Assessment of human papillomavirus and Epstein-Barr virus in lung adenocarcinoma

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Abstract. The association of human papillomavirus (HPV) and Epstein-Barr virus (EBV) infection with non-small cell lung cancer is controversial. HPV and EBV prevalence in a uniform population of lung adenocarcinoma was investigated, hypothesizing that there would be differences seen between smokers and non-smokers and between sexes. Patients involved in this study were selected from a single institution database of lung cancer. In total 497 patients with adenocarcinoma were identified and 110 patients had sufficient tissue for analysis with an *in situ* hybridization method that probed for high-risk and low-risk HPV and EBV. There were 65 males and 45 females, 78 patients with stage I-IIIA disease and 32 patients with stage IIIB-IV disease. There were similar number of smokers and non-smokers. Across all stages HPV and EBV staining was absent from all tissues examined. It is unlikely that HPV or EBV is an important etiological agent in adenocarcinoma of the lung, even among the never-smokers.

Introduction

Cigarette smoke is strongly associated with the development of lung cancer (1). While the epidemic of smoking has reduced through public health awareness programs and education, the incidence of lung cancer remains unabated. There has been a demographic change in the global pandemic with a global realignment of histological subtypes. Adenocarcinoma incidence is rising globally among both smokers and non-smokers. Up to 30% of all diagnosed non-small cell lung cancer (NSCLC) among various Asian countries, including Singapore, do not have a history of exposure to cigarette smoking. These patients tend to be predominantly female and have adenocarcinoma (2). This has given rise to different postulates as to the possible etiologies of NSCLC in this distinct of patients, ranging from exposure to cooking oil fumes (3,4), as well as diet and cancer susceptibility genotypes among Asians (5-7). Human papillomavirus (HPV) and Epstein-Barr virus (EBV) have been variously described to be associated and causative with several cancers. HPV is likely to be causal in cervical carcinoma and head and neck squamous cell carcinoma (HNSCC), and EBV in naso-pharyngeal carcinoma. There have been various reports of association between HPV and EBV with lung cancer but results have not been conclusive (8). Leveraging on the excess of non-tobacco related lung cancer in our patients we investigated HPV and EBV prevalence in a uniform population of adenocarcinoma of the lung, hypothesizing that there would be differences seen between smokers and non-smokers and between sexes.

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Patients and methods

Patients. Patients involved in this study were selected from a database of NSCLC patients, diagnosed in a single institution (SGH) from 1999 to 2002. The study had the approval of the Institutional Review Board of National Cancer Centre Singapore. The main selection criterion was a diagnosis of adenocarcinoma. Four hundred and ninety-seven patients were identified and among them, 110 patients had sufficient tissue blocks available for our analysis using *in situ* hybridization method for HPV and EBV. The tissue samples were reviewed and confirmed by the collaborating pathologist (KLC). Five-micrometer slices from paraffin embedded tissue blocks were used for the analysis.

HPV and EBV in situ hybridization method. This *in situ* hybridization procedure was done in the Department of Pathology, Singapore General Hospital. Sections from paraffin-embedded tissues were deparaffinised and subjected to a series of steps as described by Brousset *et al* (9). The probes for the various infective agents described below were used according to the manufacturer's recommendations.

For human papillomavirus (HPV) study, peptidic nucleic acid (PNA) probes from Ventana (Ventana Medical Systems, Tucson, AZ, USA) were used. HPV II family 6 probe (catalog no. 800-2220, Ventana) containing a cocktail detecting the low-risk genotypes 6 and 11 was used while HPV II family

Table I. Patient characteristics (n=110).

		n (%)
Age	Median (years)	64
Race	Chinese	110 (100)
Sex	Male	65 (59)
	Female	45 (41)
Smoker status	Never-smoker	54 (49)
	Smoker	56 (51)
Stage	I-IIIA	78 (71)
	IIIB-IV	32 (29)

16 probe (catalog no. 800-2219) containing a cocktail detecting high-risk genotypes 16, 18, 31, 33, 35, 45, 51, 52, 56, 58 and 66 was used. For Epstein-Barr virus (EBV), PNA probes from Ventana (Epstein-Barr Early RNA probe, catalog no. 780-2842) were used.

Appropriate positive and negative control tissues as well as positive and negative reagent controls were used during the procedure. A positive result was read when there was significant nuclear staining of the cells tested.

Results

One hundred and ten tissue samples were retrieved from the Department of Pathology. Patient demographics are illustrated

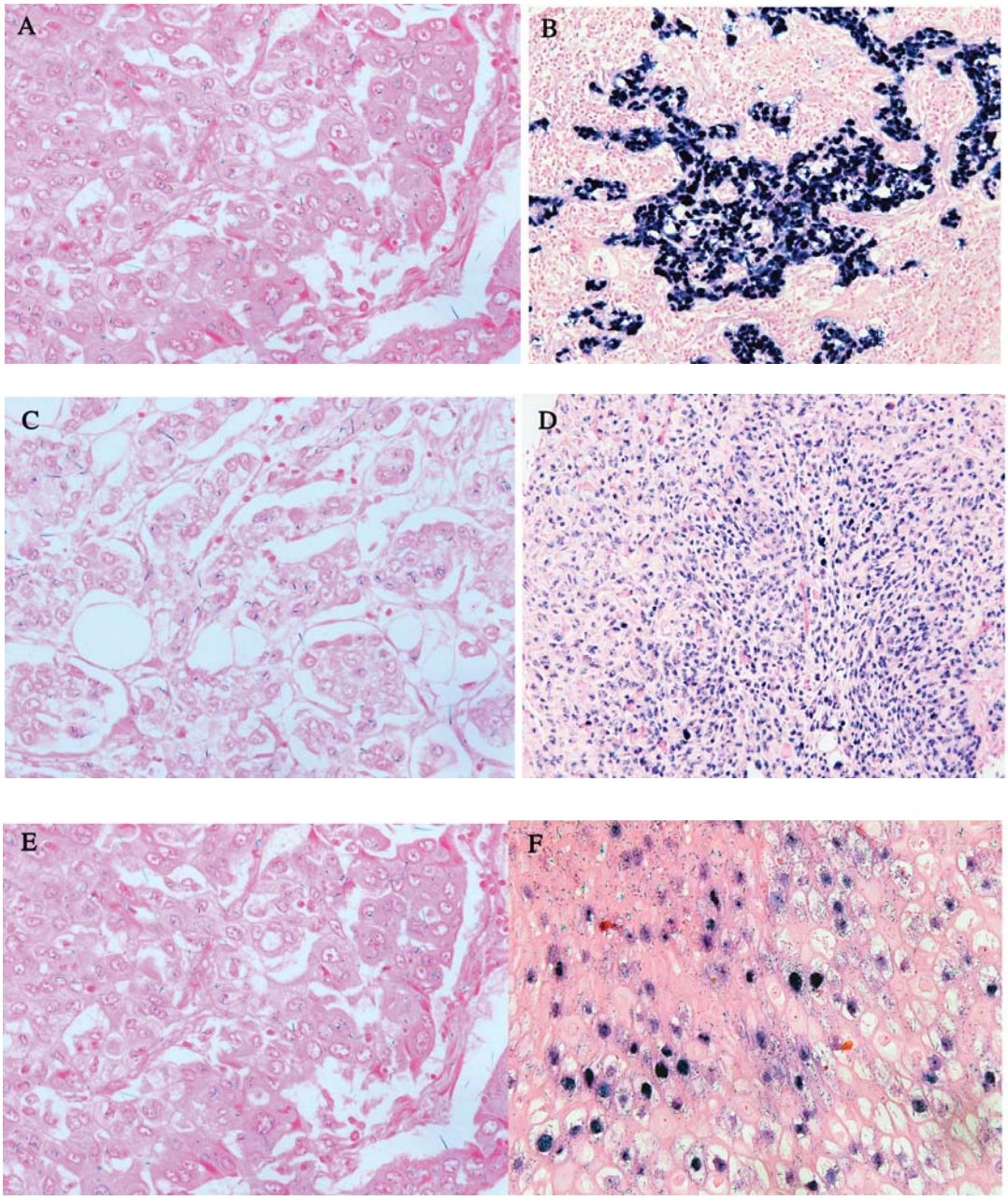

Figure 1. (A) Absence of EBV by ISH in lung adenocarcinoma (x400). (B) Paired positive control for EBER probe (x200). (C) Absence of high-risk HPV by ISH in lung adenocarcinoma (x400). (D) Paired positive control for HPV family 16 probe (x200). (E) Absence of low-risk HPV by ISH in lung adenocarcinoma (x400). (F) Paired positive control for HPV family 6 probe (x400).

Table II. Selected studies of HPV in non-small cell lung cancer.

Author/Refs.	Country	Methods	n	Histology (Adenoca./ non-adenoca.)	HPV serotypes
Syrjanen (8)	Finland	ISH	131	0/131	6 (1.5%), 16 (5.3%)
Kaya, *et al* (19)	Turkey	ISH	26	0/26	16/18 (7.7%), 6/11 (3.8%)
Gorgoulis, *et al* (20)	Greece	PCR and ISH	68	32/36	16/18 (0%)
Szabo, *et al* (22)	Japan	PCR	47	0/47	6, 11, 16, 18, 31, 33, 52b, and 58 (all 0%)
Aguayo, *et al* (23)	Chile	PCR and Southern blotting	69	Not stated	16 (29%)
Noutsou, *et al* (24)	Greece	PCR	99	41/58	16 (4%),18 (8%),11 (3%), 33 (2%)
Giulani, *et al* (25)	Italy	Blot hybridisation	78	27/51	16 (3.8%), 31 (1.3%), 6/53 (2.6%), 16/18 (1.3%)
Kinoshita, *et al* (26)	Japan	PCR	36	22/24	18 (8%)
Iwamasa, *et al* (28)	Japan	PCR and ISH	44	0/44	16/18 (57%)
Cheng, *et al* (29)	Taiwan	Nested PCR and ISH	141	83/58	16/18 (54.6%)
Fei, *et al* (30)	China	Nested PCR/ISH	73	33/40	16 (26%), 18(23.3%), 16/18 (27.7%)
Park, *et al* (31)	Korea	PCR	112	53/59	16 (10.7%), 18 (9.8%), 33 (33%)
Jain, *et al* (32)	India	PCR	40	9/31	18 (5%)
Coissard, *et al* (34)	France	Line blot assay	218	80/138	16 (1.8%)
Clavel, *et al* (35)	France	Hybrid capture II	185	60/125	High-risk (2.7%)

in Table I. There were similar number of smokers and non-smokers (56/54). There were 65 males and 45 females, 78 patients with stage I-IIIA disease and 32 patients with stage IIIB-IV disease. HPV and EBV were uniformly absent from this large population of lung adenocarcinoma screened (Fig. 1).

Discussion

The current study failed to demonstrate the presence of HPV or EBV DNA by an *in situ* hybridization method in any of the tumors that were screened. We acknowledge that *in situ* hybridization methods have only moderate sensitivity for detection of HPV, and there could be a false-negative rate (10). However, there remains no uniform technology for detection of HPV. *In situ* hybridization methods that combine polymerase chain reaction (PCR) methods while more sensitive are also prone to false-negative rates from failed amplification when fragmented DNA extracted from paraffin-embedded tissue is used, and false-positive rates from amplification of other DNA (11), and are not routinely available in service pathology labs. One significant advantage of this *in situ* hybridization method is that it allows for demonstration of the viral genome within tumor, and it can be routinely used to evaluate paraffin-embedded tissues. This is especially important in lung cancer where tissue samples are often small and limited. Although we were able to retrieve only about 25% of the intended population of adenocarcinoma, the 110 patient samples analyzed is not small and may be considered one of the larger studies. In addition, our analysis includes a high proportion of never-smokers, which is important to answer the association of these viruses with lung cancer among this distinct group of patients.

There is no strong explanation for HPV reaching and infecting lung tissue. HPV has long been thought not to have a viraemic phase in humans, although more recent data suggests that it my not be so (12,13). In lung cancer, Chiou *et al* raised the possibility of viraemic spread when they described a 70% concordance rate between HPV DNA in peripheral blood and paired lung cancer tissue (14). Even if viraemic spread were possible the low prevalence of HPV in the general population would imply a low rate of HPV infection in lung if any. HPV prevalence among women in Singapore is estimated at 5% (15), for South East Asia is low (16), and for women in Asia with normal cervical histology/cytology this rate stands at 14.4% (17). More conventionally, however, HPV spreads by local contact or seeding from nearby tissue. The development of lung squamous cell carcinomas by seeding from HPV-infected laryngeal papillomas is rare (18). Studies have also reported low or zero prevalence of HPV in lung cancer (both squamous and adenocarcinoma) when compared to HNSCC (19,20) in the same population. All this suggests a low likelihood of developing lung adeno-carcinoma from an upper respiratory source of HPV infection or from HPV viraemia.

HPV is epitheliotropic. Different studies in lung cancer have demonstrated wide-ranging differences of HPV among different world populations (Table II). It has been associated with squamous cell carcinoma and less so with adenocarcinoma

(21-25). One of the earliest and largest series was reported from Okinawa where there was high prevalence of HPV among adenosquamous and squamous cell carcinoma of the lung (26). The authors subsequently updated their series and reported a reduction in squamous cell carcinoma in Okinawa and a corresponding reduction in HPV prevalence in the tumors screened (27). This reduction was attributed to reduction in HPV prevalence in the general population, and improvement in health and socioeconomic status that are known risk factors for HPV transmission. The single study that suggested rather higher HPV prevalence among non-smokers and women in a Taiwanese population (28) is so far unique. In a subsequent study using similar methods the same group described lower prevalence in an ethnic Chinese population from Wuhan, China (29). There is the possibility that the use of nested-ISH could have led to over-amplification of HPV DNA. A recent Korean study using PCR methods for detecting HPV 16, 18 and 33 showed low prevalence of the high-risk genotypes averaging 10% and no association with smoking status or sex (30). A study from India that looked at 40 patients with lung cancer showed again low prevalence (5%) of HPV 18 and absence of HPV 16 (31). Several recent well conducted studies from Europe using fresh frozen tissue and modern methodologies have similarly shown the absence or low prevalence of HPV DNA and associated E6/E7 mRNA in lung cancer tissues (32-34). The 'burden of proof' of a causal nature lies in the ability to demonstrate conclusively and repeatedly to a high degree the presence of HPV DNA in tumor tissue that is transcriptionally active, as is the case for HPV and cervical cancer. The consistently low or zero prevalence of HPV-DNA and HPV E6/E7 transcription in lung cancer argues that a global study may be required to determine the true role of HPV in lung cancer in different populations and ethnicities. A large worldwide study in the similar vein as those conducted by the International Association for Research into Cancer (IARC) for cervical carcinoma and HNSCC using uniform methodology for HPV detection may need to be done for this purpose.

EBV on the other hand is commonly prevalent in Asian populations and is intimately related to nasopharyngeal carcinoma (35). Epstein-Barr early RNA (EBER) *in situ* hybridization is a recognized standard for identifying the association of EBV with a tumor. EBER is expressed in all three forms of EBV latent infection and EBV-infected cells carry significant copy numbers of EBER making it a sensitive test (36). To date the described associations with lung cancer have been with the rare lymphoepithelioma subtype (37). A slightly higher frequency of EBER has been described in squamous cell carcinoma and neuroendocrine carcinomas (38). A subsequent study in small cell lung cancer however showed complete absence of EBER-1 by *in situ* hybridization (39) and no staining for ZEBRA and LMP-1 and only focal expression of EBNA-1. EBV was similarly absent in mesotheliomas and lung adenocarcinomas in the study by Conway *et al* (40). The absence of EBER-ISH in this large cohort of patients makes it unlikely that EBV is involved in the pathogenesis of lung adenocarcinoma among Asians.

In conclusion HPV and EBV vary in prevalence across different world populations. The absence of HPV or EBV in lung adenocarcinoma in this study parallels that seen in several other studies. It is unlikely that HPV or EBV is an important etiological agent in adenocarcinoma of the lung, even among the never-smokers.

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