Recent findings suggest that Sonic Hedgehog (Shh), a member of the mammalian hedgehog protein family, may also contribute to nerve regeneration and repair. The hedgehog proteins were first characterized in *Drosophila* as regulators of segmental polarity during development.[11] In vertebrate development, Shh regulates the patterning of the central nervous system,[12–14] whereas the activity of another hedgehog protein, Desert Hedgehog, is restricted to the peripheral nervous system and appears to influence cellular elements of the epineural and perineural sheath.[15] In the absence of injury, Shh signaling is attenuated, at least in part, by interactions between Shh and the Shh inhibitor hedgehog-interacting protein (HIP);[16] however, Hashimoto *et al*[17] have shown that the expression of Shh increases after peripheral-nerve injury in adult rats, and that cyclopamine, which disrupts hedgehog signaling, reduces the survival of motor neurons. Furthermore, exogenous Shh protein administration has been shown to enhance angiogenesis and improve nerve function in a diabetic neuropathy model.[18]

Collectively, these observations indicate that both E2 and Shh may contribute to the neovascularization and regeneration of injured peripheral nerves in adult animals, and estrogen has recently been linked to the upregulation of Shh expression in breast-cancer cells.[19] Here, we investigated whether E2 improves functional recovery in mechanically injured nerves by activating the hedgehog-signaling pathway.

MATERIALS AND METHODS

Animal Models and Treatments

Experiments were performed in ovariectomized female wild-type (C57BL/6J) mice and in ovariectomized female heterozygous Patched-1(Ptch1)-LacZ (NLS-*Ptch1-lacZ*) or heterozygous Gli1-LacZ (NLS-*Gli1-lacZ*) mice and their wild-type littermates (The Jackson Laboratory, Bar Harbor, ME, USA) E2 in poly lactide-co-glycolide (PLGA) was kindly provided by Dr. Tabata (Kyoto University, Japan). All surgical procedures were approved by the Institutional Animal Care and Use Committee of Northwestern University's Feinberg School of Medicine and were consistent with the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health.[20] Ovariectomy was performed 2 weeks before injury and E2-PLGA, saline-PLGA, or E2 pellets (Innovative Research of America, Sarasota, FL, USA) were administered at the designated injury sight 1 week later (ie, 1 week before injury); E2-PLGA and saline PLGA were administered via subcutaneous injection, and the E2 pellets were subcutaneously implanted. Cyclopamine (50 mg/kg per day) and the estrogen receptor antagonist ICI 182,780[21] (8.3 mg/kg per day; Tocris Cookson, Bristol, UK) were intraperitoneally injected once daily from 2 days (cyclopamine) or 3 days (ICI) before injury until the time of killing. Serum E2 levels were determined with an E2-ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) as directed by the manufacturer's instructions. Nerve-crush injury was induced as described previously[22] and as summarized in the Supplementary Methods.

Nerve Function

Motor-nerve conduction velocity (MCV) and the duration of rotarod exercise were measured as summarized in the Supplementary Methods. MCV measurements in the injured limb were normalized to measurements performed in the uninjured contralateral limb. For exercise duration, the maximum of three measurements was reported for each mouse.

Vascularity and Capillary Density

Functional vascular structures were identified by injecting mice with fluorescein isothiocyanate-conjugated *Bandeiraea simplicifolia*-1 lectin (Vector Laboratories, Burlingame, CA, USA) 15 minutes before killing, and endothelial cells were identified by staining sections with anti-CD31 antibodies (BD Biosciences, San Jose, CA, USA). Capillary density was evaluated by counting double-positive tubular structures in five sections MAPK from the proximal, middle, and distal regions of both the injured and uninjured contralateral nerve (totaling 15 sections per nerve); three fields were evaluated in each section. Measurements in the injured nerve were normalized to those obtained in the uninjured nerve.

Gene Expression

For *in-vivo* assessments, the mRNA expression of Shh, smoothened (smo), Ptch1, Gli1, HIP, and vascular endothelial growth factor (VEGF) was evaluated via quantitative, real-time, reverse-transcriptase, polymerase chain reaction (qRT-PCR). Activation of Ptch1 and Gli1 expression in sections from Ptch1-LacZ and Gli1-LacZ mice was evaluated via X-gal staining. Ptch1 protein and HIP protein were identified with anti-Ptch1 and anti-HIP antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), endothelial cells were identified with anti-CD31 antibodies, and Schwann cells were identified with anti-S100 antibodies (Sigma-Aldrich, St Louis, MO, USA). Positively stained cells were counted in three sections from both the injured nerve and the uninjured contralateral nerve, three high-power fields per section; measurements in the injured nerve were normalized to those obtained in the uninjured nerve. For *in-vitro* assessments, human umbilical-vein endothelial cells (HUVECs) (Cascade Biologics, Portland, OR, USA), Schwann cells (SW-10 cells; ATCC, Manassas, VA, USA), and NIH3T3 fibroblasts (ATCC, Manassas, VA, USA) were cultured in 1×10^{-8} mol/l E2 for 6 h, then Shh, Ptch1, Gli1, and HIP mRNA expression were evaluated via qRT-PCR. Gli transcriptional activation in endothelial cells was evaluated via the Gli-luciferase assay. qRT-PCR and the Gli-luciferase assay were performed as summarized in the Supplementary Methods; qRT-PCR primer and probe sequences are provided in the Supplementary Table.

Statistical Analysis

All values are reported as mean ± s.e.m. Statistical analyses were performed with commercially available software (StatViewTM, Abacus Concepts, Berkeley, CA, USA); comparisons between two groups were tested for significance with the Mann–Whitney *U*-test, and comparisons between multiple groups were tested for significance via analysis of variance followed by *post-hoc* testing with the Tukey procedure. A P value of <0.05 was considered significant.

RESULTS

Local E2 Injection Improves Nerve Functional Recovery and Vascularity after Nerve-Crush Injury without Significantly Increasing Serum Estrogen Levels

Because of the side effects and potential health risks associated with systemically administered E2, we investigated whether the apparent benefits of systemic E2, administered as an implanted E2 pellet,[9] could be achieved with locally delivered E2. One week before surgical sciatic nerve-crush injury was induced in mice, E2 in PLGA or PLGA alone (placebo) was locally injected, or an extended-release E2 pellet was subcutaneously implanted, at the designated injury site. Local E2 administration did not significantly alter serum E2 levels, whereas serum E2 levels increased approximately 10-fold after pellet implantation (Supplementary Figure 1).

Functional recovery and motor coordination was monitored via MCV measurements performed at weekly intervals for 28 days after injury and by assessing the duration of rotarod exercise on day 28. MCV in all treatment groups was similar before nerve-crush injury and undetectable immediately afterward. MCV had improved in both E2-treatment groups on day 7 after injury, but remained undetectable in mice administered placebo, and was significantly higher in E2-treated mice than in placebo-treated mice at all subsequent time points (Figure 1a). On day 28 after injury, MCV approached pre-injury levels in both E2-treatment groups ($P<0.01$), and the duration of rotarod exercise was significantly longer in E2-treated mice than in mice administered placebo ($P<0.05$; Figure 1b). MCV and rotarod exercise measurements after E2 injection or pellet implantation did not differ significantly at any time point.

Nerve vascularity was assessed in both the injured and uninjured hind limbs of mice killed 28 days after nerve-crush injury. As observed in previous reports,[23] nerve-crush injury alone increased vascularity, even in placebo-treated limbs. Both E2 injection and E2 pellet implantation enhanced the vasculogenic response (Figure 1c), and capillary density was significantly higher ($P<0.01$) in the injured limbs of E2-treated mice than in the injured limbs of the placebo-treatment group (Figure 1d); capillary density was also significantly higher ($P<0.01$) when E2 was administered as a local injection rather than an implanted pellet.

Collectively, these observations indicate that both injected E2-PLGA and the implanted E2 pellets improve MCV, functional recovery, and nerve vascularity after injury, but the E2-PLGA injections did not lead to the significant (and potentially harmful) increases in systemic estrogen levels observed after pellet implantation. Thus, E2 was administered via subcutaneous injection, rather than pellet implantation, in subsequent experiments.

Local E2 Injection Enhances Shh-Pathway Activation after Nerve-Crush Injury

The Shh pathway is activated by nerve-crush injury[17] and contributes to vascular regeneration in response to ischemic injury.[23,24] To determine whether the E2-induced enhancement of nerve function and vascularity after injury could have occurred through elevated Shh signaling, we monitored the mRNA expression of Shh; smo, which activates downstream components of the hedgehog pathway; the Shh receptor Ptch1; the Shh transcriptional target Gli1; and VEGF, which has been linked to Shh signaling in a murine diabetic neuropathy model.[18] Injured nerves had been pretreated with local injections of E2 or placebo 1 week before injury. In the absence of injury, E2 did not alter mRNA levels of Shh (E2: 0.26 ± 0.12, placebo: 0.23 ± 0.12; normalized to 18S rRNA levels), Gli1 (E2: 59.1 ± 12.0, placebo: 60.5 ± 9.4), or VEGF (E2: 5.27 ± 2.50, placebo: 6.35 ± 4.70), and treatment of the injured limb did not alter Shh, Ptch1, smo, Gli1, or VEGF expression in the uninjured limb.

Shh expression was significantly elevated in the injured nerves of placebo-treated mice within 3 h of injury ($P<0.02$) and continued to increase through hour 24 before returning to non-injury levels on day 7 (Figure 2a). Shh expression was even higher in the injured limbs of E2-treated mice ($P<0.01$ *vs* placebo treatment from hour 6 through hour 12), but the time course of elevated Shh expression was similar in both groups. The mRNA expression of smo declined after injury but was similar in E2-treated and placebo-treated nerves at all time points (Figure 2b). Ptch1 and Gli1 expression declined in the injured nerves of placebo-treated mice through hours 6 (Ptch1) and 24 (Gli1) after injury, then slowly recovered to approximately 40% (Ptch1) and 75% (Gli1) of non-injury levels on day 7 (Figures 2c and d). The initial declines in Ptch1 and Gli1 expression were also observed in the injured nerves of E2-treated mice, but recovery was enhanced: Ptch1 expression was significantly higher ($P<0.01$) from day 3 through day 7, and Gli1 expression was significantly higher ($P<0.01$) from hour 24 through day 3, in E2-treated nerves than in placebo-treated nerves. E2 treatment was also associated with significantly higher mRNA levels of VEGF from hour 24 through day 3 (Figure 2e).

The apparent E2-induced enhancement of Shh signaling was corroborated with assessments performed in the injured and uninjured contralateral nerves of mice treated with E2 and the estrogen-receptor antagonist ICI. Three days after injury, Shh expression was significantly lower in nerves treated with both E2 and ICI than in nerves treated with E2 alone, whereas Shh expression after placebo treatment or E2-ICI co-administration was similar (Figure 2f).

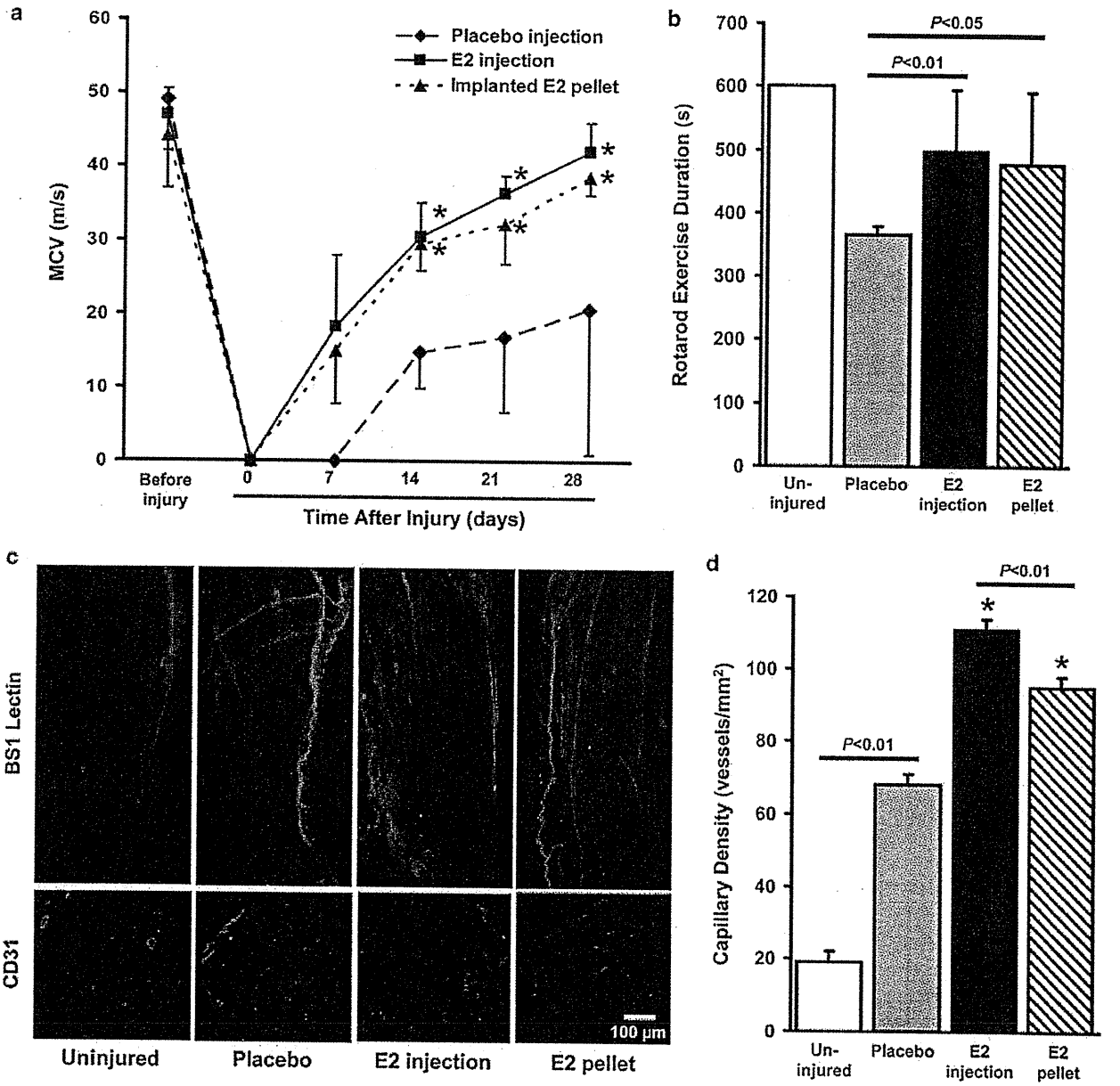


Figure 1 Local E2 injection improves the functional recovery and vascularity of injured nerves. One week before surgical sciatic nerve-crush injury, E2 (100 μg) in PLGA (to ensure extended E2 delivery) or PLGA alone (placebo) was locally injected into the designated injury site, or an extended-release E2 pellet (0.5 mg delivered over 60 days) was subcutaneously implanted into the limb. (**a**) MCV was measured before injury, immediately afterward (day 0), and at weekly intervals for the next 28 days; for clarity, only one error bar is shown per data point. (**b**) The duration of rotarod exercise was measured 28 days after injury; the duration for the uninjured group was equal to the length of the experiment (600 s, s.e.m. = 0). (**c**) Functional vasculature was identified in sections of injured nerve tissue by injecting mice with fluorescein-conjugated BS-1 lectin 15 minutes before killing on day 28. (**d**) Capillary density was evaluated by staining cross sections of the vasa nervorum from mice killed on day 28 with the endothelial-cell marker CD31; $^*P < 0.01$ *vs* placebo.

Local E2 Injection Activates Ptch1 and Gli1 Protein Expression in the Injured Nerve

To confirm that the E2-induced enhancement of Ptch1 and Gli1 mRNA expression after nerve-crush injury was accompanied by the activation of protein expression, experiments were performed in mice that carried nondisruptive insertions of a lacZ reporter gene upstream of the Ptch1- (Ptch1-LacZ mice) or the Gli1- (Gli1-LacZ mice) coding region. Assessments were performed 3 days after injury, when Ptch1, Gli1, and VEGF mRNA expression were higher in the E2-treated nerves than in the placebo-treated nerves of wild-type mice. In Ptch1-LacZ mice, Xgal staining of whole-mount sciatic nerve tissue found markedly greater evidence of Ptch1 expression at the site of nerve-crush injury in E2-treated nerves than in placebo-treated nerves (Figures 3a and b). In Gli1-LacZ mice, LacZ-expressing cells were significantly more

common in both the endoneurium and the perineurium of E2-treated nerves than in the corresponding regions of uninjured nerves ($P<0.01$), whereas only the endoneurium of placebo-treated nerves displayed evidence of Gli1 upregulation ($P<0.01$ *vs* uninjured nerves; Figures 3c and d); LacZ expression in both regions was significantly more common after E2 treatment than after placebo treatment ($P<0.01$). The expression of Gli1 mRNA was also elevated in E2-treated nerves ($P<0.01$ *vs* placebo treatment), and this enhancement was abolished by co-treatment with ICI (Figure 3e). In sections co-stained with CD31 antibodies, X-gal–CD31 double-positive cells were observed in E2-treated nerves but not in placebo-treated nerves (Figure 3f). Thus, E2 appears to enhance Shh signaling in endothelial cells after nerve-crush injury.

The E2-Induced Enhancement of Vascularity after Nerve-Crush Injury Requires Shh-Pathway Activation

To determine whether the E2-induced increase in nerve vascularity after crush injury could be attributed to enhanced Shh signaling, functional nerve vascularity and capillary density were assessed in the injured nerves of Ptch1-LacZ mice treated with placebo, with E2, with cyclopamine, which

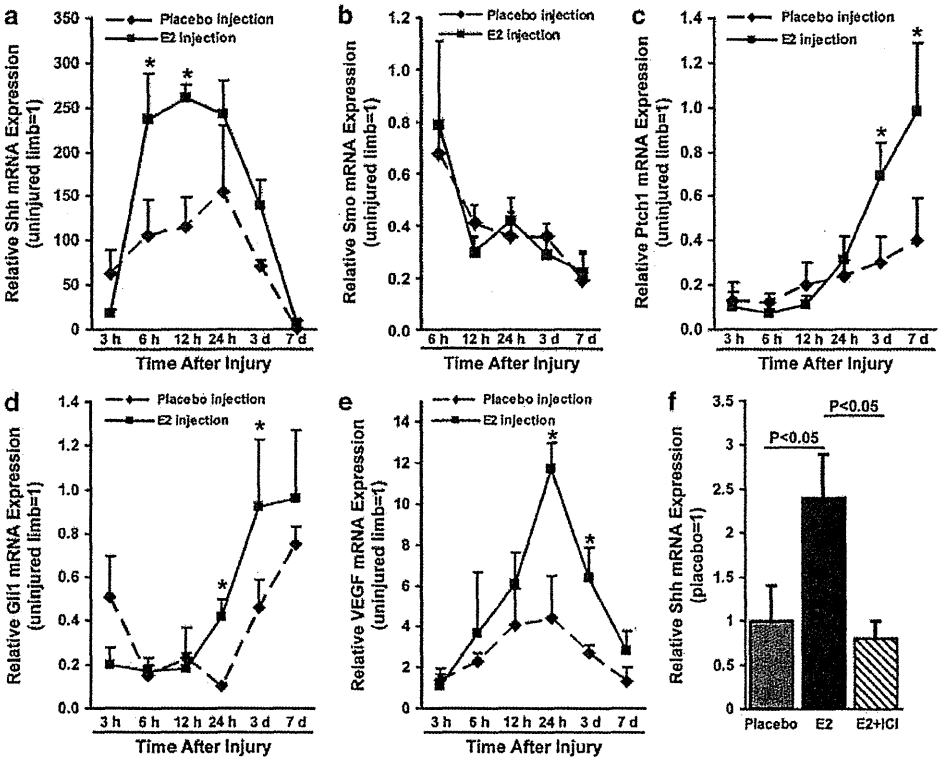
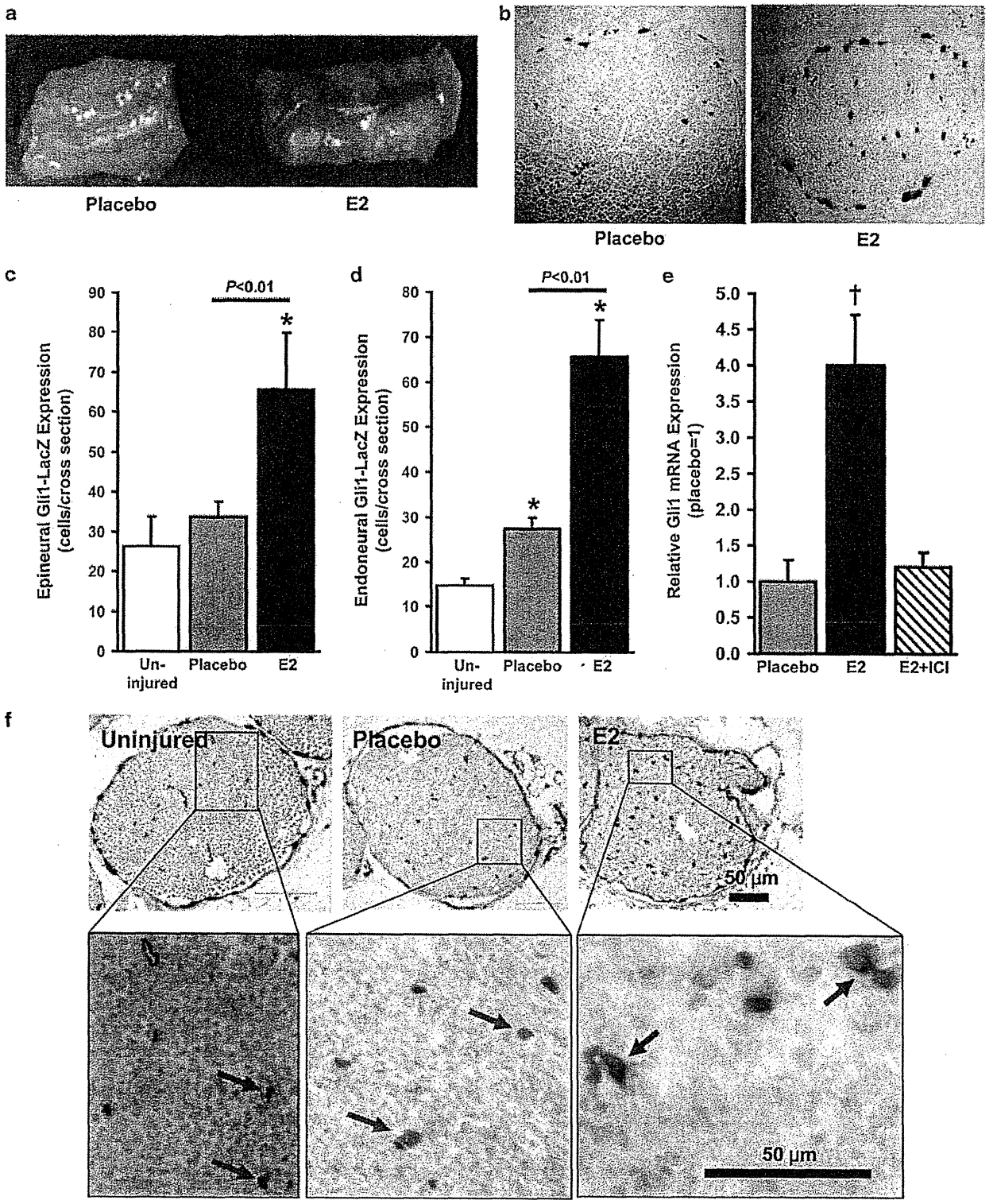


Figure 2 Local E2 injection enhances Shh-pathway activation after nerve injury. One week before surgical sciatic nerve-crush injury, E2 or saline (placebo) in PLGA was locally injected into the designated injury site. The mRNA expression of (**a**) Shh, (**b**) smo, which activates downstream components of the hedgehog signaling pathway, (**c**) the Shh receptor Ptch1, (**d**) the Shh transcriptional target Gli1, and (**e**) VEGF was evaluated for up to 7 days after injury via qRT-PCR; measurements were performed in both the injured and uninjured contralateral nerves, normalized to endogenous 18S rRNA levels, and presented relative to the values obtained in the uninjured limb; for clarity, only one error bar is shown per data point. (**f**) Shh mRNA expression was evaluated after nerve-crush injury in mice treated with placebo, with E2 injections, or with injections of E2 and the E2-receptor blocker ICI; ICI (8.3 mg/kg) was injected intraperitoneally 3 days before injury, and assessments were performed 3 days after injury, when both Ptch1 and Gli1 mRNA expression were significantly upregulated (**c, d**), via qRT-PCR, normalized to endogenous 18S rRNA levels, and presented relative to the values obtained in placebo-treated mice. *$P<0.01$.

Figure 3 Local E2 injection activates Ptch1 and Gli1 expression in injured nerves. Ptch1-LacZ mice and Gli1-LacZ mice received local injections of E2 or saline (placebo) and PLGA 1 week before sciatic nerve-crush injury; subsequent assessments were performed in mice killed 3 days after injury, when both Ptch1 and Gli1 mRNA expression were significantly upregulated in wild-type mice (Figures 2c and d). (**a, b**) The activation of Ptch1-LacZ protein expression was evaluated by staining (**a**) whole-mount tissues and (**b**) cross sections of the injured nerves from Ptch1-LacZ mice with X-gal (blue). (**c, d**) The activation of Gli1-LacZ expression was evaluated in X-gal–stained sections of (**c**) epineural and (**d**) endoneural tissue from the injured nerves of Gli1-LacZ mice. (**e**) Gli1 mRNA expression was evaluated in Gli1-LacZ mice treated with placebo, with E2 injections, or with injections of E2 and the E2-receptor blocker ICI; measurements were performed via qRT-PCR, normalized to endogenous 18S rRNA levels, and presented relative to the values obtained in placebo-treated mice. (**f**) X-gal–stained sections from uninjured limbs and from the injured limbs of placebo-treated and E2-treated mice were co-stained for expression of the endothelial-specific marker CD31 (brown) to identify endothelial cells that expressed Gli1 (blue). *$P<0.01$ *vs* uninjured; †$P<0.01$ *vs* placebo or E2 + ICI.

blocks hedgehog signaling by interfering with smo, or with E2 and cyclopamine. Twenty-eight days after injury, both vascularity and capillary density were greater in E2-treated nerves than in placebo-treated nerves, but this enhancement was not observed in mice treated with both E2 and cyclopamine (Figures 4a and b). Cyclopamine also abolished the E2-induced enhancement of Ptch1 upregulation in Ptch1-LacZ mice (Figure 4c) and the E2-induced enhancement of Gli1 upregulation in Gli1-LacZ mice (Figure 4d), which confirms that the loss of E2-induced capillary growth in mice



administered both E2 and cyclopamine was accompanied by a decline in Shh signaling.

E2 Downregulates the Expression of HIP after Nerve-Crush Injury

To determine whether Shh-pathway activation after nerve-crush injury could be triggered by a decline in expression of the Shh-inhibitor HIP, and whether E2 enhances HIP down-regulation, HIP mRNA expression was monitored in the injured and uninjured contralateral nerves of placebo-treated and E2-treated mice. In placebo-treated nerves, HIP expression declined during the first 12 h after injury and then increased through (at least) day 7, when it exceeded non-injury levels by nearly 3-fold. In E2-treated nerves, the decline was similar, but the increase was slowed: HIP levels on day 7 were similar to non-injury levels and approximately 63% lower than the levels measured in injured, placebo-treated nerves (Figure 5a). These observations were corroborated by assessments performed in mice treated with both E2 and ICI: 3 days after injury, HIP expression in placebo-treated nerves and in nerves treated with both E2 and ICI was similar and significantly higher than HIP expression in nerves treated with E2 alone (Placebo *vs* E2: $P<0.01$; E2 + ICI *vs* E2: $P<0.03$; Figure 5b).

To determine which cells in the nerve express HIP, sections of nerve tissue were fluorescently immunostained for HIP expression and for co-expression of HIP and CD31 or HIP and S100 (a Schwann-cell marker). HIP expression was observed in both endothelial cells and Schwann cells of uninjured nerves (Figure 5c), and the number of HIP-expressing cells was lower in E2-treated nerves than in placebo-treated nerves on day 3 after injury (Figure 5d); Ptch1 was also expressed by both cell types (Figure 5c). Collectively, these observations suggest that components of the Shh pathway are present in the endothelial cells and Schwann cells of uninjured nerves, but the activation of Shh signaling is inhibited by HIP.

E2 does Not Alter the Expression of Shh-Pathway Components *In Vitro*, but Downregulates HIP Expression

To identify the direct effects of E2 on the expression of Shh-pathway components, we determined whether E2 altered the mRNA expression of Shh, Ptch1, Gli1, or HIP in cultured HUVECs, fibroblasts (NIH 3T3), and Schwann cells (SW-10). E2 treatment did not significantly alter Shh (Figure 6a), Ptch1, or Gli1 (Supplementary Figure 2) expression in any of the cell lines; however, HIP expression in endothelial cells and Schwann cells declined ($P<0.01$; Figure 6b). To determine whether the E2-induced decline in HIP expression could enhance Shh signaling in endothelial cells, HUVECs were treated with or without E2, and with or without Shh, and then Shh signaling was evaluated via the Gli-luciferase assay. Shh treatment alone did not increase luciferase activity, but E2 increased activity 2-fold, and co-treatment with E2 and Shh increased activity 3.6-fold (Figure 6c). Collectively, the results from our *in-vivo* and *in-vitro* expression assessments suggest that the beneficial effects of E2 after nerve-crush injury do not evolve from direct, E2-induced activation of Shh signaling; rather, E2 reduces HIP expression and, consequently, magnifies Shh signaling in response to injury.

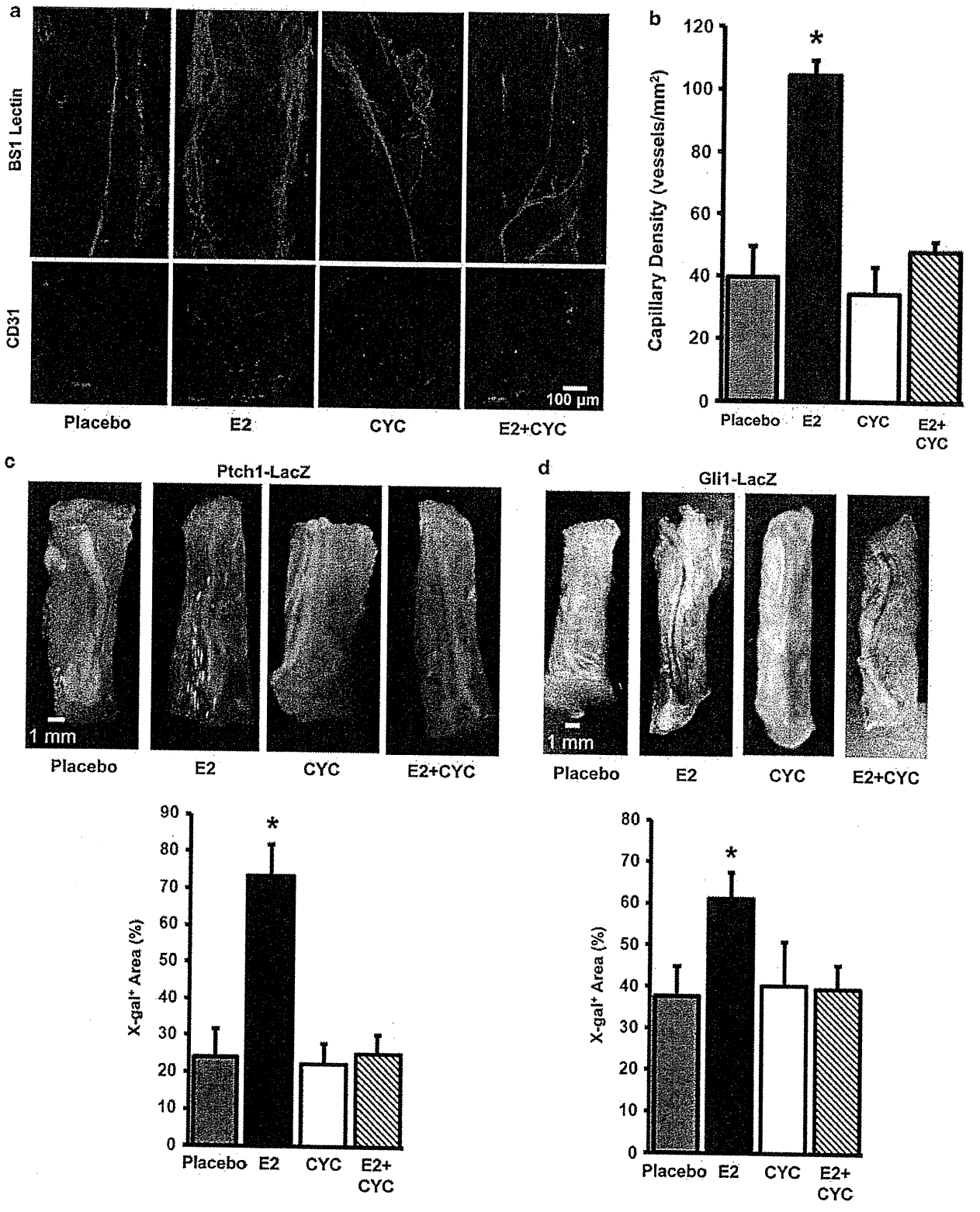
DISCUSSION

The results presented here demonstrate that local E2 injection before nerve injury promotes functional recovery and vascular growth. MCV, the duration of exercise, and capillary density were higher in mice treated with local injections of E2 than in mice administered placebo treatment, and these benefits were not accompanied by significant increases in serum E2 levels, which could alleviate some of the concerns associated with hormone replacement therapy. The enhanced vascularity appeared to evolve through upregulated hedgehog signaling: the expression of Shh, the Shh receptor Ptch1, and the Shh transcriptional target Gli1 was higher in E2-treated mice than in placebo-treated mice, and the enhanced vascularity associated with E2 treatment was abolished by co-administration of cyclopamine, which blocks hedgehog signaling by interfering with smo. E2 also downregulated HIP expression on day 7 after injury and reduced HIP expression in cultured HUVECs and Schwann cells without altering Shh expression. Thus, E2 appears to enhance Shh signaling, at least in part, by downregulating HIP, particularly at later time points when Shh expression has returned to pre-injury levels.

Shh has an important role in nerve preservation during diabetic neuropathy,[18,25] and Desert Hedgehog is expressed by Schwann cells and several other cell types in the peripheral nerves of adult mice.[15] Thus, hedgehog signaling appears to function in adult neural cells, despite the apparent lack of Shh expression in uninjured peripheral nerves. Shh expression is induced immediately after sciatic nerve crush injury, as shown both here and in a previous report,[17] which suggests that hedgehog signaling likely has an important role in the recovery of injured nerves. Nerve regeneration is crucially

Figure 4 E2-induced vessel growth in injured nerves requires Shh-pathway activation. (**a–c**) Ptch1-LacZ mice were injected with saline (placebo); with E2 (100 μg administered 1 week before sciatic nerve-crush injury); with cyclopamine (CYC) (50 mg/kg per day from 2 days before nerve-crush injury until the time of killing), which blocks hedgehog signaling by interfering with smo; or with both E2 and cyclopamine. (**a**) Functional vasculature was identified in sections of injured nerve tissue by injecting mice with fluorescein-conjugated BS-1 lectin 15 minutes before killing on day 28 after injury. (**b**) Capillary density was evaluated by staining cross sections of the vasa nervorum from mice killed on day 28 with the endothelial-cell marker CD31 and quantified. *$P<0.01$ *vs* placebo, CYC, or E2 + CYC. (**c**) Ptch1 protein expression was evaluated in X-gal–stained whole-mount tissues from injured nerves, quantified, and presented as a percentage of the whole-nerve area. *$P<0.05$ *vs* placebo, CYC, or E2 + CYC. (**d**) Gli1-LacZ mice were injected with saline (placebo), E2, cyclopamine, or both E2 and cyclopamine and sacrificed 28 days after sciatic nerve-crush injury; then, Gli1 protein expression was evaluated in X-gal–stained whole-mount tissues from the injured nerve, quantified, and presented as a percentage of the whole-nerve area. *$P<0.05$ *vs* placebo, CYC, or E2 + CYC.

dependent on angiogenesis, and Shh has been shown to induce angiogenesis after myocardial infarction in mice and swine,[24] after ischemic hindlimb injury in mice,[23] and in murine models of corneal angiogenesis[26] and wound healing,[27] perhaps by inducing expression of angiogenic factors such as VEGF, angiopoietin 1, and angiopoietin 2.[26]



Shh is a secreted protein and can rapidly induce Gli1 or Ptch1 mRNA expression through an autocrine mechanism; however, Shh secretion may be more diffuse after injury, and at least some of the secreted protein likely enters the peripheral circulation. Both of these effects would reduce the local Shh concentration, which may explain why Gli1 and Ptch1 upregulation occurred considerably later (24–72 h after injury) than Shh upregulation (6 h after injury) in our experiments. Fibroblasts respond strongly to Shh stimulation,[27] but in endothelial cells the canonical Shh-induced activation of Gli1 does not occur, although endothelial cells express the Shh receptor Ptch1. Endothelial cells also express high levels of HIP,[28] and the anti-angiogenic activity of HIP has been well documented by reports in the field of cancer

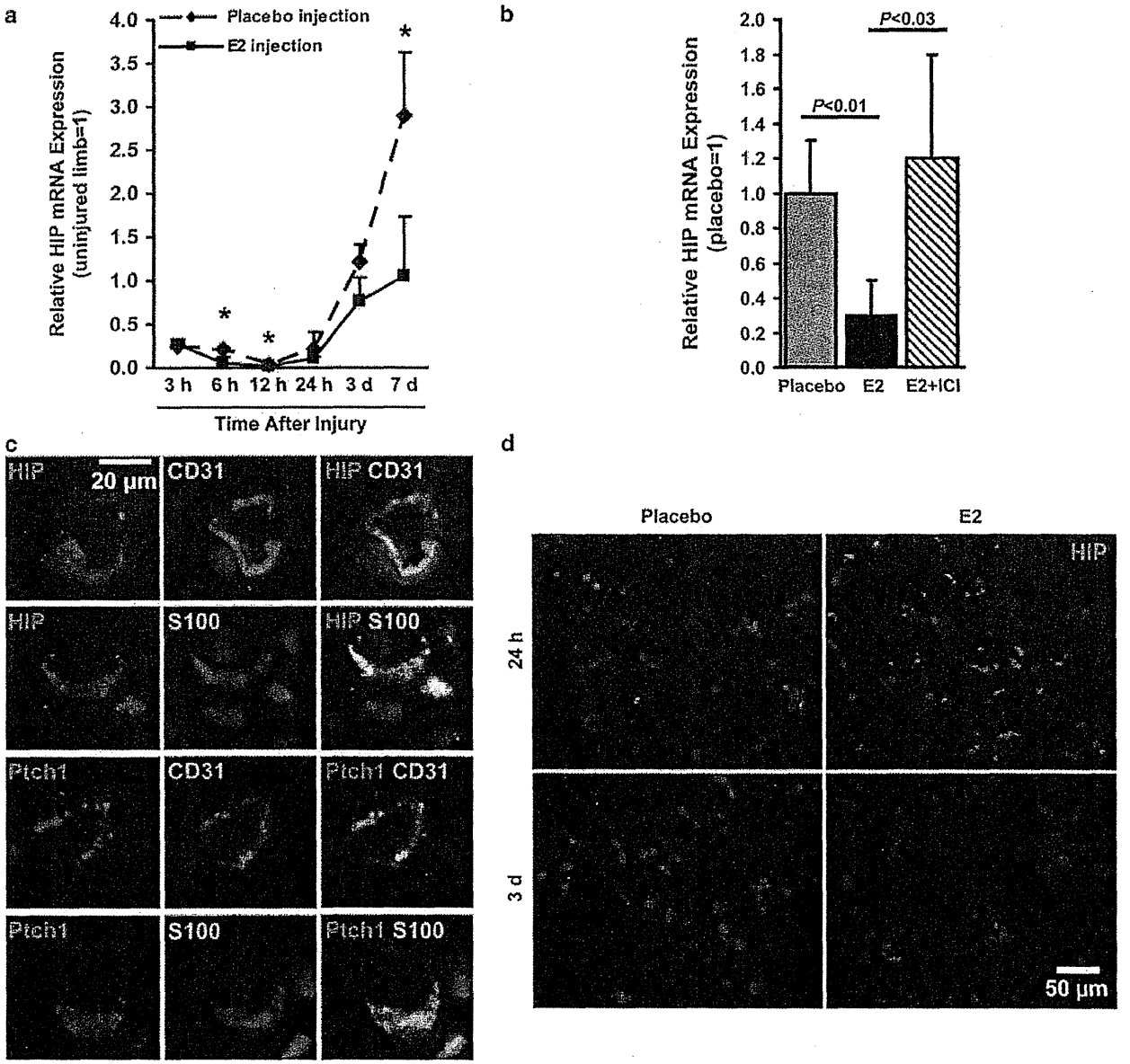


Figure 5 E2 downregulates HIP expression in injured nerves. (**a**) One week before surgical sciatic nerve-crush injury, E2 or saline (placebo) and PLGA was locally injected into the designated injury site, and the mRNA expression of HIP was evaluated from 3 h to 7 days after injury; measurements were performed via qRT-PCR, normalized to endogenous 18S rRNA levels, and presented relative to the values obtained in the uninjured limb. (**b**) HIP mRNA expression was evaluated in mice treated with placebo, with E2 injections, or with injections of E2 and the E2-receptor blocker ICI; assessments were performed on day 3 after injury, when both Ptch1 and Gli1 mRNA expression were significantly upregulated (Figures 2c and d), via qRT-PCR, normalized to endogenous 18S rRNA levels, and presented relative to the values obtained in placebo-treated mice. (**c**) Sections from uninjured nerves were stained for co-expression of (top row) HIP (red) and the endothelial-cell marker CD31 (green; second row) HIP (red) and the Schwann-cell marker S100 (green, third row) Ptch1 (red) and CD31 (green), or (bottom row) Ptch1 (red) and S100 (green); nuclei were counterstained with DAPI (blue). (**d**) Sections from placebo- and E2-treated mice killed 24 h or 3 days after injury were stained for HIP expression (red); nuclei were counterstained with DAPI (blue). *$P<0.01$.

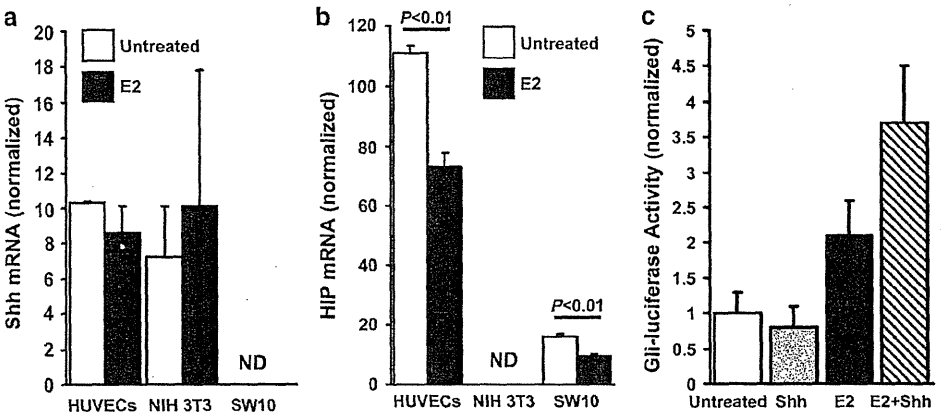


Figure 6 E2 enhances Shh signaling in endothelial cells by downregulating HIP expression. (**a**) Shh and (**b**) HIP mRNA expression were evaluated in HUVECs, fibroblasts (NIH 3T3), and Schwann cells (SW10) that had been treated with 1×10^{-8} mol/l E2 for 6 h; measurements were performed via qRT-PCR and normalized to endogenous 18S rRNA levels. (**c**) HUVECs were treated with or without E2 (6 h) and with or without 1 μg/ml Shh (16 h), and then Shh signaling was evaluated via the Gli-luciferase assay. ND indicates not detectable.

research.[28,29] HIP is a type 1 membrane-associated protein that impedes hedgehog signaling by binding and sequestering hedgehog proteins both at the cell surface and extracellularly;[16,30–33] consequently, ectopic expression of HIP inhibits hedgehog signaling, whereas declines in HIP expression enhance hedgehog signaling.[30] E2 treatment downregulated HIP expression in endothelial cells and Schwann cells, and Shh signaling in cultured endothelial cells was activated by treatment with E2 and (especially) E2 and Shh, but not by treatment with Shh alone. Thus, E2 likely increases Shh signaling in endothelial cells both intracellularly and in response to Shh produced by neighboring fibroblasts or Schwann cells, thereby increasing the contribution of endothelial cells to vascular growth.

VEGF levels after nerve injury were approximately 3-fold higher in E2-treated nerves than in placebo-treated nerves. Estrogen treatment also enhanced VEGF expression in a murine myocardial infarction model[34] and increased the mobilization of bone-marrow–derived progenitor cells after myocardial infarction[34] and arterial injury,[35] perhaps by increasing expression of endothelial nitrous oxide synthase in the bone marrow.[35] Collectively, these observations suggest that the pro-angiogenic properties of E2 could evolve, at least in part, through increases in VEGF expression and (possibly) progenitor-cell mobilization, and that E2-induced Shh signaling after nerve injury may be linked to signaling by VEGF and/or endothelial nitrous oxide synthase. Confirmation of these potential mechanisms requires additional investigation.

In conclusion, our findings demonstrate that local E2 injection before nerve injury promotes functional recovery and vascular growth afterward, and these benefits appear to evolve, at least in part, through E2-induced declines in HIP levels and a resultant upregulation of hedgehog signaling. Furthermore, local E2 injection did not significantly increase serum E2 levels, which could alleviate some of the concerns associated with hormone replacement therapy, such as elevated risk of breast cancer. Thus, local administration of an extended-release E2 preparation may be a viable strategy for enhancing angiogenesis in injured neuronal tissue.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org).

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest..

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Regular Article

Clinical outcome in Japanese elderly patients with non-valvular atrial fibrillation taking warfarin: A single-center observational study

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ABSTRACT

Background: Although a lower target prothrombin time–international normalized ratio (PT-INR) with warfarin therapy is recommended in Japan for atrial fibrillation (AF) patients ≥70 years of age, few studies have provided supporting data. The current study aimed to evaluate the clinical outcome in elderly Japanese patients with non-valvular AF who were taking warfarin.

Methods: We conducted a cohort study of 845 consecutive non-valvular AF patients ≥70 years of age who were taking warfarin (median age, 74 years; 30.5% women) with a median follow-up period of 27 months (4–69 months). Of these patients, 29.7% had a history of stoke/transient ischemic attack (TIA), and 73.1% of the patients had a $CHADS_2$ score ≥2. The occurrence of thromboembolic events, including ischemic stroke, TIA and other systemic embolisms, and major bleeding events were validated through a review of medical records.

Results: The incidence of thromboembolic and major bleeding events were 3.8 and 2.1% per year, respectively. A higher incidence of both events was observed in patients with a $CHADS_2$ score ≥3. The multivariate analysis showed that prior stroke/TIA (odds ratio 1.7, 95% CI 1.0-2.7) and diabetes (odds ratio 1.7, 95% CI 1.0-2.8) were independent risks of thromoembolic events. A HAS-BLED score ≥3 represented a risk for major bleeding (hazard ratio 2.8, 95% CI 1.7-4.6). A PT-INR of 1.5-2.5 indicated a low incidence of thromboembolic and major bleeding events in patients with a $CHADS_2$ score ≥2.

Conclusions: Our results demonstrate that a target PT-INR of 2.0 and a range of 1.5-2.5 may be safe for elderly Japanese patients with non-valvular AF.

Introduction

Atrial fibrillation (AF) is the most clinically prevalent tachyarrhythmia, and the incidence of AF increases with age [1,2]. AF is a potential risk factor for stroke, heart failure and death [3–5], and it impairs quality of life.[6] In particular, AF is a major risk for stroke and confers a fivefold increase in risk [3]. Increasing age is an important risk factor for stroke in patients with AF [7–9].

The therapeutic goals for AF patients are to reduce symptoms and prevent severe complications associated with AF [10]. AF-associated stroke is often severe and results in disability or death. Anticoagulant therapy with warfarin reduces the risk of AF-related deaths and stroke [11]. In elderly patients, warfarin therapy is associated with a higher risk of bleeding. Previous guidelines for the management of AF have suggested a lower target prothrombin time–international normalized ratio (PT-INR) range (i.e., 1.6-2.5) for elderly patients ≥75 years of age [12] than for younger patients; however, this recommendation is not based on evidence from a large trial [10]. Large cohort and randomized controlled studies have shown that warfarin therapy with a recommended PT-INR range of 2.0-3.0 was effective, even in elderly patients without an increased risk of bleeding compared to other antithrombotic therapies [13–15]. Recent guidelines recommend that a target PT-INR range of 2.0-3.0 be used for the elderly [10,16].

In Japan, however, the guidelines for pharmacotherapy of atrial fibrillation still recommend that patients 70 years of age or older be maintained with a PT-INR between 1.6 and 2.6 [17] because a PT-INR less than 1.6 increases the risk of serious ischemic stroke and a PT-INR above 2.6 increases the risk of serious bleeding complications [18,19]. However, the studies describing those risks focused on secondary prevention of stroke in Japanese AF patients taking warfarin, and not all these subjects were over 70 years of age [18,19].

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To date, there is insufficient data regarding the efficacy and safety of warfarin in Japanese AF patients who are 70 years of age or older and whether this target PT-INR is optimal. This study aimed to evaluate the clinical outcome in elderly Japanese patients with non-valvular AF who were taking warfarin.

Methods

Subjects

We retrospectively conducted a cohort study of non-valvular AF patients aged 70 years and older who were taking warfarin. We identified all patients from the automated outpatient accounting databases of the Department of Cardiology at Tokyo Women's Medical University Hospital. We included non-valvular AF patients 70 years or older receiving warfarin therapy and undergoing PT-INR measurements between May 2001 and December 2006. Non-valvular AF was confirmed in the outpatient medical records. Patients with mitral valve disease, a history of valvular repair or replacement, or concurrent hyperthyroidism were excluded. Mitral valve disease was defined according to angiographic, hemodynamic or echocardiographic findings, and moderate to severe mitral regurgitation was defined as significant. This study included 845 patients. The protocol was approved by the institutional review board of Tokyo Women's Medical University.

Data collection

The data extracted from the medical records for each patient included clinical characteristics, the indication for warfarin therapy, PT-INR values, and the duration of warfarin therapy between May 1, 2001 and December 31, 2006. Data were collected for each patient up to December 31, 2006 or the point at which they discontinued warfarin, became lost to follow-up or died. Information about deceased patients was obtained from medical records, family members, patients' general practitioners and the hospitals to which they had been admitted.

Clinical Characteristics

The patients' age, sex, traditional risk factors, underlying disease and concomitant medications were obtained from the medical records and laboratory data. Hypertension was defined as a systolic blood pressure $\geq$140 mmHg, a diastolic blood pressure $\geq$90 mmHg, or a history of treatment for hypertension. Diabetes mellitus, which had been previously diagnosed by a physician, was defined as treatment with hypoglycemic agents or a poor glycemic control (i.e., a glycohemoglobin A1c level $\geq$6.5%). Structural heart disease consisted of the following apparent cardiac disorders: left ventricular (LV) systolic dysfunction and/or marked LV dilatation (unless secondary to severe valve regurgitation), LV hypertrophy, coronary artery disease, right heart disease with right ventricular dilation of at least moderate severity and congenital heart disease. Coronary artery disease was defined as positive stress test, coronary angiography demonstrating at least 75% stenosis or coronary spastic angina documented by an acetylcholine provocation test, a history of prior myocardial infarction, or a history of revascularization procedures. Non-ischemic cardiomyopathies were defined as ventricular myocardial abnormalities in the absence of coronary artery disease or valvular, pericardial or congenital heart disease. Heart failure was defined according to the American College of Cardiology/American Heart Association criteria [20]; the patients in our study were designated stage C (current or prior symptoms of heart failure) or stage D (refractory heart failure). LV dysfunction was defined as a LV ejection fraction $\leq$40% by echocardiography, left ventriculography, or radionuclide angiography.

Warfarin therapy and intensity of PT-INR

Between May 1, 2001 and December 31, 2006, anticoagulant treatment with warfarin for AF was standardized. The warfarin dose was monitored using PT-INR at each routine visit, and patients who were taking warfarin were treated with a target PT-INR of 2.0 with an acceptable range of 1.5-2.5. The frequency of PT-INR testing ranged from once per week if a control needed to be established to every one to three months if the PT-INR was stable.

Outcomes

The occurrence of thromboembolic and bleeding events was validated with a review of medical records conducted by 3 investigators (M.N., T.S., and K.S.). Thromboembolic events included fatal or non-fatal ischemic strokes, TIAs or other systemic embolisms. An ischemic stroke was defined as the sudden onset of a new focal neurological deficit lasting more than 24 hours and not explained by other causes. A TIA was diagnosed when the neurological deficit lasted less than 24 hours. Computed tomography or magnetic resonance imaging was performed in all but two patients. Other systemic embolisms were diagnosed using computed tomography, angiography or thrombectomy and a confirmed absence of underlying atherosclerosis in the affected artery. Major bleeding events were defined as intracranial hemorrhage observed by imaging or surgery, intraocular hemorrhage leading to a substantial loss of vision, gastrointestinal hemorrhage or other severe hemorrhage that was fatal or required an endoscopic hemostasis, a surgical intervention, hospital admission, or a blood transfusion. Neurologists in our hospital diagnosed strokes, TIAs and intracranial hemorrhages. However, for patients who died from these events in other hospitals, or who were admitted to other hospitals because of these events and were not subsequently seen at our hospital, we obtained the information from those hospitals.

Statistical Analysis

Summary data are presented either as the median and range or as the numbers of patients. PT-INR values obtained at routine or extra visits at our institution were analyzed. Time in the therapeutic range (TTR) was calculated using the Rosendaal linear interpolation method [21], which is a linear interpolation of consecutive PT-INR values that calculates the percentage of time that the PT-INR is below, within or above the target therapeutic range (in this study, 1.5 to 2.5). We calculated the incidence rate (percent per patient-year) of thromboembolic and major bleeding events and the relative ratios with their 95% confidence intervals (CI) according to the different age groups. The rates of events were compared between individual groups using the chi-square test. The incident rates (percent per patient-year) of thromboembolism and major bleeding events with appropriate 95% CIs for a rate were calculated in relation to the $CHADS_2$ scores of 0, 1, 2 and $\geq$3. We also calculated the relationship between thromboembolic events and stroke risk factors, including female gender, prior stroke/TIA, heart failure, LV dysfunction, hypertension, diabetes and coronary artery disease, using the Cox proportional hazards model for multivariate analysis. Subgroup analyses using the chi-square test were prespecified for a HAS-BLED bleeding risk score of 1–2 or $\geq$3, a daily warfarin dose of <2 mg or $\geq$2 mg, and concomitant antiplatelet drug use (0 or $\geq$1).

The mean values of the PT-INR before and immediately after the events were considered associated with the events. We regarded the mean PT-INR value for the period between two measurements as representative of that period. Subsequently, the person-time at each PT-INR value was summed over all measurements for all patients. The incidence rates (per patient-years) of thromboembolic and major bleeding events with appropriate 95% CI for a rate were calculated for a PT-INR<1.50, 1.50-1.99, 2.00-2.49, 2.50-2.99 and

≥3.00 in patients with a $CHADS_2$ score of ≥2 and an indication for long-term warfarin therapy.

P values <0.05 were considered to be significant. Data analyses were performed using the SPSS statistical software (version 11.01, SPSS Inc., Chicago, Illinois).

Results

Patient characteristics

The patients' baseline characteristics are shown in Table 1. The cohort median age was 74 years (range 70–91 years), and 30.5% were women. Of the 845 patients studied, 30.9% had heart failure, 71.0% had hypertension, 35.9% had diabetes mellitus, and 62.0% had structural heart disease. The mean TTR was 73.9%, which was 15.9% below the range and 10.2% above the range. A total 36 patients (4.3%), including 29 patients who had a $CHADS_2$ score ≥2 and 7 patients who had a $CHADS_2$ score 0–1, were lost to follow up because they did not visit our hospital and provided no explanation.

Table 1
Baseline characteristics of the patients.

Number	845
Median age (range), years	74 (70–91)
Female	254 (30.5%)
Patient-years	1900
Permanent AF	341 (40.4%)
Heart failure	261(30.9%)
LV systolic dysfunction(LV ejection fraction <40%)	54 (6.4%)
Hypertension	600 (71.0%)
Diabetes mellitus	303 (35.9%)
History of TIA/Stroke	251(29.7%)
Structural heart disease	
Coronary artery disease	333 (39.4%)
Idiopathic dilated cardiomyopathy	50 (5.9%)
Hypertrophic cardiomyopathy	64 (7.6%)
Other cardiomyopathies	8 (0.9%)
Hypertensive heart disease	42 (5.0%)
Congenital heart disease	27 (3.2%)
Implantation of pacemaker/ICD	175 (20.7%)
$CHADS_2$ score	
0	54 (6.4%)
1	173 (20.5%)
2	264 (31.2%)
3	190 (22.5%)
≥ 4	164 (19.4%)
CHA_2DS_2—VASc score	
1	31 (3.7%)
2	81 (9.6%)
3	173 (20.5%)
≥ 4	560 (66.3%)
HAS-BLED bleeding risk score	
1	92 (10.9%)
2	254 (30.1%)
≥ 3	499 (59.1%)
Concomitant medications	
Aspirin	256 (30.3%)
ACE inhibitor/ARB	524 (64.0%)
Beta-blocker	353 (41.8%)
Calcium channel blocker	400 (47.3%)
Digoxin	333 (39.0%)
Statin	154 (18.2%)
Amiodarone	60 (18.2%)
Other antiarrhythmic drugs	93 (7.1%)
Bucolome	23 (2.7%)

The values are expressed as median (range) or n (%).
ACE, angiotensin-converting enzyme; AF, atrial fibrillation; ARB, angiotensin II receptor blocker; ICD, implantable cardioverter-defibrillator; LV, left ventricular; TIA, transient ischemic attack.
$CHADS_2$ = cardiac failure, hypertension, age ≥75 years, diabetes, previous stroke or TIA (doubled).
CHA_2DS_2-VASc = congestive heart failure/LV dysfunction, hypertension, age ≥75 years (doubled), diabetes, previous stroke/TIA/thromboembolism (doubled), vascular disease, age 65–74 years, female sex.
HAS-BLED = hypertension, abnormal renal and liver function (1 point each), stroke, bleeding history or predisposition, labile INR, elderly (≥65 years), and concomitant drugs or alcohol (1 point each).

Thromboembolic and bleeding events

During a median follow-up period of 27 months (4–69 months), 67 patients experienced 72 thromboembolic events (3.8%/year), and 36 patients experienced 40 major bleeding events (2.1%/year) (Table 2). Of the 72 thromboembolic events, 33 (46%) occurred in patients with a PT-INR <1.50. Eight of these events occurred when warfarin therapy was temporally interrupted for surgical or diagnostic procedures. Of the 40 major bleeding events, 11 occurred in patients with a PT-INR ≥2.50, and 11 occurred in patients with concomitant antiplatelet drug use and a PT-INR <2.50. The incidence of thromboembolic or major bleeding events did not increase with age (Table 3).

The incidence of thromboembolic and major bleeding events was highest in patients with a $CHADS_2$ score ≥3 (Table 4). To identify the more potent risk factor for thromboembolic events in patients taking warfarin, we assessed the relationship of stroke risk factors to thromboembolic events. In a multivariate analysis, prior stroke/TIA (odds ratio, 1.7; 95% CI, 1.0-2.7, P<0.05) and diabetes (odds ratio, 1.7; 95% CI, 1.0-2.8, P<0.05) were independent predictors of thromoembolic events in patients taking warfarin. The hazard ratio for major bleeding events was higher in patients with a HAS-BLED score ≥3 than in patients with a HAS-BLED score of 1–2. A low maintenance dose of warfarin (<2 mg daily) or concomitant antiplatelet drug use was not significantly associated with an increased incidence of major bleeding (Table 5).

Intensity of PT-INR and rates of thromboembolic and bleeding events

The incidences of thromboembolic and bleeding events according to the PT-INR in patients with a $CHADS_2$ score of ≥2 who had an indication for warfarin therapy are shown in Table 6. The rate of thromboembolic events was highest at a PT-INR <1.50. In contrast, the incidence of major bleeding events increased with a PT-INR ≥2.50; it was especially marked at a PT-INR ≥3.00.

Discussion

Our study in elderly Japanese non-valvular AF patients taking warfarin revealed the following findings: 1) the crude risk of thromboembolic and major bleeding events was 3.8 and 2.1% per year, respectively; 2) the incidence of thromboembolic or major bleeding events was not higher in older age groups; 3) a high incidence of thromboembolic events was observed in patients with a $CHADS_2$ score ≥3, and prior stroke/TIA and diabetes mellitus were independent risk factors in patients taking warfarin; 4) the hazard ratio for major bleeding was higher in patients with a HAS-BLED score ≥3 than in those with a score <3; and 5) PT-INR 1.5-2.5 was the lower

Table 2
Thromboembolic and major bleeding events.

Event	Number
Thromboembolism	72
Fatal ischemic stroke	4
Non-fatal ischemic stroke	48
TIA	18
Systemic embolism	2
Major bleeding	40
Fatal intracranial hemorrhage	2
Non-fatal intracranial hemorrhage	10
Gastrointestinal hemorrhage	26
Other	2

TIA, transient ischemic attack.

Table 3
Incidence rate per patient-year of thromboembolism and major bleeding events in relation to age.

Age	Patient-year	Thromboembolism			Major bleeding		
		Rate/patient-year % (N)	RR (95% CI)	P-value	Rate/patient-year % (N)	RR (95% CI)	P-value
70-74	870	3.8 (33)	1.0		2.0 (17)	1.0	
75-79	662	3.2 (21)	0.8 (0.5-1.3)	0.5	2.9 (19)	1.5 (0.8-2.6)	0.2
≥80	368	4.9 (18)	1.3 (0.8-2.0)	0.2	1.1 (4)	0.5 (0.3-1.1)	0.1

RR, relative ratio; CI, confidence interval.

incidence of thromboembolic and major bleeding events in patients with a $CHADS_2$ score ≥2.

Controlled clinical trials have shown that the TTRs in warfarin therapy were 60-65%, but "real-life" cohort studies have suggested that the TTRs might be lower [10]. An analysis from ACTIVE-W (the Atrial Fibrillation Clopidogrel Trial With Irbesartan for Prevention of Vascular Events) demonstrated that the TTR should be at least 58% to derive benefit from warfarin [22]. Our observational study indicated that a TTR of 73.9% is satisfactory (our target PT-INR 1.5-2.5).

The SPAF-II (Stroke Prevention in Atrial Fibrillation II) study reported that the incidence of thromoboembolisms was 3.6%/year in patients ≥75 years of age [23]. The BAFTA (Birmingham Atrial Fibrillation Treatment of the Aged Study) reported that the incidence of stroke and TIA was 3.1%/year in the same age group [13]. The ATRIA cohort showed that the annual rate of ischemic stroke or systemic embolism was 1.5-1.8%/year in the same age group taking warfarin, although the clinical characteristics were different [14]. Our patients exhibited a higher prevalence of heart failure (30.9%), diabetes (35.9%) and prior stroke/TIA (29.7%) compared to those in the SPAF II (heart failure, 26%; diabetes, 13%; and prior stroke/TIA, 9%), BAFTA (heart failure, 20%; diabetes, 14%; and prior stroke/TIA, 13%) and ATRIA cohort (heart failure, 34.6%; diabetes, 18.9%; and prior stroke, 12.3%) studies [13,14,23]. Hylek et al. also found a higher prevalence of diabetes, heart failure and prior stroke in patients who had an ischemic stroke and were taking warfarin at the time of their stoke compared to that of patients who were or were not taking aspirin [11]. Our study revealed that diabetes and prior stroke/TIA were independent risk factors of thromboembolism in patients taking warfarin. In our studies, more than half of thromboembolic events occurred in patients with a PT-INR within or above the target value. Because we could not rule out a purely atherothrombotic stroke from the medical-records review, ischemic stroke might include a certain number of atherothrombotic strokes.

The incidence of major bleeding was also high in elderly patients with a $CHADS_2$ score of 3–6. A recent assessment of the bleeding risk in AF patients is the HAS-BLED score in which a score of ≥3 indicates a high risk. This score is useful in managing anticoagulation in elderly AF patients [10,24]. Our results show that patients with a high HAS-BLED score ≥3 are, in fact, more likely to have major bleeding. Because there is overlap between the risk factors associated with the $CHADS_2$ and HAS-BLED scores [10], this observation is not surprising. To prevent major bleeding during warfarin therapy, drug-specific factors independent of stroke risk factors should be considered. A low warfarin dose requirement may increase the risk of major bleeding [25]. However, the majority of our subjects used low maintenance dose and low dose (<2.0 mg daily) was not associated with major bleeding. This might be related to Japanese specifically because a lower warfarin dose (30-40% less than that of Caucasian patients) is used in practice, partly because of the genetic differences in cytochrome P450 2 C9 and vitamin K epoxide reductase [26]. In our study, concomitant antiplatelet drug use increased the relative risk of major bleeding (1.3), but this finding was statistically not significant, due to the small number of events. However, one third of the patients with warfarin plus antiplatelet therapy and a PT-INR <2.50 experienced major bleeding, especially gastrointestinal bleeding. Previous large studies showed that combination therapy with warfarin and antiplatelet drugs was associated with an increased risk of bleeding in AF patients [27–29]. Warfarin combined with antiplatelet drugs, such as aspirin, should be avoided in elderly AF patients. Moreover, other factors, including hypertension, anemia, renal insufficiency, the presence of cerebrovascular or gastrointestinal disease, malignancy, polypharmacy and insufficient adherence, may contribute to major bleeding, particular in elderly patients [30]. A meta-analysis of 12 AF trials demonstrated that patient age increased the risk of ischemic stroke and serious bleeding [31]. However, the incidence of major bleeding in patients with a favorable TTR (71%) and who are ≥80 years of age was not high in a prospective observational study [32]. Our study showed that the incidence of major bleeding events did not increase in older

Table 4
Incidence rate per patient-year of thromboembolism and major bleeding events in relation to $CHADS_2$ score of 0, 1, 2 and ≥3.

$CHADS_2$ score	Patient-year	Thromboembolism		Major bleeding	
		Rate/patient-year		Rate/patient-year	
		N	% (95% CI)	N	% (95% CI)
0	109	1	0.9 (0.0-1.5)	1	0.9 (0.0-1.5)
1	396	10	2.5 (1.2-4.6)	6	1.5 (0.6-3.3)
2	592	17	2.9 (1.7-4.6)	6	1.0 (0.4-2.2)
≥3	803	39	4.8 (3.5-6.6)	23	2.9 (1.8-4.3)

CI, confidence interval.

Table 5
Bleeding risk in patients with non-valvular atrial fibrillation taking warfarin.

Risk	N	Rate (n)	HR (95% CI)	P-value
HAS-BLED score				
1-2	346	2.0 (7)		
≥3	499	5.6 (28)	2.8 (1.7-4.6)	<0.001
Warfarin daily dose				
<2 mg	647	4.3 (28)		
≥2 mg	198	3.5 (7)	0.8 (0.4-1.9)	0.75
Concomitant antiplatelet				
No	552	3.8 (21)		
Yes	293	4.8 (14)	1.3 (0.6-2.5)	0.44

HR, hazard ratio; CI, confidence interval.

Table 6
Incidence rate per patient-year of thromboembolism and major bleeding events according to PT-INR in patients with a $CHADS_2$ score of ≥2.

PT-INR	Patient-year	Thromboembolism		Major bleeding	
		Rate/patient-year		Rate/patient-year	
		N	% (95% CI)	N	% (95% CI)
≤1.49	237	30	12.6 (8.7-17.6)	4	1.7 (0.5-4.3)
1.50-1.99	663	18	2.7 (1.6-4.3)	12	1.8 (0.9-3.1)
2.00-2.49	399	11	2.8 (1.4-4.9)	6	1.5 (0.6-3.2)
2.50-2.99	117	1	0.9 (0.0-4.7)	4	3.4 (0.9-8.5)
≥3.00	35	1	2.9 (0.1-14.9)	7	20.0 (8.4-36.9)

CI, confidence interval.

age groups. Maintaining a therapeutic PT-INR is important in elderly AF patients taking warfarin [24].

The optimal level of PT-INR in elderly Japanese patients with non-valvular AF has not been determined. Our results showed that a low incidence of thromboembolic and major bleeding events among patients with a PT-INR of 1.5-2.5 and presented an indication for long-term warfarin therapy (a $CHADS_2$ score of ≥ 2). Previous reports have suggested that a PT-INR less than 1.5-1.6 increases the risk for thromboembolism, and a PT-INR above 2.3-2.6 increases the risk of major bleeding in Japanese AF patients [17,18,33,34]. You et al. found that a PTINR of 1.8-2.4 was associated with the lowest incidence of thromboembolic and major bleeding events in Chinese patients receiving warfarin [35]. A number of studies have indicated a higher baseline risk of intracranial hemorrhage in Asians as compared to Caucasians, although the mechanisms were unknown [36–40]. Odén et al. have suggested that a PT-INR of 2.0-2.5 indicates the lowest risk of stroke and death in patients with non-valvular AF [41]. This moderate anticoagulant intensity seems to be suitable for both younger and elderly patients, as well as for both Asian and Western patients. However, it is difficult to maintain this narrow range in practice. The therapeutic range of PT-INR may be lower in Japanese patients compared to the optimal level (2.0-3.0) in Western patients. To clarify these issues, large multicenter clinical investigations in Japanese patients with non-valvular AF are required.

Limitations

There are some limitations of this study. First, the study was a retrospective observational study. A total of 4.3% of patients were lost to follow up. For a number of patients, we could not determine the diagnosis of events from the medical records review. The data concerning PT-INR and other clinical parameters at the time of thromboembolic and major bleeding events were not available in all cases. A patient had a PT-INR of 3.2 28 days before the occurrence of cerebral infarction, but the PT-INR values at the time of event were not available. He had renal failure on dialysis and died due to cerebral infarction in other hospital. In addition, there was a treatment bias. The dose adjustment of warfarin, rate and rhythm therapies for AF and treatment of concomitant cardiovascular disease or other illnesses were determined according to the physician's judgment. Second, this was a single-center cohort study. Our patients had a high proportion of prior stroke/TIA, heart failure and diabetes mellitus. The clinical characteristics of our patients might not reflect those of general AF patients in Japan because our institution is a university hospital. In addition, the number of subjects was small. Our results are limited in their generalizability to the management of Japanese patients with AF. Third, we could not detect minor events. In practice, a physician might purposefully adjust the target intensity of anticoagulant therapy for patients who had minor events.

Conclusions

Our results suggested that it might be safe to set a target PT-INR of 2.0 (within a range of 1.5-2.5) for elderly Japanese patients with non-valvular AF. Prior stroke/TIA and diabetes mellitus were risks for thromboembolism, and a HAS-BLED score of ≥ 3 was a risk factor for major bleeding in elderly AF patients taking warfarin.

Competing interests

None declared.

Acknowledgments

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A case of long QT syndrome with triple gene abnormalities: Digenic mutations in *KCNH2* and *SCN5A* and gene variant in *KCNE1*

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Introduction

Long QT syndrome (LQTS) is characterized by delayed ventricular repolarization with prolonged QT interval on the surface electrocardiogram (ECG). Patients with LQTS are often children or young adults who develop cardiac arrhythmias, syncopal attacks or cardiac arrest. Since the early reports of LQTS,[1–3] molecular genetic studies have identified 13 different forms of congenital LQTS caused by mutations in the genes of potassium, sodium, and calcium channels or membrane adaptors. Recent studies also showed that common candidate gene variants are associated with QT interval duration in the general population.[4] LQTS is associated with highly variable phenotypic expression and incomplete penetrance.[5]

On the other hand, digenic mutation (2 mutations in 2 different genes) and compound mutation (2 mutations in 1 gene), including the combination of mutation and gene variant, are found in approximately 8% of the patients with LQTS and are associated with a severe phenotype.[6] Patients with similar multiple mutations need to be appropriately identified to lessen the risk of life-threatening arrhythmia and prevent potential syncopal attacks.

The present case is probably the first of LQTS with complex gene abnormalities and detailed clinical information.

Subjects

A 7-year-old son of unrelated parents was admitted to our hospital after developing convulsive seizure while sleep. On admission, the ECG showed prolongation of QT interval, with a mean QTcb (corrected QT interval [QTc] rectified using Bazett's formula) value of $0.50^{1/2}$, and notched T waves in leads V_2–V_4 (Figure 1a). The Holter record showed marked prolongation of the QT interval during episodes of bradycardia (Figure 1b). On the basis of the presence of sleep-related seizure, the attending physician requested genetic testing to exclude mutation in *SCN5A*, and mutational analysis was conducted by denaturing high-performance liquid chromatography after obtaining a written informed consent from the parents. Treatment with the antiarrhythmic drug mexiletine hydrochloride was administered. However, syncopal attacks continued to occur, which were often elicited by loud sound (particularly that of an alarm clock) and emotional stress. The patient had never required cardiac resuscitation. At 10 years of age, the patient was readmitted to our hospital for urinary incontinence elicited by loud sound from the alarm clock. The following morning, torsades de pointes was detected for 7 seconds on the Holter ECG (Figure 1C). Marked prolongation of the QT interval (QTcb: $0.62^{1/2}$) and biphasic T waves were present immediately before the appearance of torsades de pointes.

A subsequent increase in the dose of mexiletine reduced the QTcb from $0.58^{1/2}$ to $0.50^{1/2}$; however, mexiletine did not prevent the syncopal attacks, which were triggered by loud sound and emotional stress. Genetic restudy by direct DNA sequencing was performed including other members of the family.

KEYWORDS Digenic mutation; Compound mutation; *KCNH2; SCN5A; KCNE1;* molecular screening

ABBREVIATIONS ECG = electrocardiogram; **LQTS** = long QT syndrome; **PAS** = Per-Arnt-Sim; **QTc** = corrected QT interval; **QTcb** = QTc rectified using Bazett's formula (Heart Rhythm 2013;10:600–603)

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Methods

All subjects gave written informed consent in accordance with the guidelines approved by the institutional review board. Each subject underwent ECG recording and full assessment for cardiac symptoms. The QT intervals of 3 consecutive beats were measured from the onset of the Q wave to the end of the T wave in lead V_5. The QT/RR data for each of 3 consecutive beats were corrected using Bazett's formulas and the mean values for the 3 consecutive QTc were used.

Mutational analysis

Genetic screening of the complete exons for *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2*, *KCNJ2*, and *CAV3* by polymerase chain reaction and direct DNA sequencing was reperformed in the Research Laboratory of the National

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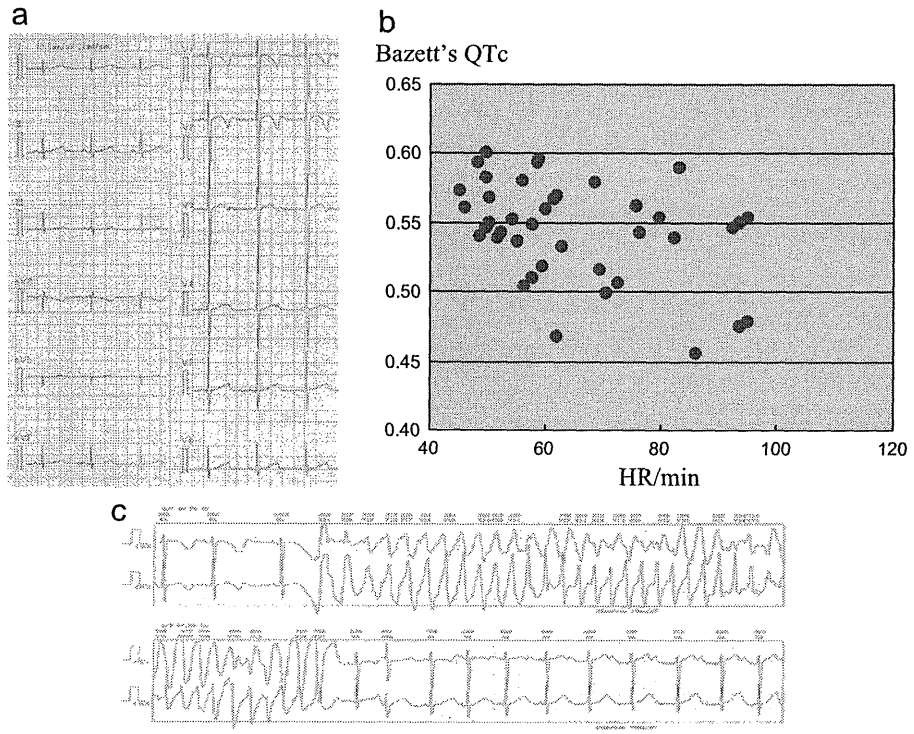

Figure 1 **a:** The entire 12-lead electrocardiogram (ECG) recorded in the proband at 7 years of age. **b:** The corrected QT interval (QTc) dynamics (QTcb; QTc rectified using Bazett's formula) at 7 years of age. **c:** An ECG strip showing the torsades de pointes recorded at 10 years of age.

Hospital Organization Kagoshima Medical Center. The exons of *CACNA1C* and *KCNJ5* were not analyzed owing to the absence of typical symptoms of LQT8 (fingers/toes abnormalities) or LQT13 (hyperaldosteronism). Furthermore, the exons of *ANKB*, *SCN4B*, *AKAP9*, and *SNTA1* were not analyzed owing to lack of reported cases of these mutations in the Japanese population. Genetic analysis included mutations or variants that alter the open reading frame, thereby affecting the primary amino acid composition of the genes. Promoter regions or introns were not analyzed. Genomic DNA was isolated using a QIAmp DNA Blood Midi Kit (Qiagen, MD). Polymerase chain reaction products were purified by AMPure (Beckman Coulter, Fullerton, CA). After treatment with BigDye Terminator v1.1 Cycle Sequence Kit (ABI, Warrington, UK) and BigDye X Terminator, direct sequencing was performed by using an ABI3130x1 Genetic Analyzer (ABI).

Results

Results of mutation analysis

Genetic analysis of the proband showed triple gene abnormalities: mutations in *KCNH2* (N45D, 133A>G), *SCN5A* (A1428S, 4282G>T), and a gene variant in *KCNE1* (D85N, 253G>A) (Figure 2). The N45D in *KCNH2* and A1428S in *SCN5A* were de novo mutations, unique to this family. Figure 3 shows the pedigree structure and phenotypic and genotypic data of the family members. The father had *KCNH2* mutation, while the mother and aunt had *SCN5A* mutation and the gene variant D85N in *KCNE1*. The youngest brother had *KCNH2* mutation and a gene variant in *KCNE1*.

Analysis of the family members showed long QTc in the youngest brother (QTcb: $0.50^{1/2}$); however, the brother had experienced no LQTS-related symptoms. The QTc values of other family members were within the normal range.

The use of a higher dose of mexiletine hydrochloride shortened the QTcb from 0.58 to $0.50^{1/2}$ at low heart rates. However, the patient continued to develop recurrent syncopal attacks twice a year, together with seizures limited to the upper limbs. Furthermore, electroencephalography revealed right frontal lobe spikes during sleep stage transition. Therefore, he was also diagnosed with right frontal lobe-related epilepsy, requiring treatment with an anticonvulsant (gabapentine). At the last follow-up examination, 3 years later, the patient was symptom-free.

Discussion

A child with a combination of digenic mutations (in *KCNH2* and *SCA5A*) and gene variant in *KCNE1* suffered repeated syncopal attacks and convulsions induced by a variety of stimuli. Incidentally, the father and the mother had mutation in different genes—*KCNH2* and *SCA5A*, respectively. Only the proband inherited both mutations. The presence of the D85N variant of *KCNE1* may have contributed to the severity of the clinical course.

Digenic mutation/compound mutation

Digenic and compound mutations are relatively common in LQTS. Schwartz et al[7] reported that 6 (4.6%) of 130 LQTS

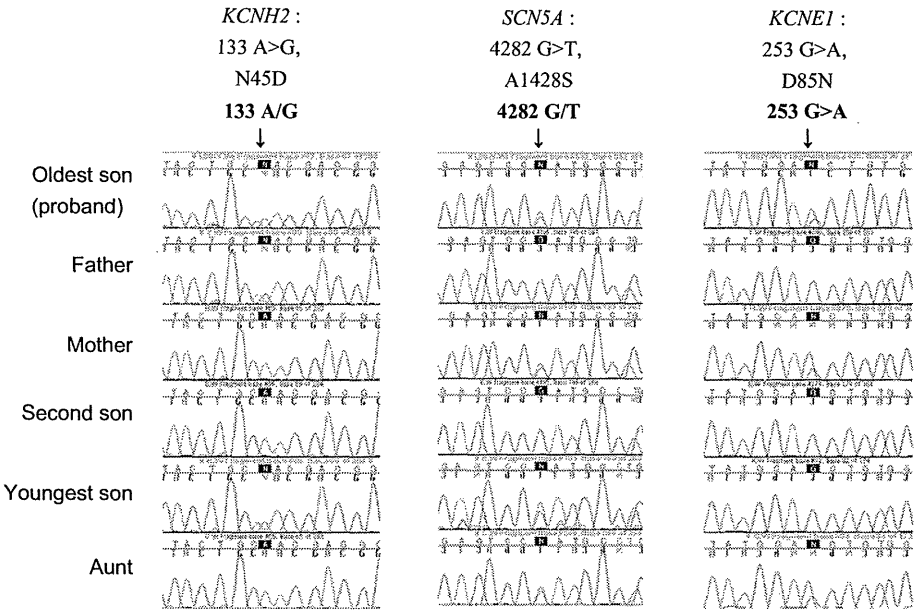

Figure 2 Mutations in the family members. Mutations in *KCNH2* (N45D, 133 A>G), *SCN5A* (A1428S, 4282G>T) and a gene variant in *KCNE1* (D85N, 253 G>A) were detected in the proband.

probands had compound or digenic mutation. Westenskow et al[8] found compound or digenic mutation in 20 (7.9%) of their 252 patients with LQTS. They reported that probands with digenic or compound mutation suffer from prolongation of QTc with associated severe symptoms such as syncope and cardiac arrest. Previous studies indicate that LQTS is associated with a low penetrance and asymptomatic mutations are sometimes found.[5] It is not surprising, therefore, to find asymptomatic mutations in this family members with normal QT interval. It is important to perform molecular screening for all known LQTS mutations when the proband and/or a family member have severe symptoms. Patients with similar gene abnormalities need to be appropriately identified to lessen the risk of life-threatening arrhythmia and prevent syncope and sudden death.

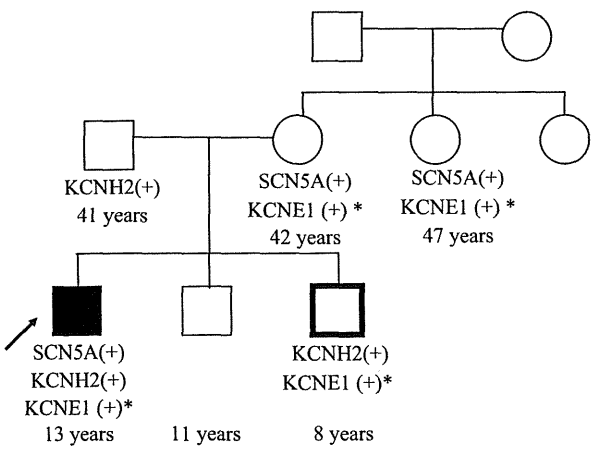

Figure 3 Family pedigree. Solid square: Family members with symptomatic long QT syndrome (LQTS). Thick-frame square: Asymptomatic family members with LQTS. Thin-frame square: Family members with a normal QT interval. Arrow: Proband. *Single nucleotide polymorphisms of *KCNE1*.

Single nucleotide polymorphisms

Genetic polymorphisms located in the *KCNQ1*, *KCNE1*, *KCNH2*, and *SCN5A* genes influence the QTc length in healthy population. Applying gender-pooled analysis, Marjamaa et al[4] reported that the *KCNE1* D85N minor allele was the variant most associated with QT prolongation. Furthermore, Nishio et al[9] demonstrated that the allele frequency of D85N, *KCNE1* polymorphism, is significantly higher in patients with LQTS and that D85N has loss-of-function effects on both I_{Kr} and I_{Ks} and acts as a disease-causing variant. The same group also showed that D85N reduced the peak tail currents of wild-type *KCNQ1/KCNE1*-encoded currents by 28% and those of wild-type *KCNH2/KCNE1*-encoded currents by 31%–36%. Nof et al[10] also reported the dominant negative effect of D85N on the *KCNH2* currents. Lahtinen et al[11] studied the effect of D85N on the duration of QT interval in patients with LQTS carrying the mutation *KCNQ1* G589D and reported that the presence of D85N was associated with QT prolongation of 26 ms ($P = .003$) in men with *KCNQ1* G589D, but not in women with G589D. They found that D85N carriers were more often probands of the family ($P = .042$) and more likely on beta-blockers ($P = .01$) than noncarriers.[11] Interestingly, D85N was described by the above groups as a disease-causing gene variant,[9] a modulator,[10] or a modifier,[11] but not a disease-causing gene. In the present report, although the proband was a heterozygous D85N carrier, he had severe symptoms probably due to the 2 variants in other genes. Thus, probands with severe symptoms must be screened for variants as well.

Administration of mexiletine alone resulted in shortening of the QT interval in our patient. Furthermore, the combination of mexiletine and an anticonvulsant resulted in the disappearance of syncopal attacks. It is postulated that the same channelopathy, *SCN5A*, may be associated with both familial LQTS and epilepsy.[12] Our case also had a mutation in *SCN5A* that was probably also involved in epilepsy.

The present study has certain limitations. We did not perform functional analysis for the 2 de novo mutations of N45D-*KCNH2* and A1428S-*SCN5A*. Asparagin^{45} in *KCNH2* is present in the Per-Arnt-Sim (PAS) domain (amino acids 41–70).[13] HERG channels have slow deactivation kinetics, which is regulated by the PAS domain.[14] Mutations in the PAS domain are present only in LQTS cases but not control subjects,[13] and the presence of mutations in the PAS domain correlates with severe LQTS phenotype.[15] Alanine^{1428} in *SCN5A* is present in the S5/S6 region of the DIII domain. A1428V, but not A1428S, in *SCN5A* was reported as a disease-causing mutation in Brugada syndrome.[16] Administration of mexiletine effectively shortened the QTc values in the proband. These findings suggest that both N45D-*KCNH2* and A1428S-*SCN5A* mutations are disease-causing mutations in the proband.

Conclusions

We presented a patient with severe LQTS phenotype who was found to have triple gene abnormality: digenic mutations in *KCNH2* and *SCN5A* and a gene variant in *KCNE1*. The present case is probably the first of LQTS with triple gene abnormalities and detailed clinical information.

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