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ORIGINAL ARTICLE

Detection Of Human Papilloma Virus In Esophageal Squamous Cell Carcinoma In Guilan Province

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ABSTRACT

Background: Despite many researches on the causes of Esophageal Squamous Cell Carcinoma (ESCC) many factors can cause but some types of HPV seem to be one of those factor. At the last studies the HPV cause 10 to 67% in some regions. Guilan is a region in Iran with high incidence of (ESCC) so it's need to study more on (ESCC).

Methods: In this study, we compared the prevalence of 3 kind of primers: general markers GP5+/GP6+ to show how much HPV are participated, mild oncogenic types of HPV 31,33,35,39,41,51,52 and high-risk oncogenic types of HPV 16 and 18 on E6/E7 gene in tumor tissues from 45 ESCC cases.

Results: In 17 of 45 ESCC (37.7%) samples were positive for general markers ,GP5+/GP6+, the HPV presence was 4 of 45 and in respect of ESCC (8.8%) samples were positive for oncogenic types and 2 of the ESCC (4.4%) samples were high risk HPV 16,18 E6/E7 gene and the 22 samples were negative for HPV types.

Conclusions: Our data are consisted with HPV DNA studies conducted in high-risk area (Guilan) for ESCC. HPV should be considered as a potential factor responsible for the increased incidence of ESCC, in spite of low incident in our study in Guilan .

Key Words: Human Papilloma virus (HPV), Esophageal Squamous Cell Carcinoma (ESCC), Guilan

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Introduction

Cancer of esophagus is the 10th most common malignancy in the world, the second ranking after heart infarction according to the last report of EMRO's regional office for the Eastern Mediterranean Organization[1], Iran is one of the areas with highest rate of Esophageal Squamous cell carcinoma (ESCC) cases and north of Iran especially Guilan province is a region with high incidence. ESCC most commonly originate from the non-keratinizing stratified mucosal epithelium and show morphological to squamous cell carcinoma of other regions of the body like cervix and esophagus. It is generally agreed that tobacco, Alcohol and hot black tea (bad habit in some regions of north east of Iran) and alcohol consumption in Europe the major environmental risk factors for developing of ESCC [2].

However some patients developed ESCC even without these 4 risk factors hence this fact suggests that additional causes such as genetic predisposition, diet, or oncogenic viruses may also help cells to override or escape the physiological mechanism of proliferation control.

The first substantial evidence suggesting an HPV infection of the esophagus was published in 1982 by Syrjanen, who found histological changes identical to those of condylomatous lesions in 40% of ESCC.The relevance of human papilloma virus HPV infection in cervical and anal cancer is well established and by way

of analogy oncogenic HPV viruses might have a role in malignant transformation of squamous epithelia of any body region[3],[4].

The aim of this study is show the prevalence of HPV in High risk area of ESCC in Guilan province .The HPV is a small epitheliotropic no enveloped DNA virus. Its genome comprises 7000 to 8000 base pairs (bp) of double-stranded closed-circular DNA. More than 120 different HPV types have been identified in humans. Some of these HPV types especially 16,18,31,33,51,52,58 seem to have specific roles in the development of cancer and are therefore named oncogenic types. Some kinds of Squamous cell carcinoma like cervical cancer infected mainly by oncogenic HPV types, whereas other mucosa such as the esophagus are infected by wide range of HPV types with varying tumorgenic potential [5],[6].

The presence of HPV genome is ESCC has been reported with various percentages and genotyping patterns. Several of these reports proposed a potential role for HPV types that are associated with high risk in the malignant transformation of esophageal mucosa stem cells [Table/Fig 1] [7].

(Table/Fig 1) HPV Infection in Esophageal Squamous Cell Carcinoma

Study (Year)	Geographic Source	HPV Detection Method(s) Used	No. Positive/ No. Tested	HPV Types Detected
Syrjänen[6] (1982)	Finland	Morphology	24/60	
Hille et al[7] (1985)	South Africa	Morphology	8/24	
Hille et al[9] (1986)	South Africa	IHC	7/70	
Kulski et al[8] (1986)	Australia	Morphology	0/120	
		FISH	5/10	11, 13, 16, 18
De Villiers[10] (1988)	Germany	FISH	2/46	6, 11, 16, 18
Kiyabu et al[11] (1989)	USA	E6 PCR	0/13	None
Mori et al[12] (1989)	China, Japan	IHC	8/46	
Brandsma et al[13] (1989)	USA	SB	0/3	None
Chang et al[14] (1990)	China (Linxian)	ISH	2/51	16, 18
Loke et al[15] (1990)	Hong Kong	ISH, slot-blot	0/37	None
Kulski et al[16] (1990)	Australia	FISH	9/39	6, 11, 16, 18
Kim et al[17] (1991)	Korea	E6 PCR	16/24	16, 18
Williamson et al[18] (1991)	South Africa	L1 PCR	6/14	Not typed
Benamouzig et al[19] (1992)	France	ISH, dot-blot	5/12	6/11, 16/18
Toh et al[20] (1992)	Japan	E6-E7 and L1 PCR	3/45	16, 18
Brachman et al[21] (1992)	USA	E6 and L1 PCR	3/30	16, 18
Chang et al[22] (1992)	China (Linxian)	ISH, E5 and L1 PCR	25/51	6, 11, 16, 18
Chaves et al[23] (1993)	Portugal	PCR	8/12	16, 18
Ashworth et al[24] (1993)	England	ISH	0/10	None
Furihata et al[27] (1993)	Japan (Kochi)	ISH	24/71	16, 18
Chang et al[26] (1993)	China (Henan)	ISH	85/363	6, 11, 16, 18, 30
Poljak and Cerar[25] (1993)	Slovenia	ISH, L1 PCR	2/20	16
Chen et al[29] (1994)	China (Fozhou)	E1 PCR	24/40	6, 16
Togawa et al[2] (1994)	Japan	Nested L1 PCR	2/20	16, 18
	France (Lyon)	Nested L1 PCR	1/8	16, 18
	Japan	Nested L1 PCR	1/4	16, 18
	Iran	Nested L1 PCR	1/8	16, 18
	USA	Nested L1 PCR	2/15	16, 18
	South Africa	Nested L1 PCR	3/18	16, 18
Lewenson et al[28] (1994)	Sweden	Nested L1 PCR	0/10	None
Akutsu et al[30] (1995)	Japan (Chiba)	SB, E6 and L1 PCR	0/33	None
Benamouzig et al[24] (1995)	France	E6 and L1 PCR	0/75	None
Cooper et al[33] (1995)	South Africa	ISH, E6 PCR	25/48	6, 16, 18
Fidalgo et al[32] (1995)	Portugal	E6 PCR	9/16	16, 18
Smits et al[31] (1995)	Netherlands	L1 and E6 PCR	0/61	None
Dillner et al[37] (1995)	Finland	Serology	8/39	16
Suzuk et al[35] (1996)	USA (Cincinnati)	ISH, E6 and L1 PCR	1/27	6, 16
	China (Beijing)	ISH, E6 and L1 PCR	3/88	16

The carcinogenic effect of these HPVs has been largely attributed to 2 viral gene product E6 and E7.It has been shown that the transforming activity of these 2 viral oncoproteins is at least partially mediated by enhancing the breakdown of the central tumor suppressor of p53 and increase mutant form and inactivating Rb. Therefore decreased levels of p53 with lower or unchanged expression of Rb is expected in HPV – transformed cells [8],[9].

Method and Materials

Sample Collection

A total of forty- nine formalin –fixed paraffin embedded samples of ESCC were collected from 2005 – 2007 surgical pathology archives of Rasht Razi Hospital, Pour sina Hospital, Golsar Hospital, Dr.Satari (Sina) Pathobiology lab and Dr.Saffari (Fouman) Pathobiology lab. The patients visited the Rasht Razi Hospital from various parts of province, and are a major center of Gastro Intestinal diseases.

The paraffin-embedded blocks were obtained from surgery (total or sub total esophagotomy) or biopsy for esophageal cancer. First, Hematoxylin and Eosin – stained slides prepared to confirm the ESCC and tumor stage by expert histopathologists then the remaining of sample were re-examined for molecular pathology. Information of age and gender was recorded.

DNA Extraction

Three to five 10 - µm thick sections from each paraffin block were placed into 2-mL micro centrifuge tubes and de waxed with sequential washes of xylene (two times) and absolute and 95% ethanol. To obtain a good extract from paraffin embedded block, we used Ether – Chloroform method for getting pure form of DNA. Pellet were dried and digested 12 hours at 37°c with 0.2mg /ml proteinase K in 10 mMTris –HCL, pH8.0, 5mM EDTA, 0.5%SDS.The proteinase inactivated with 300 µl phenol / chloroform / isoamyl alcohol solution (with ratio of 25:24:1) mixed and then centrifuged at 12,000rpm for 10 min. The aqueous layer (upper layer) transferred into a fresh tube, added 600 µl of ethanol and 30 µl 3 M CH3cooNa and left at -70° c for 30 min discard the supernatant and rinsed with ethanol 80% and then dried. Dissolve the genome DNA in 50 µl distilled sterilized water and read for DNA concentration with Nano drop Spectrophotometer at OD 260/280nm [10].

Polymerase Chain Reaction (PCR)

The samples were analyzed by PCR using three sets of primers, i.e, GP 5+: 5'–ttggatccT TTG TTA CTG TGG TAG ATA CTA C–3' - GP 6+:5'–ttggatccG AAA AAT AAA CTG TAA ATC ATA TTC–3' (general primers for HPV) and A commercial kit designed for hot start PCR was utilized for all amplification reactions,16,18, 31,33,51,52,58 and primers 16, 18 (for high risk HPV) [Table/Fig 2].

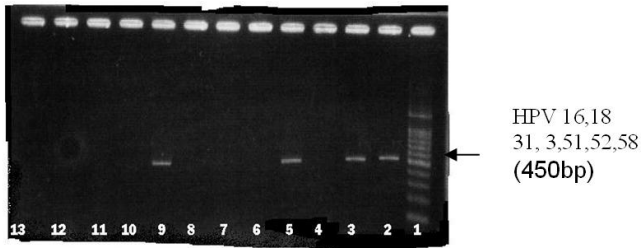
(Table/Fig 2)

Geno type	Primer sequences	Country
GP5+(150 bp)	5'–ttggatccT TTG TTA CTG TGG TAG ATA CTA C–3'	Netherlands
GP6+(150 bp)	5'–ttggatccG AAA AAT AAA CTG TAA ATC ATA TTC–3'	Netherlands
31,33,51,52,58, 16, 18(450 bp)	commercial kit	(Isogen, Russia)
16, 18(450 bp)	commercial kit	(Isogen, Russia)

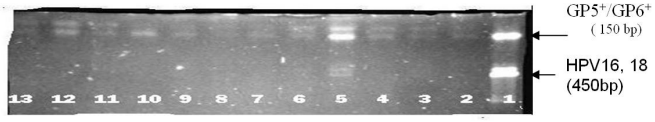
The PCR conditions for these primers were as follows: For primers $GP^{+}5/GP^{+}6$ the total 25 µl PCR reaction mixture contained 5 µl sample, 10mMtris-HCL,pH 9.0, 50mMKCL, 0.1%triton X-100, 1mMMgCL2, 200 µM deoxynucleotide triphosphate (dNTPs), 0.4pmole of each primer, and 0.2Uof Taq polymerase .The PCR thermal profile was at 95 ^{o}C for 5 min and 40 cycles at 94 ^{o}C for 30 s, 45 ^{o}C for 30s, 72 ^{o}C for 30 s, and final extension of 5 min at 72 ^{o}C.

PCR conditions for HPV 16,18 primers were the same as for the GP primers, except that for HPV 16,18 the annealing temperatures were 61 ^{o}c and 63 ^{o}c, respectively. The amplified PCR product was run on 1.5%agrose gel and stained with ethidium bromide .The PCR products were identified on the basis of their predicted fragment size.

To authenticate our results several controls were used in the study (a) to validate the PCR data, DNA from positive control tissues from i.e. cervical carcinoma samples known to be positive for HPV subtype 16,18, was subjected to PCR. (b) Negative control s was included with out DNA template to control for template contamination. [Table/Fig 3],[Table/Fig 4] represent two kinds of simple and multiplex conventional PCR .



**(Table/Fig 3) Represent the Electrophoresis of HPV PCR.Lane 1 is ladder.
Lane 2 shows positive control from cervical carcinoma (CC) specimen.
Lanes 3, 5 and 9 are positive.**



(Table/Fig 4) Represent the Electrophoresis of HPV PCR (Multiplex HPV16, 18 and $GP5^{+}/GP6^{+}$, Lane 1 shows positive control from cervical carcinoma (CC) specimen. Lanes 2-5 and 10- 12shows positive for $GP5^{+}/GP6^{+}$ and lane 5 positive for HPV16 &18.

Results

The mean age of the ESCC at diagnosis was 64 years (women 22 and men 27) (range: 35 to 81 years). The prevalence of HPV was higher among men than women .The study reported here is the first of its kind to gain an understanding of the prevalence of HPV in Guilan province with Esophageal Squamous cell Carcinoma .The work was retrospectively using paraffin-embedded ESCC taken from 2005 to 2007 from four different pathological labs.We estimated the prevalence of HPV in 45 (4 samples were too small) Esophageal Squamous Cell Carcinoma (ESCC) biopsies from Guilan province from diverse social and economic strata. Our results showed 17 out of 45 ESCC (37.7%) samples were positive for general markers of HPV, GP5/GP6 presence. 4 of 45 ESCC (8.8%) samples were positive for oncogenic types, 31,33,51,52,58 [Table/Fig 5].

(Table/Fig 5)Shows the distribution of different genotypes of HPV in 45 ESCC patients

Geno type	positive
GP5+/ GP6+(150 bp)	17 (37.7%)
31,33,51,52,58, 16, 18(450 bp)	4 (8.8%)
16, 18(450 bp)	2(4.4%)

Discussion

Esophageal Squamous Cell Carcinoma (ESCC) is one of the most prevalent human cancers worlds wide. The in incidence of ESCC has been found to have marked geographical variation .In most countries , incident rates of ESCC per 100,000 are about 2.5 to 5.0 for men and 1.5 -2.5 for women ,although in certain regions of Asia ,South Africa ,Iran, France ,and South America (high-incidence or high – risk area s) the relative risk is as high as 300 – fold [11],[12].In spite of many factor such as excessive tobacco, alcohol and black hot tea consumption even nutrition might be the cause of cancer but some types of HPV seem to be one factor . At the last studies the incidence of HPV cause 10 to 67% in some country .Infection with oncogenic HPV types is associated with 10% of human cancers worldwide. Most of these cancers originate from squamous epithelia of the anogenital and cervical cancer. The latter event is thought to be necessary for persistence and transforming activity of HPV oncogenes in tumor cell lineages,[13],[14] although the necessity of HPV integration for malignant transformation of host cells has not been firmly established in human tumors. In the course of malignant transformation early HPV proteins E6 and E7 interact with the cellular antioncogene such as Rb and P53 proteins. In particular, E6 can complex the p53 protein and E7 binds to Rb, enhances their degradation, and thereby alters the normal control of cell growth. The bypassing of the p53 and Rb checkpoints is necessary for any malignant tumor, and therefore the developing tumor is not expected to alter its early oncogenic mechanisms in consecutive phases of tumor development once inactivation of these proteins was successfully accomplished by HPV oncogene in the early phase of tumor development [15],[16].

The study reported here is the first of its kind to gain an understanding of the prevalence of HPV in Guilan province patients with ESCC .The work was performed retrospectively, using paraffin- embedded ESCC biopsies taken from 2005 – 2007 ,from a main hospital (Razi Hospital and 3 private path biology labs).The presence of HPV DNA was detected in 37.7% of ESCC cases, 13.2% of which were infected with more than one HR-HPV type (16, 18, 31, 33,51,52 and 58) and (4.4%) samples were high risk HPV 16,18 E6/E7 gene and 22 ESCC patients were negative with different types of HPV . . This frequency is in approximate agreement with previous reports mucosa and precancerous lesions but is different in other report from Turkmen Sahara, North –East of Iran Golastan province, that is more than 50% of course other factors ,mainly technical, may account for the observed difference in HPV detection rates in different PCR-based studies. This is a strong and notorious argument for expecting a carcinogenic role for HR-HPV types in esophageal cancer. However our statistical analyses failed to identify any significant difference in the variables studied between HR-HPV–positive and HR-HPV–negative ESCC patients [17]. Previous studies have also indicated unclear associations between viral infection and other known causes of esophageal cancer, such as tobacco, alcohol, and black hot tea especially in Golastan provice . In our patients the prevalence of alcohol abuse because of religious believes was very low in both HR-HPV–positive and –negative patients but use of tobacco was not studied , but HR-HPV was not significantly more frequent in the patients who were reported free of these established mutagenic risk factors. In spite of 13.2% infected with high risk HPV in 6 patients in our study and negative in 22 ESCC patients in Guilan with High incidence of ESCC in Guilan province, we must search for other factors in this province or combination of some factors cause of ESCC in Guilan province.

In conclusion, our study confirms that most ESCC originating from high risk incidence geographic areas for this carcinoma are not associated with HPV infection lonely and other pathogenic mechanisms are more important in the etiology of this carcinoma. These studies might prove useful in evaluating the potential that anti –HPV intervention measures may have in reducing morbidity caused by this disease, which currently has poor prognosis.

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Conflict Of Interest

Non declare.

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