

Pathway analysis with genome-wide association study (GWAS) data detected the association of atrial fibrillation with the mTOR signaling pathway

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ABSTRACT

Background: Genome-wide association studies (GWAS) have identified numerous loci associated with diseases and traits. However, the elucidation of disease mechanisms followed by drug development has remained a challenge owing to complex gene interactions. We performed pathway analysis with MAGENTA (Meta-Analysis Geneset Enrichment of variaNT Associations) to clarify the pathways in genetic background of AF.

Methods: The existing GWAS data were analyzed using MAGENTA. A microarray analysis was then performed for the identified pathways with human atrial tissues, followed by Gene-Set Enrichment Analysis (GSEA).

Results: MAGENTA identified two novel candidate pathways for AF pathogenesis, the CTCF (CCCTC-binding factor, $p = 1.00 \times 10^{-4}$, FDR $q = 1.64 \times 10^{-2}$) and mTOR pathways (mammalian target of rapamycin, $p = 3.00 \times 10^{-4}$, FDR $q = 3.13 \times 10^{-2}$). The microarray analysis with human atrial tissue using the GSEA indicated that the mTOR pathway was suppressed in AF cases compared with non-AF cases, validating the MAGENTA results, but not CTCF pathway.

Conclusions: MAGENTA identified a novel pathway, mTOR, followed by GSEA with human atrial tissue samples. mTOR pathway is a key interface that adapts the change of environments by pressure overload and metabolic perturbation. Our results indicate that the mTOR pathway is involved in the pathogenesis of AF, although the details of the basic mechanism remain unknown and further analysis for causal-relationship of mTOR pathway to AF is required. CTCF pathway is essential for construction of chromatin structure and transcriptional process. The gene-set components of CTCF overlap with those of mTOR in Biocarta.

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1. Introduction

Atrial fibrillation (AF) is a common arrhythmia in clinical practice [1]. The prevalence of AF was estimated 2.2 million people in the United States and 716,000 people in Japan [2]. According to the Global Burden of Disease 2010 Study, an international collaborative effort to systematically assess global data on AF, evidence from 184 different publications showed that around 33.5 million individuals have AF worldwide in 2010, with 5 million new AF cases occurring per year [3]. AF is a multifaceted condition affected by risk factors, and

co-morbidities [4] such as aging [5], genetic predisposition and acquired factors including pressure overload in atrium [6], and other cardiovascular diseases. Furthermore, atrium is exposed to numerous mediators such as metabolic perturbation [7], oxidative stress [8], and inflammation [9], ectopy of pulmonary vein, electrical and structural remodeling [10]. As for electrical remodeling based on animal experiments and clinical research [11,12], the enhanced membrane repolarization through reduced $I_{Ca,L}$, and increased I_{K1} and agonist-independent constructive $I_{K,ACh}$ promotes action potential duration shortening and re-entry, whose alteration affects Ca^{2+} handling due to the perturbation of Ca^{2+} release from sarcoplasmic reticulum Ca^{2+} store. Slow and/or heterogeneous conduction through changes in the expression, function, and localization of connexins responsible for electrical cell-to-cell coupling can promote re-entry. On the other hand, structural remodeling

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in the atrium is related to environmental stress and growth factors such as activated renin-angiotensin-aldosterone system [13], and transforming growth factor- β [14]. In individuals with genetic risk factors, acquired and environmental factors accumulate and enhance susceptibility to atrial remodeling.

Genome-wide association studies (GWAS) have been used to identify loci associated with numerous diseases and physical properties. GWAS is a comprehensive and powerful method to detect candidate molecules underlying diseases. Recent large-scale meta-analysis of AF-GWAS identified 97 loci in trans-ethnic population and 16 loci in Japanese population [15,16]. However, it is challenging to apply these association data to the elucidation of disease mechanisms because the functions of loci are often unknown. First, functional analysis of regions and genes included in a locus associated with AF has been attempted. 4q25 region including *PITX2* has been identified repetitively in GWAS and the functional analyses were performed through searching for enhancer sequence [17,18]. Second, besides these functional analysis for individual loci, pathway analysis with AF-GWAS of European population was performed and identified cardiac developmental, electrophysiological, contractile and structural pathways [15]. Bioinformatics approach including pathway analysis with GWAS data had identified new therapeutic options of existing drugs. Candidate drugs targeted for type 2 diabetes has been suggested in the approved drugs such as thiazolidinediones for rs1801282 and/or unapproved drugs such as GSK activators for rs10278336 through protein-protein interaction data and Therapeutic Targets Database using the GWAS data for type 2 diabetes [19]. The GWAS for rheumatoid arthritis also indicated existing cancer drugs as novel candidates for rheumatoid arthritis using a similar strategy [20].

Meta-Analysis Gene-set Enrichment of variaNT Associations (MAGENTA) was developed to decode the basis of complex traits [21]. MAGENTA considers all SNP data obtained by GWAS using the gene set enrichment analysis (GSEA) approach: 1) SNPs are mapped to the nearest gene, 2) SNPs with the lowest p -value are selected for each gene, 3) the p -values of the best SNPs are corrected depending on the length of the gene, 4) significant pathways are estimated by GSEA. The statistical power of MAGENTA to detect disease-associated pathways has been evaluated by computer simulation and validated using several GWAS datasets. MAGENTA was employed in the AF-GWAS in the trans-ethnic population and identified cardiac developmental pathways including *PITX2* and *TBX5* [15].

In this study, we applied MAGENTA to previously obtained AF-GWAS data and identified novel disease pathways. We assessed the finding with the following microarray analysis of human atrial tissues. The combination of bioinformatics data from GWAS and microarray experiments led to the identification of an association between the mTOR pathway and AF. These findings may be a clue in understanding the complicated AF pathogenesis.

2. Materials and methods

2.1. MAGENTA

MAGENTA (Broad Institute; <http://www.broadinstitute.org/mpg/magenta>) software was applied to identify novel pathways associated with AF. Data from a previous Japanese GWAS (843 AF cases and 3350 controls) [22] were used for the analysis. All samples were collected by BioBank Japan. Written informed consent was obtained from each study participant. The dataset included 610K SNPs genotyped using the Affymetrix GeneChip500K, annotated with GRCh37. BIOCARTA and Ingenuity were used as informative pathway resources, including data for 214 and 81 pathways, respectively. The false discovery rate (FDR) q -value and p -value were obtained to identify significantly enriched pathways.

2.2. Human atrial tissue samples

Atrial tissue samples were collected from eleven patients who underwent cardiac surgery of the mitral valve in the Department of Cardiovascular Surgery, Tokyo Medical and Dental University Hospital. This study was approved by the Ethics Committee for Genome Research at the Medical School, Tokyo Medical and Dental University.

2.3. DNA array analysis

All participants provided written informed consent. RNA was extracted from the samples and treated with RNeasy after their excision. The RNeasy Mini Kit (Qiagen, Hilden, Germany) and 3' IVT PLUS Array Kit (Affymetrix, Santa Clara, CA, USA) were used according to the manufacturers' protocols. Samples were applied to the GeneChip® Human Genome U133 Plus2.0 Array (Affymetrix) and expression levels were measured using GeneChip Scanner 3000 equipment after treatment. Data processing was performed using Affymetrix® Expression Console, Tiling Analysis, and Transcriptome Analysis Console according to standard protocols. Quality control procedures were performed according to the manufacturer's protocol (<http://www.thermofisher.com>). The quality control metrics includes hybridization controls, labeling controls, internal control genes, global array metrics, and algorithm parameters [Supplementary Figs. 1, 2A, B, Supplementary Table 1].

2.4. Gene set enrichment analysis (GSEA)

An enrichment analysis was performed for the two pathways identified by MAGENTA, the mTOR and CTCF pathways, using GSEA software (Broad Institute, <http://www.broadinstitute.org/gsea/index.jsp>) [23]. In brief, after calculating the enrichment score (ES), target genes were assessed to determine whether they are randomly distributed or exhibit statistically significant differences between two biological phenotypes, AF and non-AF. The expression dataset was processed with Expression Console 1.4.1.46 software based on MAS-5 program for the background correction and the normalization of the raw data and applied for BioCarta pathway database (c2. cp. biocarta. v5.1. entrez) and the Molecular Signatures Database (MSigDB). A 100-permutation test was performed to create a histogram of ES values. Accordingly, the criteria of significant pathways and genes was based on ES at a nominal p -value of <0.05 . The ES reflected the degree to which mTOR and CTCF pathway sets are overrepresented at the extremes of the entire ranked list. The scores were calculated by summing the observed frequencies of the gene sets of the two pathways in the ranked list using Kolmogorov–Smirnov statistics.

3. Results

3.1. Identification of the mTOR and CTCF pathways using MAGENTA

We identified novel biological or molecular pathways demonstrating significant enrichment using previous GWAS data for atrial fibrillation in the Japanese population (843 AF cases and 3350 control subjects) [22]. Although these data indicated associations of AF with *KCNN3*, *PRRX1*, *PITX2*, *CAV1*, *C9orf3*, and *ZFH3*, an *in silico* pathway analysis with DAVID (<https://david.ncifcrf.gov/>) failed to identify a particular pathway including these six loci (data not shown). MAGENTA software was applied to these GWAS data using the gene sets included in BIOCARTA and Ingenuity as information resources for biological and molecular pathways; they include 214 and 81 pathways, respectively. The pathways exhibiting statistically significant associations with AF were the mTOR ($p = 3.00 \times 10^{-4}$, FDR $q = 3.13 \times 10^{-2}$) and CTCF pathways ($p = 1.00 \times 10^{-4}$, FDR $q = 1.64 \times 10^{-2}$) [Table 1] [Supplementary Table 2].

3.2. Microarray analysis using human atrial tissue samples

We investigated the enrichment of genes in the mTOR and CTCF pathways in left atrial tissues of AF and non-AF patients using GSEA software. The clinical background of the patients is summarized in Table 2. All patients were diagnosed with mitral valve disease: a mean age of 68.7 ± 2 years, a mean left atrial diameter of 52.6 ± 2.3 mm, and different atrial fibrillation risk factors, including hypertension and heart failure. A microarray analysis was performed to estimate differential gene expression between the non-AF group and AF group. Among 54,613 genes on the microarray, 1575 genes were differentially expressed ($p < 0.05$, fold change < -2 or > 2), including 214 up-regulated genes and 1361 down-regulated genes in the comparison between the AF and non-AF groups (Supplementary Fig. 2A and B, Supplementary Table 1). A hierarchical clustering analysis after filtering data with $p < 0.001$ showed the significant difference in the top core gene sets between AF and non-AF samples (Fig. 1).

3.3. Gene set enrichment analysis

We investigated the up- or down-regulated gene sets in the overall dataset including mTOR and CTCF pathways in left atrial tissues of AF and non-AF patients using GSEA software according to the recommended parameters. All genes in the data set were ranked according to the correlation between their expression and functional category

(Supplementary Tables 3, 4). We used BioCarta as a biological pathway database and determined whether members of the gene sets were randomly distributed in the observed gene set. Based on ES estimates, we demonstrated that the mTOR pathway was significantly enriched in AF tissues with a nominal p -value < 0.05 , and was down-regulated in the AF phenotype. Most of the gene sets in the mTOR pathway were down-regulated in the AF group compared with the non-AF group (Table 3, Fig. 2). On the other hand, the CTCF pathway, which is related to signaling in immature B cells by B-cell receptor and transforming growth factor β (TGF- β), was randomly distributed with low ES.

4. Discussion

A pathway analysis of previously generated AF-GWAS data with MAGENTA identified the association between the mTOR pathway and AF, and this association was confirmed by microarray data of human atrial tissues obtained from AF and non-AF patients. In our study, we used two different datasets: (1) GWAS data for 850 AF patients and 3350 controls in Japan and (2) microarray transcriptome data for eleven mitral valve disease patients. As for the present results, mTOR pathway was identified through GWAS screening with lone AF as the possible mechanism. Microarray analysis using human atrial tissues which were exposed by pressure overload of mitral stenosis revealed that mTOR pathway was suppressed in AF. Although samples in the two

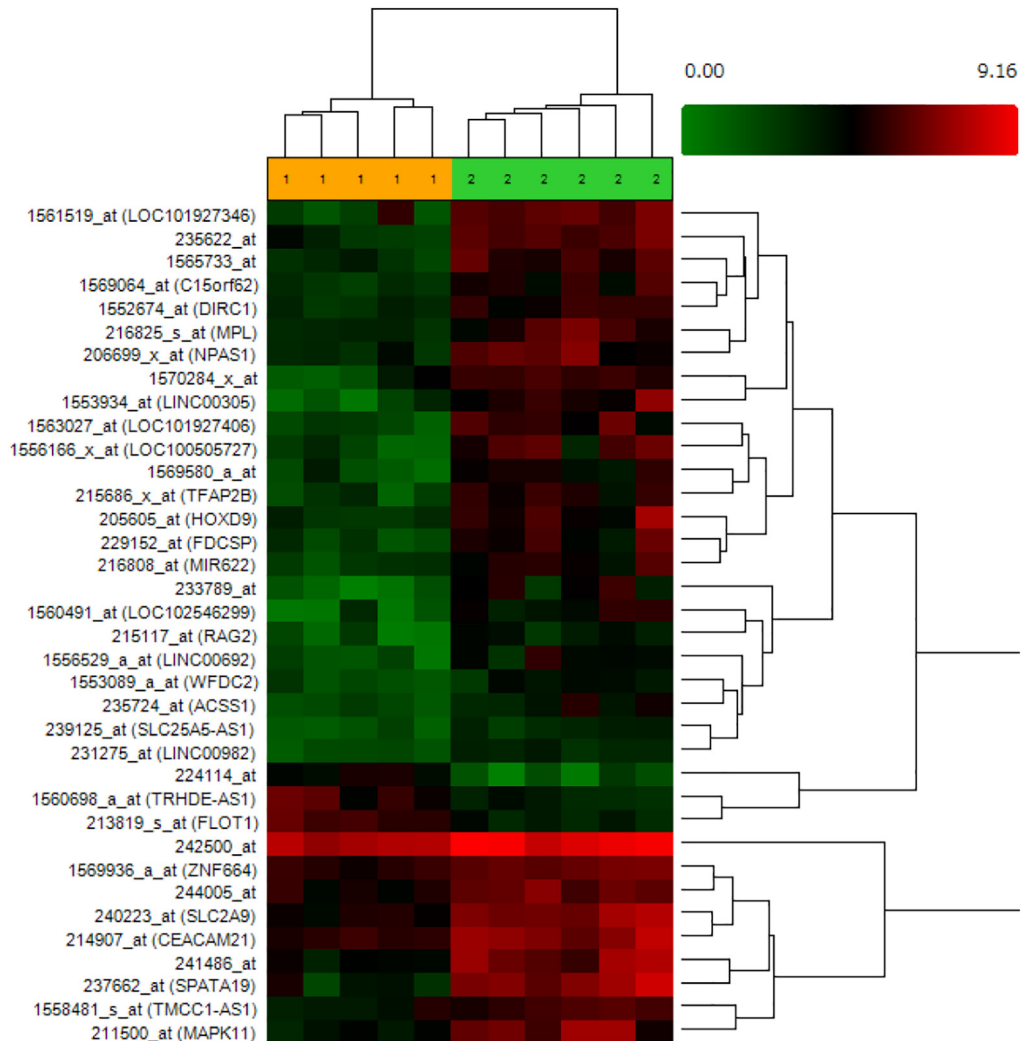


Fig. 1. Hierarchical clustering. All the genes under condition (p -value <0.001 , fold change <-2 or >2) were clustered according to the distance between the data of two genes. The number 1 and 2 indicate non-AF group and AF group, respectively.

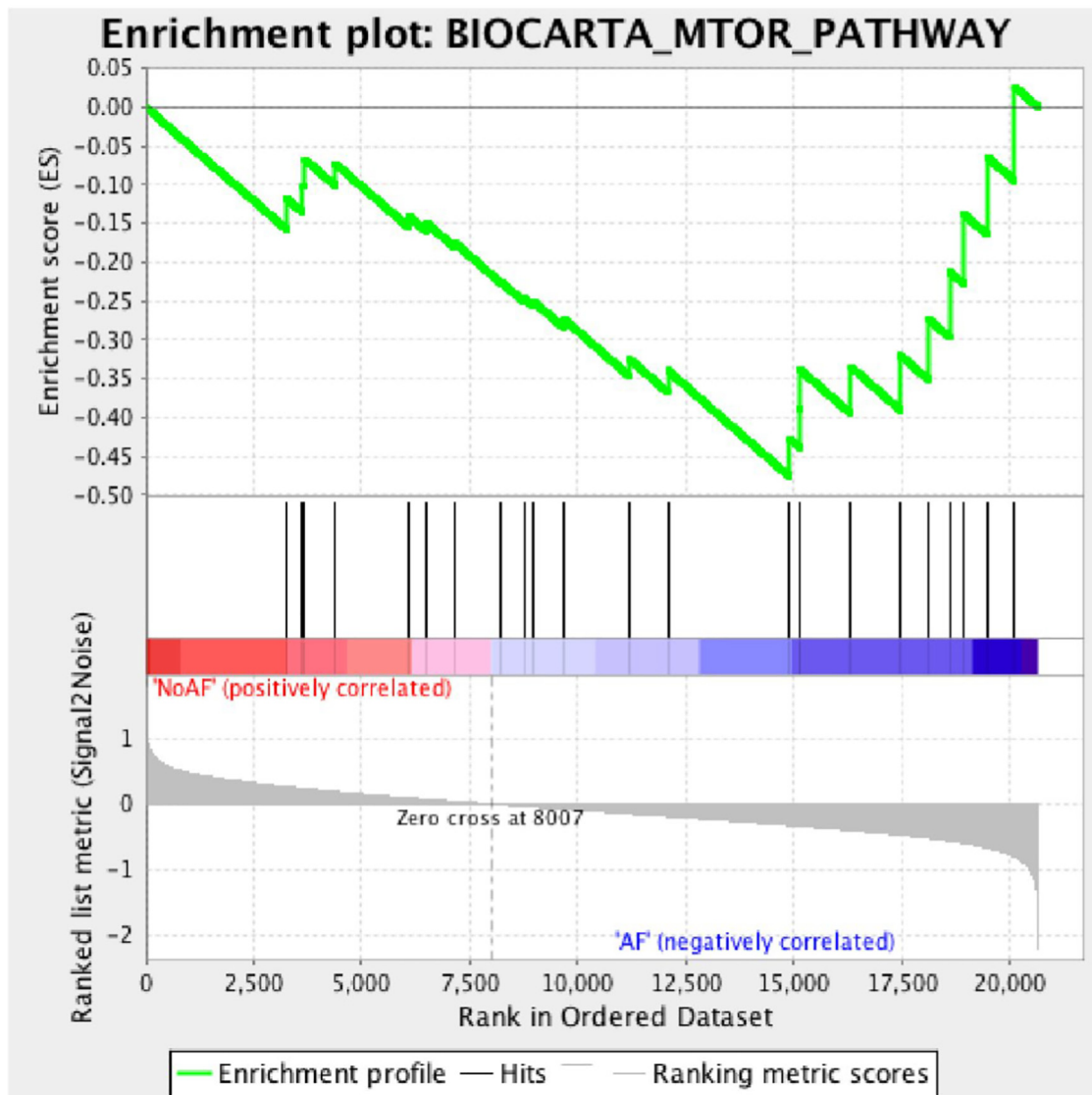


Fig. 2. Enrichment plot of mTOR pathway. Enrichment score of genes included in mTOR pathway correlated positively in non-AF tissue while correlated negatively in AF tissue.

studies were different, it indicated that mTOR pathway is associated with AF pathogenesis.

MAGENTA and GSEA were applied to GWAS data and microarray data, respectively. Both MAGENTA and GSEA are used to statistically compare the observed number of significant genes that differ between two groups in a certain gene-set with the predicted number. The previous AF-GWAS in Japanese have detected only the 4q25 locus in the case of the strict criterion of $p < 5 \times 10^{-8}$ [22]. Using the MAGENTA, we identified two new disease pathways, mTOR (mammalian target of rapamycin) and CTCF (CCCTC-binding factor). Second, we used microarray transcriptome data to confirm the MAGENTA results with a different dataset. Atrial tissues were excised from patients undergoing cardiac surgery for mitral valve disease, whose atrial tissues must receive high intracardiac pressure, transiently or persistently. We obtained both patients with AF and without AF, comparing their gene expression levels. Significant number of genes involved in the mTOR pathway were suppressed in the atrial tissues of AF patients and an enrichment analysis with GSEA showed that the pathway was also significantly suppressed. The GWAS missed the component molecules in mTOR pathway as candidates, because no variant in mTOR locus was identified. In our study, pathway analysis enabled to identify the associated pathway. The association of mTOR pathway with AF was confirmed in the transcriptome analysis of atrial tissue, while we failed to replicate the results

for the CTCF pathway obtained by MAGENTA with microarray data. CTCF expression was not associated in atrial tissue with AF under the influence of pressure overload because the difference between lone AF and AF with mitral stenosis might affect the result. CTCF pathway is essential for construction of chromatin structure and transcriptional process of gene expression change. The gene-set components of CTCF overlap those of mTOR in Biocarta. MAGENTA found CTCF pathway due to the similar composition.

mTOR is a protein kinase that is conserved in a wide variety of species as an interface of various metabolic pathways. mTOR plays crucial roles in cell metabolism, a fundamental biological reaction involved in cell development, growth, maintenance and survival [24]. The kinase is activated by extracellular stress and growth factor signaling and enhances the cytoplasmic translational process and the protein synthesis. mTOR pathway is involved in cardiac homeostasis against stress such as pressure overload in myocardium [25]. The effect of mTOR inhibition and deficiency on myocardium differs in the physiological and pathological status [26]. The cardiac-specific mTOR knockout mice are lethal in the embryonic and neonatal stage mainly due to left ventricular dilatation and dysfunction, suppressing cell growth, protein synthesis, mitochondrial function and sarcomere structure maintenance [17,18]. mTOR pathway contributes to cell proliferation, survival, and embryogenesis of cardiomyocytes from embryonic to neonatal stage. On the

Table 1
Result of MAGENTA analysis. As database for comparison, Ingenuity and BIOCARTA were used.

Database	Gene signaling	Number	MEAN	P-value	FDR	Expected	Observed
Ingenuity	Neuregulin.Signaling	27	92	3.30E-03	1.27E-01	3	8
Ingenuity	TR.RXR.Activation	62	75	5.60E-03	9.83E-02	6	13
Ingenuity	Apoptosis.Signaling	31	54	7.30E-03	1.13E-01	3	8
Ingenuity	Sonic.Hedgehog.Signaling	10	62	1.11E-02	1.52E-01	1	4
Ingenuity	Amyotrophic.Lateral.Sclerosis.Signaling	29	89	1.73E-02	1.69E-01	3	7
Ingenuity	PI3K.AKT.Signaling	29	57	2.30E-02	1.96E-01	3	7

Database	Gene signaling	Original number	MEAN	P-value	FDR	Expected	Observed
BIOCARTA	CTCF_PATHWAY	23	58	1.00E-04	1.64E-02	2	9
BIOCARTA	MTOR_PATHWAY	23	59	3.00E-04	3.13E-02	2	9
BIOCARTA	P35ALZHEIMERS_PATHWAY	11	79	2.70E-03	6.02E-02	1	5
BIOCARTA	IGF1MTOR_PATHWAY	20	76	8.30E-03	2.18E-01	2	6
BIOCARTA	WNT_PATHWAY	26	72	1.30E-02	2.43E-01	3	7
BIOCARTA	BCELLSURVIVAL_PATHWAY	16	51	1.36E-02	2.56E-01	2	5
BIOCARTA	RAS_PATHWAY	23	59	1.52E-02	2.39E-01	2	6
BIOCARTA	ACH_PATHWAY	16	72	1.61E-02	2.69E-01	2	5
BIOCARTA	PAR1_PATHWAY	37	132	1.67E-02	2.57E-01	3	8
BIOCARTA	NGF_PATHWAY	18	50	2.20E-02	2.45E-01	2	5
BIOCARTA	ALK_PATHWAY	37	72	2.28E-02	2.96E-01	4	8
BIOCARTA	EIF4_PATHWAY	24	109	2.78E-02	3.20E-01	2	6
BIOCARTA	MITOCHONDRIA_PATHWAY	21	39	2.94E-02	3.08E-01	2	5
BIOCARTA	BAD_PATHWAY	26	80	3.27E-02	2.61E-01	3	6
BIOCARTA	DC_PATHWAY	22	11	3.32E-02	2.65E-01	2	5
BIOCARTA	TOB1_PATHWAY	21	49	3.36E-02	2.81E-01	2	5
BIOCARTA	DEATH_PATHWAY	33	43	3.58E-02	2.96E-01	3	7
BIOCARTA	STATHMIN_PATHWAY	19	82	3.73E-02	2.64E-01	2	5
BIOCARTA	G1_PATHWAY	28	65	4.41E-02	2.91E-01	3	6

Number: original numbers included in signaling.
 MEAN: mean gene size.
 FDR: False discovery rate.
 Expected: expected number of genes.
 Observed: actually observed number of genes.
 The genes in Bold were significant in GSEA.

other hand, the knockout and inhibition of mTOR in aged mice have resulted in the prolonged survival interval and the suppressive effect of cardiac hypertrophy. The gradual maladaptation of mTOR in the process of aging causes ROS formation, accumulation of unfolding proteins, cellular senescence, energy expenditure, and mitochondrial insufficiency. Furthermore, mTOR inhibition reactivates cardiac autophagy and acts on myocardium in a protective manner [27].

In the current study, we found that mTOR pathway identified from GWAS with lone AF was suppressed in human left atrial tissue obtained from elderly patients of their 60s–70s. Although it is generally thought that in the myocardial senescence, the quality control of proteins that contributes to the cell maintenance through metabolic integration in the young myocardium is impaired to maladaptive state, the result

was contrary to expectations. The left atrial tissue in our microarray analysis was exposed to high pressure overload, which means that the observed changes in gene expression may not always reflect AF-related modeling. At least, this result showed that expression of several genes in mTOR pathway changed. Our findings indicated the association of mTOR dysfunction with AF. As a risk factor for atrial fibrillation, aging, cardiac hypertrophy, and metabolic perturbation are thought to be

Table 2
Clinical characteristics of patients.

Phenotypes	Non-AF	AF	Total	p-Value
	n = 5	n = 6	n = 11	
Age (years)	72.0 ± 4.1	66.0 ± 7.2	68.7 ± 2.0	NS
Female, n (%)	1(20.0)	3(50.0)	4(36.4)	NS
Drinking history, n (%)	1(20.0)	4(66.7)	5(45.5)	NS
Smoking history, n (%)	3(60.1)	2(33.4)	5(45.5)	NS
LA diameter (mm)	50.6 ± 3.0	54.3 ± 10.2	52.6 ± 2.3	NS
Dyslipidemia, n (%)	2(40.0)	1(16.7)	3(27.3)	NS
History of CAD, n (%)	2(40.0)	2(33.4)	4(36.4)	NS
Diabetes mellitus, n (%)	0(0.0)	0(0.0)	0(0.0)	NS
Heart failure, n (%)	3(60.1)	5(83.3)	8(72.7)	NS
Hypertension, n (%)	3(60.2)	2(33.4)	5(45.5)	NS
HCM, n (%)	0(0.0)	1(16.7)	1(9.1)	NS
CKD, n (%)	1(20.0)	0(0.0)	1(9.1)	NS
COPD, n (%)	1(20.0)	0(0.0)	1(9.1)	NS

LA diameter, left atrial diameter; BNP, B-type natriuretic peptide; MVD, mitral valve disease; CAD, coronary atherosclerotic heart disease; HCM, hypertrophic cardiac myopathy; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease.

Table 3
The components of mTOR pathway.

Entrez_ID	Gene symbol	Rank in gene list	Rank metric score	Running ES	Core enrichment
1982	EIF4G2	20,129	-0.797821045	0.026012314	Yes
5515	PPP2CA	19,489	-0.661122859	-0.06417525	Yes
7248	TSC1	18,951	-0.589762688	-0.13854751	Yes
5295	PIK3R1	18,619	-0.557331443	-0.21206315	Yes
8569	MKNK1	18,131	-0.51423192	-0.27309778	Yes
5170	PDPK1	17,484	-0.466402262	-0.31988618	Yes
1978	EIF4EBP1	16,332	-0.396058679	-0.33494985	Yes
207	AKT1	15,162	-0.332502306	-0.33846062	Yes
8672	EIF4G3	15,146	-0.331991702	-0.38822106	Yes
2475	FRAP1	14,916	-0.321033925	-0.42753774	Yes
1981	EIF4G1	3683	0.23271291	-0.06741249	No
5164	PDK2	4377	0.189100042	-0.07226868	No
7249	TSC2	3634	0.235626712	-0.1004224	No
1975	EIF4B	3258	0.26200974	-0.11803479	No
1977	EIF4E	6078	0.093864225	-0.14036475	No
2280	FKBP1A	6491	0.073707983	-0.14913033	No
1973	EIF4A1	7156	0.040029906	-0.17521651	No
8661	EIF3S10	8235	-0.010132063	-0.22589663	No
5728	PTEN	8753	-0.032501176	-0.24600525	No
6194	RPS6	8968	-0.042265035	-0.24995396	No
5290	PIK3CA	9683	-0.07499864	-0.2731525	No
6198	RPS6KB1	11,216	-0.145169243	-0.32532048	No
1974	EIF4A2	12,089	-0.18551822	-0.33939135	No

The genes in Bold were significant in GSEA.

under quality control of mTOR. The microarray analysis indicated the suppression of mTOR pathway in myocardium as a result of the protective process against atrial remodeling.

One of the important findings in our study is that mTOR pathway had not been identified even in the several larger GWASs, which means that we need the careful interpretation for our results, thinking of the specificity of the population. First, we used lone AF cases. The characteristics of this phenotype of lone AF could affect the difference from GWAS including AF cases with many complications. Second, we used Japanese population. According to stroke GWAS, GWAS of cardiogenic and atherosclerotic stroke identified the different loci of overall stroke [28]. Our study using GWAS with these specific population with three characteristics could provide different results. The next one is that the pathway of cardiac development including *PITX2* and *TBX5* failed to be identified in our study. The cardiac developmental pathway is important and was identified in larger GWAS [15,16]. Our existing GWAS was thought to be purely underpowered. The components of the developmental pathways were under the level of significance.

In conclusion, MAGENTA identified a novel pathway, mTOR, followed by GSEA with human atrial tissue samples, although the interpretation of the study result might be quite limited. mTOR pathway is a key interface that adapts the change of environments by pressure overload and metabolic perturbation. mTOR pathway could be associated with AF pathogenesis.

4.1. Limitation

This study has several limitations. First, to minimize bias, we recruited participants with AF who had no complication except hypertension. On the other hand, the smaller sample size indicated the possibility of false positive result. Second, the study population consisted predominantly of Japanese patients. As for these two points, multi-ethnic pathway analysis with larger sample size should be required. Third, recent study with larger population did not detect this pathway. The inconsistency is the difference of the AF property of participants in each study. Fourth, the atrial tissue samples of surgery for validation study might be different from the participants in the existing GWAS. Fifth, mTOR pathway was suppressed in the atrial tissue with MVD contrary to our expectation. The background mechanism remains unclear and is required for further basic experiments.

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Declaration of Competing Interest

No conflicts of interest to disclose.

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