GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Expression of tyrosine hydroxylase and growth-associated protein 43 in aging atrial fibrillation patients of Xinjiang Uygur and Han nationality

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Genet. Mol. Res. 12 (4): 5257-5266 (2013)
Received May 28, 2013
Accepted October 14, 2013
Published October 30, 2013
DOI http://dx.doi.org/10.4238/2013.October.30.10

ABSTRACT. The aim of this study was to explore the changes in gene and protein expressions of tyrosine hydroxylase (TH) and growth-associated protein 43 (GAP43) in aging atrial fibrillation patients of Xinjiang Uygur and Han nationality, and the significance of the changes. Real-time polymerase chain reaction and Western blot analysis were used to detect gene and protein expressions of TH and GAP43 in atrial tissues of 54 patients with valvular heart disease. mRNA and protein expressions of GAP43 and TH were significantly

different between the sinus rhythm and atrial fibrillation groups ($P < 0.05$). Protein expressions of GAP43 and TH of both nationalities differed significantly between the sinus rhythm group and the atrial fibrillation group ($P < 0.05$), whereas there was no statistical difference between the two nationalities within each group ($P > 0.05$). Protein expressions of GAP43 and TH differed significantly among different age groups of different nationalities in the sinus rhythm and atrial fibrillation groups ($P < 0.05$); only protein expression of GAP43 differed significantly in different age groups in the atrial fibrillation group ($P < 0.05$). The changes of mRNA and protein expressions of TH and GAP43 played a vital role in the process of maintaining the atrial fibrillation. Therefore, increased expression of TH and GAP43 might be a molecular mechanism for left atrial myoelectricity remodeling of aging atrial fibrillation patients, which might be potential therapeutic targets of atrial fibrillation.

Key words: Atrial fibrillation; Xinjiang Uygur; Han nationality; Aging; Tyrosine hydroxylase; Growth-associated protein (GAP43)

INTRODUCTION

Atrial fibrillation (AF) is one of the most common arrhythmias in clinical cardiac diseases, and its morbidity is closely related with the age of the patients. Although AF can attack anyone at any age, it has a high morbidity in the aged population and an extremely low morbidity in children. The morbidity rate of AF is above 0.4% overall, and the morbidity rate rises significantly with increasing age, reaching 6% for those older than 65 years, 10% for those older than 75 years, and nearly 20% for those older than 85 years (Nattel et al., 2008). The continuous existence of AF can readily cause further serious complications such as heart failure, intra-atrial thrombus, and cerebral embolism, among others, which have high disability and mortality rates (Fuster et al., 2001; Tsang et al., 2005). Currently, there are many therapeutic methods for treating AF, including drug therapy, electrical conversion, surgical maze operation treatment, radiofrequency catheter ablation, and pacemaker implantation, etc.; however, none of these methods is completely satisfactory, which calls for investigating the mechanism of AF's occurrence and maintenance.

The function of neural regeneration in AF myoelectricity remodeling has gained increasing attention in recent years. Tyrosine hydroxylase (TH) and growth-associated protein 43 (GAP43) are important nerve growth factors, and previous studies (Horikawa-Tanami et al., 2007) have indicated that the former is a signaling factor of the sympathetic nerve and that the latter is a signaling factor of the parasympathetic nerve. Therefore, TH and GAP43 may play a vital role in the process of AF formation, maintenance, and recovery. This study aimed to explain the role of changes in gene and protein expressions of TH and GAP43 in aging AF patients. Expression changes were evaluated in atrial tissues of AF patients of different ages of the Xinjiang Uygur and Han nationalities in order to provide a new theoretical basis for developing a better clinical AF therapeutic strategy.

MATERIAL AND METHODS

Patients

The study subjects were 54 patients who suffered from valvular heart disease and needed open-chest valve replacement operations in the First Affiliated Hospital of Xinjiang Medical University from 2008 to 2011. This study was conducted in accordance with the declaration of Helsinki and with approval from the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University. Written informed consent was obtained from all participants. Among these 54 cases, 28 were of Han nationality and 26 were Uygur, 22 were males and 32 were females, and they ranged in age from 43-72 (46.28 ± 9.15) years. These 54 patients were divided into two groups: the sinus rhythm group and the atrial fibrillation group. There were 26 cases, 14 male patients and 12 female patients, in the sinus rhythm group, in which the average age was 53.38 ± 12.74 years, and there were 28 cases, 8 male patients and 20 female patients, in the atrial fibrillation group, in which the average age was 55.29 ± 8.58 years. There were 8 cases of paroxysmal AF and 18 cases of chronic AF (AF lasted more than 6 months) in the atrial fibrillation group.

Inclusion and exclusion criteria

The patients' AF lasted for more than at least 6 months, which was confirmed by regular examinations such as electrocardiograms, 24-h ECG, and echocardiography, etc., before the operation. Coronary arteriography was given to a portion of the patients to exclude the possibility of coronary heart disease; none of the patients had liver or kidney function damage, electrolyte disturbance or infection, hypertension, hyperthyroidism, or diabetes mellitus, and their heart function was in the scope of II to III grades (according to NYHA Grading).

Specimen collection and preservation

The patients' clinical baseline data were registered and the informed consent forms were signed before the operation. The extracorporeal circulation was established during the operation, and approximately 200 mg left auricle tissue was taken out after cardiac arrest. The left auricle tissues were placed in liquid nitrogen immediately after the blood and fat tissues were excluded, and were preserved at -80°C until further use.

Real-time polymerase chain reaction (PCR)

One hundred milligrams left auricle tissues was taken, and total RNA was extracted by the Trizol one-step method (Invitrogen Company). One microgram total RNA was taken and reverse transcribed into cDNA according to instructions of the Reverse Transcription Kit (Promega Company A3500). The design and synthesis of the primers were performed by the TaKaRa Company (Table 1).

The real-time PCR system comprised a 20-μL total volume. For detection, specimens were subjected to cDNA 2-fold dilution, and 1 μL cDNA was taken by using 10 μL SybrGreen qPCR Master Mix (Shanghai Ruian BioTechnologies), 1 μL 10 μM upstream primer, 1 μL 10 μM downstream primer, and 7 μL ddH_2O.

Table 1. Design and synthesis of the primer.

Gene	Primer sequences (5'→3')	Amplified fragment length (bp)
β-actin	F: GCTACGAGCTGCCTGACG R: TCGTGGATGCCACAGGAC	112
TH	F: TGTTCCAGTGCACCCAGTATATC R: CCAATGTCCTGCGAGAACTG	136
GAP43	F: AGAACATAGAAGCTGTAGATGAAAC R: CCATTTCTTAGAGTTCAGGCAT	112

The reaction conditions were as follows: 10 min 95°C initial denaturation, which was followed by 40 PCR cycles of 15 s 95°C denaturation, 30 s annealing at 60°C, and 20 s of 72°C extension. Reflected light signals were collected and the $2^{-\triangle\triangle Ct}$ method was used to calculate the relative mRNA expression value of the target gene.

Western blotting

Total protein was extracted from tissues according to manufacturer instructions (Beijing Solarbio), and the total protein content was detected by the bicinchoninic acid method. The total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 50 μg sample loading per well, and the protein on the gel was then blotted to a nitrocellulose film via galvanic transferring. After being sealed and eluted with 5% non-fat dry milk/Tris-buffered saline solution, hybridization was carried out by TH-specific first resistance (Anti-rabbit IgG; Abcam Company), GAP43-specific first resistance (Anti-rabbit IgG; Abcam Company), and β-actin channel protein-specific first resistance (Anti-rabbit IgG; Wuhan Boster Company), which was followed by 4°C overnight incubation. Then, samples were incubated for 1 h by using specific second resistance (Goat Anti-rabbit IgG; Wuhan Boster Company) conjugated with horseradish peroxidase. DAB staining was carried out, and all hybridization signals were scanned quantitatively via the BIO-RAD gel imaging system.

Statistical analysis

The EXCEL2003 software was used for data collection, and the SAS JMP statistical analysis software package was used for statistical analysis. General descriptions of ages, left atrial diameter, right atrial diameter, and ejection fraction in the sinus rhythm and atrial fibrillation groups are reported as means ± standard deviations, whereas the constituent ratio and constituent rate are used to express the gender and nationality constituents. The Student *t*-test was used for the comparison of general data between the sinus rhythm group and the atrial fibrillation group under the premise of variance homogeneity. Analysis of variance (ANOVA) was also used for evaluating GAP43 and TH differences between the sinus rhythm group and the atrial fibrillation group, in which nationality and age were used as covariates. In the comparison of GAP43 and TH between different nationalities and different age groups, the diseases were used as covariates; $\alpha = 0.05$ was used as the inspection level.

RESULTS

General information

The left atrial diameter of patients in the atrial fibrillation group exceeded that of patients in the sinus rhythm group. Other clinical data, such as age, nationality, gender, ejection fraction, and cardiac functional grading (NYHA Grading), etc., showed no statistical significance between the two groups (Table 2).

Table 2. Comparison of clinical features between the two groups (means ± SD).

	Sinus rhythm group (N = 26)	Atrial fibrillation group (N = 28)	Statistic	P
Nationality, N (%)				
Han	10 (36%)	18 (64%)	$\chi^2 = 3.60$	0.058
Uygur	16 (62%)	10 (38%)		
Gender, N (%)				
Male	14 (64%)	8 (36%)	$\chi^2 = 3.567$	0.059
Female	12 (10%)	20 (90%)		
Age (years)	53.38 ± 12.74	55.29 ± 8.58	$t' = -0.641$	>0.05
LA (mm)	45.09 ± 7.73	57.90 ± 11.36	$t = 4.807$	0.000
RA (mm)	42.94 ± 8.47	45.37 ± 19.12	$t' = 0.611$	>0.05
EF (%)	63.33 ± 11.15	61.31 ± 6.60	$t' = 0.802$	>0.05

After test for homogeneity of variance, the *t*-test was used for age, RA and EF comparison due to heterogeneity of variance ($F_{age} = 2.2048$, $P_{age} = 0.0472$; $F_{RA} = 5.0958$, $P_{RA} = 0.0001$, $F_{EF} = 2.8541$, $P_{EF} = 0.0090$); Homogeneity of variance was reported in LA between two groups. LA = left atrium; RA = right atrium; EF = ejection fraction.

mRNA and protein expression

The mRNA and protein expressions of GAP43 and TH differed significantly between the sinus rhythm group and the atrial fibrillation group ($P < 0.05$) (Table 3).

Table 3. mRNA and protein expression of GAP43 and TH in the two groups (means ± SD).

	GAP43		TH	
	mRNA	Protein	mRNA	Protein
Sinus rhythm group (N = 26)	0.86 ± 0.23	0.28 ± 0.21	0.06 ± 0.03	0.64 ± 0.30
Atrial fibrillation group (N = 28)	2.19 ± 0.73	0.85 ± 0.38	0.13 ± 0.05	1.03 ± 0.42
Statistic	9.163*	6.885*	6.289*	3.900*
P	P < 0.05	P < 0.05	P < 0.05	0.000

*Heterogeneity of variance: *t*-test.

GAP43 and TH protein expressions

The difference in protein expressions of GAP43 and TH were compared between the sinus rhythm group and the atrial fibrillation group using nationality (Han or Uygur) as a covariate. The results showed that the protein expressions of GAP43 and TH differed significantly in the Xinjiang Uygur and Han nationalities between the sinus rhythm and atrial fibrillation groups ($P < 0.05$) (Table 4).

Table 4. Difference of protein expression of GAP43 and TH in the two groups (means ± SD).

	GAP43	TH
Han		
Sinus rhythm group (N = 10)	0.26 ± 0.01	0.36 ± 0.11
Atrial fibrillation group (N = 18)	0.76 ± 0.35	0.87 ± 0.52
Statistic	5.956*	4.003*
P	0.000	P < 0.05
Uygur		
Sinus rhythm group (N = 16)	0.29 ± 0.27	0.42 ± 0.19
Atrial fibrillation group (N = 10)	1.02 ± 0.40	1.05 ± 0.40
Statistic	5.5737#	4.663*
P	0.000	P < 0.05

*Heterogeneity of variance: *t*-test; #Homogeneity of variance: *t*-test.

Effect of nationality

The differences in the protein expressions of GAP43 and TH in different nationalities were evaluated, in which different diseases were used as the covariate. The results showed that the protein expressions of GAP43 and TH did not differ significantly between the two ethnic groups (P > 0.05) (Table 5).

Table 5. Difference of protein expression of GAP43 and TH in different nationalities in the two groups (means ± SD).

Group	Nationality	GAP43	TH
Sinus rhythm group	Han (N = 10)	0.26 ± 0.01	0.36 ± 0.11
	Uygur (N = 16)	0.29 ± 0.27	0.42 ± 0.19
	Statistic	0.444#	1.019*
	P	P > 0.05	P > 0.05
Atrial fibrillation group	Han (N = 18)	0.76 ± 0.35	0.87 ± 0.52
	Uygur (N = 10)	1.02 ± 0.40	1.05 ± 0.40
	Statistic	1.791#	0.947#
	P	0.085	0.352

*Heterogeneity of variance: *t*-test; #Homogeneity of variance: *t*-test.

Effect of age

The differences in the protein expressions of GAP43 and TH between the sinus rhythm group and the atrial fibrillation group were compared using the different nationalities as stratification factors, and the results showed that the difference between the groups was statistically significant (P < 0.05) (Table 6).

Table 6. Difference of protein expression of GAP43 and TH in different ages in the two groups (means ± SD).

Age		GAP43	TH
≤65 years	Sinus rhythm group (N = 22)	0.23 ± 0.46	0.51 ± 0.23
	Atrial fibrillation group (N = 14)	0.60 ± 0.24	0.76 ± 0.21
	Statistic	5.756*	3.286#
	P	0.000	0.002
>65 years	Sinus rhythm group (N = 4)	0.53 ± 0.50	0.49 ± 0.28
	Atrial fibrillation group (N = 14)	1.10 ± 0.34	1.18 ± 0.58
	Statistic	2.679#	2.268#
	P	0.017	0.038

*Heterogeneity of variance: *t*-test; #Homogeneity of variance: *t*-test.

The differences in the protein expressions of GAP43 and TH were evaluated across different age groups, in which diseases were used as the stratification factor. The results showed that only the protein expression of GAP43 in the atrial fibrillation group differed significantly across different ages ($P < 0.05$) (Table 7).

Table 7. Difference of protein expression of GAP43 and TH in the two groups in different ages (means ± SD).

Group	Age	GAP43	TH
Sinus rhythm group (N = 26)	≤65 years (N = 22)	0.23 ± 0.46	0.51 ± 0.23
	>65 years (N = 4)	0.53 ± 0.50	0.49 ± 0.28
	Statistic	1.186#	0.155#
	P	0.247	0.878
Atrial fibrillation group (N = 28)	≤65 years (N = 14)	0.60 ± 0.24	0.76 ± 0.21
	>65 years (N = 14)	1.10 ± 0.34	1.18 ± 0.58
	Statistic	4.495#	2.548*
	P	0.000	P < 0.05

*Heterogeneity of variance: *t*-test; #Homogeneity of variance: *t*-test.

DISCUSSION

Previous studies indicated that stimulating the vagus nerve and giving acetylcholine could cause significant cardiac electrophysiological changes; the former might cause shortening of the atrial refractory period, which would in turn induce AF (Brundel et al., 2004). High-frequency electrical stimulation on cardiac ganglionated plexi (GP) may cause triggered activity originating from the pulmonary veins, which would in turn induce AF (Hauerte et al., 2001). On the basis of atrial premature stimulation, stimulating epicardial fat (contained in GP) may lead to AF (Scherlag et al., 2005). Radiofrequency ablation of GP could reverse the changes of the atrial refractory period and eliminate the capacity of premature stimulation, which would influence the superior pulmonary vein, and in turn induce AF (Nakagawa et al., 2004). Further studies (Patterson et al., 2005) confirmed that the rapid discharge of the vein was the result of the combined action of sympathetic and parasympathetic neurotransmitters. Many studies have found that functional changes of autonomic nerves could induce AF. Wijffels et al. (1995) confirmed that stimulating the vagus nerve could cause shortening of the atrial muscle cells during the effective refractory period, which would in turn induce AF. Recent studies have also suggested that pulmonary vein-induced AF was likely due to the presence of special cells with electric conduction function in the pulmonary vein; these cells contained rich sympathetic cells, the activity of which caused local depolarization, which could in turn induce AF (Chen and Tan, 2007). Abnormal activity of the autonomic nerve could also induce AF by inducing cardiac intracellular calcium overload and early after depolarization (EAD). Burashnikov and Antzelevitch (2003) found that injecting Ach into the coronary artery could shorten the action potential of atrial muscle cells and cause rapid atrial pacemaking, which could easily induce AF. They also found that the sudden stop of AF or the increasing of the rapid pacemaking frequency could instantly improve the tension of atrial muscle cells, which would cause 3 phases of rapid EAD of action potential and extrasystole, which would induce AF. Patterson et al. (2006) found that the continuous increase in relaxing period tension was an important cause of EAD, in which the increasing intracellular calcium concentration of the relaxing period was the cause of inducing AF. By using an optical mapping technique,

Chou et al. (2005) detected that when dogs' atrial muscle cell membrane and pulmonary vein regeneration or nerve automatic activity decreased, the rate of forming cardiac intracellular calcium overload and EAD decreased significantly, which would promote the recovery of AF.

Recent studies have indicated that the mechanism of AF was very complex, and that the autonomic nervous system (ANS) played a vital role in triggering and maintaining AF. At present, TH and GAP43 are the main markers known to be related to the regeneration and distribution of the cardiac autonomic nerve. TH is a rate-limiting enzyme that catalyzes the synthesis of the catecholamine neurotransmitter. TH is abundantly expressed in sympathetic ganglia and in noradrenergic neurons of the sympathetic nerve; therefore, the positive expression of TH might represent the sympathetic nerve distribution in the heart. GAP43 is a fast-transport cell membrane phosphate that is expressed in sprouting axon growth hillock. GAP43 is extensively distributed in ANS neurons, and is closely related with neural development, regeneration of axons, and reconstruction of the synapsis and neurotransmitter release. In short, GAP43 is generally regarded as an inherent determinant of neuron development and regeneration, the existence of which symbolizes neural development, and could therefore be used to evaluate the ANS growth activity.

Studies in recent years have further expanded understanding of the relationship between the ANS and AF. Chang et al. (2001) established a chronic AF model by rapid atrial pacing, and the immunohistochemical method was used to detect the dogs' atrium cordis, auricular appendix, eruption of the atrial septum nerve, and distribution of the sympathetic nerve. They found significant and inhomogeneous neural eruption over the distribution of the sympathetic nerve in the dogs' atrium cordis. Furthermore, the right atrium significantly exceeded the left one, which suggested that the reconstruction of neural tissues might have played a vital role in triggering and maintaining AF. Gould et al. (2006) provided histological evidence for the reconstruction of the atrial sympathetic nerve in persistent AF patients by comparing the sinus rate and the eruption and distribution levels of the sympathetic nerve in auricular appendix tissues, which further confirmed that the reconstruction of the autonomic nerve was the partial basis of triggering AF.

Furukawa et al. (2009) made atrioventricular block dog models by applying the radiofrequency ablation method. Results showed atrial enlargement, myocardial fibrosis, and significant atrial and PV effective refractory period shortening when the sympathetic nerve was stimulated, and the atrial conduction velocity accelerated 8 weeks later, which did not appear when the vagus nerve was stimulated. In the radiofrequency ablation group, the triggering rate of persistent AF increased when the sympathetic nerve was stimulated, whereas in the sham operation group, the triggering rate of persistent AF increased when the vagus nerve was stimulated. This study indicated that the stimulation of the sympathetic nerve was the key factor for triggering AF in the reconstructed atrium, which was different from the normal atrium. The internal diameter enlargement of the left atrium was regarded as an important factor for triggering and maintaining AF. In the present study, patients in the atrial fibrillation group had significantly larger left atria than patients in the sinus rhythm group ($P < 0.05$). mRNA and protein expressions of GAP43 and TH differed significantly between the sinus rhythm group and the atrial fibrillation group ($P < 0.05$), which indicated that reconstruction of neural tissues was associated with the structure reconstruction in AF patients.

The differences in protein expressions of GAP43 and TH in the sinus rhythm group and in the atrial fibrillation group remained significant when stratifying the data based on

nationality (Xinjiang Uygur and Han) ($P < 0.05$), and there was no significant difference in protein expressions between the two nationalities within each disease group, which suggests that there is no difference in the distribution of the sympathetic nerve in the heart or automatic nerve growth activity between nationalities.

Differences in the protein expressions of GAP43 and TH were also evaluated among different age groups, in which diseases were used as stratification factors, and the results showed that only protein expression of GAP43 in the atrial fibrillation group differed significantly among age groups. This result indicated that aging plays a vital role in triggering and maintaining AF.

There are several possible reasons to explain why AF causes neural eruption and inhomogenous distribution, including: 1) electrical remodeling and structure reconstruction occurs during AF (Miyauchi et al., 2003), resulting in atrial enlargement, insufficient blood supply, and myocardial damage, which in turn impairs the nerve. GAP43 and β-NGF could help neural development and neural recovery, which promote the regeneration of impaired nerves in addition to promoting the growth of myocardial intact nerves; 2) atrial structure reconstruction in AF causes distribution disorder and regeneration of the nerves distributed in the atria, and the ANS distribution density intensifies, but is not even. Therefore, the reconstruction of the vagus nerve is accompanied by the reconstruction of the sympathetic nerve (Sakamoto et al., 2010); 3) AF causes myocardial ischemia, and neurohormones, such as cytokines and growth factors, increase in circulation, which may be the cause of atrial neural development (Yang et al., 2011).

In summary, the reconstruction of the autonomic nerve is closely related with AF. The unbalance in the autonomic nerve could cause AF, which would in turn cause reconstruction of the autonomic nerve, making it easier to maintain AF. However, the exact mechanism underlying the reconstruction of the autonomic nerve and electrical remodeling awaits further exploration. We found a close association between the ANS and AF, although there are still many open questions. Nonetheless, these results suggest that the triggering and maintaining mechanisms of AF will be further elucidated with more detailed investigations of the ANS. Reversing the reconstruction of the autonomic nerve might be a new therapeutic target of atrial fibrillation, which would guide clinical treatment and improve AF patients' prognoses.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (#30860299).

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