tools

Primary Care Evaluation of Mental Disorders (PRIME-MD) Major depressive disorder, according to DSM-5, will be diagnosed through semi-structured interview using PRIME-MD [18] by the attending physicians at baseline.

The Beck Depression Inventory-II (BDI-II) The BDI-II is a 21-item self-report instrument to measure the severity of depression. Its first version was developed in 1961 [19] and a major revision was undertaken in 1996 to make the scale more congruent with the modern diagnostic criteria for major depression [20]. Good reliability and validity have been reported for the Japanese version [21].

The time frame for evaluation is set to the past 2 weeks including the day of assessment. The BDI-II will be used for a screening tool at week 0 and participants with a total score of 10 or more will be included in the present study.

Electrocardiogram The QTc will be checked using an electrocardiogram within 1 month before week 0. The QTc will be deemed prolonged if > 450 ms for males and > 470 ms for females, respectively, and will be checked again at week 1.

Primary outcome measure

Patient Health Questionnaire-9 (PHQ-9) The PHQ-9 consists of the 9 diagnostic criteria items of the DSM-4 [15]. Each item is rated between 0 = "Not at all" through 3 = "Nearly every day," making the total score range between 0 and 27. Excellent test-retest reliability and internal consistency reliability have been reported [15, 22]. Good construct validity has been demonstrated through associations with various severity indices [23]. The sensitivity to change is as good as or better than extant scales [24].

Secondary outcome measures

Patients' satisfaction with treatment The following 2 questions will be graded on a scale of 100 by patients: 1) "To what extent has the treatment met your needs for these two months? Please answer on a scale of 1 to 100, presuming 60 as a passing mark"; and 2) "To what extent have you been satisfied with the treatment for these 2 months? Please answer on a scale of 1 to 100, presuming 60 as a passing mark."

Continuation of protocol treatment up to week 9 In order to evaluate whether the Kokoro-App may have interfered with pharmacotherapy as defined in the protocol, continuation rates of protocol pharmacotherapy at week 9 will be compared between the intervention and control groups. Discontinuation of pharmacotherapy is defined as not taking a prescribed antidepressant for more than 1 week due to any reason as judged by the attending physician.

Frequency, Intensity, and Burden of Side Effects Rating (FIBSER) FIBSER was originally used in STAR*D as a global rating scale for side effects. The FIBSER consists of 3 domains evaluating the frequency, intensity and severity of side effects, each of which has a score from 0 to 6. The reliability and validity of the FIBSER have been confirmed [25].

K6 K6 is a very short (6-item) self-report questionnaire to screen for common mental disorders and to evaluate the severity of general psychological distress [26]. Good validity and reliability have been confirmed [27], and its area under the curve compared to "gold-standard" diagnoses of

depressive and anxiety disorders was 0.94 [28]. The Japanese version has been validated [28].

Procedures

Screening

Physicians (psychiatrists) in the recruiting sites will identify patients possibly eligible for the study by checking diagnosis of major depressive disorder according to DSM-5 by means of the PRIME-MD. They will also confirm absence of a history of heart disease or current prolonged QTc by electrocardiogram within 1 month before entry, and check the other eligibility criteria (Fig. 2, Table 1).

Informed consent, installing the Kokoro-App and medication at week 0

A clinical research coordinator at a recruiting clinic (site CRC) will seek written informed consent from the candidate participant at the clinic at week 0. Participants

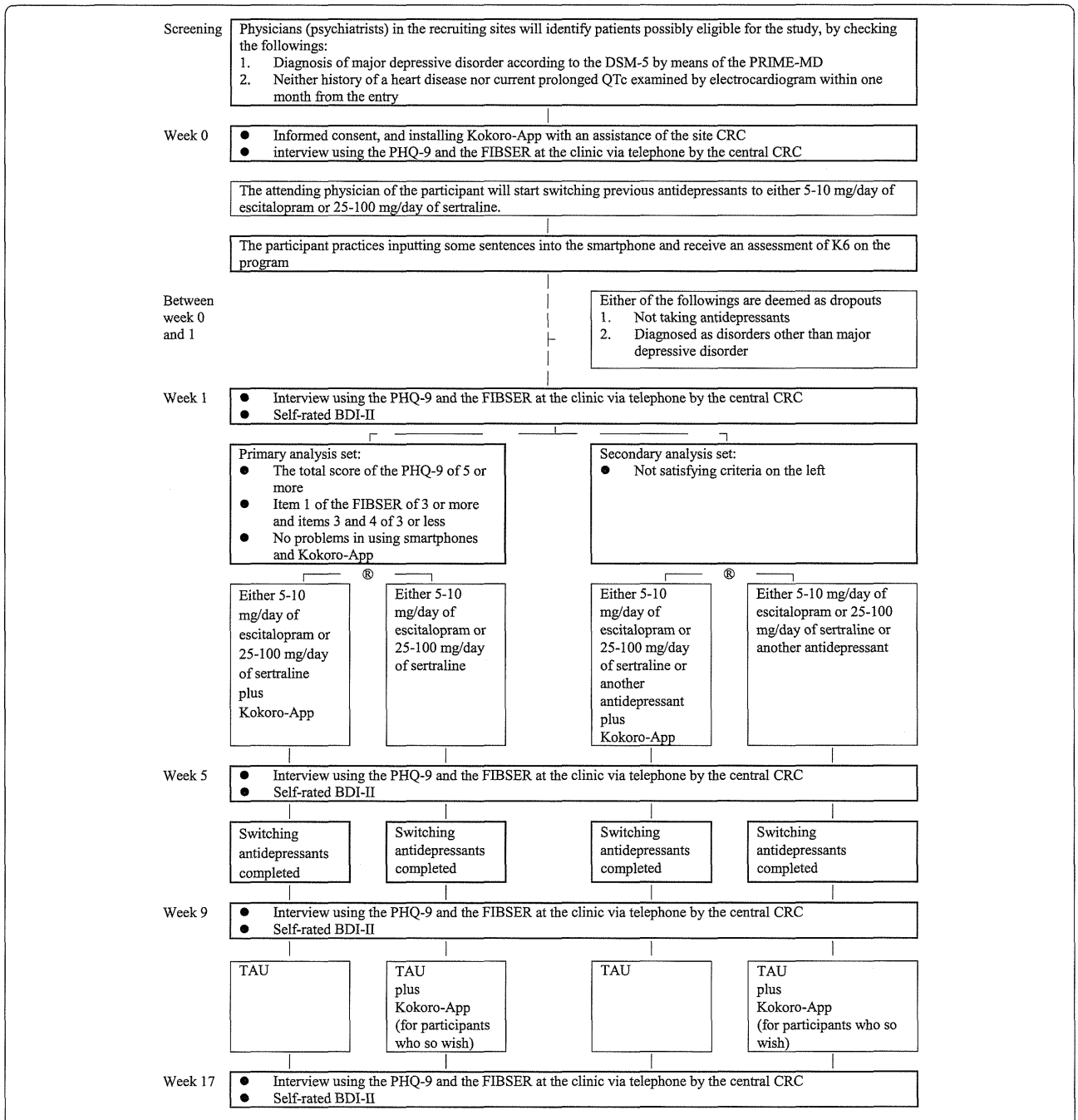

Fig. 2 Flowchart of the trial. Abbreviations: BDI-II, Beck Depression Inventory-II; CRC, clinical research coordinator; FIBSER, Frequency, Intensity, and Burden of Side Effects Rating; PHQ-9, Patient Health Questionnaire-9; PRIME-MD, Primary Care Evaluation of Mental Disorders; TAU, treatment as usual

Table 1 Schedule for the assessments

		Item	Week 0	Week 1	Week 5	Week 9	Week 17
Attending physician		PRIME-MD	√				
	Site CRC	Basic characteristics[a]	√				
		Electrocardiogram	√	√			
		Informed consent	√				
		BDI-II	√	√	√	√	√
Research CRC at the central trial office		PHQ-9	√	√	√	√	√
		FIBSER	√	√	√	√	√
		Blindness			√	√	√
		Treatment satisfaction				√	√
Administrative CRC at the central trial office		Allocation		√			
Participant	K6	√	√ (Only those allocated to Kokoro-App)	√ (Only those allocated to Kokoro-App)		√ (Only those allocated to Kokoro-App)	

BDI-II Beck Depression Inventory-II, *CRC* clinical research coordinator, *FIBSER* Frequency, Intensity, and Burden of Side Effects Rating, *K6* 6-item self-report questionnaire to evaluate the severity of general psychological distress, *PHQ-9* Patient Health Questionnaire-9, *PRIME-MD* Primary Care Evaluation of Mental Disorders
[a]Basic characteristics include sex, age, education, work status, marital status, age at first depressive episode, number of episodes, duration of the current depressive episode, information about treatment for current episode, and current history of physical illnesses

will be informed that they can withdraw their consent at any time without stating the reason and that their withdrawal will not affect the medical services they receive. The written informed consent will be obtained along with the mobile phone number of the participant, with which the central trial office can contact him or her to assess the outcomes.

Immediately after consent, all participants will install the Kokoro-App onto his or her smartphone with an assistance of the site CRC. After successful installation, the server will provide a password specific to each participant via an Email to the participant. The participant will be able to proceed to the introductory section of the Kokoro-App by entering the password. In the introduction, the participant will practice entering some sentences into the smartphone and fill in a self-report measure of psychological distress (K6). All participants will receive an interview using the PHQ-9 and the FIBSER at the clinic via telephone from the central trial office.

The attending physician of the participant will start switching the previous antidepressant at week 0 to either 5–10 mg/day of escitalopram or 25–100 mg/day of sertraline. The patient will take escitalopram if he or she has taken sertraline for the current episode, and vice versa. If the patient has taken neither escitalopram nor sertraline for the current episode, one of these two will be selected by the attending physician. The maximum dosage of escitalopram was set at 10 mg/day because: 1) the efficacy of 20 mg/day of escitalopram has not been proved to be superior to that of 10 mg/day [29, 30]; 2) the tolerability of the former has been proved to be inferior to that of the latter in terms of dropout rates due to side effects [31]; and 3) a dose response has been reported between dosage of escitalopram and prolonged

QT interval [32]. Anxiolytics and hypnotics can be used, started, increased/decreased and discontinued during the study.

Randomization and medication at week 1

An appointment at week 1 will be scheduled within 3 to 14 days from the entry. At week 1, the participant will receive an electrocardiogram and an interview, using the PHQ-9 and the FIBSER, via telephone from the central trial office. Based on these results, the participant will be classified into either of the following two groups: A) primary comparison set: those who have not responded or only partially responded to escitalopram or sertraline (defined by the total score of the PHQ-9 of 5 or more at week 1), are tolerant to escitalopram or sertraline (defined by item 1 of the FIBSER of 3 or more and items 3 and 4 of 3 or less at week 1, which means that the participant has been able to take the antidepressant for 3 days or more and the intensity and the interference with daily functions due to side effects of the medication is mild or less), and have no problems in using the smartphones and the Kokoro-App; B) secondary comparison set: those who have not satisfied any of the above criteria.

Within each group, the participants will be randomly allocated to either the combined smartphone-based CBT and antidepressant switch group (intervention group) or the antidepressant switch alone group (control group) at 1:1 ratio with a minimization method using the electronic data-capturing web program (EDC) at the central trial office. Random allocation will, therefore, be concealed. Clinics, number of antidepressants previously prescribed for the index episode (3 or more versus 2 or less), and a total score of the PHQ-9 (10 or more versus 9 or less) at week 1 will be used as stratification variables.

If the participant is allocated to the intervention group, he or she will be provided with a password to proceed to Session 1 of the Kokoro-App. If the participant is allocated to the control group, he or she will be informed that the Kokoro-App can be resumed if the participant is willing to do so after week 9.

With regard to antidepressants, if the participant is intolerant of escitalopram or sertraline prescribed at week 0 (defined by scores of 4 or more on either item 3 or 4 of FIBSER), the physician will suggest stopping the antidepressant and starting previous or new antidepressants other than escitalopram and sertraline.

Trial period: from week 1 through week 9

For all the participants, an appointment with the attending physician will be scheduled at least every 4 weeks. Visits must take place at weeks 5 and 9. The previous antidepressant must be tapered off by week 5, and either escitalopram or sertraline (or another antidepressant if the participant is intolerant of these two) must be prescribed as monotherapy at week 5. Only anxiolytics and hypnotics are allowed as psychotropic drugs other than antidepressants.

For the participants allocated to the intervention group, the physician can check the patient's progress of the Kokoro-App through the Kokoro-App Web, and discuss it with the participant during consultations. If the participants allocated to the control group express interest in CBT by week 9, the physician is allowed to suggest self-help books about CBT but not to provide any specific CBT sessions.

At weeks 5 and 9, the participant will receive an interview, using the PHQ-9 and the FIBSER, via telephone at the clinic from the central trial office. Depression severity will also be assessed with the BDI-II. At week 9, an interview using a scale on treatment satisfaction will also be administered via telephone.

Follow-up period: from week 9 through week 17

After assessments at week 9, no restrictions will be posed in terms of medications, frequency of appointments, or CBT. The participants allocated to the control group can resume the Kokoro-App if they wish to. At week 17, all the participants will receive an assessment of the PHQ-9, the FIBSER and the scale on treatment satisfaction via telephone from the central trial office as well as to fill in a self-rating scale of the BDI-II.

Stopping rules for participants

Dropouts from the trial The participants will be excluded from the intention-to-treat (ITT) cohort of the trial, if it was not possible to randomize them at week 1 or if their primary diagnosis was changed before week 1.

Deviation from protocol treatment The following cases will be considered deviation from the trial protocol; however, the participant will not be considered to have dropped out of the trial at this stage and will receive the protocol assessments:

1) When prohibited concurrent treatments or prescription of the intervention antidepressant above the maximum dosage defined in the protocol (10 mg/day for escitalopram, and 100 mg/day for sertraline) took place between week 0 and week 9;
2) When changes in treatment that are allowed to be co-administered but not to be changed between week 0 and week 9 took place;
3) If the participant cannot take any pills of sertraline or escitalopram due to side effects between week 1 and week 9;

4) If the participant develops a manic/hypomanic/mixed episode, or is diagnosed with schizophrenia or dementia between week 1 and week 9.

Discontinuation of protocol treatment If the participant meets any of the following conditions, the trial physician can stop the antidepressant or the Kokoro-App. The participant, however, will not be considered to have dropped out of the trial at this stage and will receive the protocol assessments:

1) The participant wishes to stop the protocol treatment;
2) The trial physician judges that it is difficult to continue the protocol treatment because of serious side effects;
3) The trial physician judges that the risk outweighs the benefit in continuing the protocol treatment even when no serious side effect is reported;
4) The participant becomes pregnant and the trial physician judges that the risk outweighs the benefit in continuing the protocol treatment;
5) The trial physician judges that it is inappropriate to continue the protocol treatment for some other reason.

Stopping assessment If the participant withdraws consent for assessments, he/she will not be followed up.

Blindness and reliability of assessment from the central trial office

The primary outcome (PHQ-9) and information about side effects (FIBSER) at weeks 0, 5, 9, and 17 and treatment satisfaction judged by participants at weeks 9 and 17 will be collected via telephone by central CRCs, who are kept blind to groups to which the participant has been allocated.

At week 5, 9 and 17 assessments, the blindness of the central CRCs as to the participant's treatment will be assessed by having the CRCs guess the allocated treatment by selecting one from the following: 1) I strongly believe that the patient is allocated to the combination group; 2) I guess that the patient is allocated to the combination group but am not confident; 3) I cannot tell; 4) I guess that the patient is allocated to the antidepressant alone group but am not confident; and 5) I strongly believe that the patient is allocated to the antidepressant alone group.

The inter-rater reliability of the central CRCs will be examined by comparing the assessors' ratings of audio recordings of the PHQ-9 and FIBSER, recorded in our previous study [11], with the original ratings. In addition, five recordings in the present study will be assessed again by another rater blinded to the original rating in order to evaluate the inter-rater reliability in the present study.

Reporting of adverse events and protection of participants

Definition of adverse events

An adverse event is defined as any unwanted or unintended sign (including laboratory exams), symptom or disease seen in participants of the trial. According to the "*Ethical Guidelines for Clinical Studies: Questions and Answers*," which is published by the Japanese Ministry of Health, Labor and Welfare, severe adverse events are defined as events leading to any one the following: a) death; b) threatened death; c) admission or prolongation of admission for treatment; d) enduring and severe impairment and dysfunction; or e) congenital anomaly.

When a serious adverse event occurs, the trial physician must notify the principal investigator within 48 hours, regardless of the causal relationship with the trial intervention. The principal investigator shall report to the institutional review board in Kyoto University Graduate School of Medicine within 72 hours, and notify all the co-principal investigators. The principal investigator must also notify all the collaborators at all the recruiting sites. The collaborators will take the necessary measures according to the information from the principal investigator. If it concerns an unforeseen serious adverse event, the principal investigator shall report it to the Ministry of Health, Labor and Welfare.

Foreseeable adverse events

Escitalopram Frequent side effects include: nausea (23.8 %), somnolence (23.5 %), headache (10.2 %), dry mouth (9.6 %), dizziness (8.7 %), fatigue (7.1 %), diarrhea (6.2 %), etc.

Serious side effects include: convulsion (unknown frequency), the syndrome of inappropriate anti-diuretic hormone secretion (SIADH) (unknown frequency), serotonin syndrome (unknown frequency), prolonged QT (unknown frequency), and ventricular tachycardia (unknown frequency).

Sertraline Frequent side effects include: nausea (18.9 %), somnolence (15.2 %), dry mouth (9.3 %), headache (7.8 %), diarrhea (6.4 %), dizziness (5.0 %), etc.

Serious side effects include: serotonin syndrome (unknown frequency), malignant syndrome (unknown frequency), convulsion (unknown frequency), coma (unknown frequency), liver dysfunction (unknown frequency), SIADH (unknown frequency), Lyell syndrome and toxic epidermal necrolysis (unknown frequency), and anaphylactoid symptoms (unknown frequency).