

HUMAN PAPILLOMAVIRUS (HPV) – RELATED CARCINOMAS OF THE UPPER AERODIGESTIVE TRACT

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* Epidemiologic, clinical, morphologic and molecular evidence show that high risk HPV particularly type 16 is a prerequisite for some carcinomas of the upper aerodigestive tract (UADT).

* The most common site of HPV-related carcinomas of the UADT is the Oropharynx and particularly the tonsils and base of tongue.

* HPV + tumors are distinct clinically and morphologically. Microscopically they are nonkeratinizing squamous cell carcinomas with basal cell features and have a characteristic immunohistochemical profile.

* An anti-HPV vaccine has recently been made available for prevention of cervical cancer. The impact of wide use of the vaccine on the prevalence of HPV related carcinomas of the UADT is currently not known but likely beneficial.

Background

The role of human Papillomavirus (HPV) as a prerequisite for the development of cancer of the uterine cervix has been established for many years. The prevalence of high risk (oncogenic) HPV infection in cervical cancer tissue is estimated to be in the range of 90-99%. According to the WHO, about 500,000 new cases of cervical cancer occur globally each year. The majority are identified in developing countries [26,37,39]. More recently epidemiologic as well as clinical and molecular evidence have implicated HPV particularly type 16 in the causation of some upper aerodigestive tract (UADT) carcinomas, particularly in the oropharynx and notably the tonsils and base of tongue [7-9,11,13,16,20,24,25,36].

The virus

Human papillomaviruses belong to the family of DNA Papovaviridae. They are small non-enveloped icosahedral viruses with an 8 Kbase-long double-stranded circular DNA genome. They include more than a 100 different strains or genotypes. More than 30 of

which are sexually transmitted, infecting the genital areas of men and women. Some of these viruses can cause premalignant lesions and carcinomas in the affected areas, and are called "high-risk" types. Others called "low-risk" types may cause mild cytologic abnormalities and genital warts as well as laryngeal papillomatosis and oral condyloma acuminata. The most common high-risk types are types 16 and 18, while the most common of the low risk ones are types 6 and 11.

According to the CDC epidemiologic studies, 75% of the 15-75 year-old populations acquire genital HPV infection at some point in their lives. By the age of 50 at least 80% of women will have acquired genital HPV infection [26,37,39].

Human papillomavirus genome is made up of 7 early (E) genes and 2 late (L) genes that encode the early proteins E1-E7 and late proteins L1-L2. The E proteins are nonstructural and are involved in replication and transcription of the genome, while the L proteins are the structural capsid proteins of the intact virion [3,35,39].

High-risk HPV infection

It has been known that persistent infection with high risk HPV particularly types 16 and 18 are necessary cause of high grade cervical dysplasia and cancer. However, cervical cancer is a rare complication of HPV infection. The majority of Infections in young women, (about 80%) resolve spontaneously without even giving rise to dysplastic lesions. Development of cancer requires multiple additive events both genetic and epigenetic, in addition to the persistent high risk HPV infection. On the other hand, cases that resolve may develop into a productive viral infection with new virions assembly and shedding from the fully differentiated squamous epithelial cells [3,35,39]. High grade dysplasia and cervical carcinoma often show integration of the viral genome into the host cell with a resultant deregulation and uncontrolled expression of E6 and E7 viral oncogenes. Molecular evidence in cervical as well as oropharyngeal carcinomas show that the HPV oncogenes E6 and E7 act through inactivation of p53 and retinoblastoma (Rb) tumor suppressor genes, inducing cell cycle deregulation and genomic instability. In addition E6 can directly activate telomerase and E7 induces abnormal centrosome duplication [3,22,35,39].

Clinical as well as in vitro studies suggest a model for carcinogenesis in which immortalization of the epithelial cells combined with genomic instability and accumulating genetic alterations lead to overt malignancy [3,35,39]. A familial predisposition for the development of cervical cancer has been suggested in a study in which the Swedish Family-Cancer Database was used to analyze a large number of invasive and in-situ cervical cancers in mothers and daughters [15]. It is suggested that impaired immunity may be a factor

HPV-related Carcinomas of the Head and Neck

During the last few decades there has been an increase in incidence of oropharyngeal carcinoma in young patients under 45 years of age. Using the SEER data base a statistically significant increase in incidence of carcinomas of the tonsils and base of tongue was documented during the period 1973 – 2001 in U.S. population 20-44 years of age. No similar increase occurred in other oral sites outside the oropharynx. Many of

metastatic carcinomas were identified in FNA biopsies as well as in surgical specimens by morphologic criteria and ISH for high risk HPV [40-42].

Treatment and prognosis

Accumulating body of evidence in the American as well as the international literature confirms that HPV positive carcinomas of the tonsils and base of tongue have statistically significant better prognosis, regarding disease free and overall survival, than HPV negative tumors. The favorable outcome for patients with HPV positive tumors is independent of TNM stage, nodal status, age or gender. It is suggested that the favorable outcome is attributable to increased sensitivity toward radiotherapy [5,18,19,23,27,38]. Early experimental evidence show that the broad spectrum anti DNA virus agent Cidofovir can inhibit proliferation and induce apoptosis in HPV transformed cultured cells. It also enhanced their radiosensitivity. Cidofovir is also used clinically, with some success, in treatment of laryngeal papillomatosis, when injected intralesionally [1,17,33].

Prevention:

Because HPV infection of the oropharynx is believed to be sexually transmitted, the practice of protective sexual behavior is likely to have preventive effects. Abstinence, monogamy, limiting the number of sexual partners has all been advocated for prevention of STD.

Unfortunately HPV infection can occur in the genital areas that are covered, as well as areas not covered, by a latex condom. According to NCI the efficacy of the use of condoms in prevention of HPV infection is not known, although condom use has been associated with a lower rate of cervical cancer.

The HPV Vaccine

In June 8, 2006, the Food and Drug Administration (FDA) licensed the first anti HPV vaccine. The quadrivalent vaccine, *Gardasil*, immunizes against HPV types (6,11,16,18). It is made from non-infectious viral-like particles (VLP) [4,14,34].

On June 29, 2006, the Advisory Committee on Immunization Practices (ACIP) voted to recommend this vaccine in females, ages 9-26. The vaccine has been tested in over 11,000 females of that age group in many countries around the world including the USA. These studies demonstrated 100% efficacy in preventing cervical precancers, and nearly 100% efficacy in preventing vulvar and vaginal precancers, as well as genital warts, caused by the targeted HPV types. These studies also found that the vaccine is safe and cause no side effects [14,29,34].

The Impact of wide use of anti HPV vaccines on HPV-related oropharyngeal carcinoma is currently not known. However is reasonable to conclude that a direct or indirect benefit may be achieved.

Summary

- Epidemiologic, clinical, morphologic and molecular evidence show that high risk HPV particularly type 16 - like in the case of cervical cancer- is a prerequisite for some carcinomas of the upper aerodigestive tract (UADT).
- Sexual transmission is an important mode of infection.

these patients have little or no exposure to known risk factors such as smoking or excessive drinking [12,31].

About 20 years ago high risk HPV was identified in squamous cell carcinoma of the head and neck [20]. A multitude of studies using a variety of techniques including in situ hybridization (ISH), immunohistochemistry, and polymerase chain reaction (PCR) have since been able to demonstrate the presence of HPV genome in some UADT carcinomas particularly of the tonsils and base of tongue where 18-90% of the tumors are HPV +. HPV DNA has also been identified in some laryngeal and sinonasal carcinomas (12-20%). The virus is very rarely identified in squamous cell carcinoma of the oral cavity [7-9,11,13,24,25,36].

HPV-Related Oropharyngeal Squamous Cell Carcinoma

Demographic and Clinical features

As mentioned above the prevalence of HPV DNA in oropharyngeal carcinoma has varied in different studies form 18 to 90%. In a review of 235 cases of oropharyngeal carcinomas, in all age groups, at our institution we found that 36% of tonsillar and 32% of base of tongue carcinomas were HPV related. Alternatively, 91% of tonsillar carcinomas in young patients, 40 years of age or younger were HPV type 16 positive. The male to female ratio for all age groups combined was 4:1 [8,9].

In another large study of 1670 patients who had oral or oropharyngeal carcinomas and 1732 healthy volunteers, from nine countries, the International Agency for Cancer research found that 18.3% of oropharyngeal carcinomas were HPV 16 positive. Patients with HPV positive tumors were three times as likely to report having had oral sex as those with HPV negative tumors. Patients with HPV positive tumors were also more likely to have had multiple sex partners. HPV is less frequently detected in cancer biopsies from patients who are tobacco smokers or paan chewers [12,30,31]. An analysis of the Swedish cancer registry data (1958-1996) showed that husbands of women with cervical cancer had significantly increased risk of developing tonsillar carcinoma [31].

Early asymptomatic carcinomas usually develop in the crypts of the palatine and lingual tonsils without apparent clinical manifestations. Because of their deep location neither clinical examination nor cytologic tests, analogous to the Pap smears used for cervical lesions, are useful in early detection. These small, occult tumors are not uncommonly associated with extensive cervical lymph node metastasis. In the more advanced primary tumors, patients may complain of sore throat, dysphagia, odalgia, and sensation of a foreign body in the throat.

Pathologic features

HPV-related oropharyngeal carcinomas are not only distinct clinically but also microscopically and molecularly. The tumors are characterized by nonkeratinizing, basaloid cell morphology. Microscopically the neoplastic cells are generally monomorphic, oval or spindle shaped, with hyperchromatic basophilic nuclei, inconspicuous cytoplasm and indistinct cell borders. They form cords, sheets and nests with sharply defined borders. Palisading of the peripheral cells may be present. Excessive mitosis and apoptosis are observed as well as comedo type necrosis. Keratinization and

- The most common site of HPV-related carcinomas of the UADT is the Oropharynx and particularly the tonsils and base of tongue, where they constitute one third of carcinomas in that location.
- HPV + tumors are distinct clinically and pathologically. They are more common in young patients (<40 years) with a male to female ratio of 4:1. They usually present as a small or occult primary with advanced neck disease. Tobacco use and excessive drinking are not necessary risk factors.
- Microscopically they are nonkeratinizing squamous cell carcinomas with basal cell features, excessive mitosis and comedo type necrosis.
- The tumors have a distinct immunohistochemical profile characterized by strong and diffuse p16 reactivity, low or negative p53 staining and high Ki67 labeling scores.
- HPV + Carcinomas are more radio-sensitive and have better prognosis than the classical keratinizing SCC of the UADT.
- An anti-HPV vaccine has recently been made available for prevention of cervical cancer. The impact of wide use of the vaccine on the prevalence of HPV related carcinomas of the UADT is currently not known but likely beneficial.

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keratin pearl formation is generally absent although some tend towards cell maturation may occasional be present in focal areas.

In lymph node metastasis tumor masses commonly show extensive central necrosis leading to a characteristic cystic change. The lining epithelium of the cystic structures may be so scant and bland appearing that diagnosis of a benign cyst may be erroneously made, particularly in cases in which the primary tumors are occult [8,9,36].

Immunohistochemistry:

A characteristic and distinct immunophenotype is exhibited by oropharyngeal HPV-related nonkeratinizing carcinoma. These tumors are distinguished by a strong and diffuse staining for p16INK4a (p16) antibodies, a negative or weak reactivity to p53 protein and higher Ki67 staining scores, as compared to the keratinizing type carcinomas of the same site [8,9].

Overexpression of p16 has also been extensively documented in HPV-related carcinomas of the uterine cervix and ano-rectal tract and is considered to be a surrogate marker for HPV+ carcinomas. p16 is a cell cycle protein which acts as cyclin-dependant kinase (CDK) inhibitor which is involved in tumor suppression by the retinoblastoma pathway [2,6,10,21,28]. It is believed that deregulation of pRb by HPV E7 oncoprotein results in paradoxical overexpression of p16 by feed back control. p16 immunoreactivity in keratinizing squamous cell carcinoma is usually either absent or weak [7-9].

The lack of correlation between NKCa and p53 reactivity contrasts with well documented p53 mutations identified in the majority of conventional keratinizing SCCs of the upper aerodigestive tract. In the case of HPV related carcinomas interference with p53 function is achieved by viral E6 protein which targets p53 resulting in its ubiquitination and degradation [32,35,39]. The high mitotic activity observed in NKCa is reflected in high labeling scores for the cell cycle specific protein Ki67 [7-9].

The exact mechanism by which HPV related carcinomas acquire none keratinizing basaloid histomorphology is not known. However, several of the viral oncogenes that are expressed in the tumor cells are known to interfere with cell cycle regulatory mechanisms resulting in cell immortalization with uncoupling of proliferation and differentiation. Interestingly HPV positive tumors of the larynx and sinonasal tract show identical histologic and immunophenotypic features like the HPV-related oropharyngeal carcinomas [7-9].

Detection

Currently no cytologic tests, analogous to cervical Pap smears, are used for early detection of HPV related oropharyngeal dysplasia and carcinoma. Unfortunately such techniques may not be useful because the majority of oropharyngeal lesions start at the bottom of the crypts of the palatine and lingual tonsils, and thus inaccessible to routine cytologic smears.

Advanced disease is symptomatic and manifested, clinically and radiographically, by tumor mass at the primary site as well as enlarged neck lymph nodes. Occasionally small primary tumors that are undetectable on routine clinical examination are associated with significant neck node metastasis (occult primary). We have recently shown that more than 90% of HPV positive neck metastasis arise from the oropharynx, mainly the tonsils and base of tongue, while less than 10% of those metastasis originated outside the oropharynx including the oral cavity proper, larynx and hypopharynx. HPV related

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Proliferative Verrucous Leukoplakia
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* **Proliferative verrucous leukoplakia is a clinical condition characterized by the repeated recurrence of white lesions of the oral cavity with epithelial abnormalities that progressively worsen to inexorably eventuate in oral carcinomas.**

* **Proliferative verrucous leukoplakia is predominantly a condition of women and has been associated with human papillomavirus, although HPV is not detected in all cases.**

* **Microscopically, the lesions of proliferative verrucous leukoplakia vary from non-dysplastic hyperkeratosis to various forms of carcinoma, including verrucous, squamous cell and poorly differentiated papillary carcinoma.**

* **Because of the high mortality rate with which it is associated, PVL mandates aggressive clinical follow-up and early management of white lesions. The pathologist is in a position to reduce morbidity and mortality from this condition by identifying multiple progressive lesions in the same patient and warning the appropriate clinician.**

Proliferative verrucous leukoplakia (PVL) was first described in the literature as a pathologic condition of the oral mucosa by Hansen and colleagues in 1985.¹ Their manuscript delineated a series of progressive stages through which the oral white lesions of patients would evolve over a period of time, with the eventual result of one or more carcinomas. The patient population they described consisted predominantly of elderly females and the time of progression to oral cancer varied from months to years, but the ultimate outcome was reported to be inevitable.

A decade later, in 1995, Palefsky and colleagues² reported the first association of PVL with human papillomavirus (HPV). In their study, the polymerase chain reaction was utilized on 9 lesions from 7 patients with PVL, histologically diagnosed as focal keratosis (1), papilloma (1), epithelial dysplasia (5) and squamous cell carcinoma (2). Eight were HPV positive, 7 for HPV

16. They compared these with 55 non-PVL-associated oral specimens, including 24 carcinomas. Of the non-PVL cancers, 33% were positive, 4 for HPV 16. They concluded that their data suggested that HPV 16 infection may play an important role in the pathogenesis of PVL-associated oral dysplasia/cancer, but that HPV 16 was found in only a small proportion of the more common non-PVL associated oral lesions.

The role of HPV in PVL has since been investigated with mixed results. Campisi and colleagues³ assessed the presence of HPV DNA in PVL versus conventional oral leukoplakias in exfoliated mucosal cells, utilizing nested PCR with MY09/MY11 and GP5+/GP6+ primer pairs with the HPV genotype determined by direct sequencing. HPV genotype was identified in 24.1% of PVL lesions and 25.5% of conventional oral leukoplakias. They found no statistical association between any demographic variable and HPV infection. A review of their data indicates, however, that the high risk HPV types, 16 and 18, were more common in the PVL lesions. Gopalakrishnan and colleagues⁴ compared mutated and wild-type p53 expression and HPV integration in 10 samples of normal mucosa, 10 of PVL lesions and 10 squamous cell carcinomas (SCC) and concluded that HPV infection, along with p53 expression, plays a "yet to be defined role in the pathogenesis of a limited number of PVL cases and SCC cases". Campisi and colleagues³ have reported on the use of fuzzy neural networks in modeling relationships of HPV infection with apoptotic and proliferation markers in potentially malignant oral epithelial lesions and suggest that survivin and PCNA may be involved in deregulation of epithelial maturation and that, conversely, HPV may play a role in the expression of these two markers. They conclude that the fuzzy neural network system appears to be an effective tool for analyzing correlates of oral leukoplakia and HPV infection.

In addition to and/or in conjunction with HPV infection, investigators have looked at other factors that may affect proliferation rate in PVL, including levels of transforming growth factor (TGF)-alpha and DNA ploidy. Kannan et al.⁵ found increased amounts of TGF alpha in PVL lesions and SCC compared to normal mucosa. Klancit et al.⁶ demonstrated that both conventional histopathology and ploidy anomalies were effective in predicting the site of transformation of oral leukoplakias in PVL to SCC.

In recent years, a gingival variant of the PVL condition has been reported.⁸ Lesions present as solitary or regional areas of the free and attached gingiva which start as flat lesions that become verrucous or papillary over time. Although these gingival lesions are recurrent, they are not associated with similar lesions in other locations. Bagan et al.⁹ studied a group of patients with PVL, looking at age, sex, location, recurrence, the appearance of new lesions and the frequency of development of oral cancer. A control group was formed from patients treated on the same service chosen randomly from those presenting with squamous cell carcinoma. Results showed the average age of PVL patients to be 70.97 ± 12.73 years, 80% females, 23.3% cigarette smokers. The area most affected was the lower gingiva. The control group was younger, less likely to be female and more likely to be cigarette smokers. The presence of HPV was not addressed in this study.

An examination of the literature points out a significant problem in studying PVL, and the contribution of HPV to its pathogenesis; that being the use of the term "PVL" as a microscopic diagnosis. For example, some recent current studies have stated that "x number of lesions with a

lesion consistent with the clinical condition of PVL unless a data base is searched for prior lesions and multiple oral epithelial lesions are subsequently detected. The recognition of the condition through the data base of a given lab may also be compromised by the fact that all of the patient's biopsies may not have been submitted to the same lab and/or the submitting surgeon may not give a history of previous similar lesions. Awareness of this life-threatening clinical condition on the part of the pathologist, however, should facilitate thorough clinical follow-up by the clinician and in the future may permit successful treatment of those cases associated with HPV.

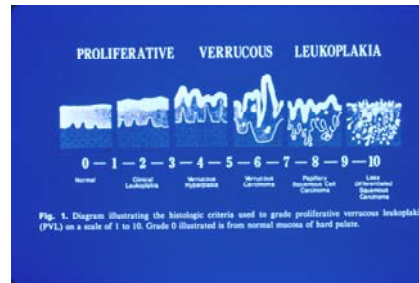


Diagram from Hansen et al., 1985.

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diagnosis of PVL were studied by this or that molecular technique". A biopsy report bottom line with a diagnosis of PVL is not appropriate as PVL is only a clinical term. In such studies, one does not know whether a hyperkeratosis, dysplasia or carcinoma was the biological lesion assessed.

The study of a time continuum for these lesions has rarely been reported. The best study addressing this is that of Silverman and Gorsky, who followed 54 patients from the time of initial biopsy for a mean period of 11.6 years. A mean number of 2.6 sites per patient were identified and the most common sites were the buccal mucosa in women and the tongue in men. The investigators reported that during a mean time of 7.7 years, over 70% of the patients developed a squamous cell carcinoma (SCC) at a PVL site. Over thirty-eight per cent of the patients died of the PVL-associated SCC.

Animal model

Reliable animal models are essential tools in the investigation of mechanisms of carcinogenesis. During the past decade, research regarding an association between HPV and oral epithelial dysplasia or OSCC has focused on the mere presence of the virus in affected tissues. This has been accompanied by debate concerning whether the variable association noted between the two is an etiologic or passive phenomenon. Another question has concerned the difference in oral versus uterine cervix response to the HPV 16 and 18 viruses.

The K14-HPV16 transgenic mouse has been proposed as an animal model of PVL by Murrah and colleagues.¹⁰ The K14-HPV16 transgenic mouse:

- **Spontaneously develops epidermal carcinogenesis that progresses through stages of hyperplasia, and dysplasia to invasive carcinoma.**
- **Spontaneously develops oral carcinogenesis that progresses through stages of hyperplasia, and dysplasia to CIS and SCC.**
- **Develops invasive cervical carcinoma in a hormone-dependent manner when exposed to chronic estrogen treatment.**

This transgenic was initially developed by Jeff Arbeitt¹¹ as a means of studying HPV-associated cervical carcinoma. In the K14-HPV16 transgenic mouse, a keratin 14 promoter targets HPV16 to basal cells. Oral epithelial changes were first described by Murrah et al.

A breeding colony of K14-HPV16 transgenic mice was established at the University of North Carolina by breeding K14-HPV16 stud males containing the HPV16 E6-E7 transgene to female FVB/N mice obtained from Jackson Laboratories (Bar Harbor, ME,

cell carcinoma: an immunohistochemical study. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1996; 82(1): 69-74.

⁷ Klancit P, Sperandio M, Brown AL, Shirlaw PJ, Challacombe SJ, Morgan PR, Odell EW. DNA ploidy in proliferative verrucous leukoplakia. *Oral Oncol*. 2006 Aug 21; [E-pub ahead of print].

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⁹ Bagan JV, Jimenez Y, Sanchez JM, Poveda R, Milian MA, Murillo J, Scully C. Proliferative verrucous leukoplakia: high incidence of gingival squamous cell carcinoma. *J Oral Pathol Med* 2003 Aug; 32(7): 379-82.

¹⁰ Murrah, V.A. and Gilchrist, E.P. Epithelial proliferation markers in dysplasias and carcinoma associated with human papillomavirus. *Oral Surgery, Oral Pathology, Oral Radiology and Endodontology*; 96: 3, 2003.

¹¹ Arbeitt JM, Manger K, Howley PM, Hanahan D. Progressive squamous epithelial neoplasia in K14 human papillomavirus type 16 transgenic mice. *J Virol* 1994; 68: 4358-4368.

¹² Murrah, V.A. and Gilchrist, E.P. Estrogen effects on progressive oral neoplasia in K14-HPV16 transgenic mice. *J Dental Res* (Spec Issue A): 0516, 2003.

¹³ Murrah, V.A. and Gilchrist, EP. Attenuation of Progressive Epithelial Dysplasia in K14-HPV16 Mice by Indole-3-Carbinol. *J Dent Res* 84 (Spec. Iss A): 1404, 2005.

¹⁴ Sethi, N and Palefsky, J. Transcriptional profiling of dysplastic lesions in K14-HPV16 transgenic mice using laser microdissection. *FASEB J*. 2004 Aug; 18(11): 1243-5. Epub 2004 Jun 4.

¹⁵ Femenao, F, Gombos, F, Scully C. Oral proliferative verrucous leukoplakia (PVL): open trial of surgery compared with combined therapy using surgery and methisoprinol in papillomavirus-related PVL. *Int J Oral Maxillofac Surg*. 2001 Aug; 30 (4): 318-22.

USA). Animals were weaned at 21 days and assigned to experimental time groups at 30 days of age, when the transgenic phenotype could be determined by the appearance of the ears. Transgenic offspring comprised the experimental group of the study and non-transgenic littermates served as the control group.

Animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Seventy mice of both sexes, 41 transgenics and 29 controls, were sacrificed at 1, 3, 6 or 9 months of age in order to assess progressive oral epithelial changes. Oral tissues from the tongue, labial and buccal mucosae were collected and processed for routine histology. Histology showed mild to severe dysplasia of the tongue, buccal and labial mucosa that progressed over time to carcinoma in situ or invasive squamous cell carcinoma. Murrah and Gilchrist have subsequently used this animal model to look at the effects of estrogen on the oral lesions¹², since PVL is predominantly a female condition, and to look at the effects of indole-3-carbinol as a preventive agent¹³.

Sethi and Palefsky have analyzed transcription patterns in this mouse model and have found that HPV transcription varies within the different layers of the epithelium¹⁴. These investigators have pointed out that understanding the patterns of HPV transcription is critical to designing and testing new HPV-specific therapeutic strategies. They looked at HPV expression in homogeneous populations of cells derived from the stratum basale, stratum spinosum and stratum corneum of these mice using PALM microlaser technology. RNA extracted from each layer was subjected to two-step gene-specific RT-PCR and real time quantitative nested PCR. High levels of E2 were detected in both basal and suprabasal layers of hyperplastic and dysplastic lesions. E7 and E6 levels also increased over time in the basal and spinous layers. The investigators concluded that the K14-HPV16 mice correctly spliced E2 transcripts and are suitable as a preclinical model to test a therapeutic strategy utilizing transcriptional regulation by the E2 protein.

Treatment of the lesions of PVL is a major clinical challenge. Both scalpel and laser excision are the current treatments for the premalignant lesions. Femenao and colleagues¹⁵ compared an open trial of surgery in 25 patients with oral HPV-positive PVL with combined therapy using surgery and methisoprinol in another 25 patients with oral PVL. Eighteen months post-operatively they found a significant difference. There were 18 recurrences of premalignant or malignant lesions in patients treated by surgery alone and only 4 recurrences in patients treated with surgery and methisoprinol, which is a synthetic drug with immunomodulatory properties and some antiviral activity against HPV.

It is conceivable that therapeutic HPV vaccines which target non-structural early viral antigens, such as E6 and E7, could also benefit patients with PVL.

Because of the high mortality rate with which it is associated, PVL mandates aggressive clinical follow-up and early management of white lesions. A major clinical problem, however, lies simply in the identification of patients with PVL. A specific isolated sample received by a pathologist is unlikely to be identified by that pathologist as

Human Papillomavirus and Proliferative Verrucous Leukoplakia

Valerie A. Murrah DMD, MS

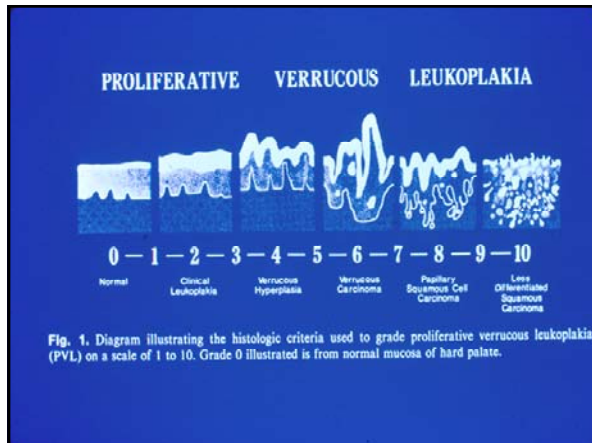
Professor and Chair, Department of Diagnostic Sciences
Director, Oral & Maxillofacial Pathology
The University of North Carolina School of Dentistry

Professor, Department of Pathology & Laboratory
Medicine
The University of North Carolina School of Medicine

BACKGROUND

Proliferative Verrucous Leukoplakia (PVL)

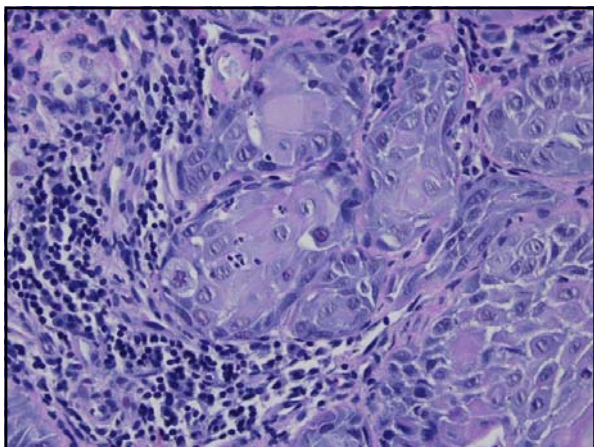
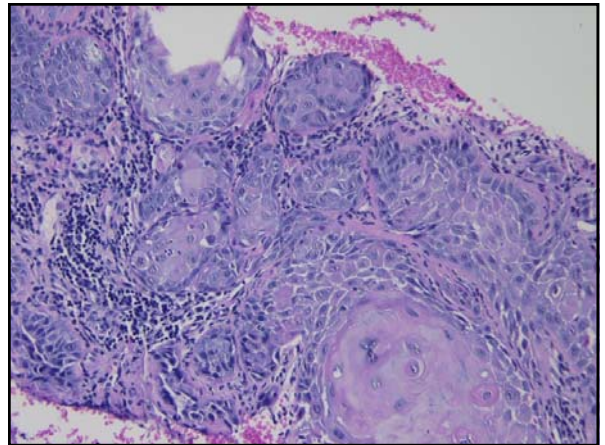
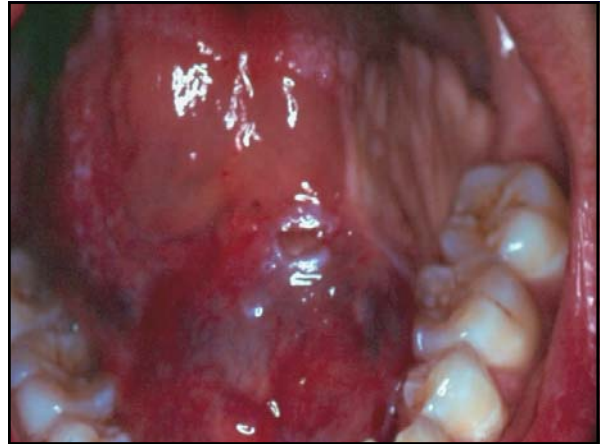
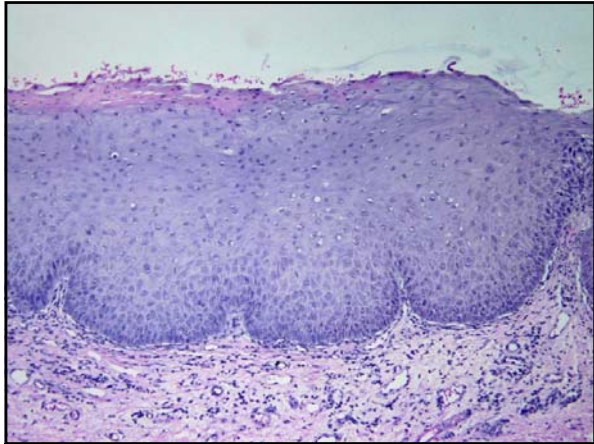
- 1) Associated with HPV
- 2) Predominance in Females
- 3) Progresses to SCC



BACKGROUND

An association between human papillomavirus (HPV) and oral cancer has been reported by multiple investigators in retrospective analyses of archival paraffin-embedded tissues.





BACKGROUND

Estrogen is suspected to be involved in carcinogenesis through the formation of superoxide free radicals, such as peroxynitrate (ONOO^-) resulting from the interaction of primary (unmethylated) 2-hydroxyestradiol (2-OHE_2) with NO, both of which are found in breast and uterus.

Garner, CE et al. *Toxicology and Applied Pharmacology* 162(2):115-23, 2003.

Yoshie, Y et al. *Free Radic Biol Med*. 24:341-48, 1998.

De Cree, C et al. *Fertility and Sterility*. 67(3):505-16, 1997.

BACKGROUND

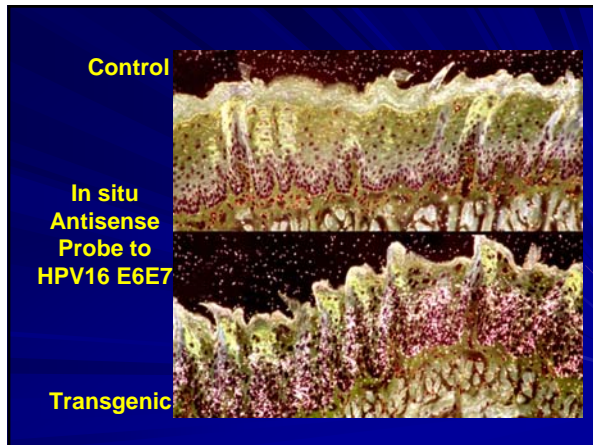
The K14-HPV16 transgenic mouse was developed and has been extensively characterized as a model of progressive squamous epithelial neoplasia in the epidermis¹, cervix², and recently in the oral cavity³.

1. Arbeit, JM. Cancer Surveys 26:7 – 34, 1996
2. Arbeit, JM et al. Proceedings of the National Academy of Sciences, USA 93:2930-35, 1996.
3. Murrain, VA et al. Journal of Dental Res 79(Special Issue):167, 2000.

BACKGROUND

The K14-HPV16 mouse:

- Spontaneously develops epidermal carcinogenesis that progresses through stages of hyperplasia, and dysplasia to invasive carcinoma.
- Spontaneously develops oral carcinogenesis that progresses through stages of hyperplasia, and dysplasia to CIS and SCC.
- Develops invasive cervical carcinoma in a hormone-dependent manner when exposed to chronic estrogen treatment.

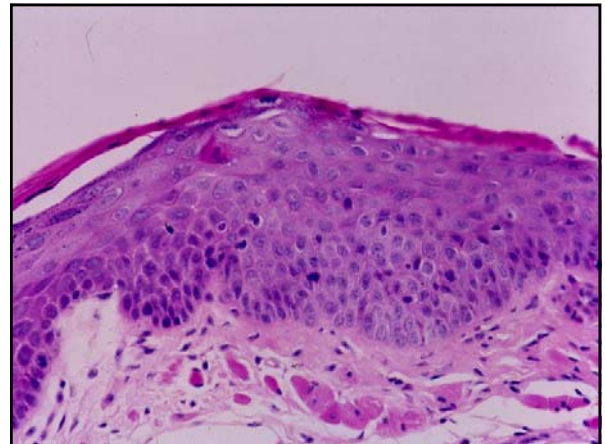
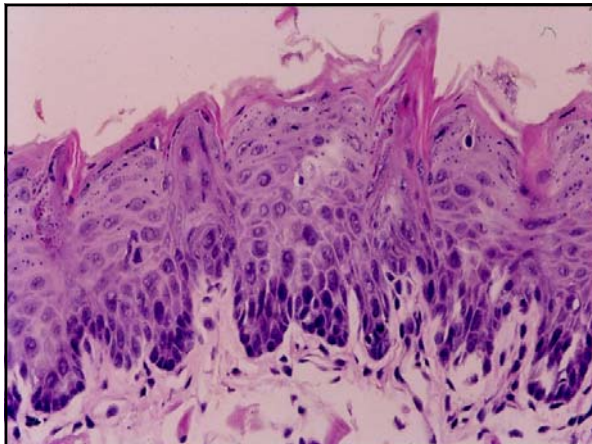
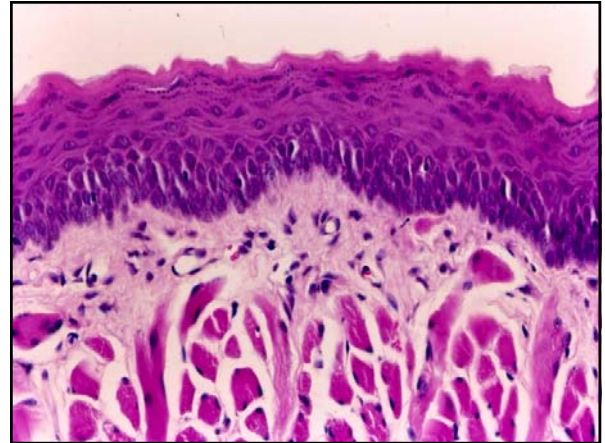
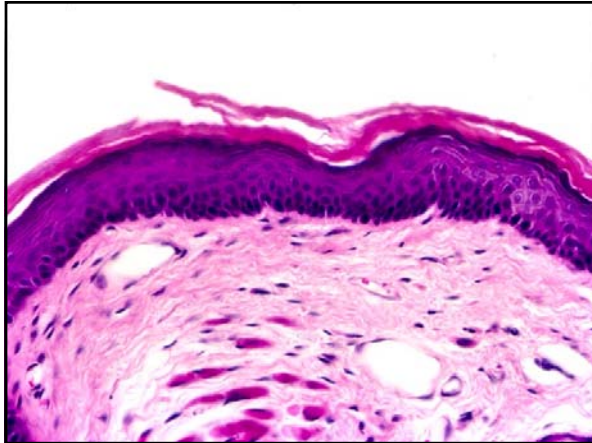


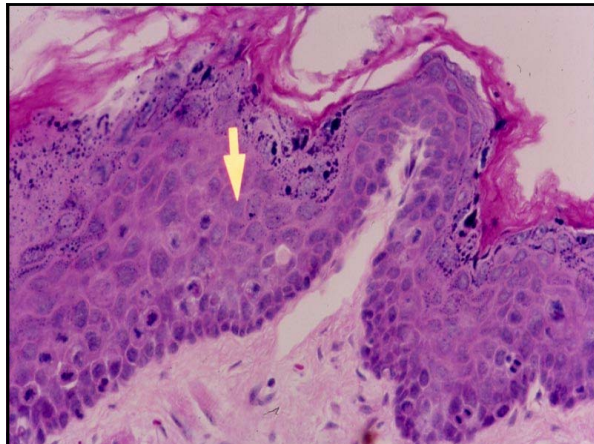
METHODS

A breeding colony of K14-HPV16 transgenic mice was maintained in the FVB/N strain, breeding non-transgenic females with stud males containing the transgene.

BACKGROUND

In the K14-HPV16 transgenic mouse, the E6 & E7 genes of HPV type 16 have been linked behind a keratin-14 promoter, targeting expression specifically to squamous epithelia.





RESULTS

BrdU Indices

	Collective	Tongue	Buccal	Labial
HPV+	16.3 ± .99	18.2 ± 1.49	12.8 ± .84	17.8 ± 2.02
CT	7.0 ± .93	9.2 ± 1.38	4.6 ± .49	4.7 ± .49
CT/Est	7.6 ± .84	9.7 ± .88	7.3 ± 1.20	6.1 ± 1.12
HPV+/Est	16.7 ± 1.22	19.8 ± 2.05	13.5 ± 1.20	16.6 ± 1.35

S-phase indices were determined by counting the number of labeled cells in a minimum of 1000 cells for each animal / site.

Buccal Histopathology

Histology	CT	HPV+	CT/Est	HPV+/Est
Normal	3	1	5	1
Norm - Mild Dys.	--	1	--	--
Mild Dys.	--	4	4	3
Mild - Mod Dys.	1	2	--	4
Moderate Dys.	--	--	--	4
Mod - Sev Dys.	--	--	--	1
Severe Dys.	--	--	--	--

Labial Histopathology

Histology	CT	HPV+	CT/Est	HPV+/Est
Normal	3	1	5	1
Norm - Mild Dys.	--	2	--	--
Mild Dys.	1	1	5	5
Mild - Mod Dys.	--	1	--	1
Moderate Dys.	--	2	--	3
Mod - Sev Dys.	--	--	--	2
Severe Dys.	--	1	--	2

Tongue Histopathology

Histology	CT	HPV+	CT/Est	HPV+/Est
Normal	3	--	5	--
Norm - Mild Dys.	--	--	1	--
Mild Dys.	1	4	4	1
Mild - Mod Dys.	--	1	--	3
Moderate Dys.	--	2	--	5
Mod - Sev Dys.	--	1	--	1
Severe Dys.	--	--	--	5

Collective Histopathology

Histology	CT	HPV+	CT/Est	HPV+/Est
Normal	3	--	3	--
Norm - Mild Dys.	--	--	1	--
Mild Dys.	1	4	7	--
Mild - Mod Dys.	--	--	--	2
Moderate Dys.	--	2	--	5
Mod - Sev Dys.	--	1	--	2
Severe Dys.	--	1	--	6

While the effects of the HPV transgene appear to be the overriding factor for collective dysplasias in the oral cavity, at one month, the effects of the exogenous estrogen appear to be additive, and approach statistical significance when analyzed by chi square:

Dysplasia	Yes	No	Totals	
HPV +	4	4	4	p =.0565
HPV+/Est	13	2	2	
Totals	17	6	23	

CONCLUSION

The K14-HPV16 mouse can be used as an appropriate model of HPV induced oral progressive epithelial neoplasia, which appears to be effective for investigating the effects of estrogen as a co-factor in oral carcinogenesis.

PVL TREATMENT

- Scalpel surgery
- Laser surgery
- Surgery + methisoprinol (an immunomodulatory drug with some antiviral activity against HPV)
- Therapeutic HPV vaccines which target E6,E7

Analysis of transcription patterns in K14-HPV16 transgenic model

- Sethi and Palefsky have found transcription variation within the epithelium:

*RNA was extracted from each layer and subjected to two-step gene specific RT- PCR and real time quantitative nested PCR.

- High levels of E2 in basal and suprabasal layers
- E6 and E7 also increased over time in basal and spinous layers

K14-HPV16 mice correctly spliced E2 transcripts and are suitable as a preclinical model to test a therapeutic strategy utilizing transcriptional regulation by the E2 protein.

Implications for the Pathologist

- The laboratory data base should be searched to identify multiple lesions on any patient with a leukoplakic lesion
- The pathologist is in a position to alert the clinician about the possibility of this condition and thereby reduce morbidity and mortality in patients with PVL.

An Update on Epstein-Barr Virus and Nasopharyngeal Carcinoma.

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Epstein-Barr Virus

Epstein-Barr virus (EBV) is a double stranded DNA γ -herpesvirus with widespread distribution in all human populations. EBV is associated with a variety of diseases including infectious mononucleosis, hairy leukoplakia, inflammatory pseudotumors, nasopharyngeal carcinoma (NPC), Burkitt's lymphoma, Hodgkin lymphoma, post-transplants lymphoproliferative disorders, HIV-associated B-cell lymphomas, some T-cell lymphomas particularly extranodal NK/T cell lymphomas of the nasal-type, and recently a subset of gastric and breast carcinomas.¹ EBV preferentially infects B-lymphocytes through the binding of the major envelop glycoprotein gp350 to the CD21 receptor on the surface of B-cells and through the binding of a second glycoprotein, gp42, to human leukocyte antigen (HLA) class II molecules as a co-receptor.² EBV has the capacity to transform resting B-cells into permanent latently infected lymphoblastoid cell lines.

EBV-transformed lymphoblastoid cell lines express a set of viral gene products referred to as latent proteins which include six EBV nuclear antigens (EBNAs 1,2, 3A, 3B, 3C, -LP) and three latent membrane proteins (LMPs 1, 2A, and 2B). Transformed lymphoblastoid cells also show abundant expression of small, non-polyadenylated, non-coding RNAs (EBER1 and EBER2)

cells in tissue sections. In NPC practically all the tumor cells show nuclear staining. EBV latent membrane protein-1 (LMP-1) immunohistochemistry is less reliable and often is weak and patchy with expression being detected in less than 40% of cases. PCR detection of EBV is unreliable since even the presence of a few non-neoplastic EBV-infected lymphocytes will yield a positive result.

Nasopharyngeal carcinoma precursor lesions

Although NPC in-situ can be identified in approximately less than 10% of conventional invasive NPC, pure nasopharyngeal carcinoma in-situ is exceedingly rare.^{11,19-22} As invasive NPC, nasopharyngeal carcinoma in-situ are positive for EBV (EBER) in keeping with the concept that EBV infection in an early event in NPC carcinogenesis.^{11,19-23} The absence of EBV-infected epithelial cells in normal nasopharyngeal mucosa from individuals at high risk of developing NPC argues against a pre-existing normal reservoir infected cells from which virus-positive carcinoma arise; however, deletions in chromosome regions 3p and 9p have been identified in low-grade dysplastic lesions and normal nasopharyngeal mucosa of individuals at high risk of developing NPC indicating that these genetic events occur early in the pathogenesis of NPC and that they might cause predisposition to subsequent EBV infection.²⁴⁻²⁶

Pathologic classification of nasopharyngeal carcinoma

which are expressed in all forms of latent EBV infection. Transcripts from the *Bam*HIA viral genome known as BART-transcripts are also detected in lymphoblastoid cells.³ EBNA2, EBNA3C and LMP1 are key in the transformation of EBV-infected cells.^{4,5} LMP1 is the main transforming protein of EBV and functions as a classic oncogene in fibroblast transformation assay.⁶ LMP1 function as an activated member of the tumor receptor (TNFR) superfamily, and activates several signaling pathways.^{7,8}

Epstein-Barr Virus and Nasopharyngeal Carcinoma

EBV latent infection of normal epithelial cells has not been detected in normal cells. The mechanisms of EBV latent infection of epithelial cells are not well understood, but serious consequence is malignant transformation resulting in the development of nasopharyngeal carcinoma (NPC), a subset of gastric and breast carcinomas and certain salivary gland carcinomas.¹ Studies of normal nasopharyngeal tissue and premalignant samples in patients at high risk for the development of NPC indicate that genetic events – particularly *RASSF1A* and *p16* inactivation – occur early in the pathogenesis of NPC, and that these might predispose the abnormal epithelium to subsequent EBV infection originating from adjacent lymphoid tissues and circulating B-cells.⁹ EBV latent-gene expression in NPC is predominantly restricted to the EBNA1 nuclear antigen, the latent membrane proteins LMP2A and LMP2B, *Bam*HIA transcripts, and the oncogenic LMP1 protein and is consistent with a type II latency.^{9,10} Southern-blot hybridization from NPC tissues demonstrates monoclonality of the viral genome indicating that the EBV infection takes place before the clonal expansion of the population of malignant cells.^{11,12}

The morphologic features of nasopharyngeal carcinoma have been well described and summarized in the 2005 WHO Classification of Head and Neck Tumors.^{27,28} Subclassification of nonkeratinizing carcinoma into undifferentiated and differentiated subtypes is optional since their distinction is of no clinical or prognostic significance and features of both types can be seen in the same biopsy material or in sequential biopsies from the same patient. Nonkeratinizing and keratinizing squamous carcinomas are almost invariably associated with EBV in all geographic areas,²⁹ whereas only a small proportion of keratinizing squamous cell carcinoma are positive for EBV in areas with low incidence of NPC.^{30,31} EBV expression in keratinizing squamous cell carcinomas tend to be low and limited to cells with a basaloid appearance with terminally differentiated squamous cells being negative. There has been an overall decline in the incidence of NPC in Hong Kong, but the decline appears to be limited to a decrease in keratinizing squamous cell carcinoma attributable to a decrease in smoking and other environmental factors.³²

Table 2. 2005 WHO Classification of nasopharyngeal carcinoma and incidence of histologic types²⁷

WHO histologic types	High incidence population	Intermediate incidence population	Low incidence population
Keratinizing squamous cell carcinoma	1-17%	8%	13-25%
Nonkeratinizing carcinoma	83-99%	92%	75%
-Undifferentiated			
-Differentiated			
Basaloid squamous cell carcinoma	<0.2%	?	?

Epstein-Barr virus detection

Table 1. Epstein-Barr virus detection in the laboratory (Gulley 2001)¹³

Test	Main applications
In-situ hybridization (EBER)	Identify EBER transcripts or EBV DNA in specific cell types in microscopic sections
EBV DNA amplification	Assess clonality of lesions Distinguish latent from replicative infection based on the episomal (circular) versus linear EBV genome
Serology (VCA, EBNA, EA, heterophile antibodies)	Measures antibody response to viral proteins in serum samples Distinguishes acute from remote infection
EBV viral load	Monitor disease status Quantitate EBV DNA in blood or fluids to monitor disease status
Immunohistochemistry (LMP1, EBNA1, EBNA2, LMP2A, BZLF1)	Identify EBV protein expression in specific cell types in microscopic sections Distinguished latent from replicative infection based on expression profiles
Culture of EBV or EBV-infected B-cells	Detect and quantify infectious virions or latently-infected B-cells
Electron microscopy	Impractical for routine clinical use Identify whole virions representing replicative viral infection Impractical for routine clinical use

Serological studies identify increased EBV-specific antibody titers in individuals living in high-incidence areas and in patients affected by NPC. The most common serologic studies employed in clinical practice include IgA antibodies against viral capsid antigen (VCA) and IgG/IgA antibodies against early antigens (EA).¹³ Antibodies to the EBV capsid antigen have proved useful in monitoring the effectiveness of therapy.¹⁴⁻¹⁶ More recent studies using real-time quantitative PCR to measure circulating tumor derived EBV DNA in the blood of patients with NPC have shown that the levels of pre-treatment EBV DNA are strongly associated with overall survival, and that post-treatment EBV DNA predict progression free and overall survival.¹⁴⁻¹⁷

The simplest and more reliable method for detection of EBV in tissues is in-situ hybridization for EBV encoded early RNA (EBER).^{13,18} In-situ hybridization allows identification of the infected

Molecular Pathology of Nasopharyngeal Carcinoma

Genetic risk

There an association between certain HLA phenotypes and increased risk of developing NPC. These haplotypes include HLA A2-B46, HLA B17, HLA A2-B38, and HLA A2-B16.³³⁻³⁵ The genetic bases of familial NPC are not well understood, but susceptibility loci have been identified in chromosome regions 3p21 and 4p15^{36,37}

GSTM1 and CYP2E1 polymorphisms

Glutathione S-transferase M1 (GSTM1) detoxifies benzo[a]pyrene and the cytochrome P450 2E1 is responsible for the metabolic activation of carcinogenic nitrosamines. Although the literature remains controversial, it has been reported that alterations of GSTM1 and CYP2E1 are associated with a moderate increased risk of NPC.³⁸⁻⁴³

Molecular genetic alterations

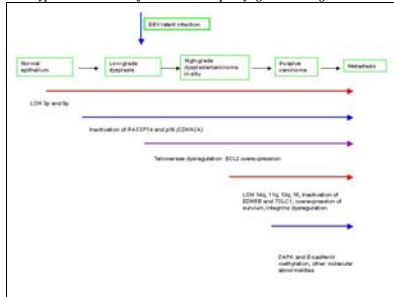
Molecular alterations in NPC are complex. Inactivation of the *RASSF1A* and *p16* tumor suppressor genes on 3p21 and 9p21 by homozygous deletions and promoter methylation have the most common alterations described in NPC.^{25,44-50} 3p and 9p abnormalities have been identified in low-grade dysplastic lesions and normal nasopharyngeal mucosa of individuals at high risk indicating that these genetic changes are early events in the pathogenesis of NPC.^{11,19,21,26} Other genes frequently inactivated by promoter methylation in NPC include *TSC1* at 11q23 and

EDNRB at 13q22, E-cadherin, and death-associated protein kinase (DAPK)^{48,51-56} Gene expression profiling has shown dysregulation of the PI3K/Akt, WNT/ β -catenin, *TGF- β* , and *MAPK* signaling pathways in NPC with upregulation of NF- κ B2, survivin, Bel-2 upregulation, nuclear accumulation of β -catenin, and dysregulation of integrins.⁵⁷⁻⁵⁹

Table 3. Common molecular abnormalities in NPC.

RASSF1A promoter methylation
<i>p16</i> homozygous deletions and methylation
<i>EDNRB</i> promoter methylation
<i>TSLC1</i> promoter methylation
E-cadherin hypermethylation
DAPK methylation
Telomerase dysregulation

Hypothetical model of EBV and nasopharyngeal carcinogenesis.^{2,25}



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Epstein Barr Virus and Upper Aerodigestive Tract Lymphomagenesis
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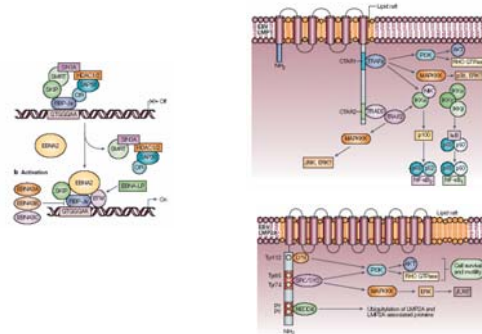
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Epstein Barr Virus and Upper Aerodigestive Tract Lymphomagenesis.

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Biology of viral infection

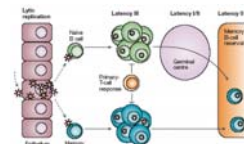


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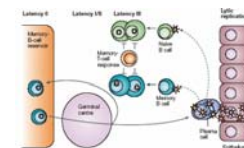
Latency Programs in EBV infection

Latency	EBER	EBNA1	LMP1	LMP2A	EBNA2	EBNA-LP	Disease
0	+	?	-	+	-	-	Memory B cells
I	+	+	-	-	-	-	Burkitt Lymphoma PEL
II	+	+	+	+	-	-	Hodgkin Lymphoma
III	+	+	+	+	+	+	PTLD

Primary Infection

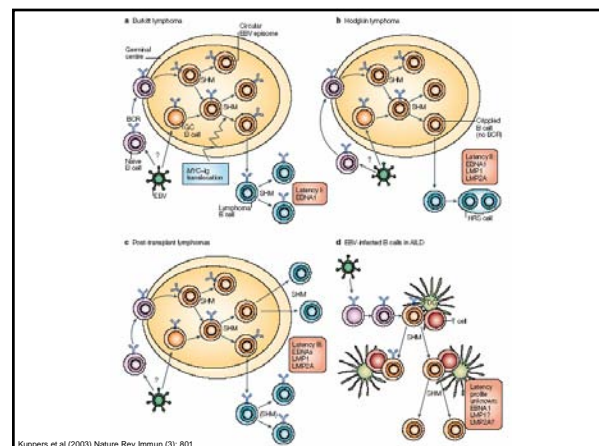


Persistent Infection

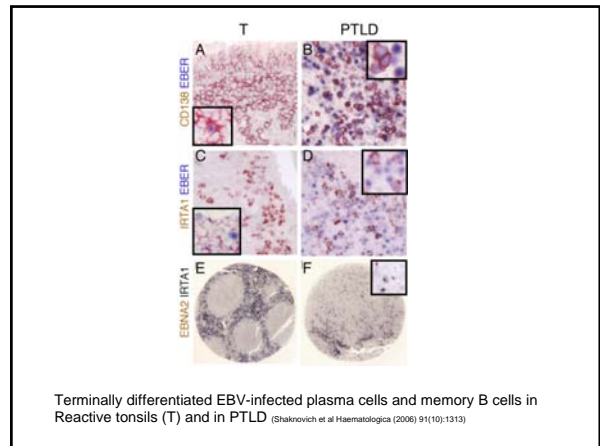
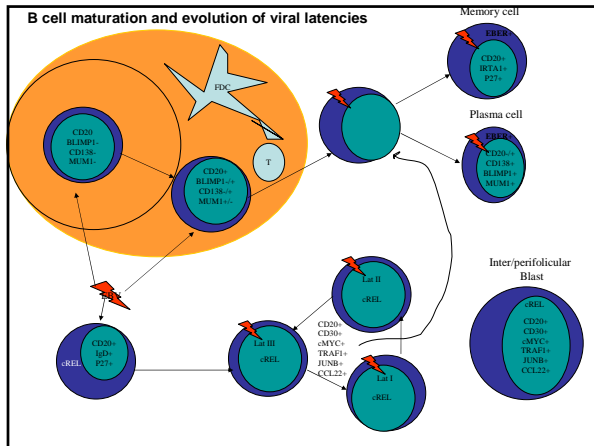


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Lymphoma	% EBV +	EBV latency	Immunophenotype	V gene mutations	Cell of origin
Burkitt Lymphoma	95-100% (endemic) 20-30% (sporadic)	I	CD20+ CD79a+BCL6+ CD10+ CD77+ slg+	Mutated V genes Ongoing SHM	Centroblast
C Hodgkin Lymphoma	40% (West) 90% (Central America)	II	Loss of B cell Phenotype slg-	Mutated V genes Ongoing SHM	Pre-germinal GC B cell
Post-transplant LPD	80% (diminishes with time)	III (mixture of latencies)	CD20+ CD79a+ BCL6- CD10+/- slg+/-	Mostly mutated V genes, mixed SHM	Variable stages of B cells
AILT	40-90%	0 (EBER+)	CD3+ CD4+ CD8- CD10+	TCR rearranged	Infected B cell and expansion of helper T cells
NK/T, nasal	most	EBER+	cyCD3, CD56+	No TCR rearrangement	NK/T cells
AIDS-associated B-cell lymphoma	30-50% BL	I	CD79a+BCL6+ CD10+ CD77+ slg+	Mutated V genes Ongoing SHM	GC
	100% PEL	I	CD20- CD138+	Mutated V genes	Post-GC cells
	90% DLBCL-IB	III	CD20+ CD138+/-	Mutated V genes, no ongoing SHM	GC or post-GC
LYG	most	EBER+	CD20+ CD79a+		Post GC B cell



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Terminally differentiated EBV-infected plasma cells and memory B cells in Reactive tonsils (T) and in PTLD (Shaknovich et al Haematologica (2008) 91(10):1313)