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Detection of Human Papillomaviruses Type 16, 18 and 33 in Bronchial Aspirates of Lung Carcinoma Patients by Polymerase Chain Reaction: A Study of 84 Cases in Croatia

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A B S T R A C T

Besides its well-known role in cervical carcinoma, HPV is also suggested to be involved in lung cancer development. A number of authors have been investigating the presence of HPV in histological materials. We used routine bronchial aspirates from 84 patients with lung carcinoma for DNA extraction and then performed polymerase chain reaction for high-risk HPV types 16, 18 and 33. The results were compared to those obtained from buccal and eyelid mucosa. Only three patients were positive for HPV in bronchial aspirates: one for HPV 16 type, one for HPV 18 type, and one for HPV 33. Our data indicated the low prevalence of HPV in patients with lung carcinomas in Croatia, therefore it seems unlikely that HPV contributes to the development of lung carcinomas in this region.

Key words: *lung carcinoma, human papilloma virus, polymerase chain reaction*

Introduction

The etiology of lung carcinoma is only partially resolved. The majority of tobacco users do not develop such tumors and at least 10–15% of lung carcinomas occur in non smokers[1]. Some studies suggested that HPV may play an aetiologic role in bronchial carcinogenesis and possibility of a latent HPV infection as a cocarcinogen cannot be excluded[2]. High-risk genotypes, including HPV 16, 18 and 33 are associated with the cancer of the uterine cervix[3] but they can be also detected in cancers of the upper aerodigestive tract, including cancers of the oral cavity, larynx and oesophagus tract[4–6]. A study in Finland[7] reported HPV integration in cancers of the hypopharynx, larynx, tongue and oral cavity and review of Syrjanen showed that HPV was detected in 22% of bronchial carcinomas with wide racial and geographic variation from 0–100%[2]. Recent article of Klein and al. reviewed 53 publications and 4508 cases showing the mean incidence of HPV in lung carcinoma was 24.5% and suggested that HPV is the second most important cause of lung cancer after cigarette smoking[8]. Those HPVs, which are considered as high risk for the cancer development, express the early oncoproteins E7 and E6. E7 inactivates the cellular tumor suppressor protein Rb, while E6 protein binds to the host cellular tumor suppressor protein p53 and triggers its degradation through the ubiquitin pathway[9]. The inactivation of p53 by HPV-E6 is considered to play a crucial role in human carcinogenesis[10]. Some studies showed that tracheal epithelial cells could be immortalised by HPV18 E6 and E7 oncogenes. Similar experiments were carried out with bronchial epithelial cells, which were immortalised by the HPV 16 E6 and E7 oncogenes and showed terminal differentiation of the keratinocytes but were not-tumorigenic in nude mice[2].

The aim of our work was to study the prevalence of high-risk HPV types 16, 18 and 33 in 84 bronchial aspirates of lung carcinoma patients by PCR and to elucidate whether the HPV infection is associated with lung cancer development in Croatia. The incidence of bronchial carcinoma in Croatia for the year 2005 was 2996 *per* 100000[11].

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HPV prevalence in Croatia is unknown as in the most other countries[12]. To our knowledge this is the first study to examine HPV prevalence in cytological bronchial specimens of lung cancers patients in Croatian population.

Materials and Methods

Patients and samples

Requests for specimens were consented by patients and approved by the local Ethical Commissions. The anamnestic data collected from the patient were: age, sex, smoking habits (pack/years, 1 pack/year corresponds to a pack of 20 cigarettes smoked daily over a period of 1 year). Cytological evaluation of 139 patients, who underwent bronchoscopy in the University Hospital for Lung Disease »Jordanovac«, Zagreb, Croatia during 2007–2008, revealed 84 lung carcinomas. Bronchoscopy was done according to the standard clinical procedure with a flexible video fiberoptic bronchoscope and routine cytological samples were taken (bronchial aspirates, bronchial brushings and/or biopsy touch smear specimens). For bronchial aspirate specimens the pulmonary physician collected fluid from endobronchial lesion. Buccal and eyelid swabs were simultaneously collected, as control DNA, just before bronchoscopy, from one side of inner cheek and inner side of one lower eyelid respectively by the cotton sticks. The cotton swabs with mucosal epithelial cells were immediately soaked in 2 ml sterile physiological solution (0.9% NaCl) in the sterile tube. On arrival at the cytology laboratory, bronchial aspirates were primarily processed for routine diagnostic purposes. The samples were centrifuged and a part of the cell pellet with remaining liquid was used to prepare conventional cytological smears. They are fixed on air and then subsequently stained according to May-Grünwald-Giemsa. According to the 2003 WHO classification of lung tumors, we classified lung cancers into four broad categories: squmous cell carcinoma, adenocarcinoma, small cell carcinoma and large cell carcinoma[13]. The residual material of bronchial aspirate, together with buccal and eyelid swabs, were transferred to another laboratory at the Croatian Institute for Brain Research, for DNA isolation and HPV DNA detection.

Detection and typing of HPV DNA

The liquid with cells obtained either from bronchial aspirates or buccal and eyelid swabs was transferred to the microcentrifuge tubes and centrifuged at maximum speed for 8 minutes. The supernatant was removed, leaving collected cells at the bottom. By combining alkaline and temperature lysis DNA was isolated[14,15]. Samples were resuspended in 75 μL alkaline mixture of 25 mM NaOH and 0.2 mM EDTA and incubated for 10 minutes at 98°C. Afterwards, samples were vortexed and incubated for 10 minutes at 98°C, followed by cooling 2 minutes on ice. Finally, the neutralizing solution containing 40 mM Tris-HCl pH 5.0 was added to every sample, the same volume as the initial volume for alkaline lysis. This corresponded to 75 μL of neutralizing solution making total of 150 μL, respectively, PCR-ready DNA solution which was stored at 4 °C. The quality of DNA was assessed by performing sex chromosomes detection with PCR[16]. The screen was based on the presence of amelogenin gene (AMEL) on both X and Y chromosomes (AMELX and AMELY), whereas AMELY contains the small deletion in the first intron, which is used to distinguish the sequences of X and Y chromosomes. Three primers used were SexX (5'-TCTCCTATACCACTTAGTCACT), SexY (5'-GCCCAAAGTTAGTAATTTTACCT), and SexCommon (5'-CAGCTTCCCAGTTTAAGCTCT) enabling the amplification of 330-bp PCR product from the X, and 218-bp from Y chromosome. HPV viral DNA was amplified with type specific primers flanking the E6 region to identify the HPV subtype[17]. The forward primer (5´AAGGGCGTAACCGAAATCGGT-3) was common to the sequence of HPV 16, 18 and 33 DNA. The reverse primer sequences for HPV 16, 18 and 33 were 5´-GTTTGCAGCTCTGTGCATA-3´, 5´-GTGTTCAGTTCCGTGCACA--3´ and 5´-GTCTCCAATGCTTGGCACA-3´ respectively, amplifying 140-bp product for HPV 16 and 18, and 141--bp for HPV 33. The final PCR product was analyzed by agarose gel electrophoresis.

Results

84 patients with the cytological diagnosis of lung cancer in bronchial aspirates and others bronhoscopic specimens were analyzed in this study. There were 64 males and 20 females from 46 to 85 years (mean 64.35±7.73 years). 73 had a history of cigarette smoking (mean 64.35±36.94 pack/years), while 11 were non-smokers.

Cytologic examination revealed 38 cases of squamous cell carcinoma, 14 cases of adenocarcinoma, 14 small cell carcinoma, 12 cases of large cell carcinoma and 4 cases of mixed type carcinoma (small and large cell carcinoma). Only three out of 84 bronchial aspirates showed the presence of HPV DNA by PCR. The bronchial specimen tested positive for HPV 16 belonged to 69-year old male, smoker (100 pack/years) with squamous and small cell lung carcinoma.The bronchial aspirate specimen positive for HPV 18 was from 71-year-old female, smoker (35 pack/years) with adenocarcinoma and her buccal mucosa specimen was also HPV 18 positive. The bronchial aspirate specimen positive for HPV 33 belonged to 72-year--old male, smoker (125 pack/years) with large cell lung carcinoma. His buccal mucosa specimen was also HPV 33 positive. Buccal mucosa specimens were HPV positive in two patients mentioned before and negative for 82 patients while eyelid mucosa specimens were HPV negative for all 84 patients (Figure 1).

Discussion

The present study on a series of 84 lung carcinoma patients indicated the low incidence of HPV infection in cytological samples of lung carcinomas in these patients. In other studies the prevalence of HPV in lung cancers ranged from 0 to 100%[2]. Sample preparation may be one

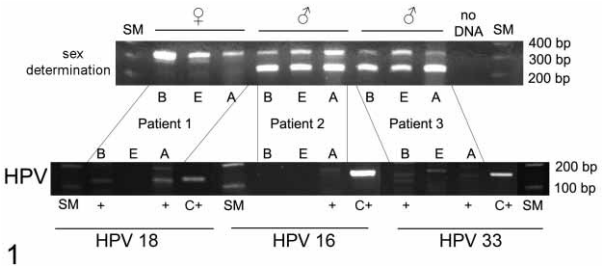

Fig. 1. Gel electrophoresis of PCR products of sex determination (upper gel) and HPV detection (lower gel) obtained from samples isolated from patients 1–3. Patient 1 was female positive for HPV 18, while patient 2 and 3 were males positive for HPV16 and 33 respectively (+denotes HPV positive samples, C+ positive control obtained from the cervix of an unrelated patient). The samples were taken from buccal (B) and eyelid (E) mucosa and bronchial aspirates (A). SM – 100 bp DNA ladder as a size marker, no DNA-negative control without DNA.

of the factors responsible for variation in HPV prevalence. All previous studies of detection of HPV DNA in lung carcinomas were made on histological tissue specimens. This is the first study of HPV DNA detection from cytological bronchoscopic specimens of lung cancer patients. When old samples were used, the detection rate of HPV DNA decreased with sample aged. Fresh samples obtained immediately yielded better results[11]. We chose to isolate DNA immediately from the fresh cytological samples of bronchial aspirates. This was expected to avoid modified or fragmented DNA frequently obtained from archival tissue due to formalin fixation of the histological samples. Moreover we implemented a simple protocol for human DNA isolation which enabled to isolate DNA solution ready for PCR in less than a hour[14,15].

The geographic and environmental factors including education, economic and weather conditions may account for the variable results reported. One study showed that Chinese who lived in different areas showed different HPV infection prevalence[18]. Our results confirmed low incidence of HPV infection in lung carcinomas in our geographic area. In most European countries (Germany 0%, France 2.7%, Turkey 5%, Greece 0%), in America 5.9% and India 5% prevalence of high-risk HPVs in lung cancers are low[19]. Another study in Greece showed completely opposite results with HPV positivity as high as 69%[20]. In northern Europe and Chile (29%) HPV infection rate seems to be higher: in Finland 41.7% in adenocarcinoma and 28.6% in squamous cell carcinoma and in Norway in 49% of lung carcinoma[19]. The highest prevalence of HPV infection was detected in Asia. In Okinawa in Japan HPV infection rates were 79% in squamous cell carcinoma and 49% in adenocarcinoma[21]. In China the prevalence of HPV16 infection was 26% and of HPV18 was 23.3% in non-small cell lung carcinoma[18].

Our samples contained normal and cancer cells, therefore it was not possible to distinguish whether HPV exclusively affected the tumor cells, a problem present also in the studies which used tissue specimens[21]. HPV is frequently ubiquitous and it infects a high percentage of people, therefore HPV infection is as well reported in normal lung with presence of HPV DNA in pneumocytes and alveolar macrophages[22]. Moreover, the carcinogenic role of HPV has been previously emphasized in vitro by immortalization of bronchial cell lines[21]. Kinjo et al.[23] has argued that HPV is not aetiologically involved in lung carcinomas development but that HPV induces squamous metaplasia in adenocarcinomas. Another explanation for a low viral load might be a »hit and run« mechanism, where the virus DNA may be lost after transformation, as shown in studies on a bovine model and as suggested in HPV 18 oncogenesis[21].

Two of our three patients, whose bronchial aspirates contained HPV DNA, had also HPV positive sample of buccal mucosa. Today little is known about oral HPV infection in the general population and in some population-based, control studies of oral cancer, the prevalence of HPV infection among control patient varied from 5.0% to 9.2%[24]. HPV has been regarded as a sexually transmitted disease but this view is challenged by frequent detection of HPV in children. Unlike in genital tract, natural history of oral HPV infection is poorly studied[25]. HPV can also be transmitted by non-sexual routes including casual physical contact and perinatal vertical transmission[26]. On the other hand, some authors suggested that the oral cavity is a significant reservoir for HPV infection that may not be entirely independent of the cervical reservoir[27]. The prevalence of HPV 16/18 was even demonstrated in the blood circulation of lung cancer patients where it was significantly higher than that of the control group. Still the possible route of HPV entering the blood cells remained an enigma[28].

In conclusion, in our experience with 84 bronchopulmonary cancers, few cases of oncogenic HPV DNA were detected suggesting HPV 16, 18 and 33 infections are unlikely to contribute to lung carcinogenesis in Croatia, similar to some other European countries. All three HPV positive patients were smokers. It would be useful to detect the presence of HPV in lung cancers in larger number of nonsmokers where we can see the effect of HPV alone in the absence of other associated carcinogenic factors. Also it would be important to find the prevalence of HPV in the general population.

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ODREĐIVANJE HUMANOG PAPILOMA VIRUSA TIPA 16, 18 I 33 U ASPIRATIMA BRONHA PACIJENATA S KARCINOMOM PLUĆA METODOM LANČANE REAKCIJE POLIMERAZOM: ISTRAŽIVANJE 84 PACIJENATA U HRVATSKOJ

SAŽETAK

Za humani papiloma virus je pretpostavljeno da može imati, osim u etiologiji karcinoma cerviksa uterusa, ulogu i u etiologiji nastanka karcinoma pluća. Prisustvo visokorizičnih tipova papiloma virusa tipa 16, 18 i 33 istraživano je u rutinskim citološkim uzorcima aspirata bronha 84 pacijenata s karcinomom pluća, nakon izolacije DNA, metodom lančane reakcije polimerazom. Rezultati su uspoređivani sa onima u uzorcima bukalne i vjeđne sluznice. Samo tri pacijenta bila su pozitivna na humani papiloma virus: jedan na HPV 16 tip, jedan na HPV 18 tip i jedan na HPV 33 tip. Naši rezultati potvrđuju nisku prevalenciju humanog papiloma virusa u pacijenata s plućnim karcinomom u Hrvatskoj što ukazuje da HPV vjerojatno ne sudjeluje u razvoju karcinoma pluća na ovom području.