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ORIGINAL ARTICLE

Functional promoter −1816C>G variant of *RANKL* predicts risk and prognosis of lone atrial fibrillation

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Abstract

Receptor activator of nuclear factor-κB ligand (*RANKL*) had been confirmed contributing to the development and progression of AF by regulating atrial structural remodeling. But the involved genetic mechanism is unknown. We intended to explore the association between the polymorphism *RANKL* −1816C>G (rs7984870) and susceptibility and prognosis of lone AF. *RANKL* rs7984870 was genotyped in a case–control study of 828 patients and 834 controls in Chinese population. The CG and/or CC genotypes had an increased lone AF risk [adjusted odds ratio (OR) 1.20 for CG, OR 2.16 for CC, and OR 1.55 for CG/CC], compared with the *GG* genotype. Moreover, patients carrying CG/CC genotypes showed a higher possibility of AF recurrence after catheter ablation, compared with patients carrying GG genotype. In a genotype–phenotype correlation analysis using 24 normal left atrial appendage samples, increasing gradients of atrial *RANKL* expression levels positively correlated with atrial collagen volume fraction were identified in samples with CC, CG and GG genotypes. The in vitro luciferase assays also showed a higher luciferase activity of the −1816 C/C allele than that of the −1816 G/G allele. These results suggested that *RANKL* rs7984870 is involved in the etiology of lone AF and thus may be a marker for genetic susceptibility to lone AF and predicting prognosis after catheter ablation in Chinese populations. Therefore, we provide new information about treatment strategies and our understanding of *RANKL* in AF.

Keywords Receptor activator of nuclear factor-κB ligand · Lone atrial fibrillation · Genetic variation · Susceptibility · Prognosis

Introduction

Atrial fibrillation (AF) is one of the most common cardiac arrhythmias and its prevalence is increasing worldwide [1]. The mechanism is not fully understood. This is particularly true for lone AF (LAF), defined as AF in the absence of heart disease or comorbidities predisposing to the arrhythmia [2]. Radiofrequency catheter ablation (RFCA) is considered an effective treatment option for this kind of patients [3], but is associated with a considerable recurrence rate [3]. According to our previous findings, atrial structural remodeling (ASR), can reflect a spectrum of pathophysiological changes, which seems to play an important role in AF development and recurrence [4–6]. Identification of biochemical marker regulating ASR extent and prediction of AF recurrence after RFCA is of high clinical importance.

The receptor activator of nuclear factor-κB ligand (*RANKL*) is the most important member if osteoprotegerin (OPG)/RANK/*RANKL* axis, which is an essential pathway in the regulation of extracellular matrix remodeling [7]. Recently, we found that interactions between OPG, *RANKL*, and its cognate receptor RANK might be implicated in the pathogenesis and recurrence of AF through different mechanisms such as prevention of matrix degradation, promoting local inflammation and apoptosis in the atria [5, 6, 8]. *RANKL* is the key factor in the whole process of regulating atrial remodeling, because the increased

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RANKL/OPG ratio, which reliably reflects activity of the axis can aggravate myocardial fibrosis by regulating matrix metalloproteinase (MMP)/tissue inhibitor of metalloproteinase (TIMP) system [9], and *RANKL* can bind to decoy receptor OPG for competitively inhibiting the interaction between *RANKL* and RANK [10]. Tan et al. find that the promoter −1816C > G (rs7984870) polymorphism affected *RANKL* expression level [11]. Therefore, we hypothesized that *RANKL* rs7984870 was associated with LAF risk and AF recurrence after catheter ablation in Chinese population. Furthermore, we also designed to explore whether this polymorphism had an effect on *RANKL* expression in vitro and left atrial appendage (LAA) tissue.

Materials and methods

Subjects

We consecutively recruited 850 lone AF patients with written informed consent admitted to the Affiliated Drum Tower Hospital of Nanjing University Medical School and the First Affiliated Hospital of Nanjing Medical University between December 2013 and May 2017. The diagnosis was based on the following criteria [12]: age at first diagnosis of AF < 60 years, no past cardiovascular history, no evidence suggesting ischemic heart disease, no cardiomyopathy, no heart failure, no valvular heart disease, no diabetes, no hypertension within 2 years of the onset of AF, and no hyperthyroidism. Sex- and age-matched 850 AF-free control subjects with written informed consent were genetically unrelated to the cases. All cases and control subjects received comprehensive transthoracic echocardiographic examination (GE VIVID5, General Electric Medical, Wisconsin, USA) excluding structural heart diseases including valvular diseases, cardiomyopathies, heart failure and so on. The study was conducted according to the Helsinki Declaration and approved by the ethics committees.

Among all the subjects, 828 lone AF cases and 834 healthy controls were finally included in the susceptibility analyses due to DNA quality or quantity. Among the 828 cases, 734 received first-time catheter ablation and were successfully cardioverted to stable SR. During the follow-up, 126 patients were lost. Therefore, a total of 608 patients had complete follow-ups and clinical information.

Follow-up

AF-free time was calculated from the date of ablation to the date of recurrence or last follow-up. Atrial arrhythmias that occurred during the first 2 months after ablation, which is considered a blanking period [13], were not counted as recurrences. All patients with documented arrhythmia and those maintained on antiarrhythmics for control of AF beyond the blanking period were counted as recurrences.

Patients had scheduled clinical visits, 12-lead ECG, and 24-h Holter monitoring at 3, 6, and 12 months after ablation and then yearly after the first year. Moreover, patients would receive ECG monitoring in local clinics at anytime if they had AF-related symptoms. AF recurrence was identified by symptoms with ECG documentation of an atrial tachyarrhythmia lasting ≥ 30 s on a 12-lead ECG or Holter monitor recording. AF recurrence during the follow-up was considered censored.

Genotyping

The *RANKL* rs7984870 was determined by commercially available TaqMan assays (Applied Biosystems, Darmstadt, Germany). The SNP TaqMan genotyping assay was designed with Primer Express 3.0 (Applied Biosystems, primer sequences available on request). All TaqMan assays were carried out with TaqMan Genotyping Master Mix (Applied Biosystems) on a StepOne Plus realtime PCR cycler (Applied Biosystems) according to the manufacturer's instructions.

LAA samples

We collected 24 LAA tissues from healthy heart donors for transplantation. They were trauma victims and were free of cardiovascular pathology and documented AF. LAA specimens were obtained before perfusion.

Real-time quantitative RT-PCR

The primers of *RANKL* (Forward primer [F]: 5′-GCCAGC AGAGACTACACCAAG-3′, Reverse primer [R]: 5′-GAG GGCCACGAACATGGAG-3′, NM_003701) and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) [forward primer (F): 5′-ATGGGGAAGGTGAAGGTCG-3′, reverse primer (R): 5′-GGGGTCATTGATGGCAACAATA-3′, NM_002046] were synthesized by Invitrogen Co., Hong Kong, China. The synthesis of cDNA was according to the manufacturer's instructions with the reverse transcriptase kit (Promega Co., US). The real-time PCR was performed using the Applied Biosystems (Darmstadt, Germany). The results were analyzed according to the manufacturer's instructions. Data of transcripts were calculated relative to GAPDH using the $2^{-\Delta\Delta Ct}$ method. The measurements of each sample were performed in triplicate.

Western blot

Frozen LAAs were used for protein isolation. Proteins (40 μg/lane) were separated by sodium dodecyl sulfate

polyacrylamide gel electrophoresis and transferred onto polylinylidene fluoride membranes using a Bio-Rad semidry transfer system (Bio-Rad). The membranes were blocked with 5% non-fat dry milk and then probed with mouse monoclonal *RANKL* (ab45039, Abcam, USA) and horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-GAPDH (KC-5G5, KangChen Biotech, China). The resulting reaction was visualized using HRP-conjugated anti-mouse secondary antibody (Santa-Cruz Biotechnology, the Netherlands), followed by incubation with ECL Western Blot Detection Kit (Amersham, the Netherlands) for 1 min. The blots were exposed to Kodak film for 5 min and immunoreactive bands developed for quantification using The Discovery Series™ image analysis software (Bio-Rad) normalized by the corresponding value of GAPDH. Experiments were repeated three times and the mean was scored.

Masson's trichrome staining

After embedded in paraffin, 4 μm serial sections were sliced and subjected to Masson's trichrome staining to highlight collagen fibers. Collagen volume fraction (CVF) was determined by the HPISA 100 chromatic color pathological analysis system (Olympus, Japan) using five random images from each slide and five slides per sample, and the mean values of CVF were obtained by one investigator blinded to the groups.

Promoter functional assay

The *RANKL* promoter region (−1 bp to −2 kb relative to the transcription start site) was previously cloned into the luciferase reporter pGL4-basic vector [11]. The plasmids for −1816 G/G in the *RANKL* promoter region were mutated into −1816 C/C using the QuikChange Lightning Site-Directed Mutagenesis kit (Stratagene). After cloning, the vectors were sequenced to confirm the orientation and integrity of the inserts of each construct. For transfections, mouse atrial fibroblasts were seeded onto 24-well plates (100,000 cells per well), and each well was transfected with 1 μg of the vector DNA with either −1816G or −1816C allele, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). As an internal standard, all plasmids were cotransfected with 8 ng pRL-SV40, which contained the Renilla luciferase gene. The pGL4-basic vector without an insert was used as a negative control. After 48 h of incubation, cells were collected and analyzed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical analysis

Differences between the cases and controls were evaluated using the Student's *t* test (for continuous variables) and χ^2 test (for categorical variables). Hardy–Weinberg equilibrium was tested using a goodness-of-fit χ^2 test. The association between *RANKL* rs7984870 and AF risk was estimated by computing odds ratios (ORs) and their 95% confidential intervals (CIs) from multivariate logistic model. An allele-specific difference in luciferase activity was also tested using the Student's t-test. For the comparison of atrial expression of *RANKL* and the degree of atrial fibrosis, one-way ANOVA test (normally distributed) or Mann–Whitney test (two groups, non-normally distributed) and Kruskal–Wallis test (n groups, non-normally distributed) were used among the three genotypes. Spearman correlation analysis was applied to assess the association between expression of *RANKL* and CVF in LAAs. The Kaplan–Meier method, log-rank test, and Cox survival regression model were used to determine factors predictive of AF outcome after ablation. $p < 0.05$ was considered statistically significant, and all statistical tests were two-sided. All the statistical analyses were performed with Statistical Analysis System software (v.9.1.3; SAS Institute, Cary, NC, USA).

Results

Characteristics of the study population

The characteristics of LAF group and control group enrolled in this study are shown in Table 1. There were no significant differences in the distribution of the age, sex, body mass index (BMI), blood pressures, cigarette smoking, alcohol intake, hypercholesterolaemia and hypertension. Left atrial dimension (LAD) was significantly larger in LAF than control. In LAF group, age at first diagnosis of AF was 47.8 ± 10.9 years, and 65.9% was paroxysmal AF. There was no one using amiodarone in control group. LAF group used more beta-blocker and angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, while used no significant proportion of calcium-channel blocker and statins, compared with control group.

RANKL rs7984870 and LAF risk

The genotype and allele distributions of *RANKL* rs7984870 in the cases and controls are shown in Table 2. The observed genotype frequencies for this polymorphism were in Hardy–Weinberg equilibrium in the controls ($\chi^2 = 0.274$, $p = 0.541$). The frequencies of the GG, CG and CC genotypes were 21.5, 40.3 and 38.2%, respectively, among the cases, and 31.2, 45.6 and 23.3%, respectively, among the controls. After adjusting for possible confounders (age, sex, body mass index, smoking status, drinking status, hypercholesteremia and hypertension), subjects carrying CG or CC or CG/CC genotypes had an increased risk of LAF [adjusted

Table 1 Clinical characteristics

Variables	LAF group	Control group	*p* value
Patient number (*n*)	828	834	–
Sex, M/F (*n*)	578/250	576/258	0.743
Age at enrollment (years)	51.6 ± 12.1	51.9 ± 10.9	0.918
Age at first diagnosis of AF (years)	47.8 ± 10.9	N/A	–
Paroxysmal/Persistent AF (*n*)	546/282	N/A	–
Body mass index (kg/m^2)	23.8 ± 2.9	23.9 ± 3.0	0.896
Diastolic pressure (mmHg)	124 ± 14	118 ± 13	0.403
Systolic pressure (mmHg)	79 ± 10	73 ± 9	0.479
Mean artery pressure (mmHg)	94 ± 10	89 ± 9	0.427
Cigarette smoking (*n*)	180	164	0.297
Alcohol intake ≥ 1 drink per day (*n*)	122	105	0.203
Hypercholesterolaemia (*n*)	52	64	0.265
Hypertension at enrollment (*n*)	213	194	0.243
Left atrial dimension (mm)	37.9 ± 6.3	31.1 ± 3.8	<0.001
Medications before enrollment (*n*, %)			
Amiodarone	164 (19.8)	0 (0)	<0.001
Beta-blocker	200 (24.2)	93 (11.2)	<0.001
ACE-I/ARB	366 (44.2)	163 (19.5)	<0.001
Calcium-channel blocker	32 (3.9)	31 (3.7)	0.882
Statins	54 (6.5)	39 (4.7)	0.102

Values are presented as mean ± SD or number of patients

ACE-I angiotensin-converting enzyme inhibitor, *ARB* angiotensin receptor blocker

OR 1.20 (1.04–1.47)] for CG, 2.16 (1.83–2.57) for CC and 1.55 (1.41–1.90) for CG/CC; $p_{trend} < 0.001$), compared with GG homozygote, and the C allele displayed a high prevalence of LAF compared with the G allele ($p < 0.001$).

Factors associated with AF recurrence after ablation

Over a median follow-up of 19 months (range 3–45 months) after ablation, 214 patients (35.2%) had AF recurrence and 394 patients (64.8%) remained in SR. In multivariate Cox proportional hazards analysis, factors associated with arrhythmia recurrence were found to be persistent AF [versus paroxysmal AF; HR 1.342 (1.184–2.041); $p = 0.021$], larger left atrial dimension [HR 1.493 (1.243–2.032); $p = 0.002$], CG/CC genotype [versus GG genotype; HR 1.615 (1.373–1.964); $p < 0.001$], while postoperative Amiodarone was found protective for stabilization of SR [0.751 (0.593–0.824); $p = 0.012$]. In addition, Kaplan–Meier survival estimates showed that LAF patients carrying different genotypes had different proportion of AF recurrence ($p = 0.003$) (Fig. 1a). Moreover, CG/CC genotypes also had a higher proportion of AF recurrence after ablation, compared with GG genotype ($p = 0.004$) (Fig. 1b).

rs7984870 *and luciferase activity*

As shown in Fig. 2a, the vector with the −1816 C/C allele had an increase in the relative luciferase activity, compared with that with the −1816 G/G allele ($p < 0.01$).

Expression of RANKL and the degree of atrial fibrosis among genotypes

In the 24 LAA specimens, 6 were of the CC genotype, 10 of the CG genotype, and 8 of the GG genotype. As shown in Fig. 2b, Real-time quantitative RT-PCR assay showed a decreasing gradient of gene expression of *RANKL* in the groups carrying CC, CG and GG genotypes, although there was merely a borderline significant difference between CG and GG groups ($p = 0.056$).

Western blot analysis (Fig. 2b) showed an decreased expression of *RANKL* in CC and CG groups than GG group, whereas the difference was borderline between CC and CG groups ($p = 0.075$).

Interstitial collagen (stained blue), revealed by Masson staining and expressed as CVF, was highest in CC group, followed by CG and GG groups (Fig. 2c).

Table 2 Genotype and allele frequencies of *RANKL* rs7984870 and their associations with risk of LAF

Genotypes	LAF (*n* = 828)		Controls (*n* = 834)		p^a	Adjusted OR (95% CI)[b]
	n	%	*n*	%		
GG	178	21.5	260	31.2		1.00 (reference)
CG	334	40.3	380	45.6	0.013	1.20 (1.04–1.47)
CC	316	38.2	194	23.3	<0.001	2.16 (1.83–2.57)
CG/CC	650	78.5	574	68.9	0.002	1.55 (1.41–1.90)
G allele	690	41.7	900	54.0	<0.001	
C allele	966	58.3	768	46.0		
p_{trend}					<0.001	

[a]Two-sided χ^2 test for either genotype distributions or allele frequencies

[b]Adjusted for age, sex, body mass index, smoking status, drinking status, hypercholesteremia and hypertension in logistic regression model

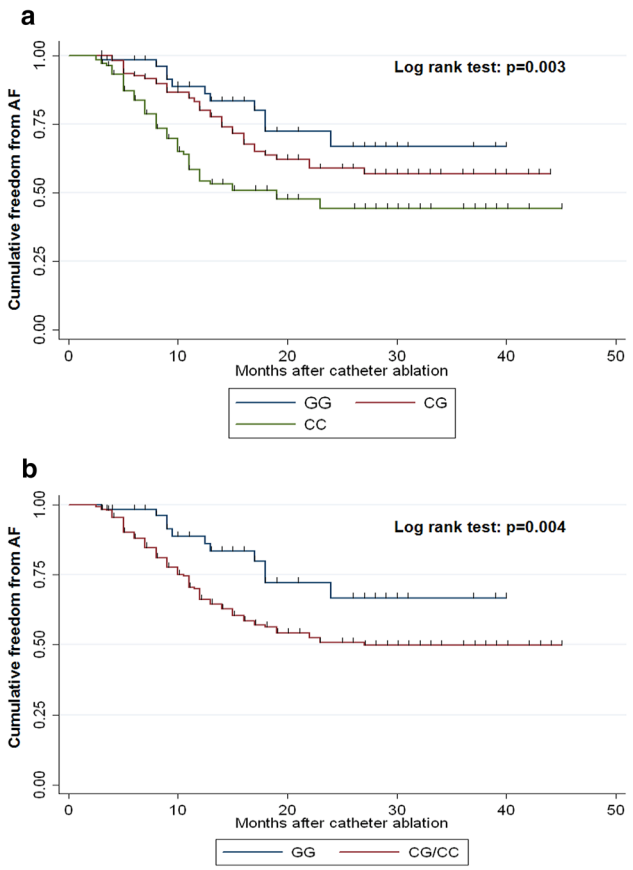

Fig. 1 Kaplan–Meier survival curves showing freedom from AF recurrence after catheter ablation according to *RANKL* rs7984870. **a** Survival free from AF recurrence in GG ($n=116$), CG ($n=226$) and CC ($n=266$) groups. **b** Survival free from AF recurrence in GG ($n=116$) and CG/CC ($n=492$) groups

The correlation test indicated a strong positive correlation between atrial protein expression of *RANKL* and CVF in LAAs ($r=0.688$, $p<0.001$).

Discussion

To our knowledge, this is the first study to evaluate the relationship between *RANKL* polymorphism and AF risk and prognosis. We found that the *RANKL* −1816C allele conferred significantly increased risk of LAF in our Chinese case–control study. Furthermore, the −1816C allele was associated with higher AF recurrence rate for 496 LAF patients after catheter ablation. These results were further supported by functional analysis. In vitro luciferase assays, it showed higher luciferase activity for the −1816C allele than for the −1816G allele. We also found that of the *RANKL* was overexpressed in LAA in individuals who carried the −1816C allele. In addition, the degree of atrial interstitial fibrosis in the CC, CG and GG three groups were significantly decreasing trend, which was positively correlated with the *RANKL* protein expression. Taken together, it suggested that *RANKL* rs7984870 is indeed a functional target involved in the susceptibility of LAF as well as a marker for predicting AF recurrence.

These findings are biologically plausible, especially in light of regulating atrial interstitial fibrosis roles of *RANKL*. The most predominant ASR in AF is atrial interstitial fibrosis, which results from an excessive accumulation of collagen fibers within atria, particularly due to disruption of the equilibrium between synthesis and degradation of collagen. The degradation of collagen is mainly regulated by MMP/TIMP system [14]. The expression of gelatinases (MMP-2 and -9) and their inhibitors (TIMP-1 and -2) is dysregulated when atrial fibrosis occurs, and the increase of the corresponding ratios can promote fibrosis [15]. Upregulation of *RANKL* is known to be contributed to enhanced MMP activity (MMP-2 and -9 in particular), as well as a modest decrease in TIMP expression in human fibroblasts, which suggested a potential mechanism by which activation of the OPG/RANK/*RANKL* axis might result in matrix degradation, adverse ventricular remodeling, and worsening myocardial function [9, 16]. More importantly, our previous study has also identified a convincingly positive correlation between MMP/TIMP system and the OPG/RANK/*RANKL* axis [8].

Rhee et al. first reported that a polymorphism in *RANKL* gene was associated with risk of cardiovascular disease [17]. However, accumulative evidence suggests that polymorphism in the promoter region could destroy transcriptional regulation factors, leading to altered transcription [17, 18]. Therefore, we designed functional experiments to determine whether *RANKL* rs7984870 could influence the transcriptional activity and expression level of *RANKL* gene in vivo or in vitro. Consistent with our functional experiment results, Tan et al. found that *RANKL* rs7984870 CC genotype might affect the promoter activity and expression level by creating a binding site to transcription factor sry-related high-mobility-group box-5 (SOX-5) in T cells [11]. In summary, we speculated that the variant of the −1816C > G polymorphism created a binding site to transcription factor SOX-5, gene transcription changed, which increased the expression level of the *RANKL* mRNA, and led to increased expression of *RANKL*. The increased *RANKL*/OPG protein ratio improved the activity of the OPG/RANK/*RANKL* axis which had also identified a convincingly positive correlation between MMPs/TIMPs ratios [8]. The biological process led to an excessive accumulation of collagen fibers in atria, and then myocardial fibrosis causing AF.

RFCA is a widely accepted strategy for eliminating AF, while the recurrence rate is considerable. Therefore, it is very important to evaluate the prognosis after ablation. Our previous study demonstrated that persistent AF, AF

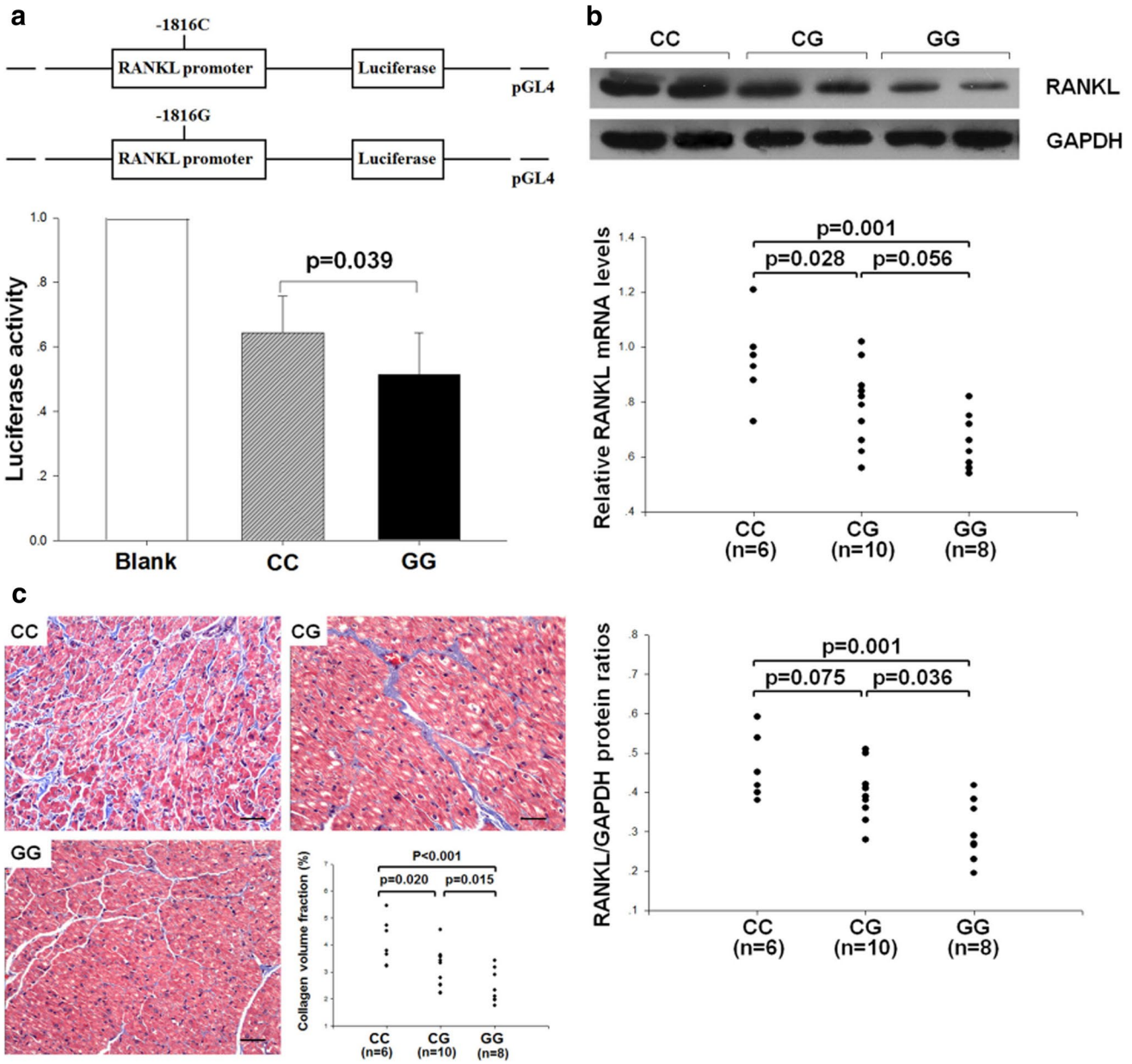

Fig. 2 Functional studies: **a** schematic representation of reporter plasmids containing the −1816C or −1816G allele, which was inserted upstream of the luciferase reporter gene in the pGL4-basic plasmid. Two constructs were transiently transfected into the mouse cardiac fibroblasts. The luciferase activity of each construct was normalized against the internal control of Renilla luciferase (blank). Values are mean ± SD. **b** Atrial gene and protein expressions of *RANKL* among different genotypes in healthy heart donors. Boxes show interquartile ranges, and bars represent the 10th and 90th percentiles. **c** Representative photomicrographs of Masson staining showing the interstitial collagen (stained blue) (×200, bar = 50 μm). Collagen volume fraction is used to evaluate the degree of fibrosis

duration, left atrial diameter, amiodarone after ablation, serum soluble *RANKL* level and soluble *RANKL*/OPG ratio independently predicted LAF recurrence after RFCA [13], which was partly consistent with our findings herein. In addition, we mainly found that patients carrying CG/CC genotypes showed a higher possibility of AF recurrence after catheter ablation, compared with patients carrying GG genotype. The patients suffering from AF recurrence indicate an excessive structural remodeling in atria [6], so the mechanism of *RANKL* rs7984870 in the prognosis of AF must be the same as that in the initial of AF. We could expect a growing trend toward linking the launch of therapeutic methods with diagnostic markers. Just like our result, *RANKL* rs7984870 may help to improve the outcome of individualized therapy as a diagnostic marker before ablation.

Clinical perspectives

ASR, particularly atrial interstitial fibrosis, limits the efficacy of existing therapies for AF. Accordingly, attenuation of ASR, the so-called upstream therapy, has increasingly become the focus of attention. Previously, we had demonstrated that the activation OPG/RANK/*RANKL* axis (reflected by *RANKL*/OPG ratio) could result in the upregulation of atrial interstitial fibrosis in vivo [5]. Therefore, regulating excessive expression of atrial *RANKL* may induce the excessive fibrosis in atria resulting in AF. Taken together, we propose that inhibiting excessive expression of endogenic *RANKL* by targeting *RANKL* rs7984870 can be a new promising upstream therapy of AF. Additionally, profile evaluation of *RANKL* rs7984870 has the potential to be used clinically as a routine pre-ablation assessment, and together with other factors including AF type and left atrial diameter, may provide a more integrated picture for physicians to evaluate the clinical status of LAF patients. Paroxysmal AF patients carrying GG genotype of *RANKL* rs7984870 without significantly enlarged left atria may be the optimal candidates for catheter ablation.

Limitations

This study has several limitations. First, although our sample size is moderate, we had 80% power at a 0.05 significance level to detect an OR of 1.4 or greater and 0.7 or smaller with an exposure frequency of 30%, given the sample size of our current study. Second, the uses of antiarrhythmic drugs were not well discussed. This was because various doses and combinations of drugs had been given to groups according to their clinical states. Third, because different combinations of antiarrhythmic drugs and various doses had been given to the postoperative patients and patients with AF recurrence according to their clinical states, it was difficult to analyse the influence of combinations, duration and doses to the rhythm. In addition, the functional studies may make a plausible biologic explanation for our epidemiologic findings. However, since all subjects enrolled in this study were ethnic Han Chinese, the function of this polymorphism in the other human races needs further validation by larger prospective studies.

Conclusion

In summary, to our knowledge, this study provided the first evidence that the *RANKL* promoter-1816C allele, compared with the −1816G allele, may contribute to an increased LAF risk in Chinese populations and higher recurrence rate in LAF patients after RFCA, possibly by increasing the *RANKL* expression level followed by the activation of OPG/RANK/*RANKL* axis that induced excessive atrial fibrosis. These findings enhanced our knowledge of the role of OPG/RANK/*RANKL* axis in AF, and suggested that the −1816C > G variant of *RANKL* could be a functional genetic target for developing new treatment strategies and guide the physicians for catheter ablation as a useful marker.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to disclose.

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