

Original Research Article

Sequences variations of Human Papillomavirus type 16 E6 and E7 genes in cervical cancer isolates from Gabon

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, E6 gene, E7 gene, cervical cancer, Gabon.

1. INTRODUCTION

Among sexually active people, infection with human papillomavirus (HPV) is one of 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 **conduce** to particularly aggressive natural history of the infection [10]

Therefore, importance of identification of HPV 16 variants **seems clear for design** newer preventives, diagnostics and therapeutics strategies[1]. However, although the HPV 16 variants in cervical cancer have been **carry out** so far in others countries, especially in European and American countries, no study related to Gabonese women have been previously realized. The aim of this study was to examine the sequences 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. MATERIAL AND METHODS

2.1. Origin of clinical specimens

DNA of twenty nine cervical cancer samples **collected to the Laboratory of** anatomy and cytology of the University of Health Sciences and in the cancer institute of Libreville was available from 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 HPV16. 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 25 µl 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 used 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 GeneAmp® PCR thermal Cyclor (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 multiple 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 shown 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 other 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).

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. The other 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 other nucleotide change conducted to alteration in the coding amino acids. These other nucleotide changes were missense mutations and led to the following amino acid variation: R10T (G132C), R10I (G132T), Q14D (C143G and G145T), and H78Y (C335T) (Table 1).

Variants lineage	Variant sublineage	Prevalence variant n(%)	E6 nucleotide position								Aminoacid changes	E7 nucleotide position			Aminoacid 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	-
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)	

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

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116 *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*
117 *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*
118 *mutation.*

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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.

3.3. E7 gene sequence variations

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 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 and 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 not with some others. Indeed, other phylogenetic 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 other 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 seem 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 findings are in adequacy with some studies conducted in Africa. Indeed, in a study conducted in Uganda, Af-1 group was identified in all the samples[19]. In Morocco, Qmichou et al. [20] reported 35.5% of the Af-1 group and 29% of the Af-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 gene product 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 its 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.

REFERENCES

1. Chimeddorj B, Pak CY, Damdin A, Okamoto N, Miyagi Y: **Distribution of HPV-16 intratypic variants among women with cervical intraepithelial neoplasia and invasive cervical cancer in Mongolia.** *Asian Pacific journal of cancer prevention : APJCP* 2008, **9**(4):563-568.
2. Harald ZH: **Human papillomaviruses in the pathogenesis of anogenital cancer.** *Virology* 1991, **184**(1):9-13.
3. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N: **Human papillomavirus is a necessary cause of invasive cervical cancer worldwide.** *The Journal of pathology* 1999, **189**(1):12-19.
4. Jiang P, Liu J, Zeng X, Li W, Tang J: **Association of TP53 codon 72 polymorphism with cervical cancer risk in Chinese women.** *Cancer genetics and cytogenetics* 2010, **197**(2):174-178.
5. Gudleviciene Z, Didziapetriene J, Ramael M, Uleckiene S, Valuckas KP: **Human papillomavirus and p53 polymorphism in Lithuanian cervical cancer patients.** *Gynecologic oncology* 2006, **102**(3):530-533.
6. Wu Y, Chen Y, Li L, Yu G, He Y, Zhang Y: **Analysis of mutations in the E6/E7 oncogenes and L1 gene of human papillomavirus 16 cervical cancer isolates from China.** *The Journal of general virology* 2006, **87**(Pt 5):1181-1188.
7. Bernard HU, Chan SY, Manos MM, Ong CK, Villa LL, Delius H, Peyton CL, Bauer HM, Wheeler CM: **Identification and assessment of known and novel human papillomaviruses by polymerase chain reaction amplification, restriction fragment length polymorphisms, nucleotide sequence, and phylogenetic algorithms.** *The Journal of infectious diseases* 1994, **170**(5):1077-1085.
8. Burk RD, Chen Z, Harari A, Smith BC, Kocjan BJ, Maver PJ, Poljak M: **Classification and nomenclature system for human Alphapapillomavirus variants: general features, nucleotide landmarks and assignment of HPV6 and HPV11 isolates to variant lineages.** *Acta dermatovenerologica Alpina, Pannonica, et Adriatica* 2011, **20**(3):113-123.

- 219 9. Chen Z, Schiffman M, Herrero R, Desalle R, Anastos K, Segondy M, Sahasrabudde VV, Gravitt
220 PE, Hsing AW, Burk RD: **Evolution and taxonomic classification of human papillomavirus 16**
221 **(HPV16)-related variant genomes: HPV31, HPV33, HPV35, HPV52, HPV58 and HPV67.** *PloS*
222 *one* 2011, **6**(5):e20183.
- 223 10. Chen Z, Terai M, Fu L, Herrero R, DeSalle R, Burk RD: **Diversifying selection in human**
224 **papillomavirus type 16 lineages based on complete genome analyses.** *Journal of virology*
225 2005, **79**(11):7014-7023.
- 226 11. Yamada T, Wheeler CM, Halpern AL, Stewart AC, Hildesheim A, Jenison SA: **Human**
227 **papillomavirus type 16 variant lineages in United States populations characterized by**
228 **nucleotide sequence analysis of the E6, L2, and L1 coding segments.** *Journal of virology*
229 1995, **69**(12):7743-7753.
- 230 12. Yamada T, Manos MM, Peto J, Greer CE, Munoz N, Bosch FX, Wheeler CM: **Human**
231 **papillomavirus type 16 sequence variation in cervical cancers: a worldwide perspective.**
232 *Journal of virology* 1997, **71**(3):2463-2472.
- 233 13. Xi LF, Kiviat NB, Hildesheim A, Galloway DA, Wheeler CM, Ho J, Koutsky LA: **Human**
234 **papillomavirus type 16 and 18 variants: race-related distribution and persistence.** *Journal*
235 *of the National Cancer Institute* 2006, **98**(15):1045-1052.
- 236 14. Xi LF, Koutsky LA, Hildesheim A, Galloway DA, Wheeler CM, Winer RL, Ho J, Kiviat NB: **Risk for**
237 **high-grade cervical intraepithelial neoplasia associated with variants of human**
238 **papillomavirus types 16 and 18.** *Cancer epidemiology, biomarkers & prevention : a*
239 *publication of the American Association for Cancer Research, cosponsored by the American*
240 *Society of Preventive Oncology* 2007, **16**(1):4-10.
- 241 15. Garbuglia AR, Carletti F, Minosse C, Piselli P, Zaniratti MS, Serraino D, Capobianchi MR:
242 **Genetic variability in E6 and E7 genes of human papillomavirus -16, -18, -31 and -33 from**
243 **HIV-1-positive women in Italy.** *The new microbiologica* 2007, **30**(4):377-382.
- 244 16. T Yamada MMM, J Peto, C E Greer, N Munoz, F X Bosch, and C M Wheeler: **Human**
245 **papillomavirus type 16 sequence variation in cervical cancers: a worldwide perspective.** *J*
246 *Virol* 1997, **71**(3):2463-2472.
- 247 17. Pande S, Jain N, Prusty BK, Bhambhani S, Gupta S, Sharma R, Batra S, Das BC: **Human**
248 **papillomavirus type 16 variant analysis of E6, E7, and L1 genes and long control region in**
249 **biopsy samples from cervical cancer patients in north India.** *Journal of clinical microbiology*
250 2008, **46**(3):1060-1066.
- 251 18. de Araujo Souza PS, Sichero L, Maciag PC: **HPV variants and HLA polymorphisms: the role of**
252 **variability on the risk of cervical cancer.** *Future Oncol* 2009, **5**(3):359-370.
- 253 19. Buonaguro FM, Tornesello ML, Salatiello I, Okong P, Buonaguro L, Beth-Giraldo E,
254 Biryahwaho B, Sempala SD, Giraldo G: **The uganda study on HPV variants and genital**
255 **cancers.** *J Clin Virol* 2000, **19**(1-2):31-41.
- 256 20. Qmichou Z, Ennaji MM, Amrani M, El Fahime EM, Melloul M, Meftah El khair M, El Mzibri M,
257 Khyatti M, Attaleb M: **Molecular Characterization of HPV16 E6 and E7 Variants among**
258 **Women with Cervical Cancer in Morocco.** *British Microbiology Research Journal* 2013, **3**
259 **(4):692-705.**
- 260 21. Tu JJ, Kuhn L, Denny L, Beattie KJ, Lorincz A, Wright TC, Jr.: **Molecular variants of human**
261 **papillomavirus type 16 and risk for cervical neoplasia in South Africa.** *International journal*
262 *of gynecological cancer : official journal of the International Gynecological Cancer Society*
263 2006, **16**(2):736-742.
- 264 22. Qmichou Z, Khyatti M, Berraho M, Ennaji MM, Benbacer L, Nejari C, Benjaafar N, Benider A,
265 Attaleb M, El Mzibri M: **Analysis of mutations in the E6 oncogene of human papillomavirus**
266 **16 in cervical cancer isolates from Moroccan women.** *BMC infectious diseases* 2013,
267 **13**(1):378.
- 268 23. Chan SY, Ho L, Ong CK, Chow V, Drescher B, Durst M, ter Meulen J, Villa L, Luande J, Mgaya
269 HN *et al*: **Molecular variants of human papillomavirus type 16 from four continents suggest**

- 270 **ancient pandemic spread of the virus and its coevolution with humankind.** *Journal of*
271 *virology* 1992, **66**(4):2057-2066.
- 272 24. Cornet I, Gheit T, Franceschi S, Vignat J, Burk RD, Sylla BS, Tommasino M, Clifford GM:
273 **Human papillomavirus type 16 genetic variants: phylogeny and classification based on E6**
274 **and LCR.** *Journal of virology* 2012, **86**(12):6855-6861.
- 275 25. Eschle D, Durst M, ter Meulen J, Luande J, Eberhardt HC, Pawlita M, Gissmann L:
276 **Geographical dependence of sequence variation in the E7 gene of human papillomavirus**
277 **type 16.** *The Journal of general virology* 1992, **73** (Pt 7):1829-1832.
- 278 26. Icenogle JP, Sathya P, Miller DL, Tucker RA, Rawls WE: **Nucleotide and amino acid sequence**
279 **variation in the L1 and E7 open reading frames of human papillomavirus type 6 and type**
280 **16.** *Virology* 1991, **184**(1):101-107.
- 281 27. Zehbe I, Voglino G, Delius H, Wilander E, Tommasino M: **Risk of cervical cancer and**
282 **geographical variations of human papillomavirus 16 E6 polymorphisms.** *Lancet* 1998,
283 **352**(9138):1441-1442.
- 284 28. Stöppler MC, Ching K, Stöppler H, Clancy K, Schlegel R, Icenogle J: **Natural variants of the**
285 **human papillomavirus type 16 E6 protein differ in their abilities to alter keratinocyte**
286 **differentiation and to induce p53 degradation.** *Journal of virology* 1996, **70**(10):6987-6993.
- 287 29. Ellis JRM, Etherington I, Galloway D, Luesley D, Young LS: **Antibody responses to HPV16**
288 **virus-like particles in women with cervical intraepithelial neoplasia infected with a variant**
289 **HPV16.** *The Lancet* 1997, **349**(9058):1069-1070.
- 290 30. Fujinaga Y, Okazawa K, Nishikawa A, Yamakawa Y, Fukushima M, Kato I, Fujinaga K: **Sequence**
291 **variation of human papillomavirus type 16 E7 in preinvasive and invasive cervical**
292 **neoplasias.** *Virus genes* 1994, **9**(1):85-92.
- 293 31. Stephen AL, Thompson CH, Tattersall MH, Cossart YE, Rose BR: **Analysis of mutations in the**
294 **URR and E6/E7 oncogenes of HPV 16 cervical cancer isolates from central China.**
295 *International journal of cancer Journal international du cancer* 2000, **86**(5):695-701.