Correlation of Viral Load, Disease Severity and High Risk-Human PapillomaVirus (HR-HPV) in Abnormalities of Cervical Samples

Hula Y. Fadhil
Dhuha S. Saleh
College of science, University of Baghdad.
Faisal G. Al-Hamdani
Head of Virology Department, the National Central Public Health Laboratory, Ministry of Health.

ABSTRACT

Clinical and epidemiological studies have confirmed that high-risk human papillomavirus (HR-HPV) infection (genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 59, and 68) is the direct cause of cervical intraepithelial neoplasia (CIN) and cancers. This study was designed to determine whether viral loads in relation with progression of HPV infections using real-time PCR. According to cytological test, 110 and 12 of suspected women with abnormal and normal cytologic changes were selected. Results showed that 35/110 and 2/12 women were positive for HR-HPV DNA. Results showed the median of viral load increasing in high-grade cervical intraepithelial neoplasia (CIN II, III and cancer) and associated with the severity of lesion (786 copies/ml equivalent for CIN II, 2,037,000 copies/ml equivalent for CIN III, and 956,840 copies/ml equivalent for invasive cancer). Meanwhile, the median of viral load in CIN I showed more 250-fold higher than that of HPV infected women with normal cytology (183,700 copies/ml equivalent for CIN I and 683 copies/ml equivalent for normal cytology). Although the viral load was increased with primary infection and specific genotypes, high viral load was accompanied with severity of disease.

KEY WORDS: Human Papillomavirus, Real-Time PCR, Viral Load.

INTRODUCTIN

Clinical and epidemiological studies have confirmed that high-risk human papillomavirus (HR-HPV) infection (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 59, and 68) is the direct cause of cervical intraepithelial neoplasia (CIN) and cancers [1, 2]. However, the relationship between HR-HPV viral load and histological severity of cervical precancerous lesions is still controversial. Some

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studies have found that different levels of HR-HPV correlate with grades of CIN [2, 3, 4, 5, 6], while others have reported no significant correlations [7, 8, 9], or only a high viral load of HPV-16 positively associated with the severity of cervical lesions [1, 10, 11, 12, 13]. Other studies revealed lower amounts of HR-HPV in the cervical samples of women with CIN3 compared with CIN2 [14]. This study was designed to determine whether viral loads in relation with progression of HPV infections using real-time PCR.

MATERIALS AND METHODS

1- Study design

According to cytological test, 110 and 12 of suspected women with abnormal and normal cytologic changes were selected for this study. All women suffered from chronic inflammation and abnormal appearance of their cervix by gynecologist examination. Meanwhile, 10 health women as a control group were mainly depending on the absence of clinical symptoms and cervical cytological changes. All cervical samples were attending Women Health Center- Al-Alwia hospital and Al-kadhmia teaching hospital in Baghdad during the period from February to December 2010.

2- Cervical cell samples

Cotton swabs were used to collect ecto-and endocervical samples from selected women. Sample was immersed in a tube containing Tris-EDTA buffer (PH 7.4). All samples were stored at -20c˚until real-time PCR tested.

3- HPV DNA detection

Real-time PCR was used for screening 13 HR-HPV (16,18,31,33,35,39,45,51,52,56,58,59 and 68) in one reaction tube (HybriBio Real-Time HR-HPV PCR Kit, HongKong). This kit was used for *in vitro* detection and viral load estimation for these HPV genotypes. DNA extraction and PCR amplification were performed according to instructions kit.

4- Viral load calculation

For performance of quantitative real-time PCR, Standard dilutions were prepared first as follows. Sterile water was used for dilution. Positive control $(1 \times 10^7$ copies/ml) was taken as the starting high standard in the first tube. $36 \mu l$ sterile water was pipetted into next six tubes, then added $4 \mu l$ in each tube of these from it prior tube, vortex, placed in water bath at 60 C° for 5 min and mixed thoroughly for each tube.

Normalized viral load was calculated threshold cycle number (Ct) values for each sample automatically then applied to standard curve to express as copies of virus per ml according to the formula described by instruction protocol:

Viral load (HPV copies/ml) = Number of HPV copies×R_2 value

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R_2: Coefficient determinant. Standard curve was generated by plotting the Ct values against the log of the copy numbers and the copy numbers for unknown samples inferred from the regression line [15].

RESULTS AND DISCUSSION

Results showed that 35/110 and 2/12 women were positive for HR-HPV DNA. Viral DNA copy number for the most HR-HPV types in cervical samples was determined from 35 women with low and high-grade cervical intraepithelial neoplasia (CIN I, II, III and SCC), and two with normal cytology. However, the viral load of the 33 samples spread over a wide range from 1×10 to 9.4×10^6 copies/ml (mean 4.17×10^4 copies/ml). In addition, remaining two samples were around the standard curve values as: one had a Ct nearby Ct of the first standard concentration (1×10^7 copies/ml) and the other sample had a Ct 5 cycles more than that of the concentration (1×10^7 copies/ml). Thus, the viral loads of these samples were approximately 5×10^7 and 1×10^9 considered, respectively according to results obtained by Mackay *et al.* (2002), who reported that amount of amplicon increases at rate of approximately one $\log_{10}$ every two-three cycles [16]. HPV viral load was classified as low or high according to median HPV DNA titer and examined for its prognostic value [17]. Consequently the HPV viral load < 10,000 copies/ml was considered as low viral load, viral load more than > 100,000 copies/ml was considered as high viral load while the range of 10,000 - 100,000 copies/ml was considered as slightly high viral load . According to data mentioned above viral load in the present study was estimated approximately 46% (16/35) constituted as high viral load, 34% (12/35) as low viral load and 20% (7/35) as slightly high viral load.

Results showed the median of viral load increasing in high-grade cervical intraepithelial neoplasia (CIN II, III and cancer) and associated with the severity of lesion (786 copies/ml equivalent for CIN II, 2,037,000 copies/ml equivalent for CIN III, and 956,840 copies/ml equivalent for invasive cancer). Meanwhile, the median of viral load in CIN I showed more 250-fold higher than that of HPV infected women with normal cytology (183,700 copies/ml equivalent for CIN I and 683 copies/ml equivalent for normal cytology) (Table 1). On the other hand, women with CIN II/III had a median amount of HPV DNA, which was more than 11-fold higher than that of HPV infected women with CIN I and approximately 3000-fold higher than that HPV positive cytologically normal women and approximately 2-fold higher than that of HPV infected women with SCC. This effect was observed in women infected with a single HPV and in those infected with multiple types. Our results are agreed with previous reports have suggested that high HPV DNA copy number is associated with cytologic abnormalities and that HPV-positive women with normal cytology are often

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observed to have very low viral loads with minimal risk of subsequent progression to cancer [18].

Table 1. The Relationship between Clinical presentation and Viral Load

Studied group	No. of cases	Mean of viral load copies/ml
CIN I	22	18.37×10^4
CIN II	3	0.786×10^3
CIN III	2	20.37×10^5
Cancer	6	95.684×10^4
Normal cytology	2	6.825×10^2
LSD value	--	344.73 *

LSD: Least Significance Differences; * Significant differences (P<0.05).

These data suggested that the increase of viral load in some cases may be resulted from the infections with multiple types or that samples were harboring the pure episomal state of the virus, which may allow accumulation of higher numbers of copies as noticed by Cheung *et al.* (2008) and García *et al.* (2011) or because of the recent replication of HPV in SIL as observed by Coutlee *et al.* (2009) [19, 20, 21].

Other cases have low viral load< 1000 copies/ml were either represented as latent infection or because of HPV integration with the host genome, which resulted in reduction of viral DNA copy number [17]. These results are supported by other studies which showed that the low HR-HPV DNA viral load found in some cervical carcinoma cell lines such as SiHa cells (1-2 copies/cell for HPV-16) and Hela cells (10-50 copies/cell for HPV-18). However, the low viral load, particularly with a common integrated viral genome and high expression of its oncoproteins, may be sufficient to lead to or promote carcinogenesis [22].

Schlecht *et al.* (2003), observed that higher crude viral load in high-grade lesions may be explained by the fact that abnormal cells express fewer intracellular adhesion molecules than the normal cells and thus are more readily exfoliated [9]. These differences in viral load observed among cytological abnormalities may indicate that varying levels of viral replication are needed to disrupt the cellular homeostasis and induce biological changes that lead to cervical disease [23].

Approximately 29% of HR-HPV infections at LSIL with high viral load may also predicate the higher risk of progression to CIN II/III, which was comparable to other observation [24]. Also, Castle *et al.* (2005), observed that

high viral loads ($>10^5$ copies) at baseline was predicting disease progression among women with LSILs [25].

There are statistically significant for viral load differences within the clinical groups (p<0.05). However, some studies suggested that the increasing amount of HPV in higher CIN stages indicates dose-response association between viral load and lesion grade [26]. Other study reported that the viral load was associated with lesion size rather than lesion severity. A high viral load could be used as a short- term marker of progression towards precancerous lesions, although lower load does not inevitably exclude progress of disease [27]. The hypothesis of Peitsaro *et al.* (2002), that high viral loads are likely to increase the risk for events initiating dysplasia such as viral integration [28]. However, HPV DNA loads showed a specific but not sensitive diagnostic marker to define cervical disease status [1].

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