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Research Article

Implication of *CYP17A1* Gene Mutations in the Association of Fibroma and Pregnancy in Senegalese Women

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Abstract

Background. Uterine fibroids are the most common gynaecological tumours and represent a significant medical and financial burden. Several genetic, hormonal and biological factors have been shown to contribute to the development and growth of these tumors. *Objective.* We aimed to evaluate the evolution of fibroma during pregnancy and understand the genetic link between fibroma and pregnancy in Senegalese women. *Methods.* We analyzed the functional impact of non-synonymous variants on the CYP17A1 protein in 20 pregnant patients with fibroids, using PCR-sequencing. First of all, mutations were detected using Mutation Surveyor, then the functional impact of non-synonymous variants was analysed using In Silico tools, the secondary and three-dimensional structure of the protein were also analysed. After, raw data were aligned using BioEdit software for doing phylogenetic analysis. *Results.* The results show that the *CYP17A1* gene is involved in the development of uterine fibroids in pregnant women, and that the c.-34T>C polymorphism plays an important role; on the one hand, some of non-synonymous mutations (p.Lys26Thr and p.Ser30Asn) have caused genetic disorders on the secondary structure, and on the other, others (p.Lys26Thr, p.Ser30Asn and p.Ser39Asn) have destabilizing effects on the protein. *Conclusion.* The c.-34T>C polymorphism is involved in fibroid cell growth during pregnancy through the effect of hormone overexpression, while non-synonymous variants lead to dysfunction in protein synthesis. The c.76A>T (p.Lys26*) mutation results in a truncated CYP17A1 protein, and cause premature loss of function. The non-synonymous variants (p.Lys26Thr, p.Ser30Asn and p.Ser39Asn) induce a change in the enzyme's biological function.

Keywords

Pregnancy, *In silico*, Uterine Fibroids, *CYP17A1*, Genetics

1. Introduction

Abnormal cell growth is a common neoplastic event (proliferation) in smooth muscle tissue, giving rise to tumors that evade normal growth control mechanisms and survive despite restrictive conditions [1]. Uterine leiomyomas represent the most common category of pelvic solid tumors in women, occurring in up to 80% of all women of childbearing age, with up to 30% of women experiencing fairly severe symptoms [2-5]. African-American women develop leiomyomas more frequently and at earlier ages than Caucasian women. Furthermore, tumors in African-American women are more ag-

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gressive, presenting with larger leiomyomas and more significant symptoms than their Caucasian counterparts [6, 7]. In line with the current trend towards delayed childbearing, the frequency of pregnant women affected by uterine fibroids is increasing dramatically [8]. Although up to 70% of pregnancies end without complication, in the remaining situations, uterine fibroids can be responsible for a multitude of obstetric problems, potentially occurring during each trimester of gestation [9, 10]. According to various authors, the risk of uterine fibroid-related complications during pregnancy may be primarily correlated with lesion size, while the exact location of tumors (submucosal, intramural or subserosal) may be responsible for different types of adverse events [11-13]. However, several genetic, hormonal and biological factors have been shown to contribute to the development, growth and maintenance of uterine fibroids. These include steroid hormones, growth factors, cytokines, chromosomal, genetic and epigenetic aberrations [14, 15]. The abrupt rises and falls in estrogen and progesterone production associated with early pregnancy and the puerperal period (the period from delivery to the return of menses) have a dramatic effect on fibrous growth [16, 17]. According to K én ém é*et al*., (2018) [18], it is now accepted that uterine fibroids are dependent on steroid hormones, including estrogen. Indeed, estrogen is considered to be the main agent inducing fibroid growth.

2. Materials and Methods

2.1. Samples

The study was carried out on 20 pregnant Senegalese patients diagnosed with uterine fibroids. Uterine fibroids are benign tumors, so surgery is performed on tumor tissue only. These samples are sent directly to the Genomics Laboratory of the Population Genetics and Management Team at UCAD, where they are preserved in tubes containing 96% alcohol for molecular analysis.

DNA Extraction, PCR Amplification and Sequencing

Total DNA from each sample is extracted using the Zymo research kit and protocol. Cytochrome P450 c17A1 (steroid 17α-monooxygenase, 17α-hydroxylase, 17-alpha-hydroxylase, 17, 20-lyase, 17, 20-desmolase), a hydroxylase enzyme encoded in humans by the *CYP17A1* gene, also known as CPT7; S17AH; P450C17, is studied. It possesses both 17α-hydroxylase and 17,20-lyase activities and is a key enzyme in the steroidogenic pathway that produces progestins, mineralocorticoids, glucocorticoids, androgens and estrogens. The *CYP17A1* gene is located on chromosome 10 (10q24.q25) and contains eight exons and seven introns [19]. The 5' untranslated region (UTR) of *CYP17A1* contains a polymorphism (T/C) 34 bp upstream of the translation start site [20] or 27 bp downstream of the transcription start site [21]. This change creates an additional SP1-type promoter site (CCACC instead of CCACT), which may lead to increased gene expression [22]. This polymorphism has been associated with increased estrogen and progesterone levels, which may be linked to the development of uterine fibroids [19]. Amplification of the promoter region (5'UTR) and exon 1 of the *CYP17A1* gene was performed on 20 DNA extracts from fibroid tissue coexisting with pregnancy with a reaction volume of 25 µl: 2 µl or 4 µl DNA extract, 18.4 µl pure PCR water or 16.4 µl for the 4 µl, 2.5 µl 10X buffer, 0.5 µl dNTP, 0.5 µl F primer (5'-TCCTGAGCCCAGATACCAT-3'), 0.5 µl R primer (5'-CCGCCCAGAGAAGTCCT-3'), 0.5 µl MgCl2, 0.1 µl Taq, 23 µl volume mix for 2 µl DNA or 21 µl volume mix for 4 µl DNA. Amplification conditions for the *CYP17A1* gene are as follows: initial denaturation (94 ℃; 12min), 35 cycles of denaturation (94 ℃; 30s), hybridization (60 ℃; 30min), elongation (72 ℃; 1min) and final elongation (72 ℃; 7min). Sequencing involves determining the nucleotide sequence of a DNA fragment. Samples are suspended in distilled water and subjected to electrophoresis in ABI 3730xl sequencer (Appled Biosystems). The gel is read by automatic laser scanning to detect the various fluorochromes coupled to the 4 ddNTPs. The result of this scan is a chromatogram representing the succession of bases making up the DNA fragment.

2.2. Genetic Studies

2.2.1. Search for Mutations

The chromatograms were submitted to Mutation Surveyor v5.2 (www.softgenetics.com). This software uses patented technology to identify DNA variants from chromatograms with particular precision. The software detects the presence of heterozygous mutations (het), insertions (ins) and deletions (del) in comparison with the reference sequence of the human genome. The resulting sequences were then carefully checked, corrected and aligned using BioEdit v7.2.5 [23] with, Clustal W algorithm [24].

2.2.2. Analysis of the Functionnal Impact of Non-synonymous Variants on Protein Stability

Influence of mutations on protein using the In Silico method

To determine the influence of non-synonymous variants on protein stability, we subjected them to I-mutant2.0, iStable 2.0 and DUET:

I-mtant2.0: is a support vector machine (SVM)-based tool for the automatic prediction of protein stability changes during point mutations. Predictions on the I-Mutant2.0 web page are made either from the protein structure or, more importantly, from the protein sequence. For the classification task and for the assignment of ΔΔG values, [25] essentially adopt an input code by identifying two labels: one represents increased protein stability (ΔΔG > 0, label is positive (+)), the other is associated with destabilizing mutation (ΔΔG < 0, label is negative (-)).

iStable 2.0: is used to evaluate the performance of the prediction model and compare it with other methods, [26] pre-

pared an independent test set from data obtained from ProTherm (thermodynamic database for proteins and mutants, last updated in 2013) [27], containing 2869 point mutations in 81 proteins [26] defined stabilizing data with a ΔΔG value greater than or equal to zero (≥0) as positive (+) and destabilizing data with a ΔΔG value less than zero (<0) as negative (-).

DUET: is an integrated computational approach to predict the effects of missense mutations on protein stability. DUET combines mCSM and SDM into a consensus prediction, consolidating the results of the separate methods into an optimized predictor using support vector machines (SVMs) trained with sequential minimal optimization [28]. Predicted results are expressed as changes in Gibbs free energy (ΔΔG) in kcal/mol, and negative values denote destabilizing mutations.

2.2.3. Relative Prediction of Solvent Accessibility and Secondary Structure

To predict the relative solvent accessibility and secondary structure of the CYP17A1 protein, we submitted its protein sequence to the NetSurfP-2.0 web server.

NetSurfP-2.0: is a new, extended version of NetSurfP, which uses a deep neural network approach to accurately predict absolute and relative solvent accessibility, secondary structure using both 3- and 8-class definitions [29], structural disorder [30], φ and ψ dihedral angles and interface residues of a given protein from its primary sequence only. As input for the analysis the protein sequence with wild-type amino acids replaced by mutated ones is submitted and as output we first have a table combining the mutant's exposed or conserved assignment information, the relative accessibility area in percent, the absolute accessibility area in Angstrom, the position of the mutant in the secondary structure (helix α, coil or Strand β), the percentage of disorder and the dihedral angles Phi and Psi (φ and ψ) and in figure the secondary structure with the mutated amino acids and the disorder based on the thickness of the grey line.

2.2.4. 3D Structure of the CYP17A1 Protein

To determine the impact of mutated amino acids on protein dynamics compared with the reference and its interactions, and the structural damage involved, we submitted each non-synonymous variant to the Dynamut2 and Missense 3D servers.

Dynamut2: DynaMut2, is a web server that combines analysis methods using a range of features, including protein dynamics (NMA), wild-type residue environment, substitution propensities and contact potential scores, interatomic interactions [31] as well as the well-validated graph-based signature approach [32-34]. The protein destabilizing effect is marked by a ΔΔG (< 0.0 kcal/mol) and the stabilizing effect by a ΔΔG (> 0.0 kcal/mol).

3D Missense: mutant and wild-type structures were analyzed to identify whether the structural consequence of the substitution should be damaging in terms of the stability of the folded protein. Based on well-established principles of protein conformation and previous studies on the structural consequences of disease-associated substitutions, [35-37] considered 17 structural features.

3. Results

We analyzed the variability of the *CYP17A1* gene in 20 cases of fibroma and pregnancy by PCR-sequencing.

3.1. Nature and Position of Mutations

Chromatogram analysis using Mutation Surveyor software shows the presence of mutations in fibroid tissue in pregnant women (Figure 1). Out of the 20 patients, only 4 showed no variability in exon 1 of the *CYP17A1* gene. A total of seventy-two mutations were found, including mutations in the intronic, promoter region with the desired polymorphism (c.34 T>C) and exonic with two synonymous mutations (p.His46His and p.Ser65Ser), two stop codon-inducing mutations (p.Lys26* and p.65S*) and five (p.Lys26Thr, p.Ser30Asn, p.Ser39Asn, p.Arg45Ile, p.Lys55Glu) non-synonymous mutations.

3.2. Influence of Non-Synonymous Mutations on Protein Stability

Analysis of the effect of non-synonymous mutations on protein stability shows that the p.Lys26Thr, p.Ser30Asn and p.Ser39Asn variants are predicted by three of the software programs to destabilize protein, with negative Gibbs energy difference values between wild-type and mutant (Table 1).

Table 1. *In Silico prediction of non-synonymous mutations on protein stability.*

			DUET		
Mutations	**I-Mutant 2.0 (ΔΔG)**	**i-Stable 2.0 (ΔΔG)**	**ΔΔG mCSM (kcal/mol)**	**ΔΔG SDM (kcal/mol)**	**ΔΔG DUET (kcal/mol)**
c.77A>C p.Lys26Thr	Destabilising (-1.87)	Destabilising (-0.94)	Destabilising (-0.477)	Destabilising (-0.26)	Destabilising (-0.28)

			DUET		
Mutations	**I-Mutant 2.0 (ΔΔG)**	**i-Stable 2.0 (ΔΔG)**	**ΔΔG mCSM (kcal/mol)**	**ΔΔG SDM (kcal/mol)**	**ΔΔG DUET (kcal/mol)**
c.89G>A p.Ser30Asn	Destabilising (-0.75)	Stabilising (0.01)	Destabilising (-0.497)	Stabilising (0.56)	Destabilising (-0.101)
c.116G>A p.Ser39Asn	Destabilising (-1.79)	Stabilising (0.60)	Destabilising (-1.187)	Stabilising (0.14)	Destabilising (-0.91)
c.134G>T p.Arg45Ile	Destabilising (-0.45)	Destabilising (-1)	Stabilising (0.64)	Stabilising (0.34)	Stabilising (0.739)
c.163G>T p.Lys55Glu	Destabilising (-1.24)	Stabilising (0.68)	Stabilising (0.192)	Stabilising (0.99)	Stabilising (0.688)

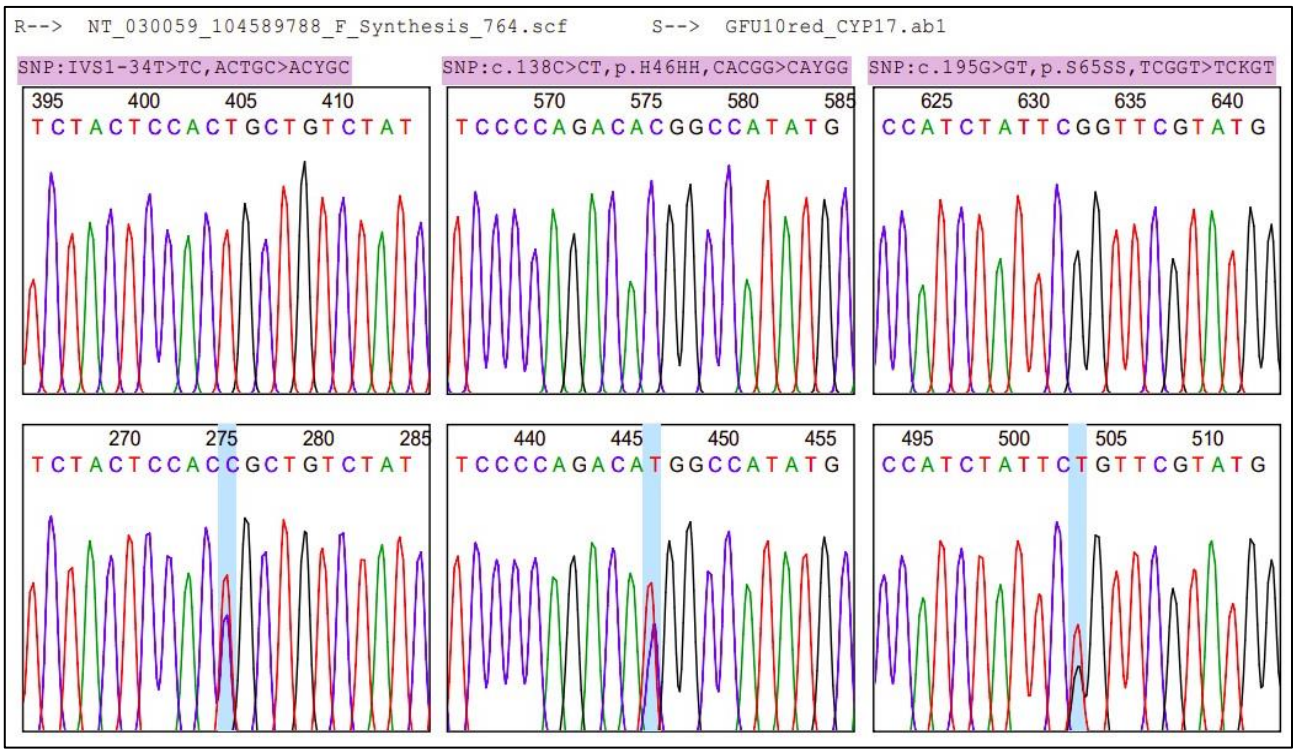

Figure 1. *Some SNPs in the CYP17A1 gene.*

3.3. Relative Prediction of Solvent Accessibility and Secondary Structure

Relative analysis of the solvent accessibility surface first shows us the presence of residue disorder based on the thickness of the grey line (Figure 2) in two (p.Lys26Thr and p.Ser30Asn) of the five variants with their respective percentages (Table 2). Only one of the five non-synonymous variants is predicted as conserved (p.Ser39Asn) compared with the others, which are exposed with large relative percentages of the accessibility surface (44 to 68%). For the secondary structure (Figure 2), only one variant (p.Lys55Glu) is located at the α-helix and the other four are located at the Coil.

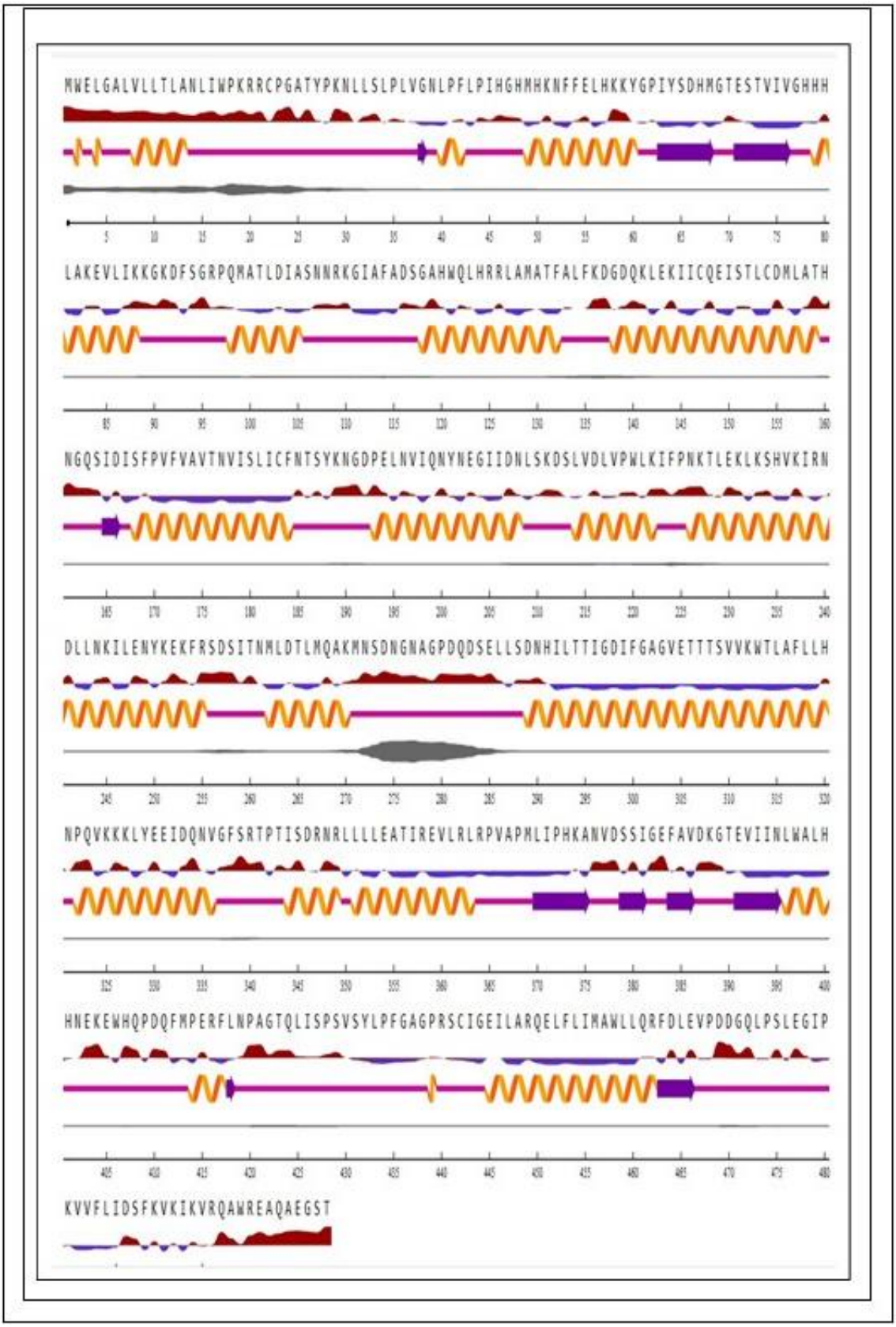

Figure 2. *Structure of the CYP17A1 protein.*

NB: Surface accessibility: The red upward elevation indicates the exposed residue, while the sky-blue downward elevation indicates the residue buried in the protein structure. Secondary structure: The straight pink line is the coil, the orange spiral is the helix and the coloured arrow (indigo) is the strand. Disorder: Below the secondary structure prediction line is the thick greyish line indicating the probability of disorder linked to this residue. The wider the line, the greater the risk of disorder.

Table 2. Relative accessibility surface and disorder prediction.

Mutations	NetSurfP-2.0					
	Assignment	RSA in %	ASA in Å	Secondary structure/P disorder (%)	Phi	Psi
p.Lys26Thr	Exposed	68	95	Coil (7)	-100	132
p.Ser30Asn	Exposed	59	87	Coil/Turn (4)	-75	43
p.Ser39Asn	Buried	13	19	Coil (0)	-102	92
p.Arg45Ile	Exposed	31	57	Coil/Turn (1)	-81	-4
p.Lys55Glu	Exposed	44	77	Helix/α helix (0)	-65	-42

3.4. 3D Structure of the Protein

Among the non-synonymous variants, only one (p.Arg45Ile) is predicted to stabilize the protein by the Dynamut2 server, with a value for the Gibbs free energy difference between the wild-type and the positive mutant. However, none of the variants is predicted to cause structural damage, so they are not classified according to the seventeen Missense 3D features. Nevertheless, analysis of the 3D structure shows differences in the types of amino acids substituted or their interactions. We note either loss of bonds, or the appearance of new bonds, or conformational changes, or substitutions by charged or hydrophobic or hydrophilic residues.

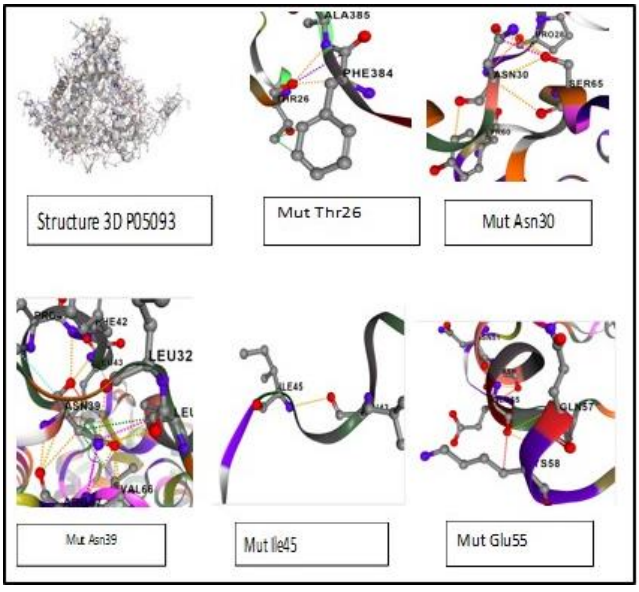

Figure 3. 3D structure of the protein.

4. Discussion

The aim of the present study is to identify the *CYP17A1* gene mutations involved in cases of uterine fibroids in pregnant Senegalese women, and the functional impact of these mutations on protein stability. A total of 20 cases of fibroids associated with pregnancy were studied. The cause of the fibroids is unknown. It seems that elevated levels of estrogen and possibly progesterone (female hormones) stimulate their growth. According to Mutch and Biest, [38], fibroids can develop during pregnancy (when levels of these hormones rise, and they tend to disappear at menopause, when these levels drop drastically). In line with the biological hypothesis that polymorphisms in genes involved in sex hormone pathways could influence estrogen levels [39], we selected one of the genes affecting estradiol metabolism, cytochrome P450, family 17, subfamily A, polypeptide 1 (*CYP17A1*). This is an enzyme that catalyzes the 17α-hydroxylation (17α-OH) of pregnenolone (Preg) to 17α-OH pregnenolone (17α-OHPreg), and subsequently, thanks to its C17,20 lyase activity, it can further convert 17α-OHPreg to dehydroepiandrosterone (DHEA). In the present study, the number of mutations found, as well as the presence of mutations in fibroid tissue and pregnancy, show that the *CYP17A1* gene is highly polymorphic, and is thought to be involved in the incidence of uterine fibroids in pregnant women. Although the triggers leading to the development of uterine leiomyomas are unknown, there is considerable evidence that sex steroid hormones are important factors in tumour growth [40, 41]. A number of single-nucleotide polymorphisms (SNPs) have shown phenotypic effects through several mechanisms, including enhanced or reduced transcription, altered post-transcriptional or post-translational activity or changes in protein structure [42, 43]. The hypothesis is that the activity of the products of these genes may affect long-term estrogen levels and/or may activate estrogens into potentially reactive metabolites and indirectly influence the risk of uterine leiomyomas, which are likely to have a hormonal basis. More than half of all mutations are in the intron, and although they generally do not directly affect the amino acid sequence of a protein, they can have indirect effects on gene regulation or the splicing process. There are numerous mutations in the *CYP17A1* gene, the majority of which are extremely rare [44, 45]. The work of Yamagushi *et al*., (1998) and Nie *et al*., (2007) [46, 47] stipulates that there are over 70 inactivating mutations in *CYP17A1*

leading to 17α-hydroxylase (17OHD) deficiency, and six of these are intronic mutations. According to Daw *et al*., (2011) [48], intronic mutations in the *CYP17A1* gene would cause cryptic splicing in a 17OHD. The presence of the c.-34T>C variant (SNP Ref: rs743572) in fibroid tissue and pregnancy corresponding to the sought-after polymorphism shows that it could influence the onset of uterine fibroids and its evolution during pregnancy by causing overexpression of the gene in the environment. Three common polymorphisms of the *CYP17A1* gene have been described [49, 50], but only one, in the 5′-untranslated region (5′-UTR), has been extensively studied. This polymorphism involves a single base pair change (T → C) in the promoter region [49]. The variant creates a recognition site for the MspAI restriction enzyme. The TC variant is thought to create an additional Sp-1 promoter site (CCACC box) [20]. It has been postulated that genetic variation in *CYP17A1* is responsible for differences in circulating hormone levels. This is a possible mechanism by which the gene could influence disease risk. Some relevant studies are consistent with the hypothesis that the TC variant, by somehow altering gene function, at least alters hormone concentrations, estrogen levels in premenopausal women. One report showed that in African women, homozygous carriers of *CYP17A1* (TC) were at high risk of developing uterine leiomyoma, whereas in Caucasian women, the *CYP17A1* genotype did not contribute to the etiology of uterine leiomyoma. The authors [51] hypothesized that higher estrogen levels in African women homozygous for the *CYP17A1* TC allele expose the myometrium to a stronger stimulatory effect, which could, in the long term, lead to spontaneous mutations and uncontrolled growth an important feature of uterine fibroids. The onset, maintenance and termination of pregnancy depend largely on the interaction of hormonal and neural factors. The fetus and placenta produce and secrete steroids and peptides into the maternal circulation, stimulating the production of maternal hormones [52, 53]. Estrogens are formed mainly in the placenta with the aid of fetal and maternal androgens, and diffuse into the maternal and fetal compartments. The rate of production increases continuously during pregnancy, reaching 100-120 mg/24 h [54]. In the study, synonymous mutations (p.His46His and p.Ser65Ser) appear to have no effect on gene expression or protein function, as the protein produced is identical. But this is now being called into question, as it has been shown that certain genetic diseases are linked to the presence of a synonymous mutation in the genome. It has also been shown that these mutations act not on the quality of the proteins produced (which are identical), but on their quantity [55]. The c.195G>T (p.Ser65Ser) variant is found only on the *CYP17A1* gene, where it is benign in steroid 17α-monooxygenase deficiency activity. The c.76A>T (p.Lys26*) mutation results in a truncated CYP17A1 protein, which could cause premature loss of function, with the 17α-hydroxylase and 17,20-lyase functions of the said gene being deficient. This deficiency is a condition that affects the functioning of certain hormone-producing glands known as the adrenal glands. The c.194C>A (p.65Ser*) mutation, pathogenic to the *MSH6* gene and located in coding exon 1, results from a C to A substitution at nucleotide position 194. This changes the amino acid of a serine this alteration should lead to loss of function through premature protein truncation or nonsense mRNA decay. As such, this alteration is interpreted as a pathogenic mutation [56]. The three variants (p.Lys26Thr, p.Ser30Asn and p.Ser39Asn) predicted to destabilize the protein could have an impact on the degradation of the protein to be synthesized. Protein stability determines conformational structure and function. Misfolding, degradation or abnormal changes in the amino acids making up the protein molecule can affect protein stability. Stabilizing variants, on the other hand, have no effect on protein function. If the wild-type amino acids are essential for protein function, then these mutations may alter the protein's ability to perform its normal function. With regard to relative surface accessibility, conserved variants appear not to induce effects in the management of the biological system, including stability, folding or both, unlike exposed variants. The latter, with their high surface accessibility, are located on the surface of the protein, which could lead to loss of interaction and structural alteration. The exposed variant (p.Lys55Glu) of the α-helix could lead to instability in the protein's structure, stability, function and ability to interact with other molecules. Polar charge is a factor in stability. An α-helix is generally positively charged at one end and negatively charged at the other, which can destabilize the protein's structure. Mutations located in the coil (p.Lys26Thr, p.Ser30Asn and p.Arg45Ile) could affect protein function and lead to tumor cell growth during pregnancy. The differences observed in the 3D structure, compared with the reference, have resulted in structural damage to the protein, which may affect its biological function, its membrane transport capacity due to the hydrophobic mutant (Isoleucine), its molecular cohesion function due to the ionic mutants creating a negative opposite charge (Asparagine and Glutamic acid) and the alteration of its electron sites due to the mutants with polar characteristics (Asparagine, Threonine). Molecular cohesion refers to the linkage between different parts of a gene. Steroidogenic enzymes fall into two groups: cytochrome P450 enzymes and hydroxysteroid dehydrogenases. A cytochrome P450 may be type 1 (in the mitochondria) or type 2 (in the endoplasmic reticulum), and a hydroxysteroid dehydrogenase may belong to the aldo keto reductase or short-chain dehydrogenase/reductase families. The activities of these enzymes are modulated by post-translational modifications and cofactors, in particular electron-donating redox partners. Elucidation of the precise roles of these different enzymes and cofactors has been greatly facilitated by the identification of the genetic basis of rare diseases of steroidogenesis [57].

5. Conclusion

The results obtained show the involvement of the *CYP17A1* gene in the incidence of uterine fibroids in pregnant women in Senegal. The presence of the mutation in the promoter region of the gene (c.-34T>C) in the patients seems to confirm the

hypothesis of Amant *et al*, (2004) that higher estrogen levels in African women homozygous for the *CYP17A1* TC allele expose the myometrium to a stronger stimulatory effect, which could, in the long term, lead to spontaneous mutations and uncontrolled growth; an important feature of uterine fibroids. The c.76A>T (p.Lys26*) mutation results in a truncated CYP17A1 protein, and cause premature loss of function. The non-synonymous variants (p.Lys26Thr, p.Ser30Asn and p.Ser39Asn) induce a change in the enzyme's biological function by inhibiting progesterone metabolism by the *CYP17A1* enzyme and thus overproducing the latter, on which pregnancy maintenance and fibroid growth depend.

Conflicts of Interest

The authors declare no conflicts of interest.

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