RESEARCH ARTICLE

Situation of HPV16 E2 Gene Status During Radiotherapy Treatment of Cervical Carcinoma

Saloua Kahla[1], Lotfi Kochbati[2], Mongi Maalej[2], Ridha Oueslati[1]*

Abstract

Background: Human papillomavirus (HPV) integration within the E2 gene has been proposed as a critical event in cervical carcinogenesis. This study concerned whether HPV16 status and E2 gene intactness are predictive of radiation response in patients with cervical cancer. Materials and Methods: Biopsies of 44 patients with cervical cancer were collected before or after radiotherapy. The presence of HPV16 was assessed by polymerase chain reaction (PCR) using specific primers for the L1 region. E2 disruption was detected by amplifying the entire E2 gene. Results: HPV16 DNA was found in 54.5% of the clinical samples. Overall, 62.5% of the HPV16 positive tumors had integrated viral genome and 37.5% had episomal genome. There was a tendency of increase of HPV16 E2 negative tumors compared with HPV16 L1 ones in advanced stages (75% versus 20% in stage III respectively). Detection of E2 gene appeared influenced by the radiotherapy treatment, as the percentage of samples containing an intact HPV16 E2 was more frequent in pretreated patients compared to radiotherapy treated patients (66.6% versus 20%). The radiation therapy caused an eight-fold [OR= 8; CI=1.22-52.25; p=0.03] increase in the risk of HPV16 genome disruption. The integration status is influenced by the irradiation modalities, interestingly E2 disruption being found widely after radiotherapy treatment (75%) with a total fractioned dose of 50Gy. Conclusions: This study reveals that the status of the viral DNA may be used as a marker to optimize the radiation treatment.

Keywords: HPV16 - PCR - L1 gene - E2 gene - radiotherapy

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Introduction

A causal association of high risk HPV persistent infections with cervical cancer is firmly established by epidemiological and experimental evidence (Parkin et al., 2005). Nearly all women will become infected with HPV during their lifetime but only minority will progress to invasive disease. Thus, additional risk factors are required which can be environmental, host- or virus-related factors such as HPV type, integration or viral load for progression (Castellsague and Muñoz, 2003). Carcinogenic progression is accompanied frequently by integration of the viral genome into the host genome, which leads to disruption of the E2 gene region (Schmitz et al., 2012; Li et al., 2013), while the E6 and E7 ORFs and long control region (LCR) remain intact. The preferential integration within E2 ORF is considered merely to be the consequence of its facile accessibility to breaks and different kind of genetic rearrangements. The papillomavirus E2 proteins have been shown to exert many functions in the viral cycle including pivotal roles in transcriptional regulation and in viral DNA replication (Bellanger et al., 2011). Indeed E2 protein controls the transcription of the oncogenes E6 and E7 which manipulate the cell cycle and the ability to undergo apoptosis (Desaintes et al., 1999; Lindel et al., 2012), and there may be a possible correlation between E2 status and radiotherapy effectiveness (Lindel et al., 2006).

Radiation therapy remains a key component of modern multimodal anticancer treatment approaches (Delgado et al., 2009; Le Tinier et al., 2012). In fact ionizing radiation exposure of cervical cells causes a spectrum of lesions within the cells and at the DNA level (Vozenin et al., 2010), these circumstances prompted us to investigate the influence of episomal virus DNA on phenotype of the tumor and the effect of radiotherapy treatment on the molecular presence of HPV-16 L1 and the E2 gene intactness.

Materials and Methods

Study population and treatment

A retrospective study was performed on cervical cells obtained from biopsies; these samples were provided by 44 patients with cervical cancer, before or after radiotherapy treatment, coming all to consult at Salah Azaiez Institute

*[1]Unit of Immunology Microbiology Environmental and Carcinogenesis (IMEC), Science Faculty of Bizerte, University of Carthage, Jarzouna, Bizerte, [2]Radio-Oncology Department, Salah Azaiez Institute, Tunis, Tunisia *For correspondence: oueslatiridha12@hotmail.fr*

between January 2007 and July 2009. The medium age of cervical patients was 57 with a range of 38-76 years. Histology consisted of 39 squamous cell carcinomas and 5 adenocarcinomas. Clinically, the tumors were classified in different stages according to the guidelines of the International Federation of Obstetrics and Gynecology (FIGO). All patients underwent pelvic external beam radiation therapy using 18MV linear accelerators (Varian Medical System). Radiation was delivered to the tumor zone in a total dose varying from 45 to 50Gy of in 25 fractions (1.8 to 2Gy/fraction) according to tumor stage.

DNA isolation

Genomic DNA was extracted from frozen tissues using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with detergent lysis buffer and proteinase K digestion. Finally target DNA was purified and quantified by spectrophotometry at 260nm.

HPV16 screening

The presence of HPV16 in cervical cells was detected by PCR using specific primers which were designed to amplify the latent L1 conserved region (synthesized by GENECUST, France). The HPV16 specific primers were as follows: Forward: 5'-GCAAGCAACAGTTACTGCGACGT-3' and Reverse: 5'-GCAACAAGACATACATCGACCGG-3' generating a 301 bp PCR product. The PCR was performed in a final reaction volume of 100μL reaction mixture containing 100 ng of DNA sample, 1.5μmol of each primer, 1.5mmol Mg Cl2, 50 mmol KCl, 10 mmol Tris HCl, 200μmol of each dNTPs (deoxynucleoside triphosphate) and 2.5 U of taq polymerase (Fermentas). Cycling parameters for all samples were as follows: an initial 5 min denaturation at 94°C, followed by 40 cycles of 94°C for 1min, 1min for 58°C, and 72°C 1min, a final elongation step of 10 min at 72°C. Each PCR experiment was performed with a negative control (water) and the appropriate positive controls for HPV16 (HPV16 Plasmids). The adequacy of the samples and the absence of PCR inhibitors were monitored by preliminary PCR amplifications with primers recognizing the human ß-globin gene (Forward: 5'-CAACTTCATCCACGTTCACC-3', Reverse: 5'-GAAGAGCCAAGGACAGGTAC-3', 268 bp product) (Yang et al., 2004). All the samples gave adequate quality of genomic DNA. At last PCR were examined by electrophoresis on 2% agarose gels stained with ethiduim bromide, visualized under ultraviolet light, and photographed.

Physical status of viral genome

The integration of HPV16 was detected essentially as already described in detail in a study on HPV physical status in genital tumors (Bhattacharjee and Sengupta, 2006). Briefly, to distinguish the pure integrated viral DNA forms from the circular DNA form, the integrity of HPV16 genomes was analysed by amplifications with type specific primers (W1: 5'-ATGAAAATGATAGTACAGAC-3', W2: 5'-CCAGTAGACACTGTAATAG-3', 1026 bp) recognizing the almost entire E2 ORF sequences that is the region most frequently disrupted or deleted during the viral integration into the cell genome.

Statistics

The associations between the variables were assessed using Fisher's exact test. Odds Ratios (OR) and corresponding 95% Confidence Intervals (CI) were estimated by logistic regression models. A stepwise logistic regression analysis was used to assess the simultaneous effect of more than one variable and to identify for possible confounding factors. In all analyses, probability values less than 0.05 were regarded as significant (SPSS, version 18.0).

Results

Prevalence of HPV16 DNA in cervical cancer cells and E2 physical status

Analyses were performed stepwise. First, the quality of the DNA preparation was assessed by PCR using β-globin primers. All DNA preparations were β-globin positive and thus adequate for further analysis. DNA from clinical samples was then assessed first by PCR using the specific primers for the HPV16 L1 region. In Figure 1 HPV16 detection results, distributions of physical forms are reported. Among the 44 DNA samples suitable for HPV analysis, HPV16 DNA was detected in 24 of 44 (54.5%) biopsies specimens. The physical status of HPV16 DNA was assessed by amplification of the E2 gene analysed by PCR assay. Among the HPV16 positive DNA samples, integrated and episomal forms were identified in 15 (62.5%) and 9 cases (37.5%), respectively. Figure 2 shows the distribution of HPV16 L1/E2 negative tumors in different stages of cervical carcinoma. In early stages the HPV16 L1/E2 negative tumors have nearly the same rates (48.7% versus 60% in stage (I,II) respectively). Conversely the HPV 16 E2 negative tumors were apparently increased in advanced stage, compared with HPV 16 L1 negative tumors (75% versus 20% in stage III respectively).

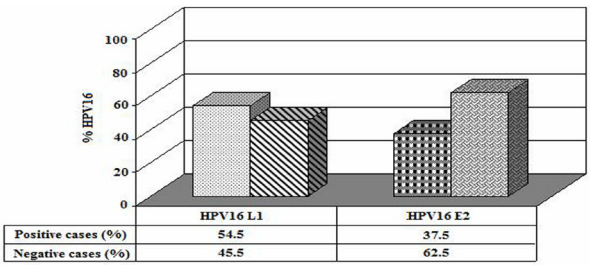

Figure 1. Frequency Distribution of HPV16 L1 and HPV16 E2 Rates in Cervical Cancer Cells

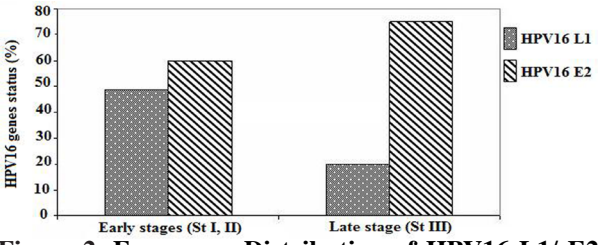

Figure 2. Frequency Distribution of HPV16 L1/ E2 Negative Tumors in Different Cervical Cancer Stages

The relationships between HPV16 status and clinical characteristics

HPV16 positive tumors were found in 54.5% (24 cases) of the patients, and HPV16 negative ones were found in 45.5% (20 cases). The relationships between HPV16 status and age, stage and histology are summarized in Table 1. Statistical analysis using the Fisher's exact test revealed no significant associations between HPV infection and clinicopathologic data (p>0.05).

Impact of radiation treatment on the molecular detection of HPV16

The study was completed by the genomic viral state analysis based on radiotherapy treatment (Table 2, Figure 3): In the subgroup treated by radiotherapy (29 patients): 51.7% showed a persistent viral presence (15/29 patients) and 48.2% were HPV16 L1 negative (14/29 patients). Whereas in the subgroup collected before treatment (15 patients): 60% of cervical tumor samples (9/15 patients) showed viral presence and 40% were HPV16 L1 negative. We observed a non-significant correlation between the presence of viral HPV16 L1 DNA and the therapeutic condition of patients (p=0.4).

Impact of radiotherapy on the intactness of the viral genome

In the subgroup treated by radiotherapy of HPV16 L1 positive patients (15 patients): 20% of the lesions (3/15 patients) contained the episomal form with an intact E2 gene while 80% (12/15 patients) showed disruption of E2 gene (Table 3). In the subgroup collected before radiotherapy treatment of HPV16 L1 positive patients (9 patients), the trend was reversed: as 66.6% (6/9 patients) showed episomal form of the HPV16 DNA, whereas 33.3% (3/9 patients) contained integrated form with E2 disruption. There was a significant correlation between the presence of the viral E2 gene of HPV16 and therapeutic condition of patients (p=0.03) (Table 3, Figure 3). The radiation therapy caused an eight-fold [OR=8; CI=1.22-52.25; p=0.03] increase in the risk of HPV16 genome disruption. Irrespective of E2 status patients treated with radiotherapy were found to harbor higher integrated forms, compared to those collected before radiotherapy treatment.

Table 1. Bivariate Analysis of HPV16 Status with Clinicopathologic Data

Patients	Total	Status of HPV16 DNA HPV	HPV	*p value
Parameter	n=44	16+(%)	16-(%)	
Age				
≤64 years	30	17 (56.6)	13 (43.3)	0.7
>64 years	14	7 (50)	7 (50)	
FIGO stage				
Early(I/II)	39	20 (51.2)	19 (48.7)	0.3
Late (III)	5	4 (80)	1 (20)	
Histology				
Squamous cell carcinoma	39	23 (58.9)	16 (41)	0.1
Adenocarcinoma	5	1 (20)	4 (80)	

*P: two-sided Fisher's exact test

Table 2. HPV16 L1 Positivity According Radiation Therapy

Patient therapeutic status	Total N	HPV16 L1 Positive(%)	Negative(%)
After radiotherapy treatment*,**	29	15 (51.7)	14 (48.2)
Before radiotherapy treatment*, **	15	9 (60)	6 (40)

*p: two-sided Fisher's exact test, p=0.4; **OR (95% CI)= 0.7 (0.2-2.51)

Table 3. HPV16 E2 Positivity According Radiation Therapy

Patient therapeutic status	HPV16 L1+ Total N	HPV16 E2 Positive(%)	Negative(%)
After radiotherapy treatment*,**	15	3 (20)	12 (80)
Before radiotherapy treatment*, **	9	6 (66.6)	3 (33.3)

*p: two-sided Fisher's exact test, p=0.03; **OR (95% CI)= 8 (1.22-52.25))

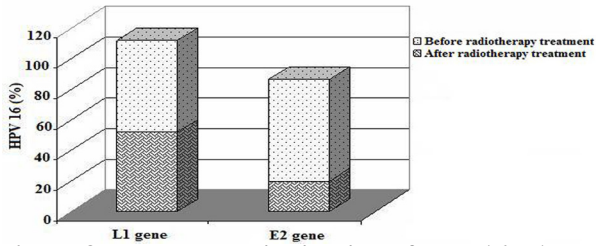

Figure 3. Frequency Distribution of HPV16 L1 and HPV16 E2 According to Radiotherapy Treatment

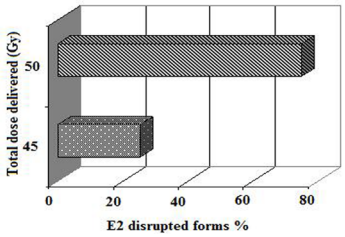

Figure 4. HPV16 E2 Disruption According to the Total Dose Delivered during Radiation Treatment

Influence of radiation doses on the genome integrity

Finally, the HPV16 E2 genome integrity according to the therapeutic modalities was reported in Figure 4. The proportion of HPV16 disruption clearly augments in accordance to the total dose delivered. Genome disruption was found in 25% at 45Gy, although the percentage of integrated forms increase with a rate of 75% at 50Gy.

Discussion

Cervical carcinomas develop as a result of multiple genetic alterations, and specific alteration lead to specific clinical behaviour. The involvement of HPV in the development of carcinomas of the uterine cervix has been firmly established (Micalessi et al., 2011). In the case of cervical carcinoma, there is an obvious need to study the effects of HPV infection on intrinsic tumor cell radiosensitivity (Harima et al., 2001). However, the literature detailing the differences in prognosis between patients with HPV 16-positive and HPV16-negative tumors has yielded conflicting results (Wang et al., 2010; Lai et al., 2013). Tumors that lack oncogenetic HPV may have a larger number of gene mutations coding for cell cycle regulating proteins in order to be transformed, and thus may be more therapy resistant. Several studies

reported a decreased rate of p35 mutations among HPV-positive tumors (Gillison et al., 2000; Lindel et al., 2005), and HPV-positive tumors appear to express significant amounts of functional p35, which may contribute to the cellular radioresponse. In our series, HPV16-positive tumors were found in 54.5% and HPV16 negative in 55.5%. In the group of cervical cancer patients, HPV16 infection seems to have a positive influence on the success of radiotherapy treatment. Although disruption of the viral E2- gene has been shown to be associated with poor outcome in patients with cervical cancer (Lindel et al. 2006). Not only in cervical cancer, but also in HPV-positive head and neck cancer E2-protein may be relevant for treatment success (Lindel et al., 2001). There is still a debate about direct influence of HPV on radiosensitivity. Because the referred interference of E2-protein with regulation of apoptosis and cell cycle control (Thierry and Demeret, 2008; Thierry, 2009). Overall, our results show 62.5% of tumors had integrated HPV16 and 37.5% had episomal HPV16. Our study showed that HPV16 displayed an integrated form in most of cervical cancer cases. The integration was 60% in early stages and 75% in late stages. In agreement with our findings, several earlier studies have stated an association between E2 disruption and tumor development and progression (Cricca et al., 2009; Wang et al., 2013). The loss of the E2-gene causes reduced proapoptotic signals, as well as deregulation of expression of the oncogenes E6 and E7 (Alazawi et al., 2004). Radioresistence may be related to aneuploidy and inhibition of apoptosis by E6 in E2-disrupted cells. Gammoh et al. could demonstrate that activity of E7 can be controlled through a direct interaction with E2, resulting in an inhibition of the activity of E7 (Gammoh et al., 2006) and that E7-induced degradation of pRb was rescued (Gammoh et al., 2009). Induction of apoptosis is caused by accumulation of E2 in the cytoplasm and involves caspase 8 activation (Thierry and Demeret, 2008). All of these observations highlight the potential influence of the E2 protein on cancer cell behaviour and the radiotherapy treatment success. Although ionizing radiation causes production of ionized molecules within biological tissues. These ionized molecules are highly reactive and trigger a rapid cascade of damage affecting all cell molecules. Low dose rate radiotherapy led to increased levels of p53 and p21 and enhanced cell cycle arrest in G1 and G2 in wt p53 expressing cells (Vozenin et al., 2010). These circumstances prompted us to investigate the impact of radiotherapy on genome integrity targeting the HPV16 L1 and E2 genes. PCR assay involves amplification of HPV16 DNA by primers that bind to highly conserved regions within the L1 open reading frame (Castle et al., 2002). In order to rule out the possibility that the HPV late 1 region did not integrate into the cellular genomic DNA, and therefore could not be detected by PCR and can be used as a control. Although, the E2 region is prone to breaks and different kinds of genetic rearrangements and therefore can be the best target for the ionizing radiation. Our findings suggest that pre and postradiotherapy patients have approximately the same percentage of HPV16 L1 (51.7% versus 60% respectively), consequently the L1 gene integrity can not be useful to be used as a biomarker of radiotherapy treatment. Nagai et al. have only found HPV prevalence (57%) among postradiotherapy patients and its association with recurrence of the disease (Nagai et al. 2000). Although, the radiation therapy caused an eight-fold [OR=8; CI=1.22-52.25; p=0.03] increase in the risk of HPV16 E2 genome disruption. Radiotherapy is the dose limited and over dosage will cause deadly damage for patients (But-Hadzic et al., 2011). However, as we analyzed a group of patients, with a range of treatment techniques and radiation doses; we determined that the viral genome disruption might be caused by the high dose of radiotherapy delivered on the tumor cells. Data showed that disruption was frequently observed with a dose of 50Gy (2Gy/ Fraction, 75%) than a dose of 45Gy (1.8Gy/ Fraction 25%). This suggests that E2-gene status after radiotherapy treatment is dose dependent, because disruption is required for a dose of 50Gy. Here we report the first study in which an HPV16 infection status has been examined with radiotherapy treatment. Santin et al. have reported that high doses of IR (12.5-100Gy) could increase E6/E7 expression in cervical carcinoma cell lines (Santin et al., 1998).

A better understanding of the molecular biology and radiobiology of cervical cancer will likely lead to the identification of new therapeutic interventions.

However, this study is the first study to analyze the impact of ionization radiation on the genome integrity, in contrast to the studies discussed above. It also point out that E2 gene status might be a promising and new target as a prognostic biomarker of radiotherapy treatment effectiveness.

In summary, further investigations will be required in order to define more precisely the impact of ionizing radiation on the quality of viral genome, then on the viral dynamic, and this might offer the best strategies for therapeutic target.

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