A review of 20 years of human papillomavirus research in Slovenia

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KEY WORDS

HPV, human papillomavirus, Slovenia

ABSTRACT

Background: Human papillomaviruses (HPV), remarkably diverse DNA viruses etiologically linked with various benign and malignant neoplastic lesions of mucosal and skin epithelium, have been the subject of intensive research for the last 30 years worldwide.

Objective: Briefly to review 20 years of HPV research in Slovenia by analyzing the articles published in journals indexed in peer-reviewed databases Medline/Pubmed, Science Citation Index/Web of Science, Embase and PsycINFO.

Methods and Results: Up until October 2011, Slovenian researchers published 73 articles in journals indexed in peer-reviewed databases, which can be divided into 15 categories: detection of HPV in archival clinical specimens, development of novel HPV tests, evaluation of various commercial tests for the detection of high- and low-risk alpha-HPV, HPV and anogenital tumours, HPV testing in routine gynecological practice, HPV and laryngeal benign tumours, HPV and laryngeal epithelial hyperplastic lesions and laryngeal cancer, HPV and tumors in oral cavity, HPV and esophageal benign and malignant tumors, HPV and inverted papillomas, genomic diversity of selected HPV types, hair follicles as an important endogenous reservoir of HPV, identification and characterization of novel HPV types, HPV vaccination and HPV basic research. Until October 2011, Slovenian HPV papers received 473 citations (self-citation excluded) and their Hirsch index is currently h=13.

Conclusion: In the last 20 years, Slovenian HPV researchers have been actively and successfully incorporated in the international HPV community and have contributed small but significant achievements in the field.

Human papillomaviruses (HPV) are remarkably diverse DNA viruses, which are etiologically linked to various benign and malignant neoplastic lesions of mucosal and skin epithelium. Currently, 148 different HPV types are officially recognized, ranging from HPV-1 to HPV-152 (HPV-46, HPV-55, HPV-64 and HPV-79, which have not met the criteria as unique HPVs, are now classified as subtypes). All known

HPV types are classified by the similarity of their genome into five genera (alpha, beta, gamma, mu, and nu) and 33 species.

To the best of my knowledge, HPV research in Slovenia started in 1990 independently at the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana (Jožica Marin), the Institute of Pathology, Faculty of Medicine, University of Ljubljana (Mario Poljak) and the Institute of Oncology, Ljubljana (Marjetka Uršič-Vrščaj). The first results of Slovenian HPV research were published in regional non-indexed medical journals from 1991-1994 (1-5), and the first two Slovenian PhD theses in the HPV field were defended in 1995 (6-7).

Only articles published in journals indexed in peer-reviewed databases Medline/Pubmed, Science Citation Index/Web of Science, Embase and PsycINFO have been considered for the present review of 20 years of HPV research in Slovenia. The initial search was performed on June 1, 2011 and repeated again on October 14, 2011. According to our search, up until October 2011, Slovenian researchers had published 73 articles in SCI or/and PubMed/Medline cited journals, on various HPV topics, ranging from basic virological research to clinical studies. According to the search of Web of Science and Scopus performed on August 23, 2011, Slovenian HPV papers received 473 citations (self-citation excluded), the top cited Slovenian HPV paper being a research report describing the cross-reactivity of a high-risk probe cocktail of Hybrid Capture 2 Test (Qiagen, Hilden, Germany) and published in *Journal of Clinical Virology* in 2002 (78 citations excluding self-citations) (8). The current Hirsch index of Slovenian HPV papers is h=13 (13 Slovenian papers have been cited in peer-reviewed journals at least 13 times).

For the purpose of this review, I divided all published Slovenian HPV studies into 15 categories and briefly describe and comment them.

Detection of HPV in archival clinical specimens

Archival clinical specimens are an invaluable resource for etiological and epidemiological studies of HPV and other viral infections in cases in which fresh or frozen tissue is not available. Papanicolaou-stained smears are the most common archival material in cytological laboratories. In the 1990s, we developed three simple and rapid procedures for the extraction of DNA from this archival clinical material, which allow efficient polymerase chain reaction (PCR) amplification: (i) proteinase K/Tween 20/NP-40 method coupled with a simplified phenol/chloroform/isoamyl alcohol protocol (9); (ii) proteinase K/Tween 20/NP-40 method coupled with a salting-out procedure using saturated NaCl (10); and (iii) modification of a commercial QIAamp procedure (Qiagen, Chatsworth, CA) originally developed for the isolation of DNA from different tissue specimens (11). All three extraction methods were found to be suitable and almost equally efficient for analyzing Papanicolaou-stained archival smears, with an overall DNA extraction efficiency of 95.5% being obtained when testing 335 samples with storage times varying from 3 months to 10 years. Additionally, only negligible differences in the amplification efficiency were observed between Papanicolaou-stained and unstained smears from the same patients (9-11). Although some authors reported that the removal of coverslips from archival smears was time-consuming, requiring 2-7 day incubation in xylene, we found out that coverslips could be successfully removed by simply incubating stained smears for 2 h at -30°C and subsequently for 10 min at 37°C (9).

Formalin-fixation and subsequent paraffin/paraplast embedding is a standard method for long-term preservation of tissue specimens in pathological departments worldwide. However, the detection of viruses in archival specimens is often challenging, due to substantial degradation of viral DNA/RNA as a result of excessive fixation or tissue ageing or the presence of substances that inhibit PCR amplification or proteinase. Since the fragmentation of DNA significantly influences the efficiency of PCR amplification, HPV methods that amplify a relatively small portion of the viral genome are most suitable when working with archival specimens. In a recent comparative evaluation of the RealTi*me* High Risk HPV test (Abbott Molecular, Des Plaines, IL) and INNO-LiPA HPV Genotyping *Extra* CE test (Innogenetics, Gent, Belgium) on alternately processed formalin-fixed, paraffin-embedded specimens, which was performed on 31 cervical cancers and 31 uterine myomas, complete agreement in the detection of 14 assay-common HPV genotypes and partial genotyping of HPV-16 and HPV-18 was observed. The tissue preparation protocol was shown to be sample-to-sample contamination safe (12).

Our experiences of evaluating different DNA extraction protocols that take less than 4 hours, in order to find the most suitable method for routine processing of formalin-fixed, paraffin-embedded tissue specimens and Papanicolaou- or Giemsa-stained smears, were summarized in a review article published in 2000 (13).

Development of novel HPV tests

In the past 20 years, we have developed several PCR protocols for the detection of a range of HPV types for research and diagnostic purposes and have

published several of them. In 1996, an original method based on MY09/MY11 PCR followed by the detection of PCR amplicons with a microtiter plate hybridization assay was developed (14). The sensitivity of the method, determined by serial log-dilutions of SiHa cells, was about 50 copies of HPV-16 per reaction. The reliability and feasibility of the method was further evaluated on 225 archival clinical specimens and almost complete agreement between the results of this method and the results of previous in-house PCRs and typing methods was obtained (14).

In 2008, an original real-time PCR assay was developed based on fluorescence resonance energy transfer hybridization probe technology, allowing very sensitive and specific detection and reliable differentiation of HPV-6 and HPV-11, as well as prototypic and non-prototypic HPV-6 genomic variants, in a single PCR reaction (15). The sensitivity of the assay, determined at a 95% detection level, was 25.3-43.4 DNA copies per reaction. When testing 200 clinical specimens, the novel assay showed complete agreement with Innogenetics INNO-LiPA HPV Genotyping *Extra* CE test (Innogenetics) results and HPV-6 E2 and E6 gene sequencing (15).

In 2010, a novel TaqMan-based HPV-52 type-specific assay was developed, mainly for confirmation of the presence/absence of HPV-52 in clinical specimens positive with a Linear Array HPV Genotyping Test (Roche Molecular Diagnostics, Branchburg, NJ) cross-reactive probe (16). The sensitivity of the assay at a 95% detection level was 3.9 DNA copies/reaction and the dynamic range was seven orders of magnitude, discriminating between 10 and 10^7 viral genome equivalents/reaction. When testing a panel of 147 clinical samples, the novel assay showed complete agreement with the results obtained with GP5+/GP6+ or PGMY09/PGMY0911 PCR-based screening and sequencing.

A PCR-restriction fragment length polymorphism assay was recently developed for the sensitive detection and reliable differentiation of five low-risk alpha-HPVs: HPV-6, HPV-11, HPV-42, HPV-43 and HPV- 44, as well as differentiation of prototypic and non-prototypic HPV-6 genomic variants (17). Testing on defined plasmid standards showed that the assay, which is based on the amplification of a 320-bp fragment of the HPV E1 gene and subsequent analysis of PCR amplicons with endonucleases *BsaJI* and *HinFI*, enabled simple and reliable identification and differentiation of five targeted HPVs and could reproducibly detect down to 10 copies of viral genome equivalents per PCR. The assay showed almost complete agreement with the previously obtained genotyping results on 265 clinical samples, containing targeted and several non-targeted alpha-HPVs.

Evaluation of various commercial tests for the detection of high- and low-risk alpha-HPV

Since persistent infection with high-risk alpha-HPV types is the major etiological factor in the development of cervical carcinoma, HPV testing has become an important part of cervical carcinoma screening and detection algorithms in many countries. Several commercial tests have consequently been developed during the past 12 years and our group has critically evaluated many of them.

In an invited review article published in 1998, we briefly reviewed the principles and important technical details of the HPV detection methods available at the time, discussed the most frequent application problems and described measures being taken to overcome them (18). In 2010, we wrote an expert review about commercially available assays for multiplex detection of alpha-HPVs, in which we described in detail and critically evaluated 33 different commercial HPV assays currently on the market and provided a personal five-year view of the field (19).

The Hybrid Capture 2 HPV DNA Test (hc2) (previously Digene Corporation, Gaithersburg, MD; currently Qiagen), the most widely used diagnostic HPV test, has several times been extensively evaluated in our laboratory. In 1999, in the first comparative evaluation of the first and second generation of Hybrid Capture assays, we showed that the second generation test - hc2, is more accurate, rapid and easier to perform and thus more appropriate for routine detection of HPV infection than its first-generation counterpart (20). The enhanced sensitivity of hc2 was mainly a result of the reformulation of reagents and, to a lesser extent, a result of the addition of the probes for new HPV types (20). In a pilot study in 2002, we established the clinical utility of hc2 in combination with conventional cytology in a group of 171 women, who were followed-up with both cytology and molecular testing for 3 years (21). In the same year, the analytical specificity and accuracy of hc2 was studied in detail by genotyping HPV isolates obtained from 325 women recognized as HPV positive using the hc2 high-risk probe cocktail (8). Our study showed for the first time that the hc2 high-risk probe cocktail detects, in addition to 13 targeted HPV types, at least 15 other HPV types, some of them classified as low-risk HPV types (8). In order further to address hc2 analytical inaccuracy, we genotyped 240 samples with repeatedly borderline/equivocal/indeterminate hc2 results (samples with repeated relative light unit/cut-off (RLU/CO) values between 0.4 and 4.0) in order to resolve their HPV status (22). A false negative rate of 11.3% and

false positive rate of 19.1% were recorded in samples with repeatedly borderline hc2 results and the hc2 false reactivity increased with proximity to the hc2 cut-off value. On the basis of our results, we recommended the introduction of an hc2 grey zone and retesting of samples with repeatedly borderline hc2 results by an alternate detection method with higher analytical specificity (22). After detailed retrospective and prospective evaluation of the Amplicor HPV Test (Roche Molecular), which is designed to detect the same 13 high-risk HPV types as the hc2 high-risk probe cocktail, we suggested that the Amplicor HPV Test can be used for final determination of HPV status in samples with repeatedly borderline hc2 results (23). In 2007, we published the first evaluation of an hc2-based probe system for HPV-16, HPV-18 and HPV-45 (24). In the study, in which 227 women were enrolled, we showed that this probe system is suitable for partial HPV genotyping in non-PCR laboratories, since it enables easy identification of the three clinically most important HPV types and provides important clinical information for further follow-up in women with both normal and abnormal cervical cytology (24). We were also the first to comparatively evaluate the Digene HPV Genotyping RH Test RUO (Qiagen) with the standard INNO-LiPA HPV Genotyping Extra CE assay (Innogenetics) (25). After parallel testing of 70 hr-HPV positive samples, a novel Digene test was identified as suitable for the detection of hr-HPV genotypes in clinical samples. Although INNO-LiPA, identified significantly more samples with multiple HPV types than the Digene test, the clinical benefit of such a difference is at present unclear (25). In a recent genotyping study, which was performed on 285 hc2 low-risk HPV-positive cervical specimens, we found that the hc2 low-risk probe cocktail, similar to the hc2 high-risk cocktail, cross-reacts with several untargeted HPV types (26). Cross-reactivity is often clinically beneficial, due to the detection of untargeted low-risk genotypes, but the total of 8.4% of hc2 low-risk positive results, usually weak, were due to cross-reactivity with high-risk types. We thus suggested a more cautious interpretation of all samples with weak low-risk hc2 signal strength and recommended the introduction of a gray zone (26).

In 2009, when a RealTi*me* High Risk HPV test (Abbott Molecular) designed to detect 14 high-risk HPVs and concurrently distinguish HPV-16 and HPV-18 within a single test was released onto the European market, we evaluated its analytical specificity in comparison with hc2 (27), on 37 samples with previously determined hc2 false-positive results and its clinical sensitivity on 362 archived routine cervical specimens collected from women with histologically confirmed cervical carcinoma or CIN3 lesions. The RealTime test showed excellent analytical specificity and no cross-reactivity with low-risk HPV types that tested positive with hc2. Clinical sensitivity of the RealTime test was comparable to hc2 (27). Recently, we prospectively compared the clinical performance of the RealTime test and hc2 in the population-based cervical cancer screening setting (4,432 women 20 to 64 years old) and showed that the clinical performance of RealTime is not inferior to that of hc2 for women >30 years old and women 20 to 64 years old (28). Excellent analytical agreement between the two diagnostic tests was obtained (kappa value 0.84), while the analytical accuracy of RealTime was significantly higher than that of hc2. RealTime displayed excellent intralaboratory reproducibility and interlaboratory agreement (28).

HPV and anogenital tumours

Approximately 40 HPV types from the HPV genus alpha are known to infect the mucosal epithelium, with a subset of 10 to 15 high-risk HPV types being associated with lesions that can progress to cancer. These HPV types are the etiological agents of virtually all cervical carcinomas and their immediate precursors — high-grade cervical intraepithelial neoplasia (CIN) lesions. In addition to cervical carcinoma, high-risk HPV types, the most frequent being HPV-16, play the leading etiological role in the development of anal cancer and a substantial proportion of vaginal, penile, vulvar and oropharyngeal (mainly tonsillar) cancers. In contrast, low-risk alpha HPV genotypes (mainly HPV-6 and HPV-11) are etiologically associated with the development of virtually all genital warts and laryngeal squamous cell papillomas of both genders.

Two research groups from Ljubljana and one from Maribor performed several cross-sectional studies in the 1990s studying the role of HPV in the etiology of different grades of CIN lesions and cervical cancer (29-32). Similar to other researchers around the world, we also showed an increasing prevalence of infection with high-risk HPV with severity of cervical lesions in Slovenia (29-32).

In order to establish the pre-vaccination distribution of HPV types in Slovenian women with CIN 3, a total of 261 cervical swabs collected from women with histologically confirmed CIN 3 were analyzed in 2009, using four genotyping methods (33). The most common HPV type found in CIN 3 lesions was HPV-16 (59.0%), followed by HPV-31 (7.5%), HPV-33 (7.1%), HPV-58 (5.0%) and HPV-51 (4.0%) (33). A similar study on a representative population of Slovenian women with cervical cancer showed that 262 of 278 cervical cancer samples (94.2%) contain HPV DNA (34). The most frequent HPV types found in

Slovenian women with cervical cancer were: HPV-16 (64.9%), HPV-18 (12.2%), HPV-33 (4.7%) and HPV-45 (4.1%) (34). Both studies showed that prophylactic HPV vaccination with currently available vaccines could prevent up to 77.1% of cervical cancers and up to 67.4% of CIN 3 lesions in Slovenia caused by HPV-16 or HPV-18. An adjunct study in which we compared the detection and distribution of HPV types in 40 paired cervical scrape samples and tissue samples in patients with cervical cancer showed that cervical scrape samples are equally useful for HPV type determination as tumor tissue samples in patients with cervical cancer (35).

We recently studied the correlation of human telomerase gene amplification and the presence of HPV infection in 101 Slovenian women with different grades of CIN and were not able to demonstrate any correlation between these two parameters (36).

In order to establish the HPV type distribution in genital warts in our country, we recently tested 55 tissue specimens collected from the same number of immunocomponent male patients (37). HPV DNA was detected in all 55 tissue specimens; HPV-6 or HPV-11 was detected in 53 cases, and HPV-44 and candHPV-91 in one tissue sample each (37). Because HPV-6 or HPV-11 was detected in 96.4% of the genital warts studied, it seems that a prophylactic quadrivalent HPV vaccine could prevent almost all incidental genital warts in our country.

Anal HPV infection commonly affects men who have sex with men and is associated with the development of anal cancer, especially in HIV positive patients. We recently showed a high prevalence of anal HPV infection in both HIV-negative (75%) and HIV-positive (95%) Slovenian men who have sex with men (38). Promiscuity and the use of "poppers" (alkyl nitrites taken for recreational purposes through direct inhalation) were found to be strongly associated with a higher prevalence of anal HPV infection (38).

HPV testing in routine gynecological practice

HPV DNA testing has recently become an important part of cervical carcinoma screening and management algorithms. The four main clinical applications of HPV DNA testing at present are: (i) triage of women with equivocal screening cytology results, in order to determine which patients should be referred for colposcopy, (ii) follow-up of women with abnormal screening cytology results who are negative at initial colposcopy/biopsy, (iii) prediction of the therapeutic outcome after treatment of high-grade CIN lesions, and (iv) primary screening of women ≥30 years old in combination with the Pap smear to detect cervical cancer precursors. Similar to other researchers around the world, we have also performed several clinically oriented studies in the last decade in order to evaluate the clinical usefulness of HPV testing in daily routine gynecological practice in Slovenia.

In 2005, we determined the prevalence and distribution of high-risk HPV types in Slovenian women with repeat mild dyskaryosis and evaluated three molecular methods for the detection of high-risk HPV genotypes that could be used as a complementary method to cytology (39-41). These studies showed that the cervical screening program in Slovenia is overburdened with mild dyskaryosis, that repeat cytology as follow-up for women with mild dyskaryosis is ineffective and that HPV testing should be introduced as a complementary method in a triage of women with equivocal screening cytology (39-41).

In a prospective study performed in Ljubljana from 2004 to 2006, we established the efficiency in eliminating HPV infection in women with precancerous cervical lesions, of three surgical procedures: laser vaporization, large loop excision of the transformation zone and cold knife conization. A total of 214 women were enrolled and 67.6%, 86.3% and 100% of women treated with laser vaporization, large loop excision of the transformation zone and cold knife conization, respectively, were hc2 HPV negative 10 months after treatment (42). In a study performed from 1999 to 2004 in Maribor, 797 consecutive patients with different grades of CIN treated with cold knife conization were closely followed (43). On the basis of HPV negative results obtained in some patients with recurrent lesions after conization, the authors recommended a combined follow-up approach using HPV testing and cytology for the detection of residual or recurrent CIN after conization (43). The most probable explanation of the slightly different results obtained in the Ljubljana and Maribor studies is that the majority of the patients with false-negative HPV results identified in the Maribor study were followed using *in situ* hybridization (ISH), which lacks clinical sensitivity as a test of cure.

In a study performed on 181 women in Ljubljana, we showed that a relatively high proportion of women surgically treated in our country are in fact overtreated, due to overestimation of the cytological and histological findings. We showed that HPV testing before treatment can significantly reduce the number of unnecessary surgical treatments (44).

In a recent study, the Maribor group evaluated the prevalence of HPV-16 and HPV-18 infection determined by ISH in patients with different grades of CIN related to the use of three different contraceptive methods: intrauterine devices, oral contraceptives and

barrier methods (45). In a study group of 1,435 patients, no significant differences in HPV-16 and HPV-18 prevalence were found among patients in relation to the contraception method used (45).

HPV and laryngeal benign tumours

Squamous cell papilloma is the commonest benign laryngeal epithelial tumor. Virtually all laryngeal papillomas are etiologically linked with HPV, mainly HPV-6 and HPV-11. In 1994, we tested a series of 79 laryngeal papillomas obtained from 36 patients for the presence of HPV, using ISH, and found HPV-6 or HPV-11 in 28 of 29 juvenile papillomas, in 26 of 30 adult multiple papillomas, and in 17 of 20 adult solitary papillomas (46). There were no clear-cut histological differences between juvenile and adult papillomas, the presence of koilocytosis was equally observed in both and there was no prevalent type of epithelial hyperplasia in either form, except that all three cases of atypical hyperplasias (precancerous lesions) were found among adult patients. During a 14 year follow-up, no malignant transformation of laryngeal papillomas was observed (46).

Testing of 103 laryngeal papillomas in 1996 using monoclonal antibody PAb 1801 revealed occasional strongly p53-positive cells in 45 cases, p53 protein immunoreactivity almost exclusively restricted to the basal epithelial cells in 26 cases, basal cell layer immunoreactivity accompanied by aggregates of p53-positive cells in the lower two-thirds of the epithelium in 11 cases and in 21 cases we failed to show any detectable level of p53 protein reactivity (47). The observed patterns of p53 immunoreactivity in the majority of cases were interpreted as a result of immunohistochemical detection of the stabilized wild-type p53 protein rather than the mutated p53 protein (47). Twenty-four cases of laryngeal papillomas were additionally tested for the expression of c-erbB-2 protein (48) and the presence of Langerhan's cells (49). Two staining c-erbB-2 patterns were observed in the hyperplastic epithelium covering laryngeal papillomas: membranous and cytoplasmic. With the increasing grade of epithelial abnormalities, cytoplasmic staining became predominant, and c-erbB-2 positivity sometimes occupied the whole epithelial thickness (48). Quantitative analysis of the presence of Langerhan's cells using CD1a and S100 antibodies showed no statistically significant differences in the mean number of Langerhan's cells per mm^2 of the cross-sectioned epithelium covering laryngeal papillomas, comparing simple, abnormal and atypical hyperplasia groups, but the mean number of Langerhan's cells per mm^2 in laryngeal papillomas substantially exceeded that of the vocal cord surface epithelium in patients with chronic laryngitis (49).

In 1997, we described a rare case of a 19-year-old female who suddenly died of asphyxiation caused by a massive laryngeal papilloma (50).

HPV and laryngeal epithelial hyperplastic lesions and laryngeal cancer

Laryngeal epithelial hyperplastic lesions, usually clinically defined as leukoplakia and chronic laryngitis, are a broad spectrum of histomorphological changes characterized by hyperplasia, with a preserved basement membrane accompanied by more or less expressed structural and cellular abnormalities. These lesions have been the subject of intensive research of our group for more than 40 years. In contrast to laryngeal papillomas, the role of HPV in the etiology of laryngeal epithelial hyperplastic lesions and laryngeal cancer is still controversial.

In 1997, we tested for the presence of HPV a series of laryngeal epithelial hyperplastic lesions, ranging from simple hyperplasia to invasive squamous cell carcinoma, using three different HPV consensus PCRs and *ISH* (51). The presence of HPV DNA was detected in only two of the 88 specimens tested: HPV-6 was detected in one case of simple hyperplasia and HPV-16 in one case of laryngeal invasive squamous cell carcinoma. Our study suggested that most laryngeal epithelial hyperplastic lesions are not associated with HPV infection and that other pathogenic mechanisms are more important in the etiology of these lesions (51).

In 1996, we investigated the immunohistochemical expression of p53 protein using monoclonal antibody PAb 1801 in a similar case series of laryngeal epithelial hyperplastic lesions (52). Overexpression of p53 was observed in 10/19 (53%), in 9/16 (56%) and in 9/13 (69%) cases of simple, abnormal, and atypical hyperplasia, respectively, and in 8/10 (80%) cases of laryngeal cancer. The proportion of p53 immunoreactive cells and staining intensity increased with the progression of the lesions but, considering the follow-up of the patients, p53 expression cannot be considered to be a reliable prognostic factor for any group of laryngeal epithelial hyperplastic lesions, regardless of the severity of the lesions (52).

An immunohistochemical analysis of overexpression of epidermal growth factor receptor, c-erbB-2 and p53 proteins was performed in 1997, on 43 biopsies of laryngeal epithelial hyperplastic lesions (53). p53 and

epidermal growth factor receptor overexpressions were detected in 28/54 (52%) and 33/54 cases (61%), respectively, and tended to increase with the degree of epithelial changes. c-erbB-2 was weakly positive in the majority of cases and changed from predominantly membranous in simple hyperplasia to cytoplasmic staining in abnormal and atypical hyperplasias. We concluded that the overexpression of each biomarker itself adds little predictive value over routine histomorphology but immunostaining patterns of EGFR and p53 in up to two-thirds or more of the epithelial thickness in atypical hyperplasia could be considered a reliable pattern that correlates with the progression to cancer (53).

In order to visualize directly the sequence of genetic changes underlying the entire spectrum of laryngeal epithelial hyperplastic lesions and laryngeal cancer, 59 tissue specimens were tested for chromosomes 7 and 17 using ISH and by immunohistochemistry for overexpression of p53 protein and epidermal growth factor receptor (54). Polysomy for both chromosomes increased in correlation with progressive grades of laryngeal lesions. The most important finding was a statistically significant difference in chromosome copy numbers between the isolated atypical hyperplasia and atypical hyperplasia associated with laryngeal carcinoma. According to our results, we concluded that numerical changes in chromosomes 7 and 17 might be associated with an upregulation of epidermal growth factor receptor and p53 genes, and could contribute to critical events in laryngeal carcinogenesis. For daily practice, cytogenetic and immunohistochemical analyses could be of assistance in distinguishing between low- and high-risk groups of atypical hyperplasia (54).

There is no internationally accepted classification of laryngeal epithelial hyperplastic lesions. The majority of current classifications follow criteria similar to those commonly used for cervical epithelial lesions. However, a different etiology of laryngeal cancer and its particular clinical and histological features requires a grading system more appropriate to this region. The Ljubljana classification of laryngeal epithelial hyperplastic lesions was devised in 1971 to cater to this requirement. Detailed criteria for histological grading in this classification were finally formulated by a working group of the European Society of Pathology in 1999 and, following this meeting, in the last decade our research group has written three comprehensive reviews summarizing and updating the major achievements in the field (55-57).

HPV and tumors in oral cavity

In order to elucidate the putative etiologic role of HPV in the development of tumors in the oral cavity, two case-control studies were performed (58-59). In a comparative study performed on tissue specimens of oral squamous cell carcinoma and histologically normal oral mucosa from individuals who matched the subjects with carcinoma in age, gender, localization of obtained tissue specimens, and drinking and smoking habits, the presence of HPV DNA was detected in 5/59 (8.4%) of oral carcinomas and 4/61 (6.6%) of normal oral mucosa specimens (58). In the second study, the presence of HPV was examined using three consensus PCRs in tissue specimens of oral squamous cell papillomas and tissue specimens of histologically normal oral mucosa obtained from individuals who matched the patients with papillomas in age, gender and localization of obtained tissue specimens (59). HPV-6 was detected in three and HPV-16 in one of 44 papillomas tested. Three out of 45 tissue specimens of normal oral mucosa were HPV DNA positive, harboring HPV-6, HPV-11 and HPV-31. Since no significant difference in the prevalence of HPV DNA was found between cases and control in both studies, we consider that HPV plays a limited role in the etiology of oral papillomas and the majority of oral cancers, at least in this part of Europe (59).

HPV and esophageal benign and malignant tumors

Five studies have been performed (60-64) to elucidate the putative etiologic role of HPV in the development of esophageal tumors in Slovenia. The etiology and pathogenesis of esophageal squamous cell papillomas, rare benign tumors of the human esophagus, was unexplained and controversial. In order to study the role of HPV infection in the etiopathogenesis of these tumours, we tested a total of 36 esophageal squamous cell papillomas from 35 patients originating from Slovenia and Poland for HPV infection, using ISH and PCR (60-61). HPV-6 was detected in two papillomas, indicating that pathogenetic mechanisms other than HPV infection are important in the etiology of these tumors. Additionally, the same tumor series was immunostained for p53 using the monoclonal antibody PAb 1801 (62). Overexpression of p53 was found only in occasional epithelial cells and in basal epithelial cells of some esophageal squamous cell papillomas. We speculated that these findings are the result of immunohistochemical detection of stabilized wild-type p53 protein, rather than mutated p53 protein (62).

Some studies, mainly from high risk areas, have suggested a possible role of HPV in the carcinogenesis of esophageal cancer. In 1993, we performed a pilot study on 22 cases of esophageal cancer and de-

tected the presence of HPV DNA in two cases (63). To elucidate further the putative role of HPV infection in the etiology of esophageal cancer, in 1998 we screened 121 esophageal cancers using eight different PCR protocols (64). We failed to detect HPV DNA sequences in any of the tumor samples tested. Our study supports the hypothesis that esophageal cancers originating from non-high-incidence geographic areas of this cancer are not associated with HPV infection (64).

HPV and inverted papillomas

Inverted papillomas are the most frequent type of sinonasal papillomas. These benign but destructive lesions are known for their high recurrence rate, probably due to incomplete excision. Testing of tissue samples from 68 patients with inverted papillomas, 5 patients with papillomas associated with squamous cell carcinoma and control group of 47 patients who had undergone septoplasty or mucotomy of the inferior turbinate, revelaled the presence of HPV DNA in 20 (30.3%), 3 (60%) and 6 (13%) patients, respectively (65). The presence of HPV DNA was not a statistically significant predictor of the recurrence of inverted papillomas (p=0.745) nor was it a statistically significant risk factor for associated squamous cell carcinoma (p=0.32) (65).

Genomic diversity of selected HPV types

Six studies, focusing on the genomic diversity of selected HPV types, have been performed in our laboratory in recent years (66-71). In a study of genomic diversity of HPV-53 (one of the three »probable high-risk« HPV types), 94 isolates obtained from 70 Slovenian women were analyzed (66). Altogether, 19 genomic variants, composed of 13 LCR, 13 E6, and 5 E7 genomic variants, were identified. A higher genomic diversity of HPV-53 was identified in an ethnogeographically closed cohort of white European women than has been reported previously on HPV-53 isolates collected worldwide. In addition, a dichotomic phylogeny of HPV-53 described previously was confirmed and it was shown for the first time that, after dichotomic split, both groups of HPV-53 genomic variants formed star-like phylogenetic clusters (66).

In a study that focused on the genomic diversity of HPV-38 (HPV type associated with skin cancer and classified taxonomically in the beta-papillomavirus genus), a total of 39 isolates obtained from hairs plucked from pubic, scrotal, perianal or eyebrow regions from 31 immunocompetent healthy male individuals were analyzed (67). In addition to 5 genomic variants identified previously, 12 novel genomic variants were characterized in our study, which was undertaken on the largest number of HPV-38 isolates to date. The presence of at least two different HPV-38 genomic sequences in four samples was demonstrated showing that co-infection with different genomic variants of HPV-38 at the same time is possible.

The prevaccination genomic diversity of HPV-6, the most important low-risk HPV type, was established in a study carried out on the largest number of HPV-6 isolates to date (68). By analyzing pooled L1, LCR, E6, E2 and E5 nucleotide data for each of the 77 clinically important HPV-6 isolates (45 obtained from genital warts and 32 obtained from laryngeal papillomas), a total of 36 different HPV-6 genomic variants was described. Several novel, potentially important mutations were identified. Non-prototypic HPV 6vc genomic variants were found in the majority of genital warts and laryngeal papillomas. The presence of serious HPV-6 genome sequence errors was confirmed and novel sequence errors were identified in sequence repositories (68). In a subsequent study, the 18 most divergent HPV-6 isolates were selected for a comparative analysis of a total of 21 full-length genome sequences (69). This study contributed the largest number of full-length HPV-6 genome sequences to date and confirmed that HPV-6 diversifies virtually equally across the entire genome.

A similar study was performed on the second most important low-risk HPV type – HPV-11 (70). In a study that included the largest number of HPV-11 isolates to date, 23 genomic variants of HPV-11 were identified after analyzing pooled L1, LCR, E6 and E5 nucleotide data for each of the 63 clinical isolates of HPV-11. Non-prototypic HPV-11 genomic variants were found in the majority of samples included in the study. Full-length genome sequences were determined for the 10 most divergent isolates, revealing more than 99% similarity to the HPV-11 prototype isolate. This was the first extensive work on the prevaccination genomic diversity of HPV-11; compared to the majority of other HPV types studied to date, including the most closely related HPV-6, HPV-11 was shown to be a substantially less polymorphic HPV genotype.

The prevaccination genomic diversity of the three most common HPV types found in Slovenian women with cervical cancer: HPV-16, HPV-18 and HPV-33, was examined in 2010 (71). A total of 26, 18, and 7 genomic variants of HPV-16, HPV-18 and HPV-33, respectively, was identified. The majority of HPV-16 and HPV-18 isolates belonged to European branches and prototypic and non-prototypic HPV-33 genomic variants were found to be equally distributed among Slovenian patients with cervical cancer (71).

Hair follicles as an important endogenous reservoir of HPV

Three studies have been conducted to investigate whether hair follicles represent an endogenous reservoir of HPV (72-74). In the first study, the presence and distribution of alpha- and beta-HPVs was investigated using three consensus PCRs in eyebrow hairs obtained from 49 immunocompetent male patients with genital warts (72). Alpha-HPVs were found in 7/49 (14.3%) and beta-HPVs in 29/49 (59.2%) eyebrow hair samples tested. The results of the study showed that the use of a combined primer approach considerably improves HPV DNA detection over individual primer sets and greatly improves the detection of different HPVs in the plucked hairs. In addition, six putative new HPV genotypes, designated SIBX1 to SIBX6, were identified (72).

In the second study, a total of 150 anogenital hairs plucked from the scrotal, pubic, and perianal regions of 51 immunocompetent healthy male individuals were investigated for the presence of beta-HPVs, using M^a/H^a nested PCR (73). Beta-HPVs were detected in 18/51 (35.3%), 13/50 (26.0%) and 7/49 (14.3%) hair samples plucked from the pubic, scrotal and perianal regions, respectively. While the prevalence of beta-HPVs in pubic hairs was significantly higher than in perianal hairs (P=0.013), the difference in the prevalence of beta-HPVs in pubic and scrotal hairs and in scrotal and perianal hairs, was statistically not significant. Difference in the lifetime-cumulative sun exposure was the most likely explanation for the observed phenomenon. In addition to several known HPVs, five partial DNA sequences suggesting putative novel HPV types were identified in this study (73).

In the third study, the presence and distribution of alpha-HPVs was comparatively investigated in anogenital hairs plucked from scrotal, pubic and perianal regions of 53 male patients with genital warts and 53 age- and sex-matched healthy control subjects (74). Altogether, HPVs were detected in 69 (43.7%) of 158 and in 7 (4.5%) of 155 anogenital hairs obtained from patients and control subjects, respectively. At least one hair sample was HPV-DNA positive for 37/53 (69.8%) patients and for 7/53 (13.2%) controls. According to the sampling sites, HPV was detected in patients in 64.2%, 39.6%, and 26.9% of hairs from the pubic, scrotal, and perianal regions, respectively. For 91.9% of patients with HPV-positive hair samples, the same HPV type was identified in genital warts and hairs from at least one sampling site. Having genital warts was found to be strongly associated with the presence in anogenital hairs of the HPV type causing the genital warts (range of odds ratios, 13.0 –20.0) (74).

Identification and characterization of novel HPV types

As mentioned above, several putative novel HPV types have been identified and partially characterized in our laboratory in the last decade. Three of them: HPV-125, HPV-150 and HPV-151, originally isolated from a cutaneous hand-wart, eyebrow hair-follicle and a scrotal hair-follicle, respectively, were sequenced, cloned, fully characterized and officially recognized as novel HPV types (75-76). Phylogenetic analysis of novel HPV types showed them to be phylogenetically related to cutaneous HPVs of *Alphapapillomavirus* species 2 (HPV-125), and *Betapapillomavirus* species 5 (HPV-150) and 2 (HPV-151). Because phylogenetic relationships among HPV types do not necessarily reflect their tissue tropism, three HPV type-specific real-time PCR assays were developed and the tissue predilection and potential clinical significance of the novel HPV types was assessed by testing a panel of clinical samples comprising important HPV-associated malignant neoplasms (cervical carcinoma, squamous cell and basal cell carcinoma of the skin), HPV-associated benign neoplasms (genital warts, laryngeal papillomas, common warts) and, additionally, hair follicles of immuno-competent individuals, a known reservoir of 'commensalic' HPV types. The obtained results indicated that HPV-125, HPV-150 and HPV-151 are relatively rare HPV types with a cutaneous tropism. HPV-125 could be etiologically linked with sporadic cases of common warts (75), while HPV-150 and HPV-151 were found in low-copy numbers in sporadic cases of common warts and squamous cell and basal cell carcinoma of the skin in immunocompetent individuals (76).

HPV vaccination

In 2006, a population-based study was conducted with the aim of determining the level of knowledge and awareness of women about cervical cancer, Pap test, HPV infection and HPV vaccination, using a computer-aided telephone inquiry on a sample of 500 women aged from 18 to 55 years from all regions of Slovenia (77). From the results the authors concluded that there is an urgent need in this country to provide the lay population and medical community with relevant and accurate information on HPV infection, on early detection of cervical cancer and on HPV vaccination.

In order to evaluate the cost-effectiveness of HPV vaccination alongside the cervical cancer screening programme in Slovenia, a previously published Mar-

kov model representing the natural history of HPV infection was adapted to the Slovenian context (78). The model followed a cohort of 12-year-old girls to 85-year-old women and the analysis was performed from the healthcare payer perspective. Vaccination with screening compared with screening alone was associated with an incremental cost-effectiveness ratio of 23,178 EUR per quality adjusted life-year gained and 54,536 EUR per life-year gained at a discounting rate of 5% (78). It was concluded that, on the basis of the cost-effectiveness thresholds adopted by the Health Council in Slovenia, HPV vaccination alongside the screening programme can be regarded as cost-effective but the cost-effectiveness of HPV vaccination would become questionable if a booster dose was needed to provide lifetime protection (78).

HPV basic research

Slovenian researchers from the University of Nova Gorica have recently identified the location of an *in vivo* sumoylation site of the L2 protein of HPV-16, at lysine 35 (79). They proposed that this modification affects the stability of the L2 protein and postulated that sumoylation of L2 may play a part in viral capsid assembly. Furthermore, they observed that L2 upregulates the expression of a small ubiquitin-related modifier in the host-cell, providing another example of the complex interactions between HPVs and the host-cell machinery (79).

In a review article, the same research group recently discussed the functions of the viral proteins that appear to be the most appropriate for the development of therapeutics aimed at the treatment of viral infection and HPV-induced cancers (80).

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