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RESEARCH ARTICLE

Prognosis and Functional Analyzes of Missense Mutations in Exon 4 of the TP53 Gene in Colorectal Cancer in the Senegalese Population

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Abstract

Colorectal cancer is a complex and multifactorial pathology, the progression of which involves genetic mutations affecting the p53 protein, a key regulator of tumor suppression. This study focused on identifying, characterizing, and evaluating the pathogenicity of missense mutations (nsSNPs) in exon 4 of the *TP53* gene in Senegalese patients suffering from colorectal cancer. The analysis of 24 patients and 24 controls revealed 13 new variants, including 11 located in the transactivation domain TAD2 one in the proline-rich domain, and one in the DNA-binding domain. Among these mutations, some alter the stability and function of the p53 protein, while others, such as the recurrent polymorphism at codon 72 (p.P72R), are considered benign. Post-translational modifications, such as the loss of phosphorylation at S46 and the gain of phosphorylation at T55, were also observed, influencing p53 activity. Although no major structural changes were predicted for the p53 protein, conformational changes at binding sites were detected for certain mutants (p.D48V, p.D48N, p.D59A, p.I50F, p.Q52E, and p.P72R). These results highlight the potential pathogenic role of these mutations in colorectal cancer in Senegal and shed light on the importance of genetic variability in colorectal tumorigenesis.

Keywords: Colorectal, Cancer, Mutation, Exon 4, *TP53*, Senegal.

1. Introduction

Nucleic acids are not composed of a canonical and fixed structure but are constantly subject to "genotoxic stresses", occurring spontaneously or induced by the environment or even by therapeutic pressure, which can affect the structure of the double helix [1]. Under the influence of these environmental or endogenous factors, the genome can undergo mutations, some of which provide a selective advantage to different cancer pathologies. According to Reynaud *and al* [2], the succession of cycles of mutations and selections followed by phases of clonal expansion can lead to the emergence of malignant cells which have freed themselves from the mechanisms limiting their proliferation and mobility. Indeed, the emergence of cell clusters results in an imbalance between cell division and death. The balance between these two processes regulates the number of cells in the tissue, and the disruption of this balance causes the development of cancer cells which escape the proper functioning of the body [3]. Thus, cancer pathologies can be defined as genetic diseases which are characterized by an imbalance between cell division and death.

The process of carcinogenesis is very complex during which tumor cells gradually acquire new biological properties following multi-step events accompanied by multiple genetic alterations [4]. Such molecular lesions can be specific and affect genes involved in

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the control of cell proliferation and differentiation, notably oncogenes or tumor suppressor genes which have been associated with neoplastic transformation and even tumor progression [5].

The tumor suppressor protein p53 plays an important role in maintaining genome integrity, particularly by activating and repressing the expression of certain genes. Numerous studies have demonstrated that alterations of the p53 protein or its regulators play a fundamental role in the resistance of cancer cells to apoptosis. Indeed, p53 is a transcription factor playing a central role in the initiation of the apoptotic process, particularly in response to genotoxic stress. Exon 4 of the *TP53* gene encodes the transactivation domain (TAD) of the p53 protein, essential for its role in activating target genes involved in cell cycle arrest and apoptosis. Deleterious mutations in the p53 protein are found in more than 50% of human tumors [6, 7] and also in approximately 40 to 50% of colorectal tumors [8]. The impact of exon 4 mutations could help in the development of personalized therapies such as molecules restoring p53 function. Despite important studies carried out on this gene, questions arise about their importance in the study of colorectal tumors in the Senegalese population.

This is in the context of a better understanding of the genetic mechanisms that govern these pathologies that our study took place, which aimed to determine the involvement of genetic alterations of *TP53* in the evolution of colorectal cancers in senegalese patients.

2. Methodology

2.1 Study Population

The study was carried out on 24 patients with CRC and 24 control cases were included for comparison. These patients were recruited in the general surgery and oncology departments of the Aristide le Dantec hospital, the Principale hospital of Dakar and Grand-Yoff. For each operated patient, a sample from a fresh surgical specimen was taken from the full tumor, collected in a dry tube and stored at 20°C, accompanied by its clinical information sheet. After sampling, tumour tissues were directly sent to the Genomics Laboratory of the Animal Biology department of the Faculty of Science and Technology of the University of Dakar where the tissues were preserved in 96% alcohol for the various molecular analyses.

2.2 Extraction, Amplification and Sequencing of Exon 4 of the *TP53* Gene

DNA extraction was carried out on 24 tumor tissue samples and 24 control blood samples using the standard Zymo Research kit protocol. Exon 4 gene was amplified in a reaction volume of 25 µl using the primers: F 5'- TCCCCCTTGCCGTCCCAA-3' and R 5'- CGTGCAAGTCACAGACTT-3'. These primers cover a region of 279 bp. PCR was carried out in a thermal cycler at conditions of initial denaturation at 94°C for 5 minutes, followed by a repeat of 35 cycles with denaturation at 94°C for 30 seconds, primer annealing at 58°C for 45 seconds, elongation of complementary DNA strands at 72 °C for 40 seconds. The final elongation lasted at 72 °C for 10 minutes. The presence of amplicons was verified by performing electrophoretic migration on a 2% agarose gel. Sanger sequencing was performed to determine the nucleotide succession of the gene of interest.

2.3 Identification of Mutations

The presence of point mutations as well as their position at the chromosomal level was identified by submitting raw sequencing data to Mutation Surveyor software version 5.0.1 [9]. Mutation Surveyor software uses anti-correlation technology that compares sample sequences to a reference sequence from chromatograms. The analysis uses a patented algorithm that detects mutations and calculates a mutation confidence score based on peak intensity, drop factor (vertical), overlap factor (horizontal base spacing), and ratio signal to noise of each peak. Variants with a Phred score $\geq$ 20 were listed and considered significant, a value for which the precision is 99%.

2.4 Functional Domain Detection of SNPs

The functional domains of the p53 protein were predicted with the database named MobiDB[1]. This database provides comprehensive information, analysis and tools to help understand the structure, function and dynamics of proteins. The protein ID of *TP53* was submitted to the MobiDB server to determine the domains to which the found SNPs belong.

In order to determine the characteristics of the mutations, each nsSNP was submitted to the *TP53* database[2] which is a server helping to interpret genetic mutations identified on the human genome. It

[1] (https://mobidb.bio.unipd.it)

[2](https://tp53.isb-cgc.org/search_gene_by_var)

compiles various types of data and information from the literature and general databases on variations in the human *TP53* gene linked to cancer. For each mutation found, characteristics such as nomenclature according to HGVS (Human Genome Variation Society), location, nature and functional activity were collected.

2.5 Analys of the Phylogenetic Conservation of SNPs

The phylogenetic or evolutionary conservation of an amino acid extremely influences the structure and function of a protein. This type of protein attribute is essential for interpreting the functional impact of missense mutations [10]. To do this, ConSurf [3] [11] and MobiDB two bioinformatics tools allowing to accurately estimate the rate of protein family evolution were used.

2.6 Analysis of Missense Mutation Pathogenicity

The functional effect of SNPs on the protein was studied by submitting missense variants to Polyphen-2, Mutation Taster and PROVEAN software.

Polyphen-2[4] is a multiple sequence alignment server that aligns sequences using structural information. The input to the server is either a protein sequence or a SWALL database ID or accession number with the position of the sequence with two amino acid variants [12]. It performs functional annotation of single nucleotide polymorphisms (SNPs) and then estimates the probability that the missense mutation is damaging based on a combination of all these properties [13]. It estimates the position-specific independent count (PSIC) score for each variant and then determines the difference between them, the higher the PSIC, the higher the functional impact of the amino acid on protein function can be. For each amino acid substitution, a qualitative prediction ("probably damaging ", "possibly damaging ", "benign" or "unknown") is provided with a score ranging from 0 to 1 [13].

MutationTaster[5] uses a Bayesian classifier to predict the pathogenic potential of an alteration. It makes it possible to characterize the alterations and calculates the probabilities that the alteration is a pathogenic mutation or a simple polymorphism. The MutationTaster (MT) score value is the probability of the prediction, i.e. a value close to 1 indicates high «security» of the prediction, a value less than 0.5 indicates that the classifier came to a different conclusion.

PROVEAN[6] (Protein Variation Effect Analyzer) is a bioinformatics tool for predicting functional effects of protein sequence variations. It uses an approach based on the similarity of the query sequence with a set of its related protein sequences taking into account the indicated variants. Indeed, a clustering of BLAST hits is performed by CD-HIT with an overall sequence identity parameter of 75%. The top 30 groups of closely related sequences form the set of supporting sequences, which will be used to generate the prediction. A delta alignment score is calculated for each support sequence. Scores are then averaged within and across clusters to generate the final PROVEAN score. If the PROVEAN score is equal to or lower than the predefined threshold (-2.5), the protein variant is expected to have a "deleterious" effect. If the PROVEAN score is above the threshold, the variant should have a "neutral" effect.

2.7 Stability Analysis of Mutant Proteins

A missense variant or nsSNP can change the stability of a protein, which has a direct effect on the function of the protein. It is therefore essential to evaluate changes in the stability of mutant proteins. To do this, the ProtParam[7] tool available in the World Wide Web server ExPASy[8] (Expert Protein Analysis System), DynaMut[9] and I-mutant[10] were used.

The ProtParam program calculates various physicochemical properties that can be deduced from a protein sequence. The ProtParam entry can be specified either as an accession number or a Swiss-Prot/TrEMBL ID, or as a raw sequence. Among the calculated parameters, the instability index provides an estimate of protein stability. A protein with an instability index below 40 is considered stable, a value above 40 predicts that the protein may be unstable.

DynaMut performs computer simulation using static structures to assess the impact of mutations on protein structure and function. The analysis was carried out

[3](https://consurfdb.tau.ac.il/)
[4](http://genetics.bwh.harvard.edu/pph2/)
[5](https://www.genecascade.org/MutationTaster2021/#transcript)
[6](http://provean.jcvi.org/seq_submit.php)
[7](https://web.expasy.org/protparam/)
[8](http://www.expasy.org)
[9](https://biosig.lab.uq.edu.au/dynamut2/)
[10](https://folding.biofold.org/cgi-bin/i-mutant2.0.cgi)

using the identifier of the chain (A) and that of the 3D structure (8F2I) of the p53 protein. This approach predicts the effects of mutations on protein stability and flexibility. DynaMut uses a very strict significance threshold ($p < 0.001$) to ensure the reliability of its predictions regarding the effects of mutations on proteins. This helps minimize the risk of errors and increases confidence in the results obtained.

I-Mutant2.0 is optimized to predict the change in protein stability upon mutation. The analysis was carried out from the protein structure and the nsSNPs were submitted one by one to bring out the values of the reliability index (RI) and the Gibbs free energy of deployment (DDG). It consists of predicting the sign (increase +, decrease -) of the change in free energy (DDG) or its value (+/- DDG) of mutant variant compared to wide type. The RI value is calculated only when the sign of the stability change is predicted and the DDG value is calculated from the unfolding Gibbs free energy value of the mutated protein minus the unfolding Gibbs free energy value of the wild type (Kcal/mol). Values of DDG<0 are considered decreazing the stability of the mutant protein and.

2.8 Prediction Analysis of the Effect of nsSNPs on the Structure and Function of p53

Residues on the surface of proteins that are not directly involved in function are generally more tolerant of amino acid substitutions than residues affecting the buried core of a protein. It is established that missense variants at the core of the tertiary structure are more often associated with disease than those located on the surface [14]. To predict the detrimental effect of variants in exon 4 of the *TP53* gene and also understand the molecular mechanisms by which they affect protein structure/function, the Missense3D server and MutPred2 were used.

MutPred2[11] is a machine learning-based method and software package that integrates genetic and molecular data to reason probabilistically about the pathogenicity of amino acid substitutions. It models a wide range of structural and functional properties, including helix loss, strand gain, gain/loss of nitrogen-linked glycosylation and sulfation, transmembrane protein modification, accessibility gain relating to the solvent and the modification of the ordered interface. It provides a general prediction of pathogenicity and generates a ranked list of specific molecular alterations potentially affecting the phenotype.

MutPred2 prediction is based on a scoring system where the probability threshold value is 0.5. When the MutPred2 probability score is greater than 0.5, the mutation is determined to be pathogenic, but when the MutPred2 probability score is less than 0.5, the mutation is determined to be neutral [15]. MutPred2 prediction is based on the scoring system where the probability threshold value is 0.5. When the MutPred2 probability score is greater than 0.5, the mutation will be determined as pathogenic, but when the MutPred2 probability score is less than 0.5, the mutation will be determined as neutral [15].

2.9 Prediction Analysis of Post-Translational Modification Sites

Post-translational modifications (PTMs) involve a change of amino acids with the addition of various side chains in proteins. These changes or modifications occur due to procedures that govern various cellular activities. PTMs therefore have a major influence on the structure and function of proteins. Interferences in these PTM sites can direct the disruption of important biological mechanisms, which can cause several diseases [16]. MusiteDeep server[12] [17] and NetPhos 3.1 server[13] were used to predict PTM sites.

MusiteDeep is an online resource providing a deep learning framework for the prediction and visualization of protein post-translational modification (PTM) sites. The FASTA sequence of the exon 4 protein of the *TP53* gene was submitted for analyse. Predicted PTMs are labeled using their abbreviations at their positions on the protein sequence. Multiple labels are displayed above a position if that position is planned to have multiple PTMs.

The NetPhos 3.1 server predicts serine, threonine, or tyrosine phosphorylation sites in eukaryotic proteins using sets of interaction networks. Generic and kinase-specific predictions are made. Predictions are made for the following 17 kinases : ATM, CKI, CKII, CaM-II, DNAPK, EGFR, GSK3, INSR, PKA, PKB, PKC, PKG, RSK, SRC, cdc2, cdk5 and p38MAPK.

A threshold of 50% was used for these analysis.

2.10 Modeling Analysis of p53 Mutant Proteins

Structural changes of point mutations were determined using I-TASSER web server. It was designed to

[11](http://mutpred.mutdb.org/)
[12](https://www.musite.net)
[13](https://services.healthtech.dtu.dk/services/NetPhos-3.1/)

explain the molecular origin of a phenotype linked to pathologies caused by mutations in protein sequences. The I-TASSER[14] server generates three-dimensional (3D) atomic models from multiple thread alignments and iterative structural assembly simulations [18]. The output of a typical server run contains full secondary and tertiary structure predictions, as well as functional annotations on ligand binding sites, enzyme commission numbers, and gene ontology terms. An estimate of the prediction accuracy is provided based on the modeling confidence score. Five models are generated and the best model is assessed based on the highest C-score estimate and the residual-specific accuracy of each. The 3D three-dimensional models for the mutant-like protein models were constructed by using the reference protein sequence of the *TP53* and additting manually the mutations found.

3. Results

3.1 Nature and Position of Mutations Detected

Comparison of each chromatogram to that of the reference sequence revealed the presence of five mutants causing changes in the amino acid sequence. Among these variants, 50% (12/24) were detected in controls and 95.3% (23/24) in cancerous tissues.

The presence of a recurrent non-synonymous transversion type mutation (c.215C>G) was detected where proline is replaced by arginine at codon 72 (p.P72R) with a frequency of 29.16% (7/24) in healthy individuals and 50% (12/24) in cancer subjects (dbSNP rs1042522). Figure 1 illustrates some chromatograms showing point mutations.

Ten new variants were found only in cancer tissue sequences. These variants are presented in Table 1.

Table 1. *Mutations in exon 4 of the TP53 gene identified on Mutation Surveyor*

Pos. chromosomal	DNA Variant	Protein variant	Affected tissues	dbSNPs
17:7676154	c.215C>G	p.P72R	7TS/12TC	rs1042522
17:7676004	c.365T>G	p.V122G	2TC	
17:7676229	c.140C>G	p.P47R	4TS	-
17:7676229	c.140C>T	p.P47L	TC	-
17:7676226	c.143A>T	p.D48V	TS	-
17:7676227	c.142G>A	p.D48N	TC	-
17:7676226	c.143A>G	p.D48G	TC	-
17:7676223	c.146A>C	p.D49A	TC	-
17:7676220	c.149T>G	p.I50S	TC	-
17:7676221	c.148A>T	p.I50F	TC	-
17:7676215	c.154C>G	p.Q52E	TC	-
17:7676214	c.155A>T	p.Q52L	TC	-
17:7676211	c.158G>C	p.W53S	TC	-

TC= Cancerous tissues ; TS= Healthy tissues

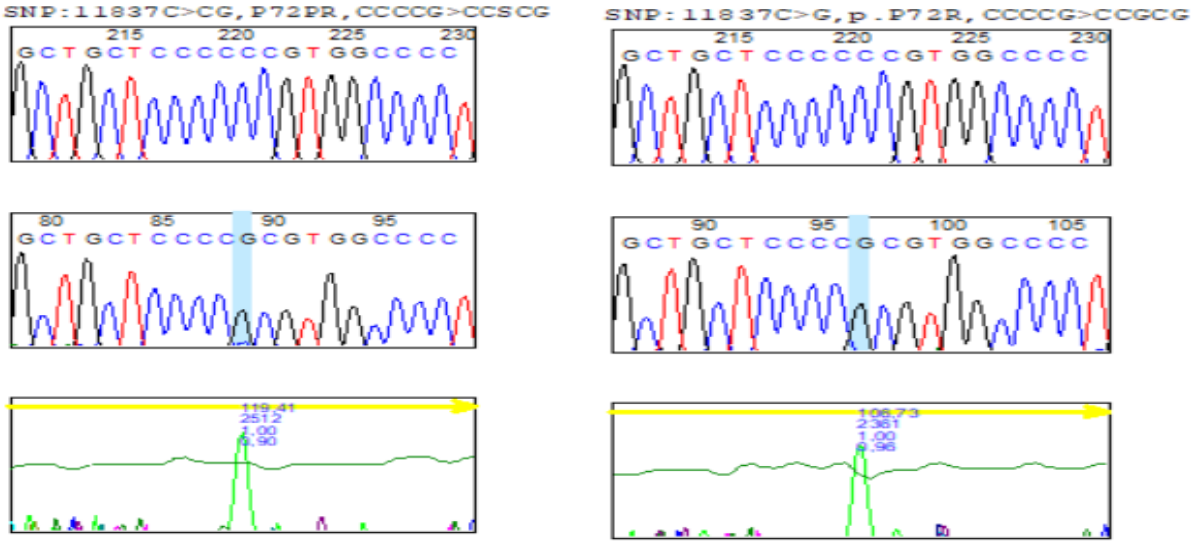


Figure 1. *Chromatogram profiles of the rs1042522 polymorphism*

3.2 Functional Domain Detection of SNPs

Exon 4 of the *TP53* gene consists of 93 residues. Functional analysis of this protein sequence predicted the presence of transactivation domain 2 from residue 35 to residue 59. The amino acids from residue 33 to residue 99 were predicted to be an intrinsically disordered region. All nsSNPs detected except the V122G variant are found in these regions. The V122G variant localizes in a region of transcription factors responsible for DNA binding. These results are presented in Table 2.

Mutational characteristics of nsSNPs on the *TP53* database revealed eleven variants located in the TAD2 transactivation domain whose residues are capable of activating gene transcription. Among these residues, three mutants (p.P47L, p.I50S and p.W53S) exhibit partially functional and supertrans transcriptional activity for arginine at codon 47 (p.P47R). The codon 72 (p.P27R) polymorphism was located in a rich proline residues region (SH3/Pro-rich). It does not have transactivation activities. Only the variant (p.V122G) located in the DNA binding zone has transcriptional activity (TA) and is non-functional with an L1/S/H2 type structural motif. These results are presented in Table 3.

Table 2. *Localization of nsSNPs in the functional regions of the TP53 gene with MobiDB*

Residues	Predictions	References
35 - 59	Transactivation domain 2	InterPro IPR040926 / Pfam PF18521
97 - 125	DNA-binding domain superfamily/p53-like transcription factors	InterPro IPR008967 / SUPERFAMILY SSF49417
33 - 99	prediction of consensus disorder	MOBIDB_LITE mobidb-lite

Table 3. *Description of the characteristics of mutated proteins in the TP53 database*

Protein description	Structurel motif	Domain Function	Class TA
p.P47R	N-terme/Transactivation	Transactivation TAD2	supertrans
p.P47L	N-terme/Transactivation	Transactivation TAD2	partially functional
p.D48V	N-terme/Transactivation	Transactivation TAD2	fonctional
p.D48N	N-terme/Transactivation	Transactivation TAD2	fonctional
p.D48G	N-terme/Transactivation	Transactivation TAD2	fonctional
p.D49A	N-terme/Transactivation	Transactivation TAD2	fonctional
p.I50S	N-terme/Transactivation	Transactivation TAD2	partially functional
p.I50F	N-terme/Transactivation	Transactivation TAD2	fonctional
p.Q52E	N-terme/Transactivation	Transactivation TAD2	fonctional
p.Q52L	N-terme/Transactivation	Transactivation TAD2	fonctional
p.W53S	N-terme	Transactivation TAD2	partially functional
p.P72R	type SH3/Pro-riche	type SH3/Pro-riche	-
p.V122G	L1/S/H2	DNA binding	non fonctional

TA= transcriptional activity ; TAD2=Transactivation Domain 2

3.3 The Evolutionary Conservation of the p53 Protein

nsSNPs located in a highly conserved region may have more effect on protein structure and function than nsSNPs located in a less conserved region. The results provided by ConSurf are presented in Figure 2. It reveals that all the mutations are located at hot spots, that is to say in the highly variable regions of the p53 protein with the exception of the variant at site 122 which shows a very preserved character with very significant scores. These results are presented in Table 4. Figure 2 gives an overview of the different regions of the p53 protein with the variability score ranging from 1 to 4 and conservation ranging from 6 to 9.

The MobiDB server has also identified conserved regions of the p53 protein going from residue 97 to residue 182. It is in this highly conserved region that we find the single-nucleotide variation replacing valine with glycine at codon 122. Except for this variant, all the mutations found are located in the very variable interval from the first to residue 96. It is in this zone that a linear interaction of the peptides is observed. Only residues (69 – 90) showed a disorganized structure and a proline-rich region. This area contains the nsSNPs (I50S, I50F, Q52E, Q52L, W53S and P72R). These results are presented in Figure 3 and their three-dimensional profiles in Figure 4.

Table 4. *Consurf results showing conservation scores of TP53 nsSNPs*

Residual change	Prediction	Retention Score
P47R	Highly exposed	1
P47L	Highly exposed	1
D48V	Exposed	3
D48N	Exposed	3
D48G	Exposed	3
D49A	Exposed	3
I50S	Highly exposed	1
I50F	Highly exposed	1
Q52E	Highly exposed	1
Q52L	Highly exposed	1
W53S	Highly exposed	1
P72R	Exposed	4
V122G	Buired	8

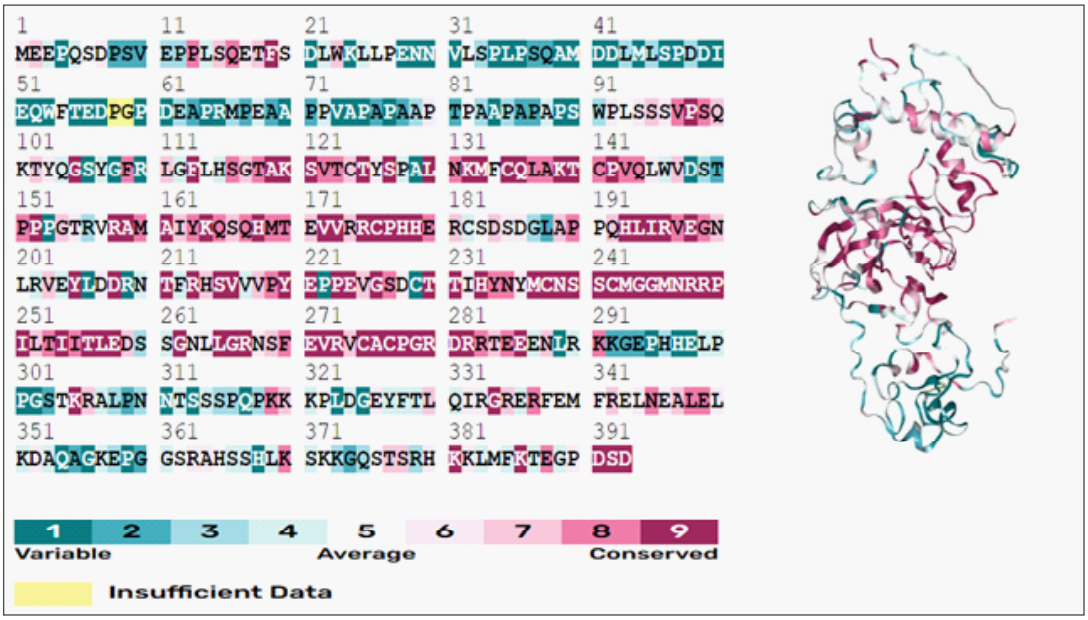
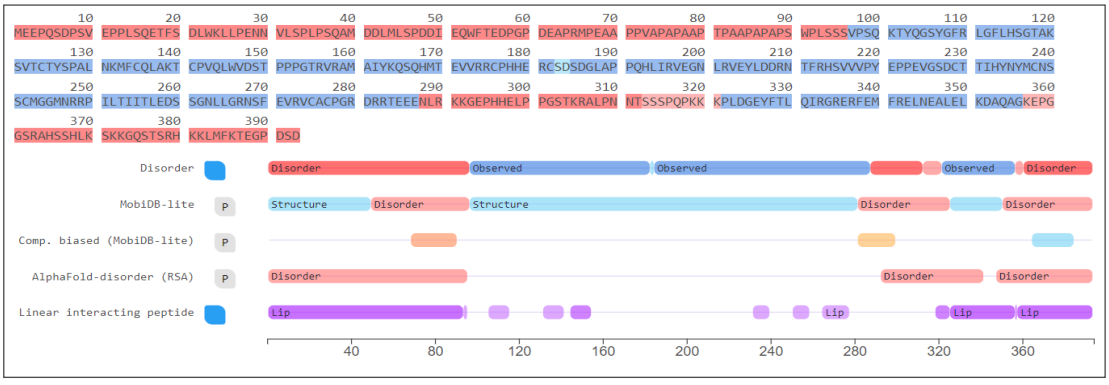


Figure 2. *Prediction of evolutionary conservation of individual residues of the p53 protein by conSurf*



Cloudy residues with integrated annotation from a database (Coverage 39.2%)

Cloudy residues with an annotation predicted by a sequence-based method (Coverage 3.3%)

Residues preserved with annotation automatically derived from PDB structures (57.0% coverage)

Structured residues annotation predicted by a sequence-based method

Protein-rich region (Coverage 5.6%)

Linear interacting peptides (Coverage 23.7%)

Figure 3. *Evolutionary conservation of p53 protein residues by MobiDB*

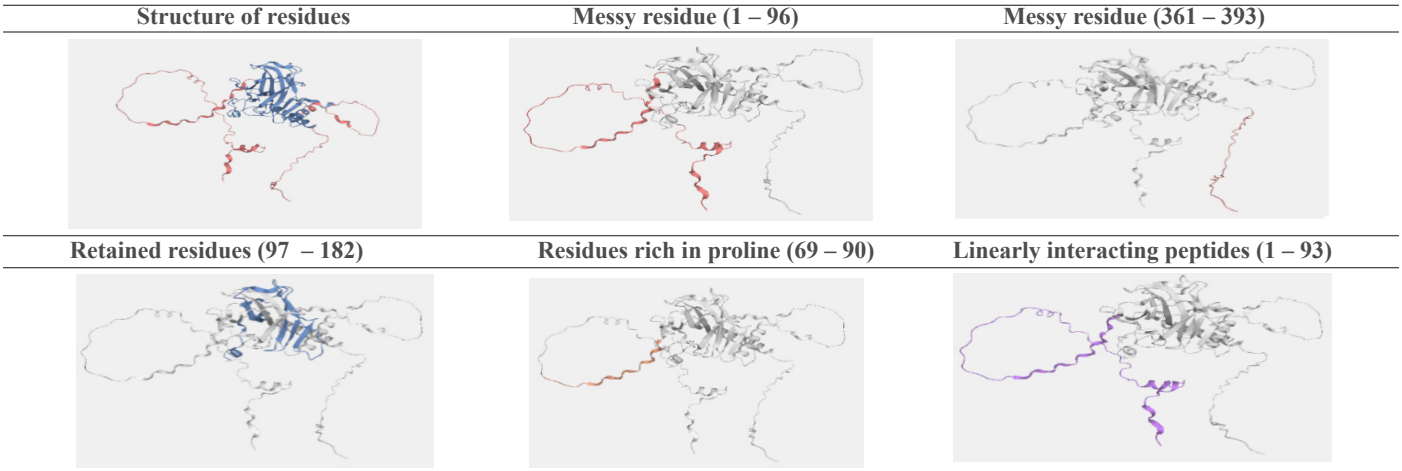


Figure 4. *3D structure of the p53 protein showing variable residues, conserved residues and linearly interacting residues.*

3.4 Pathogenicity Prediction of nsSNPs

The 3 reference tools, namely, PolyPhen-2, Mutation Tester and PROVEN were used to highlight the damaging variants from the missense variants. The PolyPhen-2 algorithm listed 5 SNPs as Potentially Damaging. On the other hand, Mutation Tester and PROVEN classified all missense variants without impact in relation to the pathology. These results are presented in Table 5.

Table 5. *Pathogenicity analysis of missense mutations*

DNA description	Protein description	Polyphen-2		Mutation Taster		PROVEAN	
7676229C>G	p.P47R	Potentially damaging	0.947	Benign	0.05	Neutral	-0 .270
7676229C>T	p.P47L	Potentially damaging	0.848	Benign	0.04	Neutral	0.569
7676226A>T	p.D48V	Benign	0.113	Benign	0.04	Neutral	-0.433
7676227G>A	p.D48N	Benign	0.153	Benign	0.04	Neutral	-0.095
7676226A>G	p.D48G	Potentially damaging	0.633	Benign	0.04	Neutral	0.082
7676223A>C	p.D49A	Potentially damaging	0.945	Benign	0.04	Neutral	0.090
7676220T>G	p.I50S	Benign	0.163	Benign	0.08	Neutral	0.546
7676221A>T	p.I50F	Benign	0.000	Benign	0.04	Neutral	-0.430
7676215C>G	p.Q52E	Benign	0.000	Benign	0.05	Neutral	-0.488
7676214A>T	p.Q52L	Benign	0.006	Benign	0.04	Neutral	-0.797
7676211G>C	p.W53S	Potentially damaging	0.995	Benign	0,04	Neutral	0.298
7676154C>G	p.P72R	Benign	0.93	Benign	0.04	Neutral	-0.230
7676004T>G	p.V122G	Potentially damaging	01	Benign	0.04	deleterious	0.04

3.5 Stability of Mutated Proteins

The function of a protein is associated with its stability, which is why it is important to identify the change in stability of a protein due to nsSNPs. The Expasy server's ProtParam tool predicted that most of the mutant residues carry negative charges. The analyzes also revealed that all proteins were unstable with instability index above 40 (Table 6).

Table 6. *Physical and chemical properties of SNPs using ProtParam*

Protein variants	Residues negatively	Residues positively	Instability index (II)
p.P47R	50	47	73.16*
p.P47L	50	46	72.67*
p.D48V	49	46	73.89*
p.D48N	49	46	73.78*
p.D48G	49	46	73.78*
p.D49A	40	46	73.59*
p.I50S	50	46	73.46*
p.I50F	50	46	72.28*
p.Q52E	51	46	73.55*
p.Q52L	50	46	73.71*
p.W53S	50	46	74.71*
p.P72R	50	47	72.42*
p.V122G	50	46	73.59*

*= *unstable protein*

Stability search with Dynamut2 determines a destabilizing prediction of six mutations (P47L, D48G, I50S, I50F, Q52E, W53S) with energy exchanges well below 0.001. The results of these predictions are presented in Table 7.

Table 7. *Prediction of protein stability with Dynamut2*

Protein variant	Chain	ID-PBD	Free energy exchange (ΔΔG)	Prediction
p.P47R	A	8F2I	0.43 kcal/mol	Stabilizing
p.P47L	A	8F2I	-0.18 kcal/mol	Destabilizing *
p.D48V	A	8F2I	0.04 kcal/mol	Stabilizing
p.D48N	A	8F2I	0.21 kcal/mol	Stabilizing
p.D48G	A	8F2I	-0.018 kcal/mol	Destabilizing *
p.D49A	A	8F2I	0.06 kcal/mol	Stabilizing
p.I50S	A	8F2I	-3.03 kcal/mol	Destabilizing *
p.I50F	A	8F2I	-1.79 kcal/mol	Déstabilisant*
p.Q52E	A	8F2I	-0.32 kcal/mol	Destabilizing *
p.Q52L	A	8F2I	0.92 kcal/mole	Stabilizing
p.W53S	A	8F2I	-3.24 kcal/mol	Destabilizing *
p.P72R	A	8F2I	0.18 kcal/mol	Stabilizing
p.V122G	A	8F2I	0.01 kcal/mol	Stabilizing

**= unstable protein*

I mutant 2.0 predicted the extent to which nsSNPs alter p53 protein stability. All nsSNPs were predicted to decrease p53 protein stability except (I50F and Q52L). The nsSNPs with the highest RI values may be involved in greater damage to p53 protein stability. Predictions of changes in stability of exon 4 mutants of the *TP53* gene are given in Table 8.

Table 8. *Stability of the p53 protein depending on nsSNPs with I-mutant*

Protein variant	Stability	DDG value	RI
p.P47R	Decreased	-0.32	6
p.P47L	Decreased	0.60	2
p.D48V	Decreased	-0.60	4
p.D48N	Decreased	-1.81	5
p.D48G	Decreased	-1.79	8
p.D49A	Decreased	-1.36	2
p.I50S	Decreased	0.75	8
p.I50F	Decreased	0.29	7
p.Q52E	Decreased	-0.11	2
p.Q52L	Decreased	0.21	2
p.W53S	Decreased	-1.64	5
p.P72R	Decreased	-0.46	1
p.V122G	Decreased	-3.34	9

DDG: unfurling Gibbs free energy ; RI= Reliability index

3.6 Impact of nsSNPs on the Structure and Function of the p53 Protein

Physicochemical properties reflect the functional and structural characteristics of a protein. The analysis of these physicochemical properties revealed no structural damage with Missense 3D (Table 9) for all nsSNPs and therefore on their molecular evolution. An impact of nsSNPs on the structure and function of

Table 9. *Structural damage detected using Missense 3D*

Protein variant	Structural damage	Protein variant	Structural damage
p.P47R	No structural damage detected	p.I50F	No structural damage detected
p.P47L	No structural damage detected	p.Q52E	No structural damage detected
p.D48V	No structural damage detected	p.Q52L	No structural damage detected
p.D48N	No structural damage detected	p.W53S	No structural damage detected
p.D48G	No structural damage detected	p.P72R	No structural damage detected
p.D49A	No structural damage detected	p.V122G	No structural damage detected
p.I50S	No structural damage detected	-	-

the p53 protein was revealed with MutPred2. These modifications of the protein structure are characterized by a disordered interface of the three-dimensional structure on four variants (p.I50S; p.I50F; p.Q52L and p.W53S). Among these variants, p.I50S and p.I50F also caused a loss of phosphorylation at S46 as well as a gain of function of factor B only linked to p.I50S. A conformational change through loss of helix was observed following the change from glutamine to glutamic acid at codon 52 of the tumor protein p53 and a gain of phosphorylation at T55 at codon 53 where tryptophan is replaced by serine. These results are presented in Table 10 with statistically significant P-values.

Table 10. *Effects of nsSNPs on structural and functional properties of TP53 with MutPred2*

Protein variant	Probability of substitution	PROSITE Patterns / ELM concerned	Molecular mechanisms	P value <= 0.05
p.P47R	0.410	-	-	
p.P47L	0.391	-	-	
p.D48V	0.428	-	-	
p.D48N	0.246	-	-	
p.D48G	0.441	-	-	
p.D49A	0.443	-	-	
p.I50S	0.591	ELME000053 / ELME000239	Modified messy interface	4.0e-03
			Loss of phosphorylation at S46	0.02
			B factor gain	9.0e-03
p.I50F	0.547	ELME000328	Modified messy interface	0.02
			Loss of phosphorylation at S46	0.03
p.Q52E	0.516	-	Propeller loss	0.05
p.Q52L	0.630	-	Modified messy interface	0.03
p.W53S	0.807	ELME000064 / ELME000147 / PS00006	Modified messy interface	6.6e-03
			Phosphorylation gain at T55	0.05
p.P72R	0.303	-	-	
p.V122G	0.407	-	-	

ELM= Eukaryotic Linear Motifs for functional sites in proteins

3.7 Post-Translational Modification Sites in Mutated Proteins

The phosphorylated sites in exon 4 of the *TP53* gene predicted by MusiteDeep and NetPhos 3.1 are mentioned in Table 11. Musite Deep predicted 12 residues and NetPhos predicted 8 residues with phosphorylation potential. The highlighted colors of the predicted sites correspond to their prediction confidence levels. By hovering over the predicted sites, the detailed information of the prediction will be displayed by MusiteDeep. Only four amino acid residues at position 38, 75, 87 and 89 are not found to be phosphorylated by both Musite Deep and NetPhos. The NetPhos 3.1 server predicted that the I50S mutation resulted in the loss of the CKII kinase phosphorylation site, while an unspecified kinase activity was predicted for the W53S variant.The formation of Phosphoserine was found in these two variants by Musite Deep.

Table 11. *Prediction of phosphorylation sites by MusiteDeep and NetPhos 3.1*

Residues	MusiteDeep		NetPhos-3.1		
	Prediction	Score	Prediction		Score
33	Phosphoserine	0.846	Kinase p38MAPK	Oui	0.529
37	Phosphoserine	0.573	Kinase DNAPK	Oui	0.584
38	Pirrolidone_Carboxilic_Acide	0.704	-	-	-
46	Phosphoserine	0.826	Kinase unsp	Oui	0.996
50	Phosphoserine	0.746	Kinase CKII	Non	0.492
53	Phosphoserine	0.752	Kinase unsp	Oui	0.907
55	Phosphoserine / O-Linked_Glycoslation	0.612 / 0.517	Kinase CKII	Oui	0.598
75	Hydroxyproline	0.628	-	-	-
81	O-Linked_Glycoslation	0.673	Kinase unsp	Oui	0.704
87	Hydroxyproline	0.509	-	-	-
89	Hydroxyproline	0.646	-	-	-
90	O-Linked_Glycoslation	0.576	Kinase GSK3	Oui	0.505

unsp= unspecified

3.8 Structural Modeling of I-TASSER Mutated Proteins

The 3D structures of wild type *TP53* and 13 of its mutants were predicted by I-TASSER and the results are shown in figure 5. Compared to the wild-type 3D structure, no structural deformation of the mutant proteins was observed and to a large extent no change in the biological activity of the p53 protein in the tumor cell. I-TASSER also generated predictions of ligand binding sites that form complex non-covalent substances or reversible bonds by matching the target model to proteins. Modifications to the ligand-binding site of the p53 protein were observed in the presence of the p.D48V, p.D48N, p.D59A, p.I50F, p.Q52E, and p.P72R mutations

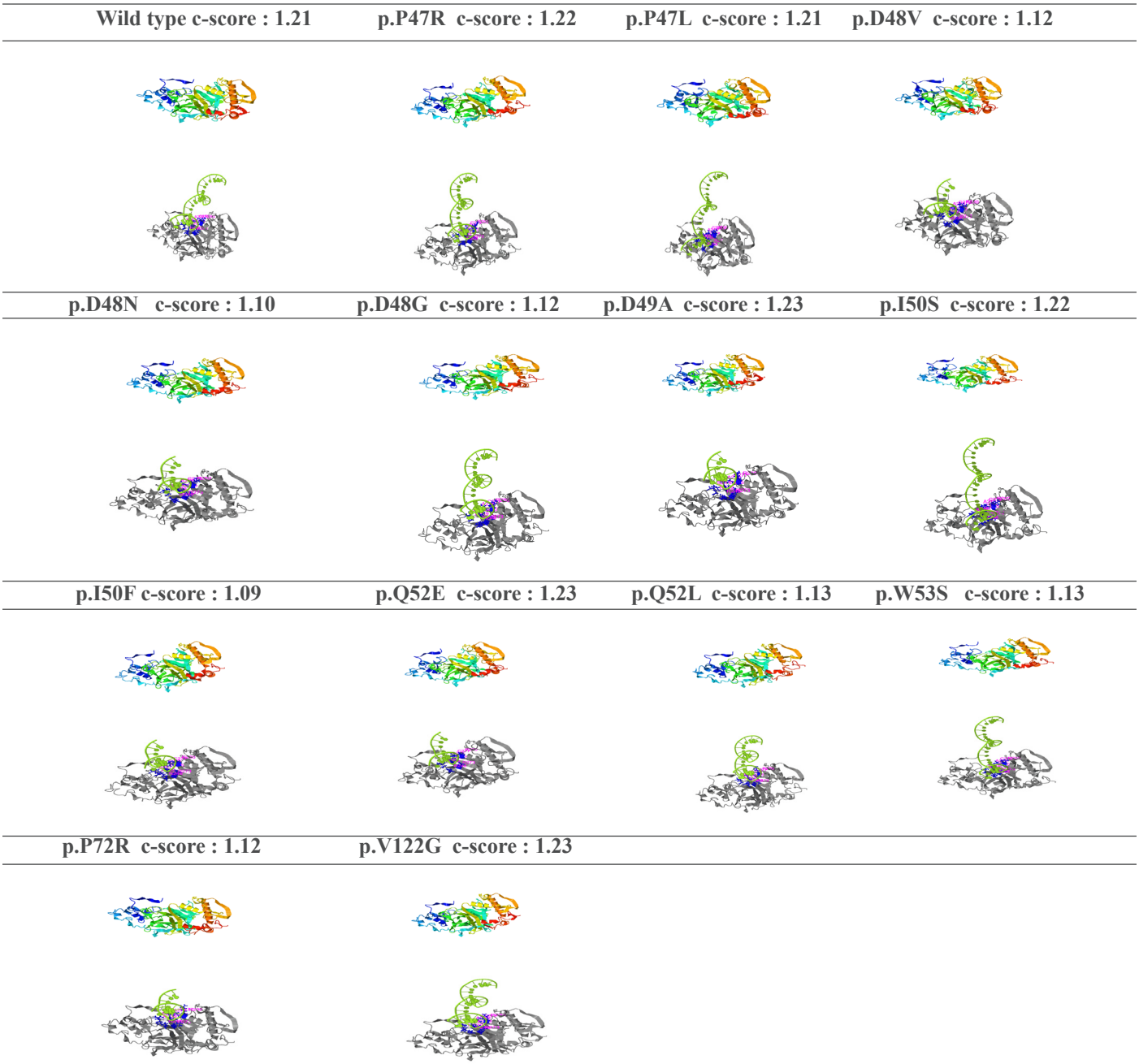


Figure 5. *3D structure of the wild-type p53 protein and that of mutants as well as the binding of protein ligands*

4. Discussion

The crucial differences between normal and cancer cells arise from discrete changes in specific genes controlling tissue proliferation and homeostasis. Tumor suppressor genes are critical sites of DNA damage vulnerability, due to their physiological barrier functions [19]. These barriers act against clonal expansion or genomic mutability and are expected to impede the growth and even metastasis of cells driven to uncontrolled proliferation by oncogenes. Thus, p53 protein activity is ubiquitous in human cancer. Its tumor suppressor activity can be

disrupted due to genome damage caused by mutation, chromosomal rearrangement and nondisjunction, gene conversion, imprinting, or mitotic recombination. In this study, the mutability of exon 4 of the *TP53* gene was investigated in 24 cases of colorectal tumors in comparison with 24 control cases as well as the probable damage of mutations on the structure and function of the corresponding protein. Identification ofmutations revealed the presence of 10 variants present in cancerous tissues and two others found in controls. A mutation was found in both cases (12 cancer tissue sequences and in 7 controls). These 10 tumor variants are located in the Transactivation 2 (TAD2) domain, three of which (p.P47L, p.I50S and p.W53S) have partially functional TAD2 activity. Transactivation 2 (TAD2) has residues capable of independently activating gene transcription and is an intrinsically disordered protein domain that was revealed by consurf and MobiDB. This disordered structure could provide inherent flexibility allowing TAD2 residues to adapt and bind to a wide range of proteins [20]. Indeed, an accumulation of mutations in these areas, each of which confers one or the other a growth advantage, can lead to the progressive conversion of normal human cells into cancer cells. In addition, the work of Olive *et al*., [21] describes that under certain physiological conditions, the expression of the p53 protein, carrying missense point mutations, goes beyond the simple loss of function of p53 and that it considerably increases the oncogenic potential of tumor cells. It has also been shown that the transactivation activity of wild-type p53 protein is influenced by four critical hydrophobic amino acids in its N-terminus (Leu 22, Trp 23, Trp 53 and Phe 54) [22]. When these four residues are mutated to polar amino acids (L22Q; W23S; W53Q and F54S), the transactivation capacity of wild-type p53 is completely abolished.

A variant, where valine is replaced by guanine at codon 122 (p.V122G) and found in three cancerous tissues, is located in the DNA binding domain having non-functional transcriptional activity (TA) and a structural motif of type L1/S/H2. These regions of the p53 protein have been predicted to be highly conserved. Interactions of the DNA-binding domain of p53 with its N-terminus may contribute to the stability of the p53 tetramer. And the conserved structure supports the hypothesis that DNA binding is essential for the biological activity of p53 and provides a framework for understanding how mutations inactivate it. Mutations in the DNA binding domain could cause conformational changes or even changes in the specificity of p53 target DNA sequences. Bouaoun *et al*., [23] argue that the propensity for carcinogenic mutations residing in this domain highlights its importance in the tumor suppressive and homeostatic functions of p53. Therefore, mutations in these residues could have an impact in the areas of contact with the DNA and can cause the formation of hetero-oligomerization of the mutant protein to the wild one which subsequently can compromise the transcriptional activity of p53 [24]. Indeed, the role of transcription factor played by the p53 protein requires the formation of a homotetrameric structure [25]. Also, the amino acids of the central domain provide distinct functions. Some residues are involved in maintaining the three-dimensional structure of the DNA-binding domain while others ensure DNA binding [26]. Mutations in these residues contribute directly to the generation of proteins for which the DNA binding capacity is partially or even completely abolished.

Our results also showed a recurrent mutation in the *TP53* gene (p.P72R) found in both affected and healthy tissues and has a disordered structural pattern. Its presence among controls indicates that this variant would not be involved in the occurrence of colorectal cancers. The work of Olschwang and her collaborators [27] on the comparison of the characteristics of this frequent polymorphism of the *TP53* gene in patients with colon cancer and a control population was unable to demonstrate any evidence showing that this polymorphism is associated to a marked predisposition to colorectal cancer. However, Rodrigues *et al*., [28] as well as Dastjerdi, [29] showed that overexpression of p53 was synonymous with mutation, which could explain the high frequency of this mutation in both groups of tissues. Added to this are the reflections of Wan *et al*., [30] on this single nucleotide polymorphism (SNP) at codon 72 of the *TP53* gene which resulted in an association of the polymorphism with the risk of development of various neoplasms. This codon 72 polymorphism is located in the proline-rich domain connecting TAD2 to the DNA binding domain. A proline-rich region is thought to be necessary to regulate *TP53* stabilization and apoptosis because it is involved in the regulation of important cellular pathways, such as cell proliferation, migration, and cytoskeletal changes. Protein-protein interactions mediated by these proline-rich motifs are involved in the regulation of many important signaling cascades. Indeed, they can modify the 3D structure of a protein

by isomerization of proline (PIN1, cyclophilins) in the process of regulating the orientation and interaction angles of its functional domains [31]. Therefore, the presence of this mutation could be considered as a relatively late event in tumorigenesis.

Although p53 function may help inhibit tumor progression by protecting cells against DNA damage and genome instability, disruptions in its function are common in human cancers. According to Leroy *et al*., [32] point mutations can lead to the weakening of p53 function or even affect the amplification of Mdm2 (mouse double minute), encoding a negative regulator of p53, or the inactivation of arf (alternative reading frame), encoding an MDM2 inhibitor. This phenomenon is likely to induce a reduction in sensitivity to apoptosis [33] since p53 is a transcription factor whose stabilization and activation leads to an increase in the expression of genes that coordinate stopping proliferation [34]. Thus, the protein fluctuations of the five mutations (p.P47L, p.D48G, p.I50S, p.Q52E and p.W53S) predicted by both the three tools (ProtParam, Dynamut2 and I mutant 2.0) showed an effect which would reduce the stability of the p53 protein. This decrease in stability could induce a steric obstacle (intermolecular shock) or even repulsive interactions which can be correlated with the mechanisms of colorectal cancer genesis.

Functional p53 activity for transcriptional activation of p53 target genes requires protein stabilization, recruitment and binding of p53 to specific DNA sequences [34]. These different phases of p53 activity are influenced by the post-translational modifications to which the p53 protein is subject. Protein post-translational modifications (PTMs) are biochemical alterations of amino acids that modify the physicochemical properties of target proteins. These modifications lead to structural changes and thus regulate protein-protein interactions as well as cellular signal transduction in neoplasm developmental pathways [35]. They take place during or after translation, reversibly or not, and thus allow the regulation of protein activity. The variants found at codon 50 of the p53 protein transactivation domain accumulate a loss of phosphorylation at S46 and therefore a loss of function of enzymes such as DYRK2, PKCδ, p38K, HIPK2, AMPKα, CDK5 responsible for the modification [36]. The fact that several kinases can phosphorylate S46 provides information on the importance of this site in p53 function and makes understanding its regulation more difficult. However, the main effect of phosphorylation of the transactivation domain is the stabilization of p53, since it influences the interaction between p53 and negative regulator (Mdm2) [37]. Indeed, under conditions of moderate DNA damage, the p53 negative regulator Mdm2 induces the degradation of the HIPK2 enzyme. Severe DNA damage results in reduced MDM2 levels, rendering HIPK2 stable which favors phosphorylating p53 at S46 to induce cell death [38]. Thus, on the one hand, the phosphorylation of p53 on Ser46 by HIPK2 only occurs in cases of severe and non-reparable DNA damage that irreversibly leads cells to apoptosis. Therefore, the loss of phosphorylation at the S46 site could be correlated with the inactivation of apoptosis genes regulated by p53. On the other hand, in unstressed cells, T55 is constitutively phosphorylated [39], which promotes p53 inactivation [40] and its degradation [41]. Activation of p53-mediated transcriptional programs after DNA damage is benefited by pT55 dephosphorylation [41]. According to Wu and colleagues [42] subsequent rephosphorylation of T55 by TAF1 kinase in the late DNA damage response inactivates p53-mediated transcription and promotes dissociation of p53 from p21 and other promoters that regulate cell cycle arrest and apoptosis. Thus, the gain in T55 phosphorylation found turns out to be involved in the inactivation of p53.

Added to this is the activation of the p38MAPK, DNAPK, unsp and GSK3 kinases. DNA-dependent protein kinase (DANPK) is one of the main players in the repair of radiation-induced double-strand breaks, but also those induced by chemotherapy. The p38 MAPK (mitogen-activated protein kinase) signaling pathway allows cells to interact with a wide range of external signals and respond appropriately by generating a multitude of different biological effects [43]. Its activation could generate a key signal transduction cascade advantageous for cancer cells to detect and adapt to a multitude of environmental stimuli. According to Zou & Blank [44], p38MAPK activation can act as an onco-suppressive pathway and also argue that its signaling is very active in different types of cancers, promoting tumor growth. Unsp or unspecific phosphorylation could indicate phosphorylation that occurs in a non-specific manner and without a precise target.

Previous studies have shown that local motifs surrounding a phosphorylation site interact with the kinase binding domain to enable phosphorylation [45]. Targeting of phosphorylation substrates by kinases is controlled by a wide variety of processes

within the cell that ensure kinase-substrate fidelity. To this end, there are numerous kinase-specific phosphorylation site predictors that take advantage of the sequence specificity of kinases to predict kinase-specific phosphorylation sites [46, 47], as well as phosphorylation sites in a non-kinase specific manner [48]. Analyses of post-translational modifications revealed mutants at residues 46 and 53 of the p53 protein favor the activation of kinases with non-specific phosphorylation site prediction (Kinase unsp).

CK2 is a constitutively active kinase, which acts paradoxically in several regulatory processes. It is able to use phosphoserine residues as consensus determinants, which suggests that CK2 can act permanently with other kinases [49]. This adhesion to phosphoserine residues is found to be inactivated at codon 50 of the p53 protein in our study. Its expression at codon 53 of the p53 protein, where tryptophan is replaced by serine and predicted to be probably damaging by PolyPhen-2. This activation of CK2 suggests its involvement in the occurrence or progression of colorectal cancer. Trembley *et al*., [50] suggest that elevated levels of protein kinase CK2 have long been associated with increased cell growth and proliferation, both in normal and cancer cells. The activity of CK2 can be correlated with that of GSK3 kinase. Glycogen synthase kinase-3 (GSK3) is an integral component of various signaling pathways that regulate crucial cellular processes such as proliferation, differentiation, apoptosis, and metabolism. Shakoori *et al*., [51] and Guil-Luna *et al*., [52] suggested that in colorectal tumor cells, the expression levels of GSK3, particularly of the GSK-3β isoform, and the amounts of its active form were higher in tumor cells than in their normal counterparts; which can be matched to its activation revealed at codon 90 of the p53 protein.

Distinguishing the mutations that cause pathologies also requires understanding the impacts of missense mutations on the expression and regulation of genes, or even on the disruption of protein stability, interaction with proteins, or even small molecule ligands. The modeling analysis predicted no structural changes to the p53 protein. This implies that SNPs have a very small impact on the overall protein structure. Nevertheless, further analysis of individual variants revealed conformational changes of the ligand site of mutants p.D48V, p.D48N, p.D59A, p.I50F, p.Q52E and p.P72R compared to the type protein savage. These modifications can influence binding affinities which represent non-covalent interactions between biomolecules, such as hydrogen bonds, electrostatic and hydrophobic interactions as well as Van der Waals forces between the two molecules [53]. However, these conformational changes of the ligand sites do not seem to affect the specificity of DNA binding but may lead to an altered mechanism of the biological function of the p53 protein. Neutralization of the destructive functions of p53 can lead to escape from tumor suppression and cancer. Indeed, this neutralization is often the consequence of a mutation of a copy of p53 and a dominant negative effect of the mutant on the product of the normal copy of p53 and possibly on those of p63 and p73 [54]. According to Levine & Oren, [55], such mutations, generally on a single amino acid, occur rather late in tumorigenesis. And thus the neutralization of the destructive functions of p53 can cause mutations in p53 effectors or regulators and possibly occur earlier [56].

5. Conclusion

The objective of this study was to identify the most pathogenic missense variants that could alter the function of the p53 protein in colorectal cancers. The study identified nine nsSNPs in the coding region of exon 4 of the *TP53* gene compared to controls which had two variants and a recurrent mutation at codon 72. These nsSNPs belong to the transactivation domain 2, the binding domain of p53, three of which (p.P47L, p.I50S and p.W53S) have partially functional TAD2 activity. They can play a major role in tumor progression because they are involved in the decrease of p53 protein stability and the loss of the potential phosphorylation site. Most mutants possessing these nsSNPs showed a distortion in the structure of the ligand to the p53 protein compared to the wild type. These nsSNPs can contribute to the promotion and progression of cancer depending on the cell type in which the mutation occurred. The high level of mutated p53 within cancer cells, compared to healthy cells, provides an interesting avenue for the development of anti-proliferative agents. These nsSNPs may be important for therapeutic strategies and personalized medicine and can be used for further experimental research to study the role of these nsSNPs in the pathogenesis of related diseases.

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Author contributions

Anna Ndong performed the molecular analyses and genetic analyses. Bineta Kénémé was responsible for the design and writing of the manuscript. Fatimata Mbaye revised the manuscript and read and approved the submitted version. Mbacké Sembène supervised the study, the selection of the biological material, and the gene studied.

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All data relevant to the study are included in the article. For additional information or materials related to this project, please contact the corresponding author

Conflicts 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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