Determination of the Physical Status (Episomal/Integral) of HPV by qPCR in Esophageal Squamous Cell Carcinoma

Fariborz Soheili1,2, Bahram Nikkho1, Mazaher Khodabandehloo1

1Cellular and Molecular Research Center, Kurdistan University of Medical Sciences, Sanandaj, Iran
2Department of Marine Biology, Faculty of Marine Sciences, Chabahar Maritime University, Chabahar, Iran

Abstract
Background: In cervical cancer, the carcinogenic mechanism of human papillomavirus (HPV) occurs through the integration of viral DNA into the host genome. This process initiates with a disruption in the E2 open reading frame (ORF) of the viral genome. Disruption of E2 ORF results in an increased expression of the viral oncoproteins, E6 and E7, by removal of E2 suppression effect on their promoters. E6 and E7 interfere with the normal cell cycle by degrading the p53 and pRb tumor suppressor proteins, respectively.

Objectives: The objective of this study was to determine the physical status (episomal/integral) of HPV genome in esophageal squamous cell carcinoma (ESCC).

Materials and Methods: The rate of copy numbers of E2 and E6 genes in HPV-18 and HPV-16 positive samples were analyzed by quantitative polymerase chain reaction (qPCR) in order to assess the physical status (episomal/integral) of HPV. DNA extracts from HeLa cell line were used as the positive control.

Results: The E2 gene was detected in 1 sample, co-infected with HPV-16 and HPV-18. While, E6 gene was detected in all 11 HPV positive samples. The qPCR analysis showed the presence of integrated form of viral DNA in all HPV positive samples and only 1 mixed episomal-integrated form was detected.

Conclusion: The presence of integrated forms of high risk HPV-16 and HPV-18 genomes might reflect a crucial process towards malignant transformation of ESCC.

*Corresponding Author: Fariborz Soheili; Tel/Fax: +98 54 31272342; Email: fariborz.soheili@gmail.com

Received October 17, 2016; Revised December 1, 2016; Accepted December 4, 2016

Background
Human papillomaviruses (HPVs) located in the viral family, papillomaviridae, are DNA containing tumor viruses with small nonenveloped icosahedral capsids. HPVs infect epithelial cells of skin and mucosa and generally produce benign hyper proliferative lesions which have the potential to undergo malignant transformation.1 High risk HPV types are ethologic cause of cervical cancer.2 Recent studies have reported HPV DNA detection in extragenital cancers as well; even though the etiological association of HPV in those malignancies is still contentious.3-5

One of the main procedures of HPV which induce carcinogenesis is the integration of its genome into the host DNA.6,7 However, the physical status (episomal/integral) of HPV genome is not absolute for the carcinogenesis and confirmation of insertional mutagenesis.8

The mechanisms of carcinogenesis for HPV in the cervical cancer, which involves DNA integration into the host genome, is attributed to a disruption of the viral genome in the E2 open reading frame (ORF).9-10 The papillomavirus E2 gene is translated into sequence-specific DNA binding proteins which regulate viral genes expression.11 The disruption of E2 gene results in the overexpression of viral oncogenic early proteins (E6 and E7) by the exclusion of E2 suppression effect on their promoters.12,13 The cellular p53 tumor suppressor protein is the target for HPV E6 oncoprotein. Binding of this oncoprotein to p53 stimulates its degradation through ubiquitin-dependent mechanism.14 E7 protein is recognized for its interaction and decay of the cellular pRb protein.15 Therefore E6 and E7 oncoproteins intervene in the normal cell cycle via degrading the p53 and pRb tumor suppressor proteins, respectively.16

Some studies have found HPV infection in extra-genital tumors including esophageal squamous cell carcinoma (ESCC),4,17 although the etiological contribution of the HPV in these malignancies is still argued.
Objective
The aim of this study was to determine the physical status (episomal/integral) of HPV genome in order to investigate the carcinogenic mechanism of HPV using quantitative polymerase chain reaction (qPCR) in ESCC. The study was performed on the patients from Kurdish and Kermanshah provinces of Iran.

Materials and Methods
Patients and Clinical Samples
In this study, 59 subjects from Kermanshah province and 44 subjects from Kurdistan province were participated. The patients were diagnosed with the HPV virus during 2007 to 2013. A total number of 11103 biopsies (HPV positive samples) were obtained, and then formalin fixed and paraffin embedded (FFPE). The blocks diagnosed with ESCCs were in the Kurdish population.

DNA Extraction
The samples were cut into small pieces (5 μm in thickness) and collected in sterile tubes. To avoid contamination, a new disposable microtome blade was used for each sample. DNA was extracted (QIAamp DNA FFPE Tissue Kit, Qiagen, Germany) according to the manufacturer’s instructions. DNA quality and the absence of PCR inhibitors in the extracted DNA samples were analyzed by PCR for β-globin (110bp) gene using PCO3, 5’-ACA CAA CTG TGT TCA CTA GC-3’ and PCO4 5’-CAA CAT AGC TGG GCA CT-3’ primers. The PCR conditions were adjusted as follows: initial denaturation at 95°C for 5 minutes, 30 cycles (95°C for 30 seconds, 52°C for 45 seconds, 72°C for 45 seconds), extension at 72°C for 5 minutes, and final hold at 4°C.

Quantitative PCR for Detecting the Ratio of E2 and E6 Sequences
To determine the physical status (episomal/integral) of HPV genome in the infected samples with HPV, the real time PCR (qPCR) was performed. The E2 and E6 genes were amplified4 (4) using the Rotor-gene 6000 and 2x QuantiFast SYBR® Green PCR kit (Qiagen, Germany). The rate of copy numbers of E2 and E6 sequences determine the physical status. This study was a relatively comparative study in that we used DNA extracts from HeLa cell line with a mixed status and physical status (episomal-integral) as standard.

The amplification conditions were as 95°C for 5 minutes, followed by 45 cycles (2 steps) (95°C for 10 seconds and 60°C for 30 seconds ). Primer sets for E2 and E6 sequences detection in HPV-18 positive samples were as follows: 5’-AGA AGC AGC ATT GTG GAC CG-3’ and 5’-GTT CGC TAT GTT TTC GCA AT-3’ for E2, and 5’-TCA CAA CAT AGC TGG GCA CT-3’ and 5’-CTTGTGTTTT CTCTGCGTCGT-3’ for E6. The sizes of the E2 and E6 amplicons were 167 and 91 bp, respectively.16 Primer sets for E2 and E6 sequences detection in HPV-16 positive samples were as follows: 5-AAC GAA GTA TCC TCT CCT GAA ATT AG-3 and 5-CCA AGG CGA CGG CTT TG-3 for E2, and 5-AGA AAC TGC AAT GTT TCA GGA CC-3 and 5-TGT ATA GTT GTT TGC AGC TCT GTG C-3 for E6. The amplicon size for E2 was 76 bp and for E6 was 81 bp.19 DNA extracts from HeLa cell line were used as the positive control for HPV-18 which showed the mixed physical status (episomal-integral) and its curves.

Results
The ratios of E2/E6 genes in 11 HPV-16 or HPV-18 positive samples were analyzed by qPCR in order to assess the integration status of HPV genomes. The presence of E2 gene associated with E6 gene was only observed in one HPV-16 and HPV-18 co-infected sample with well differentiated grade tumor, whose ratio of E2/E6 (0.31) showed mixed (episomal-integral) status (Figuer 1).

While, E6 gene was detected in the rest of HPV positive samples without E2 gene, which indicated the presence of integrated form of viral DNA in the entire HPV positive samples (Table 1).

Discussion
In this study, we examined the physical status of the virus genome and found mostly integrated status of HPV sequences in the infected samples.

In our previous study, the association of HPV-16 and HPV-18 with ESCC was shown in Kurdish population of the west of Iran (4), where the incidence of the disease is low (7 and 8.1 in 100 000 in Kurdistan9 and Kermanshah provinces,8 respectively) in comparison with an incidence of 1/1000 in some populations of Iran such as high-risk Turkmen population in the north of Iran.22

Using real-time PCR, we examined the presence of HPV-18 and HPV-16 and also the integration status of the virus, since HPV integration is considered to result in the deletion of E2 gene.8,10 Disruption of E2 causes an increased expression of the viral oncogenic proteins, E6 and E7, by removal of E2 suppression effect on their promoters.12,13 E6 and E7 proteins interfere with the normal cell cycle by degrading the p53 and pRb tumor suppressor proteins, respectively.16

We determined the physical status of HPV genome in

![Figure 1. Melt Curve Shows The Presence of E2 Gene Associated With E6 Gene, Indicating Mixed (Episomal-Integral) Status.](www.SID.ir)
the host cells on the basis of the E2 and E6 ratio. In the present study, the integrated form of HPV was detected in all the HPV-positive specimens when the lack of HPV E2 genome was considered the integration of HPV genome into the host genome. While, the presence of both episomal and integrated form was detected in only one sample, where E2 and E6 were both detected by qPCR and this ratio was larger than zero and smaller than unity. When the E2/E6 ratio was equal to or higher than one, all the HPV genome was considered to be in an episomal form which was not detected in none of the samples.

In this study, the integration of HPV was detected in all HPV positive samples which show integration is strongly associated with the neoplastic process. In other studies, similar to ours, it was shown that viral genome integration is restricted to the neoplastic and the transforming efficacy of dysplastic tissue and is not observed in normal epithelium; so, no consequence has been detected in the field cancerization in HPV-positive tumors. This useful method is a sensitive technique to evaluate the physical status of HPV genome and is suitable to predict the progression of disease.

In conclusion, the results found in this study support the statement that the genome integration of high risk HPV (HPV-16 and HPV-18) might reflect a crucial process towards malignant transformation in dysplastic esophagus lesions and may be an indicator of the risk of ESCC, at least for patients in the Kurdistan and Kermanshah provinces of Iran.

**References**

1. Zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer. 2002;2(5):342-50. doi:10.1038/nrc798.
2. Gomez DT, Santos JL. Human papillomavirus infection and cervical cancer: pathogenesis and epidemiology. Communicating Current Research and Educational Topics and Trends in Applied Microbiology. 2007;1:680-8.
3. Snijders Pj, Steenbergen RD, Meijer CJ, Walboomers JM. Role of human papillomaviruses in cancer of the respiratory and upper digestive tract. Clin Dermatol. 1997;15(3):415-425.
4. Soheili F, Heidary N, Rahbar M, et al. Human papillomavirus and its clinical relevance in oesophageal squamous cell carcinoma in a Kurdish population in the west of Iran. Infect Dis (Lond). 2016;48(4):270-273. doi: 10.3109/23744235.2015.1109134.
5. Castillo A, Aguayo-F, Koriyama C, et al. Human papillomavirus in esophageal squamous cell carcinoma in Colombia and Chile. World J Gastroenterol. 2006;12(38):6188-6192.
6. Schmitz M, Driesch C, Jansen L, Runnebaum IB, Dürst M. Non-random integration of the HPV genome in cervical cancer. PloS One. 2012;7(6):e39632. doi:10.1371/journal.pone.0039632.
7. Thorland EC, Myers SL, Gostout BS, Smith DI. Common fragile sites are preferential targets for HPV16 integrations in cervical tumors. Oncogene. 2003;22(8):1223-1237.
8. Ziegert C, Wentzensen N, Vinokurova S, et al. A comprehensive analysis of HPV integration loci in anogenital lesions combining transcript and genome-based amplification techniques. Oncogene. 2003;22(25):3977-3984.
9. Kalantari M, Karlsen F, Kristensen G, Holm R, Hagmar B, Johansson B. Disruption of the E1 and E2 reading frames of HPV 16 in cervical carcinoma is associated with poor prognosis. Int J Gynecol Pathol. 1998;17(2):146-153.
10. Kalantari M, Blennow F, Hagmar B, Johansson B. Physical state of HPV16 and chromosomal mapping of the integrated form in cervical carcinomas. Diagn Mol Pathol. 2001;10(1):46-54.
11. Thierry F. Transcriptional regulation of the papillomavirus oncogenes by cellular and viral transcription factors in the context of oncogenesis. Arch Virol. 2001;146(10):2257-2272.

**Table 1. Detection of Copy Numbers of HPV E2 and E6 Genes in High Risk HPV Positive samples of ESCC Using Real-Time PCR**

| Sample No. | Province Name | HPV Genotype | E6 Sequence | E2 Sequence | HPV Genome Status | Degree of Tumor |
|------------|--------------|--------------|-------------|-------------|-------------------|-----------------|
| 1          | Kermanshah   | 16           | Positive    | Negative    | Integrated        | Well differentiated |
| 2          | Kermanshah   | 16           | Positive    | Negative    | Integrated        | Poor differentiated |
| 3*         | Kermanshah   | 18           | Positive    | Negative    | Integrated        | Moderate differentiated |
| 4          | Kermanshah   | 16           | Positive    | Negative    | Integrated        | Poor differentiated |
| 5*         | Kermanshah   | 18           | Positive    | Positive    | Episomal/integrated | Well differentiated |
| 6          | Kermanshah   | 16           | Positive    | Negative    | Integrated        | Moderate differentiated |
| 7          | Kermanshah   | 18           | Positive    | Negative    | Integrated        | Poor differentiated |
| 8          | Kurdistan    | 18           | Positive    | Negative    | Integrated        | Poor differentiated |
| 9          | Kurdistan    | 18           | Positive    | Negative    | Integrated        | Poor differentiated |
| 10         | Kurdistan    | 16           | Positive    | Negative    | Integrated        | Well differentiated |
| 11         | Kurdistan    | 16           | Positive    | Negative    | Integrated        | Poor differentiated |

Abbreviations: ESCC, esophageal squamous cell carcinoma; HPV, Human papillomavirus; PCR, polymerase chain reaction.
cervical carcinoma. Virology. 2009;384(2):375-379.

12. Gammoh N, Grm HS, Massimi P, Banks L. Regulation of human papillomavirus type 16 E7 activity through direct protein interaction with the E2 transcriptional activator. J Virol. 2006;80(4):1787-1797. doi:10.1128/JVI.80.4.1787-1797.2006.

13. Kahla S, Kochbati L, Hammami S, Chanoufi MB, Maalej M, Oueslati R. Sequence variation in the E2-binding domain of HPV16 and biological function evaluation in Tunisian cervical cancers. Biomed Res Int. 2014;2014:639321. doi:10.1155/2014/639321.

14. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell. 1990;63(6):1129-1136.

15. Boyer SN, Wazer DE, Band V. E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. Cancer Res. 1996;56(20):4620-4624.

16. Vousden K. Interactions of human papillomavirus transforming proteins with the products of tumor suppressor genes. FASEB J. 1993;7(10):872-879.

17. Qi Z, Jiang Q, Yang J, et al. Human papillomavirus (HPV) infection and the risk of esophageal squamous cell carcinoma. Dis Esophagus. 2013;26(1):61-67. doi:10.1111/j.1442-2050.2012.01334.x.

18. Damay A, Didelot-Rousseau MN, Costes V, et al. Viral load and physical status of human papillomavirus (HPV) 18 in cervical samples from female sex workers infected with HPV 18 in Burkina Faso. J Med Virol. 2009;81(10):1786-1791.

19. Peitsaro P, Johansson B, Syrjänäk S. Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. J Clin Microbiol. 2002;40(3):886-891.

20. Veisani Y, Delpisheh A, Sayehmiri K. Demographic and histological predictors of survival in patients with gastric and esophageal carcinoma. Iran Red Crescent Med J. 2013;15(7):547-553. doi:10.5812/hrmjr.11847.

21. Najafi F, Mozaffari H, Karimi M, Izadi B, Tavafzadeh R, Pasdar Y. Trends in incidence of gastrointestinal tract cancers in Western Iran, 1993-2007. Iran Red Crescent Med J. 2011;13(11):805-810.

22. Kamangar F, Malekzadeh R, Dawsey SM, Saidi F. Esophageal cancer in Northeastern Iran: a review. Arch Iran Med. 2007;10(1):70-82.

23. Shuyama K, Castillo A, Aguayo F, et al. Human papillomavirus in high-and low-risk areas of oesophageal squamous cell carcinoma in China. Br J Cancer. 2007;96(10):1554-1559. doi:10.1038/sj.bjc.6603765.

24. Hudelist G, Manavi M, Pischinger Kl, et al. Physical state and expression of HPV DNA in benign and dysplastic cervical tissue: different levels of viral integration are correlated with lesion grade. Gynecol Oncol. 2004;92(3):873-880.

25. Beguro S, Cao D, Gillison M, Zahurak M, Westra WH. Tissue distribution of human papillomavirus 16 DNA integration in patients with esophageal carcinoma. Clin Cancer Res. 2005;11(16):5694-5699.

26. McGovern SL, Williams MD, Weber RS, et al. Three synchronous HPV-associated squamous cell carcinomas of Waldeyer's ring: Case report and comparison with Slaughter's model of field cancerization. Head Neck. 2010;32(6):1118-1124. doi:10.1002/hed.21171.

27. Zhang D, Zhang Q, Zhou L, et al. Comparison of prevalence, viral load, physical status and expression of human papillomavirus-16,-18 and-58 in esophageal and cervical cancer: a case-control study. BMC Cancer. 2010;10:650. doi:10.1186/1471-2407-10-650.