RESEARCH ARTICLE

Role of *Human papilloma virus* Infection and Altered Methylation of Specific Genes in Esophageal Cancer

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Abstract

Background: Evaluation of *Human papilloma virus* (HPV) and its association with promoter methylation of candidate genes, p53 and Aurora A in esophageal cancer. Materials and Methods: One hundred forty-one esophageal tissue samples from different pathologies were evaluated for HPV infection by PCR, while the promoter methylation status of p53 and Aurora A was assessed by methylation-specific restriction based PCR assay. Statistical analyses were performed with MedCalc and MDR software. Results: Based on endoscopy and histopathology, samples were categorized: cancers (n=56), precancers (n=7), esophagitis (n=19) and normals (n=59). HPV infection was found to be less common in cancers (19.6%), whereas its prevalence was relatively high in precancers (71.4%), esophagitis (57.8%) and normals (45.7%). p53 promoter methylation did not show any significant difference between cancer and normal tissues, whereas Aurora A promoter methylation demonstrated significant association with disease (p=0.00016, OR:5.6452, 95%CI:2.18 to 14.6) when compared to normals. Aurora A methylation and HPV infection was found in a higher percentages of precancer (66.6%), esophagitis (54.5%) and normal (45.2%) when compared to cancers (14.2%). Conclusions: Aurora A promoter methylation is significantly associated with esophageal cancer, but the effect of HPV infection on this epigenetic alteration is not significant. However MDR analysis showed that the hypostatic effect of HPV was nullified when the cases had Aurora methylation and tobacco exposure. Further HPV sub-typing may give an insight into its reduced prevalence in esophageal cancer verses normal tissue. However, with the present data it is difficult to assign any significant role to HPV in the etiopathology of esophageal cancer.

Keywords: *Human papilloma virus* (HPV) - p53 - Aurora A - methylation - esophageal cancer

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Introduction

The global burden of cancer is increasing; this is considered to be largely because of change in life-style, an increasing adoption of cancer-causing behaviors and exposure to carcinogens in the environment. Based on IARC statistics, 12.7 million cancer cases are diagnosed and 7.6 million cancer deaths are estimated world-wide (Ferlay et al., 2008). Esophageal cancer is the sixth most common cause of cancer-related deaths with an estimated incidence of 482,300 new cases and 406,800 deaths every year (Jemal et al., 2011).

Etiology of esophageal cancer is not yet clear, but it is a multi-step progressive process. Major risk factors for esophageal cancer are not well-characterized, but may include poor nutritional status, low intake of fruits and vegetables, drinking beverages at high temperatures, tobacco usage and alcohol consumption (Engel et al., 2003; Islami et al., 2009; Wu et al., 2009). Earlier work from our group indicates that exogenous agents like coffee, tobacco, alcohol and non-vegetarian diet affect the methylation status of specific gene promoters in esophageal tissue and trigger epigenetic changes which may initiate the carcinogenic process (Mohan et al., 2006; Chava et al., 2011).

It has also been indicated that esophageal mucosa is susceptible to injury by mycotoxins and viruses apart from alcohol and nitrosyl compounds (Suzuk et al., 1996). Syrjanen (1982) first reported that Human papillomavirus (HPV) infection caused pathological lesions in the esophagus. Other studies have implicated HPV in the development of squamous cell carcinomas at different sites, including the cervix, anogenital region, colon, tongue, lung and upper aerodigestive tracts (zur Hausen et al., 1994; Buyru et al., 2006; Janet et al., 2009; Shukla et al., 2009; Gravitt et al., 2010; Mancilla et al., 2011; Xian et al., 2011; Ahmed et al., 2012). HPV E6 protein targets p53 for ubiquitin-mediated degradation while E7

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interferes with the Rb protein activity, contributing to host cell immortalization; high-risk HPVs differ from the low risk sub-types in the transforming potential of their E6/E7 proteins (Zandberg et al., 2013). Until now, studies examining the relationship between HPV and esophageal carcinoma have been inconclusive (Dabrowski et al, 2012; Yahyapour et al, 2012; Haeri et al, 2013). There is some evidence that HPV mediated epigenetic changes in specific genes may be important in cervical carcinogenesis (Bahnassy et al., 2006; Henken et al., 2007; Janet et al., 2009; de Wilde et al., 2010). However, there are no studies on this aspect in esophageal cancer. Hence, in this study, along with HPV screening we have evaluated its association with the methylation status of two specific genes, p53 and Aurora A, which have been implicated in the etiology of esophageal cancer.

p53 gene responds to diverse cellular stresses and regulates target genes to induce cell cycle arrest, apoptosis, senescence and DNA repair. Its level and post-translational modification state get altered in response to exogenous factors leading to hypoxia, spindle damage etc (Zhao et al., 2009). Epigenetic modifications such as methylation and acetylation are known to affect p53 expression and protein function (Mass et al., 1997; Schroeder and Mass, 1997; Hodge et al., 2005; Ibrahim et al., 2010). Aurora A also interferes with p53 suppressor function by MDM2-mediated degradation of p53 in cancer cell lines causing inactivation of its transcriptional activity (Katayama et al., 2004; Liu et al., 2004; Yang et al, 2013). Gillison et al. (2000) reported that p53 mutation was predominant in HPV negative tumors as compared to HPV positive tumors, indirectly relating the presence of HPV with disease outcome.

Aurora A gene, a member of a family of mitotic serine/threonine kinases, is associated with centrosome maturation and separation, regulates spindle assembly and stability; its dysregulation has been associated with cancers in different tissues (Lin-Yu et al., 2008; Burum-Auensen et al., 2010). High frequency of chromosomal aneuploidy, attributed to an impaired Aurora A gene reported in many cancers, was also seen in esophageal cancers and precancers as reported from our group (Marumoto et al., 2003; Mohan et al., 2007). It has been suggested that p53 interacts with Aurora-A to suppress its oncogenic activity in a transactivation-independent manner (Chen et al., 2002). Hence, we have assessed the methylation status of these two candidate genes to gain insight on whether HPV alone or in interaction with other exogenous factors plays a role in epigenetic modification of specific genes leading to esophageal carcinogenesis.

Materials and Methods

Study population and sample collection

The study was conducted on a total of 141 patients presenting with upper gastrointestinal tract symptoms, who were referred for endoscopy at two separate Gastroenterology units; data from 74 samples has been included from an earlier study of our group (Chava et al., 2011). The additional endoscopic biopsy samples obtained were sent for histopathological analysis and part of it was used for DNA isolation and subsequent molecular evaluation as described (Chava et al., 2011). The patients were categorized on the basis of endoscopy and histology reports into four groups: *i*) Esophageal cancer; *ii*) Precancer; *iii*) Esophagitis with/without GERD; and *iv*) Normals as described in the paper by Mohan et al. (2006). The normal controls were those patients who sample showed absence of any pathology upon investigation.

The data about the patient's personal history which included age, gender, tobacco usage, alcohol intake, dietary habits and tea/coffee consumption, along with clinical details were collected. The study was approved by the institutional ethical committee.

DNA extraction from tissue

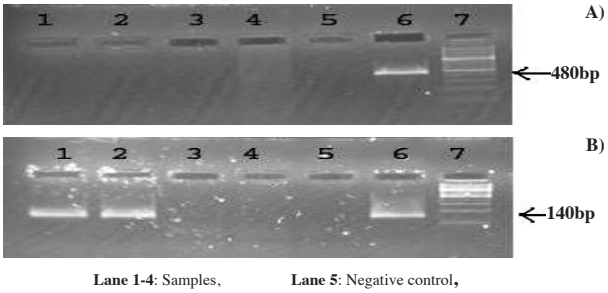
Fresh tissue biopsy samples were collected at the time of endoscopy and DNA was isolated as described in our earlier paper (Mohan et al., 2006).

HPV detection by PCR

HPV detection was carried out using HPV-PCR amplification kit (Bioserve, Hyderabad, India) according to the manufacturer's instructions: two rounds of PCR were performed for each sample, a positive (Provided by manufacturer) and negative control (Sterile water for injection, Nirlife, Gujrat, India) was used every time PCR was set up. Two sets of primers specifically designed in the conserved region of the HPV genome were used for detection of 480bp amplicon in 1st round of PCR, the product of which was used as a template for a 2nd round of PCR to generate a 140bp product. Presence of band in both rounds or atleast the 140bp product indicates HPV positivity. PCR products were checked on ethidium bromide stained, 2% agarose gels and bands were analyzed on a UVI gel documentation system using a DNA size marker (Figure 1). The use of nested PCR in our study increases the chances of detecting low copy numbers of the HPV in our samples. And we have taken care to avoid cross contamination by doing work (pre and post amplification) at two different laboratories. To rule out the product carry-over contamination, while performing PCR we maintained a negative control. And the strength of our study is we used fresh tissue biopsy DNA sample, which increased the chances of HPV detection by PCR method compared to the archival samples.

Methylation specific restriction assay (MSRA)

Equal amounts of amplifiable DNA from tissue



Lane 1-4: Samples, **Lane 5**: Negative control, **Lane 6**: Positive control, **Lane 7**: 100 bp DNA marker

Figure 1. A) 1st Round PCR Amplification and B) 2nd Round PCR

samples were subjected to digestion using methyl sensitive restriction endonuclease HpaII (20 units of HpaII and 0.5 μg of DNA in 10μl reaction; MBI Fermentas, USA). In parallel, a mock digest was set up with digestion buffer and template DNA without the restriction enzyme as done earlier (Shetty et al., 2010). All the samples were incubated for 14 hours at 37°C. A three-step PCR by the method reported from our group was carried out with the digested and undigested DNA with appropriate primers. The primer sequence of gene, annealing temperatures and the expected band size are as described in our earlier paper (Chava et al., 2011). Thirty-five cycles were performed in a thermal cycler with initial denaturation 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C, 60°C for 30 seconds, 72°C for 45 seconds and a final extension at 72°C for 5 minutes. PCR products were checked on ethidium bromide stained, 2% agarose gels and bands were analyzed on a UVI gel documentation system.

Statistical analysis

Statistical analysis was performed using the Chi square for comparisons and significance by MedCalc software (12.2.1 version), to determine differences in the HPV positivity, Aurora A and p53 gene methylation between the groups studied; values with $p<0.05$ were considered statistically significant (Table 4). Multifactor-dimensionality reduction software (2.0 beta 8.3 version) was used to perform logistic regression analysis for the evaluation of gene-gene (p53 and Aurora) and gene environment (p53, Aurora with HPV, Tobacco and Alcohol) interactions.

Results

Of the 141 esophageal biopsies collected, 56 patients were identified as cancer (45 squamous cell carcinoma and 11 Adeno carcinoma), 7 precancers, 19 esophagitis with/without GERD (7 esophagitis and 12 esophagitis with GERD), while 59 showed normal histopathology. Clinco-pathological and demographic details are presented in Table 1. The cases investigated had higher male representation (male to female ratio: 1.8:1) with mean age of 58.3 years (SD±13). 60.3% of cancer patients had history of tobacco usage, 35.8% alcohol consumption, 56.6% had non-vegetarian diet while 83% of the cases consumed hot beverages; whereas, among the individuals with a normal histopathology, 20.3% were tobacco users, 15.25% alcohol consumers, 49.1% have a non-vegetarian diet while 57.6% of the cases consumed hot beverages like tea/coffee.

Table 1. Clinico Pathologic and Demographic Characteristics of Esophageal Pathologies

		Cancer	Precancer	Esophagitis with/without GERD	Normals
Patient n=141(%)		56 (39.7%)	7 (4.9%)	19 (13.47%)	59 (41.8%)
Gender	Male	36 (64%)	4 (57%)	11 (58%)	40 (68%)
	Female	20 (36%)	3 (43%)	8 (42%)	19 (32%)
	Age	58.3 (SD±13)	53 (SD±17.7)	48 (SD±22)	44.9 (SD±15.5)

HPV prevalence in esophageal samples

Presence of HPV was seen in 38.2% of the total esophageal samples evaluated, which included 11 cancers (19.6%), 5 precancer (71.4%), 11 esophagitis with/without GERD (57.8%) (4 esophagitis and 7 esophagitis with GERD) and 27 normal histopathologies (45.7%) (Table 2, Figure 2).

Gene promoter methylation in different pathological groups and correlation with HPV infection

MSR analysis showed that p53 promoter was methylated in 80% of the esophageal specimens evaluated, which included 76.7% of cancer samples, 71.4% precancer, 83.3% esophagitis with/without GERD, and 75.8% of normal histopathologies. Of the p53 methylated samples, 42% showed HPV positivity; 25.58% of these were cancers, 80% precancers, 66.6% esophagitis with/without GERD and 45.4% normal cases.

Seventy point eight percent of the esophageal tissue samples evaluated showed Aurora A promoter methylation, which included 87.5% cancer samples, 85.7% precancers, 61% esophagitis with/without GERD and 55.5% normals. Of all the samples showing Aurora A promoter methylation, 31.9% showed HPV positivity of which 14.2% were cancers, 66.6% precancers, 54.5%

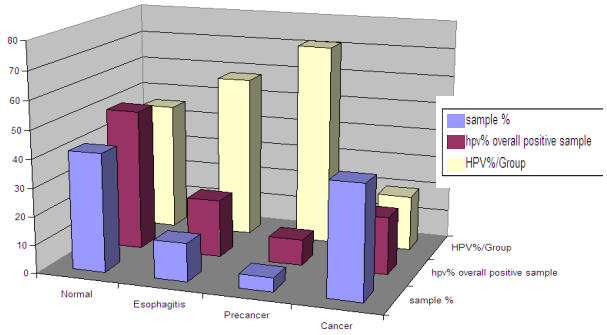


Figure 2. Histogram Showing Sample Size as Percentage of Total Samples Analyzed ( ). Represents the percentage of HPV positive samples in the particular group as against the total HPV positives (n=54) seen in the study ( ). ( ) represents the percent of HPV positive cases in each group

Table 2. Presence of HPV and Methylation Status of p53 and Aurora A Gene Promoters in Different Esophageal Pathologies

Pathological Condition	No of samples	HPV ve+	p53 Methylation	p53 Methylation & HPV	AuroraA methylation	Aurora A Methylation & HPV
Cancer	56 (39.7)	11 (19.6)	43/56 (76.7)	11/43 (25.58)	49/56 (87.5)	7/49 (14.2)
Precancer	7 (4.9)	5 (71.4)	5/7 (71.4)	4/5 (80)	6/7 (85.7)	4/6 (66.6)
Esophagitis+GERD	19 (13.47)	11 (57.8)	15/18 (83.3)	10/15 (66.6)	11/18 (61)	6/11 (54.5)
Normal	59 (41.84)	27 (45.7)	44/58 (81.15)	20/44 (45.4)	31/56 (55.35)	14/31 (45.16)
Total	141	54 (38.2)	107/139 (76.97)	45/107 (42.05)	97/137 (70.8)	31/97 (31.9)

*Figures in the parenthesis are percentages

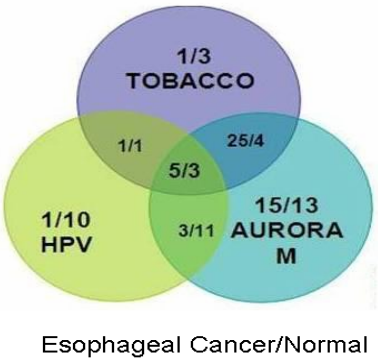


Figure 3. In Venn Diagram, Each Colored Circle Represents the Individual Factor Studied. Whereas the circles which show interaction between the factors are depicted in the overlapping areas. Tobacco and Aurora A methylation (25/4) shows highest risk followed by Aurora methylation, tobacco and HPV(5/3)

Table 3. Summary of Multi-Factor Dimensionality (MDR) Results

Model	Training Accuracy	Testing Accuracy	CVC
Tobacco	0.7048	0.668	9/10
HPV-Aurora Meth	0.7312	0.6422	6/10
HPV-Aurora Meth-Tobacco*	0.7489	0.7003	9/10

*Selected as best model and the data represented in Venn diagram

Table 4. Statistical Analysis of MSR Assay Carried Out for 141 Esophageal Tissue Samples*

Genes	Methylated	Un-methylated	Chi square	OR	p value
p53: Cancer	43/56	13/56	0.01345	1.0524	0.9077
Normals	44/58	14/58			
Precancer	5/7	2/7	0.06616	0.7955	0.3985
Normals	44/58	14/58			
Esophagitis	15/18	3/18	0.4416	1.591	0.2532
Normals	44/58	14/58			
Aurora A:					
Cancer	49/56	7/56	14.18	5.6452	0.00016
Normals	31/56	25/56			
Precancer	6/7	1/7	2.366	4.839	0.06204
Normals	31/56	25/56			
Esophagitis	11/18	7/18	0.1838	1.267	0.3341
Normals	31/56	25/56			

*p<0.05 consider to be significant

esophagitis with/without GERD and 45.1% normals (Table 2).

MDR analysis to assess interactions

The methylation status of Aurora A and p53 genes, along with exogenous factors was included in the MDR analysis. This method includes a combined cross validation/permutation-testing procedure which minimizes the false positive result that may arise from multiple examinations of the data. 10 testing accuracies were averaged and the cross-validation consistency (CVC) values were taken (Table 3). Best CVC values (9/10) were obtained for tobacco, HPV and Aurora A gene promoter methylation. further we analyzed to indicate the high risk groups and low risk group for the various factors/attributes studied; and the best graphical model was generated and represented as a Venn diagram (Figure 3).

Discussion

Esophageal cancer etiology and risk factors need to be established to help reduce the burden of this cancer which has a high mortality. In the present study, there was a higher male representation (male to female ratio: 1.8:1), which, however, did not reach the expected 3:1 ratio reported by other groups; the mean age of cancer patients was 58.3 years (SD±13) which is similar to the reports on esophageal cancer by Puttawibul et al. (2001) from Thailand and Nordenstedt et al. (2011) from USA.

Several reports are available on mutations and polymorphisms involving p53 and Aurora A gene in esophageal carcinogenesis (Katiyar et al., 2004; Chen et al., 2009; Yang et al., 2013). However, epigenetic changes in specific genes in the esophageal tissue due to its direct interaction with exogenous factors are limited (Chava et al., 2011). Different studies have reported about the role of tobacco, alcohol, diet and hot beverages in the etiology of esophageal carcinogenesis (Engel et al., 2003; Islami et al., 2009; Wu et al., 2009; Toh et al., 2010). Using MDR analysis, an earlier paper from our group has shown that non-vegetarian diet, alcohol consumption and tobacco intake confer a high-risk of esophageal cancer, whereas tea reduced this risk. Exogenous factors in our study show a slightly different level of significance with disease status when compared to our earlier study (Chava et al., 2011) which may be due to an increased sample size and also the fact that we have divided the samples into four pathological groups instead of the earlier three. In the present study, our interest was to know if HPV was prevalent in esophageal tissue from our population and whether it can be considered as an important exogenous factor that affects the methylation status of selected candidate genes, with or without, interaction with other established risk agents.

A total of 38.2% of the esophageal samples studied were HPV positive, only 19.6% of these were cancers. In esophagitis and precancer samples, a high percentage of infection was observed, but the number of samples in this category were less. Figure 2 shows that presence of HPV is more in the three remaining groups i.e., 71.4% in precancers, 57.8% in esophagitis with or without GERD and 45.7% normal samples. Our report on HPV positivity in the esophageal tissue falls in the range of 0-67% as given by de Villiers et al. (2004). Goto et al. (2011) showed that 9.4% of esophageal cancers from China, Japan and Korea were HPV positive; while Kagoshima, a city from Japan, exhibited a significantly higher prevalence of HPV (24.1%) in esophageal carcinoma. A review from India reported that the highest HPV frequency in esophageal specimens was from Dibrugarh, Assam (44%) followed by Kashmir (33%); HPV was not detected in esophageal cancer patients from New Delhi (Shukla et al., 2009). Vaiphei et al. (2013) from Chandigarh, India report 87% (20/23) esophageal cancer samples were HPV positive, many with multiple infections, whereas none of the normal controls (autopsy mid-esophageal tissue) showed HPV positivity. To the best of our knowledge, ours is the first

study from South India, demonstrating a 19.6% HPV positivity in esophageal carcinoma samples; a comparison of HPV positive cancer and normal (endoscopic biopsies with no evident esophageal pathology) samples showed a significant difference (p=0.002) with high prevalence in non-cancerous esophageal tissue. A study from Iran on individuals undergoing upper GI tract endoscopy showed, unlike our data, a higher HPV positivity in esophageal squamous cell carcinoma samples (36.8%) when compared to controls (13.2%) (Farhadi et al., 2005) and a study from the same country showed no HPV positivity in esophageal squamous cell carcinoma (Haeri et al., 2013). However, from all of the above studies it is clear that detection rates of HPV DNA in this cancer shows varied level of prevalence. Differences in the detection methods, sample types and geographical regions of the sample origin have been suggested as potential causes of this discrepancy.

From our results, we suggest the possibility that HPV in the esophagus may be behaving like an opportunistic commensal/pathogen, thereby explaining the higher prevalence in normal esophagus. It is possible that some subtypes (so-called low-risk) of HPV may be living in synergy with the esophageal microenvironment, however in the presence of certain factors like exposure to harmful diet, tobacco, alcohol, free radicals, weakened immune system etc, they may be replaced by the pathogenic forms (how that transition takes place will need investigation). Dolgin (2011) has reported the presence of commensal viruses by sequencing based methods. It may be worth examining this kind of a possibility by looking at cases with multiple infections, what sub-types they are, the individual histopathology and follow-up on response and survival. Studies from Kashmir, New Delhi, Japan, Iran Australia and South Africa report that HPV may not be an important etiologic factor for developing esophageal squamous cell carcinoma (Furihata et al., 1993; Abdulvahab et al., 2006; Mir et al., 2007; Koshiol et al., 2010; Sitas et al., 2012; Schafer et al., 2013). De Villers et al. (2004) reported that the prevalence of the high risk type HPV 16 was 9.8% in normal/inflammatory esophageal mucosa but was 47.6% in ESCC, where as the low risk HPV 11 was present in 28.9% of the carcinoma, 37.3% of normal/inflammatory, 66.7% of dysplastic samples. Viral load of HPV was not significantly different in the esophageal cancer tissue from different geographical locations and this does not seem to contribute to high incidence of ESCC (Castillo et al., 2013; Liu et al, 2013). Persistence of high-risk types in a small number of cases which progress to esophageal cancer could account for a lower percentage of HPV in our cancer samples, but further details can be given about it after performing HPV sub-typing in our positive samples, which is our future plan of work.

The mutation and methylation of p53 has been implicated in etiology of several cancers including that of the esophagus (HE et al., 1997; Kang et al., 2001; Woodson et al., 2001; Katiyar et al., 2004). Our earlier data of a smaller sample size (n=74) showed that p53 promoter methylation status was not statistically different between normal and cancer samples; inspite of increasing the sample size, in this study our results remain the same.

The promoter methylation of Aurora A gene was reported to be altered in esophageal cancer when compared to esophageal tissue from non-cancerous pathologies (Chava et al., 2011). In the present study, we reconfirmed on a larger sample size that Aurora A methylation is altered and may be important in the etiology of esophageal cancer. It is likely that presence of HPV affects the methylation status of specific genes like Aurora A in esophageal tissue, altering the spindle assembly and affecting mitotic stability. Cogliano et al. (2005) reported that high-risk HPVs can induce mitotic abnormalities through mitotic spindle checkpoints, while this is not seen in low-risk HPV. Since p53 methylation did not show any significant difference between normal and cancer tissue, further analysis to assess the effect of HPV on methylation, was evaluated for Aurora A. Thirty-two percent Aurora A methylated samples were HPV positive. It was found that the highest positivity was seen in non-cancerous pathologies, with 66.6% in precancer, 54.5% in esophagitis and 45.16% in normals as against 14.2% in cancers, suggesting that HPV does not have a significant role in altering the methylation status of Aurora A to promote carcinogenesis.

There is some evidence that agents like tobacco and alcohol interact with HPV affecting genetic and epigenetic changes. It was shown that tobacco carcinogens like benzopyrene, may enhance the activation of calcium-sensitive protein kinase C pathways which boost HPV virion synthesis/uptake (Ho et al., 1998; Alam et al., 2008; Schafer et al., 2013). For assessing high-risk combination for esophageal carcinogenesis, the best models of MDR (refer to Venn diagram: Figure 3) showed that subjects presented with Aurora methylation along with tobacco consumption fall under high risk group (25 cancer/4 controls) for esophageal carcinogenesis; where as HPV infection alone (1 cancer/10 controls) showed a lesser effect. It is noteworthy that when both Aurora A methylation and tobacco exposure are present in the subject, the reduced effect of HPV is nullified (5 cases/3 controls). Schafer et al. (2013) reported that HPV 18 pseudovirion uptake was enhanced in benzopyrene treated cells in culture. Probably, HPV works in synergy with the other two factors, tobacco and methylated Aurora, to play a part in esophageal carcinogenesis.

Aurora A promoter methylation showed significant association with disease; HPV seems to have a role in esophageal cancer only in the presence of altered promoter methylation of Aurora A and tobacco use. Due to its higher prevalance in normals when compared to cancers, investigations are warranted to explore the possibility of looking at viral entities like HPV to have a synergistic or purely antagonistic behavior in the esophageal tissue. Further, sub-typing of HPV may give an insight into its varied prevalence in cancer verses esophageal normal tissue in our cases. With the present data, it is difficult to assign any significance to HPV in the etiopathology of esophageal cancer.

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