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Sequence Variations of Human Papillomavirus Type 16 E6 and E7 Genes in Cervical Cancer Isolates from Gabon

Samira Zoa Assoumou[1,2], Luc Magloire Anicet Boumba[1,2], Angelique Ndjoyi Mbiguino[3], Barthelemy Mabika Mabika[4], Ernest Belembaogo[5], Abdelkrim Khattabi[2] and Moulay Mustapha Ennaji[1*]

[1]Laboratoire de Virologie, Microbiologie et Qualité/ETB, Faculté des Sciences et Techniques de Mohammedia, Université Hassan II Casablanca, B.P. 146, Mohammedia 20650, Maroc.
[2]Laboratoire d'Agroalimentaire et Santé, Département de Biologie Appliquée, Faculté des Sciences et Techniques, Université Hassan 1Er Settat, B.P. 577 Settat, Maroc.
[3]Laboratoire de référence MST/Sida, Laboratoire de Référence Rougeole, Rubéole et Fièvre Jaune, Département de Bactériologie et Virologie, Faculté de Médecine et des Sciences de la Santé, Université des Sciences de la Santé, Libreville, Gabon.
[4]Département d'Anatomie et de Cytologie Pathologiques, Faculté de Médecine et des Sciences de la Santé, Université des Sciences de la Santé, Libreville, Gabon.
[5]Institut de Cancérologie d'Agondjé, Libreville, Gabon.

Authors' contributions

This work was carried out in collaboration between all authors. Author SZA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors LMAB managed the analyses of the study and literature searches. Author ANM, BMM, EB, AK, MME critically revised the manuscript. All authors read and approved the final manuscript.

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**Corresponding author: E-mail: m.ennaji@yahoo.fr;*

ABSTRACT

Aims: HPV-16 variants distribution is reported to differ geographically and in their oncogenic potential for progression to cervical cancer. In this study, we investigated the HPV 16 variants distribution among women from Gabon.

Methodology: Amplification of E6 and E7 genes of 29 HPV-16 isolates was performed by using type-specific primers PCR and then directly sequenced. The sequences obtained were aligned with the HPV-16 GenBank reference sequences.

Results: Out of the 29 samples investigated, 25 were successfully amplified. In the 25 samples analyzed 9 and 3 nucleotide changes in E6 and E7 gene respectively were found. In the E6 gene, the most frequently observed mutation were C143G, G145T, T286A, A289G, C335T which led Q14D and H78Y non- synonymous amino acid variations. The others mutations found C109T; G132C; G132T; A403G were detected in 24% (6 of 25), 68% (17 of 25), 32% (8 of 25), 32% (8 of 25) respectively. The E7 gene appears to be better conserved than the E6 gene. Only 3 mutations were detected of which two were silent: T789C and T795G and the third A647G was missense mutation with substitution of Asparagine to Serine (N29S). This mutation was present in 32% (8 of 25) samples. All the variants detected in this study belonged to the Af1 (68%; 17 of 25) and Af2 (32%; 8 of 25) lineages

Conclusion: This study reported for the first time the distribution of HPV-16 E6 and E7 genetic variants in cervical cancer cases in Gabon. Our results highlight the predominance of African lineage in Gabonese population.

Keywords: HPV 16 variants; E6gene; E7 gene; cervical cancer; Gabon.

1. INTRODUCTION

Among sexually active people, infection with human papillomavirus (HPV) is a common sexually transmitted infection [1]. Epidemiological studies have clearly established HPV persistent infections with high risk (HR) oncogenic genotype as the causative etiological factor in the development of cervical cancer [2,3]. However, only a small fraction of women infected with HPV develop cervical cancer. Thus, it is admitted that additional viral or non viral risk factors are determinant in the development of malignancy [4-6]. Among the viral risk factors, a number of studies have focused on HPV 16 intratypic variations due to the high frequency of this HPV genotype in cervical cancer.

Intratypic variants have been previously defined as HPV which differ in nucleotide sequence by no more than 2% compared to the reference viral prototype [7]. However, a recent nomenclature proposal define major variants lineage by approximately 1% difference between HPV full genomes of the same type, with difference of 0.5-0.9% designated sub-lineages [8-10]. These variants are classified into six phylogenetic branches: European (E), Asian (As), Asian-American (AA), African 1(Af1), African 2 (Af2) and North-American (NA1) based on geographical origin of the population in which they were originally isolated [11,12].

Several studies based on the sequencing of E6 and E7 genes, the two major oncoprotein involved in the HPV carcinogenesis, have demonstrated that some HPV variants can affect their oncogenic potential and contribute more than others to HPV persistence infection and cervical cancer development [13,14]. Indeed any change in the sequences of these genes may lead to altered biological function in the protein encoded by these two genes and conduct to particularly aggressive natural history of the infection [10].

Therefore, importance of identification of HPV 16 variants seems to be clear for design newer preventives, diagnostics and therapeutics strategies [1]. However, although the HPV 16 variants in cervical cancer have been studied so far in others countries, especially in European and American countries, no stud related to Gabonese women has been previously conducted. The aim of this study was to examine the sequence variations in E6 and E7 genes of HPV 16 and identified the variants circulating in a population of women from Gabon, a Sub-Saharan African country.

2. MATERIALS AND METHODS

2.1 Origin of Clinical Specimens

DNA of twenty nine cervical cancer samples which were collected in the Laboratory of anatomy and cytology of the University of Health Sciences and in the cancer institute of Libreville was available in our laboratory DNA bank (Laboratory of Virology, Microbiology and Quality/ETB, Faculty of Sciences and Technics of Mohammedia, Morocco). All these samples were determined to be positive for HPV 16. Briefly, all these samples were Squamous cervical carcinoma. During samples collection, ethical approval was obtained from the Ministry of Health of Libreville under the number N° 00287/MS/SG after reviewed the study protocol.

2.2 PCR Amplification and DNA Sequencing of HPV16 -E6 and E7 Genes

Amplification of HPV 16 E6 and E7 genes specific PCR was performed as described by Garbuglia et al. [15] with primers flanking outside of the coding region of HPV E6 ORF (nt: 41-579): 5'-ATCGGTGAACCGAA-3' and 5'-AGGTGTATCTCCATGCA-3' and HPV16 E7 ORF (nt: 483-911): 5'-ATATAAGGGGTCGGTGGA-3' and 5'-TTACATCCCGTACCCTC-3'. The two PCR reactions were performed separately in 25µl volume containing 1x PCR buffer, 2.5mM MgCl2, 10 mM of DNTPs, 10 µM of forward and reverse primers, 100 ng genomic DNA and 1U of GoTaq® DNA polymerase (Promega, USA).The PCR thermal profile was: 95°C for 10 min, following by 40 cycles of 95°C for 1 min, 49.5°C for E6 or 53.5°C for E7, for 1 min, 72°C for 1 min, and final extension of 7 min at 72°C. For every reaction, ultrapure water (DNase/RNase free) was use as a negative control and DNA of SiHa cell lines was used as positive control. The amplification reactions were performed in a Perkin Elmer 2400 GeneAmpR® PCR thermal Cycler (Scientific Support, Inc, Hayward, CA). All PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide and visualized by UV light.

For DNA sequencing, the PCR products were purified by using the ExoSaP-IT clean up system (USB, USA) and the sequencing reaction was performed (with the E6 or E7 forward primer)according to the manufacturer's protocol with the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3130 XL DNA analyzer (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol in molecular and functional genomics platform (UATRS-CNRST, Rabat, Morocco).

2.3 Sequence Analysis and Phylogenetic Tree

The full length of E6 and E7 genes were aligned with the European prototype (HPV16-R; accession numbers: K02718, NC_01526) available through the GenBank database (NCBI, National Institute of health, Bethesda, MD, USA) by multiples sequences alignment using BioEdit Sequence Alignment Software v7.0.4.1 through the Clustal W program.

HPV16 variant identity was established according to the data published by Yamada et al. [16]. Phylogenetic tree was built with nucleotide sequences of the full Open reading frame of E6 (nt 104-559) by the computer software MEGA 6 package. The distance based criterion Tamura 3-parameter was used as the substitution model and Maximum Likelihood algorithm with bootstrap proportions were calculated with 1000 replicates to test the robustness of the major phylogenetic groups.

2.4 GenBank accession numbers

The nucleotide sequences from each region sequenced in this study were deposited into GenBank Database (http://www.ncbi.nlm.nih.gov/GenBank), under the accession numbers KP677553 to KP677555 for E6 and KP677556 to KP677557 for E7 genes.

3. RESULTS

3.1 HPV 16 Variants Identification

Of the 29 DNA samples evaluated, successful amplification and sequencing of E6 and E7 genes was obtained for 25 samples and 4 samples failed to be amplified. The nucleotide changes and variants in E6/E7 genes (ORF 104 to 559 for E6 region and ORF 562 to 854 for E7) of the 25 adequate samples are show in table 1. All the variants detected in this study belonged to the Af1 (68%; 17 of 25) and Af2 (32%; 8 of 25) lineages. The others lineages were not detected in our samples. Phylogenetic tree was built from three sequences of E6 region from this study and other HPV-16 published sequences available in GenBank (Fig. 1).

Table 1. Nucleotide sequence variations at E6 and E7 genes, predicted amino acid isolates among 25 HPV 16 insolates from Gabon

			E6 nucleotide position								Amino acid changes	E7 nucleotide position			Amino acid changes
			1	1	1	1	2	2	3	4		6	7	7	
			0	3	4	4	8	8	3	0		4	8	9	
			9	2	3	5	6	9	5	3		7	9	5	
		HPV-16 Ref	**T**	**G**	**C**	**G**	**T**	**A**	**C**	**A**		**A**	**T**	**T**	**-**
Variants lineage	**Variants sublineage**	**Prevalence variant n(%)**													
Af-1	Af-1a	17(68.0)		C	G	T	a	g	T	-	R10T/Q14D/H78Y	-	c	g	-
Af-2	Af-2a	6(24.0)	c	T	G	T	a	g	T	g	R10I/Q14D/H78Y	G	c	g	N29S
Af-2	Af-2a	2(8.0)		T	G	T	a	g	T	g	R10I/Q14D/H78Y	G	c	g	N29S
		Prevalence mutation n (%)	6 (24)	25 (68/32)	25 (100)	25 (100)	25 (100)	25 (100)	25 (100)	8 (32.0)		8 (32.0)	25 (100)	25 (100)	

Phylogenetic lineages are noted Af-1 for African 1 and Af-2 for African 2. Capital letters indicate variants with an amino acid change. Lower letters indicate silent mutations. In the Amino acid changes column, the letter preceding the amino position refers to the reference sequence and the letter after refer to the substitution. Dashes indicate no mutation

3.2 E6 Gene Sequence Variations

The E6 gene revealed 9 nucleotide variations and all isolates showed a common pattern of 5 mutations, namely C143G, G145T, T286A, A289G and C335T (Fig. 2). The others mutations found C109T; G132C; G132T; A403G were detected in 24% (6 of 25), 68% (17 of 25), 32% (8 of 25), 32% (8 of 25) respectively. Concerning the coding amino acids, the nucleotide change at position 109 (T109C), 286 (T286A), 289 (A289G) and 403 (A403G) were silent mutations at codon 2 (Phenylalanine), 61 (Alanine), 62 (Valine) and 100 (Leucine) respectively. The others nucleotide change conducted to alteration in the coding amino acids. These others nucleotide change were missense mutations and lead to the following amino acid variation: R10T (G132C), R10I (G132T), Q14D (C143G and G145T), and H78Y (C335T) (Table 1).

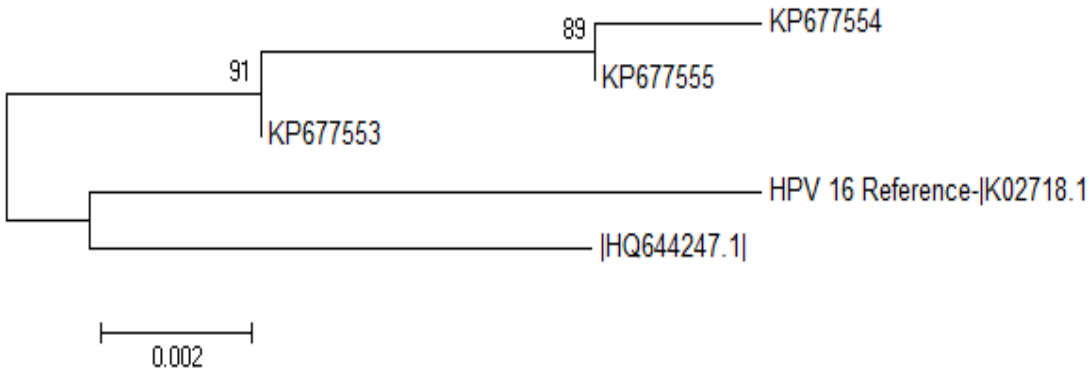

Fig. 1. Phylogenetic tree of HPV 16 E6 sequences isolate from Gabonese women

Study sequences are labeled in KP GenBank accession numbers. Others are reference GenBank sequences, K02718.1 (HPV-16 Reference) and HQ644237 (Lineage Asian-American). Phylogenetic trees were constructed by the Maximum Likelihood method and the Kimura 3-Parameter model by MEGA package. Bootstrap proportions were calculated with 1000 replicates.

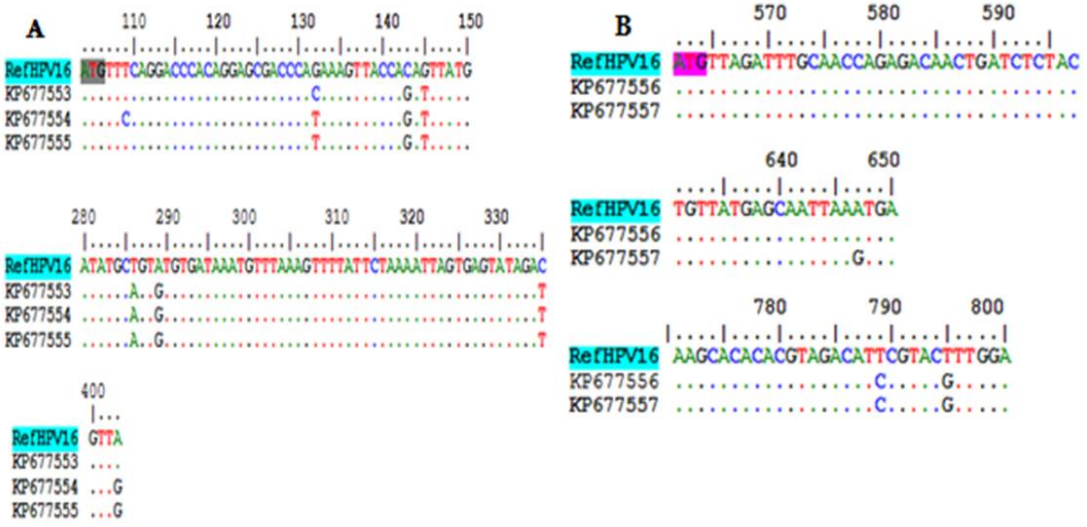

Fig. 2. Multiple sequence alignment by clustal W program

A: E6 gene, the nucleotide changes are in the position 109, 132, 143, 145, 286, 289, 335 and 403. B: E7 gene, the nucleotide changes are in position 647, 789 and 795. The codon start in the E6 gene is in grey. The codon start in the E7 gene is in pink. Points represent no mutation. The HPV 16 reference is in blue

3.3 E7 Gene Sequence Variations

The E7 gene appears to be better conserved than the E6 gene. Only 3 mutations were detected (Fig. 2) of which two were silent: T789C and T795G for the amino acids Isoleucine and Threonine at codon 76 and 78 respectively. These two mutations were found in all samples. The remaining one nucleotide change, A647G was missense mutation with substitution of Asparagine to Serine (N29S). This mutation was present in 32% (8 of 25) samples.

4. DISCUSSION

Data on identification of HPV variants are important for the development of improved HPV diagnosis tests and would facilitate the design of therapeutics and vaccines [17]. In Gabon, no previous study related to the HPV variant distribution has been conducted. Then, the main objectives of this study were to sequence HPV 16 E6 and E7 genes to show the distribution patterns of HPV 16 variants among Gabonese women for the first time.

Gabon is a sub Saharan Africa country located in the middle of Africa and has a population of around 1.5 million people. Our country is close to Equatorial Guinea in northwest, Congo-Brazzaville on the East and South, and Cameroun to the North. Previous studies have demonstrated that the distribution of HPV variants is related to geographic or race distribution [13,18] and therefore, owing to the geographic location of Gabon, we expected the predominance of African variants. Analysis of the E6 ad E7 nucleotide sequences of the 25 samples clearly demonstrated the predominance of African lineage in Gabon. Our results are in line with some of the few African studies [19,20] but no with some others. Indeed, others phylogenic variants were also found in several African countries. In South Africa, Tu et al. [21] reported a predominance of European variants in 79% of cases followed by African and Asian-American variants respectively in 14% and in 7% of cases. The predominance of European variant was also reported by Qmichou et al. [22] in cervical cancer cases in Morocco followed by African (30.1%) and North American (11.6%). Authors have reported that the introduction of some HPV variants from one continent to another can be a result of the people migration [23]. The fact that only African variants were isolated from cervical cancer cases in our country could indicate that other variants seems to be not responsible of enhancing oncogenic potential in our population. However, further investigations by a large population-based study are needed to clarify this hypothesis.

In our study, the two main subgroups of the African lineage (Af-1 and Af-2) were detected respectively in 68% and 32% of samples. In line with this, our finding are in adequacy with some studies conducted in Africa. Indeed, in a study conducted in Uganda, Afr-1 group was indentified in all the samples [19]. In Morocco, Qmichou et al. [20] reported 35.5% of the Afr-1 group and 29% of the Afr-2 group. These results confirm those found by Cornet et al. [24] in his study conducted worldwide and showed the predominance of Af-1 subgroup in our continent.

HPV16 E6 and E7 gene analysis revealed that all the cases exhibited at least one specific nucleotide variation in the E6 gene. This finding is in agreement with published results reporting that approximately 90% of the E6 gene in cervical cancer cases contained variations while the E7 gene seems to be more conserved [17, 25-27]. E6 nucleotide variations showed in all samples a pattern of 5 mutations which characterized the African lineage: C143G, G145T, T286A, A289G and C335T. Among these mutations, three are significant and lead to non-synonymous amino acid changes Q14D(C143G and G145T) and H78Y (C335T). The others non-synonymous E6 variants identified among our samples were located at codon 10 (R10T, G132C and R10I, G132T) in 32% of samples. The amino change at codon 10 and 14 might induce strong selective pressure upon the E6 gene by acting in the ability of the E6 oncoprotein to interact with p53. This affinity significantly altered the degradation of E6 by p53 [28,29].

Compared with E6 gene variations, the HPV 16 E7 geneproduct is more conservative in terms of its amino acid change. Our results showed only one spot of E7 nucleotide variations in 25% of samples: nt A647G resulting in N29S to amino acid change. The two others mutations found were silent (T789C and T795G). The N29S mutation was particularly reported in some Asian countries [6,30,31] but was also found in some African studies [20,25]. This mutation is likely to be significant and may be associated with a higher oncogenic risk because it location is in an immunoreactive region.

5. CONCLUSION

The present study constitutes the first database about HPV 16 variant in Gabon and may form a basis for further investigations with more samples in our country. In the present study it was demonstrated the exclusive distribution African lineage in Gabonese cervical cancer samples. We suggested that these variant could have enhanced oncogenic potential. However, future studies related to the relations about HPV 16 variants and the developments of cervical cancer in Gabon are needed.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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