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Targeting Mutational Landscape of TP53 in patients diagnosed with Oral Cancer living in Senegal

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Abstract

Introduction

Genomic mutations in *TP53* gene in association with etiological risk factors have been associated with oral carcinogenesis. Herein, we screened for genomic variants of *TP53* predisposing to oral cancers in Senegalese patients.

Methodology

88 patients with confirmed diagnostic were recruited after informed consent. Blood samples were collected from each patient to perform DNA extraction, PCR amplification of all coding exons of *TP53* followed by Sanger Sequencing of PCR products. Nucleotide sequences were analysed with Genalys software. 94 blood donors with no cancer diagnosis were also recruited as controls for association study between the most common variants identified in patients and predisposition to oral cancers.

Results

Sequence analysis showed that 52.27% of patients carry at least one mutation in *TP53*. Eleven genomic variants were identified, 7 variants already reported in databases and 4 new variants. The most

recurrent variants in this study already reported as cancer -related variants were Pro72Arg (rs1042522; Arginine frequency estimated at 31.26%) and a 16 bp insertion in intron 3 (rs59758982; allelic frequency estimated at 26.25%). Haplotype analysis between these variants showed a strong linkage disequilibrium (D' = 0.999, r^2 = 0.153 and p-value < 0.05). However, association study did not find any significant association with susceptibility to oral cancer (p-value > 0.05).

Conclusion

Our study highlighted that despite the absence of association between the two most common cancer-related variants in Senegalese patients diagnosed with oral cancer, their strong LD suggested that they could be transmitted together in a common haplotype which may be implicated in oral carcinogenesis.

Introduction

With an estimated incidence of nearly 300.000 new cases per year and a mortality rate around 50%, oral cancers are major challenge in oncology and represent the sixth leading cancer worldwide, according to GLOBOCAN report 2018 [1].

The main risk factors are exposure to genotoxic agents such as tobacco, alcohol and betel nut chewing [2-4]. These agents induced genetic alteration in genes controlling the cell cycle such as *TP53* [5-7]. Also, poor oral hygiene, bad set of teeth, inadequate diet and poor nutrition, or immunosuppression, may increase oral cancer risk by promoting chronic infections of the oral mucosa with high risk Human Papilloma Virus (HPV), by inducing loss of function of *TP53* encoded protein [8-10].

Mutations of *TP53* gene have been reported as the main driver event of carcinogenesis [11]. Missense variants have been reported in the coding region of the DNA Binding Domain of p53 protein which specifically binds to the promoters of targeted genes.

Eight hotspot amino-acid substitutions in the DNA Binding Domain of p53, characterizing almost 27% of mutant proteins, were identified in human cancers while they are not directly cancer associated (Arg175His, Gly245Ser, Arg248Gln, Arg248Trp, Arg249Ser, Arg273His, Arg273Ser, and Arg282Trp) [12]. In mice model, the introduction of the Arg172His mutation corresponding to human Arg175His, has been associated with the functional inactivation of p63 and p73 proteins [13, 12].

Other variants are located in a 1kb region spanning intron 3 to intron 4 containing the 6 cancer-related variants reported in IARC *TP53* database [14]: a 16 bp insertion in intron 3 (NG_017013.2:g.16185_16200Ins, rs59758982), two variants located in exon 4 coding for the Proline-rich domain of p53 (Pro47Ser : NG_017013.2:g.16321C>T, rs1800371) and Pro72Arg : NG_017013.2:g.16397C>G, (rs1042522)), and 2 variants located in intron 4 (NG_017013.2:g.17032T>C (rs1794287) and NG_017013.2:g.17198G>A (rs35850753) (Figure 1).

Effect predictions of these cancer related variants in Varsome database have shown benign effect (rs1800371, rs78378222 and rs35850753) or uncertain significance (rs59758982, rs1042522 and rs1794287), although functional/association studies are in favour of cancer predisposition (Table 1).

The 16 bp insertion in intron 3 (rs59758982) has been associated with increased risk of colorectal cancer in a case-control study and correlated with a reduced level of *TP53* mRNA in lymphoblastoid cell-lines [15]. The Pro47Ser variant (rs1800371) has been reported in populations of African ancestry (allelic frequency estimated at 2-4% in Africa and 1.2% in African-Americans) [16] and have been associated with increased risk of breast cancer among pre-menopausal women [17]. The Pro72Arg (rs1042522) is a non-conserved amino acid with reported altered electrophoretic mobility, conformation and function of the mutant protein [18]. This variant shows significant ethnic bias with Arginine allelic frequencies estimated at ~72% for Europeans compared to ~38% for African and African-Americans and ~51% for Asian populations (gnomAD database: https://gnomad.broadinstitute.org/variant/17-7579472-G-C?dataset=gnomad_r2_1). In a

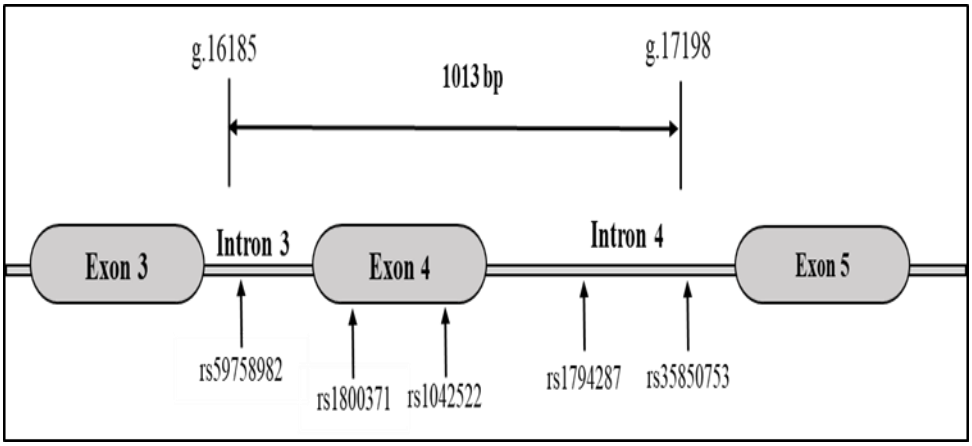

Figure 1. Cancer-related variants of TP53 encompassing exons 4

Table 1. VarSome effect predictions and functional study findings on cancer-related variants reported in IARC TP53 database

Genetic variations	Location	rs number	Effect Prediction	Functional/Association study findings
NG_017013.2:g.16185_16200Ins	Intron 3	rs59758982	VUS	Association with increased risk of colorectal cancer and reduced level of *TP53* mRNA in lymphoblastoïd cell-lines [15]
NG_017013.2:g.16321C>T (p.Pro47Ser)	Exon 4	rs1800371	Benign	Association with breast cancer risk [17]
NG_017013.2:g.16397C>G (p.Pro72Arg)	Exon 4	rs1042522	VUS	Association with altered electrophoretic mobility and, a conformational and functional modifications of the p53 mutant protein [18]
NG_017013.2:g.17032T>C	intron 4	rs1794287	VUS	Affect the activity of *TP53* internal promoter [20]
NG_017013.2:g.17198G>A	intron 4	rs35850753	Benign	Association with neuroblastoma [21]
NG_017013.2:g.24117A>C	3' UTR	rs78378222	Benign	Association with neuroblastoma [21]

UTR: Untranslated region VUS: Variant of Uncertain Significance

previous study, we showed in an ethnicity-stratified meta-analysis that this variant is associated with oral cancers risk in Asian population (OR = 1.31; $CI_{95\%}$ =1.09 - 1.58; p=0.004) whereas any significant association was observed in Africans and Caucasians [19].

Two other cancer-related variants (rs1794287) and (rs35850753) are located in intron 4 and were shown to affect the activity of *TP53* intron 4 internal promoter by changing its affinity with several transcription factors [20]. The last variant (rs78378222) maps to the polyadenylation signal located in 3′ UTR of *TP53*. This variant is in linkage disequilibrium with rs35850753 in intron 4 and both have been associated with n euroblastoma (rs35850753: OR = 2.7, $CI_{95\%}$ = [2.0 - 3.6]; rs78378222: OR = 2.3, $CI_{95\%}$ = [1.8 - 2.9] [21].

In Sub-Saharan Africa, very few studies have investigated genetic variability of *TP53* and its association with cancer risk. Here we screened for *TP53* genomic variants predisposing to oral cancers in patients living in Senegal.

Population and Methods

The study was carried out from January 2018 to September 2021 through a collaboration between the Department of Stomatology and Maxillofacial Surgery of Aristide Le Dantec Hospital in Dakar for patients recruitment, the Senegalese National Blood Transfusion Center for the recruitment of healthy blood donor controls, the department of Human Genetics of the Faculty of Medicine, Pharmacy and Odontology of Cheikh Anta Diop University Dakar for DNA extraction and PCR amplification, and the Functional Genomics Platform of the National Center for Scientific and Technical Research (CNRST) of Rabat (Morocco) for Sanger sequencing of PCR products. The research protocol was approved by the Ethics Committee of Cheikh Anta Diop University under reference 0320/2018/CER/UCAD.

Study Population

Patients with confirmed diagnosis of oral cancer undergoing treatment at the Department of Stomatology and Maxillofacial Surgery of Aristide Le Dantec Hospital were recruited after informed consent. A questionnaire was filled out by each patient providing information about age, gender, ethnicity, alcohol and tobacco status, tumor location and histology, and disease stage.

94 healthy blood donors without cancer were also recruited as controls. For each individual a blood sample was collected for DNA extraction.

DNA Isolation, PCR Amplification of TP53 Coding Exons

DNA was isolated from blood samples using Quick-DNA™ MiniPrep (Zymo Research) following manufacturer's protocol. DNA extract were quantified with a spectrophotometer (SimpliNano™, Biochrom). PCR amplifications of the 10 coding exons (exons 2 to 11) of *TP53* gene were processed with seven primer sets at specific annealing temperatures (Table 2). PCR conditions were as follow: initial denaturation at 95°C for 10 min; 40 cycles of denaturation at 94°C for 30s, primer annealing for 30s, and extension at 72°C for 30s. A final extension step followed at 72°C for 10 min. PCR products were visualized by electrophoresis in a 1.5% agarose gel.

The 16 bp insertion in intron 3 was genotyped in control population by PCR amplification using forward (TGCTCTTGTCTTTCAGACTTCCT) and reverse primer (GAGCAGTCAGAGGACCAGGTC) at 62°C annealing temperature during 30s. PCR products were visualized in a 3% agarose gel after electrophoresis at 100 volts for an hour. Two fragments were observed (114bp for the insertion allele and 130bp for the wild type allele).

The Pro72Arg variant (c.215C>G) was genotyped in controls by PCR-RFLP as previously reported [22].

Sanger Sequencing of PCR Products and Sequence Analysis

PCR products were cleaned with PureLink™ Quick Gel Purification kit (Invitrogen) according to manufacturer's protocol. Cleaned PCR products were sequenced with BigDye™ Terminator kit (Applied Biosystems) and loaded on a SeqStudio™ Genetic Analyzer System 4 capillary (Applied Biosystems).

Sequences analysis were performed with Genalys Software (version 3.3.42a) and mapped to the reference sequence of *TP53* gene (NG_017013.2) for variants

detection. Clinical significance of identified variants was checked on ClinVar, dbSNP, VarSome and gnomAD databases. For newly identified variants, we predicted their biological effects with SIFT and PolyPhen2 tools.

Statistical Analysis

Linkage Desequilibrium (LD) was tested with Haploview Software (version 4.1). The association between the most common variants and predisposition to oral cancers was checked in a case/control study. Association parameters (odds ratio (OR), 95% confidence interval ($CI_{95\%}$) and p-value) were estimated through an online dedicated website [23].

Results

Characteristics of Study Population

The study population included 88 patients diagnosed with oral cancers. Mean age at diagnosis was 51.90 ±17.98 years with ages ranging from 18 to 85 years. Sex ratio was in favor of females (0.76) and most of our patients (73%) had a negative alcohol-tobacco status. Tumor histology showed that 90.9% were squamous cell carcinoma. Tumors were preferentially located in the tongue (22.7%), followed by gum (20.5%), cheek mucosa (19.3%), facial massif (8%), lips (6.8%), oral floor (3.4%), palate (2.3%), mandible (2.3%) and maxillary (1.1%) (Table 3).

TP53 Mutation Screening

The proportion of patients carrying at least one mutation in *TP53* was 52.27%. Sequence analysis identified 11 genomic variations, 10 Single Nucleotide Variant (SNV) and one Copy Number Variation (CNV) located in intron 3. Among identified variants, seven have been reported in Clinvar, dbSNP or gnomAD databases: c.63C>T (p.Asp=, rs1800369); c.215C>G (p.Pro72Arg, rs1042522); c.692C>T (p.Thr231Ile, rs1555525564); c.745A>T (p.Arg249Trp, rs587782082); c.773A>T (p.Glu258Asp, rs2073239956); c.776A>T (p.Asp259Val, rs745425759) and the CNV (GGGCTGGGGACCTGGA, NG_017013.2:g.16185_16200Ins, rs59758982).

We have identified four new variants not yet reported in variant databases: c.62A>C (p.Asp21Ala); c.64C>G (p.Leu22Val); c.268T>A (p.Ser90Thr) and c.773A>T (p.Glu258Val). Each of them was detected in one patient in the study population. Effect prediction with SIFT and PolyPhen highlighted possibly damaging effect (c.62A>C (p.Asp21Ala); c.773A>T (p.Glu258Val)) and benign effect (c.64C>G (p.Leu22Val); c.268T>A (p.Ser90Thr) (Table 4).

The most recurrent variants were two cancer-related variants reported in IARC *TP53* database: p.Pro72Arg (rs1042522; Arginine allelic frequency estimated at 31.26%) and the CNV located in intron 3 (rs59758982; alternative allelic frequency estimated at 26.25%). Since these variants are located closely in the genomic region spanning intron 3 to exon 4, we then tested for linkage desequilibrium (LD) and found a strong LD (Table 5).

Case/controls association study between the two most frequent variants and oral cancer predisposition did not raise any significant association in the study population (Table 6).

Discussion

Clinical characteristics of Senegalese patients diagnosed with oral cancers have been described by Touré et al in 2005 and highlighted early age at diagnosis (52.6 years), a sex ratio in favor of females (0.67), a negative alcohol-tobacco status (62.9% non-smokers and 83.8% non-alcoholics) and a poor oral hygiene [24]. This profile is quite similar to what observed in our patients. These results highlighted that tobacco and alcohol are not the common risk factors and emphasized the hypothesis that exposure to HPV and genetic factors may be associated with oral carcinogenesis in Senegalese population.

The predominant tumor histology in our patients was squamous cell carcinoma. This type represents 95% of all oral cancers tumors worldwide and is characterized by altered proliferation of dysplastic squamous cells on the surface of the epithelial layer [25, 26]. As well, the most common tumor typologies we observed were respectively tongue, gum and cheek mucosa while others studies reported tongue and palate [27, 28].

Table 2. Primer sets and annealing temperature of TP53 coding exons

Fragments length in pb	**Sequence of Primer sets**	**Annealing temperature**
Exon 2 355	**E_2F :** CAGCCATTCTTTTCCTGCTC **E_2R :** TCCCACAGGTCTCTGCTAGG	62ºC
Exons 3 & 4 765	**E_3F :** CCCCCTCTGAGTCAGGAAACA **E_4R :** ACAGGAGTCAGAGATCACACA	55ºC
Exons 5 & 6 668	**E_5F :** TGAGGTGTAGACGCCAACTCT **E_6R :** GGGAGGTCAAATAAGCAGCA	55ºC
Exon 7 237	**E_7F :** CTTGCCACAGGTCTCCCCAA **E_7R :** AGGGGTCAGCGGCAAGCAGA	60ºC
Exons 8 & 9 473	**E_8F :** TTGGGAGTAGATGGAGCCTG **E_9R :** AAACAGTCAAGAAGAAAACGGC	60ºC
Exon 10 343	**$E_{10}F$:** GCTGTATAGGTACTTGAAG **$E_{10}R$:** GCTTTCCAACCTAGGAAGGCAG	55ºC
Exon 11 324	**$E_{11}F$:** GATTTGAATTCCCGTTGTCC **$E_{11}R$:** CAAGGGTTCAAAGACCCAAA	55ºC

Table 3. Characteristics of recruited patients

Characteristics	**Parameters**	**Values**
Age	Mean age ± standard deviation	51.90 ± 17.98
	Median age [extreme values]	55 [18 - 85]
Sex-ratio		0.76 (38/50)
Smoking and alcohol status	-	73 %
	+	5.5 %
	Not specified	21.5 %
Tumor location	Tongue	22.7 %
	Gum	20.5 %
	Cheek mucosa	19.3 %
	Facial massif	8 %
	Lips	6.8 %
	Oral floor	3.4 %
	Palate	2.3 %
	Mandible	2.3 %
	Maxillary	1.1 %
	Multiple locations	11.3 %
	Not specified	2.3 %
Tumor histology	Squamous cell carcinoma	90.9 %
	Adenocarcinoma	1.1 %
	Burkitt lymphoma	1.1 %
	Not specified	6.9 %

Table 4. TP53 mutations identified in patients with oral cancer and their clinical significance

c(g)DNA description	**rs number**	**Genomic location** (GRCh38)	**Exon/ Intron location**	**Protein effect**	**Variant Clinical significance (ClinVar, dbSNP & gnomAD) vs. Effect Prediction (SIFT & PolyPhen)**	**Alternative allele frequency**
c.62A>C	**---**	**17:7676533**	**Exon 2**	**p.Asp21Ala**	**Possibly damaging (PolyPhen)**	**0.57 % (01/88)**
c.63C>T	rs1800369	17:7676532	Exon 2	p.Asp21=	Conflicting interpretations of pathogenicity (ClinVar & dbSNP)	0.57 % (01/88)
c.64C>G	**---**	**17:7676531**	**Exon 2**	**p.Leu22Val**	**Benign (PolyPhen)**	**0.57 % (01/88)**
g.16185_16200Ins	rs59758982	17:7676351_7676366Ins	Intron 3	---	Benign ; Likely-benign ; Hereditary cancer-predisposing syndrome (ClinVar & dbSNP)	26.25 % (18/40)
c.215C>G	rs1042522	17:7676154	Exon 4	p.Pro72Arg	Uncertain clinical significance ; pathogenic (ClinVar & dbSNP) ; Tolerated (SIFT) ; Benign (PolyPhen)	31.26 % (23/48)
c.268T>A	**---**	**17:7676101**	**Exon 4**	**p.Ser90Thr**	**Benign (PolyPhen)**	**1.02 % (01/49)**
c.692C>T	rs1555525564	17:7674271	Exon 7	p.Thr231Ile	Uncertain clinical significance (ClinVar & dbSNP) ; Deleterious (SIFT) ; Probably damaging (PolyPhen)	0.98 % (01/51)
c.745A>T	rs587782082	17:7674218	Exon 7	p.Arg249Trp	Uncertain clinical significance ; likely pathogenic ; Deleterious (SIFT) ; Possibly damaging (PolyPhen)	2.94 % (03/51)
c.773A>T	**---**	**17:7674190**	**Exon 7**	**p.Glu258Val**	**Probably damaging (PolyPhen)**	**0.98 % (01/51)**
c.773A>T	rs2073239956	17:7674189	Exon 7	p.Glu258Asp	Clinical significance not reported in Clinvar & dbSNP ; Probably damaging (PolyPhen)	1.96 % (02/51)
c.776A>T	rs745425759	17:7674187	Exon 7	p.Asp259Val	Uncertain significance (ClinVar & dbSNP) ; Deleterious (SIFT) ; Possibly damaging (PolyPhen)	0.98 % (01/51)

Table 5. Linkage desequilibrium calculation between the two most frequent variants

Markers	D	D'	r^2	p-value
rs59758982 vs. rs1042522	-0.079	0.999	0.153	0.000551698

Table 6. Case/control association study of rs59758982 and rs1042522 with predisposition to oral cancer

Variants	Allelic frequencies		Test of association
	Cases	Controls	
16 bp insertion rs59758982	26.25 %	27.5 %	OR = 1.07 CI $_{95\%}$ = [0.57 – 2] p = 0.841
Arg allele rs1042522	31.26 %	32.8 %	OR = 0.93 CI $_{95\%}$ = [0.51 – 1.68] p = 0,823

OR: Odds ratio CI: confidence interval

Among oral cancer etiological factors genetic alterations in specific genes controlling the cell cycle such as *TP53* have been reported. In this study, 52.27% of patients carried at least one mutation in *TP53* (46/88), similar to data from Poeta *et al* in the US (53.3%, 224/420) [29] and Yamamoto *et al* in Japanese patients (49.5%, 91/194) [30].

We identified eleven *TP53* variants in our patients including ten Single Nucleotide Variants (SNV) and one Copy Number Variation (CNV). Among these variants, four have been newly identified in this study. They were not reported in any variant database (ClinVar, dbSNP, Varsome…). Effect prediction by SIFT and PolyPhen tools showed possible clinical significance of 2 missense variants on p53 function (c.62A>C (p.Asp21Ala); c.773A>T (p.Glu258Val)). Further studies are required to assess their functional significance.

The other seven variants identified in this study have been reported in Clinvar, dbSNP and gnomAD databases. Two of them are the most frequent (p.Pro72Arg, rs1042522) and the 16 bp insertion (rs59758982). They are both cancer related-variant and their functional impact in p53 have been reported [14]. The first one (p.Pro72Arg, rs1042522) has been extensively studied as a potential risk factor for the development of malignancies and is the most common variant associated with predisposition to oral cancers. Allelic and genotypic distribution of rs1042522 are known depending on geographic latitude and ethnicity throughout the world [31-35]. The variant has been associated in case/control studies with a higher risk of oral, nasopharyngeal, lung, thyroid, skin, cervical, prostate, bladder, gastric, colorectal, and hepatic cancers [20, 36-39]. Arg72 allele frequency was estimated at 31.26% in our patients, similar to frequencies observed in African and African American populations as reported in gnomAD database (31.4% and 32.1% respectively). In contrast, frequencies reported for Asian and Caucasian populations are higher (58.4% and 73.6% respectively) confirming the ethnic and geographic bias observed in the distribution of this variant throughout the world.

For the 16 bp insertion in intron 3 (rs59758982), its implication on carcinogenesis has also been highlighted in several cancers: colorectal, breast, cervical, gastric, head

and neck, lung, bladder, esophagus, and in glaucoma [20, 40, 15]. Allelic frequency of 12% has been reported in Caucasian diagnosed with colorectal cancer [40] whereas in our study, the frequency was 2 times higher (26.25%). In contrast this variant was not detected in 425 Japanese patients with primary open angle glaucoma [41].

Association study between these two cancer-related variants did not find any significant association in our population as we previously reported in a meta-analysis [19]. However, LD test showed that they are strongly linked and could be transmitted together in a common haplotype predisposing towards oral cancers. It is therefore necessary to study the haplotype structure and investigate the role of neighboring variants spanning intron 3 to intron 4 region, especially intron 4 where an internal promoter encoding nine p53 isoforms has been located. Mutations in this promoter have been reported to affect the transcription and expression level of p53 isoforms [20, 42]. Eiholzer and colleagues have recently demonstrated in 2020 that this genomic region harbor several haplotypes blocks that could increase the level of expression of the Δ133_*TP53* transcript, which at certain levels of expression can have pro-tumor effects [43]. Therefore it would be of interest to investigate by long read target sequencing and functional analysis this genomic region in patients diagnosed with cancer, and definitely address its implication on carcinogenesis.

Conclusion

Our study highlighted that despite the absence of association between the two most common cancer-related variants of TP53 observed in Senegalese patients diagnosed with oral cancer, their strong LD suggests that they are transmitted together in a common haplotype and may be implicated in oral carcinogenesis. Further functional and genomic studies are needed to explore the implication of the genomic region spanning intron 3 to intron 4 of TP53 gene where most cancer-related variants are located.

Conflict of Interest

No potential conflicts of interest were disclosed.

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