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Nasopharyngeal carcinoma: molecular pathogenesis and therapeutic developments

Qian Tao and Anthony T.C. Chan

Nasopharyngeal carcinoma (NPC) is a prevalent tumour in southern China and southeast Asia, particularly in the Cantonese population, where its incidence has remained high for decades. Recent studies have demonstrated that the aetiology of NPC is complex, involving multiple factors including genetic susceptibility, infection with the Epstein–Barr virus (EBV) and exposure to chemical carcinogens. During development of the disease, viral infection and multiple somatic genetic and epigenetic changes synergistically disrupt normal cell function, thus contributing to NPC pathogenesis. NPC is highly radiosensitive and chemosensitive, but treatment of patients with locoregionally advanced disease remains problematic. New biomarkers for NPC, including EBV DNA copy number or methylation of multiple tumour suppressor genes, which can be detected in serum and nasopharyngeal brushings, have been developed for the molecular diagnosis of this tumour. Meanwhile, new therapeutic strategies such as intensity-modulated radiation therapy and immuno- and epigenetic therapies might lead to more specific and effective treatments.

Nasopharyngeal carcinoma (NPC) is a malignancy with an unusual geographical and ethnic distribution across the world (Ref. 1). It was documented more than 100 years ago in the endemic areas in southern China (including GuangDong province and Hong Kong), with a peak incidence of 30 cases per 100 000 per year (~20% of all cancers), and it remains a serious healthcare problem in these regions. In Hong Kong, NPC is the fourth leading cancer in men, but the leading one in men aged 20–44 years (Hong Kong Cancer Registry 2002, The Hong Kong Hospital Authority; http://www3.ha.org.hk/cancereg/). Earlier work showed that Cantonese 'boat people' in southern China had the highest incidence of NPC (54.7/100 000/

Cancer Epigenetics Laboratory, State Key Laboratory in Oncology in South China, Sir YK Pao Centre for Cancer, Department of Clinical Oncology, Hong Kong Cancer Institute and Li Ka Shing Institute of Health Sciences, Chinese University of Hong Kong.

Corresponding authors: Qian Tao, Rm 315, Cancer Center, PWH, Chinese University of Hong Kong, Shatin, Hong Kong. Tel: +852 2632 1340; Fax: +852 2648 8842; E-mail: qtao@clo.cuhk.edu.hk; Anthony T.C. Chan, Department of Clinical Oncology, Chinese University of Hong Kong, Shatin, Hong Kong. Tel: +852 2632 2119; Fax: +852 2649 7426; E-mail: anthonytcchan@cuhk.edu.hk.

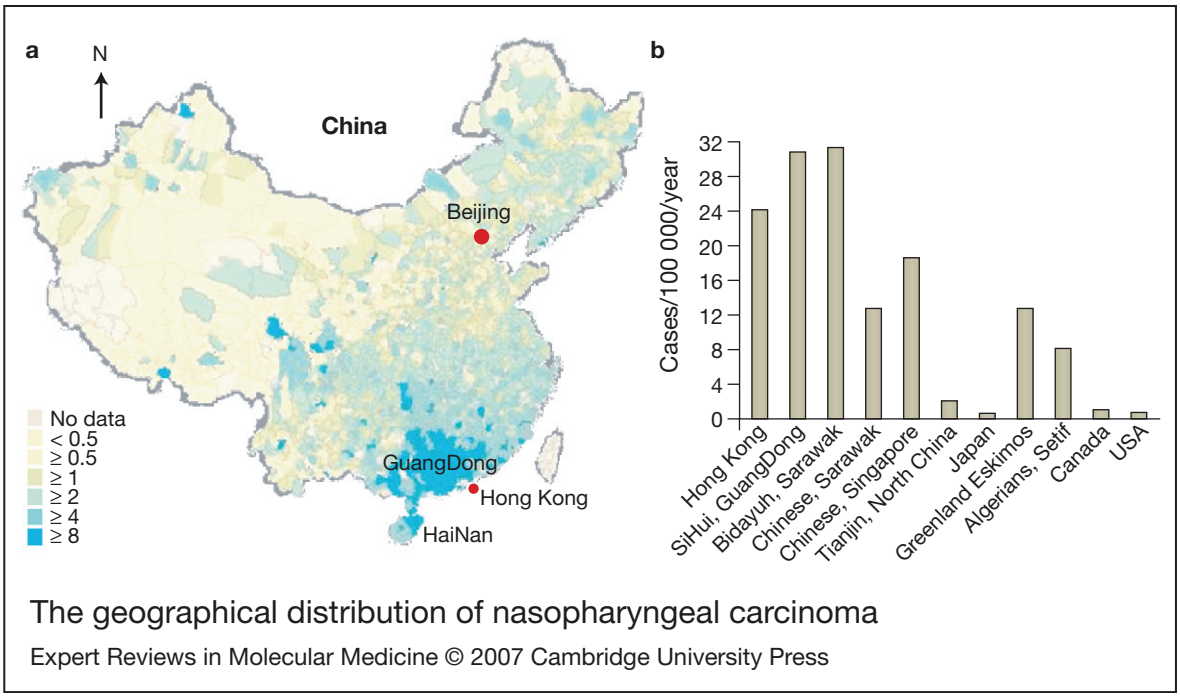
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year), although it is impossible to identify such a pure ethnic community of Cantonese boat people nowadays. Other provinces in mainland China next to GuangDong, such as GuangXi, HaiNan, HuNan and FuJian, also have a relatively high incidence of NPC. Within China, the incidence decreases from south to north with only ~2 cases per 100 000 per year among men in northern provinces (Ref. 2) (Fig. 1a).

By contrast, the incidence of NPC in neighbouring oriental countries such as Korea and Japan is very low (Fig. 1b), although in other areas of southeast Asia, including the Philippines, Malaysia and Singapore, its incidence is also relatively high (~18/100 000/year in the Chinese population in Singapore). A previous study comparing the incidence among different ethnic groups living in Singapore (Chinese, Malays) showed that the Cantonese-speaking group had a much higher NPC incidence than that of the Teochew- or Hokkien-dialect group (Ref. 3). A recent interesting study showed that a distinct ethnic group – the native Bidayuh in Sarawak, East Malaysia – has a high incidence of NPC (23.1/100 000/year), comparable with the endemic southern China areas (Ref. 4). However, NPC is a rare disease in most countries of the world, except for an intermediate incidence in Alaskan Eskimos and northern Africans in the Mediterranean basin (~10/100 000/year) (Ref. 2) (Fig. 1b). Its incidence in the USA and Canada is <1/100 000/year, representing only 0.2% of all tumours.

The International Agency for Research on Cancer (IARC) reported no demonstrable change in NPC incidence among Chinese in China or southeast Asia for 50 years before the early 1980s (Ref. 2). Recently, a widely quoted report showed that the age-standardised incidence rates of NPC have decreased during the past 25 years in Hong Kong (Ref. 5).



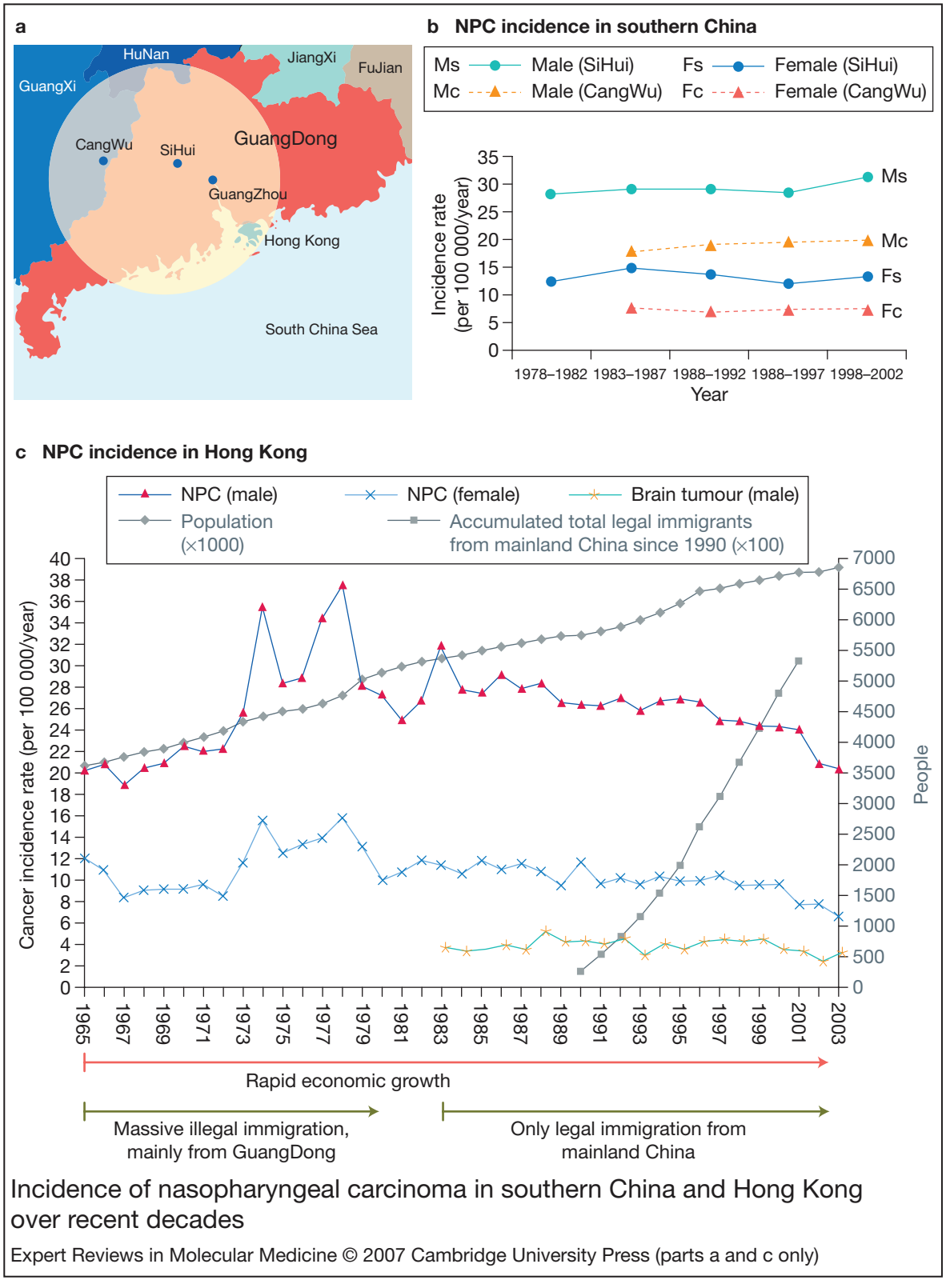
The geographical distribution of nasopharyngeal carcinoma

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Figure 1. The geographical distribution of nasopharyngeal carcinoma. (a) Nasopharyngeal carcinoma (NPC) mortality rate (male, world standardised rate) in China in the 1970s (China Cancer Database, Ministry of Science and Technology of China; http://cancernet.cicams.ac.cn). Within China, the mortality rate of NPC shows great variations from north to south. The dark-blue colour indicates regions with significantly higher mortality rates than the national average, which are mainly located in southern China. (b) The annual incidence of NPC worldwide in males. In addition to the association of high incidence of NPC with Cantonese populations, high incidence was also reported recently in the native Bidayuh in Sarawak, East Malaysia. Alaskan Eskimos and northern Africans in the Mediterranean basin show an intermediate prevalence of the disease.

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Incidence of nasopharyngeal carcinoma in southern China and Hong Kong over recent decades

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Figure 2. Incidence of nasopharyngeal carcinoma in southern China and Hong Kong over recent decades. *(See next page for legend.)*

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Similarly, in Singapore, little change in the NPC incidence rate from 1973 to 1992 was followed by a slight decrease of NPC incidence rates in both sexes in Singapore Chinese from 1993 to 1997 (Ref. 2). These drops in NPC incidence in recent years might be partially real, attributed to a gradual change in lifestyle, declined use of salted fish to feed young children and decreased exposure to traditional preserved food after World War II as these areas underwent rapid economic development. However, these results also need to be viewed cautiously in the context of population shifting as a result of constant immigration, as discussed for Hong Kong in detail in Figure 2c. In poorly developed, more closed Cantonese areas (e.g. SiHui in GuangDong and CangWu in GuangXi) that have much more stable populations and also a high NPC incidence, the NPC incidence rates have not changed for 35 years (Ref. 6) (Fig. 2b). Thus, in this context, further careful epidemiological studies of the NPC incidence in Hong Kong and Singapore, which consider the possibility of population shifting, are needed.

Histology of NPC

NPC is a squamous cell carcinoma that develops from the epithelium of the nasopharynx (Fig. 3); it normally originates from the fossa of Rosenmuller in the nasopharynx, often in the recess. Histologically, it has a high background of reactive lymphocytes, and used to be called lymphoepithelioma. NPC has been shown to be multifocal in origin in its early pathogenesis in some cases (Ref. 7). As inflammation and infection are very common in the upper respiratory tract, mild hyperplasia is common in normal nasopharyngeal epithelium, and is a reversible mild lesion. Early lesions of NPC including severe dysplasia or carcinoma in situ (CIS) have been described, but are difficult to differentiate from each other and extremely rare (reported in 11 out of 5326 biopsies in one report) (Refs 7, 8, 9, 10). At the molecular level, these lesions are infected with clonal Epstein–Barr virus (EBV) (see below) and express the same EBV viral proteins as NPC, and are therefore already clonal tumours (Ref. 10).

The World Health Organization (WHO) classification system (1978) acknowledges three

Figure 2. Incidence of nasopharyngeal carcinoma in southern China and Hong Kong over recent decades. (*Legend; see previous page for figure.*) (a) More-detailed map of the highest nasopharyngeal carcinoma (NPC) incidence regions (within the shaded circle) in China: these are the Cantonese areas centered around SiHui and GuangZhou, including Hong Kong. (b) The annual incidence of NPC in SiHui and CangWu (1978–2002), which are the poorly developed, more closed Cantonese areas in southern China, with stable populations. The incidence of NPC (both male and female) has not changed for ~30 years (up to 2002) in SiHui, which has the highest NPC incidence (30/100 000), and in CangWu (on the border between GuangXi and GuangDong), which has a high incidence of 20/100 000. Graph adapted from Ref. 6, with permission from Drs W-H. Jia and Y-X. Zeng (State Key Laboratory of Oncology in Southern China, Cancer Center, Sun Yat-sen University, GuangZhou, China). (c) NPC incidence in Hong Kong (1965–2003). Interpretation of NPC incidence in Hong Kong (data from the Hong Kong Cancer Registry, The Hong Kong Hospital Authority; http://www3.ha.org.hk/cancereg/) is complicated by the waves of immigration from mainland China, which have resulted in the population in Hong Kong increasing by 49% from 3.6 million in 1965 to 6.97 million in 2006. From the mid-1960s, as a result of the Cultural Revolution, poor living conditions in China and the rapid growth of the Hong Kong economy, a huge number of illegal immigrants from mainland China flooded to Hong Kong (up to 450 per day). This immigration was mainly from the GuangDong areas near Hong Kong and thus predominantly of Cantonese origin, with a high risk of NPC. The population increased by 1 million from 1972 to 1980, which was the fastest growth period in Hong Kong history after 1945–1946, and this might explain the abnormal peak of NPC incidence in males and females during 1973–1980. By contrast, a stricter immigration policy from 1980, with only legal immigrants allowed to stay and limited issuing of visas (75/day 1983–1993, 105/day in 1993–1995, and 150/day from 1995) led to immigration from all areas of mainland China. From 1990 to 2001, a total of 533 552 such immigrants came to Hong Kong, of which ~60% were females. It is most likely that this second peak of continuing immigration after 1990s, which was not Cantonese-dominant, diluted the high NPC incidence rate in Hong Kong and contributed to the reported decline of incidence rates in recent years (Ref. 5). The incidence of another tumour (brain), which is not specifically associated with Cantonese, as a comparison, has been quite stable in Hong Kong for years.

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types of NPC based on the differentiation status of tumour cells: type I, differentiated, keratinising squamous cell carcinoma (25% of NPCs in North America; ~2% of NPCs in southern China) (Refs 11, 12); type II, nonkeratinising carcinoma (12% of NPCs in North America; ~3% of NPCs in southern China); and type III, undifferentiated carcinoma (63% of NPCs in

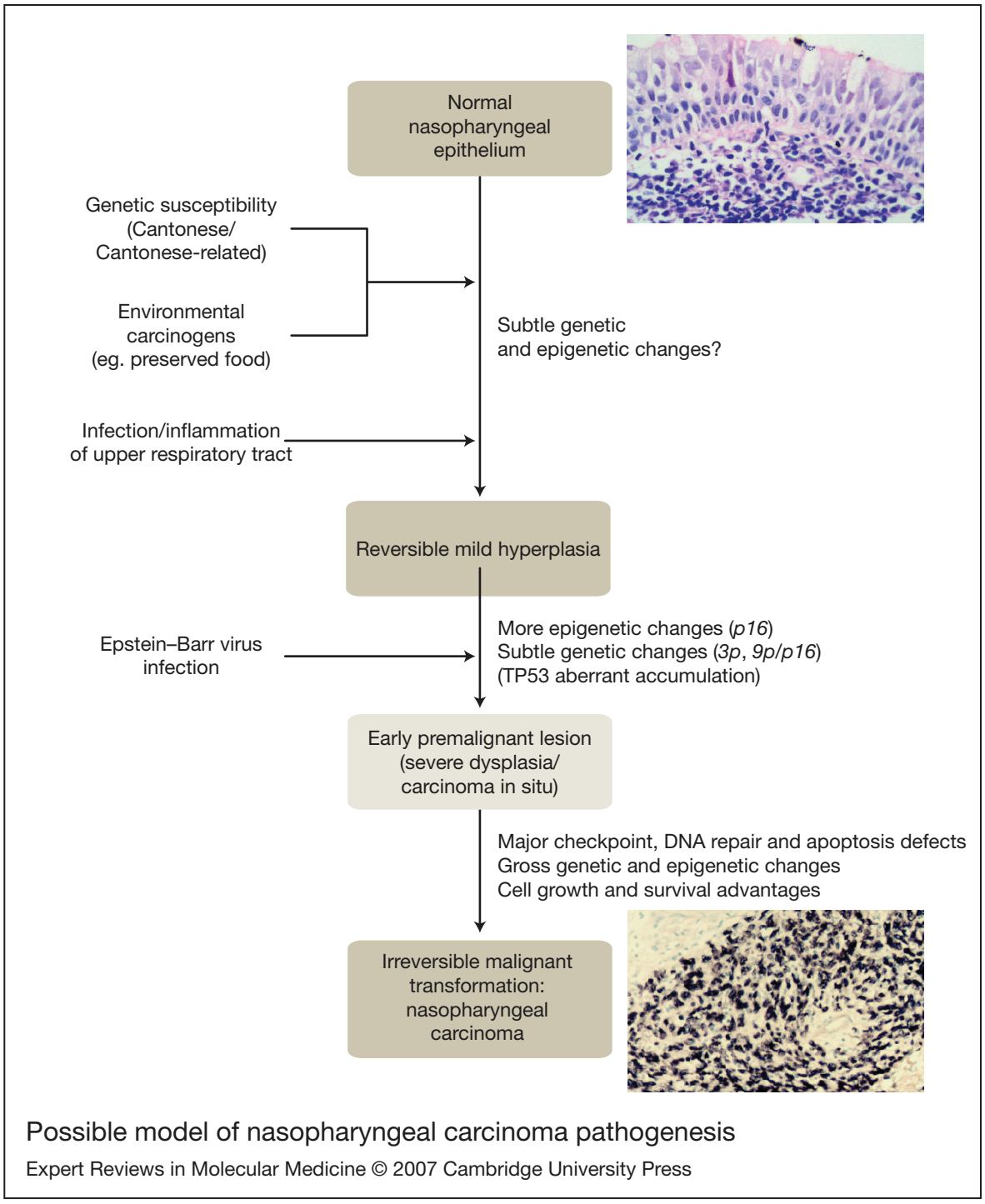


Figure 3. Possible model of nasopharyngeal carcinoma pathogenesis. *(See next page for legend.)*

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North America; 95% of NPCs in southern China). WHO types II and III can be considered together as undifferentiated carcinomas of the nasopharyngeal type (UCNT), which have similar treatment response and prognosis.

Aetiology of NPC

Although the molecular basis of NPC pathogenesis is still poorly understood, it has been suggested that the pathogenesis and development of NPC is a multistep process (Refs 13, 14) (Fig. 3), similar to that of other carcinomas as elegantly proposed by Vogelstein and Kinzler (Ref. 15). Previous epidemiological studies suggest three major aetiological factors for NPC: genetic susceptibility, early-age exposure to chemical carcinogens (particularly Cantonese salted fish), and latent EBV infection (Refs 2, 16, 17).

Genetic susceptibility

The predisposition to NPC among southern Chinese (Cantonese) strongly suggests the involvement of both genetic susceptibility and environmental factors. The high NPC incidence is retained by second-generation Cantonese who migrate to other, nonendemic countries. The NPC clustering in families in endemic areas further suggests a strong ethnic and genetic influence (Refs 18, 19). In fact, ~10% of NPC cases have a family history of the disease.

Earlier linkage study on Chinese sib pairs with NPC identified an NPC susceptibility locus at the *HLA* (human leukocyte antigen) region (Ref. 20). Study of HLA susceptibility among Chinese has shown that people with HLA A*0207 or B*4601, but not A*0201, have an increased NPC risk (Ref. 21). Recent genome-wide linkage analyses of high-risk Chinese familial NPC pedigrees identified two candidate NPC susceptibility loci – 4p15.1-q12 (Ref. 22) and 3p21.3 (Ref. 23) – with another suspected locus reported at 5p13-15 [L.F. Hu (MTC, Karolinska Institute, Sweden), pers. commun.]. However, since these chromosomal regions are very large with many candidate genes, the relevant NPC gene(s) are still far from identified.

Environmental factors

For many years, NPC has been reported to be associated with environmental factors other than EBV. The most relevant environmental exposure among Cantonese is salted fish and other preserved foods containing volatile nitrosamines, which are mutagenic chemicals (Ref. 24). Consumption of salted fish during childhood is related to increased NPC risk in southern Chinese (Refs 2, 25). This has been experimentally supported by animal-model studies showing that rats fed on a diet of salted fish developed carcinomas in the nasal cavity in a dosage-dependent manner (Refs 26, 27). A recent review of extensive epidemiological literature outlined the association between adulthood intake of preserved and nonpreserved vegetables and NPC risk in multiple countries (Ref. 28). Preserved vegetable intake is associated with a twofold increase in NPC risk, while high nonpreserved vegetable intake is associated with a 36% decrease, consistent between vegetable types and countries.

Occupational exposure to toxic pollutants (formaldehyde) in the air or wood dust is also linked to increased NPC incidence (Refs 29, 30). A very recent report on female textile workers in Shanghai, China also revealed increased NPC risk with accumulative exposure to cotton dust, acids, caustics or dyeing processes (Ref. 31). Tobacco smoking is also mildly associated with increased NPC risk (Ref. 2). These studies indicate that a prolonged exposure to pollutants

Figure 3. Possible model of nasopharyngeal carcinoma pathogenesis. (*Legend; see previous page for figure.*) Although the whole pathogenesis of nasopharyngeal carcinoma (NPC) might take as long as ~40 years, the transition from early premalignant lesion (severe dysplasia/carcinoma in situ) to an NPC appears to be quick (shown as lighter shading to indicate this short window), probably due to the tumour-promoting potentials of EBV infection. The image of normal nasopharyngeal mucosa is a haematoxylin- and eosin-stained section (method in Ref. 33; magnification 400x) kindly provided by Professor Zifen Gao (Department of Pathology, Peking University Health Science Center, China). The image of NPC tumour tissue is a digoxigenin-labelled in situ hybridisation of Epstein–Barr virus EBER-RNA (method in Ref. 34; magnification 200x), showing an island of EBER-positive NPC tumour cells, kindly provided by Professor Gopesh Srivastava (Department of Pathology, University of Hong Kong).

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contributes to NPC pathogenesis. Other environmental factors associated with NPC risk have also been reported, such as Chinese herb usage, presence of heavy metal (nickel) in the endemic area, alcohol and even fungus infection in the nasal cavity (Ref. 2).

Epstein–Barr virus (EBV) infection

Unlike all other head and neck squamous cell carcinomas, NPC is strongly associated with EBV, a human B-lymphotropic herpesvirus that is associated with a variety of tumours (Refs 16, 17). The link between NPC and EBV was discovered in 1966 from serological studies, and later supported by the demonstration of EBV DNA and EBV nuclear antigen (EBNA) proteins in NPC tumour cells (Ref. 32). Clonal viral genome was detected in NPC tumour DNA by Southern blot hybridisation, suggesting that EBV infection occurred before clonal expansion of tumour cells (Ref. 16). It is proposed that EBV plays an important role (probably indispensable in endemic areas) in NPC pathogenesis, since, as mentioned above, the earliest NPC lesion detected (severe dysplasia/CIS) is already EBV-positive, with latent and clonal viral genomes and expressing viral oncoproteins such as latent membrane protein 1 (LMP1) (Ref. 10), while EBV infection is not present in normal nasopharyngeal epithelium nor in low-grade dysplasia (Refs 33, 34).

The WHO type III NPC is always associated with EBV, regardless of geographical distribution, ethnic origin and local prevalence of the disease (Refs 16, 17). The other two types of NPC are less frequently associated in nonendemic areas, with EBV positivity in less than 50% of cases (Refs 16, 35); however, in endemic NPC areas (Malaysia and Hong Kong), all the type I NPC tumours were EBV-associated (Refs 16, 35, 36, 37). In NPC tissues, EBV exists in every tumour cell in a latent form, but rarely in the surrounding normal cells (Refs 33, 35, 38).

The pattern of EBV latent gene expression in normal and tumour cells can be classified into different forms of latency. The pattern in EBV-transformed lymphoblastoid cell lines (LCLs) is often referred to as latency III, with the expression of six different EBNAs (EBNA1, EBNA2, 3A, 3B, 3C and -LP) from the EBV Cp (and/or Wp) promoter, three LMPs (LMP1, 2A and 2B), the *Bam*HI A rightward transcripts (BARTs), and two abundant small nonpolyadenylated (noncoding) RNAs (EBER 1 and 2) (Refs 14, 17). However, the EBV gene expression pattern in most tumours is either latency I or latency II, except for post-transplant lymphomas and some AIDS-associated lymphomas, which show a latency III pattern. Latency I is characterised by restricted viral gene expression involving only EBNA1, EBERs, LMP2A and BARTs, as observed in Burkitt lymphoma, gastric carcinoma and possibly normal peripheral blood mononuclear cells (PBMCs). Latency II is present in most EBV-positive tumours, including NPC, Hodgkin lymphoma, nasal natural killer (NK)/T-cell lymphoma and some T-cell lymphomas, with the expression of EBNA1 (from the Qp promoter), EBERs, BARTs, BARF1 (except for Hodgkin lymphoma), LMP1 and LMP2 (Refs 14, 17). Recently, multiple EBV-encoded microRNAs (up to 17) have also been detected in EBV-infected B cells and carcinomas including NPC and gastric carcinoma, although the pathobiological functions of these small regulatory RNA molecules are still not very clear (Refs 165, 166).

In NPC (latency II), EBNA1 and EBERs are expressed in all EBV-positive NPC cases, while expression of the oncoprotein LMP1 is highly variable (Ref. 17); this is different from Hodgkin lymphoma and nasal NK/T-cell lymphoma, which almost always express LMP1 (Refs 39, 40, 41). Expression of EBV viral oncoproteins would confer tumour cell growth and survival advantages, maintaining the malignant phenotype; however, the rate of LMP1 detection in NPC tumour tissues varies substantially (~20–60% of cases) (Refs 17, 36). The only EBV-positive NPC cell line, C666-1, barely expresses LMP1 and the promoter is methylated (Refs 17, 42). Expression of LMP2A mRNA has been reported by reverse-transcriptase PCR and more recently by sensitive immunostaining (Ref. 43). The expression of BARTs and BARF1 [a transforming EBV oncogene (Ref. 44)] was detected in virtually all NPC tumours, suggesting a pathogenic role for these RNAs/proteins in NPC (Refs 45, 46). The expression of the viral early lytic protein BZLF1 has been reported in some cases (Ref. 47), although there is virtually no virus replication in NPC tumour cells, indicating that viral lytic activation, if it exists, is abortive in tumour cells.

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Table 1. Major genetic alterations detected by microsatellite marker analysis, CGH and array-CGH in NPC cell lines and primary tumours[a]

Chromosome	Deletions	Amplifications
1	1p36, 1p34	1q24.3-32.1, 1q42-44
2	2p12	2q24-31
3	3p25.3-26.3, 3p22-21.3, 3p14-12	3q26, 3q27.3-28
4	4q28	4q12-21
5	5q11-14, 5q31-33	5q21
6		6q14-22
7	7q31.3-36	7p15-14, 7q11.2-21
8		8q21.1-22, 8q23-24
9	9p21-23, 9q22.33-q31.2	
10	10p	
11	11q13-14, 11q14-23, 11q25	11p15, 11q13.1-13.3
12		12p12-13, 12q13-15, 12q22-24.1
13	13q12-14.3, 13q21-32	
14	14q11-13, 14q24-31	
16	16q21, 16q22.3-23.1, 16q24.1	
17	17p13.3, 17q11	17q21, 17q25
18	18p11.21, 18q23	18q12-22

[a]Data from Refs 13, 17, 23, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 59, 60, 61.

Abbreviations: CGH, comparative genomic hybridisation; NPC, nasopharyngeal carcinoma.

Somatic genetic and epigenetic alterations

Cytogenetic changes

Multiple genetic changes are present in NPC. Earlier cytogenetic analyses of NPC cell lines and xenografts showed hypo- or hyperdiploidy, with further chromosomal abnormalities such as deletion of 3p also observed (Refs 48, 49). Later, spectral karyotyping was used to characterise the genome-wide genetic alterations in NPC cell lines (Ref. 50). Microsatellite analyses also revealed various regions with frequent allelic imbalance in primary tumours (Refs 51, 52). Comparative genomic hybridisation (CGH) can detect genetic lesions more accurately than conventional cytogenetics, and was also used in NPC (Ref. 50). Through these studies, numerous genetic abnormalities have been detected on multiple chromosomal regions in NPC tumours and cell lines. Several minimal deleted regions were mapped to 3p14.1-22, 11q13.3-24, 13q14.3-22, 14q24.3-32.1 and 16q22-23.

As the best resolution of conventional CGH is only ~10 Mb (Ref. 53), higher-resolution array-based CGH has been recently used to detect genome-wide genetic abnormalities in NPC. A commercial CGH microarray containing 58 known oncogenes commonly amplified in cancers was applied to 15 NPC samples (Ref. 54). Amplification of a series of oncogenes, including *MYCL1*, *TERC*, *PIK3CA*, *NRAS* and *MYB*, was detected. A high-density cDNA microarray containing 21 632 cDNAs was also used to examine gene copy number changes and expression levels in five Chinese NPC cell lines, with the detection of amplification and overexpression of the oncogene *EVI1*, as well as deletion and downregulation of the transcription regulator *RYBP* (Ref. 55). A recent study using both conventional CGH and array-based CGH detected chromosomal alterations in a series of Mediterranean NPC xenografts and biopsies, including frequent gains associated with overexpression at 1q25-qter and 12p13, and losses at 11q14-q23, 13q12-q31, 14q24-q31 and 3p13-p23. Compared with Asian NPC, Mediterranean NPC has higher frequencies of 1q gain and 13q loss (Ref. 56). Another study, using 3 Mb array-based CGH (University of

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Table 2. Candidate oncogenes involved in nasopharyngeal carcinoma pathogenesis

Candidate oncogene (full name)	Location[a]	Functions	Alterations in NPC	Refs
A20/TNFAIP3 (Tumour necrosis factor, alpha-induced protein 3)	6q23	Zinc finger protein: inhibits NF-κB activation and TNF-mediated apoptosis; negative regulator of TLR-mediated inflammatory response	Overexpressed, induced by LMP1	122, 123
BCL2 (B-cell CLL/lymphoma 2)	18q21.33	Integral outer mitochondrial membrane protein: anti-apoptotic – interacts with activated Bax and neutralises its pro-apoptotic activity	Overexpressed	124, 125, 126
CCND1 (Cyclin D1)	11q13	Regulator of cyclin-dependent kinases: regulates cell cycle G1–S transition; interacts with pRb and affects its function	Amplified, overexpressed	57, 127, 128
DeltaNp63/TP73L (Tumour protein p73-like, p63 splicing variants lacking NH(2)-terminal transactivating domain)	3q28	Dominant-negative to p53 and p63: regulator of cell cycle, apoptosis and notch signalling, transcription factor	Mutated, overexpressed	129, 130, 131
EGFR (Epidermal growth factor receptor)	7p12.1	Transmembrane receptor protein with tyrosine kinase activity: involved in signal transduction, cell communication, regulation of cell cycle, proliferation, adhesion, migration, stress response, regulation of nitric oxide synthase activity	Amplified, overexpressed, induced by LMP1	54, 132, 133, 134
EVI1 (Ecotropic viral integration site 1)	3q26.2	DNA-binding nuclear protein, DNA-dependent transcription factor: involved in chromatin remodelling; induces myelodysplastic syndrome (MDS) in mice	Amplified, overexpressed	55
HER2/ERBB2 (v-*erb*-b2 erythroblastic leukaemia viral oncogene homologue 2)	17q12	Member of the EGF receptor family of receptor tyrosine kinases: positive regulator of MAPK activity and epithelial cell proliferation; regulator of angiogenesis and phosphoinositide-mediated signalling	Overexpressed, no amplification	133, 135

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Table 2. Candidate oncogenes involved in nasopharyngeal carcinoma pathogenesis *(continued)*

Candidate oncogene (full name)	Location[a]	Functions	Alterations in NPC	Refs
HRAS (v-Ha-*ras* Harvey rat sarcoma viral oncogene homologue) *NRAS* [Neuroblastoma RAS viral (v-*ras*) oncogene homologue]	11p15.5 1p13.2	GTP-binding and GTPase activity: involved in cell-surface-receptor-linked signal transduction, small-GTPase-mediated signal transduction, regulation of cell cycle progression, cell motility	Overexpressed, mutations uncommon Amplified	54, 136, 137
ID1 (Inhibitor of DNA binding 1; dominant negative helix–loop–helix protein)	20q11	Helix–loop–helix transcription factor: represses transcription, regulates cell growth, senescence, and differentiation	Overexpressed	138
INT2/*FGF3* [Fibroblast growth factor 3; murine mammary tumour virus integration site (v-*int*-2) oncogene homologue]	11q13	Member of the FGF family: involved in embryonic development, cell growth, morphogenesis, tissue repair, tumour growth and invasion	Amplified	139
MDM2 [Mdm2, transformed 3T3 cell double minute 2; p53-binding protein (mouse)]	12q15	Binds and inhibits p53, has E3 ubiquitin ligase activity (which targets p53 for proteasomal degradation), interacts with pRb and ribosomal protein L5, affects cell cycle and apoptosis	Overexpressed, infrequently amplified, alternatively spliced?	140, 141
MET (*Met* proto-oncogene; hepatocyte growth factor receptor)	7q31	Hepatocyte growth factor receptor with protein-tyrosine kinase activity: involved in cell proliferation and signal transduction	Overexpressed	142, 143
MYC [v-*myc* myelocytomatosis viral oncogene homologue (avian)]	8q24.21	Nuclear phosphoprotein, transcription factor: positive regulation of cell proliferation, cell cycle progression, apoptosis and cellular transformation; iron ion homeostasis	Overexpressed, amplified	125, 137, 139
PIK3CA (Phosphoinositide 3-kinase, catalytic, alpha polypeptide)	3q26.32	Phosphoinositide 3-kinase signal transduction	Amplified, overexpressed, infrequently mutated	54, 144, 145

[a]Chromosomal location.

Abbreviations: EGF, epidermal growth factor; FGF, fibroblast growth factor; LMP1, latent membrane protein 1; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; pRB, retinoblastoma protein; TLR, Toll-like receptor; TNF, tumour necrosis factor.

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California at San Francisco arrays) containing 1803 BAC (bacterial artificial chromosome) clones was performed on three NPC cell lines, two xenografts and 21 primary tumours (Ref. 57). Frequent gains on 1q, 3q, 8q, 11q, 12p and 12q, and losses on 3p, 9p, 11q, 14q and 16q, were found. Moreover, several minimal regions of gains including 3q27.3-28, 8q21-24 and 11q13.1-13.3 were identified, with cyclin D1 (*CCND1*) verified as an amplified and overexpressed oncogene at 11q13.3. Recently, we also examined the genome-wide chromosomal alterations of NPC cell lines using high-resolution (1 Mb) array-based CGH with Sanger Institute whole-genome arrays containing 3040 BAC clones (Ref. 58) (http://www.sanger.ac.uk/Projects/Microarrays/) (Refs 59, 60, 61). Multiple alterations were detected, notably deletions of 3p12-14, 8p22, 10p and 18q and amplification of 3q26. These loci provide us with good candidate regions for the further identification of candidate tumour suppressor genes and oncogenes associated with NPC.

Table 1 summarises the major genetic alterations commonly detected by microsatellite marker analysis, CGH and array-CGH in NPC cell lines and primary tumours.

Oncogenes

Specific amplifications in NPC identified by cytogenetic or array-based CGH studies have implicated several putative oncogenes in NPC. Oncogenes identified include *BCL2*, *CCND1*, *EGFR*, *EVI1*, *HER2/ERBB2*, *HRAS*, *NRAS*, *MDM2*, *MYC* and *PIK3CA*, which may show amplification, overexpression or gain-of-function mutations (summarised in Table 2). For some of these genes, such as *CCND1*, encoding cyclin D1 (Ref. 57), functional studies have demonstrated their oncogenic potential in NPC cells.

Tumour suppressor genes (TSGs)

Although alterations of the well-established TSGs (such as *TP53*, encoding p53, and *RB1*, encoding the retinoblastoma protein) are relatively rare in NPC tumours (but relatively more common in NPC cell lines) (Refs 62, 63), multiple genetic and epigenetic abnormalities of various other TSGs have been detected. The most commonly affected TSG regions are 9p21 and 3p, which have as high as 85–95% deletion rates in invasive tumours. TSGs affected include those at 9p21 (*p16*, *p15* and *p14ARF*) (Refs 52, 64, 65) and 3p21.3 (*RASSF1A*, *BLU/ZMYND10* and *CACNA2D2*) (Refs 66, 67), which were found to be defective as a result of either promoter methylation, or deletions and mutations or both (Table 3). Ectopic expression of *p16*, *RASSF1A* or *BLU/ZMYND10* in NPC cell lines lacking expression resulted in significant inhibition of cell growth, colony formation or tumour growth in immunodeficient animals (Refs 17, 68), providing strong evidence that these genes function as tumour suppressors for NPC.

Through more extensive epigenetic studies, many more putative TSGs were found to be silenced by epigenetic alterations in NPC than previously thought, indicating that the molecular pathogenesis of NPC is rather complex. The epigenetic abnormalities of *GADD45G* (9q22.2) (Ref. 69), *TSLC1* (11q23.3) (Ref. 70), *DLC1* (8p22) (Ref. 59), *DLEC1* (3p22.3) (Refs 17, 71), *WIF1* (12q14.3) (Ref. 72), *CHFR*, *PRDM2/RIZ1*, *RARRES1/TIG1*, *SCGB3A1/HIN1*, *THY1/CD90*, and even new genes such as *PCDH10* (4q28.3) (Ref. 61), have been increasingly reported (Table 3). These abnormalities would disrupt multiple normal cellular regulatory and signalling processes and contribute to the pathogenesis of NPC. Moreover, the methylation of these TSGs could serve as epigenetic biomarkers, as indicated below.

Clinical implications – molecular diagnosis and therapy

Molecular diagnosis

As early detection of NPC will greatly help the treatment and improve the survival of patients, various detection systems have been developed for the early molecular diagnosis of this tumour by taking advantage of the specific presence of EBV. Higher EBV antibody titres, particularly of the IgA class, occur in approximately 90% of NPC patients, but in less than 10% of normal people. These antibody levels rise with the tumour burden regardless of different geographical locations and ethnic groups, which can be seen several years prior to the development of NPC, and correlate with tumour remission and recurrence (Ref. 73). Furthermore, the high copy number of circulating, cell-free EBV DNA in the peripheral blood sera of NPC patients has been

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Table 3. Candidate tumour suppressor genes involved in nasopharyngeal carcinoma pathogenesis

Candidate TSG (full name)	Chromosomal location	Functions	Alterations in NPC	Refs
BLU/ZMYND10 (Zinc finger, MYND-type containing 10)	3p21.3	Stress response, transcription factor	Deleted (LOH), methylated	67, 146
BRD7 (Bromodomain containing 7)	16q12	Transcriptional regulation, cell cycle regulation	Downregulated	147
CASP8 (Caspase 8, apoptosis-related cysteine peptidase)	2q33.1	Apoptosis	Methylated	148
CDH1 [Cadherin 1, type 1, E-cadherin (epithelial)]	16q22.1	Classical cadherin: calcium dependent cell–cell adhesion, proliferation, invasion, metastasis	Methylated	148, 149, 150, 151
CDKN1B/p27 [Cyclin-dependent kinase inhibitor 1B (p27, Kip1)]	12p13.2	Cyclin-dependent kinase inhibitor: cell cycle regulation	Downregulated	152
CDKN2A/p16 (Cyclin-dependent kinase inhibitor 2A) ARF/p14 (Alternate open reading frame)	9p21	Cell cycle regulation p16: inhibits CDK4 kinase ARF: stabilises p53, interacts with MDM2	p16: mutated, methylated, deleted ARF: methylated, deleted	64, 65, 148. 149, 150, 152
CDKN2B/p15 (Cyclin-dependent kinase inhibitor 2B)	9p21	Cyclin-dependent kinase inhibitor for CDK4 and CDK6: cell cycle regulation	Methylated	65, 148, 149, 150
CHFR (Checkpoint with forkhead and ring finger domains)	12q24.33	Mitotic checkpoint protein early in G2–M transition: cell cycle regulation	Methylated	153
DAPK1 (Death-associated protein kinase 1)	9q34.1	Positive mediator of interferon-γ-induced apoptosis	Methylated	65, 149, 150
DLEC1 (Deleted in lung and oesophageal cancer 1)	3p22.3	Possibly cell communication, signal transduction	Methylated	17, 71
DLC1/ARHGAP7 (Deleted in liver cancer 1)	8p22.3	Cell cytoskeleton organisation, GTPase activator, signal transduction, cell adhesion, invasion	Methylated, deleted (LOH)	59
EDNRB (Endothelin receptor type B)	13q22	G-protein-coupled receptor	Methylated	154

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Table 3. Candidate tumour suppressor genes involved in nasopharyngeal carcinoma pathogenesis *(continued)*

Candidate TSG (full name)	Chromosomal location	Functions	Alterations in NPC	Refs
FHIT (Fragile histidine triad gene)	3p14.2	Cell cycle regulation: G1–S phase checkpoint, DNA damage response, nucleotide and nucleic acid metabolism	Deleted, aberrant transcripts, methylated	155
GADD45G (Growth arrest and DNA-damage-inducible, gamma)	9q22.2	DNA damage response	Methylated, no mutation	69
GSTP1 (Glutathione *S*-transferase pi)	11q13	Apoptosis, metabolism, energy pathways	Infrequently methylated	65
H19 (H19, imprinted maternally expressed, untranslated mRNA)	11p15.5	Non-coding (?), RNA interference (?)	Methylated	156
IGSF4/TSLC1/CADM1 (Immunoglobulin superfamily, member 4)	11q23.2	Ca^{2+}/Mg^{2+}-independent cell–cell adhesion, apoptosis	Methylated	70
MGMT (*O*-6-methylguanine-DNA methyltransferase)	10q26	DNA repair, sensing and integrating DNA damage/repair-related signals with replication, cell cycle and genomic stability	Methylated	65, 148
MLH1 [MutL homologue 1, colon cancer, nonpolyposis type 2 (*E. coli*)]	3p22.3	DNA mismatch repair protein, cell cycle G(2)/M arrest	Methylated	148, 149
PCDH10 (Protocadherin 10)	4q28.3	Protocadherin, cell–cell adhesion, apoptosis, cell signalling	Methylated	61
PRDM2/RIZ1 (PR-domain-containing 2, with ZNF domain)	1p36.21	Histone methyltransferase, chromatin remodelling, transcriptional regulation	Methylated	157
RARB (Retinoic acid receptor, beta)	3p24	Thyroid-steroid hormone receptor, transcriptional regulator, retinoic acid signalling	Methylated	65, 148
RARRES1/TIG1 [Retinoic acid receptor responder (tazarotene induced) 1]	3q25.32	Retinoid acid receptor response, cell signalling, negative regulation of cell proliferation	Methylated	158

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Table 3. Candidate tumour suppressor genes involved in nasopharyngeal carcinoma pathogenesis *(continued)*

Candidate TSG (full name)	Chromosomal location	Functions	Alterations in NPC	Refs
RASSF1A [Ras association (RalGDS/AF-6) domain family 1]	3p21.3	Ras effector protein, Ras signalling, cell cycle arrest, apoptosis, DNA repair, inhibits the accumulation of cyclin D1	Methylated, mutated	65, 66, 67, 148, 149, 150
RASSF4 [Ras association (RalGDS/AF-6) domain family 4]	10q11.21	Ras signalling, cell cycle, apoptosis, cell migration and invasion	Methylated	159
RBL2/Rb2 [Retinoblastoma-like 2 (p130)]	16q12.2	Cell cycle regulation, DNA damage response	Downregulated, mutated	160
SCGB3A1/HIN1 (Secretoglobin, family 3A, member 1; High in normal 1)	5q35.3	AKT signalling pathway, cell communication	Methylated	161
THBS1 (Thrombospondin 1)	15q15	Adhesive glycoprotein, cell–cell and cell–matrix interactions, angiogenesis, cell signalling, cell motility	Methylated	148
THY1/CD90 (Thy-1 cell-surface antigen)	11q23.3	Cell adhesion, cell cytoskeleton, angiogenesis, immune response	Downregulated	162
TP53 [Tumour protein p53 (Li-Fraumeni syndrome)]	17p13.1	Cell cycle, DNA damage response, apoptosis	Infrequently mutated, frequently accumulated	62, 130, 133, 163, 164
TP73 (Tumour protein p73)	1p36.3	Cell cycle, DNA damage response, apoptosis, transcription factor	Methylated	148
WIF1 (WNT inhibitory factor 1)	12q14.3	WNT-signalling pathway, binds and inhibits WNT proteins, protein-tyrosine kinase activity	Methylated	72

Abbreviations: LOH, loss of heterozygosity; WNT, wingless-type MMTV integration site family member.

successfully used as a powerful diagnostic tumour marker (Refs 74, 75). Quantitative analysis of cell-free EBV DNA in plasma samples of NPC patients is found to be highly sensitive and specific (96% and 93%, respectively).

In addition, quantitative analysis of EBV DNA or carcinoma-specific viral BARF1 mRNA, or even promoter methylation of multiple TSGs, from brushing samples collected directly from the nasopharynx has been shown to be a noninvasive and sensitive diagnostic test

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for NPC, allowing direct investigation of disease progression in the nasopharynx (Refs 76, 77, 78).

In the near future, these molecular diagnostic systems will help to detect early tumour development, monitor early recurrence, and predict prognosis and treatment response of NPC patients.

Radiotherapy and chemotherapy

Following the definitive diagnosis of NPC by endoscopic biopsy, magnetic resonance imaging is the standard imaging for local disease and facilitates high-precision radiotherapy planning, the mainstay treatment for NPC. With the use of intensity-modulated radiation therapy (IMRT), the local control rate exceeds 90% (Refs 79, 80). However, for locoregionally advanced stages, at which most patients present, there remains significant rates of distant metastases, and attempts to improve outcome by the addition of systemic chemotherapy have met with mixed success. Randomised Phase III studies of pure neoadjuvant or adjuvant chemoradiotherapy have not resulted in improvement in overall survival compared with radiotherapy alone. However, randomised studies of concurrent chemoradiotherapy with or without adjuvant chemotherapy have consistently resulted in improvement in progression-free and overall survival (Refs 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94). A meta-analysis using original data of 1753 patients demonstrated a highly significant benefit from concurrent chemoradiotherapy (Ref. 95). Hence, the current standard for locoregionally advanced patients is concurrent cisplatin–IMRT with or without adjuvant cisplatin–5-fluorouracil (5-FU). The use of neoadjuvant chemotherapy remains experimental.

Despite improvements in the primary treatment, 30% of patients with locoregionally advanced disease will subsequently fail with distant metastases (Ref. 96). The median survival figure after distant metastases is around 9–12 months and is influenced by disease, treatment and patient characteristics. Cisplatin-based chemotherapy is the standard first-line treatment, with response rates of up to 80% (Ref. 97). Novel therapeutics using targeted agents are being actively investigated and early results using the monoclonal antibody against epidermal growth factor receptor (EGFR) (Cetuximab) are promising (Refs 97, 98).

Novel therapies

Since EBV is present exclusively in every NPC tumour cell and rarely in normal cells (Refs 17, 34), it is not only a very specific diagnostic biomarker but also a specific therapeutic target. Various approaches in immunotherapy, gene therapy and epigenetic therapy have been developed to target EBV in NPC cells. Epigenetic changes in TSGs might also provide therapeutic opportunities.

EBV-based immunotherapy

HLA class I-restricted cytotoxic T lymphocytes (CTLs) play a major role in controlling EBV infections, and also in the pathogenesis of EBV-associated tumours including NPC. Adoptive transfer of EBV-specific CTLs has been used for the treatment of EBV-positive tumours, such as post-transplant lymphoproliferative disorders (PTLDs) (Refs 99, 100, 101). As only LMP2A and LMP1, but none of the immunodominant EBV antigens (EBNA2, EBNA3 family), are expressed in NPC (latency II), which is different from the situation in PTLDs (latency III), immunotherapy targeting EBV in NPC might not work as well as in PTLDs. The first trial in NPC patients with advanced disease showed that infusion with autologous EBV CTLs was safe, and led to increased CTL levels and a reduced plasma EBV level, indicating that immune intervention of NPC patients is feasible (Ref. 102). Adoptive transfer of allogeneic EBV-specific CTLs boosted the LMP2-specific immune response in an NPC patient with advanced-stage disease (Ref. 103). Later, when ten patients with stage IV NPC received autologous EBV-specific CTLs (which were reactivated and expanded ex vivo from PBMCs stimulated with EBV-transformed autologous LCLs), control of disease progression and increased frequency of LMP2-specific immunity were observed (Ref. 104). A recent trial of using autologous CTLs to treat NPC patients showed remarkable antitumour results. At 19 to 27 months after infusion, four out of ten patients treated in remission from locally advanced disease remained disease-free, while other patients with refractory disease had either complete responses and remained in remission or partial remission or stable disease; only two patients had no response (Ref. 105). Therapeutic vaccination with LMP2-peptide-pulsed dendritic cells also boosted epitope-specific $CD8^+$ T-cell

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responses and resulted in partial tumour reduction in some NPC patients (Ref. 106). The recently developed dual antigen stimulation strategy using a chimaeric construct (part of EBNA1 fused with LMP2) to boost both $CD4^+$ and $CD8^+$ EBV-specific T-cell responses will be of better therapeutic value to NPC (Ref. 107). Thus, the use of EBV-specific immunotherapy along with other standard therapies in the future treatment of NPC looks promising.

Epigenetic therapy

CpG methylation plays important roles in NPC pathogenesis, including the silencing of TSGs and EBV immunodominant antigens (EBNA2, 3A, 3B, 3C) (Refs 108, 109). It also suppresses LMP1, immediate-early lytic antigens Zta and Rta, and lytic cycle viral kinases, and thus is an important regulatory process in the EBV life cycle and latency in tumour cells (Refs 17, 109). As CpG methylation is reversible with pharmacological demethylation using epigenetic agents, epigenetic treatment can be explored either as a novel therapeutic strategy or as a combinational intervention with other therapies. Reactivating methylated and silenced cellular genes (mostly TSGs) and immunodominant tumour/viral antigens would be expected to restore normal cell growth control, induce apoptosis in tumour cells, or induce cell immunity. Demethylation would also reactivate the expression of EBV early and lytic genes in latently infected NPC cells, which will lead to further tumour cell death. Furthermore, the lytic cycle viral kinases involved in the phosphorylation of ganciclovir and other antiviral nucleoside analogues that are of therapeutic value to NPC (see below) will also be activated (Refs 110, 111).

Epigenetic agents include DNA methyltransferase inhibitors – nucleoside analogues such as 5-aza-2′-deoxycytidine/Decitabine/DAC, 5-azacitidine and the newly developed Zebularine (Ref. 112) – and various histone deacetylase (HDAC) inhibitors (TSA, SAHA and PXD101). Earlier clinical trials using such agents have been carried out on cancer patients with colon, head, neck, renal and lung tumours, with only a partial response observed in some patients (Refs 113, 114). In our recent clinical trial of azacitidine in patients with NPC and EBV-positive AIDS-associated Burkitt lymphoma (Ref. 115), all the latent and early lytic EBV promoters [Cp, Wp, LMP1p (ED-L1), Zp, Rp] examined in post-treatment tumour samples showed significant demethylation, with the reactivation of viral antigen expression (Zta) detected by immunostaining. This study demonstrated the potential of epigenetic therapy for NPC.

EBV-based gene therapy

As targeting EBV is tumour-specific, gene therapy strategies with EBV-specific targeting to deliver cytotoxic proteins have been developed for NPC and other EBV-associated tumours. The EBV C promoter has been used to drive the expression of suicide genes (thymidine kinase), as EBV-specific targeting therapy (Ref. 116). Moreover, the utility of EBV oriP to drive p53 (Ref. 117) or a mutant noncleavable form of FasL (Ref. 118) in a replication-deficient adenovirus vector system has also been explored. EBV itself can also be used as a gene therapy vector, with the incorporation of therapeutic genes into a transformation-defective EBV deleted for transforming genes (EBNA2, LMP1) (Ref. 119). The major problem of gene therapy is how to target every individual tumour cell, although activation of EBV kinases (or delivery of thymidine kinase by gene therapy) together with ganciclovir and AZT will result in bystander killing to surrounding tumour cells (Refs 110, 111, 120).

Conclusions

There has been impressive achievement in understanding the molecular pathogenesis of NPC. Like other solid tumours such as colon, cervical and lung cancers, for which stepwise pathogenic models have been proposed (Refs 15, 121), the pathogenesis of NPC is also thought to be a multistep process: indeed, a similar model of NPC pathogenesis has been suggested (Ref. 13), although most of the details of individual steps in the model are still missing. The increasing number of genetic and epigenetic changes uncovered by recent studies (Tables 1, 2 and 3) suggests that NPC pathogenic events are more complex than initially thought. Furthermore, NPC has pathobiological features distinct from other solid tumours, suggesting different pathogenic mechanisms, which may not be exactly stepwise as in the linear progression model

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applied to other common cancers such as colon and cervical cancers. For example, NPC shows a unique peak incidence age plateau of ~40–50 years, which is distinct from the constantly increasing incidence of colon, lung and gastric cancers with age. As mentioned earlier, NPC does not have clearly defined early pathogenic steps as seen in other common carcinomas like colon, cervical or lung carcinoma. The premalignant/CIS lesion is very rare (<1/1000 cases), and most diagnosed NPC tumours are either preinvasive or invasive lesions. As NPC is strongly viral driven, it is conceivable that early EBV infection in premalignant NPC cells would promote and speed up the malignant transformation to a full-scale carcinoma, leaving only a very short window of premalignant/CIS lesion that is not easily detectable.

Nevertheless, it is now believed that a combination of environmental, genetic, epigenetic and viral factors drives the normal nasopharyngeal epithelial cells to preinvasive, then invasive tumour stages, as marked by various genetic and epigenetic abnormalities (Fig. 3). However, many questions remain to be addressed. When does EBV infect normal nasopharyngeal epithelium? Mild hyperplasia is common and also reversible in normal nasopharyngeal epithelium: is there any subtle genetic and epigenetic change in these lesions? Is the EBV gene expression pattern in the early infection of nasopharyngeal epithelial cells the same as that in NPC? Why is it so difficult to establish EBV-positive NPC cell lines and EBV is lost in virtually all the NPC cell lines except for C666-1, which is a special subline from a xenograft in athymic nude mice?

As for other solid tumours, early diagnosis is still the key to treatment success for NPC. Progress in the molecular studies of this tumour should lead to novel diagnostic and therapeutic strategies, as already indicated by approaches arising from EBV and epigenetic studies of this tumour.

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Further reading, resources and contacts

The IARC Cancer Epidemiology Database provides epidemiology information of NPC worldwide:

http://www.iarc.fr/ENG/Databases/index.php

The China Cancer Database (Ministry of Science and Technology of China) provides epidemiology information on NPC in China:

http://cancernet.cicams.ac.cn

The Cancer Registry of the Hong Kong Hospital Authority provides epidemiology information on NPC in Hong Kong:

http://www3.ha.org.hk/cancereg/

The Epstein–Barr Virus Society provides EBV-related information:

http://www.bcm.edu/ebvassociation/index.htm

The US National Center for Infectious Diseases provides background information on EBV infection:

http://www.cdc.gov/ncidod/diseases/ebv.htm

Epigenetics Society provides information on cancer epigenetics:

http://www.dnamethsoc.com/

Recent review book on EBV and its associated tumours:

Robertson, E.S., ed. (2005) Epstein-Barr Virus, Caister Academic Press, Norwich, UK (Publisher site: http://www.horizonpress.com/hsp/books/ebv.html)

Features associated with this article

Figures

Figure 1. The geographical distribution of nasopharyngeal carcinoma.
Figure 2. Incidence of nasopharyngeal carcinoma in southern China and Hong Kong over recent decades.
Figure 3. Possible model of nasopharyngeal carcinoma pathogenesis.

Tables

Table 1. Major genetic alterations detected by microsatellite marker analysis, CGH and array-CGH in NPC cell lines and primary tumours.
Table 2. Candidate oncogenes involved in nasopharyngeal carcinoma pathogenesis.
Table 3. Candidate tumour suppressor genes involved in nasopharyngeal carcinoma pathogenesis.

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