antibody and Alexa Fluor 647-conjugated anti-mouse IgG (H+L) antibody (Invitrogen). Images were acquired with a confocal microscope (LSM 700; Carl Zeiss). The enzymatic activity of calpains was measured using the Calpain-Glo Protease Assay (Promega) according to the manufacture's protocol.

Western Blot Analysis—Protein samples were fractionated with SDS-PAGE and transferred to PVDF membrane (GE Healthcare). The blotted membranes were incubated with primary antibodies followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories). Immunoreactive signals were detected with the ECL Plus Western blotting Detection System (GE Healthcare) and developed onto a film (Hyperfilm ECL; GE Healthcare Biosciences) or visualized using a lumino-image analyzer (ImageQuant LAS 4000 mini; GE Healthcare).

Primary Antibodies—The following antibodies were used: mouse monoclonal anti-N-cadherin antibody (clone 3B9, Invitrogen) raised against the intracellular domain of chicken N-cadherin, rabbit polyclonal anti-N-cadherin antibody (Santa Cruz Biotechnology, Inc.) raised against the extracellular domain of human N-cadherin, rabbit polyclonal anti-β-catenin antibody (Abcam), mouse monoclonal anti-Cx43 antibody (clone CX-1B1, Invitrogen), rabbit polyclonal anti-Cx43 antibody (Millipore), mouse monoclonal anti-sarcomeric α-actinin antibody (clone EA-53, Sigma), rabbit polyclonal anti-collagen 1 antibody (Abcam), mouse monoclonal anti-α-tubulin antibody (clone DM1A, Sigma), rabbit monoclonal anti-GAPDH antibody (clone 14C10, Cell Signaling Technology). A rabbit polyclonal antibody specific to the calpain cleavage site of the N-terminal 135-kDa fragment of αII-spectrin was described previously (25).

Real Time RT-PCR Analysis—Total RNA was extracted by using TRIzol reagent (Invitrogen), and single-stranded cDNA was transcribed by using QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's protocol. We conducted quantitative real-time PCR analysis using Light Cycler TaqMan Master Kit (Roche Applied Science) with the target-specific primers and the matching probes designed by the Universal ProbeLibrary System (Roche Applied Science). Amplification conditions were initial denaturation for 10 min at 95 °C followed by 45 cycles of 10 s at 95 °C and 25 s at 60 °C. Individual PCR products were analyzed by melting point analysis. The expression level of a gene was normalized relative to that of *Gapdh* by using a comparative Ct method. The primer sequences and Universal Probe numbers were designed with the ProbeFinder software as following: *Nppa*, 5′-cacagatctgatggatttcaaga-3′ and 5′-cctcatcttctaccggcatc-3′, no. 25; *Nppb*, 5′-gtcagtcgtttgggctgtaac-3′ and 5′- agacccaggcagagtcagaa-3′, no. 71; *Acta1*, 5′-aatgagcgtttccgttgc-3′ and 5′-atccccgcagactccatac-3′, no. 94; *Gapdh*, 5′-tgtccgtcgtggatctgac-3′ and 5′-cctgcttcaccaccttcttg-3′, no. 80.

Statistics—The results are expressed as the mean ± S.E. Differences in measured values were analyzed using an unpaired 2-tailed Student's *t* test for two-group comparison and a 1-way analysis of variance followed by the Bonferroni's method for multi-group comparison. We estimated survival curves after MI by the Kaplan-Meier method and compared the two groups by the log-rank test. Values of $p < 0.05$ were considered statistically significant.

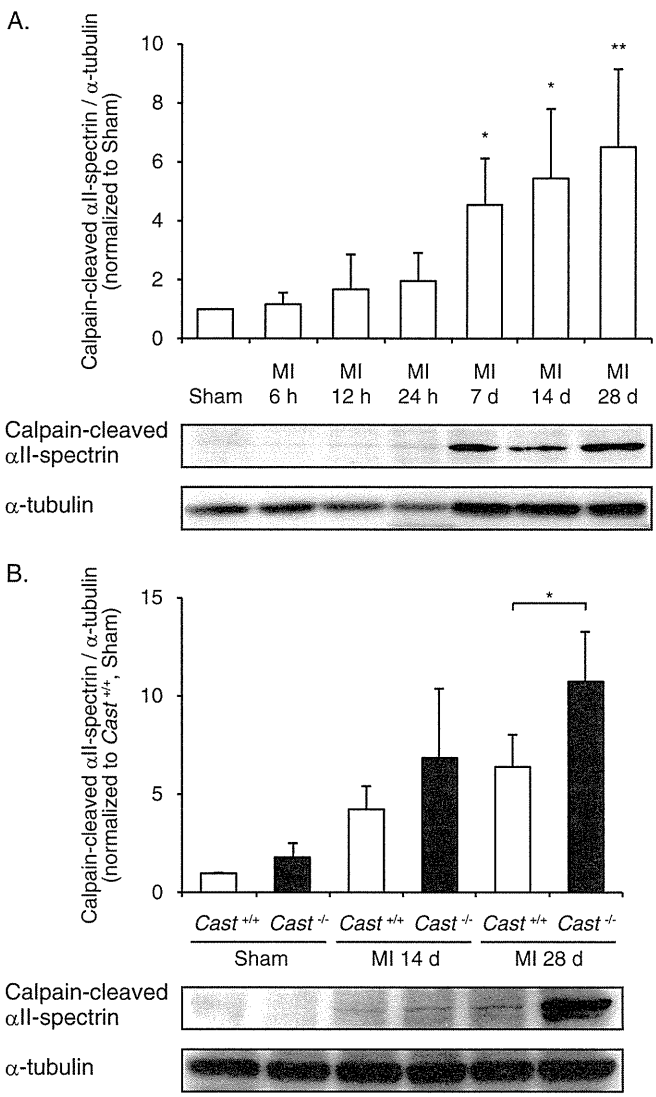


FIGURE 1. **Calpain activity in MI hearts of *Cast*$^{-/-}$ and *Cast*$^{+/+}$ mice.** *A*, time course of calpain activation after MI, determined by immunoblot analysis of calpain-cleaved αII-spectrin in the hearts of wild-type mice ($n = 5$). α-Tubulin was used as an internal control for the amount of loaded protein. The calpain-cleaved αII-spectrin/α-tubulin ratios were quantified by densitometry and plotted (*upper panel*). Data are presented as the mean ± S.E. *, $p < 0.05$; **, $p < 0.01$ *versus* Sham. *B*, comparison of calpain activation after MI between *Cast*$^{-/-}$ and *Cast*$^{+/+}$ mice ($n = 3 \sim 4$). The calpain-cleaved αII-spectrin/α-tubulin ratios were quantified by densitometry and plotted (*upper panel*). Data are presented as the mean ± S.E. *, $p < 0.05$.

RESULTS

Calpains Are Activated in the Chronic Phase but Not in the Acute Phase after MI—First, we examined the time course of change in calpain activity after MI in wild-type mice by using an antibody specific to the calpain-cleaved N-terminal 135-kDa fragment of αII-spectrin (25). Although it has been reported that intracellular Ca^{2+} is elevated immediately after myocardial ischemia (15), the level of spectrin proteolysis was unchanged within 24 h after MI (Fig. 1*A*). However, at 7 days after MI, spectrin proteolysis was significantly increased and sustained

TABLE 1

Basal heart rates, blood pressures, and echocardiographic parameters of $Cast^{-/-}$ and $Cast^{+/+}$ mice

Values are the mean ± S.E. LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; IVSth, intraventricular septal thickness; PWth, left ventricular posterior wall thickness; FS, fractional shortening; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; bpm, beats per min.

Parameters	$Cast^{+/+}$	$Cast^{-/-}$	*P*
Number	4	4	
Age (weeks)	8.60 ± 0.08	8.60 ± 0.20	0.54
Heart rate (bpm)	640 ± 13.6	646 ± 32.6	0.79
SBP (mm Hg)	98.9 ± 5.08	97.9 ± 5.73	0.79
DBP (mm Hg)	58.2 ± 4.26	52.6 ± 3.12	0.76
LVEDD (mm)	3.48 ± 0.03	3.46 ± 0.27	0.90
LVESD (mm)	1.71 ± 0.02	1.69 ± 0.04	0.51
IVSth (mm)	0.86 ± 0.11	0.89 ± 0.04	0.62
PWth (mm)	0.81 ± 0.07	0.90 ± 0.05	0.13
FS (%)	50.7 ± 1.28	50.9 ± 2.47	0.91

thereafter (Fig. 1*A*). These results suggest that calpains are activated not in the acute phase but in the subacute and chronic phase after MI.

Calpain Activation in the Chronic Phase after MI Is Enhanced in $Cast^{-/-}$ Mice—Next, we compared the activities of cardiac calpains after MI between $Cast^{-/-}$ and $Cast^{+/+}$ mice. It has been reported that basal activity of calpain is important for protein homeostasis in unstressed hearts (29), but the calpain activity in sham-operated hearts, as assessed by the level of spectrin proteolysis, was indistinguishable between $Cast^{-/-}$ and $Cast^{+/+}$ mice (Fig. 1*B*). Essentially, $Cast^{-/-}$ mice showed normal development, fertility, and life span (25). In addition, $Cast^{-/-}$ mice exhibited normal heart rates, blood pressures, and cardiac function under the physiological conditions as assessed by echocardiographic evaluation (Table 1). These results suggest that calpastatin deficiency has little effect on basal activity of calpain under the physiological conditions. However, as compared with $Cast^{+/+}$ mice, $Cast^{-/-}$ mice showed a significant increase in spectrin proteolysis at 28 days after MI (Fig. 1*B*), suggesting that calpastatin deficiency exaggerates activation of calpains when calpain activation is induced under stressed conditions.

LV Remodeling after MI Is Enhanced in $Cast^{-/-}$ Mice—At 14 days after MI, histological analysis with Masson's trichrome staining and echocardiographic examination revealed no significant difference in LV geometry and function between $Cast^{-/-}$ and $Cast^{+/+}$ mice (Fig. 2, *A* and *B*). However, at 28 days after MI, $Cast^{-/-}$ mice showed more severe LV dilatation and dysfunction than $Cast^{+/+}$ mice (Fig. 2, *C* and *D*). Because there was no significant difference in infarct size, determined either by area measurement or by length measurement, between $Cast^{-/-}$ and $Cast^{+/+}$ mice (Fig. 2, *E* and *F*), LV remodeling was promoted independently of the infarct size in $Cast^{-/-}$ mice. As a consequence, the survival rate was significantly lower in $Cast^{-/-}$ mice than in $Cast^{+/+}$ mice ($p < 0.05$), although the early death within 14 days after MI was comparable ($p = 0.058$) (Fig. 2*G*). These results suggest that enhanced activation of calpains in the chronic phase enhances LV remodeling in $Cast^{-/-}$ mice, leading to death possibly due to heart failure.

Myocardial Cell Death after MI Is Comparable between $Cast^{-/-}$ and $Cast^{+/+}$ Mice—Calpains have been implicated in the execution of cell death (1). Thus, we compared the prevalence of cardiomyocyte apoptosis in $Cast^{-/-}$ and $Cast^{+/+}$ mice by TUNEL staining. There was no significant difference in the number of TUNEL-positive cardiomyocytes in the infarct area of $Cast^{-/-}$ and $Cast^{+/+}$ hearts at 24 h after MI (Fig. 3, *A* and *B*). Throughout the period from 3 to 28 days, apoptotic cardiomyocytes were scarcely observed in the infarct or non-infarct area of both genotypes, and the number was not significantly different between $Cast^{-/-}$ and $Cast^{+/+}$ mice (Fig. 3, *C* and *D*). In addition, the infarct area of $Cast^{-/-}$ mice was not significantly different from that of $Cast^{+/+}$ mice at 28 days after MI (Fig. 2, *E* and *F*). These results suggest that calpastatin deficiency has little impact on myocardial cell death both in the acute phase and in the chronic phase after MI.

Calpain Activation Is Associated with a Decrease in N-cadherin Expression in the Border Zone of $Cast^{-/-}$ Hearts—To gain insights into the mechanism of how calpain activation leads to exacerbation of LV remodeling, we utilized immunofluorescence staining and assessed the cellular and subcellular localization of calpain activation in the infarct and border zones. In sham-operated hearts of $Cast^{+/+}$ mice, calpain-cleaved αII-spectrin was primarily localized in cardiomyocytes (Fig. 4*A*). Especially in cardiomyocytes, the fluorescence signals showed a characteristic cross-striated pattern that is typically seen with sarcomeric proteins, and intense fluorescence was colocalized with N-cadherin at the intercalated discs (Fig. 4*A*). A similar localization pattern of spectrin proteolysis with comparable intensity was observed in $Cast^{-/-}$ and $Cast^{+/+}$ mice (Fig. 4*A*). At 28 days after MI, the levels of calpain-cleaved αII-spectrin were indistinguishable in the non-infarct zone between $Cast^{-/-}$ and $Cast^{+/+}$ mice (Fig. 4*B*). However, we observed exaggeration of calpain-mediated spectrin proteolysis exclusively in cardiomyocytes at the border zone, adjacent to the infarct zone (Fig. 4*C*). $Cast^{-/-}$ mice showed significantly broader area of cardiomyocytes with intense immunofluorescence for calpain-cleaved αII-spectrin at the border zone than $Cast^{+/+}$ mice (Fig. 4, *C* and *D*). Notably, in cardiomyocytes at the border zone of $Cast^{-/-}$ mice, profound activation of calpains was associated with a considerable decrease in the expression of N-cadherin (Fig. 4*C*). N-cadherin is a Ca^{2+}-dependent transmembrane glycoprotein that enhances cell adhesion by binding to α-, β-, and γ-catenins at adherens junction (30) and stabilizes gap junction formation by maintaining connexin 43 (Cx43) at intercalated discs (31). In parallel with the decrease in N-cadherin expression, the expression levels of β-catenin and Cx43 were decreased in cardiomyocytes at the border zone of $Cast^{-/-}$ mice (Fig. 4, *E* and *F*). Next, we isolated RNA from the border zone of MI hearts for quantification of the expression levels of fetal cardiac genes. $Cast^{-/-}$ mice showed a significant increase in the expressions of *Nppa*, *Nppb*, and *Acta1* as compared with $Cast^{+/+}$ mice (Fig. 5*A*). Furthermore, immunohistochemical analysis revealed that the expression of collagen 1 was significantly increased in the border zone of $Cast^{-/-}$ mice as compared with $Cast^{+/+}$ mice (Fig. 5*B*). These results suggest that molecular and cellular remodeling in the border zone was more prominent in $Cast^{-/-}$ mice than in $Cast^{+/+}$ mice. Collectively, we speculate that unregulated activation of calpains in cardiomyocytes may disassemble the cadherin-mediated structures of adherens junction and gap junction of cardiomyocytes

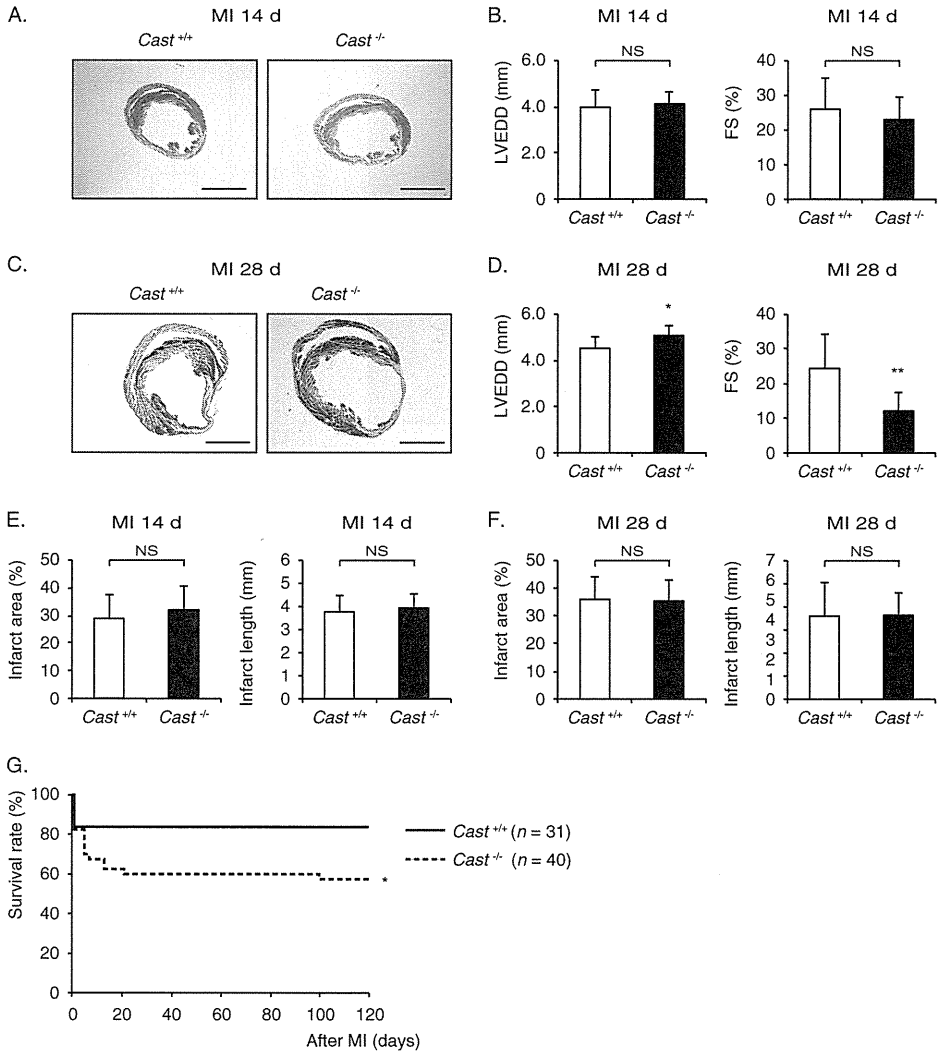


FIGURE 2. **LV remodeling after MI in *Cast*$^{-/-}$ and *Cast*$^{+/+}$ mice.** *A*, Masson's trichrome staining of *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts at 14 days after MI. *Scale bars*, 2 mm. *B*, echocardiographic parameters of *Cast*$^{-/-}$ and *Cast*$^{+/+}$ mice at 14 days after MI. *C*, Masson's trichrome staining of *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts at 28 days after MI. *Scale bars*, 2 mm. *D*, echocardiographic parameters of *Cast*$^{-/-}$ and *Cast*$^{+/+}$ mice at 28 days after MI. *E* and *F*, infarct area (*light panels*) and infarct length (*right panels*) of *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts at 14 days (*E*) and 28 days (*F*) after MI. *G*, Kaplan-Meier survival curves of *Cast*$^{+/+}$ ($n = 31$) and *Cast*$^{-/-}$ mice ($n = 40$) after MI. *LVEDD*, LV end-diastolic dimension; *FS*, fractional shortening. Values represent the mean ± S.E. of data from 10 mice in each group. *NS*, not significant. *, $p < 0.05$; **, $p < 0.01$ *versus* *Cast*$^{+/+}$ mice.

in the border zone and thereby lead to progression of LV remodeling after MI.

Calpain Activation Causes N-cadherin Cleavage and Disassembles Cadherin-based Cell Adhesions in Cultured Cardiomyocytes of Neonatal Rats—To examine whether calpain activation was sufficient for disruption of N-cadherin-based cell adhesions, we stimulated cultured cardiomyocytes of neonatal rats with the Ca^{2+} ionophore ionomycin. Direct measurement of the enzymatic activity of calpains using a luminescent assay revealed that calpain enzymatic activity was significantly enhanced after stimulation with ionomycin, which was significantly, but not completely, repressed by pretreatment with the cell-permeable calpain inhibitor MDL28170 (Fig. 6*A*). Western blot analysis showed that the expression levels of full-length N-cadherin (140 kDa) were significantly decreased by stimulation with ionomycin, which was attenuated by pretreatment with MDL28170 (Fig. 6, *B* and *C*). In parallel with a decrease in the levels of full-length N-cadherin, ionomycin significantly increased the generation of the 106- and 37-kDa fragments, as detected by anti-N-cadherin antibodies raised against the N-terminal extracellular domain and the C-terminal intracellular domain, respectively (Fig. 6*B*). Similarly, the expression levels of β-catenin and Cx43 were significantly decreased by stimulation with ionomycin, which was attenuated by pretreatment with MDL28170 (Fig. 6, *B* and *C*).

Confocal immunocytochemistry revealed reduction in the expression levels of N-cadherin and β-catenin at the intercalated discs when cardiomyocytes were stimulated with ionomycin (Fig. 7*A*). Meanwhile, Cx43 immunofluorescent signals showed reduction at the intercalated discs and concomitant intracellular redistribution in response to ionomycin stimulation (Fig. 7*B*). Ionomycin-induced effects on the amount and spatial distribution of these intercalated disc proteins were prevented by pretreatment with MDL28170 (Fig. 7, *A* and *B*). These results suggest that calpain activation is sufficient to cause degradation of N-cadherin and to dis-

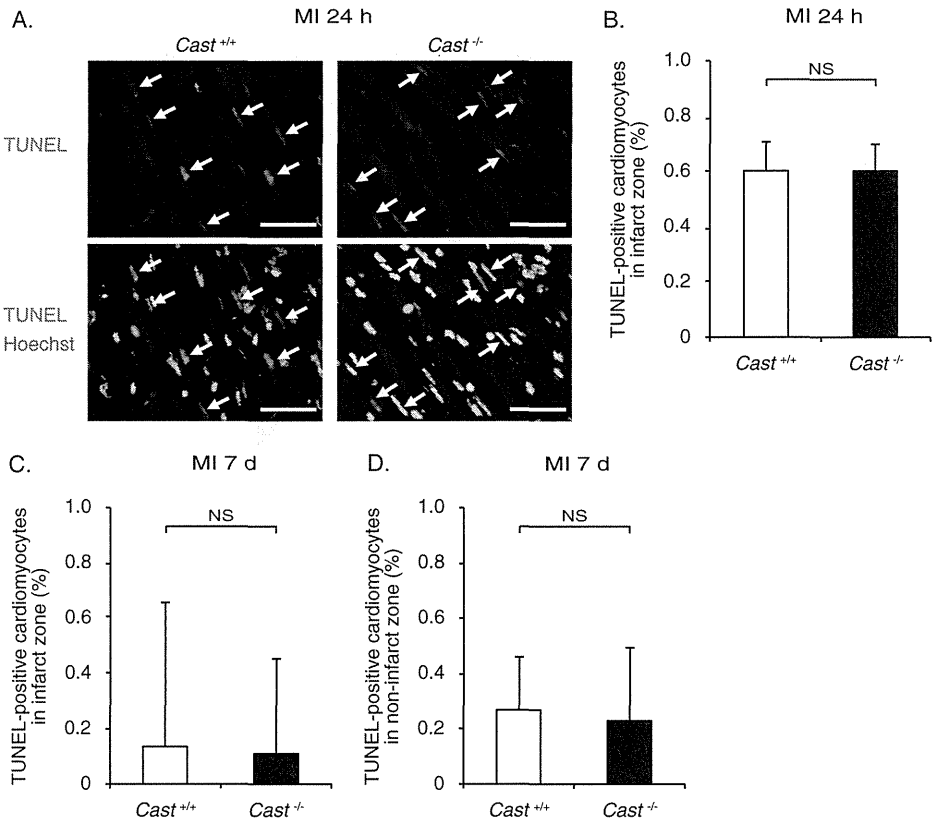


FIGURE 3. **Cardiomyocyte apoptosis after MI in $Cast^{-/-}$ and $Cast^{+/+}$ mice.** *A*, TUNEL staining with nuclear staining with Hoechst 33258 in the infarct zone of $Cast^{-/-}$ and $Cast^{+/+}$ mice at 24 h after MI. *Scale bars*, 50 μm. *B*, percentage of TUNEL-positive cardiomyocytes in the infarct zone of $Cast^{-/-}$ and $Cast^{+/+}$ mice at 24 h after MI was calculated. Values represent the mean ± S.E. (500 cardiomyocytes sampled from 12 visual fields from 3 mice in each group). *NS*, not significant. *C* and *D*, percentage of TUNEL-positive cardiomyocytes in the infarct zone (*C*) and non-infarct zone (*D*) of $Cast^{-/-}$ and $Cast^{+/+}$ mice at 7 days after MI was calculated. Values represent the mean ± S.E. (800 cardiomyocytes sampled from 12 visual fields from 3 mice in each group). *NS*, not significant.

assemble cadherin-based cell adhesions in rat neonatal cardiomyocytes.

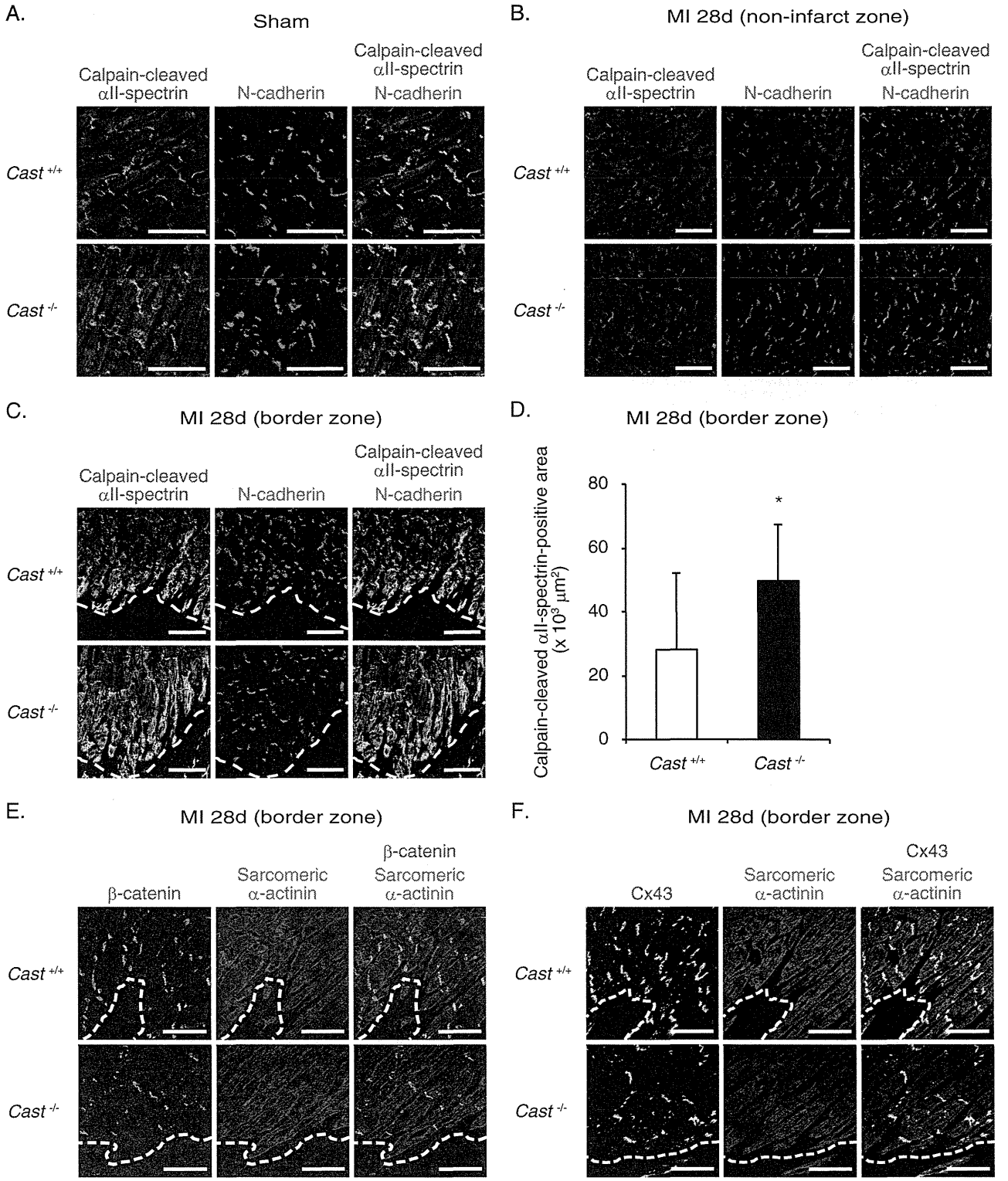
DISCUSSION

In the present study we provided experimental evidence that calpains impaired the cell-cell interactions through degradation of cadherin-associated protein complex and thereby promoted LV remodeling after MI. Calpain-mediated proteolysis was increased in the chronic phase (7 days and later), not in the acute phase (before 24 h), after MI, and profound activation of calpains exacerbated LV remodeling without affecting myocardial cell death in $Cast^{-/-}$ mice. In the border zone of MI hearts, $Cast^{-/-}$ mice showed a decrease in N-cadherin expression concomitant with an increase in calpain activation and prominent myocardial remodeling. In cultured cardiomyocytes calpain activation caused degradation of N-cadherin and disorganization of cadherin-based cell adhesions.

The pathogenic role of calpains in MI hearts has remained unclear despite the efforts of many laboratories. One of the major obstacles for the study of calpains has been the lack of reliable methods to measure calpain activity accurately *in vivo*. Previous reports demonstrated that expression levels or enzymatic activities of calpains were increased in the hearts after MI (17–21), but these results must be interpreted cautiously. Obviously, the protein content of calpains does not necessary correlate with their proteolytic activity, and the *in vitro* enzymatic assays using homogenized tissue samples after the addition of Ca^{2+} merely indicate the proteolytic capacity of calpains, not the calpain activity *in situ*. However, in our study the use of a specific antibody against calpain-cleaved fragment of αII-spectrin has allowed for direct observation of the proteolytic activity of calpains in *in vivo* hearts in temporal and spatial terms. Another obstacle has been the lack of specific and effective calpain inhibitors (1). The best approach for identifying the role of calpains in cellular function is to introduce calpastatin, the specific and endogenous inhibitor of calpains. However, according to a recent study, cardiac overexpression of calpastatin inhibited basal calpain activity and resulted in spontaneous and progressive heart failure (29). Thus, we have analyzed $Cast^{-/-}$ and $Cast^{+/+}$ mice to examine the consequences of exaggerated calpain activation in the hearts after MI.

In a variety of mammalian cells, activated calpains mediate cell death by multiple mechanisms. For example, calpains activate p53 (32), and pro-apoptotic BH3-only protein Bax (33) and Bid (34) inactivate anti-apoptotic Bcl-xL (35), facilitate the release of apoptosis-inducing factor (AIF) from mitochondria (36, 37), induce caspase-12 activation (35) and release of lysosomal cathepsins (38), cleave autophagy regulating protein Atg5 (39), degrade cytoskeletal proteins (40), disrupt ion homeostasis (9, 40, 41), and increase plasma membrane permeability (40). *In vivo* transgenic overexpression of calpain 1 in the heart

has induced heart failure, which is associated with cardiomyocyte necrosis and mononuclear cell infiltration (29). In addition, profound calpain activation in *Cast*$^{-/-}$ mice has exacerbated excitotoxicity by kainite, leading to neuronal DNA fragmentation in the brain (25). However, the prevalence of cardiomyocyte apoptosis and the infarct size did not differ between *Cast*$^{-/-}$ and *Cast*$^{+/+}$ mice throughout the observation period after MI (Figs. 2 and 3). These results suggest that calpastatin deficiency has little effect on myocardial cell death in MI hearts. Previous reports demonstrated that pharmacological inhibition of calpains reduced the infarct size (22, 23). In addition, recent reports showed that overexpression of *Capn1*



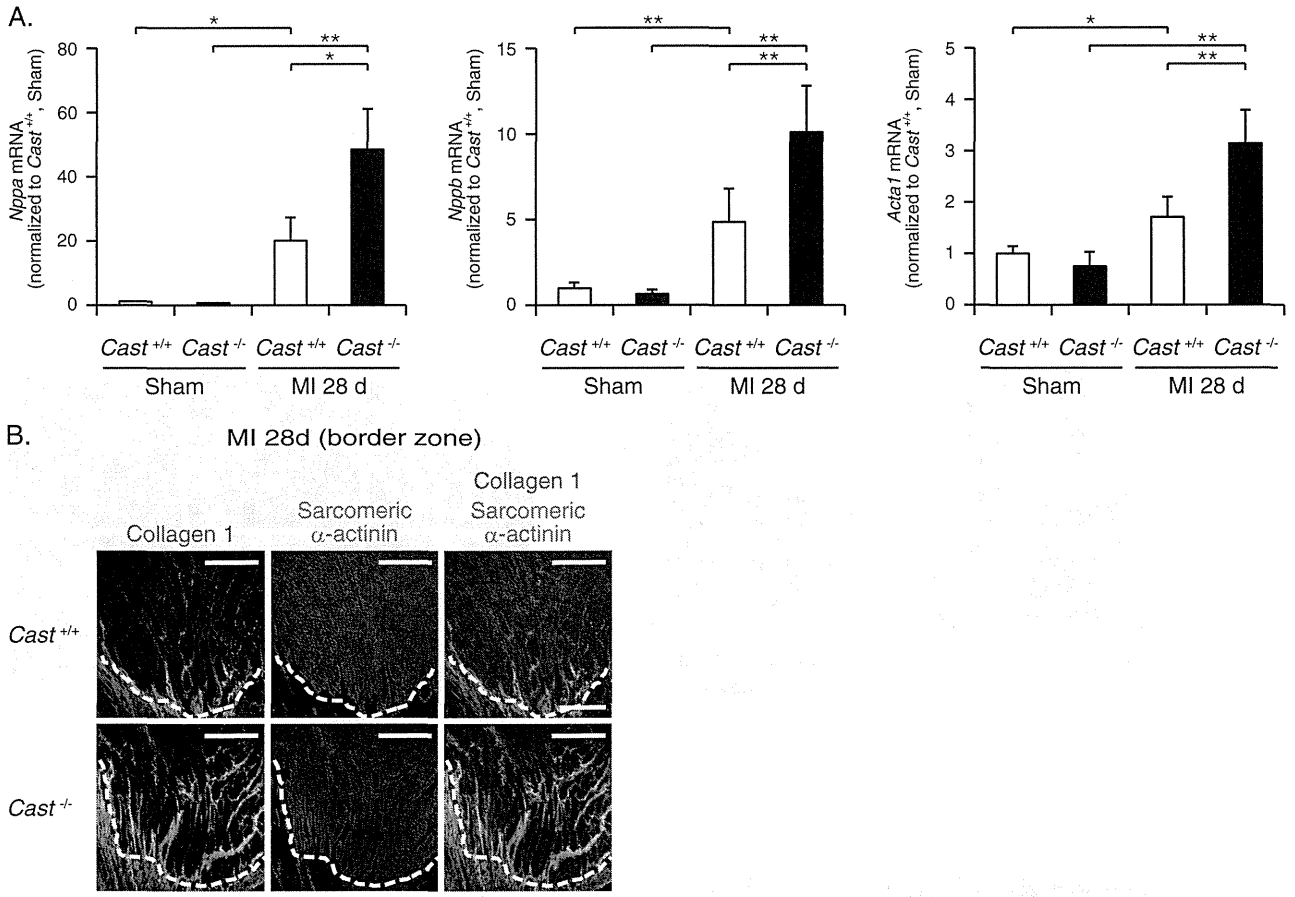
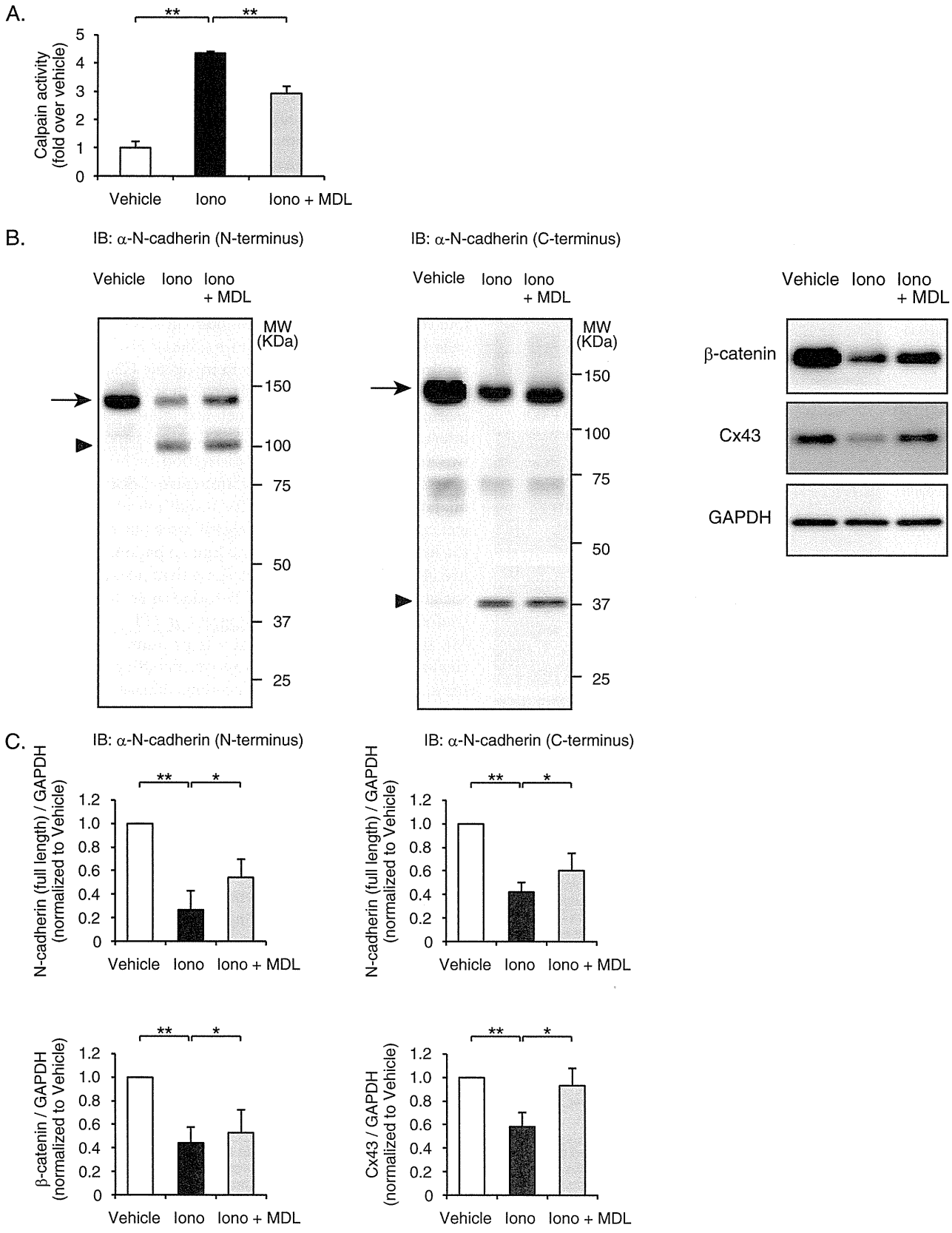


FIGURE 5. **Molecular and histological changes in the border zone of *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts after MI.** *A*, the mRNA expressions of *Nppa*, *Nppb*, and *Acta1* in the border zone of *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts at 28 days after MI and in sham-operated *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts ($n = 3 \sim 5$, in each group). Values represent the mean ± S.E. *, $p < 0.05$; **, $p < 0.01$ *versus* sham-operated *Cast*$^{+/+}$ mice. *B*, immunofluorescence of the border zone in *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts at 28 days after MI ($n = 5$, in each group). Collagen 1 and sarcomeric α-actinin are represented in *green* and *red*, respectively. *Scale bars*, 200 μm. The *dotted lines* indicate the boundary between the border zone where viable cardiomyocytes remain (*above the dotted line*) and the infarct zone (*below the dotted line*).

increased infarct size and enhanced myocardial cell death after MI (10), whereas genetic disruption of *Capn1* or *Capns1* provided the reciprocal results (10, 42). However, cardiac-specific *Capns1* knock-out mice were susceptible for hemodynamic stress-induced myocardial injury because of defective membrane repair, indicating that calpains play a beneficial role as well (43). Because calpain activity in sham-operated hearts was indistinguishable between *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts (Fig. 1*B*), our study presented experimental evidence that inhibition of calpain activation without affecting the basal activity had little impact on myocardial cell death after MI.

Temporal and spatial analyses of calpain-cleaved αII-spectrin revealed that calpain activation was induced at the border zone of MI hearts in the chronic phase (Fig. 4*C*). After MI, the border zone expands in response to increased wall stress, promoting further LV dilatation and contractile dysfunction (44). Because calpain activation was followed by progressive contractile dysfunction and LV dilatation (Figs. 1 and 2), we speculate that profound activation of calpains at the border zone may contribute to exacerbation of LV remodeling in *Cast*$^{-/-}$ mice. According to *in vitro* assays, activated calpains cleave a large number of proteins including cytoskeletal proteins, membrane-associated proteins, signaling mediators, and transcription factors (1). Recent reports showed that calpain activation induced IκB degradation and NF-κB activation (45) and that disruption of *Capns1* inhibited NF-κB signaling and inflamma-

FIGURE 4. **Cellular and subcellular localization of calpain activation in the border zone of *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts after MI.** *A*, immunofluorescence of sham-operated *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts ($n = 5$, in each group). Calpain-cleaved αII-spectrin and N-cadherin are represented in *green* and *red*, respectively. Co-localization of calpain-cleaved αII-spectrin and N-cadherin at the intercalated discs is indicated by the *yellow merging of the green and red labels*. *Scale bars*, 50 μm. *B*, immunofluorescence of the non-infarct zone in *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts at 28 days after MI. Calpain-cleaved αII-spectrin and N-cadherin are represented in *green* and *red*, respectively. *Scale bars*, 100 μm. *C*, immunofluorescence of the border zone in *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts at 28 days after MI ($n = 5$, in each group). Calpain-cleaved αII-spectrin and N-cadherin are represented in *green* and *red*, respectively. *Scale bars*, 100 μm. *D*, calculated areas of cardiomyocytes with intense immunofluorescence for calpain-cleaved αII-spectrin at the border zone in *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts at 28 days after MI ($n = 5$, in each group). Data are presented as the mean ± S.E. *, $p < 0.05$. *E*, immunofluorescence of the border zone in *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts at 28 days after MI ($n = 5$, in each group). β-Catenin and sarcomeric α-actinin are represented in *green* and *red*, respectively. *Scale bars*, 100 μm. *F*, immunofluorescence of the border zone in *Cast*$^{-/-}$ and *Cast*$^{+/+}$ hearts at 28 days after MI ($n = 5$, in each group). Cx43 and sarcomeric α-actinin are represented in *green* and *red*, respectively. *Scale bars*, 100 μm. The *dotted lines* (*C*, *E*, and *F*) indicate the boundary between the border zone where viable cardiomyocytes remain (*above the dotted line*) and the infarct zone (*below the dotted line*).



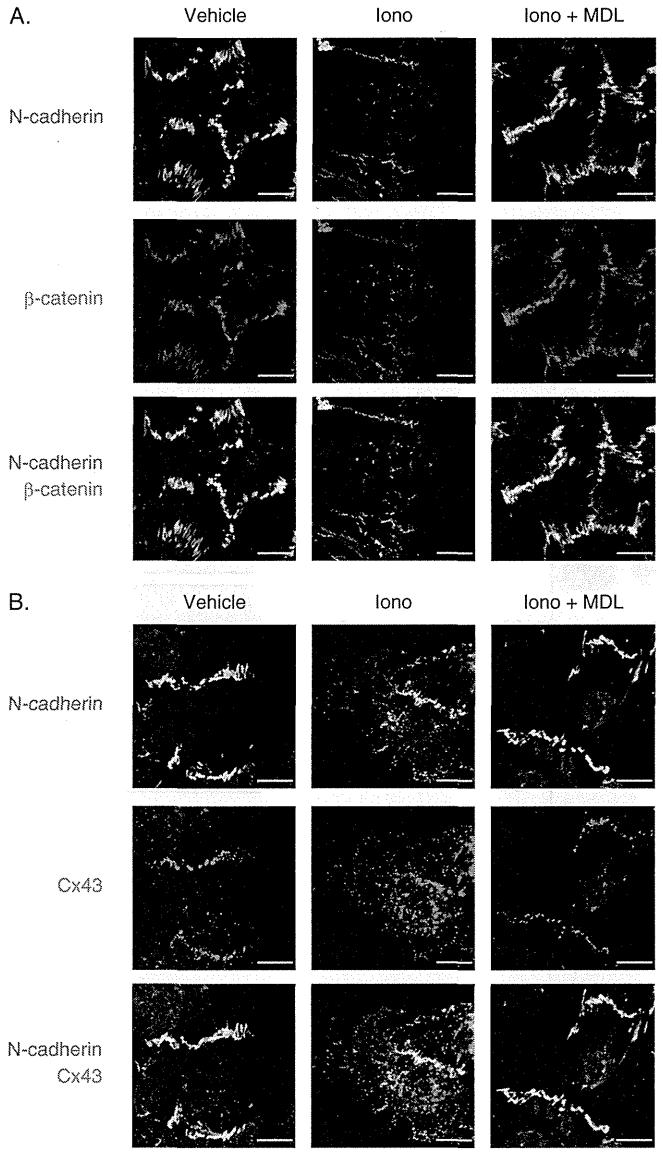


FIGURE 7. **Calpain-mediated disassembly of intercalated disc proteins in rat neonatal cardiomyocytes.** *A*, immunofluorescence of N-cadherin and β-catenin in rat neonatal cardiomyocytes. Cells were pretreated with MDL28170 (*MDL*, 10 μM) and stimulated with ionomycin (*Iono*, 10 μM) for 10 min. N-cadherin and β-catenin are represented in *green* and *red*, respectively. Experiments were repeated three times in triplicate, and the representative images are shown. *Scale bars*, 10 μm. *B*, immunofluorescence of N-cadherin and Cx43 in rat neonatal cardiomyocytes. Cells were pretreated with MDL28170 (10 μM) and stimulated with ionomycin (10 μM) for 10 min. N-cadherin and Cx43 are represented in *green* and *red*, respectively. Experiments were repeated three times in triplicate, and the representative images are shown. *Scale bars*, 10 μm.

tion, leading to improvement of LV remodeling after MI (42). In an attempt to explore potential calpain substrates in MI hearts, we found that calpain-cleaved αII-spectrin was co-localized with N-cadherin at the intercalated discs (Fig. 4, *A–C*). Importantly, increased calpain proteolysis was associated with a decrease in N-cadherin expression in cardiomyocytes at the border zone of $Cast^{-/-}$ hearts (Fig. 4*C*). In cultured cardiomyocytes, N-cadherin was cleaved in the presence of ionomycin, resulting in the generation of at least two fragments, an N-terminal 106-kDa and a C-terminal 37-kDa fragment. A calpain inhibitor MDL28170 significantly but not completely suppressed ionomycin-stimulated activation of calpains (Fig. 6*A*) and thereby prevented a significant degree of N-cadherin cleavage (Fig. 6*B*). In contrast to the degradation of full-length N-cadherin, the generation of cleaved fragments was not repressed by pretreatment with the calpain inhibitor (Fig. 6*B*). We speculate that the cleaved fragments may become unstable and susceptible to further degradation by undefined proteases. Previous reports demonstrated that calpains cleaved at least four regions of the intracellular domain of N-cadherin in neural cells (46, 47) and that calpain-mediated N-cadherin cleavage suppressed cell-cell adhesion in myogenic C2C12 cells (47). In cultured cardiomyocytes, calpain activation by ionomycin disassembled cadherin-based cell-cell adhesion consisting of intercalated disc proteins such as β-catenin and Cx43 (Fig. 7). It was reported that targeted disruption of N-cadherin in the hearts caused disassembly of intercalated discs, leading to LV dilatation and dysfunction (48). In addition, abnormal mechanical coupling through intercalated discs has been observed in the hearts of animal model and human patients with heart failure (49–51). Therefore, we assume that calpain activation-associated down-regulation of N-cadherin at the border zone is profoundly involved in the progression of LV remodeling after MI. It has been reported that a large number of proteins are cleaved by calpains in *in vitro* assays, including cytoskeletal proteins, membrane-associated proteins, kinases and phosphatases, and transcription factors (1). To prove the pathogenic significance of calpain-mediated cleavage of N-cadherin, our observation must be further investigated in future studies using knock-in mice of N-cadherin that is resistant to calpain cleavage for testing if these mice would rescue the exacerbated LV remodeling after MI in $Cast^{-/-}$ mice.

It remains an open question of what triggers calpain activation at the border zone cardiomyocytes in the chronic phase after MI. One of the candidates upstream of calpain activation is the renin-angiotensin system. It is well established that the local renin-angiotensin system is activated during the remodeling process after MI and that pharmacological or genetic blockade of renin-angiotensin system prevented LV remodeling after MI in many animal models and human patients (52). In cultured vascular smooth muscle cells, stimulation with angiotensin II (Ang II) has increased the calpain activity through transactivation of epidermal growth factor receptor and systemic overexpression of calpastatin blunted cardiac hypertro-

FIGURE 6. **Calpain-mediated degradation of N-cadherin and down-regulation of intercalated disc proteins in rat neonatal cardiomyocytes.** *A*, ionomycin-induced calpain activation in rat neonatal cardiomyocytes. Cells were pretreated with MDL28170 (10 μM) and stimulated with ionomycin (10 μM) for 10 min, and calpain activity was determined by a luminescent assay. Experiments were repeated three times in triplicate, and data are shown as -fold induction over vehicle control (mean ± S.E.). *Iono*, ionomycin; *MDL*, MDL28170. **, $p < 0.01$. *B*, immunoblot (*IB*) analysis of N-cadherin, β-catenin, and Cx43 in rat neonatal cardiomyocytes. Besides the full-length N-cadherin (*arrows*), the degraded N-terminal fragments (*arrowhead*) and C-terminal fragments (*arrowhead*) were detected by anti-N-cadherin antibody raised against the extracellular domain and intracellular domain of N-cadherin, respectively. *C*, quantitation of the N-cadherin (N terminus)/GAPDH ($n = 6$), N-cadherin (C terminus)/GAPDH ($n = 7$), β-catenin/GAPDH ($n = 3$), and Cx43/GAPDH ($n = 4$) are shown as *bar graphs* (*right panel*). Data are presented as the mean ± S.E. *, $p < 0.05$; **, $p < 0.01$.

phy and perivascular inflammation in Ang II-infused mice (53). A recent study showed that calpain activation mediated Ang II-induced endothelial dysfunction in rodents (54). It is intriguing that activation of Ang II receptor signaling by locally produced Ang II and mechanical stress (28) may lead to calpain activation and thereby promote LV remodeling after MI. Even so, we have to assume that additional undefined factors play a regulatory role in activating calpains in a temporally and spatially restricted manner in MI hearts.

In conclusion, calpains are activated in the chronic phase after MI, and profound activation of calpains may exacerbate LV remodeling possibly through the alterations of intercalated disc organization in cardiomyocytes at the border zone. Therefore, pharmacological intervention of the calpain-calpastatin system may emerge as a promising strategy in the treatment of LV remodeling after MI unless it hampers the basal calpain activity.

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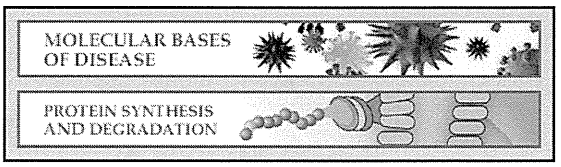
Calpain-dependent Cleavage of N-cadherin Is Involved in the Progression of Post-myocardial Infarction Remodeling



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Reduced risk of recurrent myocardial infarction in homozygous carriers of the chromosome 9p21 rs1333049 C risk allele in the contemporary percutaneous coronary intervention era: a prospective observational study

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ABSTRACT

Objectives: Chromosome 9p21 single nucleotide polymorphism (SNP) is a susceptibility variant for acute myocardial infarction (AMI) in the primary prevention setting. However, it is controversial whether this SNP is also associated with recurrent myocardial infarction (ReMI) in the secondary prevention setting. The purpose of this study is to evaluate the impact of chromosome 9p21 SNP on ReMI in patients receiving secondary prevention programmes after AMI.

Design: A prospective observational study.

Setting: Osaka Acute Coronary Insufficiency Study (OACIS) in Japan.

Participants: 2022 patients from the OACIS database.

Interventions: Genotyping of the 9p21 rs1333049 variant.

Primary outcome measures: ReMI event after survival discharge for 1 year.

Results: A total of 43 ReMI occurred during the 1 year follow-up period. Although the rs1333049 C allele had an increased susceptibility to their first AMI in an additive model when compared with 1373 healthy controls (OR 1.20, 95% CI 1.09 to 1.33, $p=2.3*10^{-4}$), patients with the CC genotype had a lower incidence of ReMI at 1 year after discharge of AMI (log-rank p=0.005). The adjusted HR of the CC genotype as compared with the CG/GG genotypes was 0.20 (0.06 to 0.65, p=0.007). Subgroup analysis demonstrated that the association between the rs1333049 CC genotype and a lower incidence of 1 year ReMI was common to all subgroups.

Conclusions: Homozygous carriers of the rs1333049 C allele on chromosome 9p21 showed a reduced risk of 1 year ReMI in the contemporary percutaneous coronary intervention era, although the C allele had conferred susceptibility to their first AMI.

Strengths and limitations of this study

- This is the first study to clearly show a change of the susceptibility risk of the 9p21 variant to acute myocardial infarction between the primary and secondary prevention settings in a percutaneous coronary intervention era.
- Data regarding the mechanism and culprit lesion of remyocardial infarction were not available.
- Replication studies with a larger sample are warranted to confirm our observations.

INTRODUCTION

Acute myocardial infarction (AMI) is one of the leading causes of death and disability worldwide.[1] AMI is associated with a positive family history as well as several traditional coronary risk factors including diabetes, hypertension, dyslipidemia and smoking, suggesting that the pathogenesis of AMI has a substantial genetic component.[2] Genome-wide association studies (GWAS) have identified several genetic loci that confer susceptibility to AMI and coronary artery disease (CAD) in the primary prevention setting.[3–7] Among these genetic variants, single nucleotide polymorphisms (SNPs) on chromosome 9p21 are the most common and significant susceptibility risk factors for AMI and CAD, regardless of race.[3–11] However, it remains controversial whether chromosome 9p21 SNPs are associated with recurrent myocardial infarction (ReMI) in patients with post-AMI receiving the evidence-based secondary prevention programmes.[12–14]

For example, the Italian Genetic study and TexGen registry revealed that 9p21 genetic variation was not associated with ReMI events after early-onset myocardial infarction and acute coronary syndrome (ACS), respectively,[13–14] while the Global Registry of Acute Coronary Events (GRACE) genetic study showed that risk allele carriers of 9p21 SNPs had a higher incidence of ReMI after ACS.[12] One possible explanation for this discrepancy among these three studies is an involvement of in-hospital ReMI as an end point. It is reported that 9p21 SNPs increase the risk of AMI onset by promoting the development and progression of coronary plaque deposition, rather than increasing susceptibility to plaque rupture.[9–14] Thus, inclusion of acute phase ReMI might have made the interpretation difficult in these 9p21 variant studies, as most of the ReMI occurring during the acute phase of AMI were most likely caused by 9p21-independent mechanisms, such as reocclusion of the culprit lesion and/or thrombosis. Therefore, to simply assess the susceptibility impact of 9p21 to ReMI in the secondary prevention settings, it may be better to include patients with post-AMI only who survived the acute stage and received the state-of-the-art secondary prevention programmes after discharge.

The aim of the present study was to investigate the susceptibility impact of 9p21 genetic variation on ReMI in consecutive 2022 patients with a first AMI who were registered in the Osaka Acute Coronary Insufficiency Study (OACIS),[15–19] treated with emergent percutaneous coronary intervention (PCI) and discharged alive.

METHODS

The OACIS

The OACIS is a multicentre, prospective observational registry for AMI in Japan that was initiated in April 1998 among 25 collaborating hospitals. It is designed to assess patient demographics including genomic information, therapeutic procedures and subsequent clinical events in patients with AMI. All study candidates were informed about data collection, blood sampling and genotyping, and provided written informed consent. Research cardiologists and trained research nurses recorded data using a specific reporting form. The diagnosis of AMI was based on the WHO criteria,[20] which required two of the following three criteria to be met: (1) clinical history of central chest pressure, pain or tightness lasting ≥30 min; (2) ST segment elevation >0.1 mV in at least one standard and (3) a rise in serum creatinine phosphokinase concentration to more than twice the normal laboratory value. The OACIS is registered to the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) in Japan (ID: UMIN000004575). Other details of the OACIS are described elsewhere.[15–19]

Study design

Among 10 074 consecutive patients with AMI registered in the OACIS between April 1998 and April 2011, 2045 patients who had a first AMI, underwent emergent PCI, survived to discharge and gave written informed consent to the study were enrolled in this study. Exclusion criteria included a history of previous myocardial infarction or PCI, in-hospital death cases and lack of written informed consent for genetic study and DNA sampling. Genomic DNA was extracted from peripheral blood samples using a commercially available kit (QIAamp DNA Blood Midi Kit; Qiagen, Hilden, Germany). Patients were genotyped for the rs1333049 SNP of chromosome 9p21 using the multiplex-PCR-based invader assay as previously described.[21] We focused on the rs1333049 because it is the most widely studied 9p21 genetic variant in the primary and secondary prevention settings.[8 10–12 14] We also confirmed that rs1333049 is in linkage disequilibrium with other major 9p21 SNPs in the OACIS registry (see online supplementary figure S1). Finally, the genotyping success rate for rs1333049 was 98.9% and 2022 patients were successfully genotyped and analysed for susceptibility to ReMI within a year after survival discharge (figure 1). To validate the association of the rs1333049 SNP with the first AMI, we performed a case–control association study between the present study population and healthy Japanese controls. Control blood samples of healthy Japanese adults (n=1373, mean age, 38.6 years old, 59% male) were obtained from the Health Science Research Resources Bank (Osaka, Japan). The patient backgrounds and primary preventive medications were not adjusted in this case–control association study in the primary prevention setting, since these data were not available in commercially obtained healthy controls and medications before first AMI were not available in our study population in detail.

Statistical analysis

Categorical variables were compared by the χ^2 test, and continuous variables were compared by the Kruskal–Wallis test. The impact of the rs1333049 genotype on the onset of AMI was assessed in the primary and secondary prevention settings. The impact of rs1333049 on the onset of AMI was calculated as ORs and 95% CIs in an additive model (OR per C allele increase). In the secondary prevention analysis, the Kaplan-Meier method was used to estimate event rates. Since the Kaplan-Meier analysis revealed that the incidence of ReMI differed between the CC and CG/GG genotypes of rs1333049 (figure 2), the differences between the CC and CG/GG genotypes were assessed by the log-rank tests. In addition, a Cox regression model was used to compare the 1 year prognostic impacts between the rs1333049 CC and CG/GG genotypes based on the estimated HRs and 95% CI. Multivariate Cox regression analysis was performed to reduce the confounding effects of variations in patient backgrounds using age, gender, body mass index, ST-elevation myocardial infarction (STEMI), diabetes, hypertension, dyslipidemia, smoking, target lesion, multivessel disease, peak creatinine phosphokinase and prescription of ACE

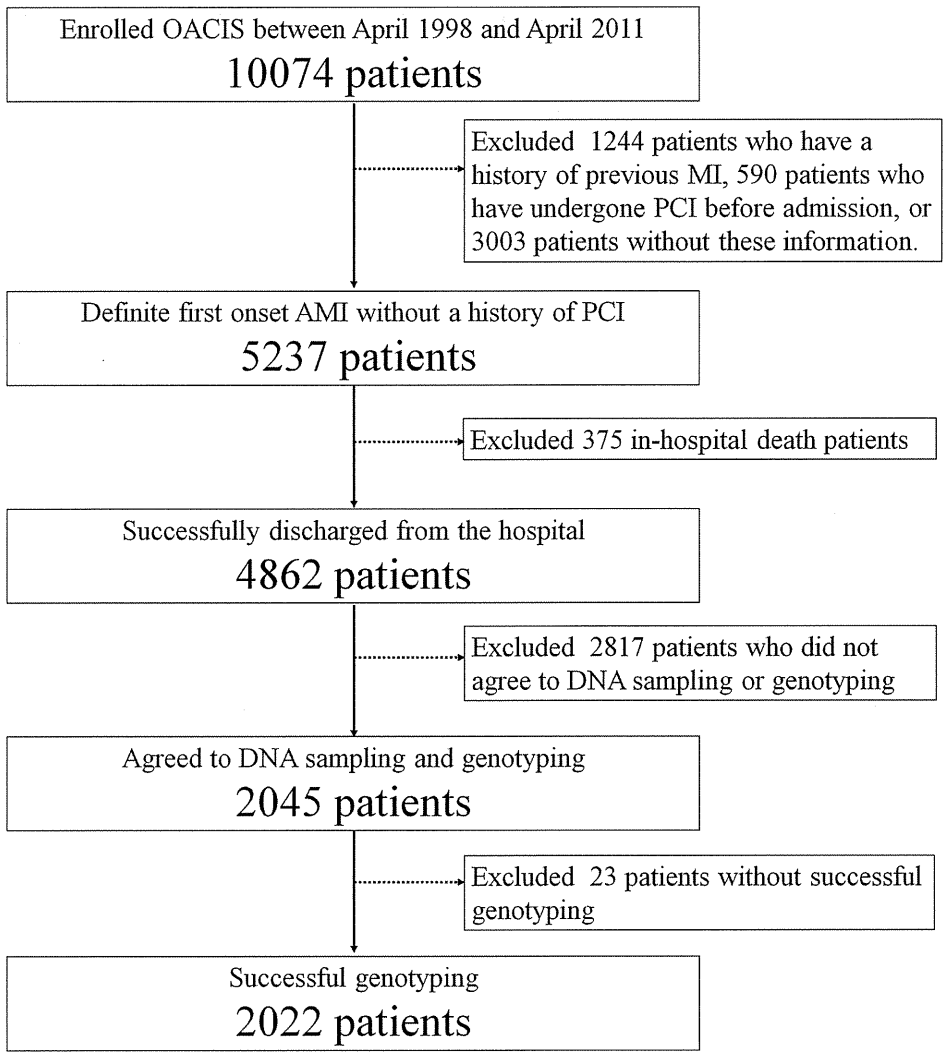


Figure 1 Patient selection flow chart. AMI, acute myocardial infarction; MI, myocardial infarction; OACIS, Osaka Acute Coronary Insufficiency Study; PCI, percutaneous coronary intervention.

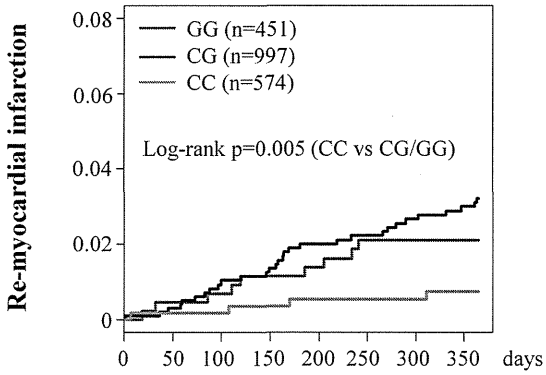
inhibitor, angiotensin receptor blocker, β-blockers, calcium channel blockers, statins, diuretics and dual antiplatelet agents as covariates. Hence, the final multivariate model included all the aforementioned covariates regardless of the univariate results shown in online supplementary table S1 because we assumed that even non-significant differences in these covariates could be confounders and should be adjusted. Gene–drug interactions were evaluated using p for the interaction between genotype and each drug tested. Statistical significance was set as $p<0.05$ for comparison of patient background or gene–drug interaction. Bonferroni correction for multiple testing was employed during the secondary prevention analysis and statistical significance was set as $p<0.025$ (0.05 divided by the number of independent testing; log-rank test and multiple Cox regression analysis). All statistical analyses were performed using SAS V.9.3 (SAS Institute Inc, Cary, North Carolina, USA) or R software packages V.2.15.1 (R Development Core Team).

RESULTS

Patient characteristics and medications at discharge are shown in table 1. The median age was 65 years, 76.8% were male and 87.7% had ST-elevation myocardial infarction. No significant differences in patient background based on rs1333049 genotypes were detected.

In the primary prevention setting, the rs1333049 C allele was associated with increased susceptibility to AMI (OR 1.20 per C allele increase, 95% CI 1.09 to 1.33, $p=2.3*10^{-4}$; and OR 1.38 CC vs CG/GG, 95% CI 1.17 to 1.62, $p=8.7*10^{-5}$) compared with 1373 healthy Japanese controls (figure 3). The frequencies of the CC, CG and GG genotypes of rs1333049 were 28.4% (574/2022), 49.3% (997/2022) and 22.3% (451/2022), respectively, among the study population, and 22.4% (307/1373), 52.3% (718/1373) and 25.3% (348/1373), respectively, among healthy controls.

In the secondary prevention setting, 43 ReMI (4 for CC, 30 for CG and 9 for GG genotypes) occurred during a 1-year follow-up period after survival discharge



Number at risk

Day	0	100	200	300
GG	451	428	419	408
CG	997	933	909	887
CC	574	562	530	530

Figure 2 Kaplan–Meier estimates of remyocardial infarction event.

for their first AMI. The Kaplan-Meier analysis revealed that the incidence of ReMI differed between patients with the CC and CG/GG genotypes (log-rank p=0.005; figure 2). Multivariate Cox regression analysis revealed that the CC genotype was associated with a lower risk of ReMI after survival discharge compared with the CG/GG genotypes (adjusted HR 0.20, 95% CI 0.06 to 0.65, p=0.007). Subgroup analysis demonstrated that the association between the rs1333049 CC genotype and a lower incidence of 1 year ReMI was common to all subgroups, and no significant gene–drug interactions were detected (figure 4).

DISCUSSION

The present study demonstrated that, in the secondary prevention setting of AMI, homozygous carriers of the rs1333049 risk allele (CC genotype) on chromosome 9p21 had a reduced incidence of ReMI, whereas the C allele did have conferred susceptibility to their first AMI. This result is of clinical importance because this is the first study to clearly show a change in the susceptibility risk of the 9p21 variant to AMI between before and after the first AMI, namely, between the primary and secondary prevention settings.

Historically, 9p21 SNP was identified as a susceptibility variant of CAD with GWAS using data from the Wellcome Trust Case Control Consortium in 2007.[3] Many other GWAS have also revealed the same association between 9p21 SNP and CAD and/or myocardial infarction.[3–8] In addition, one report by Chan *et al*[11] suggested the presence of a common pathway to develop CAD and myocardial infarction via 9p21 SNP. Thus, the 9p21 SNP is now considered as one of the most robust susceptibility variants of myocardial infarction and/or CAD in the primary prevention setting. To date, three major studies have assessed the association between 9p21 genetic variation and ReMI rates after ACS (table 2)[12–14]: the Italian genetic study and TexGen registry reported a lack of association with ReMI events after early-onset myocardial infarction and ACS (fraction unknown), respectively,[13–14] while the GRACE genetic study suggested a susceptibility risk of 9p21 SNPs for ReMI after ACS (STEMI) 27.2%, non-STEMI 43.3%, and unstable angina 29.5%).[12] Since Buysschaert *et al*[12] also reported that the statistical significance of the susceptibility risk of 9p21 disappeared after full adjustment for patient background in the GRACE genetic study, it is possible to interpret the results of these three studies as a lack of 9p21 susceptibility to ReMI in post-ACS patients.[12–14] These findings are of clinical significance because they suggested a modification of the genetic risk of 9p21 by secondary prevention programmes after ACS. However, these studies are limited to discuss modification of genetic risk with secondary prevention programmes since they only examined the susceptibility impact of 9p21 SNPs to the ReMI without comparison with that to the first AMI (ACS) in their study cohort.

From this point of view, it is noteworthy that the present study clearly showed a change in the 9p21 susceptibility risk to AMI between before and after the first onset of AMI in the same population. In the present study, the results showed that the rs1333049 C allele was associated with onset of the first AMI (OR 1.20, 95% CI 1.09 to 1.33, $p=2.3*10^{-4}$), which was consistent with the results of several previous studies in the primary prevention settings.[3–8] Interestingly, however, the present study also demonstrated that patients with the CC genotype had a lower incidence of 1 year ReMI (adjusted HR, 0.20, 95% CI 0.06 to 0.65, p=0.007) as a novel finding. These observations suggested that the risk of the 9p21 variant seen in the primary prevention setting of the present study population was modified in the secondary prevention setting. Although it is speculative, it is considered that modification of genetic risk of the rs1333049 C allele might have unmasked the risk of the rs1333049 G allele in the secondary prevention setting (see online supplementary figure S2). However, it should be discussed why the 9p21 rs1333049 CC genotype was associated with a reduced incidence of ReMI in the present study, while not in the previous three studies.[12–14] One possible explanation for this discrepancy between the previous studies and our current study is that we only include patients with post-AMI who were treated with emergent PCI on admission and survived to discharge, whereas all previous studies included all of the patients hospitalised for AMI or ACS and thus include ReMI during the acute stage of ACS as an end point. Considering that ReMI occurring during the acute stage of ACS was most likely associated with lesion-related or procedure-related backgrounds such as reocclusion of the culprit lesion due to a thrombus or mechanical acute closures rather than genetic background, inclusion

Table 1 Patient background based on the rs1333049 Genotype

Parameter	Overall (n=2022)	CC (n=574)	CG (n=997)	GG (n=451)	p Value
Age, years	65 (57–73)	65 (57–73)	65 (57–73)	65 (58–73)	0.986
Male, %	76.8	78.6	75.2	77.8	0.265
BMI, kg/m^2	23.8 (21.9–25.9)	23.9 (21.8–25.7)	23.7 (21.8–25.8)	23.8 (22.0–26.0)	0.653
STEMI, %	87.7	88.1	86.6	89.6	0.272
Coronary risk factor					
Diabetes, %	31.6	33.0	29.7	33.7	0.227
Hypertension, %	60.1	61.0	59.5	60.3	0.829
Dyslipidemia, %	46.5	43.8	47.2	48.5	0.267
Smoking, %	64.3	63.6	64.4	65.2	0.868
CAG Findings					
Target lesion					0.153
Left main trunk, %	1.0	1.0	1.0	1.1	
LAD, %	45.1	43.2	43.7	50.3	
Diagonal branch, %	2.9	3.0	2.7	3.1	
RCA, %	35.8	37.3	35.6	34.4	
LCx, %	14.7	14.8	16.5	10.6	
Graft, %	0.1	0.3	0.0	0.0	
Unknown, %	0.4	0.3	0.4	0.4	
Stenting, %	88.8	90.1	87.6	90.0	0.207
Multivessel disease, %	40.2	38.6	40.1	42.4	0.473
Peak CPK, IU/L	2269 (1027–4006)	2304 (1005–4087)	2242 (1026–4041)	2345 (1104–3882)	0.898
Medication at discharge					
ACEI, %	44.6	46.2	44.2	43.5	0.650
ARB, %	40.4	38.2	41.4	41.0	0.425
Beta-blocker, %	62.0	59.9	62.5	63.4	0.466
Calcium-blocker, %	13.5	13.2	13.2	14.4	0.814
Statin, %	53.5	50.7	54.1	55.7	0.249
Diuretics, %	24.7	22.6	26.0	24.4	0.333
Dual antiplatelet, %	80.8	80.8	80.6	80.9	0.990

Categorical variables are presented as a percentage and continuous variables are presented as a median (25–75 percentiles).
ACEI, ACE inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; CAG, coronary angiography; CPK, creatine phosphokinase; LAD, left anterior descending artery; LCx, left circumflex artery; RCA, right coronary artery; and STEMI, ST-elevation myocardial infarction.

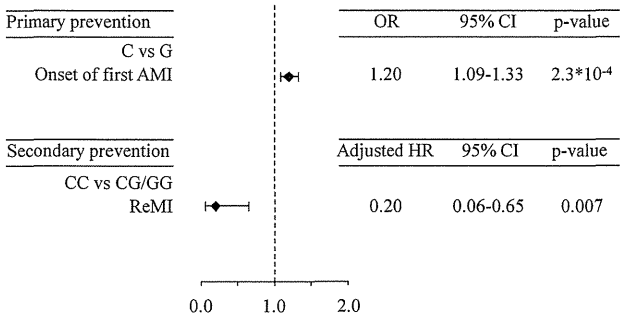


Figure 3 Impact of the rs1333049 genotype on onset and 1 year remyocardial infarction. AMI, acute myocardial infarction; (CC vs CG/GG); (C vs G per allele); ReMI, recurrence of myocardial infarction.

of these ReMI might have made the interpretation of the results difficult in the previous studies. In addition, limiting patient selection to those treated with primary PCI for the first AMI in the present study might have clearly elucidated the 9p21-treated susceptibility to ReMI in the secondary prevention cohort.

The 9p21 locus is adjacent to the tumour suppressor genes CDKN2A and CDKN2B.[8] Although the mechanism by which variation in the 9p21 locus increases AMI susceptibility in the primary prevention setting remains unclear,[8] the evidence-based secondary prevention programmes might have masked the susceptibility risk of the 9p21 rs1333049 C allelle to ReMI after ACS in the present study (figure 2), possibly via stabilising coronary plaques.[9–14 22–23] Indeed, Do *et al*[24] reported that the impact of 9p21 genetic variation can be modified by increasing the dietary intake of vegetables, suggesting a role of secondary prevention programmes including dietary practice. Thus, further studies are warranted to investigate whether and how secondary prevention programmes with evidence-based medication and lifestyle modification can reduce the risk of ReMI in patients with 9p21 genetic variants in the near future. In particular, the potential interaction of the rs1333049 SNP with secondary prevention medications warrants further investigation, because gene–drug interactions have already been detected in cardiovascular patients treated with warfarin, clopidogrel and statin.[25–27]

The present study has several limitations that warrant mention. First, since our study population only consisted of patients who provided written informed consent at survival discharge, there may have been selection bias as well as a survival bias since high-risk patients carrying the C allele might have died more frequently than patients with the GG genotype during hospitalisation. Second, the number of ReMI was relatively small and our study lacked a replication cohort to validate our observations. Therefore, replication studies with a larger sample are warranted to confirm our observations. However, it is often difficult to have a validation cohort in a prospective

Figure 4 Subgroup analysis of the impact of rs1333049 genotype on 1 year remyocardial infarction rate. ACEI, ACE inhibitor; ARB, angiotensin receptor blocker; DAPT, dual antiplatelet therapy; NA, not assessed due to the insufficient number of events in the subgroup analysis; and STEMI, ST-elevation myocardial infarction.

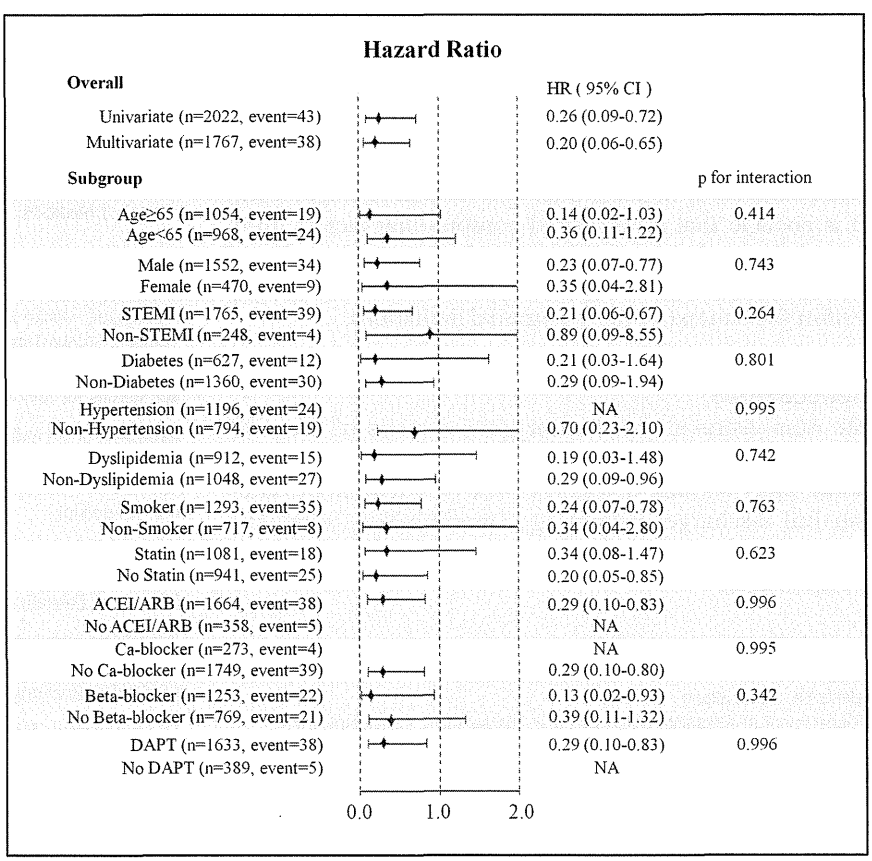


Table 2 Summary of studies examining the association between 9p21 variants and remyocardial infarction events after acute coronary syndrome

	OACIS registry	GRACE genetic study	Italian genetic study	TexGen registry
Reference	–	12	13	14
Year	–	2010	2011	2012
SNP	rs1333049	rs1333049	rs1333040	rs1333049
Pt number	2022	2942	1508	2067
Design	Prospective	Prospective	Prospective	Prospective
Follow-up	1 year	6 months	9.95 years	3.2 years
Population	Japan	UK, Belgium, Poland	Italy	USA
Background disease	MI (STEMI 87.7%, non-STEMI 12.3%)	ACS (STEMI 27.2%, non-STEMI 43.3%, UA 29.5%)	Early-onset MI	ACS (fraction unknown)
PCI	100%	47.5%	0%	63.6%
End point	ReMI after survival discharge	ReMI including in-hospital events	ReMI including in-hospital events	ReMI including in-hospital events
Conclusion	Low event rate with homozygous carriers of risk allele	High event rate with risk allele carriers (univariate)	No association	No association
Replication	None	None	None	None

ACS, acute coronary syndrome; CAD, coronary artery disease; DES, drug-eluting stent; GRACE, Global Registry of Acute Coronary Events; MI, myocardial infarction; OACIS, Osaka Acute Coronary Insufficiency Study; PCI, percutaneous coronary intervention; Pt, participants; ReMI, recurrence of myocardial infarction; STEMI, ST-elevation myocardial infarction; UA, unstable angina.

observational study design.[28] Indeed, all studies presented in table 2 did not include a replication cohort. Third, therapeutic regiment of AMI varied across the study period. Fourth, data regarding the mechanism and culprit lesion of ReMI were not available. Since ReMI can occur through a variety of mechanisms such as acute stent thrombosis of the culprit lesion, excessive intimal proliferation of stented vessels, and plaque rupture of new atherosclerotic lesions, a detailed analysis for the mechanism of ReMI is ideal. Fifth, patient backgrounds and primary preventive medications were not adjusted in the case–control association study in the primary prevention setting. Finally, it is possible that unmeasured confounding factors influenced the study outcomes due to the inherent nature of observational registry. The data should be interpreted within the context of these potential limitations.

CONCLUSIONS

We demonstrated that homozygous carriers of the AMI susceptibility variant rs1333049 SNP C allele on chromosome 9p21 showed a reduced risk of 1 year ReMI after survival discharge, suggesting a modification of genetic susceptibility of AMI with secondary prevention programmes.

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Contributors All authors participated in the study conception and design, as well as acquisition, analysis and interpretation of data, drafting of the article and its critical revision for important intellectual content, and final approval of the manuscript.

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Ethics approval The study protocol complied with the Helsinki Declaration and the guidelines for genomic/genetic research issued by the Japanese government. The study was approved by the institutional ethical committee of each participating institution.

Patient consent Obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement No additional data are available.

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Reduced risk of recurrent myocardial infarction in homozygous carriers of the chromosome 9p21 rs1333049 C risk allele in the contemporary percutaneous coronary intervention era: a prospective observational study

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