ORIGINAL ARTICLE

ASSOCIATION OF RELATIVE TELOMERE LENGTH AND RISK OF HIGH HUMAN PAPILLOMAVIRUS LOAD IN CERVICAL EPITHELIAL CELLS

Albosale A H[1,2*], Mashkina E V[2]

*Corresponding Author:** Dr. Abbas Hadi Albosale, Genetics Department, Southern Federal University, 344090, Stachki, 194/1, Rostov-on-Don Province, Russia. E-mail: abbashammadi4@gmail.com

ABSTRACT

Importunate high-risk HPV (HR-HPV) infection is the most common trigger for the cervical carcinogenesis process. In this respect, the presence of cancer can be imputed to telomere lengthening or shortening. This paper explores the possible correlation between relative telomere length and viral load in two groups of women, namely: those with high-risk HPV infection and those who do not have this infection. Thus, samples comprising of 50 women in each group were evaluated for this research. The Amplisens HPV HCR screen-titre-FRT PCR kite was employed for quantitative analysis. Relative telomere length was quantified by real-time PCR. In each of the two HPV load groups, there was no correlation between age and telomere length. Telomere shortening was found in the cervical cell samples of women with high HPV loads, compared with women in the control group. Telomere shortening is associated with elevated HPV loads.

Keywords: Telomere; Human papillomavirus; Viral load; Cervical cancer; Real-time PCR.

INTRODUCTION

Telomeres are comprised of complexes of nucleoprotein that enclose chromosome ends and not only offer protection against fusion and other injuries but also contribute to the preservation of genomic stability [1]. Each subsequent division of the cell reduces the length of the 5'-TTAGGG-3' sequence of DNA telomere repetitions, the outcome being either apoptosis or senescence [2]. When atypical conditions cause the telomeres to reduce in length, the possibility of telomere end fusions arises, which could result in an increased level of chromosome instability. This is a primary triggering event in many cancers, including colon, prostate, and cervical cancers [3–5]. Research in this field has postulated that a potential correlation exists between different cancer stages and telomere length. Specifically, telomeres that are shorter in length are linked to the onset of cancer. However, they gradually lengthen as cancer progresses as a consequence of telomerase activation or alternative telomere lengthening (ALT) [6,7]. Telomerase acts to safeguard the telomere against shortening. The telomere is comprised of two subunits, namely: TERC (telomerase RNA component) and hTERT (human telomerase reverse transcriptase). TERC functions as a blueprint for telomere sequence amplification, whereas hTERT serves as a reverse transcriptase subunit [8]. In 85-90% of human cancers, telomerase activation causes telomeres to expand in length [9]. Thus, it is reasonable to assume that the stabilization of telomere length occurs at a later point in the tumorigenesis process. Specifically, it takes place following a period of active proliferation cycles and reduced telomere length [10]. Sexual contact is the most common vehicle for the transmission of the human papillomavirus (HPV). HPV infection has affected a considerable number worldwide, although both men and women are infected with HPV, adolescent girls and women under the age of 25 are the most infected [11,12]. HPV is a diminutive virus. It penetrates cells with its capsid proteins, with the infection commencing at the basal cell layer, after which it disseminates to elevated tiers of the cervical epithelium [13]. Dysplasia resulting from the relentless HR-HPV infection can ultimately develop into cancer [14].

[1] Department of Medical Laboratory Techniques, Al-Dour Technical Institute, Northern Technical University, Mosul, Iraq

[2] Genetics Department of Academy of Biology and Biotechnology, Southern Federal University, Rostov-on-Don, Russia

The principal causative agents for different cancer types are HR-HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) [15]. HR-HPV has been implicated in cancers of the cervix, vulva, vaginal, penis, anus, and oropharyngeal cancers [16]. However, cervical cancer is the fourth most common malignant tumor in women globally, and it is associated with a significant mortality rate in women [17,18]. The cellular telomerase complex can be triggered by the hTERT transcription, which is stimulated by the HPV E6 protein [19]. Moreover, the cell immortalization that occurs during long-term high-risk human papillomavirus infection is dependent upon telomerase activation, which leads to cervical malignant neoplasm development [20]. The development of cancer passes through multiple stages, and one of the most common features is modifications to telomere length [21]. There is practically no data in the literature on the relationship between telomeric DNA length and the risk of the development of elevated viral load in cells infected with HPV. Therefore, the primary objective of the current study is to ascertain the existence of any disparities between comparative telomeric DNA lengths in the cervical epithelial cells of women with high HPV load and telomeric DNA lengths in the control group.

MATERIALS AND METHODS

Sample description. To conduct the molecular investigation, 100 samples were obtained from the epithelial cells scraped from the women's urogenital tracts of the participants in the two research groups. Material for the research was obtained from the clinical diagnostic laboratory Nauka (Rostov-on-Don, Russia). Participants who were HPV-positive were subdivided into two subgroups: participants with an HPV load of 4-5 lg HPV genomes per 100 thousand human cells (n = 21), and participants with an HPV load of over 5 lg HPV genomes per 100 thousand human cells (n = 29). HPV-negative women (n = 50) were in the control group. All participants in this research project were aged 30 or over. Three inclusion criteria were applied to participants in the control group, namely: uterus negative biopsy, normal colposcopy, and HPV negative PCR testing. The case study group included women with a range of symptoms, such as abnormal menstrual bleeding, abnormal vaginal discharge, a positive uterine biopsy, and an HPV-Positive PCR-test with an HPV viral load of over 10^4 DNA copies per 10^5 human cells. Informed written consent was required from all participants before their inclusion in this research. Ethical approval was sought and secured from the Bioethics Committee of the Academy of Biology and Biotechnology of the Southern Federal University (Protocol No. 2 of March 29, 2016). Any clinical testing included in this study was conducted according to the ethical protocols issued by the World Medical Association (Helsinki Declaration).

Quantitative analysis for HPV DNA. The total DNA was isolated from scrapings of epithelial cells from the cervical canal of women according to the protocols of the AmpliSens DNA-sorb-AM (InterLabService, Russia) reagent kit. The protocols specified for the AmpliSens-HPV HCR screen-titre-FRT and AmpliSens HPV HCR-genotype-FRT (Interlabservice, Russia) were employed for the genotyping and quantifying of the DNA for HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59). The PCR mixture for the AmpliSens-HPV HCR screen-titre-FRT contains 7 µl of PCR mix-1-FRT HPV screen titer; 8 µl mix of PCR buffer Flu and Taq polymerase and 10 µl of a DNA solution. The amplification program is as follows: 1 cycle at 95 °C for 15 minutes, followed by 1 cycle at 65 °C for 2 minutes, 93 °C for 20 seconds, 64 °C for 25 seconds, 65 °C for 55 seconds, followed by 5 cycles at 95 °C for 15 seconds, 60 °C for 25 seconds, 65 °C for 25 seconds, and 25 cycles registration. Fluorescence is measured at 60 °C in 4 channels: Fam, Joe, Rox, and Cy5 [22]. The interpretation of the viral load was performed as follows: log ≤3 per 10^5 human cells indicated low clinical significance, 3–5 log per 10^5 human cells signified clinical significance and risk of dysplasia, whereas > 5 log per 10^5 human cells was clinically significant with a strong risk of dysplasia, as per the guidelines and clinical reports issued by the manufacturer [22]. The PCR mixture for the AmpliSens HPV HCR-genotype-FRT contains 3.5 µl of one of the four variants of PCR-mixture-1-FRT HPV (four variants of the mixture differ in primers for specific types of HPV), 4.5 µl of a mixture of PCR-buffer-FRT and polymerase (TaqF), and 5 µl of DNA. The amplification program is as follows: 95°C for 15 minutes; 45 cycles: 95°C for 15 seconds, then 60°C for 30 seconds. Fluorescence is measured at 60 ° C [23].

Telomere and real-time polymerase chain reaction (PCR). With slight modifications, an approach was employed to ascertain telomere length according to the Cawthon method for telomere measurement by quantitative PCR [24]. The mix for the amplification reaction (25 ml) included 0.7 µl 10 mmol /L of either the telomere primers (teloF and teloR) or primers for the *36B4* gene (*36B4F* and *36B4R*). The *36B4* gene, which functions as a reference gene that encodes acidic ribosomal phosphoprotein (PO). (Table 1) presents the primer sequence. The PCR mixture incorporated 2.5 µl 25 mM MgCl2, 2.5 µl 2, 5 мM of dNTP, 2.5 µl PCR buffer, 0. 5 µl Taq-polymerase (5 U/µl), universal SYBR-Green 0.3 µl, 14 µl ddH2O (Syntol, Russia) and 2 µl DNA. Test tubes for telomeres and reference gene (*36B4*) (single-copy gene) were tested individually, with the findings only being calculated once

an equilibrium had been established for the reaction efficiency. A real-time system (Roter-Gene, QIAGEN) was adopted for the amplification. The (*36B4*) thermal cycling profile included one cycle (95°C for 15 seconds) followed by 40 cycles (95°C for15 seconds, 57°C for 1 minute), while the telomere cycling included one cycle (95°C for 10 minutes) followed by 50 cycles (95°C for 15 seconds, 58°C for 1 minute).

Measurement of telomere length. In the case of each DNA sample, the following calculation was made: The T/S ratio (telomere (T) was calculated for a single-copy gene (S), Telomere length was expressed as the relative T/S ratio and the relative quantification (not the absolute length) of telomeric DNA was evaluated. In each cycle, the quantity of PCR product roughly doubles. The T/S ratio was computed via the Ct (cycle threshold), which was formulated as follows: $[2^{Ct (telomeres)}/2^{Ct (36B4)}]^{-1} = 2^{-\Delta Ct}$. Furthermore, the fold-change was calculated following formula: $2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct(group 1) - \Delta Ct(group2))}$ from the average of group 1 (control group) subtracted from the average of group 2 (experimental group). The data from the $2^{-\Delta\Delta Ct}$ method comprises the fold change, and the analysis of the $2^{-\Delta\Delta Ct}$ for the case samples compares the changes in the telomere length fold with the control samples. $2^{-\Delta\Delta Ct} > 1$ indicates that the average telomere length for the case samples is longer than that of the control sample, whereas $2^{-\Delta\Delta Ct} < 1$ suggests that the DNA samples have an average telomere length that is shorter than the control DNA samples [25].

Statistical analysis. A Pearson rank correlation coefficient (rs) was employed to evaluate the correlation between the telomere length and different parameters, including age and viral load, with a p-value less than 0.05 being deemed significant. Thus, it was possible to identify statistical differences in the telomere length in the three groups (the control group, the case study group, and the viral load group) using Student's t-test. GraphPad InStat software (version 3.05) was utilized for all the statistical analyses.

RESULTS

The average age of the 50 participants in the HPV-positive group was 38.4 ±5.3 years, whereas the average age of the 50 HPV-negative participants was 42.8 ±6.6 years. The maximum HPV DNA loads among the 50 HPV-infected women were 8.5 log, the middle HPV DNA loads were 5.4 log, and the lowest loads were 4.1 log of HPV genomes per 10^5 human cells. The molecular analysis revealed that 8 participants in the case group (16% of the case group) had HPV co-infections. That is, these participants had two or more types of high-risk HPV. The most common type of mono-infection (44%) was HPV 16. HPV 18 accounted for 10% of the cases, as did HPV 33, whilst the HPV 31 type represented 8% of the cases. In addition, other HR-HPV genotypes, such as HPV 51, 56, 59, 45, and 39 were estimated to be present in 2-4% of cases (Figure 1). Comparatively longer telomeric DNA length (T/S ratios) was identified in participants in the cervical scrapings taken from the control group when a comparison was made with those from the HPV group (T/S ratios=1571.21 versus 1144.06 respectively). This is shown in (Table 2). Neither the risk HPV group (r=0.1976, p=0.1690) nor the control group (r= -0.1822, p=0.2052) revealed any significant correlation between age and telomere length (T/S ratio). However, a significant correlation was evident between HPV load and T/S ratio (r=0.4967, p=0.0002). The participants with HPV loads were subdivided into two groups to facilitate the evaluation of the extent to which the telomeric DNA length varied according to the viral load. The two groups were comprised of one group whose participants presented with an HPV load of 4-5lg and another group in which participants had HPV loads that exceeded 5lg. The analysis indicated that for participants with HPV loads of 4-5lg, the average telomere length in cervical epithelial cells (T/S ratios =1035.12) was less than that of the sample cells of participants in the HPV load (> 5lg) group (T/S=1222.93), as can be seen in
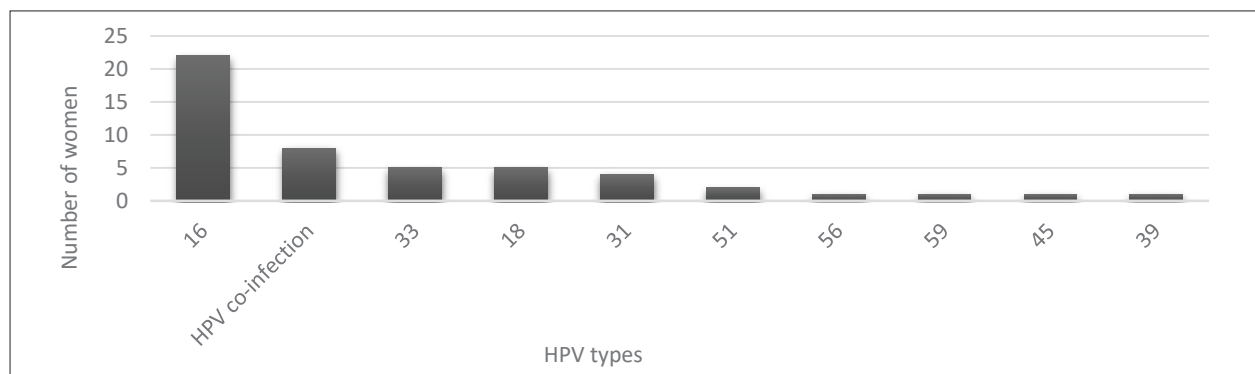

Figure 1. The prevalence of high-risk HPV types in HPV-positive women.

Table 1. The sequence of primers used in real-time PCR.

Primer/oligomer	Sequence
Primer Telo F	5′- cgg-ttt-gtt-tgg-gtt-tgg-gtt-tgg-gtt-tgg-gtt-tgg-gtt-3′
Primer Telo R	5′- ggc-ttg-cct -tac-cct -tac-cct -tac-cct-tac-cct-tac-cct-3′
Primer 36B4 F	5′- cag-caa-gtg-gga-agg-tgt-aat-cc-3′
Primer 36B4 R	5′-ccc-att-cta -tca-tca-acg-ggt-aca-a- 3′

Table 2. The relative telomere lengths among the studied groups.

HPV group		Control group		$2^{-\Delta\Delta Ct}$	p-value
Number	Mean of T/S ± SDE	Number	Mean of T/S ± SDE		
50	1144.06 ± 64.085	50	1571.21 ± 111.55	2.5925×10^{-129}	0.0014
HPV load 4-5lg					
21	1035.12 ± 65.926			4.1809×10^{-162}	0.0001
HPV load >5lg					
29	1222.93 ± 98.052			1.4454×10^{-105}	0.0217

SDE: Standard Error of mean; p-value <0.05 was regarded as statistically significant.
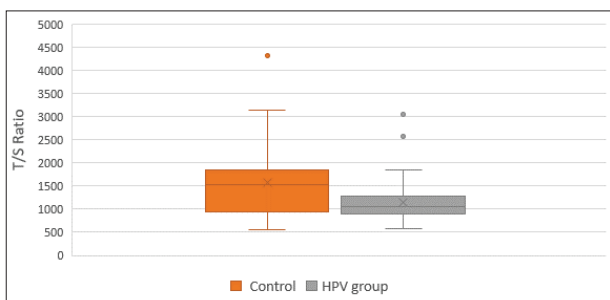

Figure 2. Relative telomere length in control and HPV-positive women. Data are shown as box plots which represent a five-number summary of the data (the minimum, lower quartile, median, upper quartile, and maximum). A small circle indicates an outlier sample.

(Table 2) (Figure 2). Despite this finding, it should be noted that the difference between the HPV load groups (4-5lg) and (> 5lg) was not statistically significant (p= 0.118).

DISCUSSION

Persistent high-risk human papillomavirus infection is the primary cause of cervical malignant neoplasm lesions [26]. As viral loads increase, in response to protracted exposure, the risk of epithelial cell dysplasia also increases. Thus, there is a discernible correlation between elevated viral loads and the incidence of abnormal lesions and cervical malignant neoplasms [27].

In the current study, cell samples taken from 50 participants with high HPV loads were investigated to ascertain whether telomere length could be employed as a potential diagnostic tool for women who are high-risk HPV positive. Thus, this study has enabled a comparative analysis of the relative telomere lengths in the DNA samples from cervical cell samples taken from participants with high HPV loads with those taken from participants in a control group. No significant differences were reported between participants with HPV loads of 4-5lg and those with HPV loads of >5lg. However, in both HPV load groups, the telomeres were shorter than what was observed in the control group. Thus, telomere shortening could be an initial molecular modification in advance of the development of high-risk HPV carcinogenesis [28]. The results emerging from the current study indicate that telemetric DNA undergoes shortening in cervical cell samples where the HPV load exceeds 4 lg. Early cervical tumorigenesis is characterized by either telomere dysfunction (the loss of telomere capping function) or excessive telomere erosion, both of which generate chromosomal aberrations that lead to oncogenesis [29].

Studies indicate that there is a link between substantially shortening telomeres and both the activation of the DNA damage response pathway (Chk2 phosphorylation) and an increase in cervical cell proliferation [30]. Shortened telomere length is associated with complex, nonreciprocal chromosomal translocations, genome amplification, deletion via breakage–fusion–bridge (BFB) cycles, and the development of epithelial carcinomas and other malignancies [31,32].

Marginal increases or predispositions to increase have been observed in association with viral loads that exceed 5 lg. The viral loads represent the number of infected cells and viral copies in individual cells. Thus, increased viral loads are linked to increased incidences of cervical cancer. Studies have shown that there is a rise in telomerase activity that corresponds with lesion development, ranging from low-grade squamous intraepithelial lesions (LSIL)

to high-grade squamous intraepithelial lesions (HSIL), and onwards to invasive cervical cancer. This indicates that telomere length abnormalities constitute a common genetic change that transpires during the many stages in the development of malignant transformation. This correlates with dysplasia and telomerase activity. Furthermore, it increases with the progression of lesions [20,33–37].

There are several limitations to the current research project, the first of which is that the sample included no participants with either cervical cancer or histological lesions. This rendered it impossible to examine telomere alterations in lesions that were at a more advanced stage. The second major limitation pertains to the comparatively small size of the study sample, which might preclude generalization. It is necessary, therefore, to confirm the findings of this study in a larger population.

In conclusion, the current study has revealed that shortened telomeres are present in cervical samples taken from participants who are HR-HPV positive and have elevated viral loads. Thus, telomere length has a potential application in future triaging, monitoring, and screening of women with high-risk HPV infections. It must be reiterated, however, that additional research with a larger sample group is needed before this hypothesis can be confirmed.

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