Focus on nasopharyngeal carcinoma

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Epidemiology and incidence

Nasopharyngeal carcinoma (NPC) has a remarkably distinctive ethnic and geographic distribution. In the year 2000, a total of 64,798 new cases were registered worldwide, and more than 80% of those were reported from China, Southeast Asia, and some Asian countries (Ferlay et al., 2001). NPC is a rare malignant disease, with an incidence well under 1 per 100,000 persons per year in Caucasians from North America and other Western countries. In contrast, the highest incidence is found among Southern Chinese (~25–30 per 100,000 persons per year), especially those of Cantonese origin. Southern Chinese immigrants also have a higher risk of NPC as compared to the local Western population. Early-age onset of NPC is observed in high-risk populations. Independent of race/ethnicity, men are 2- to 3-fold more frequently affected than women (Yu and Yuan, 2002). The dramatic difference in the incidence among populations and geographic areas suggests a strong association of NPC with genetic and environmental factors. An unusually early-age onset in the high-risk populations implies that early events in life may be important. There are recent changes in the epidemiology as shown by decreasing incidences of NPC (~30%) in Hong Kong over the past 20 years (Lee et al., 2003), which may be related to changes in environmental factors, as discussed later.

A distinctive type of head and neck cancer

NPC is a distinctive type of head and neck cancer. The World Health Organization (WHO) classification distinguishes three histopathological types of NPC based on the degree of differentiation. Type I is keratinizing squamous cell carcinoma (SCC), similar to other head and neck cancer. Type II is nonkeratinizing carcinoma, and Type III is undifferentiated carcinoma (Shanmugaratnam and Sobin, 1991). The undifferentiated carcinoma has a typical morphology with a prominent lymphoplasmacytic infiltrate, and is also referred as "lymphoepithelioma." Different prevalent histologic types of NPC are found in endemic and nonendemic regions. In endemic areas such as Southern China, WHO Type III accounts for more than 97%, while keratinizing SCC is more common in the Western countries (~75%) (Marks et al., 1998). Aside from differences in histological features, latent Epstein-Barr virus (EBV) infection is uniquely present in almost all, if not all, NPC from endemic regions, but absent in WHO Type I NPC from nonendemic regions (Figure 1) (Raab-Traub, 2002). Alternative pathogenic processes of EBV-negative NPC, especially WHO Type I from Western populations, may be involved.

Etiologies

The ethnic clustering of NPC in Southern Chinese strongly suggests the involvement of genetic susceptibility and environmental factors in its development. Three major etiological factors, including genetic, environmental, and viral factors, are defined here.

Genetic factors

Early linkage analysis on Chinese sib pair studies of NPC suggested the association of susceptibility HLA haplotypes with NPC development. The investigators hypothesized that some of the HLA antigens have reduced efficiency in activating host immune response to EBV infection, which plays a critical role in the pathogenesis of NPC. Most studies conducted among Chinese showed that individuals with HLA-A2 are at increased

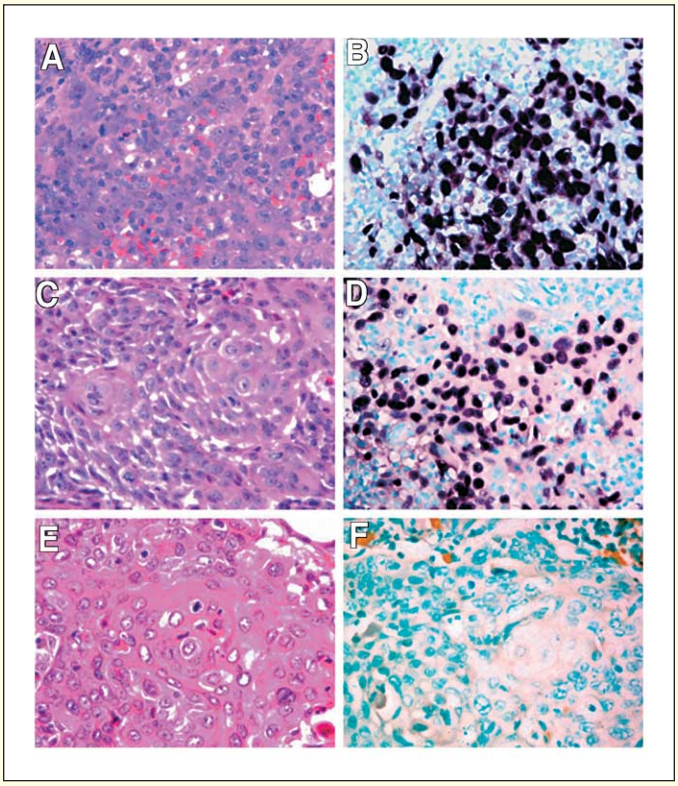

Figure 1. EBV latent infection in nasopharyngeal carcinoma

A and B: Typical nasopharyngeal undifferentiated carcinoma (WHO type III). **A:** The carcinoma cells exhibit prominent nucleoli and arrange in syncytial clusters. Dense infiltration of lymphocytes and plasma cells is evident (hematoxylin and eosin stain [H&E], 400×). **B:** In situ hybridization (ISH) for EBV RNA (EBER) reveals distinct nuclear positivity, indicative of latent infection in carcinoma cells (400×).
C and D: Squamous cell carcinoma (SCC, WHO type I) from endemic region. **C:** SCC with intercellular bridges and keratinization is seen (H&E, 400×). **D:** ISH-EBV is positive (400×).
E and F: SCC (WHO type I) from nonendemic region. **E:** SCC with intercellular bridges, keratinization, and keratin pearl are noted (H&E, 400×). **F:** ISH-EBV is negative (400×).

risk. A recent high-resolution genotyping study has detected a consistent association between NPC and the prevalent Chinese A2 subtype (HLA-A*0207), but not the prevalent Caucasian subtype (HLA-A*0201) (Hildesheim et al., 2002). Supported by affected sib pair haplotype sharing analysis and association study on HLA regions, a NPC susceptible gene/locus closely linked to the MHC region but distinct from the HLA genes was proposed (Lu et al., 1990). However, recent linkage analysis of Chinese NPC pedigrees using highly polymorphic microsatellite markers further identified two susceptibility loci on chromosomes 4p15.1-q12 and 3p21, respectively, but not on MHC region, from the Guangdong and Hunan provinces in China (Feng et al., 2002a; Xiong et al., 2004). Polymorphisms of genes for carcinogen metabolism (CYP2E1), detoxification (GSTM1), and DNA repair (XRCC1 and hOGG1) were also reported to be associated with increased risk of NPC (Hildesheim et al., 1997; Nazar-Stewart et al., 1999; Cho et al., 2003).

Environmental factors

The traditional foods of Southern Chinese, such as Cantonese-style salted fish and other preserved foods containing volatile nitrosamines, are an important carcinogenic factor for NPC. The childhood consumption of salted fish has been shown to be related to an increased risk of NPC in Southern Chinese (Yu and Yuan, 2002). In animal studies, nasal and nasopharyngeal tumors could be induced in rats by feeding them Chinese salted fish (Huang et al., 1978). These traditional diets may act as chemical carcinogens that induce genetic damage in nasopharyngeal epithelial cells. The decreasing trend in NPC incidences ($\sim$30%) in Hong Kong may be attributed to the change of traditional lifestyle, particularly the avoidance of feeding young children salted fish. The use of Chinese medicinal herbs has been suggested to increase the risk for NPC by reactivating EBV infection in the host. The association of NPC with other nondietary factors such as cigarette smoking or formaldehyde exposure is either weak or controversial.

Epstein-Barr virus (EBV)

In contrast to other head and neck cancer and epithelial malignancy in general, a unique feature of NPC is its strong association with EBV. Higher EBV antibody titers, especially of IgA class, are observed in most NPC patients. Latent EBV infection is identified in cancer cells of virtually all cases of NPC in endemic regions. The clonal EBV genome is consistently detected in invasive carcinomas and high-grade dysplastic lesions (Raab-Traub and Flynn, 1986; Raab-Traub, 2002). Such observations imply that viral latent infection may have taken place before the expansion of the malignant cell clone. A current hypothesis proposes that EBV plays a critical role in transforming nasopharyngeal epithelial cells into invasive cancer.

Clinical features, staging, and treatment

The majority of NPCs arise in the lateral walls, especially from the fossa of Rosenmuller and Eustachian tube cushions. Neck mass, blood-tinged sputum, nasal obstruction and increasing nasal discharge, aural symptoms such as tinnitus, stuffiness, and hearing loss, frequent headache, and neurological symptoms are common clinical presentations. Since the nasopharynx has an abundant supply of regional lymphatic vessels, metastasis is frequently found, and cervical lymphadenopathy is often the only clinical manifestation of NPC patients.

By incorporating all the major prognostically significant tumor parameters (nasopharynx [T], regional lymph node [N], and distant metastasis [M])—parameters previously used for forming several staging systems—a new UICC/AJCC staging classification was introduced in 1997 (Lee et al., 1999). The new system assigns patients to more uniform-size stage groupings and correlates better with prognosis than previous staging systems; e.g., Ho 1978 system and 1992 UICC/AJCC system. Moreover, in addition to computed tomography (CT) scans, magnetic resonance imaging (MRI) has been utilized to better delineate the skull base and brain involvement, a factor of considerable treatment and prognostic significance. The role of new imaging approaches, including positron-emission tomography (PET), is still being investigated.

Since NPC is highly radiosensitive, radiotherapy (RT) has always been the main treatment of choice for this cancer. With conventional practice and two-dimensional planning techniques, the local control rates were in the order of 80%, taking all T stages together. Intensity-modulated radiotherapy (IMRT) provides superior local control and better delineation of tumor target volume and spares the adjacent vital organs (Teo and Chan, 2002). With excellent local control, IMRT has replaced conventional RT as standard practice in centers where the technique is available. Although overall survival after RT for early staging is encouraging, there are significant rates of local failure and distant metastases subsequent to RT in the advanced stage disease. Attempts have been made to improve treatment results by integrating RT with some form of chemotherapy. The current standard of care is RT for early disease and concurrent chemotherapy-RT in locally advanced disease (Teo and Chan, 2002). Preliminary results on the use of neoadjuvant chemotherapy followed by concurrent chemoradiation are highly encouraging. The additional benefit of neoadjuvant chemotherapy in combination with concurrent chemotherapy-RT is being investigated in prospective randomized studies.

Molecular alterations and pathogenesis

Genetic and epigenetic events

The genetic, environmental, and viral causative factors, either acting alone or in combination, would lead to multiple genetic and epigenetic alterations. By the comprehensive genome-wide studies, multiple genetic defects have been identified in this EBV-associated cancer. Consistently high frequencies of genetic losses are observed on chromosomes 3p, 9, 11q, 13q, 14q, and 16q, while recurrent chromosomal gains were identified on chromosome 12 (Hui et al., 1999; Lo et al., 2000a). In particular, the inactivation of tumor suppressor genes on 3p, 9p, and 14q appears to be a critical event, since deletion of these chromosomal regions was detected in almost all microdissected NPC samples (Lo et al., 2000a). Genes located on chromosomes 9p21 (*p14*, *p16*) and 3p21.3 (*RASSF1A*) were found to be defective due to deletion or promoter hypermethylation (Lo et al., 1996, 2001; Kwong et al., 2002). The tumor suppressor properties of *p16* and *RASSF1A* have also been demonstrated in NPC cells (Wang et al., 1999; Chow et al., 2004).

Frequent promoter hypermethylation of cancer genes is an important feature of NPC. Apart from the common tumor suppressors, such as *RASSF1A* and *p16*, genome-wide aberrant methylation disrupts multiple cellular functions through the inactivation of retinoid signaling pathway (e.g., *RARB2*), endothelin-1 pathway (e.g., *EDNRB*), cell adhesion (e.g., *E-cadherin*, *TSLC-1*), and other novel pathways (e.g., *HIN-1*) (Lo and Huang, 2002; Kwong et al., 2002; Hui et al., 2003). Such widespread hypermethylation in the NPC genome may imply a "methylator" phenotype of this EBV-associated cancer. Induction of epigenetic alterations of cellular genes was pro-

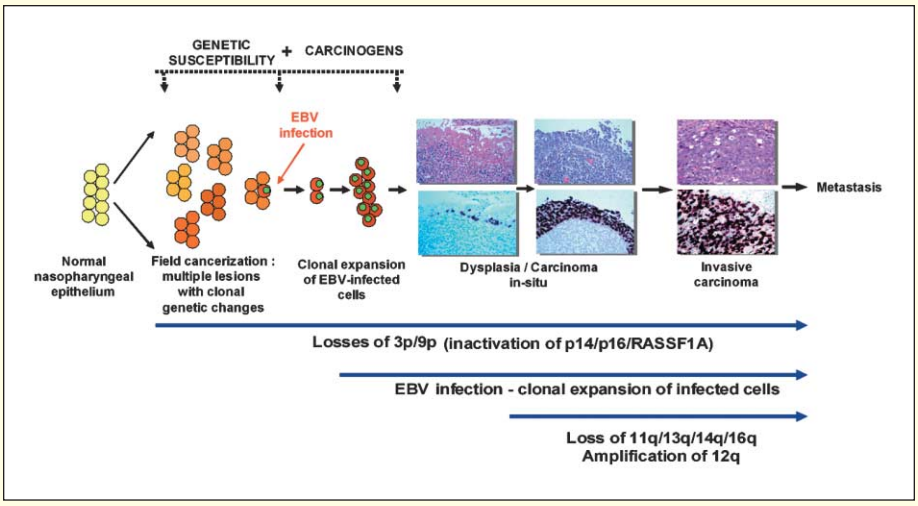

Figure 2. Multistep carcinogenesis of nasopharyngeal carcinoma

Hematoxylin and eosin staining and EBER in situ hybridization of the dysplastic lesions and invasive carcinoma are shown in the upper and lower panels, respectively.

posed as one of the mechanisms for enhancing the transformation of nasopharyngeal epithelial cells by EBV infection (Lo and Huang, 2002).

Alterations of cellular mechanisms

In NPC, multiple genetic abnormalities result in the disruption of various cellular mechanisms (Table 1). Alteration of cell cycle regulation by disrupting Rb and p53 pathways appears to be a critical event for NPC. The *p16* gene, an important cell cycle regulator for G1 restriction checkpoint, is inactivated in 62%–86% of primary tumors. Loss of p16 may result in constitutional Rb phosphorylation and uncontrolled proliferation of NPC cells (Lo et al., 1996). On the other hand, in spite of the absence of *p53* mutation, functional disruption of *p53* pathway through inactivation of *p14* and overexpression of truncated ΔN-isoform of *p63* was common in this cancer (Kwong et al., 2002; Crook et al., 2000). Since p14 binds MDM2 and inhibits the ubiquitination of p53 protein, the inactivation of *p14* may facilitate p53 degradation in NPC cells. Alternatively, ΔN-p63, as a p53 homolog, can block p53-mediated transactivation through its dominant negative function. Transcriptional silencing of the newly identified tumor suppressor, *RASSF1A*, is found in the majority of NPCs, although it is rarely involved in other head and neck cancers. Novel tumor suppressor function has been proven in NPC cells both in vitro and in vivo (Chow et al., 2004). Although *RASSF1A*-associated pathways in NPC are still unclear, our recent microarray study suggests that the gene may play roles in G protein signaling and TGF-β pathway in this particular cancer.

Aside from cell cycle regulation, multiple abnormalities associated with apoptosis and growth signals are found. Consistent upregulation of Bcl2 in precancerous lesions and invasive tumors suggests that alterations in the apoptotic response are early events in the transformation pathway (Lu et al., 1993). Overexpression of metallothionein (MT) and Id1, and loss of DAP-kinase may also contribute to the inhibition of apoptosis (Jayasurya et al. 2000; Kwong et al., 2002; Wang et al. 2002). Specific activation of NF-kB p50 homodimer and overexpression of Bcl3 may increase the proliferative capacity of NPC cells through transcriptional upregulation of target genes, such as EGFR (Thornburg et al., 2003). Furthermore, the c-Met tyrosine kinase, metalloproteinases (MMPs), and the hypoxia proteins such as HIF-1 α and CA IX were found to be overexpressed in the majority of NPCs and associated with the progression or metastasis of this cancer (Qian et al., 2002; Hui et al., 2002; Lu et al., 2003). The elevation of EGFR, MET, and hypoxia protein expression provides attractive targets for the development of novel chemotherapeutic strategies.

Roles of EBV latent infection

As a cancer-associated herpesvirus, EBV infects over 90% of the world's population. After primary infection at early age, persistent EBV latent infection is found in some resting B cells, but not in the nasopharyngeal epithelia of healthy individuals. The mechanisms for EBV entry into epithelial cells and maintenance of latency remain poorly understood. Recent evidence demonstrated the association of distinct lytic promoter sequence variation with NPC in Southern Chinese and suggested the participation of a lytic-latent switch of EBV in NPC carcinogenesis (Tong et al., 2003). In NPC cells, the virus is in the form of episome and not integrated into the host genome. EBV adopts a specific form of latent infection, latency II, in NPC cells. Only limited viral genes, including *EBERs*, *EBNA1*, *LMP1*, *LMP2*, *BARF1*, and several BamHI A transcripts are expressed

Table 1. Major gene alterations in nasopharyngeal carcinoma

Gene	Mechanisms	Frequencies	Chromosome regions
Cell cycle regulation			
p16/CDNK2A	Homozygous deletion and hypermethylation	62%–86%	9p21
p14/ARF	Homozygous deletion and hypermethylation	54%	9p21
ΔN-p63	Overexpression	100%	3q27-28
Apoptosis			
Bcl 2	Overexpression	80%	18q21.3
DAP-kinase	Hypermethylation	76%	9q34.1
Signal transduction			
Bcl 3	Overexpression	60%	19q13.1-13.2
EGFR	Overexpression	85%	7p12
Cell adhesion			
E-cadherin	Hypermethylation	52%	16q22.1
TSLC 1	Hypermethylation	34.2%	11q23.2
Other novel pathways			
RASSF1A	Hypermethylation and mutation	67%–83%	3p21.3
RARB2	Hypermethylation	80%	3p24

(Raab-Traub, 2002). LMP1 and BARF1 have profound effects on cellular gene expression and may contribute to EBV-mediated tumorigenesis. *LMP1* is considered to be a viral oncogene, since it shows transforming activity in various cell types in vitro. Expression of LMP1 in immortalized nasopharyngeal epithelial cells induces an array of genes involved in growth stimulation, enhanced survival, and increased invasive potentials (Lo et al., 2004). BARF1 is able to immortalize primate epithelial cells and enhance growth rate of the transfected cells (Wei et al., 1997). Nevertheless, in vitro EBV infection of nasopharyngeal epithelial cells did not result in apparent growth stimulation or changes in cell cycle and apoptosis (our unpublished data). In contrast, enhancement in invasiveness and migration of the infected cells was observed. It appears that EBV infection may promote tumorigenicity of NPC cells by enhancing its invading activity. Moreover, EBV-infected normal nasopharyngeal epithelial cells are not able to form tumors in nude mice. Thus, in addition to EBV, other somatic genetic changes or epigenetic events appear to be necessary for the malignant transformation of nasopharyngeal epithelial cells.

Multistep carcinogenesis

Preinvasive lesions in the nasopharynx are rarely encountered in clinical settings; however, such preinvasive dysplastic lesions are considered to precede the development of invasive NPC. High frequencies of chromosome 3p and 9p deletions are found in both low-grade and high-grade dysplastic lesions (Chan et al., 2000, 2002a). However, EBV infection is consistently observed in NPC and high-grade but not low-grade dysplastic lesions. These findings suggest that the involvement of those specific genetic changes precedes EBV infection during the initiation of NPC development. Furthermore, chromosome 3p and 9p deletions were also commonly observed in histologically normal nasopharynx epithelia from Southern Chinese, the high-risk population. The presence of multiple lesions with clonal allelic deletions in nasopharyngeal mucosa provides the evidence for field cancerization during the initiation of this cancer. Disruption of the NPC-associated tumor suppressor genes on these critical regions, such as *p16* and *p14* on 9p21 and *RASSF1A* on 3p21.3, may lead to immortalization and clonal proliferation of multiple genetic lesions in the nasopharyngeal mucosa during the initiation stage. On the other hand, early genetic changes may predispose the epithelial cells to EBV infection or persistent maintenance of latent cycle. Expression of latent genes in the EBV-infected cells may enhance its transformation capacities, and subsequently, clonal expansion may result in the rapid progression to invasive carcinoma (Figure 2).

Recent advances in cancer detection

NPC shows a remarkably high cure rate for early-stage disease, and early detection is critical to improve the overall prognosis of these patients. However, the clinical presenting features of NPC are often nonspecific, and examination of nasopharynx requires expertise and renders early detection difficult.

Since EBV shows a strong link to NPC, and a wide spectrum of EBV antigens are consistently elevated in the sera of NPC patients, EBV-specific antibody-based assays have been used for at least two decades as diagnostic markers in high-risk regions. EBV-related antibodies in NPC, such as IgA antibodies against viral capsid antigen (VCA), early antigen (EA) and nuclear antigen (EBNA), and DNase assay have been found to be useful markers in clinical practice and correlate well with disease status. A mass screening study in Southern China showed that the interval between raised IgA anti-VCA detection and clinical onset of Stage I cancer ranges between 8 and 30 months (Zeng, 1985). A recent study of 9,699 Taiwanese with 16 years of followup has also revealed that seropositivity for IgA anti-EBV VCA and neutralizing antibodies against EBV DNase are predictive of NPC (Chien et al., 2001). The raised titers identify high-risk individuals for further diagnostic evaluation and may improve the chance of early cancer detection.

The serological tests have limited roles in monitoring disease progress and detecting recurrent diseases. A novel sensitive molecular test for NPC, based on detecting circulating EBV DNA in blood plasma, has recently been developed (Lo et al., 1999). Using real-time quantitative PCR, cell-free EBV DNA was found in the plasma of 96% of NPC. Pretreatment EBV DNA levels in plasma or serum are correlated with tumor stage and overall survival. Moreover, posttreatment plasma EBV DNA levels can accurately reflect the posttreatment residual tumor load and predict recurrence and prognosis (Lo et al., 2000b; Chan et al., 2002b). Plasma EBV DNA becomes one of the most promising diagnostic and prognostic molecular markers for routine clinical use in NPC patients.

Tumor-specific methylation has also been detected in the serum of NPC patients, but with less sensitivity (Wong et al., 2004). The detection of multiple gene methylation and EBV copy number from brushing samples collected directly from the nasopharynx was shown to be a noninvasive and sensitive diagnostic test for NPC (Tong et al., 2002). Such an approach allows us to directly investigate the disease progression in the nasopharynx. By combining with plasma EBV DNA, the sensitivity may further improve to near 100%.

Molecular targets and gene therapy

EBV-targeting therapy

The presence of EBV in tumor cells provides a potential therapeutic target specific for NPC. EBV-targeting therapeutic strategies have been developed for the control of advanced stage NPC. By either pharmacologic agents or adenovirus expressing EBV-early immediate lytic proteins, induction of lytic cycle protein expression in EBV-positive NPC cell lines has been demonstrated (Feng et al., 2002b, 2002c). The results support further clinical trials by the induction of lytic infection and antiviral agents.

Cellular immunotherapy with EBV-specific cytotoxic T lymphocytes (CTLs) represents another approach for EBV-targeting therapy. Despite limited EBV protein expression in NPC cells, LMP-2 and EBNA-1 from NPC cells were demonstrated to be the targets for CD8+ and CD4+ T cells, respectively. Intratumoral functional CTLs and LMP2-specific CTL responses can be detected in the untreated NPC patients. The intact antigen-processing pathways in NPC cell lines also support the use of therapeutic vaccination (Lee et al., 2000). To enhance cellular responses to viral antigens, autologous dendritic cells were pulsed with HLA-restricted LMP2 epitope peptides and injected into inguinal lymph nodes of patients with advanced NPC (Lin et al., 2002). The treatment elicited epitope-specific CD8+ T cell responses detectable in the peripheral blood of NPC patients. Partial clinical response was seen in some selected patients. The results encourage further development of more effective vaccine strategies.

Gene therapy

Several gene-based strategies targeting common genetic abnormalities (e.g., p53 and p16) of NPC have been explored

(Liu, 2002). Recent exciting development of the "transcriptional targeting strategy" and utilization of potent membrane-based apoptotic genes (e.g., FasL) as target have shown promising results in both in vitro and in vivo studies. To express the target gene in NPC cells, a recombinant adenovirus containing EBV oriP promoter at the upstream of the target gene has been generated. Since oriP sequence is transactivated by EBV latent protein EBNA1, the vector expresses the target gene only in the EBV-positive NPC cells (Li et al., 2002; Liu, 2002). The preliminary findings suggest the high feasibility of using gene therapy as an alternative treatment for advanced-stage patients.

Future prospects

The high susceptibility of Southern Chinese to develop this disease is still a puzzle. With recent advances in single nucleotide polymorphism and haplotype analyses, genome-wide screening and association studies may help to decipher the inheritable genetic components for this enigmatic cancer. The cellular genes involved in DNA damage and its association with EBV entry or latency should be focused upon and further explored. Despite the crucial role of EBV in NPC, information about functions of the EBV genes in nasopharyngeal epithelial cells is limited. Establishment of immortalized normal nasopharyngeal epithelial cell lines allows us to characterize the EBV gene functions in this specific host environment. It should enhance our understanding of how the virus adapts itself to the epithelial cell environment and exerts its effects on malignant transformation. The cellular genes regulated by EBV are of particular interest, as they may serve as specific tumor markers and targets for novel therapy strategies.

Previous molecular studies have focused on the NPC genome, but few on transcriptomic and proteomic profiles. Small biopsy material and heavy infiltration of noncancer cells present major difficulties for these studies. With advances in microdissection and preamplification technology, comprehensive expression profiling of this lymphoepthelioma is possible.

Early detection is of paramount importance in improving the prognosis of NPC patients. Selective application of EBV DNA and epigenetic markers in a serological screening protocol may prove to be both cost-effective and accurate. The application of different screening strategies could be tested in high-risk populations such as Southern Chinese. For NPC treatment, major challenges remain in improving the survival rate of patients with advanced and recurrent diseases. In addition to the novel treatments mentioned earlier, clinical application of anti-EGFR antibodies has also been under investigation. The high frequencies of epigenetic alterations in this cancer suggest the potential application of novel inhibitors targeting DNA methylation and histone acetylation. The emerging novel approaches may brighten up the outcome of NPC patients with poor prognosis.

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