

## Original Article

# Regulation of calcium pump through Notch/Jagged/Hes signaling pathway in canine model of chronic atrial fibrillation

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**Abstract:** Objective: Using a canine model of atrial fibrillation (AF) induced by chronic pacing of left atrial fibrillation, the present study aimed to investigate the protein expression and content change of Notch-1 and its downstream target genes including Hes1, Jagged1, and SERCA2a in the atrial myocardium of canines with chronic AF. Furthermore, the correlation between Notch-1/Hes1/Jagged1 and the SERCA2a calcium pump was also analyzed. Methods: Ten healthy Beagle dogs including both males and females, aged 7-9 years were randomly divided into a sham group (n = 5) and AF group (n = 5). The AF group underwent minimally invasive surgery with implantation of a pacemaker into the left atrial appendage for induction of AF. After 8 weeks of pacemaker implantation, animals were euthanized and specimens from the right and left atrial free walls were excised for histologic and biochemical analyses. Results: After 8 weeks' pacemaker implantation, immunohistochemical expression of Notch-1, Hes1, Jagged1 and SERCA2a in the sham group was positive. Compared with the sham operation group, the intensity of Notch-1, Hes1 and Jagged1 in AF group was stronger with a significant increasing trend in the intensity of the color, respectively. The expression of SERCA2a was weak; the intensity decreased significantly ( $P < 0.05$ ). Pearson correlation analysis revealed that in the AF group, Notch-1 was negatively correlated with SERCA2a ( $r = -0.77$ ,  $P = 0.028$ ), and was positively correlated with Hes1 and Jagged1 ( $r = 0.92$ ,  $P = 0.014$ ;  $r = 0.73$ ,  $P = 0.030$ ) proteins, respectively. Conclusion: The activation of the Notch signaling pathway was associated with a decrease in SERCA2a protein expression and contributes to the development and maintenance of electrical remodeling in AF through modulation of calcium pump function and calcium homeostasis.

**Keywords:** Atrial fibrillation, notch-1, sarcoplasmic reticulum,  $Ca^{2+}$  ATPase

## Introduction

Atrial fibrillation (AF) represents the most prevalent chronic tachyarrhythmia encountered in clinical practice. AF is characterized by rapid AF-related ion-channel remodeling that may occur due to pathologic alteration of several ion channels or by concomitant changes of multiple channels. Overall, activation of the atrium with diverse alterations, including electrical and structural remodeling, neurohormonal, or molecular alterations, contributes to AF. Notably, the role of calcium overload and electrical remodeling in AF have been implicated as critical pathologic mediators and have gained increasing attention. As a hallmark of AF pathogenesis, calcium overload occurs due to pertur-

bations in intracellular calcium cycling in the sarcoplasmic reticulum (SR) calcium store, including decreased L-type  $Ca^{2+}$  current, defective regulation of cardiac sarcoplasmic reticulum  $Ca^{2+}$ -ATPase (SERCA2a) activity, and altered Ryanodine receptor (RyR)-mediated calcium release. These abnormalities and coordination disorders are primarily involved in promoting initiation and maintenance of calcium overload in atrial myocytes, which leads to a spectrum of structural alterations and electrical remodeling in AF. SERCA2a regulates intracellular  $Ca^{2+}$ -homeostasis and thus, plays an essential role during cardiac contraction and relaxation. During systole, action potentials promote the influx of a small amount of extracellular  $Ca^{2+}$  into the cell through the voltage-gated

L-type  $\text{Ca}^{2+}$  channel on the myofiber membrane. Subsequently, this calcium binds to the RyR2, inducing a massive release of  $\text{Ca}^{2+}$  from the SR; this process is known as calcium-induced calcium release. This nearly 10-fold increase in intracellular  $\text{Ca}^{2+}$  concentration activates the binding of  $\text{Ca}^{2+}$  and troponin C, which then results in cardiomyocyte contraction. The concentration of free intracellular  $\text{Ca}^{2+}$  determines the cardiomyocytes' excitability in order to regulate myocardial contractility. During diastole,  $\text{Ca}^{2+}$  is rapidly uptaken by SERCA2a into the SR, while the  $\text{Na}^+/\text{Ca}^{2+}$  exchangers and  $\text{Ca}^{2+}$  pumps on the cell membrane facilitate transport of  $\text{Ca}^{2+}$  to the extracellular membrane, and the  $\text{Ca}^{2+}$  concentration in the cytosol decreases significantly, leading to the relaxation of cardiomyocytes. The rapid decline in  $\text{Ca}^{2+}$  concentration in SR is decisive for the relaxation of cardiomyocytes. Notch signaling performs a critical role in cardiogenesis and in the pathogenesis of cardiovascular disease. Defects in Notch signaling are associated with developmental abnormalities in cardiac valves, endocardial cushions, cardiac conduction systems, and coronary artery formation [1]. However, the functions of several receptors, ligands, and downstream target genes of the Notch signaling pathway in AF remains poorly understood. A comprehensive understanding of the specific mechanism of Notch signaling and the cross-talk between associated pathways involved in the occurrence and development of AF could provide significant evidence for Notch signaling as a new clinical practice strategy for management of AF. Therefore, using a canine model of AF induced with chronic pacing of left atrial fibrillation, the present study aimed to investigate the protein expression and of Notch-1 and its downstream target genes including Hes1, Jagged1, and SERCA2a in the atrial muscle of canines with chronic AF. Furthermore, the correlation between Notch-1/Hes1/Jagged1 and SERCA2a calcium pump was also analyzed.

### Materials and methods

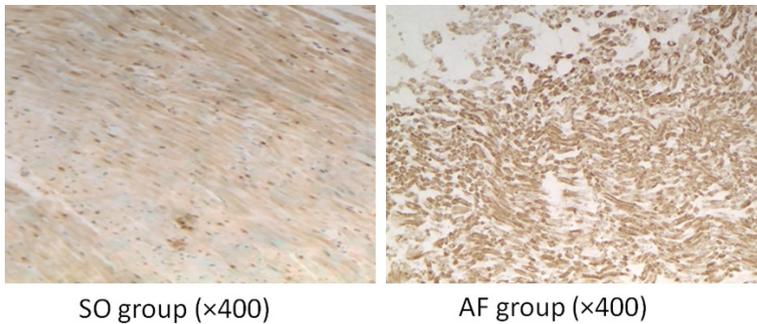
#### *Experimental materials*

*Animal Ethics approval:* All animals were managed in accordance with the *Guide for the Care and Use of Laboratory Animals*. The study protocols were reviewed and approved by the Animal Ethics Committee.

*Experimental canines groups:* Ten healthy Beagle dogs including both male and female, weighing ( $18 \pm 2.66$  kg), aged 7-9 years were randomly divided into two groups. The sham group ( $n = 5$ ) underwent minimally invasive thoracotomy without implantation of a pacemaker. While the AF group ( $n = 5$ ) underwent minimally invasive surgery with implantation of a pacemaker 8 W into the left atrial appendage for induction of AF.

#### *Experimental methods*

*Modeling of AF induced by rapid atrial pacing (RAP) in canines:* Canines were fasted from water and food for 12 hours prior to surgery. Routine disinfection of surgical instruments, surgical gowns, and surgical environments was carried out. Surgery was performed strictly in accordance with aseptic technique. Following intramuscular administration of ketamine hydrochloride injection (20 mg/kg) for basal anesthesia, the venous access was established. Respiration in anesthetized dogs was maintained by an endotracheal tube and a mechanical ventilator. Oxygen flow rate was adjusted to 4~6 L/min with the tidal volume of 20 mL/kg and the respiratory pressure of 0~2 Kpa. The normal II-lead electrocardiogram (ECG) of the canine was recorded dynamically with LEAD-2000 multichannel EP recorder. In the right lateral position, the canine's hair of left chest was shaved off. After the cut was disinfected with iodine and alcohol, minimally invasive cardiac surgery technique was performed to make a small incision under left axilla, with a length of about 5-7 cm. An incision was made at the intercostal space between the fourth and fifth ribs. The skin and subcutaneous muscular layer were carefully dissected layer by layer, and the incision was distracted with a suitable thorax retractor. The pericardium sac was opened in layers by thoracotomy to expose the left atrial appendage. Purse-string suture was implanted and fixed at the base of the left atrial appendage. The human atrial wing-shape pacing electrode was introduced and the proximal end of the pacing lead was connected with the high-frequency pacemaker unit buried in the experimental canine. The magnet was placed close to the pacemaker to test its pacing effect. The surface ECG showed well-transmitted pacing signal when the pacemaker functioned appropriately. The pacemaker was turned off when there was no bleeding



**Figure 1.** Changes of Notch-1 expression in atrial tissue of two groups of dogs at 8 weeks.

in the pericardium and thoracic cavity. Subsequently, the pacemaker was implanted into the subcutaneous pocket of the chest. Inter-costal sutures were used to close the chest, followed by suture placement in the subcutaneous tissue and skin layer by layer. After surgery, all animals were watched until they recovered completely; signs and symptoms were observed. After the general condition of the canines became stable, the pacemaker was programmed to 400 bpm (150 ms) and maintained at this rate for 8 weeks. AF was defined as a spontaneous irregular atrial rhythm for longer than 5 sec. The ECG of AF showed that the P-wave was substituted with f-wave which was uneven in size and intervals and of different morphologies, with a frequency of 450~600 beats/min and RR intervals lasting for more than 10 s. One week after the surgery, a single intramuscular injection of the 3 g Cefazolin Sodium dissolved in 250 mL of 0.9% Sodium Chloride was administered to prevent incision-related infection. Nutritional support and wound care were also provided.

At the end of the protocol, animals were euthanized for the heart explantation and post-mortem examination. Small portions of the right and left atrial free walls were excised for histologic and biochemical analyses.

**Immunohistochemistry assay:** The left atrial tissues from the two groups were embedded in paraffin and sectioned. Immunohistochemical staining of Notch-1, Hes1, and Jagged1 was performed using SP immunohistochemical staining kit. In the negative control group, the primary antibody was replaced with PBS. The cytoplasm of positively stained cells exhibited brownish-brown stain for Notch-1, Hes1, and

Jagged1 staining. Five non-repeating high-power fields (40 × 10) were selected to count the number of positively stained cells separately.

**Determination of Notch-1, Hes1, Jagged1, and SERCA2a concentration in tissues through double antibody sandwich (ELISA):** Microplates were coated with canine protein antibody to prepare solid phase antibody. The extracted canine atrial tissue protein

was successively added to microplates coated with monoclonal antibody, which then bound to Horseradish Peroxidase (HRP)-conjugated antibody to form antibody-antigen-enzyme labeled antibody complex. After thorough washing, substrate TMB was added for development of the color. TMB was catalyzed by HRP to produce a blue color product that turned into yellow after adding acidic stop solution. The intensity of the color was positively correlated with the presence of protein in the sample. The absorbance (OD) intensity was measured with a microplate reader at a wavelength of 450 nm and the concentration of canine protein in the sample was calculated based on the standard curve.

#### Statistical treatment

All statistical analyses were performed using SPSS 17.0. The data were expressed as mean ± standard deviation. The within-group comparisons were performed using two-tailed t-tests. Pearson correlation test was used to determine the correlation among variables between the two groups. A P-value of < 0.05 was considered significant.

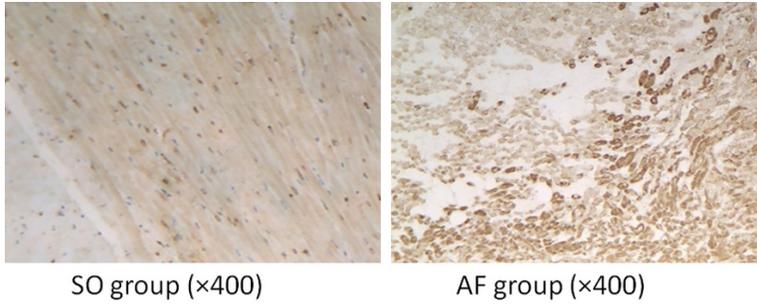
## Results

#### Model establishment

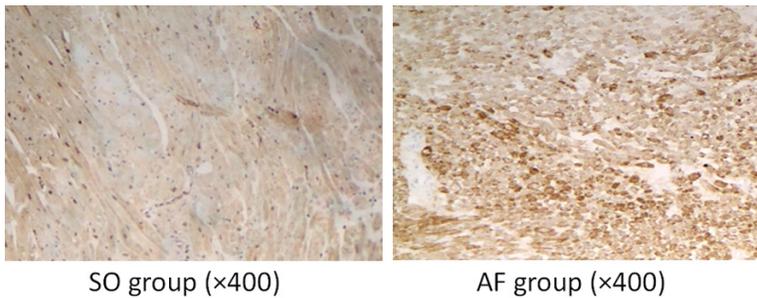
8 weeks after pacemaker implantation, the success rate for the establishment of a canine model with chronic AF in the AF group reached 100%. After the pacemaker was turned on, AF was successfully induced by rapid pacing of the left atrial appendage in the AF group by ECG test.

After 8 weeks' experiment, the color expression of Notch-1, Hes1, Jagged1, and SERCA2a in the

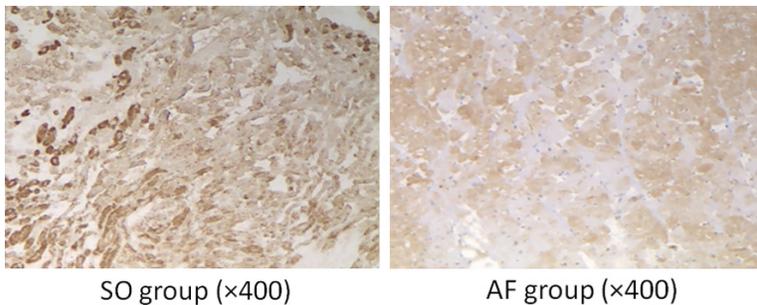
## Notch/Jagged/Hes signaling pathway and calcium pump



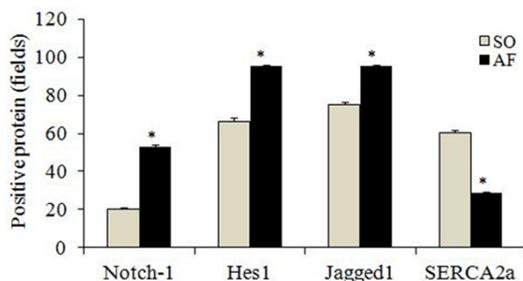
**Figure 2.** Changes of Hes1 expression in atrial tissue of two groups of dogs at 8 weeks.



**Figure 3.** Changes of Jagged1 expression in atrial tissue of two groups of dogs at 8 weeks.



**Figure 4.** Changes of SERCA2a expression in atrial tissue of two groups of dogs at 8 weeks.



**Figure 5.** Expression of Notch-1/Hes1/Jagged1 and SERCA2a in canine atrial tissue in two groups. Note: Compared with SO group, \* $P < 0.05$ .

sham operation group exhibited brown color. Compared with the sham operation group, the color expression of Notch-1, Hes1, and Jagged1 in the AF group showed a dark brown color with a significant increasing trend in the intensity of the color, respectively; the protein SERCA2a expression was weak. The expression decreased significantly with a statistically significant difference ( $P < 0.05$ ), as shown in **Figures 1-5** and **Table 1**.

After 8 weeks, the concentrations of Notch-1, Hes1, and Jagged1 proteins in AF group significantly increased compared with those in the sham group ( $P < 0.05$ ), while the concentration of SERCA2a significantly decreased ( $P < 0.05$ ) compared to the sham group, as presented in **Table 2** and **Figure 6**. Pearson correlation test showed that, in AF group, Notch-1 was negatively correlated with SERCA2a ( $r = -0.77$ ,  $P = 0.028$ ) and Notch-1 was positively correlated with Hes1 and Jagged1 ( $r = 0.92$ ,  $P = 0.014$ ;  $r = 0.73$ ,  $P = 0.030$ ), as reported in **Table 3**.

### Discussion

Notch signaling regulates the fate of one cell with that of a neighboring cell through physical interactions; thus, canonical Notch signaling is initiated when a cell surface-expressed ligand binds in trans to the Notch receptor expressed on neighboring cells. When Notch is activated, its receptors undergo two proteolytic cleavages upon recognition of its extracellular ligand (members of the Delta or Serrate/Jagged family). These cleavages release its intracellular activation domain, which enters the cells and binds with the positive regulator, recombination signal binding protein for immunoglobulin  $\kappa$ J (RBPJ $\kappa$ ) to form a complex that regulates transcription of Notch target genes [2]. Notch

## Notch/Jagged/Hes signaling pathway and calcium pump

**Table 1.** Expression of Notch-1/Hes1/Jagged1 and SERCA2a in canine atrial tissue in two groups (fields,  $\bar{x} \pm s$ )

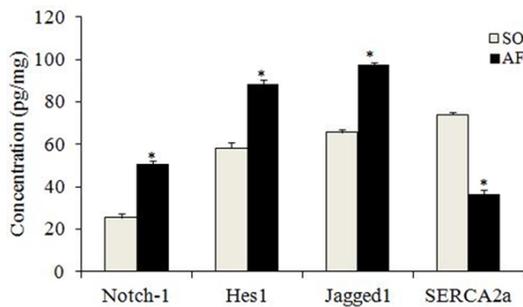
Group	Notch-1	Cav1.2	SERCA2a
SO group	20.28±1.12	38.70±1.65	30.35±1.73
AF group	53.15±2.30*	18.54±0.85*	88.63±2.20*

Note: Compared with SO group, \*P < 0.05.

**Table 2.** Concentration changes of Notch/Hes/Jagged and SERCA2a in two groups (pg/mg,  $\bar{x} \pm s$ )

Group	Notch-1	Hes1	Jagged1	SERCA2a
SO group	25.34±2.10	60.10±1.05	25.34±2.10	60.10±1.05
AF group	48.35±1.12*	110.23±2.66*	48.35±1.12*	110.23±2.66*

Note: Compared with SO group, \*P < 0.05.



**Figure 6.** Concentration changes of Notch-1/Hes1/Jagged-1 and SERCA2a in two groups. Note: Compared with SO group, \*P<0.05.

signaling is recognized to play an essential role in the development of the cardiovascular system and in the pathogenesis of cardiovascular disease by regulating multiple signaling pathways. However, its specific regulatory mechanisms in the pathogenesis of cardiovascular disease remain elusive. Mounting evidence indicates that the Notch signaling system in the myocardium may be reactivated under pathological conditions. Moreover, increased Notch-1 signaling transduction activity has been identified in models of atherosclerotic plaque, myocardial infarction, and myocardial hypertrophy, as well as heart failure [3-6]. However, presently, no relevant reports on AF and Notch signaling pathway have been reported. Therefore, a canine model of chronic AF was successfully established in the present study, and changes in protein expression of target molecules of the Notch signaling pathway and SERCA2a were confirmed. Furthermore, a significantly negative correlation between SERCA2a and Notch-

1 was also observed in the AF group. The findings of the present study indicated that the Notch signaling pathway may contribute to the development and maintenance of electrical remodeling in AF through modulation of myocardial calcium pump and calcium homeostasis.

In AF, the atrial effective refractory period was shortened, and the re-entrant wavelength of AF declined, which promoted a continuous state of AF. Studies suggest that electrical remodeling

is associated with intracellular  $Ca^{2+}$  overload [7-9]. Thus, the abnormal intracellular  $Ca^{2+}$  handling induced by aberrant myocardial SERCA2a function is one of the major factors leading to intracellular  $Ca^{2+}$  overload with subsequent myocardial damage. Clinical studies have shown that the mRNA expression of atrial SR  $Ca^{2+}$ -ATPase is significantly decreased in patients with AF; the longer the duration of AF, the greater decrease in mRNA expression. Thus, aberrant expression of  $Ca^{2+}$ -ATPase may be a predominant mechanism for the maintenance of AF [10-12]. Rapid atrial depolarization induced during fibrillation due to intracellular calcium overload with simultaneous dysfunction of SR  $Ca^{2+}$ -ATPase for a long-time and abnormal expression of SR calcium regulatory proteins may significantly contribute to atrial dysfunction, which leads to sustained AF. Conversely, Notch signaling is also involved in the protection of myocardial cells. For instance, the activation of Notch can promote myocardial injury repair and recovery of electrical function, as well as minimize the myocardial fibrosis. However, the role of the Notch signaling pathway in the pathogenesis of AF and its association with calcium pump in AF remain elusive. The findings of this study demonstrated a significant decrease in SERCA2a positive protein expression and serum concentration in the AF group resulted in dysfunction of calcium pump and impaired myocardial calcium homeostasis, consistent with other studies. Furthermore, a significant increase in the protein expression and serum concentration of Notch-1, Hes1, Jagged1, was observed in the AF group. The

**Table 3.** Pearson correlation analysis of Notch-1 with Hes1, Jagged1, and SERCA2a concentrations in AF group

Group	Hes1		Jagged1		SERCA2a	
	r	P	r	P	r	P
AF group Notch-1	0.83	0.038	0.66	0.025	-0.52	0.03

results indicated that the target molecules of the Notch signaling pathway were activated and involved in the electrical remodeling mechanism in AF. This study further validated the correlation between the Notch signal pathway and SERCA2a. The results suggested that Notch-1 was negatively correlated with SERCA2a but positively correlated with Hes1 and Jagged1. In conclusion, the findings of the present study indicated that the activation of the Notch signaling pathway was associated with a decrease in SERCA2a protein expression and was involved in the development and maintenance of electrical remodeling through regulation of calcium pump function.

Several mechanisms have been implicated in the perpetuation of AF, which involves multiple signaling pathways. However, calcium pump abnormality, mediated by the interplay among manifold signaling pathways lead to electrical remodeling in AF, has been one of the most studied mechanisms. Thus, the implication of target molecule expression and functional changes in Notch signal pathway in the occurrence and maintenance of AF needs further investigation to provide effective targets for the prevention and treatment of AF.

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#### Disclosure of conflict of interest

None.

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