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requests for materials
should be addressed to
N.D.
(ningzhengdong@
suda.edu.cn) or Q.W.
(wuq@ccf.org)* These authors
contributed equally to
this work.

A Corin Variant Identified in Hypertensive Patients That Alters Cytoplasmic Tail and Reduces Cell Surface Expression and Activity

Yue Zhang^{1*}, Hui Li^{1*}, Jianping Zhou², Aili Wang^{3,6}, Junhua Yang⁴, Can Wang¹, Meng Liu¹, Tiantian Zhou¹, Li Zhu^{1,6}, Yonghong Zhang^{3,6}, Ningzheng Dong^{1,6} & Qingyu Wu^{1,5,6}

¹Cyrus Tang Hematology Center, MOE Engineering Center of Hematological Disease, MOH Key Lab of Thrombosis and Hemostasis, Jiangsu Institute of Hematology, the First Affiliated Hospital, and Collaborative Innovation Center of Hematology, Soochow University, Suzhou, China, ²Department of Gerontology, First Hospital of Yancheng, Yancheng, China, ³Department of Epidemiology, School of Public Health, Soochow University, Suzhou, China, ⁴Department of Cardiology, First Affiliated Hospital, Soochow University, Suzhou, China, ⁵Molecular Cardiology, Cleveland Clinic, Cleveland, Ohio, ⁶Jiangsu Key Laboratory of Preventive and Translational Medicine for Geriatric Diseases, Soochow University, Suzhou, China.

Corin is a membrane-bound protease that regulates blood pressure by activating the natriuretic peptides. *CORIN* variants have been associated with hypertension and heart disease in African Americans. In this study, we conducted targeted exome sequencing and identified an insertion variant, c.102_103insA, in exon 1 of the *CORIN* gene. Analysis of two independent cohorts showed that the variant was preferentially present in hypertensive patients (38/795 or 4.78% vs. 4/632 or 0.63% in normal individuals, $p=4.14E-6$). The insertion shifted the reading frame, resulting in a corin variant with a truncated cytoplasmic tail. In cell-based studies, the corin variant exhibited poor trafficking in the Golgi, reduced cell surface expression and zymogen activation, and low natriuretic peptide processing activity. Compared with normal individuals with the wild-type allele, individuals with the variant allele had lower levels of plasma corin [0.59 ± 0.07 ng/mL ($n=25$) vs. 0.91 ± 0.02 ng/mL ($n=215$), $p<0.001$] and higher levels of plasma N-terminal pro-atrial natriuretic peptide (NT-pro-ANP) [2.39 ± 3.6 nmol/L ($n=21$) vs. 0.87 ± 0.6 nmol/L ($n=48$), $p=0.005$]. These results indicate that the variant altered corin structure and impaired the natriuretic peptide processing activity *in vivo*. The results highlight corin defects as an important underlying mechanism in hypertension.

Hypertension is a major cardiovascular disease, which increases the risk for stroke, myocardial infarction, and heart failure. Genetic factors play an important role in hypertension^{1–4}. To date, molecular defects in most hypertensive patients remain unclear^{2,3}, suggesting that more contributing factors are yet to be identified. Atrial and B-type natriuretic peptides (ANP and BNP) are cardiac hormones that regulate sodium homeostasis and blood pressure^{5,6}. In cardiomyocytes, these peptides are synthesized in pro-forms, *i.e.* pro-ANP and pro-BNP, which are converted to active ANP and BNP by corin, a membrane-bound serine protease⁷. The importance of corin in regulating blood pressure has been shown in corin knockout mice, which exhibited reduced sodium excretion and salt-sensitive hypertension due to impaired natriuretic peptide processing^{8–10}.

Structurally, corin belongs to the type II transmembrane serine protease family, a group of trypsin-like enzymes involved in diverse biological processes^{11,12}. All these membrane-bound proteases consist of an N-terminal cytoplasmic tail, a single-span transmembrane domain and an extracellular region containing various modules and a C-terminal protease domain. The transmembrane domain anchors the proteases on the cell surface, where proteolytic processes take place^{11,12}. It has been shown that cell surface targeting is important for corin zymogen activation and biological activity^{13–15}.

Human *CORIN* gene consists of 22 exons and spans >200 kb in length on chromosome 4¹⁶, making it one of the largest protease genes in the human genome. In principle, natural variants and mutations are more likely to occur in large genes. To date, *CORIN* variants have been associated with hypertension in African Americans^{17–20}. *CORIN* mutations that reduce corin activity also have been found in patient families with hypertension and



preeclampsia^{21–24}. It remains unknown if additional *CORIN* variants are present in general populations that may contribute to hypertension.

In this study, we conducted targeted exome sequencing and identified a novel insertion variant in the *CORIN* gene that was preferentially present in patients with hypertension. Biochemical experiments showed that the variant altered corin cytoplasmic tail sequence and reduced corin cell surface expression, zymogen activation, and pro-ANP processing activity. Our results suggest that naturally occurring *CORIN* variants may reduce corin activity and contribute to hypertension in patients.

Results

Identification of *CORIN* Gene Variant. We sequenced *CORIN* exons and intron-exon boundaries in genomic DNA samples from normal and hypertensive individuals and identified a variant allele with a single adenine insertion at nucleotide position 102 (c.102_103insA, referred to as “insA variant” hereafter) in exon 1 of the *CORIN* gene (Fig. 1A)^{16,25}. The insertion shifted the reading frame, creating a down-stream stop codon (Fig. 1B).

The variant allele occurred more frequently in hypertensive patients than normal individuals. In the first study cohort, 23 of 401 hypertensive patients (5.74%) were heterozygous for this variant allele, whereas 2 of 217 normal individuals (0.92%) were heterozygous for the variant allele ($p=0.0037$). We verified this finding in a second study cohort. Among 394 hypertensive patients, 15 or 3.81% were heterozygous for the variant allele, whereas 2 of 415 normal individuals (0.48%) were heterozygous for the variant allele ($p=0.00098$). When the data of both cohorts were combined, 38 of 795 hypertensive patients (4.78%) and 4 of 632 normal individuals (0.63%) were heterozygous for the variant allele ($p=4.14E-6$). We searched the 1000 genome database (www.1000genomes.org) and found the insA *CORIN* allele with a minor allele frequency of 0.0037 (8 alleles in 1094 individuals), which is similar to that in our normal controls (0.0032; 4 alleles in 632 individuals; $p=0.81$). The results indicate that the variant allele is present in general popu-

lations but its occurrence is particularly high in hypertensive patients. In agreement with this, multivariate logistic regression analysis showed consistent association of the insA variant with hypertension in both study cohorts (Supplementary Tables S1 and S2).

Characterization of insA Variant Corin. The insertion of an adenine at nucleotide position 102 shifts the reading frame (Fig. 1B), which is expected to prevent corin expression. We made the plasmid, pcDNAinsA, containing the adenine insertion and transfected it in HEK293 cells. WT corin (positive) and inactive mutants R801A and S985A (negative) were used as controls (Fig. 1B). Surprisingly, Western analysis detected the insA variant expression in transfected HEK293 cells (Fig. 2A).

To test if the insA variant corin is biologically active, a pro-ANP processing assay was performed. The variant exhibited pro-ANP processing activity, although the level was markedly reduced compared to that of WT (Fig. 2B). In negative controls (vector, mutants R801A and S985A), little pro-ANP processing activity was observed (Fig. 2B). By densitometric analysis, the variant had $41.3 \pm 8.5\%$ activity compared with that of WT ($p<0.01$) (Fig. 2C).

Zymogen Activation of Corin Variant. One possible explanation for the insA variant expression is that a down-stream methionine was used as the translation initiation site. Indeed, there is a methionine at position 30 that may be used as an alternative initiation site (Fig. 1B). To test this hypothesis, we expressed the variant in HEK293 cells together with a corin isoform that contains methionine 30 (Met-30) but lacks methionine 1 (Met-1)¹⁵. By Western analysis, similar expression levels of the insA variant, the Met-30 isoform (Met-30), WT, and mutants R801A and S985A were observed (Fig. 3A). On the Western blots, an ~40-kDa band (corin-p), representing the corin protease domain cleaved at the activation site Arg-801 (Fig. 1B), was detected in WT (Fig. 3A, lane 2). This band also was detected in the catalytic site mutant S985A (Fig. 3A, lane 5), but not in mutant R801A, in which the cleavage site is abolished (Fig. 3A, lane 4). The intensity of this band in the insA variant was markedly

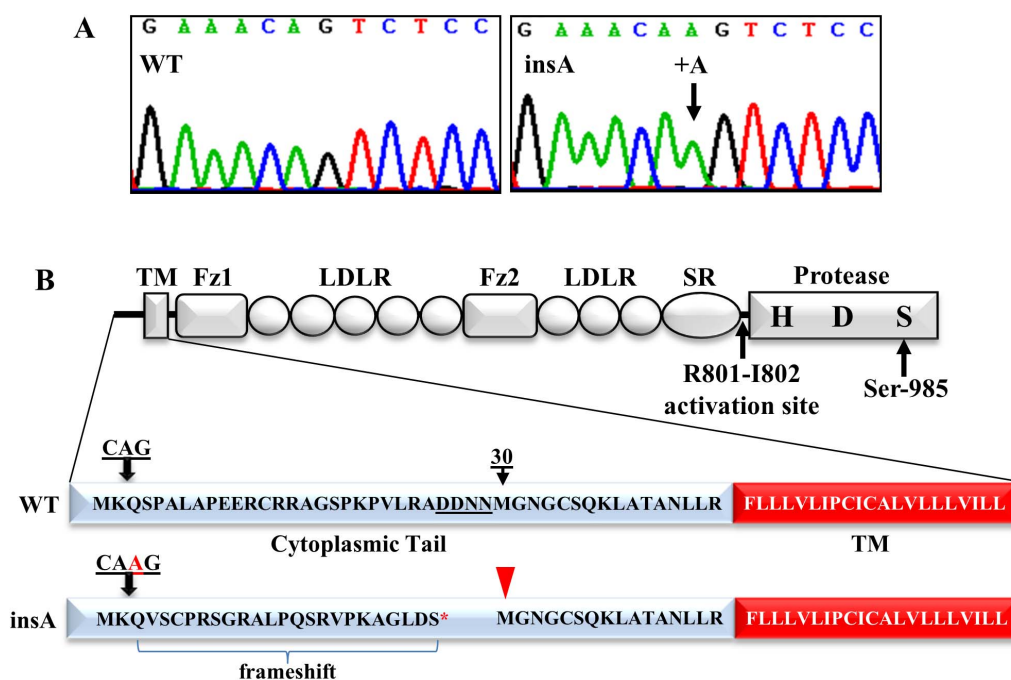


Figure 1 | Genetic variant altering corin cytoplasmic tail. (A) *CORIN* gene sequencing data from a WT allele (left) and a variant allele with an insertion (right). (B) Corin domain structure. TM, transmembrane; Fz, frizzled; LDLR, LDL receptor; SR, scavenger receptor. The catalytic sites His (H), Asp (D) and Ser-985 (S) in the protease domain are shown. The activation cleavage site R801-I802 is indicated. The insertion causes frameshift, creating a down-stream stop codon (*) in the cytoplasmic tail. An alternative initiation site, Met-30, is indicated by an arrowhead.

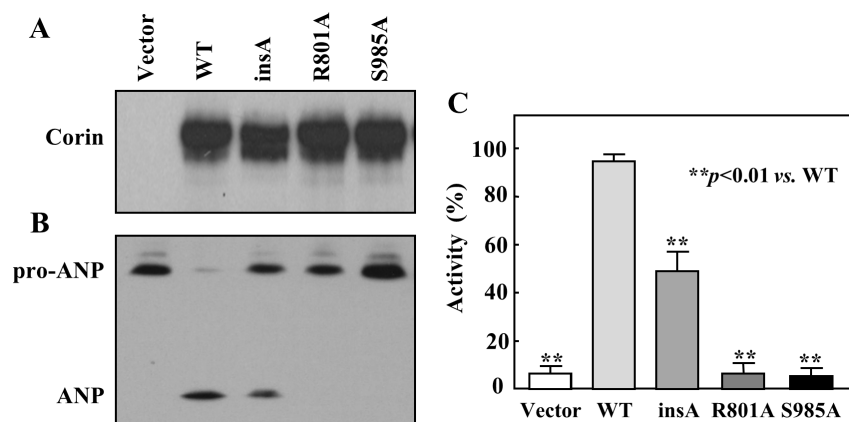


Figure 2 | Expression and activity of the insA variant in HEK293 cells. (A) Western analysis of corin WT, the insA variant, and mutants R801A and S985A protein levels in transfected HEK293 cells. (B) Pro-ANP processing activity of the insA variant. WT and inactive mutants R801A and S985A were positive and negative controls, respectively. (C) Quantitative data of pro-ANP processing activity were from densitometric analysis. The cropped blots are used in the main figures and full-length blots are included in the supplementary information (Supplementary Fig. S1). Data are representative of at least four independent experiments.

reduced (Fig. 3A, lane 3). Similar results were observed in the Met-30 isoform (Fig. 3A, lane 6). By densitometric analysis, the ~40-kDa band in WT represented $12.73 \pm 1.97\%$ of the total corin protein in lysate, whereas the band in the insA variant was $4.04 \pm 1.02\%$, significantly lower than that in WT ($n=5$, $p<0.01$) (Fig. 3B). In the Met-30 isoform, this band was $3.99 \pm 2.67\%$ of the total corin protein, which was similar to that in the insA variant (Fig. 3B). The data support that the insertion resulted in a variant corin with a shorter cytoplasmic tail and reduced zymogen activation and activity.

Corin Expression on Cell Surface. Corin is a membrane-bound protease and its activation depends on cell surface expression¹⁴. Previous studies indicated that cytoplasmic tail sequences may alter corin cell surface expression¹⁵. We examined the variant expression in biotin-labeled HEK293 cells. Surface expression of the insA variant was much less abundant than that of WT (5.5 ± 1.5 vs. $31.5 \pm 8.4\%$, $n=3$, $p<0.01$) (Fig. 4A and B). Similarly reduced cell surface expression was observed in the Met-30 isoform ($6.7 \pm 3.1\%$, $n=3$, $p<0.01$ vs. WT) (Fig. 4A and B). As controls, the cell surface expression of mutants R801A and S985A was similar to that of WT (Fig. 4A). In cell lysate, total corin levels were comparable in all samples (Fig. 4A, right panel). As expected, GAPDH was absent in labeled cell surface proteins, but present at similar levels in all lysate samples (Fig. 4A, lower panels).

We verified these results by flow cytometry. In HEK293 cells expressing WT, $32.7 \pm 8.5\%$ were corin-positive on the surface (Fig. 4C). In contrast, only 7.9 ± 3.7 and $6.9 \pm 6.4\%$, respectively, were corin-positive in cells expressing the insA variant and the Met-30 isoform ($n=3$; both p values <0.01 vs. WT).

Subcellular Localization of insA Variant. We next examined the intracellular distribution of the variant by immunostaining. In HEK293 cells expressing WT, predominant corin staining was on the cell surface (Fig. 5A). Such staining was absent in vector-transfected cells. In cells expressing the insA variant, corin staining was more prominent intracellularly. By co-staining with TGN46, a Golgi marker, a significant portion of the insA variant was found in the Golgi (Fig. 5A). In parallel experiments of co-staining with PDI, an ER marker, no significant staining of WT or the variant was found in the ER (Fig. 5B). The data indicate that the insA variant was less efficient in trafficking through the Golgi network.

Soluble Corin in Cultured Cell Medium. As reported previously²⁶, cell surface corin undergoes proteolytic shedding, generating three fragments of ~180-, ~160-, and ~100-kDa, respectively, in the conditioned medium. The ~180-kDa fragment was from a disintegrin and metalloprotease (ADAM)-mediated cleavage, whereas the ~160- and ~100-kDa fragments were from corin autocleavage²⁶. We examined corin shedding in HEK293 cells. Three bands of the

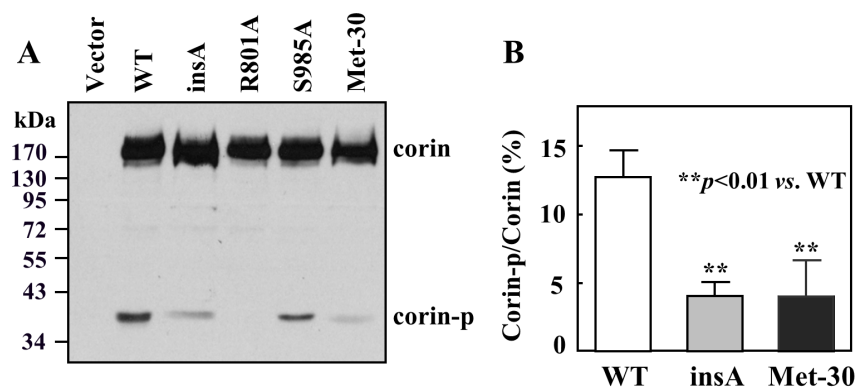


Figure 3 | Zymogen activation of the insA variant in HEK293 cells. (A) Western analysis of corin proteins in cell lysate. The ~40-kDa band represented the corin protease domain (corin-p) from activation cleavage. This band was absent in mutant R801A, in which the cleavage site was abolished. Data are representative of five independent experiments. (B) Percentage of the activated protease domain fragment (corin-p) vs. corin zymogen protein (corin) was estimated by densitometry. Data are mean \pm S.D. from five independent experiments.

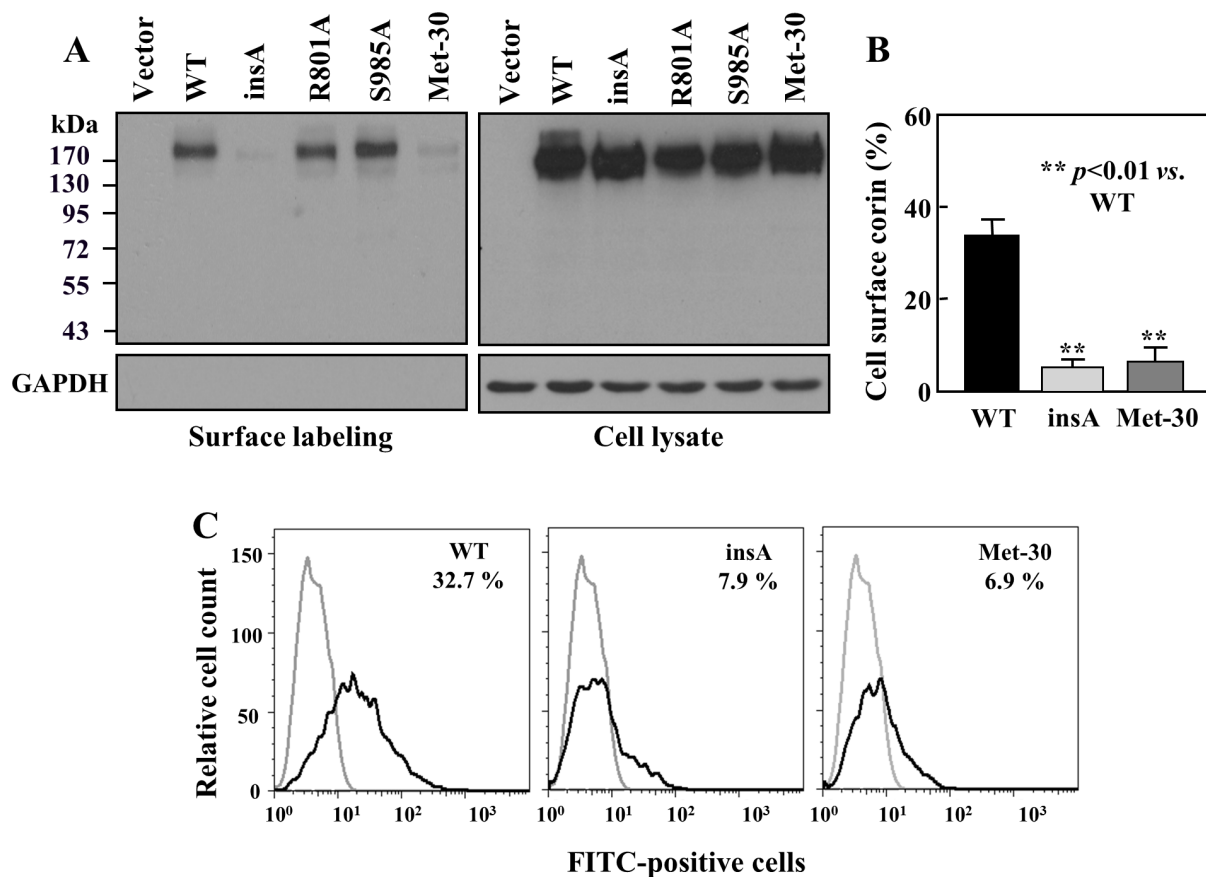


Figure 4 | Cell surface expression of corin proteins. (A) Western analysis of biotin-labeled corin proteins on the cell surface (left panel) and in cell lysate (right panel). GAPDH was used as a control (lower panels). The cropped blots are used in the main figure and full-length blots are included in the supplementary information (Supplementary Fig. S2). (B) Corin bands on Western blots were analyzed by densitometry. Percentage of corin proteins on the cell surface was estimated. Data are mean \pm S.D. from three independent experiments. (C) Analysis of cell surface corin proteins by flow cytometry. Data are representative of three independent experiments.

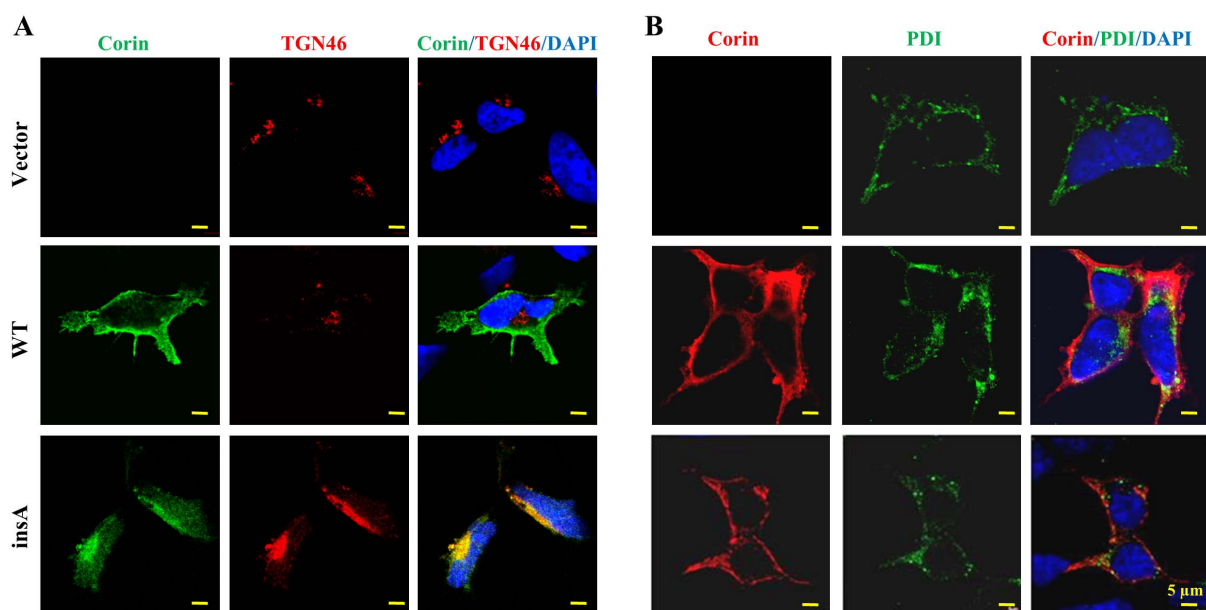


Figure 5 | Subcellular distribution of corin proteins. (A) HEK293 cells expressing WT and the insA variant were stained for corin (green) and TGN46, a Golgi marker (red). (B) HEK293 cells expressing WT and the insA variant were stained for corin (red) and PDI, an ER marker (green). In both experiments, cell nuclei were stained with DAPI (blue) and vector-transfected cells were used as a negative control. Bar: 5 μ m.



predicted sizes were found in the conditioned medium from cells expressing WT (Fig. 6A). In cells expressing the insA variant and the Met-30 isoform, the ~180-kDa fragment was less abundant and the ~100-kDa fragment was hardly visible (Fig. 6A). By densitometric analysis of the Western blots, the percentage of the ~180-kDa fragment (Fig. 6B) or all three soluble corin fragments (Fig. 6C) was significantly lower than that of WT ($n=4$, p values <0.01). In inactive mutants R801A and S985A, only the ~180-kDa ADAM-cleaved fragment was detected, but not the ~160- and ~100-kDa autocleavage fragments, as predicted. The results indicate that the insA variant had reduced shedding, likely due to impaired cell surface targeting and zymogen activation.

Plasma Soluble Corin and NT-pro-ANP Levels. We also measured plasma corin in individuals with WT and the insA variant *CORIN* alleles. The levels in 215 normal individuals with the WT allele were 0.91 ± 0.02 ng/mL, whereas the levels in 25 individuals with the variant allele were significantly lower (0.59 ± 0.07 ng/mL, $p<0.001$) (Fig. 6D). When the 25 individuals with the insA variant allele were separated into normotensive and hypertensive groups, the levels in two normotensive individuals (mean \pm range: 0.91 ± 0.04 ng/mL) were higher than that in 23 hypertensive individuals (mean \pm SD: 0.57 ± 0.07 ng/mL). However, the sample size in the normotensive group was too small for statistical analysis. In a subset of samples from individuals with the WT allele, plasma corin levels were lower in hypertensive individuals (0.62 ± 0.09 ng/mL, $n=60$, $p<0.01$) than that in normotensive individuals (0.86 ± 0.43 ng/mL, $n=60$).

We next measured plasma levels of NT-pro-ANP in individuals with WT and the insA variant alleles (Fig. 6E). Plasma NT-pro-ANP levels in 48 normal individuals with the WT allele were 0.87 ± 0.6 nmol/L, whereas the levels in 21 individuals with the variant allele were 2.39 ± 3.6 nmol/L, significantly higher than that in the normal control ($p=0.005$).

Discussion

In this study, we performed targeted exome sequencing and identified an insertion variant, c.102_103insA, in the *CORIN* gene. The occurrence of this variant allele in the normal controls from two independent cohorts (4/632 or 0.63%) is similar to that in the 1000 genome database (8/1094 or 0.73%, $p=0.81$). This allele, however, is much more prevalent in hypertensive patients (23/401 or 5.74% in the first cohort and 15/394 or 3.81% in the second cohort, both p values <0.001 vs. normal controls in respective cohorts), suggesting that the variant allele may contribute to hypertension in the patients. Previously, a corin minor allele, T555I/Q568P, is linked to hypertension and heart disease^{17–19}. The T555I/Q568P variant allele appears unique in African Americans, as it is mostly absent in other ethnic groups¹⁷. Here we have identified a novel corin variant allele that is associated with hypertension in different ethnic populations.

The insertion of a single adenine at nucleotide position 102 shifts the reading frame, causing a down-stream stop codon. In principle, the insertion may result in a null allele. In functional studies, however, we found that the insA variant was expressed at a similar level to that of WT in HEK293 cells, suggesting that the variant may use Met-30 as an alternative initiation site to produce a corin protein with a

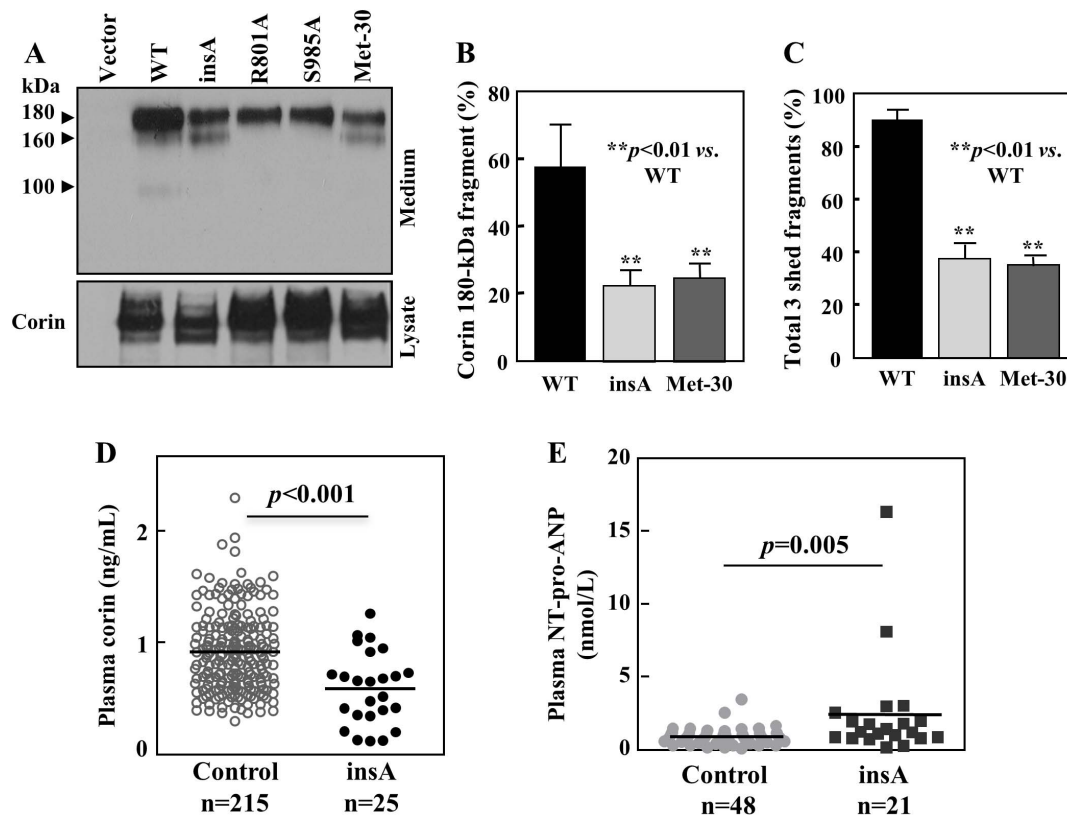


Figure 6 | Shed corin fragments in conditioned medium and plasma levels of soluble corin and NT-pro-ANP. (A) Western analysis of corin fragments in the conditioned medium from cells expressing corin WT, the variant and mutants (upper panel). Corin proteins in cell lysate are shown as a control (lower panel). The cropped blots are used in the main figure and full-length blots are included in the supplementary information (Supplementary Fig. S3). Data are representative of four independent experiments. Western blots were analyzed by densitometry. Percentages of the ~180-kDa corin fragment (B) or all three soluble corin fragments (C) were estimated. Data are mean \pm S.D. from four independent experiments. (D) Plasma levels of soluble corin in individuals with WT (Control) and the variant (insA) alleles. (E) Plasma levels of NT-pro-ANP in individuals with WT (Control) and the variant (insA) alleles.



shorter cytoplasmic tail. On Western blots, the variant and WT corin migrated similarly, indicating that Western blots are not sensitive enough to detect such a small size difference in corin cytoplasmic tails. This hypothesis was supported by a similar migration pattern in the Met-30 isoform, which contains Met-30 but lacks Met-1 (Fig. 3A).

Previously, we found that different cytoplasmic tail sequences may alter corin expression and activity on the cell surface¹⁵. In our functional studies, the insA variant exhibited low pro-ANP processing activity (Fig. 2), which was likely due to reduced cell surface expression (Fig. 4) and impaired zymogen activation (Fig. 3). By immunostaining, we found that the variant was partially retained in the Golgi, reducing its cell surface targeting (Fig. 5). Since corin zymogen activation occurs on the cell surface but not intracellularly¹⁴, these results may explain the reduced zymogen activation and activity of the variant. Earlier, a protein motif, DDNN, which precedes Met-30 in human corin cytoplasmic tail (Fig. 1B), was found to be important for corin cell surface targeting¹⁵. Consistent with these data, the insA variant lacking the DDNN motif exhibited reduced cell surface expression and zymogen activation in our experiments.

As a mechanism to regulate corin activity on the cell surface, corin is shed by autocleavage and ADAM-mediated proteolysis²⁶. Similar shedding has been reported in other membrane-bound proteases^{27–30}. In this study, we found reduced corin shedding fragments in HEK293 cells expressing the insA variant, consistent with the reduced cell surface expression and zymogen activation of the variant. In agreement with this finding, plasma corin levels were significantly lower in individuals with the variant allele than with WT allele, suggesting that the variant may have poor cell surface expression, impaired zymogen activation, and reduced shedding *in vivo*.

Low levels of plasma corin antigen and activity have been reported in patients with hypertension and heart disease^{31–33}. Low levels of serum corin also were found to be a predictor for poor outcomes in patients with coronary heart disease³⁴. In this study, low plasma corin levels were found in hypertensive patients with the variant allele. Consistently, plasma levels of NT-pro-ANP were much higher in these individuals. These data indicate that the activity of the insA variant is lower *in vivo*, which may impair natriuretic peptide processing in the individuals with the variant alleles.

The ANP signaling pathway is important for salt-water balance and normal blood pressure. Genetic variants and mutations in the genes coding for ANP or its receptor have been found in patients with hypertension and heart disease^{35–41}. Corin acts as the pro-ANP convertase at the top of the ANP pathway^{7,24,42,43}. Here we report a novel *CORIN* variant allele that is present preferentially in hypertensive patients. In functional studies, the variant exhibited poor intracellular trafficking, reduced cell surface expression, and impaired zymogen activation, which may explain low plasma corin and higher plasma NT-pro-ANP levels in the patients with the variant allele. Together, these results indicate that corin defects that impair the ANP pathway may represent an important mechanism underlying hypertension.

Methods

Blood Samples. The ethics committee of the First Affiliated Hospital of Soochow University approved this study. Written informed consent was obtained from all study participants. All experiments were performed in accordance with the approved guidelines and regulations. Blood samples were collected from normal individuals and hypertensive patients. Normal individuals had systolic blood pressure <140 mmHg and diastolic blood pressure <90 mmHg with no history of hypertension, heart disease and other diseases. Patients with hypertension had either systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg, or having a history of hypertension and taking anti-hypertensive drugs. The first study cohort consisted of 217 normal individuals and 401 patients with hypertension. The second cohort consisted of 415 normal individuals and 394 hypertensive patients. The basic characteristics of the normal controls and hypertensive patients of these two cohorts are shown in Supplementary Tables S3 and S4, respectively.

Plasma Soluble Corin and N-terminal pro-ANP. Plasma corin levels were measured by ELISA (R&D Systems)^{44,45}. Briefly, plasma samples were added to 96-well plates

coated with an anti-corin antibody. After 2 h at room temperature, the plates were washed with phosphate-buffered saline. A biotin-labeled anti-corin secondary antibody was added for 2 h. After washing, horseradish peroxidase-conjugated streptavidin was added. After incubation and washing, a horseradish peroxidase substrate (3,3',5,5'-tetramethylbenzidine) was added. The reaction was stopped by adding H₂SO₄ and the optical density in wells was recorded by a spectrometer at 450 nm. A ELISA kit (Biomedica Group) was used to measure plasma N-terminal pro-ANP (NT-pro-ANP), *i.e.* pro-ANP 1–98⁴⁶. Experimental procedures were similar to those of the corin ELISA, as indicated by the manufacturer's instructions.

DNA Sequencing. Genomic DNA was isolated from white blood cells using a Qiagen kit. The samples were used in PCR to amplify *CORIN* gene exons and intron-exons boundaries¹⁶. Amplified PCR products were sequenced. Nucleotide polymorphisms found were verified by independent PCR followed by subcloning the PCR fragments and DNA sequencing.

Plasmid Constructs. Plasmid pcDNA3.1 (Invitrogen) was used to express human corin wild-type (WT) and mutants¹⁴. Plasmids expressing corin activation cleavage site mutant R801A and active site mutant S985A were described previously^{14,15}. The corin proteins encoded by the plasmids had a C-terminal V5 tag for detection by Western blotting using an anti-V5 antibody (Invitrogen). Plasmid expressing corin insA variant was made using WT corin plasmid as a template. By site-directed mutagenesis, a single base of adenine was inserted at nucleotide position 102²⁵. The resulting plasmid, pcDNainsA, and all other plasmids were verified by DNA sequencing.

Cell Transfection and Western Blotting. Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum at 37°C. Plasmids were transfected into HEK293 cells using TurboFect reagents (Thermo Scientific). Conditioned medium was collected after 48 h and the transfected cells were lysed with a lysis buffer¹⁴. Procedures for immunoprecipitation and Western analysis of corin proteins in the conditioned medium and cell lysate were described previously¹⁴.

Pro-ANP Processing Assay. Corin pro-ANP processing activity was examined by a cell-based assay¹⁴. Briefly, corin WT and mutants were expressed in HEK293 cells. Conditioned medium containing human pro-ANP from stable cells was added to the cells at 37°C. After 1 h, the conditioned medium was collected. Pro-ANP processing was analyzed by immunoprecipitation and Western blotting^{14,15}. In these experiments, WT corin was used as a positive control and inactive mutants R801A and S985A were used as negative controls.

Corin Expression on Cell Surface. To examine corin cell surface expression, HEK293 cells expressing corin proteins were labeled with membrane impermeable Sulfo-NHS-SS-biotin (Thermo Scientific). The surface-labeled cells were lysed with a lysis buffer¹⁴. Biotin-labeled proteins were precipitated with avidin-agarose beads and analyzed by Western blotting. The optical density of corin bands on the blots was measured by densitometry, and the percentage of the activated corin fragments and corin fragments on the surface or conditioned medium was calculated, as described previously¹⁵.

Flow Cytometry. HEK293 cells expressing corin on the cell surface were analyzed by flow cytometry using an anti-V5 antibody, followed by an FITC-conjugated secondary antibody, as described previously¹⁵. Life-cell gating was performed using pyridinium iodide (Sigma). Data were acquired on Calibur flow cytometer (BD Biosciences) and analyzed by FlowJo v7.6.5 software (Tree Star).

Immunostaining. Corin-expressing HEK293 cells on coverslips were fixed with 4% paraformaldehyde or cold acetone and co-stained with a mouse anti-V5 antibody and a rabbit anti-TGN46 antibody (Sigma), or a rabbit anti-corin antibody and a mouse anti-protein disulfide isomerase (PDI) antibody (BD Biosciences). Secondary antibodies were conjugated with Alexa Fluor 488 (green) or 594 (red) (Invitrogen). DAPI (4',6-diamidino-2-phenylindole dihydrochloride) was used to stain cell nuclei. Immunostaining images were examined under a confocal microscope (Olympus).

Statistical Analysis. Data were analyzed using the Prism 5 (GraphPad) and SPSS18.0 software, and presented as means ± S.D. Comparisons between two groups were analyzed by Student's *t*-test. Multi-group comparisons were analyzed by ANOVA. Comparisons between two populations were analyzed by chi-squared test. Multivariate logistic regression analysis was done using SPSS 18.0 software. A *p* value of <0.05 was considered to be statistically significant.

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Author contributions

Y.Z., H.L., N.D. and Q.W. conceived and designed the experiments. Y.Z., H.L., C.W., M.L. and T.Z. performed the experiments. Y.Z., H.L., N.D. and Q.W. analyzed the data. J.Z., J.Y. and Z.L. contributed reagents/materials/analysis tools. A.W. and Y.-H.Z. provided DNA samples of the second cohort study. Y.Z., H.L., N.D. and Q.W. wrote the paper. All authors reviewed the manuscript. Y.Z. and H.L. contributed equally to this work.

Additional information

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