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IN SILICO PREDICTION OF *PITX2* GENE MUTATIONS IN ATRIAL FIBRILLATION

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ABSTRACT

Objective: This study aimed to analyze the *PITX2* gene using two exons, 4 and 5, in individuals with atrial fibrillation (AF) in order to assess, through in silico prediction, the potential impact of the mutations identified.

Methods: Forty-six (46) patients with AF and twenty (20) controls were included. Sequencing of the *PITX2* gene identified several variants. These were evaluated using bioinformatic functional prediction tools to determine their likely consequences on the structure and activity of the *PITX2* protein.

Results: The variants identified are not classified as pathogenic according to reference databases and current classification criteria. However, most of the mutations were found to affect protein function and DNA binding, suggesting a possible role in modulating the molecular mechanisms involved in FA.

Conclusion: Although the identified mutations are not associated with known pathogenicity, their functional effects on *PITX2* could contribute to individual susceptibility to atrial fibrillation. These results highlight the value of in silico prediction approaches for exploring the functional variability of candidate genes and better understanding the genetic complexity of AF.

Keywords: Atrial fibrillation; Genetics; *PITX2* gene; Mutations

1 INTRODUCTION

Atrial fibrillation (AF) is one of the most common major arrhythmias, characterized by an irregular heartbeat. It usually occurs in adults. However, although age is a major risk factor, it is not the most surprising one. As the sixth leading cause of cardiovascular disease [1], it increases the risk of heart failure (HF), myocardial infarction, chronic kidney disease, stroke and death [2]. AF often occurs due to ectopic electrical impulses originating in the pulmonary veins (PV). Sequence variants in the activators controlling the expression of the transcription factor *PITX2,* expressed in PV and left atrial cardiomyocytes, have been implicated in the predisposition to AF [3]. The atrial-selective transcription factor *PITX2* is an upstream transcriptional regulator of atrial electrical function and cardiogenesis [4] [5]. Complete loss of *PITX2* function can lead to malformation of the pulmonary veins, which are well-known sites of ectopic activity

promoting spontaneous AF [6]. *PITX2* deficiency increases cellular and functional heterogeneity in atrial cardiomyocytes, which impairs mitochondrial function and metabolism by altering gene and protein expression [7]. The aim of this study is to conduct an in-silico analysis of the *PITX2* gene focusing on two of its exons (exon 4 and exon 5) to identify mutations and their impact on the development of atrial fibrillation in a cohort of 46 individuals affected by the condition. The in-silico analysis was deliberately restricted to exons 4 and 5 of *PITX2* as they encode the highly conserved and functionally critical homeobox domain. These exons account for the majority of potentially deleterious variants reported in the literature and are subject to strong selective pressure, making functional predictions more relevant. Exon 4 is particularly large and characterizes the *PITX2*C isoform, which is the largest isoform with 324 amino acids [8]. Exon 5 contains the homeodomain, which is essential for DNA binding, specificity and the transactivation potential of *PITX2* [8], a region critical for the function of the transcription factor. Exons, mature sequences of the gene after splicing, contain the information necessary for protein production. Their instability would have serious consequences on protein production.

1.1 Structural analysis within functional areas

The *PITX2* gene (**title 1**) encodes a homeodomain transcription factor of the Bicoid family, which is essential for the regulation of gene expression and embryonic development, particularly in relation to left-right asymmetry, cardiac development, and certain craniofacial and ocular structures. There are several isoforms—*PITX2*a and *PITX2*b, generated by alternative splicing, and *PITX2*c, which utilizes an alternative promoter located in intron 3 and is the isoform primarily implicated in FA [9] [10]. Exon 4 is particularly important as it determines the size of the isoform, with *PITX2*c being the largest of the isoforms. The homeobox domain enables PITX2 to bind to specific DNA elements to regulate transcription. The gene binds to bicoid and bicoid-like elements in target promoters, such as the DLX2 promoter, and activates this promoter up to 30-fold in Chinese hamster ovarian cells [11]. Mutations in the homeodomain have major functional consequences: specific *PITX2* mutations associated with Axenfeld-Rieger syndrome retain similar DNA-binding specificity but lose the ability to transactivate target promoters [11].

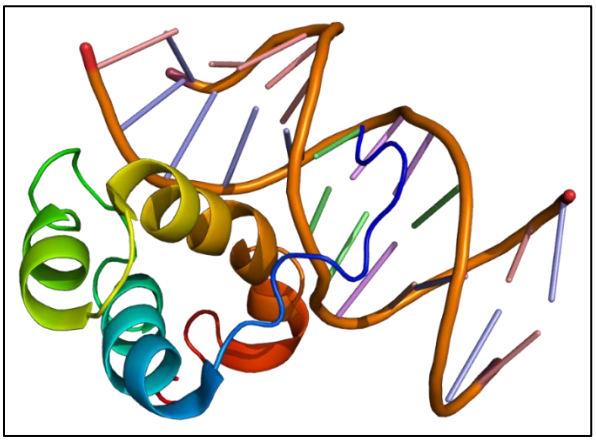

Figure 1: Protein PITX2 PDB 1yz8

1.2 Genes in Atrial Fibrillation

The figure (**title 2**) shows that genome-wide association studies (GWAS) for atrial fibrillation have explored common variants and established numerous associations with modest to low effect sizes on disease risk. Whole-exome and whole-genome sequencing studies seek to further identify rare and low-frequency variants within genes or across the genome, respectively. The workflow for establishing the genotype-phenotype link involves a hierarchical staging of analytical methods, which may include fundamental genetic differences between controls and cases, relevant patterns and correlations in the data, or mechanisms of atrial fibrillation in the context of *PITX2* alteration that may explain apparent genetic differences [12].

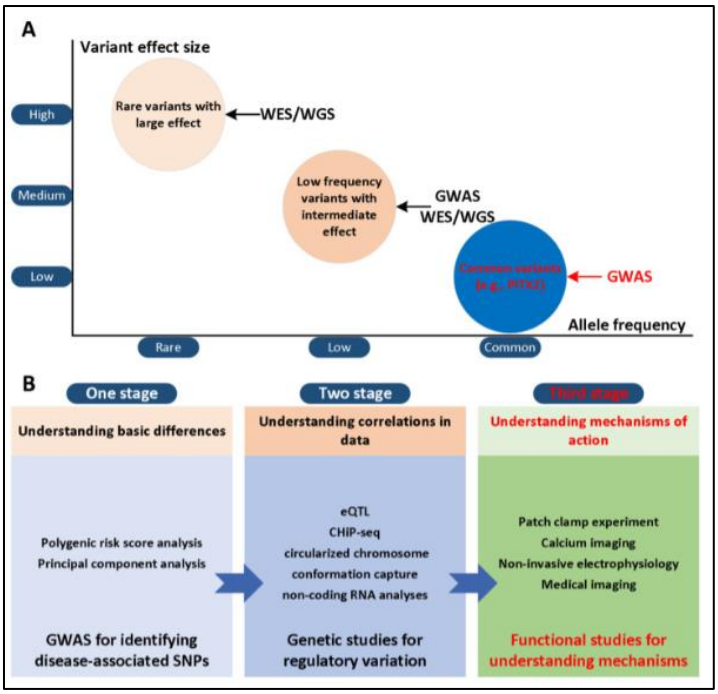

Figure 2: Role of genes in AF based on GWAS from locus to gene and their mechanism

1.3 Impact of mutations on DNA binding

The transcriptional regulation of the *PITX2* gene is modulated by several mechanisms. The C-terminal tail of *PITX2* contains an inhibitory domain that suppresses DNA binding. However, this suppression is lifted when transcription factors such as Pit-1 bind to the C-terminus of *PITX2*. Furthermore, the transcriptional activity of *PITX2* is negatively regulated by N-terminal phosphorylation and positively regulated by C-terminal phosphorylation. Mutations in the *PITX2* gene profoundly affect the protein's ability to bind to DNA, thereby compromising its function as a transcription factor. Studies clearly demonstrate that mutations in the *PITX2* homeodomain (particularly in the regions corresponding to exons 4 and 5) can significantly reduce binding affinity [13] [14] [15] and, paradoxically, increase affinity whilst reducing function [14]. These alterations in DNA binding generally result in a loss of transactivation capacity, thereby contributing to the development of malformations (Axenfeld-Rieger syndrome) or cardiac arrhythmias (atrial fibrillation) [16].

2 MATERIAL AND METHOD

A retrospective study of patient records at the FANN Hospital in Dakar. It included all patients admitted with atrial fibrillation. No distinction was made between different types of AF. All patients included in the study had rheumatic mitral valve disease associated with atrial fibrillation, which constituted an indication for surgical mitral valve replacement. The pathophysiological relationship between rheumatic mitral stenosis and atrial fibrillation is widely recognized: the chronic overload imposed on the left atrium leads to its remodeling and progressive dilation, thereby promoting the onset of this arrhythmia. The diagnosis was based on a combination of clinical, echocardiographic and surgical findings. For each patient who underwent surgery, a fresh tissue sample was taken from the surgical site (the junction of the left atrium and the right superior pulmonary vein), placed in a dry tube and stored at 20°C. After undergoing a complete clinical examination, the clinical records and DNA extract samples were sent to the laboratory where extraction, PCR and gene amplification were performed. For each of the two exons 4 and 5 at our disposal, we worked on 46 patients and 20 controls. These consisted of biological samples that were already available at the laboratory of the Faculty of Science and Technology at Cheikh Anta Diop University in Dakar at the time of the study. They were stored blood samples taken from apparently healthy subjects with no known history of heart disease or atrial fibrillation. The study has been approved by the UCAD Ethics and Research Committee (reference number: Protocol 0274/2018/CER/UCAD).

The volumes of F/R primers used were 0.3 µl, the volume of MgCl2 was 1 µl, and the temperature was raised to 57°C. After preparing the two master mixes, they were distributed into the strips, followed by the DNA volumes from each individual. The strips were then placed in the thermocycler set to the amplification conditions. On pre-prepared agarose gel, the extracts mixed with dye were deposited in the gel wells and migrated at 100 V for 25 minutes. The results showed successful PCR in all individuals for each exon. The presence of a few multi-bands was noted with exon 5. Subsequently, the PCR products to which the primers bound were sequenced. Complete exome sequencing was

performed on all individuals using the F. Sanger method (1977), which is based on a specific PCR reaction using modified nucleotides in addition to compounds: Di deoxyribonucleotides (ddNTPs) [17]. Sequencing reactions were carried out in an MJ Research PTC-225 Peltier-type thermal cycler using the ABIPRISM BigDye™ Terminator Cycle kits. Each sample was sequenced using the sense primer, with 10 µl of PCR products. The fluorescent fragments were purified using the BigDye Xterminator purification protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

The results are presented in the form of an electropherogram (or chromatogram) representing the sequence of bases comprising the DNA fragment.

3 RESULTS

3.1.1 Gene identification in exon 4 and exon 5

The chromatograms obtained were submitted to the Mutation Surveyor software [18] in order to identify the different mutations. Several mutations have been identified in both exons (120 for exon 5 and more than 200 for exon 4), but only the non-synonymous ones are of concern. Not all individuals had mutations (7 for exon 4 and 24 for exon 5). The most frequent and repetitive mutations were synonymous mutations. However, these are not taken into account as they have no functional relevance. We identified four non-synonymous mutations in both exon 4 and exon 5 (**table 1**). Similarly, the variants found in healthy subjects were considered neutral polymorphisms. The chromatogram files (.ab1) were analysed using the Mutation Surveyor software (SoftGenetics, version X.X). Each sample chromatogram was aligned to the reference sequence NC_000004.12 and subjected to an automated signal subtraction analysis. Deletions and duplications were identified on the basis of frameshift motifs, double-peaked signals and deviations from the reference chromatogram. Variants were retained when they were detected on both the forward and reverse sequencing strands, with a mutation score ≥ 0.5 and a flanking base quality of Q ≥ 20. All reported variants were manually reviewed on the chromatogram viewer prior to validation. Prediction software was used to determine the impact of these mutations on the protein, its function and its pathogenicity. **Table 1** below shows the results obtained. The conversion of genomic positions to chromosomal coordinates was carried out taking into account the orientation of the PITX2 gene on the antisense strand of chromosome 4, using the formula: Chr4 position = 110 642 353 − NG_007120.1 position. The chromosomal positions obtained are listed in **table 1**. These coordinates were validated using the Mutalyzer and Ensembl VEP tools.

Table 1: Non-synonymous mutations in the two exons

Exons	Chromosomal location	Genomic position	Statue	Amino acids	Nature	ID-dbSNP
Exon 4	Chr4:110621082	21271G>T	homozygous	Q67H	Not a synonym	News
	Chr4:110,621,081	21272C>G	homozygous	Q68E	Not a synonym	News
	Chr4:110,620,969	21384A>G	homozygous	Q105R	Not a synonym	News
	Chr4:110,620,968	21385G>T	homozygous	Q105H	Not a synonym	News
Exon 5	Chr4:110,618,104	24249C>T	homozygous	P235L	Not a synonym	News
	Chr4:110,618,089	24264A>C	homozygous	N240T	Not a synonym	News
	Chr4:110,617,966	24387T>G	homozygous	L281R	Not a synonym	News
	Chr4:110,617,951	24402T>C	homozygous	L286P	Not a synonym	News

3.2 Presence of mutations in pathogen databases

The frequency of the variants was assessed in the gnomAD database to determine their rarity in the general population. They were also checked against the MITOMAP database. These databases also draw on data from other sources such as ClinVar, dbSNP and ClinGen for known variants. None of the mutations are listed in these databases; they are therefore considered to be novel. Variants that are absent or very rare are considered to be potentially pathogenic.

3.3 Structural prediction by AlphaFold

In order to obtain a precise structural context for the various mutations, the AlphaFold software [19] was used. Based on homology, it analyses the protein encoded by the gene and provides information on the location of the mutation

(Homeobox domain, C-terminal, Helix, etc.), the pLDDt score (Predicted Local Distance Difference Test), which estimates the local reliability of the predicted structure, the position of the mutations (buried or exposed) and their chemical change (conservative or non-conservative). These information allow to determine whether a mutation can alter the stability or function of the protein. The results are illustrated using 3D visualizations generated by the Chimerax software, showing that the mutations located in the Homeobox domain (aa 50 and 110) are buried, whilst others in the C-terminal region are exposed, with the exception of the L281R mutation.

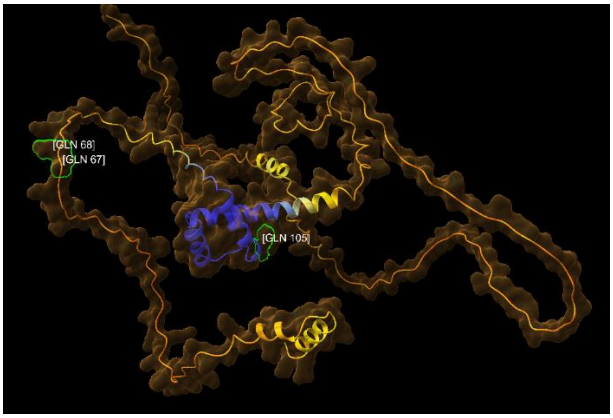

Figure 3: Mutations in Homeobox domain

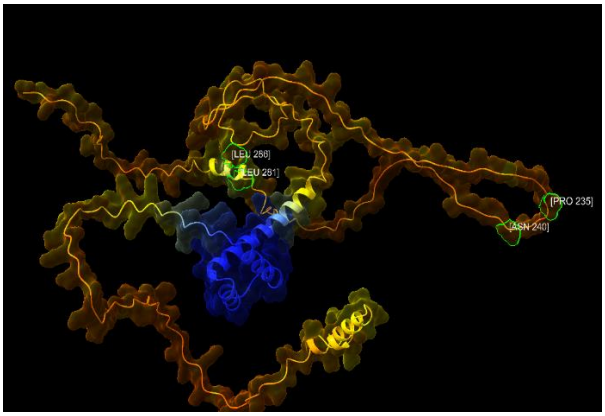

Figure 4: Mutations in C-Terminal domain

The results in **table 2** present that the most critical mutations are concentrated in the homeobox domain (DNA-binding function), that hidden mutations are more deleterious than exposed ones, and that changes in charge within the protein core are particularly disruptive.

Table 2: Structural analysis of non-synonymous mutations by AlphaFold

Exon	Mutations	Domain	pLDDT	Surface/Center	Chemical Change
Exon 4	Q67H	Homeobox	Unreliable	Buried	Non conservative
	Q68E	Homeobox	Unreliable	Buried	Non conservative
	Q105R	Homeobox	Reliable	Buried	Non conservative
	Q105H	Homeobox	Reliable	Buried	Non conservative
Exon 5	P235L	C-terminal	Reliable	Exhibited	No load (changes the local configuration)
	N240T	C-terminal	Moderate	Exhibited	Conservative
	L281R	C-terminal	Moderate	Buried	Major non-conservative
	L286P	C-terminal	Moderate	Exhibited	Major non-conservative

3.4 Analysis of the effects on protein stability by DynaMut2

Analyses using the Dynamut2 software made it possible to predict whether or not the mutation of interest causes a change in the three-dimensional structure (leading to its instability), which in turn leads to its malfunction and, consequently, the onset of the disease. These results shown in **table 3** therefore revealed that five (5) mutations (i.e. 62.5% of mutations) in the *PITX2* gene have a destabilizing effect on the protein's tertiary structure.

Table 3: Predicting the effect of non-synonymous mutations on protein stability

Exon	Type Sauvage	Position	Mutant	Chain	ΔΔG: Kcal/mol)	Prediction
Exon 4	Q	67	H	A	*-0.07 kcal/mol*	Destabilizing
	Q	68	E	A	0,01 kcal/mol	Stabilizing

	Q	105	R	A	*-0.23 kcal/mol*	Destabilizing
	Q	105	H	A	*-1.1 kcal/mol*	Destabilizing
Exon 5	P	235	L	A	*-0.49 kcal/mol*	Destabilizing
	N	240	T	A	*0.02 kcal/mol*	Stabilizing
	L	281	R	A	*0.7 kcal/mol*	Stabilizing
	L	286	P	A	*-0.07 kcal/mol*	Destabilizing

3.4.1 Analysis of the effects on protein function

The homeobox transcription factor *PITX2* has a very specific expression profile during embryogenesis. Gain-of-function and loss-of-function experiments have revealed its essential role in left-right signalling. The results showed that most of the mutations obtained are homozygous. Analysis shows that they are mainly damaging in terms of protein function and structure. Bioinformatic analyses using Mutation Taster software reveal that these mutations are not pathogenic. Using the option that distinguishes between disease-causing mutations and neutral polymorphisms, mutations in exon 4 were all damaging, particularly mutations 21384A>G and 21385G>T. According to bioinformatic analysis, these mutations are responsible for anatomical disorders, endocrine system diseases and general medical disorders [20]. Other variants such as deletions, duplications and one case of insertion were also identified. These mutations are more prevalent in exon 4, which itself has more mutations overall. The results are shown in **table 4**.

Table 4: In silico prediction of mutations in exons 4 and 5 of the *PITX2* gene

	Mutations	**P a.a**	**Statue**	**Polyphen-2**	**SIFT**	**Faithmm**	**Mutation taster**
Exon 4	21271G>T	67Q>H	homozygous	Benign	Affects protein function	Harmful	Non-pathogenic
	21272C>G	68Q>E	homozygous	Benign	Tolerable	Harmful	Non-pathogenic
	21384A>G	105Q>R	homozygous	Probably harmful	Affects protein function	Harmful (-3.93)	Non-pathogenic
	21385G>T	105Q>H	homozygous	Probably harmful	Affects protein function	Harmful (-4.00)	Non-pathogenic
Exon 5	24249C>T	P235L	homozygous	Probably harmful	Affects protein function	Harmful	Non-pathogenic
	24264A>C	N240T	homozygous	Probably harmful	Tolerable	Harmful	Non-pathogenic
	24387T>G	L281R	homozygous	Probably harmful	Affects protein function	Harmful	Non-pathogenic
	24402T>C	L286P	homozygous	Benign	Affects protein function	Harmful	Non-pathogenic

4 DISCUSSION

The study focused on in-silico prediction of mutations in two exons of the *PITX2* gene in order to identify the mechanisms linking *PITX2* gene mutations to AF in 46 patients and 20 controls.

The Q67H and Q68E mutations are located in the buried core of the *PITX2* homeobox domain helix, with a moderate pLDDT score, indicating a structured but slightly flexible region. Q67H introduces a potentially charged residue, whilst Q68E introduces a negative charge into a hydrophobic environment. These substitutions are likely to locally alter the stability of the helix, but probably do not directly affect DNA binding. The pLDDT score indicates that this prediction is moderately reliable.

The Q105R mutation is located at the heart of a helix within the homeobox domain, in a region predicted to have a high pLDDT score. The substitution of a neutral glutamine with a charged and bulky arginine is likely to introduce an unfavorable electrostatic constraint, which may alter the local stability of the helix and potentially compromise the structural integrity of the domain. The Q105H mutation, located within a buried helix of the homeobox domain, introduces a histidine that is likely to be partially charged. Although the change is non-conservative, the expected structural impact is probably less severe than that observed for Q105R, due to the more moderate size and charge of histidine.

The P235L mutation is located in the disordered C-terminal region of *PITX2*, characterized by a low pLDDT score. This region lacks a stable secondary structure, suggesting that the substitution may have a limited impact on the overall stability of the protein. The N240T mutation is located in the C-terminal region of *PITX2*. The substitution of an asparagine with a threonine constitutes a conservative change between two uncharged polar residues, suggesting a likely limited structural impact, particularly if the region is disordered. The L281R mutation introduces a highly charged and bulky residue within a buried region. The replacement of a hydrophobic leucine with a charged arginine is non-conservative and is likely to disrupt the hydrophobic interactions that stabilize the local structure, suggesting a potentially deleterious effect. L286P is less severe than the L281R mutation and, with an exposed residue, is likely to cause moderate local disruption.

In summary, mutations in the homeobox domain carry a risk of impairing DNA binding and may have a significant functional impact. C-terminal mutations, on the other hand, tend to affect transcriptional activation, and their impact is more modulatory.

Although neutral, these non-pathogenic mutations may well play a role in genetic diversity and evolution. The *PITX2* gene plays a crucial role in the development of body structures, including the heart. In the context of these syndromes, *PITX2* dysfunction can lead to cardiac malformations. For example, one study observed a strong association between *PITX2* mutations and dental and umbilical abnormalities, as well as cardiac abnormalities [21]. Most mutations in exon 4 strongly suggest that mtDNA mutations, particularly small deletions, may play a crucial role in atrial dysfunction in patients with AF. Several articles have reported that mitochondrial DNA (mtDNA) deletion mutations in human atrial tissue are associated with chronic atrial fibrillation [22] [23]. Furthermore, according to Tian and al. 2024, truncated variants in exon 3-4 of *PITX2* are the most common mechanism for causing gene dysfunction with a broader phenotypic spectrum [24]. *PITX2*-dependent metabolic changes may contribute to the structural and functional abnormalities observed in *PITX2*-deficient atria [7].

5 CONCLUSION

Despite the many aetiological factors underlying atrial fibrillation, significant progress has been made in understanding the complex genetics underlying the disease. The mutations identified in the PITX2 gene have no known pathogenic effect. However, functional analyses indicate an impairment in protein activity, particularly regarding the structural integrity of the domain. These findings suggest a potential impact on the biological regulation of the gene, although there is currently no evidence of a direct association with AF. Although mostly neutral, these non-pathogenic mutations may well play a role in genetic diversity and evolution. Current results from genetic testing in early-onset AF are modest; nevertheless, an increasing number of genes responsible for potentially fatal cardiomyopathies and ventricular channelopathies may initially manifest as AF.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

The authors declare that the research was conducted in the absence of any commercial relationships that could be construed as a potential conflict of interest.

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