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BASIS FOR RECLASSIFICATION OF NASOPHARYNGEAL CARCINOMA

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Stockholm 2012

Published by Karolinska Institutet. Printed by Larserics Digital Print AB, Sweden.

ISBN: 978-91-7457-699-3

To my mother, my father and my family

ABSTRACT

Nasopharyngeal carcinoma (NPC) shows broad differences in racial and geographical distribution, radiosensitivity, and a multifactorial etiology. This thesis aims to identify molecular biomarkers with potentially valuable for prognostic implications in NPC.

This thesis involved a series of studies which were performed on cohorts of patients with NPC to investigate the genetic alterations, Epstein-Barr virus (EBV) infection, and gene expression profiles, and to assess their correlations with clinicopathological parameters and survival of NPC patients. The results indicated that overexpression of caveolin-1 (Cav-1) and extracellular matrix metalloproteinase inducer (EMMPRIN/CD147) in NPC were significantly associated with TNM stage, metastasis, and poor prognosis (Paper I). Loss of heterozygosity (LOH) on 9p21, 16q and 19q13 may be responsible for tumor aggression behavior and progression of NPC, with a possible interaction between allelic loss and EBV infection in the etiology of NPC (Paper II). EBV latent membrane protein (LMP) 1 overexpression was significantly correlated with p53 accumulation in NPC, $CD8^+$ T cell infiltration, and matrix metalloproteinase (MMP) 9 overexpression in NPC cells. Moreover, plasma EBV DNA was detectable at a high frequency in primary NPC (96%). Higher plasma EBV-DNA levels were positively correlated with advanced TNM stages, lymph node metastasis, and NPC relapses (Papers III-V). Overexpression of LMP1 regulated the mTOR signaling pathway in NPC, possibly through phosphorylation of AKT/mammalian target of rapamycin (mTOR)/phospho-P70S6 kinase (P70S6K)/4EBP1. LMP1 expression was closely correlated with expression of p-mTOR, p-P70S6K and p-4EBP1 in NPC tumors, while expression levels of p-P70S6K, p-4EBP1 and LMP1 were significantly correlated with overall survival in NPC patients (Paper V).

Paper VI presents a new molecular NPC-space vector modulation (SVM) classifier, which integrates sex and seven genes, including LMP1, CD147, Cav-1, p-P70S6K, MMP11, survivin, and secreted protein acidic and rich in cysteine (SPARC). This NPC-SVM classifier could refine the classification of NPC patients into high- and low-risk groups, which demonstrated significant differences in 5-year disease-specific survival (DSS) rates in a group of 411 validation patients (86.2% *vs.* 37.6%, $p<0.001$). Paper VII presents a new histological classification study, which was developed mainly on the basis of morphological characteristics and tumor cell differentiation. Of 3,839 tumors, 2,057 (53.6%) were histologically classified as undifferentiated epithelial cell carcinoma (UECC), 942 (24.5%) as undifferentiated mixed epithelial-sarcomatoid cell carcinoma (UESCC), 640 (16.7%) as undifferentiated sarcomatoid cell carcinoma (USCC), and 200 (5.2%) as squamous cell carcinoma (SCC). Based on the new histological classification system, the 5-year DSS rates were 76.4% for UECC, 66.0% for UESCC, 56.0% for USCC, and 32.7% for SCC. Stratified according to the new classification, patients with UECC and UESCC who received radiochemotherapy (RCT) showed better 5-year DSS rates than those who received radiotherapy (RT) alone.

In summary, the results of these studies indicate that LOH, differentially-expressed genes, and EBV markers can act as prognostic biomarkers in NPC patients. The NPC-SVM classifier and the new proposed histopathological classification provide better discriminative prediction of NPC prognosis than the current WHO classification, as well as a means of monitoring the therapeutic efficacy of RCT and RT in advanced-stage NPC patients.

LIST OF PUBLICATIONS

I. Zi-Ming Du, Chun-Fang Hu, Qiong Shao, Ma-Yan Huang, Chang-Wei Kou, Xiao-Feng Zhu, Yi-Xin Zeng, **Jian-Yong Shao***. Upregulation of caveolin-1 and CD147 expression in nasopharyngeal carcinoma enhanced tumor cell migration and correlated with poor prognosis of the patients. **Int. J. Cancer.** 2009 Oct 15;125(8):1832-41.

II. **Jian-Yong Shao,** Xiao-Ming Huang, Xing-Juan Yu, Li-Xi Huang, Qiu-Liang Wu, Jian-Chuan Xia, Hui-Yun Wang, Qi-Sheng Feng, Zhe-Fang Ren, Ingemar Ernberg, Li-Fu Hu, Yi-Xin Zeng. Loss of heterozygosity and its correlation with clinical outcome and Epstein-Barr virus infection in nasopharyngeal carcinoma. **Anticancer Res.** 2001 Jul-Aug;21(4B):3021-9.

III. **Jian-Yong Shao,** Ingemar Ernberg, Peter Biberfeld, Heiden T, Yi-Xin Zeng, Li-Fu Hu. Epstein-Barr virus LMP1 status in relation to apoptosis, p53 expression and leucocyte infiltration in nasopharyngeal carcinoma. **Anticancer Res.** 2004 Jul-Aug;24(4):2309-18.

IV. **Jian-Yong Shao**, Yu-Hong Li, Hong-Yi Gao, Qiu-Liang Wu, Nian-Ji Cui, Li Zhang, Gang Cheng, Li-Fu Hu, Ingemar Ernberg, Yi-Xin Zeng. Comparison of Plasma Epstein-Barr Viruses DNA Level and Serum EBV VCA/IgA Antibody Titers in Nasopharyngeal Carcinoma. **Cancer,** 2004, 100: 1162-1170

V. Jing Chen, Chun-Fang Hu, Jing-Hui Hou, Qiong Shao, Li-Xu Yan, Xiao-Feng Zhu, Yi-Xin Zeng, **Jian-Yong Shao*.** Epstein-Barr virus encoded latent membrane protein 1 regulates mTOR signaling pathway genes which predict poor prognosis of nasopharyngeal carcinoma. **J Transl Med.** 2010, 26;8:30

VI. Hai-Yun Wang, Bing-Yu Sun, Zhi-Hua Zhu, Ellen T Chang, Ka-Fai To, Jacqueline SG Hwang, Hao Jiang, Michael Koon Ming Kam, Gang Chen, Shie-Lee Cheah, Ming Lee, Zhi-Wei Liu, Jing Chen, Jia-Xing Zhang, Hui-Zhong Zhang, Jie-Hua He, Fa-Long Chen, Xiao-Dong Zhu, Ma-Yan Huang, Ding-Zhun Liao, Jia Fu, Qiong Shao, Man-Bo Cai, Zi-Ming Du, Li-Xu Yan, Chun-Fang Hu, Ho Keung Ng, Joseph TS Wee, Weimin Ye, Ingemar Ernberg, Hans-Olov Adami, Anthony T Chan, Yi-Xin Zeng, and **Jian-Yong Shao***. Eight-signature Classifier for Prediction of Nasopharnyngeal Carcinoma Survival. J**ournal of Clinical Oncology**, 2011, Dec 1; 29(34):4516-4525

VII. Jia-Xing Zhang, Kai-Fo To, Jacqueline Hwang, Yih-Leong Chang, Cheng-Ping Wang, Kam Koon Ming Michael, Sherry Lee, Ming Li, Li Gao, Hao Jiang, Hai-Yun Wang, Hui-Zhong Zhang, Jie-Hua He, Xiao-Dong Zhu, Liang Zeng, Pei-Qing Ma, Chun-Yan Chen, Li-Xu Yan, Ma-Yan Huang, Ding-Zhun Liao, Jia Fu, Qiong Shao, Man-Bo Cai, An-Jia Han, Hai-Gang Li, Chun-Kui Shao, Bin Chu, Hong Du, Zuo-Fang Hao, Min-Zuo, Xiao-Mei Wang, Yi-Sheng Lu, Zi-Ming Du, Mu-Sheng Zeng, Chao-Nan Qian, Tai-Xiang Lu, Chun-Fang Hu, Ho Keung Ng, Joseph TS Wee, Hans Olov Adami, Yi-Xin Zeng, Anthony Chan and **Jian-Yong Shao*.** New Histopathological Classification of Nasopharyngeal Carcinoma and Its Clinical Implication. Manuscript in submission

Related publications:

I. Zi-Ming Du#, Chang-Wei Kou#, Chun-Fang Hu, Jing Chen, Hai-Yun Wang, Li-Xu Yan, Li-Fu Hu, Ingemar Ernberg, Yi-Xin Zeng and **Jian-Yong Shao***. Clinical Significance of Elevated Spleen Tyrosine Kinase Expression in Nasopharyngeal Carcinoma. ***Head Neck***. 2012 Jan 27. PMID: 22287277

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III. Yan Zhang#, Li-Xu Yan#, Qi-Nian Wu#, Zi-Ming Du#, Jing Chen, Ding-Zhun Liao, Ma-Yan Huang, Jing-Hui Hou, Qiu-Liang Wu, Mu-Sheng Zeng, Wen-Lin Huang, Yi-Xin Zeng and **Jian-Yong Shao***. miR-125b is Methylated and Functions as A Tumor Suppressor by Regulating the ETS1 proto-oncogene in Human Invasive Breast Cancer. ***Cancer Res***. 2011 May 15;71(10):3552-62. PMID: 21444677

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LIST OF ABBREVIATIONS

NPC	Nasopharyngeal carcinoma
NPE	Nasopharyngeal epithelia
EBV	Epstein-Barr virus
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
TNM	Minimal deletion region
WHO	World Health Organization
LMP1	Latent membrane protein 1
LMP2	Latent membrane protein 2
KSCC	Keratinized squamous cell carcinoma
NKDC	Non-keratinizing differentiated carcinoma
NKUC	Non-keratinizing undifferentiated carcinoma
EMT	Epithelial-mesenchymal transition
VEGF	Vascular endothelial growth factor
ESCC	Esophageal squamous cell carcinoma
IAPI	In situ apoptotic protein inhibitor
GEF	Growth-enhancing factor
TDGF1	Tumor-derived growth factor 1
PDGFA	Platelet-derived growth factor A chain
Cav-1	Caveolin-1
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamacin
PI3K/AKT	Phosphoinositide 3-kinase
IHC	Immunohistochemistry
P70S6K	Ribosomal protein S6 kinases,
4E-BP1	Eukaryotic initiation factor 4E (eIF4E)-binding protein,
RNA	Ribonucleic acid
NSCLC	Non small cell lung cancer
SVM	Support vector machines
VCA	Viral capsid antigen
EA	Early antigen
CSCs	Cancer stem cells
SP	Side population
EBNA1	Epstein-Barr virus nuclear antigen 1
LMP2A	Latent membrane protein 2A
Syk	Spleen tyrosine kinase
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
IGF-1	Insulin-like growth factor 1
NF	Nuclear factor
UECC	Undifferentiated epithelial cell carcinoma
UESCC	Undifferentiated epithelial-sarcoid cell carcinoma
USCC	Undifferentiated sarcoid cell carcinoma
SCC	Squamous cell carcinoma
ERCC	excision repair complementing defective repair in Chinese hamster

WBC	White blood cells
EMMPRIN/CD147	Extracellular matrix metalloproteinase inducer
EBER	EBVencoded early RNAs
MDR	Minimal deletion region
CYP2E1	Cytochrome P4502E1
GWAS	Genome-wide association study
LOD	Linking open data
HNSCC	Head and neck squamous cell carcinoma
FAL	Fractional allelic loss
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
CTARs	COOH-terminal activation regions
TRADD	TNFR-associated death domain protein
TRAFs	Tumor necrosis factor receptor (TNFR)-associated factors
HD	Hodgkin's disease
GMT	Geometric mean titer
4E-BP1	4E (eIF4E)-binding protein
RT	Radiation therapy
RCT	Radiochemotherapy

INTRODUCTION

Epidemiology and etiology of nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) shows broad differences in racial and geographical distribution, radiosensitivity, and a multifactorial etiology (Feng *et al*, 2002; Henderson *et al*, 1976; Lin *et al*, 2004). NPC is a common malignancy in areas of the Mediterranean, Central Africa, Southeast Asia, and Southern China, with an incidence rate of 25–40 per 100,000 persons per year among the Southern Chinese especially those of Cantonese origin (Licitra *et al*, 2003; Titcomb, 2001). In contrast, NPC has an incidence of well under 1 per 100,000 persons per year in Caucasians from North America and other Western countries. 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 other Asian countries (Ferlay, 2001). Southern Chinese immigrants also have a higher risk of NPC compared to the local Western population. Independent of race/ethnicity, men are 2- to 3-fold more frequently affected than women (Yu & Yuan, 2002). However, recent changes in the epidemiology of NPC are reflected by a decreasing incidences of NPC (around 30%) in Hong Kong over the past 20 years (Lee *et al*, 2003), possibly related to changes in environmental factors. The dramatic differences in the incidence of NPC among populations and geographic areas are shown in Figures 1 and Figures 2.

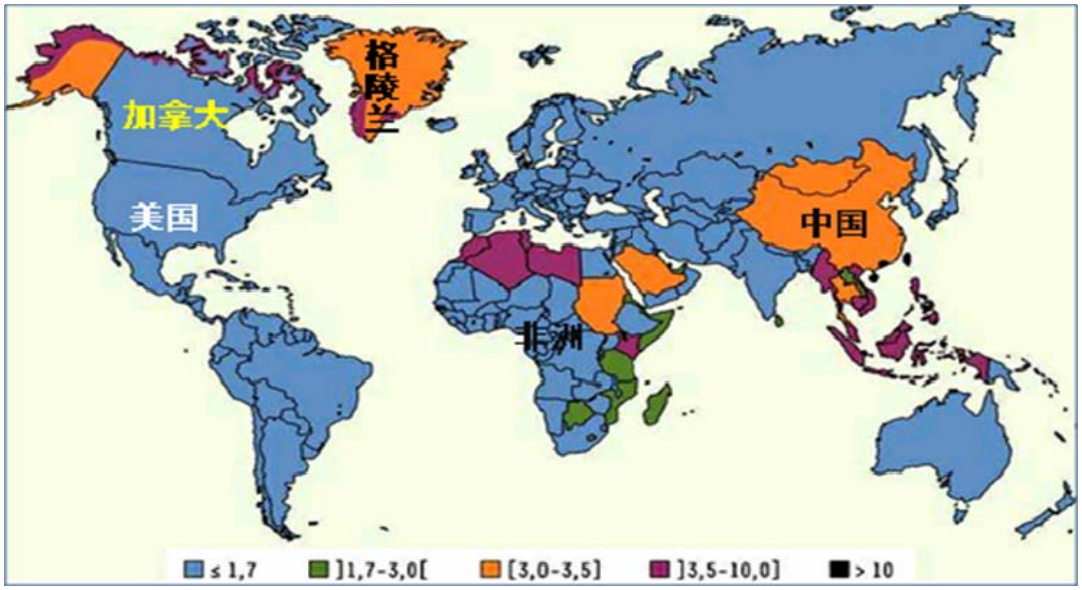


Figure 1. Differences in the incidences of NPC worldwide. Modified from IARC：GLOBOCAN, 2008

NPC is the most prevalent tumor in southern China

Constitutive order of cancer in China

Order no.	Whole China	Southern China	Province of G.D City, Guangzhou	Incidence (1964-1981)	%
1.	Lung	Liver	NPC	47848	32
2.	Liver	NPC	Liver	18609	12.5
3.	Stomach	Lung	Lung	15207	10.2
4.	Esopha-	Esopha	Esopha-	8366	5.6
5.	Breast	Breast	Breast	7507	5.3
6.	NPC		Cervix	7426	4.9
7.	Cervix		Stomach	6944	3.4
8.	Colon		Lymphoma	4883	3.3
9.	Leukemia		Colon	4422	2.9
10.	Other			30141	20.2
				149535	100 %

* NPC: Nasopaharyngeal carcinoma

	Incidence	Mortality.
Male	25.29	12.46 (3.4)
Femal	12.17	5.0 (1.7)

Figure 2. High incidence of NPC in Southern China. Modified from "Atlas of Cancer Mortality in the Peoples Republic of China (1973-1976). Beijing, China Map Press, Pages 81-82 (1979)

Epstein-Barr virus infection and NPC

Epstein-Barr virus, EBV, which ubiquitously infects more than 90% of the world's population, was the first human tumor virus identified to be causally associated with various lymphoid and epithelium malignancies (Epstein *et al*, 1966; Young & Murray, 2003). The association between EBV infection and NPC is well documented, and the EBV genome is presented in virtually all NPC cells. The underlying mechanism whereby EBV infects normal healthy carriers is summarized in Figure 3. After primary

infection at an early age, persistent latent EBV infection is found in some resting B cells, but has not been detected in the nasopharyngeal epithelia (NPE) in healthy individuals (Babcock *et al*, 1998; Tao *et al*, 1995). However, EBV infection has been demonstrated in *in situ* carcinomas of the nasopharynx, which are presumed to be precursor lesions of NPC (Niedobitek *et al*, 1996). These findings suggest that EBV infection occurs before invasive growth begins, but probably does not represent the first step in the pathogenesis of NPC.

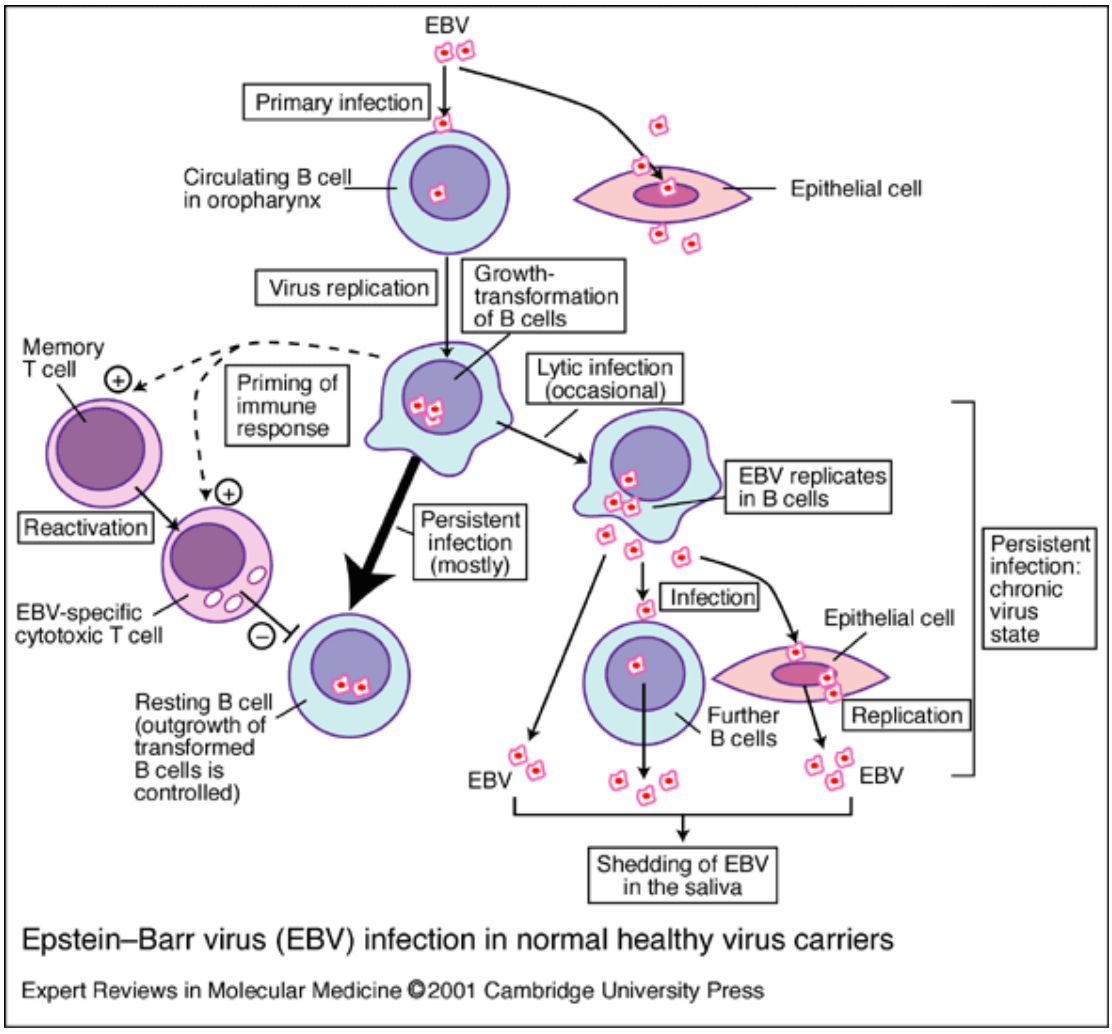


Figure 3. EBV infection in normal healthy virus carriers. Modified from: Expert Reviews in Molecular Medicine(Young, 2001).

NPC, particularly the undifferentiated type, is the most commonly known EBV-associated cancer (Young & Murray, 2003) and four EBV latent proteins can be expressed in these tumors (Andersson-Anvret *et al*, 1979; Lo *et al*, 2004). BARF 1 and the three latent membrane proteins (LMPs 1, 2A, 2B). EBNA-LP is transcribed from variable numbers of repetitive exons. EBNA1, the primary role of which is to enable replication of the viral episomal genome (Yates *et al*, 1985), is the most widely-expressed protein in NPC. LMP2A and LMP2B are composed of multiple exons located on either side of the terminal repeat region, which is formed during the circularization of the linear DNA to produce the viral episome. Although both LMP1 and LMP2A are detectable in NPC samples, most recent research has focused on LMP1 because of its known oncogenic properties in B cells (Hung *et al*, 2001; Thorley-

Lawson, 2001). However, LMP2A has also been detected in more than 95% of NPC samples at the mRNA level, and in about 50% of these specimens at the protein level, whereas LMP1 could be detected in only about 65% and 35% of NPC samples at the mRNA or protein levels, respectively (Busson *et al*, 1992; Chen *et al*, 1995; Heussinger *et al*, 2004; Niedobitek *et al*, 1992; Young *et al*, 1988). In addition, one study found that high levels of LMP2A expression in NPC samples were correlated with a poor survival outcome, although this study was carried out using only a small cohort (Pegtel *et al*, 2005). EBER1 and EBER2 are highly-transcribed non-polyadenylated RNAs, and their transcription is a consistent feature of latent EBV infection. A pivotal biologic property of the virus is its ability to alter B-lymphocyte growth *in vitro*, leading to permanent growth transformation.

Although LMP1 was demonstrated in all examined premalignant in situ lesions by immunostaining in and is thought to precede NPC (Pathmanathan *et al*, 1995; Permeen *et al*, 1990), only 35–65% of NPCs were LMP1-positive by immunoblotting, while up to 90% were positive by sensitive PCR (Chen *et al*, 1995). We previously demonstrated that LMP1 status affected clinical outcome in terms of tumor growth, therapy response, invasiveness, and risk of recurrence. Clinical and follow-up data from 74 NPC patients showed that LMP1-postive NPCs grow faster and more expansively than LMP1-negative tumors (Hu *et al*, 1995). To further elucidate the impact of LMP1 on the natural history of NPC, we examined markers related to tumor cell proliferation, apoptosis, leukocyte infiltration, and metastasis in NPC biopsies, in relation to LMP1 status.

Genetic alterations in NPC

The distinct geographic variations in the incidence of NPC indicate genetic and/or environmental contributions to its development. In support of the influence of genetic factors, Southern Chinese are an ethnically-distinct population that may be related to Aleutean Indians, another ethnically distinct, (middle) high-risk group. Possible environmental or cultural factors include the ingestion of Cantonese-style salted fish, especially during childhood. Several carcinogenic volatile nitrosamines have been detected in Chinese salted fish, although their precise role in inducing NPC remains to be determined (Henderson *et al*, 1976; Yu *et al*, 1988).

Genetic susceptibility There is clear evidence for genetic susceptibility to NPC, and the existence of susceptibility genes at the HLA and cytochrome P4502E1 (CYP2E1) loci has been demonstrated by linkage analysis. These genes could account for the majority of cases of this cancer. The association between specific HLA antigens and NPC was first reported by Simons et al. in an investigation of 144 Chinese patients and 236 controls. Early evidence for a genetic determinant among Chinese was the identification of an HLA-associated increased risk of NPC associated with the joint occurrence of HLA-A2 and HLA-BSin2 (relative risk = 2.35) (Simons *et al*, 1976). Numerous studies conducted among Chinese NPC patients have indicated an association between HLA-A2/Bw46 and NPC (Chan *et al*, 1983; Simons *et al*, 1975),

but several studies conducted in non-Chinese NPC patients have reported associations with antigens other than HLA-A and HLA-B (Burt *et al*, 1994; Chan *et al*, 1985). In addition, the HLA types Aw19, Bw46, and B17 are associated with increased risk, while A11 is associated with a decreased risk of developing NPC. Ooi et al. reported that the NPC susceptibility gene may lie within the centromeric end of the class-I and the telomeric end of the class-III regions of the MHC, near the D6S1624 microsatellite locus, where the presence of allele 4 of the microsatellite conferred a 3.5-fold increase in the risk of NPC. This represents the highest reported risk for a single locus, while the presence of allele 1 of the same microsatellite conferred a highly significant protective effect against NPC (Ooi *et al*, 1997). A recent genome-wide association study (GWAS) an additional three new susceptibility regions. This large GWAS, comprising approximately 5,000 patients and 5,000 controls of Southern Chinese descent, established beyond doubt that the HLA complex is a primary location for NPC risk, comprising multiple risk regions, with the top single nucleotide polymorphism (SNP) showing one of the highest statistically significant values of any published GWAS study (Bei *et al*, 2010).

Other potential genetic markers in addition to HLA have been examined. Two studies have investigated the relationship between NPC and CYP2E1. A strong association was observed between the restriction fragment length polymorphisms detected by DraI and RsaI digestion of CYP2E1 in NPC (Hildesheim *et al*, 1995). A further case-control study conducted in 364 NPC patients and 320 control subjects reported that individuals homozygous for an allele of the CYP2E1 gene detected by RsaI digestion (C2 allele) had an increased risk of NPC (relative risk [RR] = 2.6; 95% confidence interval [CI] = 1.2–5.7), suggesting that CYP2E1 genotype is a determinant of NPC risk (Hildesheim *et al*, 2002). A preliminary linkage study using 382 microsatellite polymorphism markers was performed in 23 Cantonese-speaking NPC pedigrees, and the susceptibility locus was mapped to chromosome 4p15.1-q12, strongly suggesting a putative NPC-susceptibility/related gene located at this region (Feng et al., 2002). Another linkage study by using multipoint linkage analysis, four loci (2q, 5p, 12p, and 18p) showed LOD scores above 1.5. They reported one locus on 5p13 showed an increased LOD of 2.1 suggested a region on 5p13 may harbor a susceptibility gene for NPC(Hu *et al*, 2008).

Comparative genomic hybridization (CGH) CGH has been used to investigate the genomic imbalance in many types of solid tumors (Kallioniemi, 2008; Kallioniemi *et al*, 1996; Kallioniemi *et al*, 1993), and numerous previously unrecognized recurrent genomic alterations have been detected using this approach, at sites potentially harboring oncogenes and putative TSGs. CGH has been applied by three different laboratories to detect chromosomal imbalances in NPC (Chen *et al*, 1999; Chien *et al*, 2001; Fang *et al*, 2001; Hui *et al*, 2002). The most frequent chromosomal gains and allelic losses analyzed by CGH in NPC were summarized in Table 1. Comparison of these CGH results indicates that chromosome gains on chromosomes 1q, 3q, 11q, 12q and 17q are common genetic events in NPC. The high incidence of genetic amplification at multiple chromosomal regions strongly suggests that putative

oncogenes related to NPC tumorigenesis may map to these regions. CGH studies also identified allelic loss on several chromosome arms in NPC. These different CGH studies can be summarized in a similar result of allelic loss on 1p, 3p, 9p, 9q, 11q, 13q, 14q, and 16q in NPC tumors, and were therefore in agreement with each other, and also in agreement with previous results of allelotyping analysis (Lo et al., 2000a; Shao et al., 2001; Shao et al., 2000) and LOH studies (Chan et al., 2002; Chan et al., 2000; Lo & Huang, 2002; Lung et al., 2001; Mutirangura et al., 1996; Tsang et al., 1999). These data strongly suggest that candidate TSGs in the deleted regions which might be involve in NPC pathogenesis and progression.

Loss of heterozygosity (LOH) Cytogenetic studies of NPC xenografts identified changes on chromosomes 1, 3, 9, 11, 12, and 17. Consistent deletions on the short arms of chromosomes 3 and 9 suggest the presence of human suppressor genes residing in these regions and contributing to the malignant phenotype when the normal copy of the gene is deleted (Hui *et al*, 1999). Earlier LOH studies on primary NPCs from different laboratories observed high frequencies of allelic losses on chromosomes 3p (Deng *et al*, 1998; Lo *et al*, 1994; Lo *et al*, 2000a; Shao *et al*, 2000), 9p (Chan *et al*, 2002), 11q (Lung *et al*, 2004), 13q (Mutirangura *et al*, 1999; Shao *et al*, 2002; Tsang *et al*, 1999), and 14q (Cheng *et al*, 1997; Shao *et al*, 2002). These studies also revealed minimal deletion regions (MDRs) on high-frequency LOH autosomal arms that may contain tumor suppressor genes (TSGs) that contribute to NPC tumorigenesis. MDRs were 3p26 (homozygous deletion), 3p25.3-26.3, 3p25, 3p14.3-24.1, and 3p14.2 on chromosome 3p (Chan *et al*, 2000; Deng *et al*, 1998; Lo *et al*, 1994; Lo *et al*, 2000a; Shao *et al*, 2000; Sung *et al*, 2000); 9p21-22 on chromosome 9p (Chan *et al*, 2002); 11q13.3-22 and 11q22-24 on chromosome 11q (Guo *et al*, 2001; Harn *et al*, 2002; Mutirangura *et al*, 1996); 13q12, 13q14, 13q14.3-22, and 13q31-34 on chromosome 13q (Tsang *et al*, 1999); 14q11, 14q12-13, and 14q32-ter on chromosome 14q (Cheng *et al*, 1997). In addition, a high frequency of LOH on 3p has been reported in histologically-normal NPE(73.9%) and dysplastic NP lesions (75%) in Southern Chinese, whereas a significantly lower frequency of LOH on 3p was observed in normal NP from low-risk groups compared to high-risk groups (Chan *et al*, 2000). The presence of such genetic alterations in histologically-normal NP and dysplastic lesions suggests that it is an early event in tumor development.

To further investigate the critical genetic events leading to tumor evolution, recent genome-wide allelotype analysis of primary NPCs revealed high frequency of LOH on chromosomes 1p, 3p, 3q, 9p, 9q, 11q, 13q, 14q, and 17q (Shao *et al*, 2000), with the highest frequencies of allelic deletions on 3p and 9p. In addition, LOH was also common on 4q, 5q, 8p, 11p, and 12p in NPC (Lo *et al*, 2000a; Shao *et al*, 2000). The detailed mapping of these autosomal allelic losses and gains are summarized in Table 1. These high-resolution allelotyping and LOH analyses of NPC have generated an accurate and clear-cut profile of the chromosomal abnormalities in NPC, which should further investigations into the localization of putative tumor suppressor genes (TSGs) associated with the pathogenesis of NPC. The identification of multiple genetic losses in NPC tumors is consistent with a multi-step model of tumorigenesis, as in most other

solid tumors. Multiple chromosomal-region deletions in NPC may indicate multiple aberrations of TSGs or cancer-related genes located on these chromosomal arms, which may play important roles in the development and progression of NPC.

Table 1. Summary of genome-wide genetic alterations in NPC

Original data	Frequency of allelic losses	MDRs	Frequency of gains	MORs (CGH)
Shao JY, et al. 2000	1p (65%), 2p (61%), 2q (74%), 3p (91%), 3q (71%), 5q (70%), 9p (60%), 9q (69%), 11q (77%), 13q (78%), 14q (79%) and 17q (60%).	1p36, 2p25-p24, 3p14-p21, 3p24-p26, 5q11-q14, 5q31-q33, 9p21-p23, 9q33-q34, and 19q13.		
Lo KW, et al., 2000a	12q (70.4%), 13q (55.6%), 14q (85.2%) , 16q (55.6%),1p (37.0%), 5q (44.4%), and 12p (44.4%)	3p14-24.2, 11q21-23, 13q12-14, 13q31-32, 14q24-32, and 16q22-23		
Fang Y, et al., 2001	16q (55%), 14q (45%), 1p (43%), 3p (43%), 16p (40%), 11q (36%), and 19p (34%)	14q24-qter, 1pter-p36.1, 3p22-p21.3, 11q21-qter, and the distal region of 19p	12q (51%), 4q (36%), 3q (34%), 1q (32%), and 18q (32%)	3q21--q26.2, 4p12--q21, 8p, and 12q14--q15
Chien G, et al., 2001	3p14-p21 (20%), 11q23-qter (20%), 16q21-qter (17%) and 14q24-qter (13%)	3p12-14, 3p25-26, 9p21-23, 13q21-32, 14q12-21, and 11q14-23	12p11.2-p12 (36%), 12q14-q21 (33%), 2q24-q31 (23%), 1q31-qter (20%), 3q13 (20%), 1q13.3 (20%), 5q21 (17%), 6q14-q22 (13%), 7q21 (13%), 8q11.2-q23 (13%) and 18q12-qter (13%)	12p12-13, 1q21-22, 17q21, 17q25, 11q13, and 12q13
Chen YJ, et al., 1999	3p (53%), 9p (41%), 13q (41%), 14q (35%), and 11q (29%	3p12-14, 3p25-26, 9p21-23, 13q21-32, 14q12-21, and 11q14-23	12p (59%), 1q (47%), 17q (47%), 11q (41%), and 12q (35%).	12p12-13, 1q21-22, 17q21, 17q25, 11q13, and 12q13

Abbreviations: LOH, loss of heterozygosity; CGH, comparative genomic hybridization; MDR, minimal deletion region; MOR, minimal overlapping region; NPC, nasopharyngeal carcinoma

Epigenetic changes in NPC

Epigenetics refer to alternate phenotypic states that are not based on differences in genotype, and are potentially reversible, but are generally stably maintained during cell division. Among the epigenetic events, DNA hypermethylation has become one of the most dynamic and rapidly developing branches of molecular biology. Changes in DNA methylation are recognized as important events in normal and pathological cellular processes, contributing both to normal development and differentiation as well as cancer and other diseases. It has recently been suggested that cancer even be initiated as an epigenetic process before any mutations (Feinberg *et al*, 2006)

In cancer, silencing of tumor suppressor genes or activation of oncogene is a main mechanism for carcinogenesis. It often coincides with the aberrant methylation of CpG dinucleotides in CpG islands, frequently located in promoters and transcription start sites of genes involved in various fundamental pathways, such as apoptosis, DNA damage repair, tumor invasion and metastasis. Aberrant methylation of tumor suppressor genes was frequently found in NPC. DNA methylation also plays an important role in the maintenance of specific EBV latency programs in the NPC cells. Thus, methylation profile of certain TSGs may serve as a complementary marker for identifying early cases. Many TSGs have been found to be frequently methylated in NPC, and the high detection rate in body fluids, such as saliva, brushings and plasma, suggested its potential application in non-invasive screening of NPC or detection of residual carcinoma after treatment (Chang *et al*, 2003). Combined analysis of five methylation markers (RASSF1A, p16, WIF1, CHFR and RIZ1) in brushings showed a good discrimination between NPC and non-NPC with a detection rate of 98% in a high risk population (Hutajulu *et al*). Moreover, hypermethylated promoter DNA of at least one of the three genes (CDH1, DAPK1, and p16) was detectable in post-treatment plasma of 5 of 13 (38%) recurrent NPC patients and none of the patients in remission, which suggested that cell-free circulating methylated gene promoter DNA is a potential useful serological marker in assisting in screening of potentially local or regional recurrent NPC (Wong *et al*, 2004). Multiplex methylation specific PCR (MMSP) for early diagnosis of NPC was developed to DNA derived from nasopharyngeal (NP) swabs. A panel of markers including two EBV genes (EBNA1 and un-methylated LMP1), and two-three cellular methylated TSGs (Rasff1A /DAPK and Rassf1A/DAPK/CHFR1) were simultaneously applied in this NPC-specific-MMSP assay through a single PCR reaction. The results showed that MMSP patterns of NPC swab were largely consistent with those of corresponding biopsies and significantly distinguished themselves from those of noncancerous volunteers. The sensitivity of detecting NPC from NP swabs is 98% (49 NPC and matched swabs, and 20 normal controls from Chinese), and 90% (37 NPC and 19 normal from Morocco) (Zhang, 2012, In press).

In summary, NPC development may involve susceptibility gene mutations (major genes) and gene polymorphisms (minor-effect genes). In some familial cases, inherited genetic alterations (major gene transmission) could be the first “hit”, and

EBV infection may contribute to the second "hit". Therefore, familial cases usually have a much younger age of onset. However, some other familial cases and probably most sporadic cases may get the first "hit" from both inherited genetic alterations (minor-effect genes, such as HLA, CYP2E1) and somatic genetic changes. In the high prevalence areas like south China, most of the NPC cases belong to this type and they usually have older age of onset than the familial cases with a major gene transmission (Figure 4) (Zeng & Jia, 2002).

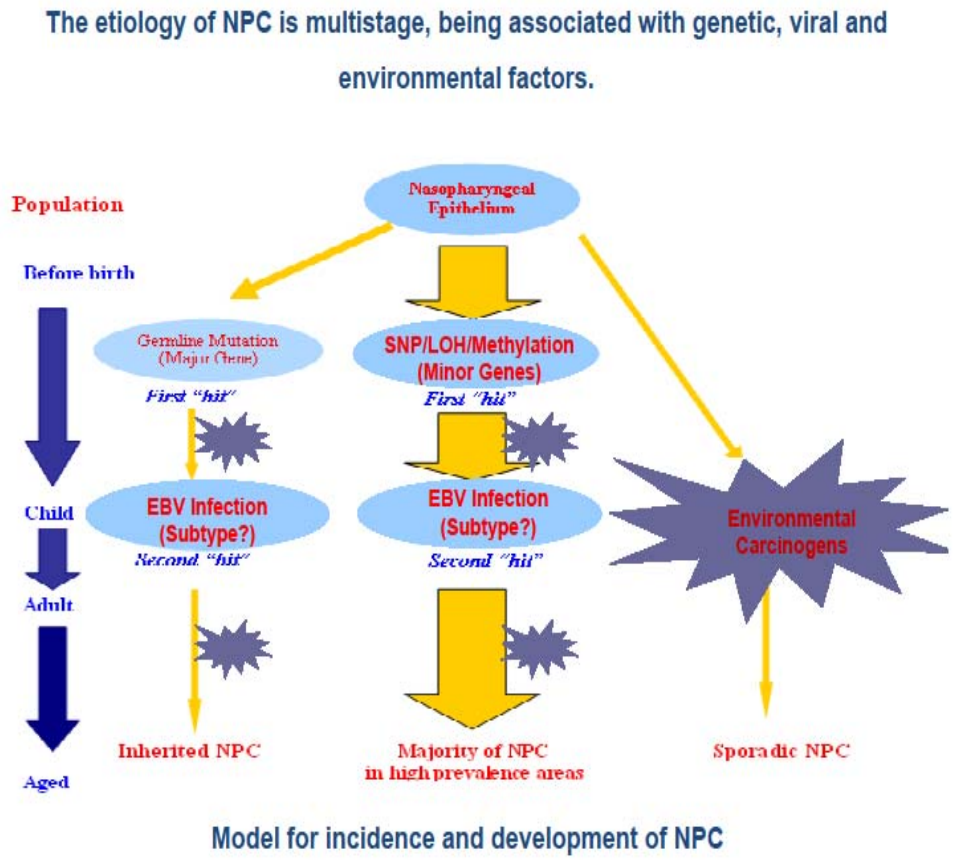


Figure 4. Putative model of genetic alterations, EBV infection and environmental factors involved in NPC development. Modified from "Pathology & Genetics, Head and Neck Tumor" (J.K.C. Chan, 2005; Zeng & Jia, 2002)

Molecular Markers and Prognosis of NPC

Genetic markers Certain genetic alterations in tumor cells can change the behavior of these cells, and these changes can be expected to be associated with certain clinical features. Cancer develops, at least in part, by an accumulation of genetic alterations that disrupt the normal processes of cell growth and differentiation. Previous molecular genetic and cytogenetic studies have demonstrated associations between LOH and tumor cell aggression, metastasis, clinical stage, and tumor differentiation in several types of cancers (Harada *et al*, 1999). A previous study found that progression of papillary renal cell carcinoma was associated with allelic loss on chromosome 9p21 (Schraml *et al*, 2000). Rosin et al. performed an important LOH study on head and

neck squamous cell carcinoma (HNSCC), and reported that nearly 60% of premalignant lesions with LOH at 3p and/or 9p plus LOH at any other tested region developed HNSCC; among the lesions that later progressed to HNSCC, more than 70% exhibited this type of LOH profile (Rosin *et al*, 2000). This provides strong evidence for the effective use of LOH profiles to augment routine histopathological evaluation of oral premalignant lesions. Another LOH study of HNSCC reported that certain LOH at 9p21, 3p and 17q13 tended to occur earlier in the progression pathway, whereas LOH at 13q11 and 8 usually occur late in the time course of progression. These results indicate that recurrent premalignant lesions arise from a common clonal progenitor, followed by outgrowth of clonal populations associated with progressive genetic alterations and phenotypic progression to malignancy (Califano *et al*, 1996; Chen & Chen, 2008). Nawrodz et al. reported that the presence of microsatellite alterations in serum DNA (shifts or LOH) was closely associated with advanced stages, metastasis, and poor prognosis in HNSCC patients, suggesting that the detection of microsatellite alterations in circulating tumor cell DNA may be useful for assessing tumor burden, metastatic status and overall prognosis (Nawroz *et al*, 1996). In a CGH study on HNSCC, Bockmühl et al. found that overrepresentations of 2q12, 3q21-29, 6p21.1, 11q13, 14q23, 14q24, 14q31, 14q32, 15q24, 16q22, and deletions of 8p21-22 and 18q11.2 were significantly associated with both shorter disease-free interval and disease-specific survival (DSS). Gains of 3q21-29, 11q13, and loss of 8p21-22 were independent prognostic markers carrying a higher significance than nodal status, as the only clinicopathological parameter with statistical importance. In addition, these three markers allowed a molecular classification of patients with low clinical risk (pN0 and pT2 tumors). Thus, genomic data derived from the evaluation of primary HNSCC has enabled patients to be stratified into subgroups with different survivals, highlighting the necessity of a genetically-based tumor classification system for refining the diagnosis and treatment of HNSCC patients (Bockmuhl *et al*, 2000).

NPC is distinguished from other head and neck cancers by a number of epidemiological, histopathological and clinical characteristics. Few previous LOH studies have focused on the correlation between genetic alterations and clinical parameters in NPC. Recently, however, studies in our laboratory found that genetic alterations at certain chromosomal regions were associated with progressive clinical parameters in NPC. LOH analysis revealed that higher-frequency allelic losses at 9p21 (56%) and/or 19q13 (50%) in NPC were correlated with primary tumor stage T3+T4 and advanced TNM stage (III+IV). High fractional allelic loss (FAL) value plus high antibody titers of EBV IgA/VCA and/or IgA/EA were significantly correlated with T3+T4 stage, distant lymph node metastasis, and advanced TNM stage in NPC. NPC patients with high titers of IgA/VCA and IgA/EA showed high frequencies of LOH on 16q (48%) and 19q13 (48%), and higher frequencies of LOH on 4q21 and 14q11-q12 were also found to be correlated with WHO type-III NPC histopathology (Shao *et al*, 2000). In a CGH study, Fang et al. reported that gain of 1q, 8q, 18q, and loss of 9q were significantly associated with advanced clinical stage in NPC (Fang *et al*, 2001). In addition, Lo et al. detected high frequency of LOH at 3p in normal NPE (73.9%) and dysplastic lesions (75%) in Southern Chinese patients,

suggesting that LOH at 3p may be an early genetic event in NPC tumorigenesis (Chan *et al*, 2000).

Plasma EBV-DNA marker In addition to genetic and environmental factors, EBV infection has also been associated with the etiology of NPC (Liebowitz, 1994; Raab-Traub *et al*, 1983). The detection of tumor-derived DNA in the plasma and serum of cancer patients suggests that polymerase chain reaction (PCR) amplification of EBV DNA may provide a feasible, minimally-invasive method for detecting and monitoring NPC. Using this method, Mutirangura et al. recently found EBV DNA in the serum of NPC patients, while Lo et al. detected circulating EBV DNA in 96% of NPC patients using real-time PCR technology (Lo *et al*, 1999b; Mutirangura, 2001).

EBV-DNA level appears to be a prognostic factor, independent of any of the above-mentioned factors, and it is thus likely that this parameter will be routinely assessed in the future, so increasing prognostic accuracy. The demonstration that tumor-derived DNA is detectable in the plasma and serum of cancer patients raises the possibility of non-invasive detection and monitoring of NPC. Using real-time quantitative PCR, cell-free EBV DNA was found in the plasma of 96% of NPC patients and 7% of controls, while patients with advanced-stage NPC had higher plasma EBV-DNA levels than those with early-stage disease (Lo *et al*, 1999b). Further studies have demonstrated that EBV DNA may be a valuable tool for monitoring NPC patient response during radiotherapy (RT) and chemotherapy, as well as for the early detection of tumor recurrence (Lo *et al*, 1999a). In a cohort of 139 NPC patients treated with a uniform RT technique and followed up for a median of 5.55 years, serum circulating EBV DNA was found to be a significant prognostic indicator associated with NPC-related death according to Cox regression analysis, with a RR of 1.6 for each 10-fold increase in serum EBV-DNA concentration (Lo *et al*, 2000b). Quantitation of EBV DNA thus appears to allow improved prognostication of NPC. The sensitivity and specificity also suggest the potential use of EBV DNA as a screening test in areas where NPC is endemic.

Histopathological Classification of NPC

NPC has a dominant clinicopathological behavior characterized by easy invasion and metastasis, which differs from other head and neck cancers (Farias *et al*, 2003). Locoregional recurrence and distant metastasis are the two major reasons for failed treatment of NPC. Prognosis is currently based primarily on clinical TNM (Tumor, Node, Metastasis) staging (Heng *et al*, 1999; Hong *et al*, 2000; Sakata *et al*, 1999), but NPC is a heterogeneous cancer, and the clinical course can vary significantly among patients with the same clinical stage, suggesting that the TNM staging system is insufficient for precisely predicting disease outcomes. It is therefore necessary to identify molecular biomarkers that can help clinicians improve the prognostic prediction and develop therapeutic interventions for NPC patients.

The current World Health Organization (WHO) histological classification system is insufficient for making a precise prognosis in NPC patients (Chan *et al*, 1998; Krueger

et al, 1981; Shanmugaratnam, 1978). The WHO classification defines NPCs as either keratinizing squamous cell carcinomas (KSCC) (2%) or non-keratinizing carcinomas (98%), with the latter subdivided into non-keratinizing differentiated carcinoma (NKDC) and non-keratinizing undifferentiated carcinoma (NKUC) (Shanmugaratnam *et al*, 1979). However, experienced pathologists have observed that NPC tumor cells exhibit obvious morphological variations; cells can be small and round, large and round, spindle-shaped, have vesicular nuclei, or be a mixtures of round and spindle-shaped cells. In light of observant these morphological heterogeneities, some pathologists have proposed a novel NPC histological classification system based on tumor cell morphology (Cammoun *et al*, 1978; Hsu *et al*, 1987; Shanmugaratnam *et al*, 1979; Sugano *et al*, 1978). However, these proposed histological classifications have not been accepted by NPC clinicians because the studies have involved limited numbers of cases, been single-center studies, or have lacked prognostic implications. Clinicians often appeal to pathologists to propose a new NPC histological classification system with improved prognostic accuracy that would permit more precisely personalized treatment.

Molecular prognostic markers could potentially be represented by changes in gene-copy number, mRNA, or protein expression levels. In the past decade, immunomarkers for tumor angiogenesis (lymphoangiogenesis) (Li *et al*, 2008; Ma *et al*, 2003), tumor cell proliferation, and apoptosis (survivin) (Ma *et al*, 2003; Taheri-Kadkhoda *et al*, 2009), and tumor microenvironment factors including EBV infection, matrix metalloproteinase (MMPs) and their regulators CD147 and Cav-1 (Du *et al*, 2009; Yip *et al*, 2006), either alone or in combination, have been reportedly correlated with prognosis in NPC patients. However, despite extensive studies, these immunomarkers have produced inconsistent results, suggesting suboptimal prognostic values. Thus some clinicopathological features or immunomarkers have only weak, or controversial, prognostic value in NPC, and more specific clinicopathological features or immunomarkers are needed to enhance the prognostic value. This hypothesis has been tested at the mRNA level, whereas there is paucity of reliable IHC markers for predicting prognosis in other malignancies (Chen *et al*, 2007; Potti *et al*, 2006).

Several supervised methods, such as decision trees, have been applied to the analysis of cDNA microarrays for refining prognosis in non-small cell lung cancer (NSCLC) (Boutros *et al*, 2009). A small subset of highly discriminating genes was recently shown to provide reliable cancer classifiers, by applying state-of-the-art support vector machines (SVM) classification algorithms, which are also effective for identifying informative features or attributes (such as critically important genes) (Spinosa & Carvalho, 2005). Using supervised SVM-based methods, we successfully developed three immunomarker-SVM-based prognostic characteristics that are closely associated with overall survival among patients with stage IB NSCLC (Zhu *et al*, 2009) . To date, supervised learning methods have not been used to develop highly predictive prognostic classifiers for NPC. To this end, we developed an immunomarker-SVM–based NPC prognostic classifier (NPC-SVM classifier) for predicting survival of patients with NPC.

AIMS OF THIS THESIS

The general objectives of this study were to characterize the morphological features of NPC; to identify common molecular markers involved in the pathogenesis and progression of NPC; and to propose an improved histological and molecular classifications of NPC.

The specific aims were:

1. To screen molecular biomarkers of loss of heterozygosity, gene expression and EBV related markers in NPC tumor. To evaluate these biomarkers (or biomarker panels) and their correlation to the clinical outcome and prognosis of the NPC patients.

2. To develop an immunomarker-SVM-based prognostic classifier for NPC (NPC-SVM-classifier) based on immunostained differentially expressed genes in NPC and the support vector machines (SVM)-based methods

3. To investigate the expression of LMP1 in NPC cells and its functional role in development and progression of NPC.

4. To propose a new histological classification system for NPC based on morphological characteristics, tumor cell differentiation, and epithelial-mesenchymal transition (EMT) morphology.

RESULTS AND DISCUSSION

Caveolin-1 and CD147 Expression in NPC (Paper I)

Cav-1 is a major structural component of caveolae, which are involved in several cellular functions, including vesicle trafficking, cholesterol homeostasis and signal transduction (Anderson, 1993; Okamoto *et al*, 1998). Reduced Cav-1 expression has been reported in ovarian cancer (Wiechen *et al*, 2001), and lung cancer (Sunaga *et al*, 2004). In contrast, Cav-1 overexpression has been observed in bladder cancer (Sanchez-Carbayo *et al*, 2002), prostate cancer (Li *et al*, 2001) and esophageal squamous cell carcinoma (ESCC) (Kato *et al*, 2002). Furthermore, recent evidence suggests a central role for Cav-1 in the regulation of cellular invasion and metastasis (Li *et al*, 2001; Lu *et al*, 2003; Williams *et al*, 2004; Zhang *et al*, 2000). Cav-1 overexpression in tumor cells has also been correlated with poor prognosis in patients with ESCC (Kato *et al*, 2002), renal clear cell carcinoma (Joo *et al*, 2004), prostate cancer (Yang *et al*, 1999), lung cancer (Yoo *et al*, 2003), and pancreatic ductal adenocarcinoma (Suzuoki *et al*, 2002). In the current study, Cav-1 overexpression in NPC tumor cells was significantly correlated with recurrence ($p = 0.038$) and metastasis ($P = 0.025$) of the disease, and with poor prognosis.

Extracellular matrix metalloproteinase inducer (EMMPRIN), also named CD147, is a glycoprotein that belongs to the immunoglobulin superfamily (Biswas *et al*, 1995). CD147 is composed of two extracellular Ig domains, a single transmembrane domain, and a short cytoplasmic domain. The first Ig domain is required for counter receptor-binding activity, which is involved in MMP induction and oligomerization, while the second Ig domain is known to associate with Cav-1 (Tang *et al*, 2004a). CD147 is enriched in the plasma membrane of tumor cells and triggers the production or release of MMPs in surrounding mesenchymal and tumor cells (Guo *et al*, 2000; Kanekura *et al*, 2002). Several recent studies have found that overexpression of CD147 is correlated with poor prognosis in human cancers, including ESCC (Ishibashi *et al*, 2004), breast carcinoma (Reimers *et al*, 2004), serous ovarian carcinoma (Davidson *et al*, 2003) and gastric carcinoma (Zheng *et al*, 2006). Overexpression of CD147 in tumor cells has been reported to be correlated with metastasis of breast cancer (Reimers *et al*, 2004) and oral squamous cell carcinoma (Bordador *et al*, 2000), and with poor prognosis in patients with ESCC (Ishibashi *et al*, 2004), breast cancer (Reimers *et al*, 2004), serous ovarian cancer (Davidson *et al*, 2003) and gastric cancer (Zheng *et al*, 2006). CD147 overexpression in NPC tumor cells was significantly correlated with metastasis ($P = 0.017$) and poor prognosis in NPC patients in the present study.

NPC patients overexpressing both Cav-1 and CD147 in tumor cells had significantly poorer prognoses and significantly lower 5-year overall survival rates relative to NPC patients with lower expression levels of both Cav-1 and CD147 (45.17% *vs.* 68.32%, $P = 0.004$). To the best of our knowledge, this represents the first report of Cav-1 and CD147 overexpression and their significance with respect to metastasis and prognosis in NPC. These results are consistent with previous studies of Cav-1 and CD147 expression in other malignancies. This is interesting in view of that these two genes

may play key roles in the invasion and metastasis of NPC, and correlate with poor prognosis in NPC patients.

The results of the current study showed that siRNA-mediated inhibition of Cav-1 expression in human NPC cell lines led to significant downregulation of CD147 protein expression (45%, CNE1; 58%, CNE2), while Cav-1 overexpression led to significant upregulation of CD147 protein expression (2.8-fold, CNE1; 1.7-fold, CNE2). Cav-1 expression positively correlated with CD147 expression in NPC tumor cells ($\rho = 0.330$, $P = <0.001$). These results indicate that Cav-1 regulates the expression of CD147 in NPC cell lines, and one of the roles of Cav-1 in NPC carcinogenesis may thus be partly mediated by upregulation of CD147 expression. Growing evidence suggests a novel association between Cav-1, CD147 and the expression or secretion of MMPs. Overexpression of Cav-1 in HEK293 cells decreased MMP-1 secretion in a co-culture assay with primary human fibroblasts (Tang & Hemler, 2004), and overexpression of Cav-1 in metastatic mammary tumor cells could inhibit MMP-2 and MMP-9 secretion, although the expression of MMP-2 and MMP-9 in whole cell lysates was not altered. In contrast, Cav-1 induced MMP-11 secretion and invasive potential in a murine hepatocarcinoma cell line (Jia *et al*, 2006). CD147 is a tumor-cell-derived MMP inducer that is expressed on the tumor cell surface and triggers the production or release of MMP-1, MMP-2, MMP-3, MMP-9, MT1-MMP and MT2-MMP in the surrounding mesenchymal and tumor cells (Guo *et al*, 2000; Kanekura *et al*, 2002; Sameshima *et al*, 2000; Tang *et al*, 2004b; Yang *et al*, 2003). The current study found that suppression of Cav-1 and CD147 expression led to decreased MMP3 and MMP11 secretion in CNE1 and CNE2 cells, whereas overexpression of Cav-1 in CNE1 and CNE2 cells promoted MMP3 and MMP11 secretion. Transwell migration assays further revealed that loss of Cav-1 and CD147 expression inhibited CNE1 and CNE2 cell-migration ability, whereas Cav-1 overexpression promoted cell-migration ability. These results indicate that Cav-1 and CD147 overexpression in NPC tumors can promote tumor-cell migration by stimulating MMP3 and MMP11 secretion by NPC tumor cells.

In summary, this study demonstrated that overexpression of Cav-1 and CD147 (MMP regulators in tumorigenesis) were closely correlated with local relapse, metastases, and poor prognosis in NPC patients. These biomarkers render tumor cells somewhat resistant to the conventional therapies including radiotherapy and/or chemotherapy. One way out of this would be to consider therapy with targeted antagonists of these molecules.

Correlation between LOH, Clinicopathological Parameters and EBV Infection in NPC (Paper II)

NPC is distinguished from other head and neck cancers by a number of epidemiological, histopathological and clinical characteristics. Certain genetic alterations in tumor cells can change their behavior, and these changes might thus be expected to be associated with certain clinical features. Cancer develops, at least in part, as a result of an accumulation of genetic alterations that disrupt the normal processes of cell growth and differentiation. It has been proposed that chromosomal

loss is often correlated with tumor histopathology, staging and other tumor clinical characteristics in a number of human cancers. A previous study showed that progression of papillary renal cell carcinoma was associated with allelic loss on chromosome 9p21 (Schraml *et al*, 2000), and LOH at chromosome 18q has been reported to be associated with poor prognosis in cancer of the cervix (Kersemaekers *et al*, 1998).

To demonstrate a comprehensive profile of LOH in NPC, we applied a large panel of 400 microsatellite polymorphism markers in 98 cases of sporadic primary NPC. Of the 335 informative markers, 83 loci showed high levels of LOH (present in more than 30% of cases) and most of the high-frequency loci were clustered to chromosomes 1p36 and 1p34, 3p14-p21, 3p24-p26, 3q25-q26 and 3q27, 4q31 and 4q35, 5q15-21and 5q32-q33, 8p22-p23, 9p21-p23 and 9q33-q34, 11p12-p14, 13q14-q13 and 13q 31-q32, 14q13-q11, 14q24-q23 and 14q32. Higher frequencies of LOH were found on chromosomes 3, 5, 9 and 11 (≥50%), while medium frequencies of LOH were found on chromosomes 1, 4, 6, 14, 17 and 19 (49–49%). Several new regions showing high frequencies of LOH were found on chromosomes 1p36, 3q25-q26, 3q27, 5q15-q21, 8p22-p23, 9q and 11p12-14 (Shao *et al*, 2000). The detailed allelic deletion map for NPC is shown in Figure 5.

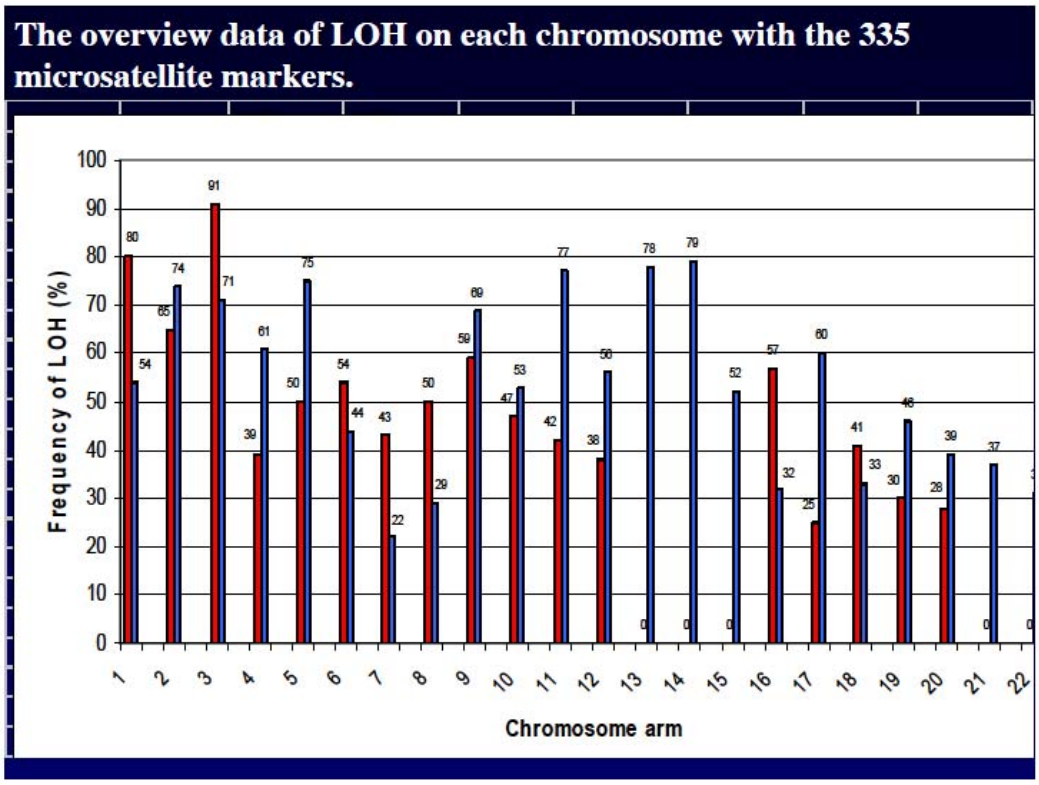


Figure 5. Detailed allelic deletion map of chromosomal arms in NPC (Shao *et al*, 2001).

Our study found a significantly higher incidence of LOH on chromosomes 9p21 and 19q13 in T3+T4-stage NPC compared to T1+T2-stage NPC, suggesting that allelic loss in these regions may correlate with the invasive progression of NPC (Figure 6A). We

also found a significantly higher mean fractional allelic loss (FAL) value (0.56 ± 0.11) in NPC stage T3+T4, compared to NPC stage T1+T2 (0.48 ± 0.1). In addition, one locus on 19q13 (D19S210) had a significantly higher LOH frequency in advanced stage (III+IV) NPC (46%, 12/26), whereas no LOH was observed in 13 cases of early-stage NPC (I+II) at this locus (p=0.002) (Figure 6C). These results suggest that the accumulation of LOH at specific chromosomal regions in NPC may result in a more aggressive population of tumor cells, which may be correlated with specific TSG inactivation and progression of NPC.

In this study, significantly higher frequencies of LOH were observed on 16q and 19q13 in NPC patients with high antibody titers of EBV IgA/VCA (≥1:640) and/or IgA/EA (≥1:80) compared to patients with low antibody titers of EBV IgA/VCA (≤1:320) and/or EA/IgA (≤1:40) (Figure 6D). In addition, tumors in the NPC group with both higher FAL values (≥0.52) and higher antibody titers of EBV IgA/VCA (≥1:640) and/or IgA/EA (≥1:80) showed more aggressive behavior at T, N, and TNM stages, compared to NPC tumors with lower FAL values (<0.52) and/or lower antibody titers of EBV IgA/VCA (≤1:320) and/or EA/IgA (≤1:40). Because both EBV infection and genetic alterations play important roles in the etiology of NPC, these data suggest a possible counteracting role of genetic factors in NPC tumorigenesis. It is possible that LOH may be a more important factor than EBV infection in the development of NPC in those patients with lower serological antibody titers of EBV IgA/VCA and IgA/EA, and with higher frequencies of LOH at specific chromosomal regions. In contrast, EBV infection may be more important than LOH in NPC tumorigenesis in patients with higher serological antibody titers of EBV IgA/VCA and IgA/EA and lower frequencies of LOH at specific chromosomal regions. The correlation between LOH (at loci with LOH frequency ≥30%) and NPC clinicopathological parameters is shown in Figure 6.

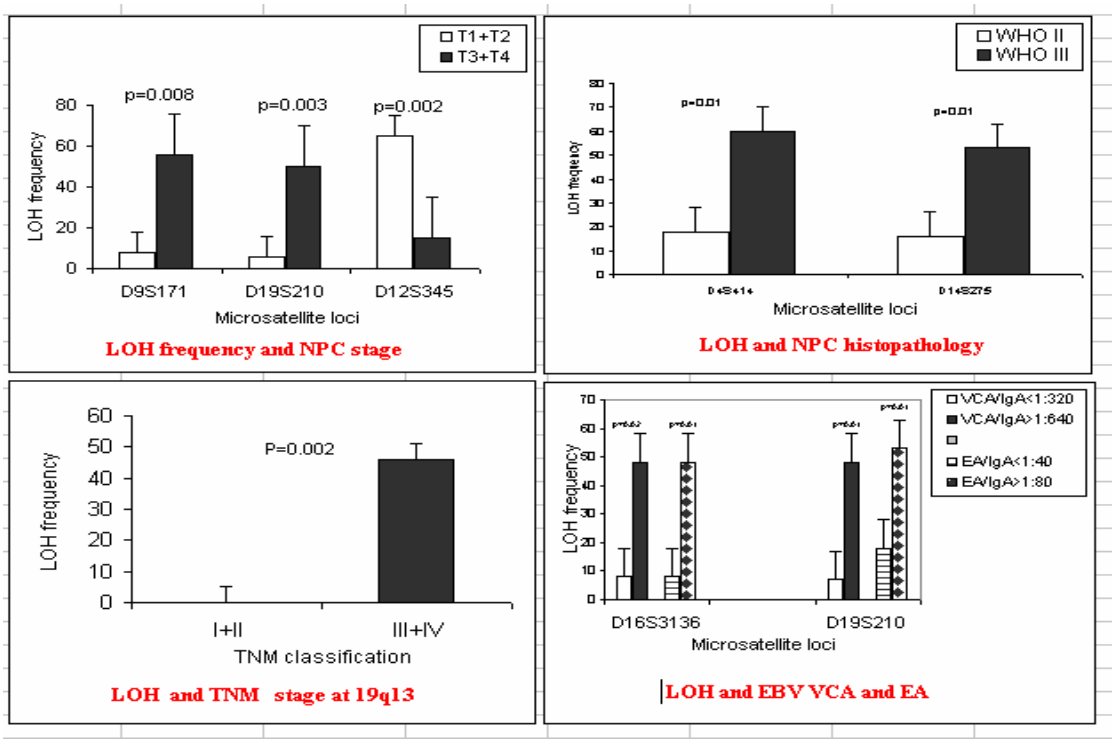


Figure 6, Correlations between LOH and clinicopathological parameters in NPC (Shao *et al*, 2001).

FAL was calculated for each tumor according to the number of autosomal arms displaying LOH, divided by the number of informative autosomal arms. FAL reflects the quantity of genetic abnormalities in each tumor (Choi *et al*, 1998; Field *et al*, 1996). The FAL values varied among the 61 NPCs, ranging from 0.23 (9/39) to 0.77 (30/39), with a median value of 0.51 and a mean value of 0.52 ± 0.12. This indicates that, on average, each tumor showed LOH on 52% of its autosomal arms. FAL values were significantly correlated with T stage in NPC, i.e., the mean FAL value was 0.56 ± 0.11 in stage T3+T4 NPC, compared to 0.48 ± 0.1 in stage T1+T2 NPC (p=0.01).

In conclusion, high frequencies of LOH (≥60%) were observed in NPC. LOH at specific chromosomal regions has been shown to correlate with a number of clinical features in NPC, i.e., aggressive and progressive behavior, histopathology, and tumor differentiation. Determination of the specific genetic markers by LOH, CGH, and linkage analysis will not only improve our understanding of the genetic epidemiology of NPC, and also provide additional indicators for earlier diagnosis and prognosis of this cancer. Moreover, accumulation of these genetic markers may be useful for the development of an NPC molecular staging system, which could improve the current clinicopathological classification and staging systems.

EBV Infection and its Relations to Apoptosis and Lymphocyte Infiltration in NPC (Paper III)

LMP1 is known to modulate several key pathways controlling transcriptional activity and cell life/death relevant to tumor cell biology, such as the nuclear factor (NF)-kB (Brinkmann *et al*, 2003), AP-1 (Kilger *et al*, 1998), JNK (Eliopoulos *et al*, 1999), Jak/STAT (Gires *et al*, 1999) and TRADD/TRAF pathways (Kieser, 2008; Schneider *et al*, 2008). LMP1 can thus protect B cells and epithelial cells from apoptosis (Fries *et al*, 1996). Furthermore, sequence variations found in the LMP1 gene in NPC tumors may reflect mutations affecting LMP1 function and/or immune surveillance, with significance for NPC tumorigenesis (Hu *et al*, 2000). In this study, we explored the expression of markers related to cell proliferation, apoptosis, immune response, stromal interactions, and EBV-gene expression in a large group of NPC biopsies, in relation to LMP1 expression, using a qualitative and semi-quantitative IHC approach.

The results demonstrated high levels of p53 expression in most NPC biopsies, together with Ki67, which correlated with LMP1 expression and a reduction in apoptosis. Thus increased p53 did not appear to lead to either cell cycle arrest or an increase in apoptosis. This suggests overexpression of a seemingly inactive p53, as has often been observed in other tumors (Levine, 1990; Lutzker & Levine, 1996)Levine et al., 1997). However, the mechanisms leading to p53 overexpression are unclear. Crook et al. (2000) found that p53-related p63 was invariably expressed in NPC biopsies in a truncated form, called the deltaN-isotype, which is

able to block p53-mediated transactivation. p63 was therefore suggested to be a suppressor of wild-type p53 function in NPC tumors, but this is not known to be LMP1-related (Crook *et al*, 2000). However, LMP1 may block p53-triggered apoptosis by induction of A20, as seen in epithelial cells *in vitro* (Codd *et al*, 1999; Fries *et al*, 1996).

In addition to measuring the apoptotic index by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, the steady state expression levels of five key regulators of apoptosis, Fas-ligand (FasL), Fas, Bcl-2, Bax, and caspase 3, were also analyzed *in situ* in NPC biopsies. FasL, Bax and caspase 3 expression may all relate to increased apoptosis, while Bcl-2 blocks apoptosis. Bcl-2 and Bax are opposing modulators of the former, while the Fas-FasL system represents the latter type of system. Both types of pathways converge to activate cleavage of caspase 3, which is the main downstream effector of many of the molecular and phenotypic effects seen in apoptosis. Our results indicated that apoptosis was significantly reduced in LMP1-expressing NPCs compared to LMP1-negative ones. As already stated, this in turn correlated with increased p53 expression, probably due to overexpression of a functionally-modified or inactive p53. This suggests that caspase 3 expression might be increased in LMP1-negative tumors, as found. However, a steady state level of caspase 3 does not necessarily have to correlate with a higher apoptotic activity, because activation requires cleavage; caspase 3 was also increased in LMP1-negative tumors with high level of FasL.

The observed negative correlation between FasL and LMP1 is interesting. LMP1 is known to modulate and interact with one of the apoptosis pathway channeled through the tumor necrosis factor (TNF)-receptor complex, because LMP1 physically binds via COOH-terminal activation regions (CTARs) to TNFR-associated death domain protein (TRADD) and tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) (Kieser *et al*, 1999). Fas-FasL operates through a parallel pathway, which overlaps with the TNF-receptor pathway downstream. In an *in vitro* transfected epithelial cell system, we observed that LMP1 may show an additive effect to Fas-mediated apoptosis (unpublished data), suggesting that LMP1-positive tumors show selection against FasL-expressing cells.

The number of $CD8^+$ T lymphocytes infiltrating within tumor cell nests was significantly higher in LMP1-positive compared to LMP1-negative tumors. $CD25^+$ and TIA-1^+ cells were not increased in the cancer nests, compared to normal tissue, indicating that the infiltrating T cells were mostly not activated, which is in agreement with the lower levels of apoptosis in these LMP1-positive tumors. However, activated $CD8^+$ cells were consistently seen in close proximity to apoptotic bodies or cells with nuclear DNA fragmentation ($TUNEL^+$), suggesting cell-mediated killing of some neoplastic target cells. This differs from the situation in Hodgkin's disease (HD), where predominantly $CD4^+$ T cells were found (Frisan *et al*, 1995). LMP1-positive cells can be targets of specific killer T cells but, as

observed in HD (Dolcetti *et al*, 1995), the T cells may be predominantly anergic. In contrast to NPC, $CD8^+$ T cells in EBV-associated gastric cancer showed a relatively high level of proliferative activity, suggesting an activated state (Kuzushima *et al*, 1999).

Blocking of apoptosis has been shown to be an important factor in tumor progression. LMP1-positive tumors showed reduced apoptosis correlated with lower caspase 3 levels, overexpression of p53, and reduced FasL expression. This may explain the prognostic difference in clinical phenotypes between LMP1-positive and LMP-negative tumors. The role of the large fraction of infiltrating T cells is unclear, but our findings suggest that they are predominantly inactive and may thus not kill the tumor cells. However, as already suggested, they may contribute locally via cytokines that could promote tumor growth. In addition, the correlation of LMP1 positivity with higher MMP-9 expression supports our earlier observation, that LMP1-positive tumors grow larger and show a higher degree of invasiveness (Hu *et al*, 1995).

As mentioned, a difference in the growth pattern and clinical course of EBV-LMP1 expressing and non expressing NPCs has been observed (Hu et al., 1995). In this study, we are not able to link the MMP-9 expression results with invasion and metastasis because of the lack of clinical data. Our results may provide one of mechanism of LMP1 function via induction of invasion by elevated expression of MMP9.

Plasma EBV DNA as a Prognostic Predictor in NPC (Paper IV)

The presence of tumor-derived DNA in the plasma and serum of cancer patients raises the possibility for an approach to minimize invasive methods in monitoring of this disease (Nawroz *et al*, 1996). Because NPC has a close association with EBV, measurement of plasma EBV DNA represents a potentially feasible method of identifying these tumors. We demonstrated that EBV DNA could be detected in the plasma of 96% of patients with primary NPC, and 100% of those with locally-recurrent and distant metastatic tumors. In contrast, EBV DNA was detected in much lower percentages in control subjects and patients with clinically-remissive NPC. These results are consistent with those of Lo et al. (Lo *et al*, 2000b; Lo *et al*, 1999b), who detected plasma EBV DNA in 96% of NPC patients. This suggests that EBV DNA could be used as a serological marker for NPC diagnosis and prognosis. In the current study, two of the control subjects had high levels of plasma EBV-DNA, and it is worth noting that both these subjects were later diagnosed with NPC by histological examination of biopsy specimens. This further suggests that quantitative analysis of plasma EBV DNA may provide a sensitive method for screening patients for NPC. This would be especially valuable in areas where this disease is endemic, such as in the Guangdong province of southern China.

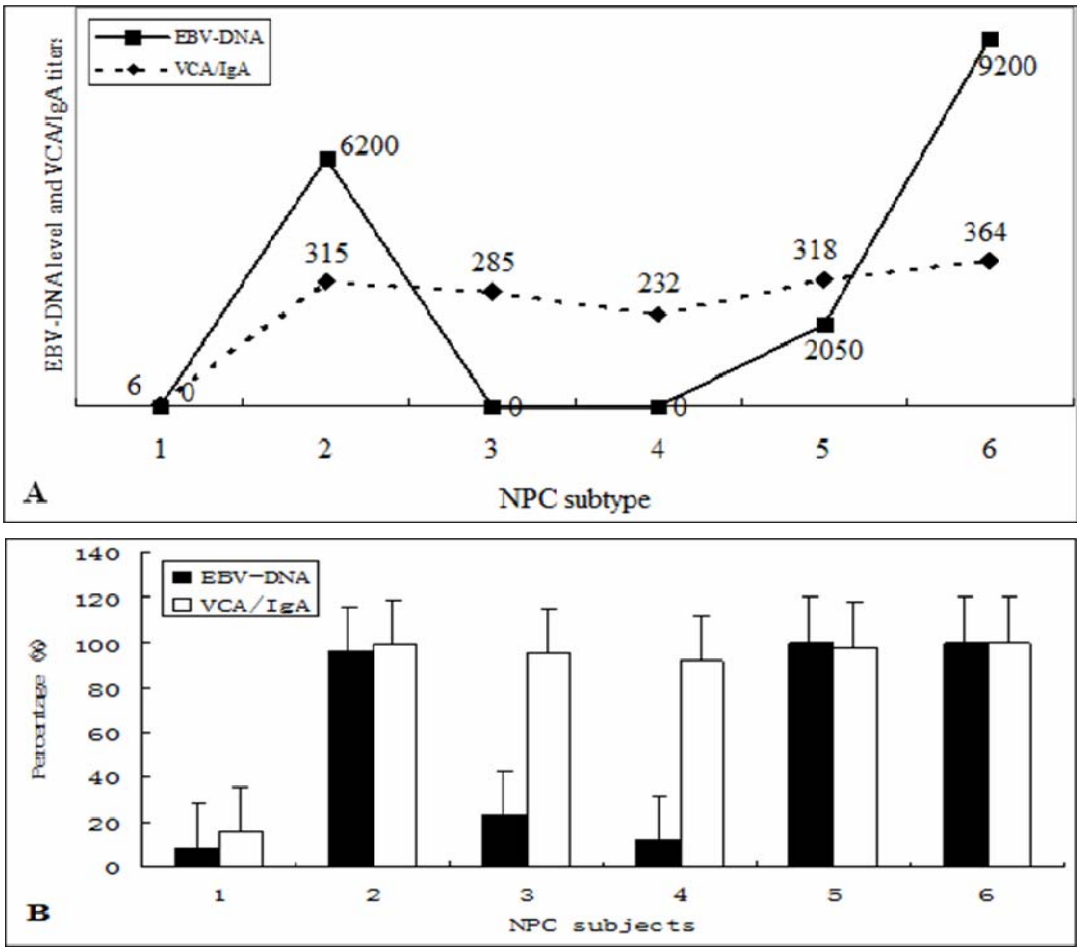


Figure 7. Variations in plasma EBV-DNA levels and EBV IgA/VCA antibody titers among different NPC subjects. Plasma EBV-DNA levels declined to 0 copies/ml in clinically-remissive NPC (subject 4) and in patients after completion of RT (subject 3), but increased to high levels after local recurrent (subject 5) and distant metastatic NPC (subject 6). However the EBV IgA/VCA antibody titer remained at high levels in all NPC groups (7A). The plasma EBV-DNA could only be detected in 23% of NPC after completion of radiotherapy and 12% of clinically remissive NPC, while all the NPC subjects maintained high IgA/VCA antibody prevalence (7B) (Shao *et al*, 2004b).

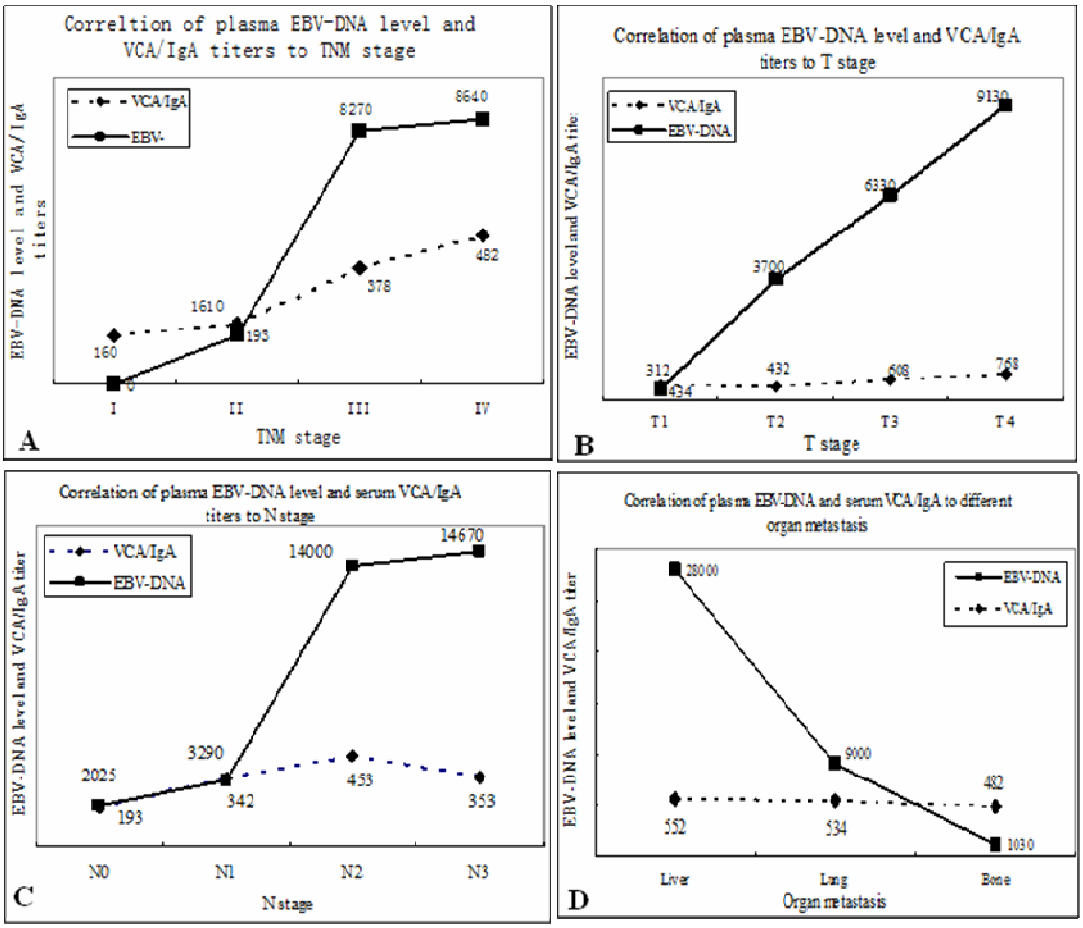


Figure 8. Plasma EBV-DNA levels and VCA/IgA titers (geometric mean) in NPC patients with different TNM stages. Plasma EBV-DNA levels increased significantly with tumor progression according to TNM stage (8A), T stage (8B), and N stage (8C). NPC patients with different organ metastases presented different plasma EBV-DNA concentrations (8D). Though VCA/IgA increased significantly in advanced TNM stage NPC, it shows no difference in different T and N stage NPC as well as in patients with distant metastasis (8A–8D) (Shao *et al*, 2004b).

Serological surveys and follow-up studies of NPC in China have been widely used to verify VCA/IgA and IgA/EA titers as valuable markers for the screening and early diagnosis of NPC (Deng *et al*, 1995; Zeng *et al*, 1983). The current results demonstrated a positive correlation between plasma EBV-DNA concentration and serum EBV VCA/IgA antibody titers in patients with primary NPCs. However, while RT and clinical remission of NPC reduced plasma EBV-DNA concentrations, in some cases to undetectable levels, EBV VCA/IgA levels remained high in these patients. These results indicate that plasma EBV DNA is a more sensitive and valuable marker than serum VCA/IgA for monitoring therapeutic effects and prognosis in NPC.

The China 92 TNM staging system has been used to determine the prognosis of NPC in China. The 5-year survival figures for stages I, II, III, and IV NPC have been reported to be 95%, 78%, 49%, and 23%, respectively (Hong *et al*, 2000; Min *et al*, 1994). We found a positive correlation between plasma EBV-DNA titer and TNM stage, as well as between plasma EBV-DNA concentration and T or N stage. In contrast, although

VCA/IgA geometric mean titer (GMT) was higher in advanced TNM stage compared to early-stage NPC, there was no significant correlation between VCA/IgA GMT and T or N stage NPC. The finding that plasma EBV-DNA concentration could be correlated with tumor progression, especially in primary tumor T stage, strongly suggests that plasma EBV DNA may be an independent prognostic indicator for staging of NPC. In addition, the strong correlation between plasma EBV-DNA concentrations and TNM stages, especially T stage, suggests that the level of circulating EBV DNA may reflect tumor burden. Thus, plasma quantitative analysis of EBV DNA may be more useful than VCA/IgA for enhancing the traditional TNM staging system at the molecular level.

NPC is a radiosensitive cancer and RT is usually the most effective treatment modality for this cancer. To date, however, there has been no accurate way of evaluating the radiotherapeutic effects on NPC. We observed that plasma EBV DNA disappeared in 77% of NPC patients after RT, and the mean concentration decreased sharply from 13,330 copies/ml prior to treatment to 0 copies/ml after completion of RT. This sharp reduction or disappearance of plasma EBV DNA in NPC patients after RT suggests that kinetic analysis of circulating EBV DNA during treatment may provide a powerful tool for evaluating the *in vivo* response of NPC to anti-neoplastic treatments.

Local tumor recurrence, regional lymph node involvement, and distant metastasis of NPC are events that result primarily from failing treatment response. It has been estimated that about 80% of NPC patients succumb to tumor with recurrence and/or distant metastases die (Chua *et al*, 2001). Thus, earlier detection of tumor recurrence or distant metastases is crucial for improving the overall survival rates of NPC patients. The detection of plasma EBV DNA in all eight NPC patients with recurrent tumors, but in only 12% of those with clinically-remissive disease, suggests that monitoring of plasma EBV DNA may provide a useful method for the early detection of tumor recurrence or metastasis. Of the seven clinically-remissive NPC patients with high plasma EBV-DNA concentrations, three were later confirmed histologically to have local tumor recurrence, while one patient had clinically-confirmed liver metastasis within 3 months of follow-up. Lo et al. (Lo *et al*, 1999b) also reported a gradual increase in serum EBV-DNA concentrations in individuals who later developed tumor recurrence. Our results thus confirm the fact that elevated plasma EBV-DNA levels precede the histological signs of tumor recurrence or progression in patients with NPC. Our investigation also enlisted 21 NPC patients with clinically-confirmed liver, brain, lung and bone metastases. The plasma EBV-DNA levels in NPC patients with liver metastases were about 3-fold higher than in those with lung metastases, and 16-fold higher than in those with bone metastases alone. These results indicate that regular assessment of plasma EBV-DNA levels in NPC patients after RT may enable earlier detection of local recurrence and distant metastasis.

EBV LMP1 Regulates Mammalian Target of Rapamycin (mTOR) Signaling-Pathway Molecules in NPC (Paper V)

The mammalian target of rapamacin (mTOR) is an evolutionarily-conserved serine/threonine protein kinase with an important role in cell growth and proliferation,

which acts through regulation of ribosome biogenesis and protein translation (Tsang *et al*, 2007). PI3K/AKT is considered a critical upstream mediator of the mTOR signaling pathway. The characterized downstream effectors of mTOR are ribosomal P70S6K, and eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1), with eIF4E dissociating from 4E-BP1 to initiate translation after 4E-BP1 phosphorylation, while P70S6K translates mRNA transcripts with a 5'-TOP motif following hyperphosphorylation by mTOR (Goberdhan & Boyd, 2009; Hay & Sonenberg, 2004).

As a well-known oncogene, one of the functions of LMP1 is to promote cell proliferation in NPC (Dirmeier *et al*, 2005; Faqing *et al*, 2005). The mTOR signaling pathway is also a major effector in cell growth, cell proliferation and cell survival, through regulation of protein synthesis, while P70S6K and 4EBP1 play particularly important roles in the growth-acceleration function of the mTOR signaling pathway (Tsang *et al*, 2007). Our findings suggest that activation of P70S6K and 4EBP1 requires LMP1, and that when these genes are phosphorylated via LMP1, activated P70S6K and 4EBP1 initiate a sequence of events that promotes protein synthesis, cell growth and proliferation. Further studies are needed to investigate the mechanism whereby LMP1 regulates mTOR signaling in NPC tumorigenesis.

Previous studies have reported the involvement of LMP1 in several signaling pathways, including NF-κB, AP-1, JAK/STAT, PI3K/AKT and ERK-MAPK (Dawson *et al*, 2008; Dawson *et al*, 2003; Mainou *et al*, 2005; Zheng *et al*, 2007). LMP1 activates the PI3K/AKT/mTOR signaling pathway in B lymphocytes (Lambert & Martinez, 2007), and the mTOR signaling pathway has been identified as a downstream component of the PI3K/AKT pathway in the LMP2A-transfected NPC cell lines HONE1 and AD/AH (Moody *et al*, 2005). The mTOR signaling pathway might also positively regulate cyclin D1 expression in NPC (Huang *et al*, 2009). In our study, microarray analysis of the NPC HONE1 cell line stably-transfected with LMP1 identified several differentially-expressed genes associated with mTOR signaling pathways. This is the first report to demonstrate that LMP1 can regulate the mTOR signaling pathway in NPC. Furthermore, LMP1 overexpression and knockdown studies confirmed that LMP1-regulated genes were involved in the mTOR signaling pathway, and that LMP1 expression was essential for the activation of p-mTOR and p-4EBP1 in NPC cell lines. In addition, our *in vitro* studies found that LMP1 expression was positively correlated with overexpression of p-mTOR, p-P70S6K and p-4EBP1 in NPC tumors.

Deregulation of the mTOR signaling pathway has been reported in many malignancies, and some of the signaling molecules in this pathway are predictors of prognosis in different types of cancers. Cytoplasmic p-mTOR expression correlates with poorer survival in gastric cancer and cervix adenocarcinoma (Faried *et al*, 2008; Murayama *et al*, 2009), while high expression of p-mTOR, p-P70S6K and p-4EBP1 correlate with poor outcome in glioblastoma (Pelloski *et al*, 2006). Our results revealed that NPC patients with high p-P70S6K and p-4EBP1 expression levels had a significantly shorter overall survival than those with low p-P70S6K ($p = 0.049$) and p-4EBP1 ($p = 0.010$) expression levels. These results are in accordance with previous studies on

malignancies. High expression levels of p-P70S6K and p-4EBP1 in NPC tissues might result in high levels of protein synthesis and cell proliferation, as well as poor prognosis in NPC patients.

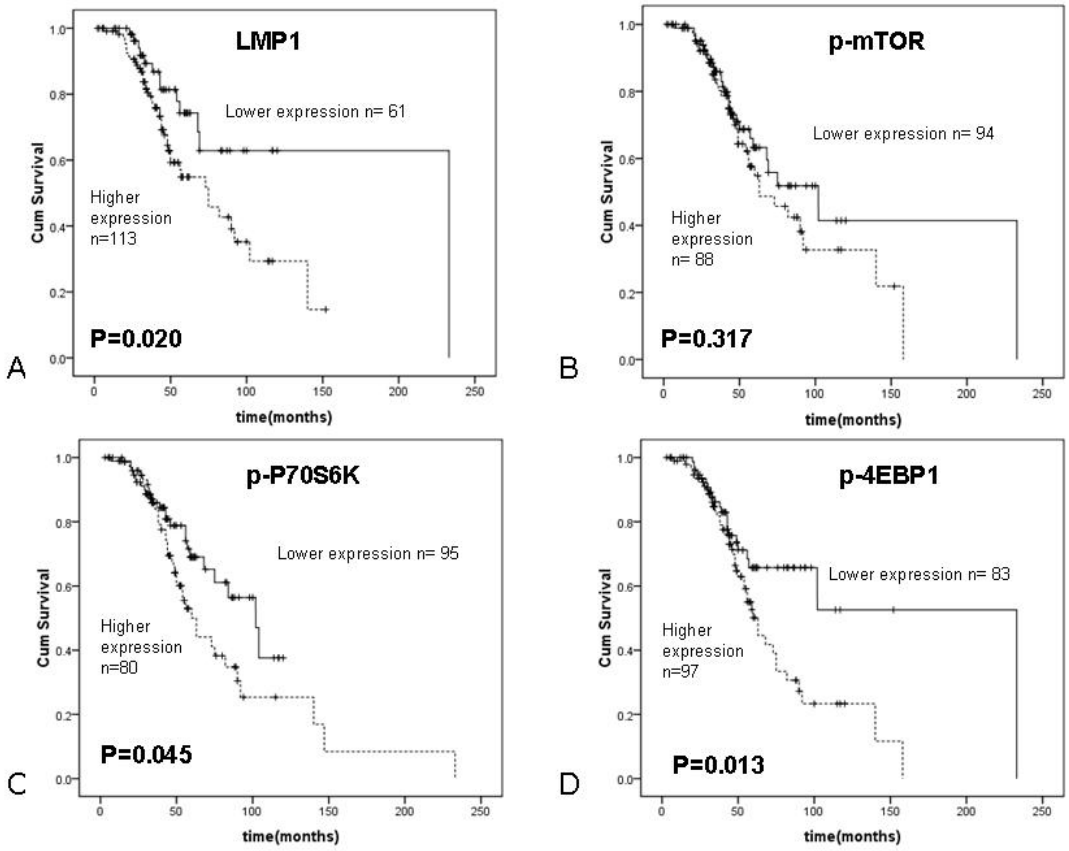


Figure 9. Kaplan-Meier curves of overall NPC patient survival. A, Five-year overall survival rates were 54% in patients with NPC tumors expressing high levels of LMP1 (n = 141), and 68% in patients with low LMP1 (n = 83) (p = 0.020). B, Five-year overall survival rates were 55% in patients with NPC tumors expressing high levels of p-mTOR (n = 109), and 62% in patients with low p-mTOR (n = 114) (p = 0.311). C, Five-year overall survival rates were 49% for patients with NPC tumors expressing high levels of p-P70S6K (n = 106), and 69% for patients with low p-P70S6K expression (n = 118) (p = 0.049). D, Five-year overall survival rates were 49% in patients with NPC tumors expressing high levels of p-4EBP1 (n = 128), and 71% in patients with low levels of p-4EBP1 (n = 95) (p = 0.010).

In this study, IHC staining of LMP1 was performed in a large sample of NPC cases, and LMP1 overexpression was detected in 62.9% (141/224) of NPC tumors, in accordance with previous studies (Horikawa *et al*, 2001; Jeon *et al*, 2004; Shao *et al*, 2004a). Interestingly, LMP1 overexpression was significantly associated with poorer overall survival in NPC patients (p = 0.020). This result differed from previous reports, which found that LMP1 overexpression was associated with a better prognosis in NPC patients (Hu *et al*, 1995), or that LMP1 was not an effective indicator of NPC outcomes (Sarac *et al*, 2001). These differences might be attributable to different sample sizes, regional distributions, or different LMP1 variants. Although high expression levels of LMP1, p-P70S6K and p-4EBP1 were associated with poor survival in NPC patients,

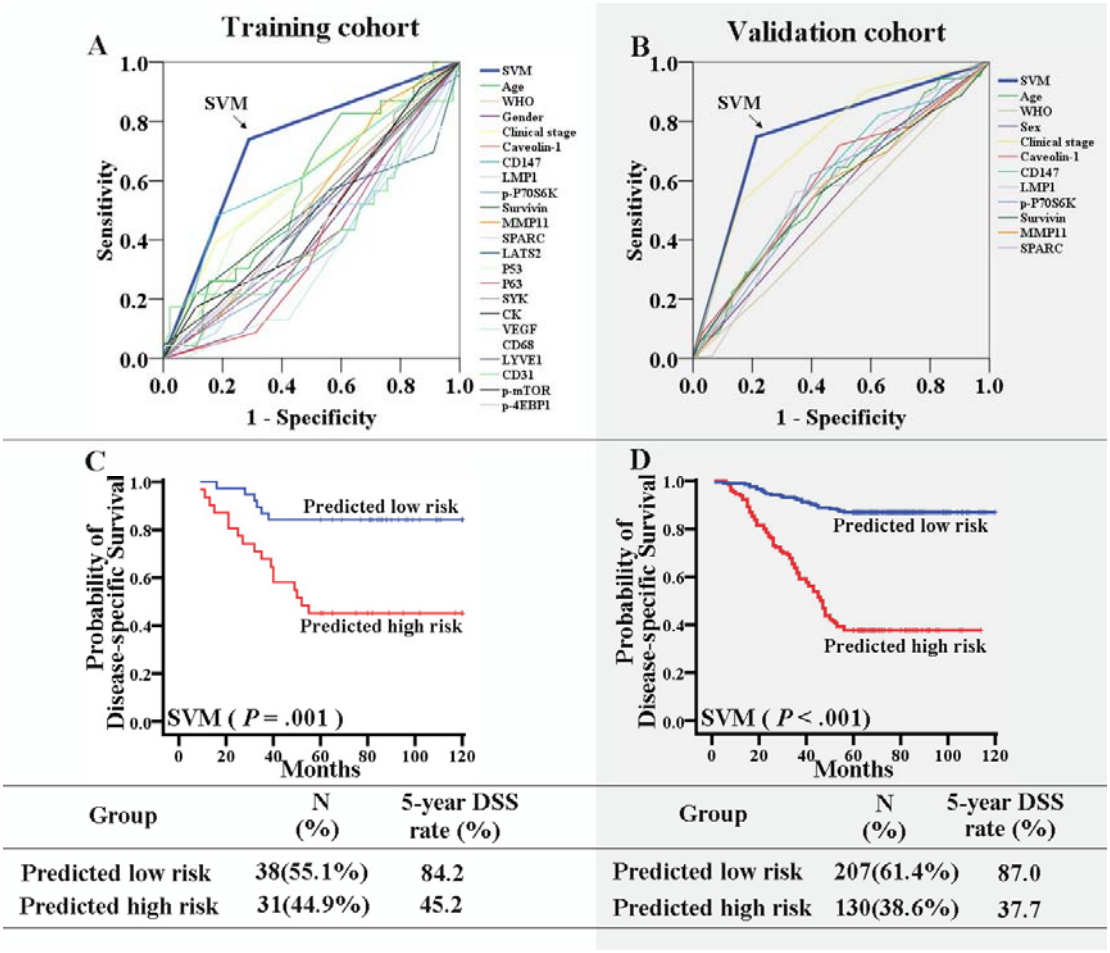
multivariate analysis only identified LMP1 expression, as well as gender ($p = 0.014$) and metastasis ($p = 0.003$), as independent prognostic factors. The mTOR signaling pathway was triggered by LMP1, supporting that LMP1 may have a more important role than mTOR signaling molecules in the carcinogenesis and development of NPC.

Molecular Classification of NPC (Paper VI)

SVM has recently shown promise for the analysis of microarray data. Compared to other machine-learning algorithms such as decision trees, artificial neural networks, and nearest-neighbor classifiers, SVM is well suited to managing classification problems, including high-dimensional data and limited number of training samples. Another important use of SVM is to select several efficient features from all available features (Vapnik & Chapelle, 2000; Vapnik, 1999). A single gene expression does not have enough predictability power. Genes are not independent from each other; only with several genes can we obtain satisfactory and reliable prediction of prognosis. With SVM, clinicopathological features can be combined with predominant genes to predict the outcome of patients. Moreover, it also excludes the problem of cut-off point of immunomarkers.

The ability to treat patients according to their molecular characteristics is becoming a trend in cancer research (Boutros *et al*, 2009; Potti *et al*, 2006; Sotiriou & Piccart, 2007). NPC is a clinically heterogeneous disease and outcomes vary even among patients with similar clinical stages; some are cured, whereas the cancer recurs in others. In our study, we developed an immunomarker-SVM-based NPC prognostic classifier (NPC-SVM classifier) for predicting survival in NPC patients. The reported NPC-SVM classifier integrates seven genes: SPARC, survivin, MMP11, caveolin-1, CD147, LMP1, and p-P70S6K, together with gender. The NPC-SVM classifier was closely correlated with clinicopathological outcomes in NPC patients. These results suggested that the NPC-SVM-classifier of NPC could select powerful factors predictors of prognosis of NPC patients.

The developed eight-signature NPC-SVM classifier can categorize NPC patients into high-risk and low-risk groups with significantly different prognoses in terms of 5-year DSS. This classifier also has high sensitivity (79.5%) and specificity (82.4%) for predicting survival of NPC patients. These results indicate that the NPC-SVM classifier can better reflect the nature of disease progression in NPC patients. Moreover the NPC-SVM classifier retained significant prognostic value after stratification by gender, clinical stage, age, and therapeutic method, except in NPC patients with early-stage tumors, and in patients with radiochemotherapy (RCT) treatment. These results provide clinicians with a credible, applicable molecular classification for better prognostic prediction in NPC patients. This represents the first large-sample study on the molecular classification of NPC.



Group	N (%)	5-year DSS rate (%)
Predicted low risk	38(55.1%)	84.2
Predicted high risk	31(44.9%)	45.2

Group	N (%)	5-year DSS rate (%)
Predicted low risk	207(61.4%)	87.0
Predicted high risk	130(38.6%)	37.7

Figure 10. Receiver operating characteristic (ROC) curves and Kaplan-Meier survival estimates of the predicting NPC cases from both the training and validation cohorts. ROC curves for molecular markers, age at diagnosis, WHO histological classification, gender, clinical stage, and the NPC support vector machine (NPC-SVM) classifier as predictors of death from NPC within 5 years in the training cohort (A) and the validation cohort (B). Kaplan-Meier survival estimates for low-risk and high-risk NPC patients as defined by the NPC-SVM classifier. DSS curves of the predicting patients in the training cohort (C) and the validation cohort (D. The log-rank test was used to calculate *p* values(Wang *et al*).

Studies have consistently failed to show that distinguishing between NKDC and NKUC NPC subtypes of NPC (98% of NPC in endemic area of Southern China) has any clinical relevance (Cho, 2007). However, the NPC-SVM classifier can easily identify NPC patients with a good or bad prognosis, and will thus make a valuable complement to the WHO classification. The NPC-SVM classifier provides a new strategy and approach for optimal clinical decisions. With this prognostic tool, clinicians can identify low-risk NPC patients (91% of early-stage and 59% of advanced -stage patients), and give them mild treatment to avoid unnecessary radical therapy. In contrast, higher-dose radiation, adjuvant therapy, or molecular targeted therapy may have additional therapeutic effectiveness in high-risk patients (9% of early-stage, and 41% of advanced-stage patients).

These results should be interpreted in the light of some limitations, including the limited number of genes screened in the training cohort, which in turn resulted in a smaller panel of genes integrated in the NPC-SVM classifier than in some other gene-expression-profiling studies by cDNA array in other cancers (Dave *et al*, 2006; Liu *et al*, 2006; Sanchez-Carbayo *et al*, 2006). Although the NPC-SVM classifier integrated seven informative genes and was a highly accurate predictor of DSS, the inclusion of other molecular markers may increase the precision and prognostic value of the classifier. In addition, new markers are being found and new techniques developed every year. Thus, the NPC-SVM classifier may be further improved by including expression data for additional genes. Other limitations of the study included incomplete follow-up for some patients and the inclusion of cases predominantly from a single clinical center in southeastern China, which may have reduced the validity of generalizing from our results. Further prospective studies are therefore needed to validate these results, including prospective studies with complete patient follow-up, as well as studies in other NPC-endemic areas, such as northern Africa, and in non-endemic populations. Further studies on high-risk patients are needed to test the efficacy of more radical treatments and therapies targeted at the angiogenesis pathway, with the potential for opening up a new era of NPC treatment.

In conclusion, the present study demonstrated that IHC- and SVM-based approaches can be used to distinguish accurately between NPC patients with substantially different clinical outcomes, even after adjustment for clinical stage, histological subtype, and age. Thus, the NPC-SVM classifier offers considerable improvement over existing methods for the prognostic classification of NPC patients, and has the potential to provide clinicians with useful, readily available information for personalizing therapy aimed at NPC targets.

New Histopathological Classification of NPC (Manuscript)

Many classification schemes have been proposed for NPC since the early 20th century. The latest classification proposed by the WHO divides NPC into two main types:-non-keratinizing carcinoma and KSCC. In practice, however, there is a gradual transition between these two histological types and the prognostic significance of the subdivision remains unclear. Though that the WHO classification happened to correlate pretty well with EBV-positivity, type III being EBV positive and type I EBV-negative. NPC is, however, a heterogeneous malignancy, and clinical outcome can vary substantially among patients at the same clinical stage. Limitations of the WHO classification therefore include unclear boundaries between the categories and insufficient prognostic value.

In this multicenter study, we proposed a new histological classification for NPC based on tumor cell morphology, cell differentiation, and epithelial-mesenchymal transition (EMT) marker expression. We enrolled 3,839 previously untreated patients with biopsy-proven NPC from 40 clinical centers, who were treated at nine cancer centers between January 1995 and December 2005. According to their morphological features**,**

NPCs could be histologically classified into four subtypes: undifferentiated epithelial cell carcinoma (UECC), undifferentiated mixed epithelial- sarcomatoid cell carcinoma (UESCC), undifferentiated sarcomatoid cell carcinoma (USCC), and squamous cell carcinoma (SCC). Representative features of the novel NPC classification subtypes are shown in Figure 11**.**

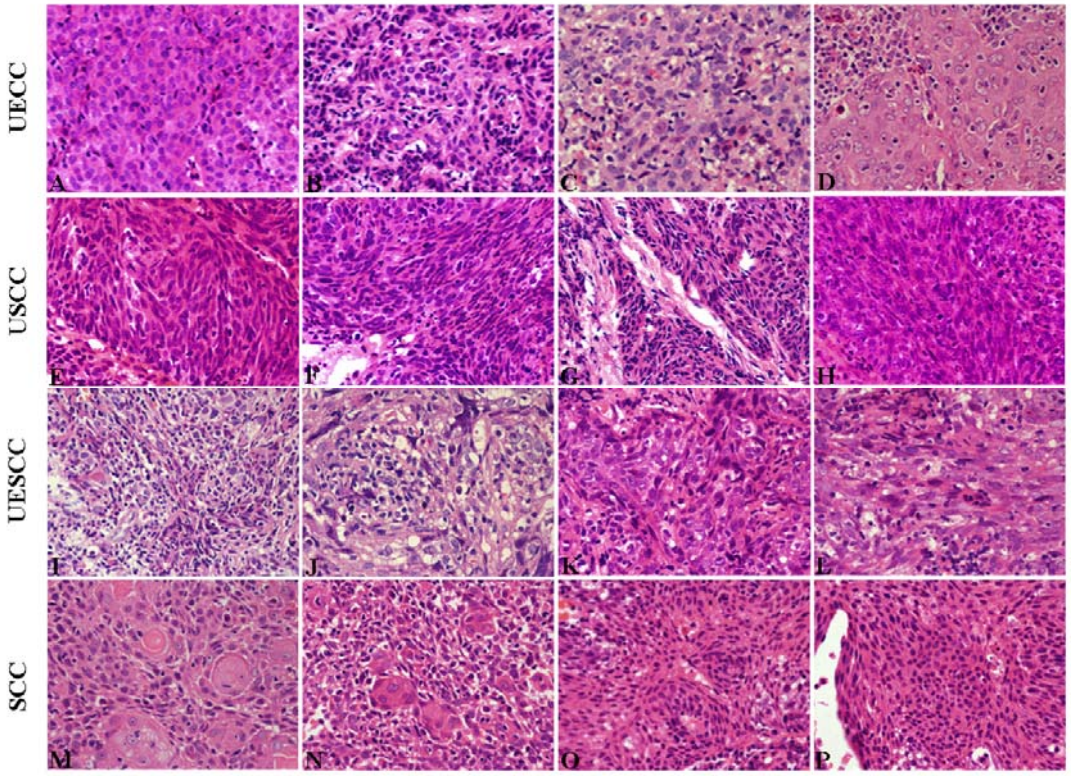


Figure 11. Representative morphological features of novel NPC classification subtypes (all stained with hematoxylin and eosin (H&E), ×400.)

UECC subtype (Figure 11A–D). This phenotype is characterized by smaller, round tumor cells showing cellular stratification and pavementing, often with a lower nuclear-cytoplasmic ratio, chromatin-rich nuclei, and unprominent nucleoli; or larger, round cells, characterized by syncytial-appearing large tumor cells with indistinct cell borders, round-to-oval vesicular nuclei, and large central nucleoli; or a vesicular nuclei carcinoma phenotype, in which more than 75% of the tumor cells are characterized by a round shape with vesicular nuclei and prominent nucleoli.

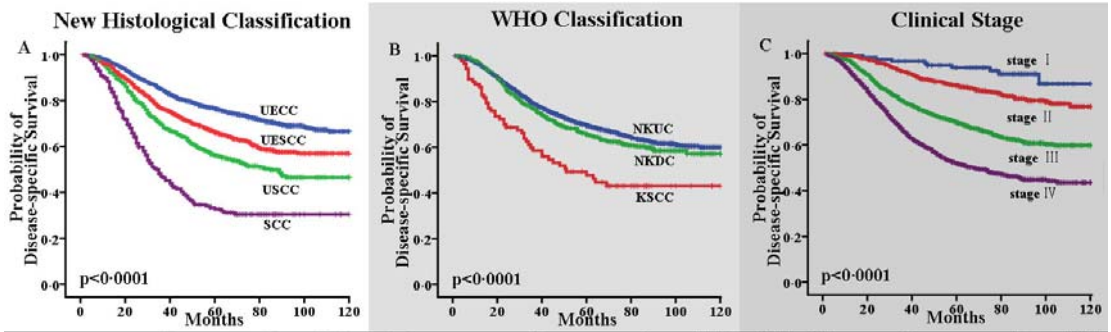
USCC subtype (Figure 11E–H). More than 50% of the tumor cells in this subtype are spindle-shaped, fusiform, or in interlacing bundles (fibrosarcomatous pattern). The tumor is composed of irregular small cells, large hyperchromatic cells, or both; or else uniformly medium-sized spindle cells. The nucleoli of the spindle cells are often not as prominent as in the syncytial-appearing cells. In some cases, tumor cells appear shrunken with dark, smudged nuclei and a dense amphophilic or eosinophilic cytoplasm.

UESCC subtype (Figure 11I–L). Morphologically, this subtype shows large, round cell nests or scattered large, round cell infiltrates in the spindle cell carcinomatous

tissue (30–50%). In most cases, no obvious boundaries are seen between the tumor and interstitial lymphoid tissue.

SCC subtype (Figure 11M–P). Well-differentiated keratinizing SCC is diagnosed when a large number of whorls and keratin are present, and low- and moderately-differentiated SCC is diagnosed when a number of single-cell, keratinized spine cells and a small number of basal-like cells are visualized.

According to the new NPC classification category, of the 3,839 tumors, 2,057 (53.6%) were classified histologically as UECC, 942 (24.5%) as UESCC, 640 (16.7%) as USCC, and 200 (5.2%) as SCC. The 5-year disease-specific survival (DSS) rates in NPC patients differed clinically and significantly among the newly-defined NPC histological subtypes, with 5-year DSS rates of 76.4% for UECC, 66.0% for UESCC, 56.0% for USCC, and 32.7% for SCC. Thus, the minimum difference in 5-year DSS between newly defined histological subtypes was 10%, while a more substantial difference of 20.4% was detected between UECC and USCC, which together comprised 70% of patients in the cohort. Using the WHO classification, the 5-year DSS rates were 69.5% for NKUC, 65.5% for NKDC, and 47.8% for KSCC. Thus, 5-year DSS differed by only 4% ($p = 0.06$) between the two WHO histological subtypes that together accounted for 98% of patients in the cohort. Using the TNM clinical staging system, the 5-year DSS rates were 93.9% for stage I, 86.0% for stage II, 69.7% for stage III, and 51.9% for stage IV.



Subtype	N (%)	5-year DSS rate (%)
UECC	2057(53·6%)	76·4
UESCC	942(24·5%)	66·0
USCC	640(16·7%)	56·0
SCC	200(5·2%)	32·7

Subtype	N (%)	5-year DSS rate (%)
NKUC	3203(83·4%)	69·5
NKDC	548(14·3%)	65·5
KSCC	88(2·3%)	47·8

Clinical stage	N (%)	5-year DSS rate (%)
Stage I	119(3·1%)	93·9
Stage II	865(22·7%)	86·0
Stage III	1637(43·0%)	69·7
Stage IV	1186(31·2%)	51·9

Figure 12. Kaplan-Meier estimates of DSS curves for NPC patients. DSS curves for NPC patients according to newly-defined histological subtype (panel A); according to WHO subtype (panel B); and according to clinical TNM stage (panel C).

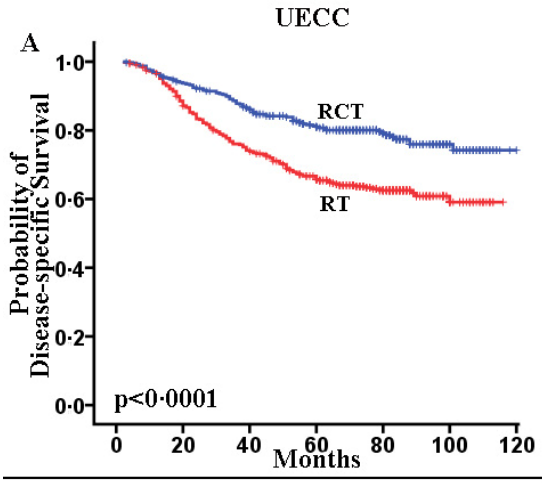
The limitations of the current WHO classification in terms of its clinical application include boundaries between the categories that are not always clear, sub-optimal intra- and inter-observer reproducibilities, (Shanmugaratnam, 1978; Shanmugaratnam, 1980) and insufficient prognostic power (Hsu *et al*, 1987; Liu & Yeh, 1998; Yu *et al*, 2005). Considerable controversy exists over the available histological NPC classifications and their prognostic significance (Baker & Wolfe, 1982; Cellai *et al*, 1982; Hua *et al*, 2009; Neel *et al*, 1985; Shanmugaratnam *et al*, 1979; Zhang *et al*, 1989). The WHO KSCC subtype is identified in one-third to one-half of NPCs in low-risk Western populations,

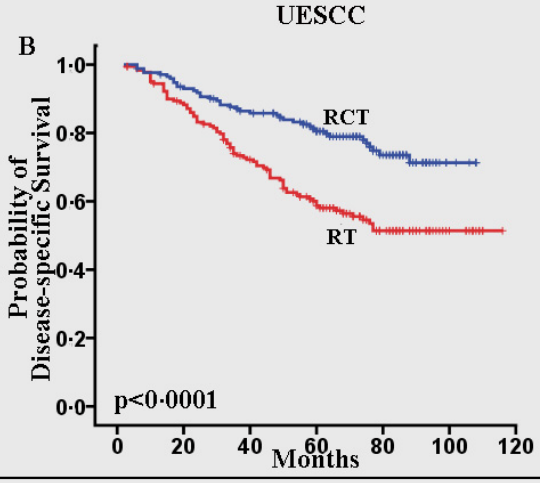
and is reportedly associated with a significantly poorer prognosis than non-keratinzing carcinoma (Fu, 1980; Mesic *et al*, 1981; Neel *et al*, 1985). However, studies have consistently failed to show that the distinction between NKDC and NKUC has any clinical relevance. These two subtypes are identified in 98% of NPC in endemic areas of China (Yu & Yuan, 2002). In this multicenter study with a large sample size, the proposed new histopathological classification of NPC showed significant differences in clinical outcomes and survival among the different groups. The UECC NPC subtype had the best prognosis, with lower rates of loco-regional recurrence and distant metastasis events, and higher 5-year DSS rates. The USCC and UEMC subtypes had poorer prognoses, and the SCC NPC subtype showed the worst prognostic outcome, similar to the WHO classification. Although the 5-year DSS rates for SCC NPC were significantly different from those for non-keratinizing NPC, they were not significantly different between the NKDC and NKUC subtypes of WHO classification. After stratifying by gender, clinical stage, age, and therapeutic method, the proposed 2010 NPC classification system retained significant prognostic value. The results provide clinicians with an applicable histological classification for better prediction of prognosis in NPC patients. To our knowledge, this is the first multicenter, large-sample study on the histological classification of NPC.

Our results demonstrated that spindle-shaped NPC tumors (USCC and UESCC) showed significant loss of E-cadherin expression and increased expression of the mesenchymal markers N-cadherin, CD44v6, Twist, Snail and cyclin D1, suggesting the occurrence of an EMT process during the development and progression of NPC; the spindle-shaped cells in USCC and UESCC tumors have an EMT phenotype. The acquisition of EMT characteristics in USCC and UESCC NPC may give the tumor cells a higher invasive potential, as extensive reductions in E-cadherin expression may lead to the loss of cell-cell adhesion, resulting in invasive and metastatic behavior. Indeed, acquisition of EMT-marker expression and reduction of E-cadherin expression in NPC were closely correlated with USCC and UESCC subtypes, local recurrence, distant metastasis, and advanced NPC stage. In addition, patients with EMT-marker overexpression and loss of E-cadherin expression had significantly poorer prognoses. These results regarding EMT events in NPC have been confirmed by recent studies (Horikawa *et al*, 2007; Lin *et al*, 2009; Song *et al*, 2009). Combining EMT morphological characteristics and EMT-marker expression analysis will provide new insights and approaches for improved prediction of recurrence, metastasis, and prognosis. Further studies are needed to determine if the results represent EMT or merely EMT-like expressional changes, and to identify the mechanism of EMT in NPC progression and development.

Although a number of improvements have been achieved in terms of both the technology and equipment for RT, treatment outcomes remain disappointing for advanced-stage disease treated with RT alone. The landmark Intergroup 00-99 study, as well as other studies, demonstrated a clear survival benefit of RCT for patients with stage III–IV disease (Baujat *et al*, 2006). In accordance with the previous trails, our retrospective study confirmed the beneficial effect of RCT compared to RT alone in

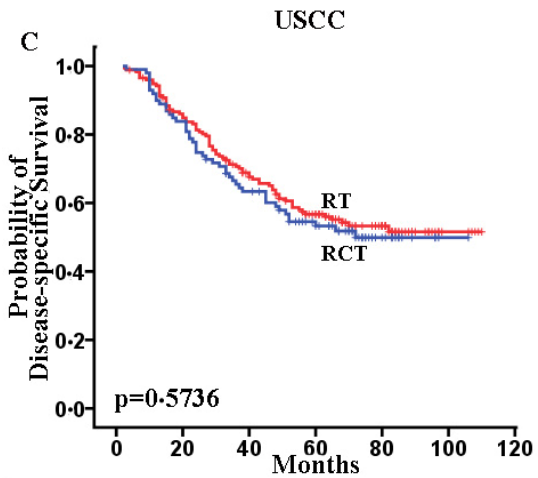
advanced-stage disease. Interestingly, UECC and UESCC subtypes showed significantly better survival outcomes with RCT than with RT alone, even after mutual adjustment. Meanwhile, the USCC and SCC subtypes showed inferior survival outcomes, and RCT treatment had no additional therapeutic gains in this subtype. These results indicate that the new histological classification system provides a new strategy and approach for optimal clinical decision making. Based on this classification, clinicians can avoid using unnecessary radical therapy in low-risk NPC patients identified with the UECC subtype (54% of NPC), whereas high-risk NPC patients with the USCC (17% of NPC) or SCC subtype (5% of NPC) may be more effectively treated with higher-dose radiation, adjuvant therapy, surgical operation, or molecular targeted therapy on the EMT markers, which may yield additional therapeutic gains. However, data from clinical trials are needed to confirm if different treatment regimens are appropriate for the various histological subtypes classified according to our new system.

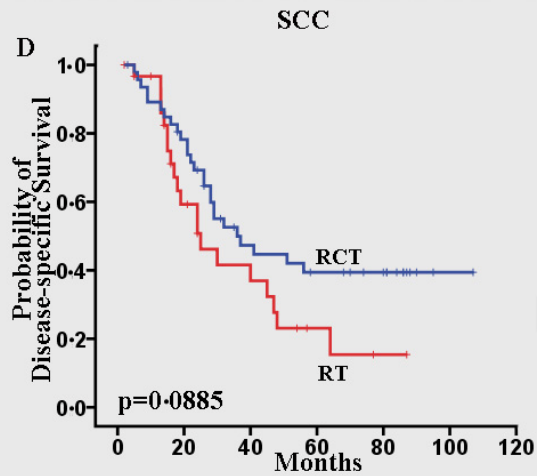




Subtype	N (%)	5-year DSS rate (%)
RT	433(52·0%)	66·6
RCT	399(48·0%)	81·3

Subtype	N (%)	5-year DSS rate (%)
RT	181(51·3%)	59·9
RCT	172(48·7%)	81·1





Subtype	N (%)	5-year DSS rate (%)
RT	174(63·7%)	56·5
RCT	99(36·3%)	54·2

Subtype	N (%)	5-year DSS rate (%)
RT	31(39·7%)	22·0
RCT	47(60·3%)	39·3

Figure 13. Kaplan-Meier estimates of DSS curves stratified by the new classification in advanced stage NPC patient. DSS curves of NPC patients according to therapy model for NPC patients: UECC subtype (panel A); UESCC subtype (panel B); USCC subtype (panel C); and SCC subtype (panel D).

To the best of knowledge, the present study represents the first large, multicenter study to propose a new NPC histological classification system that can significantly distinguish among NPC subtypes by prognosis and EMT-marker expression levels. These results should be further validated by prospective studies with complete patient follow-up, as well as studies in other NPC-endemic areas, such as northern Africa, and in non-endemic populations. Although the novel NPC classification was correlated with EMT markers, we are aware that other molecular evidence may further extend the precision and prognostic value of the classification system.

In conclusion, our proposed novel NPC classification system reliably distinguishes between prognostically and clinically-distinct groups of NPC patients more effectively than the existing WHO classification. This study provided a good opportunity to investigate the efficacy of RCT in different subtypes of NPC based on the new classification system, and may herald a new era of NPC treatment with improved outcomes.

CONCLUSIONS

1. Overexpression of Cav-1 leads to CD147 upregulation in NPC, both of which are associated with tumor recurrence, metastasis, and poor prognosis. Collectively, detection of expression levels of these biomarkers might aid in the stratification of NPC, and these biomarkers may represent new potential therapeutic targets in NPC.
2. LMP1 expression is essential for the activation of the mTOR signaling pathway in NPC. LMP1 activates the AKT/mTOR/P70S6K/4EBP1 axis in NPC tumors, and high expression levels of LMP1, p-P70S6K and p-4EBP1 predict poor prognosis in NPC patients.
3. Plasma EBV DNA is a more sensitive and specific marker than EBV VCA/IgA antibody. Thus, measurement of plasma EBV DNA may be used to enhance clinical staging, as well as for early diagnosis, monitoring local recurrence and distant metastasis, and predicting prognosis in NPC.
4. The developed NPC-SVM classifier based on immunomarkers is closely associated with overall survival among patients with NPC, and can reliably predict prognosis in NPC patients. The results will provide clinicians with useful information for personalizing therapy aimed at NPC targets.
5. The proposed novel NPC histological classification system based on morphological features is more accurate than the existing WHO classification for predicting prognosis in NPC patients. Further studies on the application of this novel NPC classification to identify high-risk patients with USCC and SCC subtypes, and to test the efficacy of more radical treatment and therapies targeted at the EMT pathway, may open a new era of NPC treatment.

SUMMARY AND FUTURE PROSPECTS

With recent advances in genomics, proteomics and bioinformatics in recent decades, more understanding of the disease etiology, carcinogenesis and progression has been gained in NPC. Despite the crucial role of EBV in NPC, the cellular genes regulated by EBV are of particular interest, as they may serve as specific tumor markers and targets for novel therapy strategies. Research into the genetic alterations, gene expression, EBV and its regulated cellular genes in NPC may unravel the pathways in NPC development and potentially decipher the molecular characteristics of the malignancy. In the era of molecular medicine, specific treatment to the potential target using technologies such as targeted gene therapy, immunotherapy and RNAi becomes formulating from bench to bedside application and thus makes molecular biomarker discovery more meaningful for NPC management. The molecular biomarker discovery and progress in NPC reported in this thesis can be potentially implicated with respect to the diagnosis, monitoring, treatment and prognostication of the disease. This is summarized and presented in Figure 14.

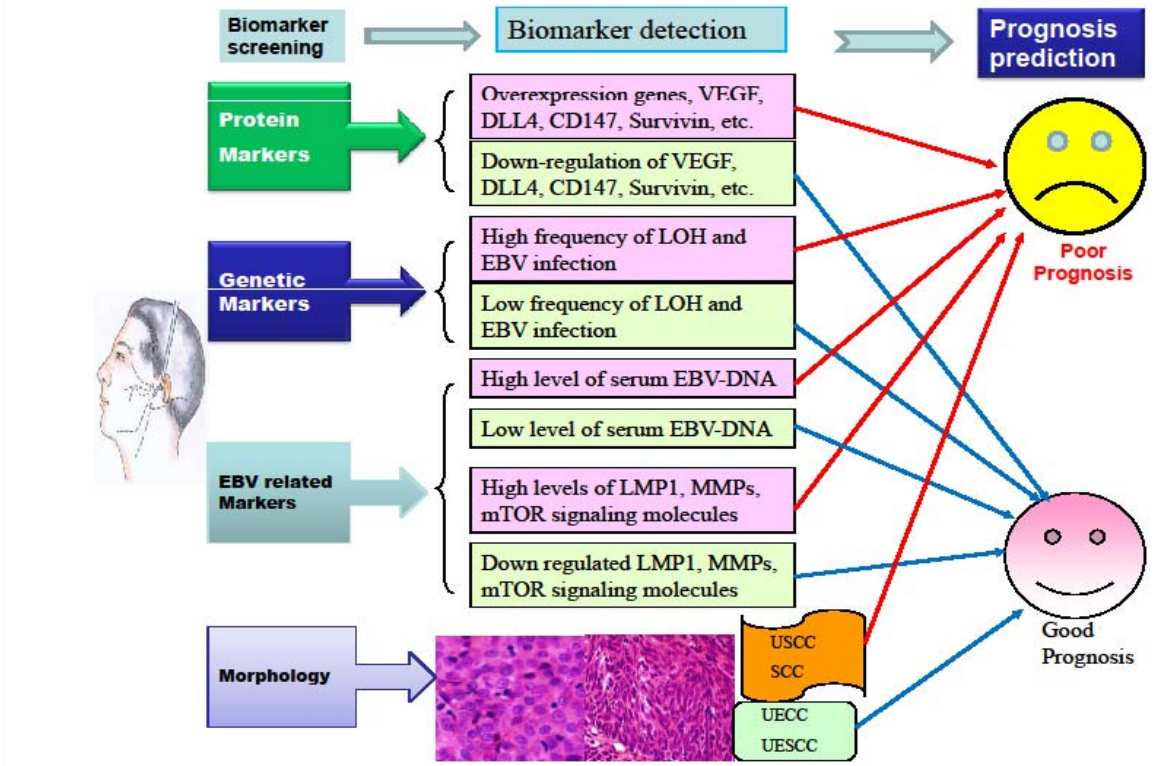


Figure 14. Biomarkers screening and their prognostic prediction in NPC

For NPC treatment, major challenges remain in improving the survival rate of patients with advanced and recurrent diseases. In this thesis, the newly developed NPC-SVM classifier based on tumour associated biomarkers will facilitate patient counseling and individualize management of NPC patients. The newly proposed novel histological classification will offer more discriminative prediction of NPC

prognosis than the current WHO classification and therapeutic efficacy of RCT and RT on advanced-stage NPC patients. The two classifications enable the clinician to identify high-risk patient, and to give more effective therapeutic approaches may brighten up the outcome of NPC patients with poor prognosis. The reclassification of NPC and its implication in the personalized treatment of the patients is summarized in Figure 15. Ultimately, the mission of clinical researchers is to find the best way of applying these new strategies into clinical practice, and in this regard one cannot overemphasize the importance of enlisting multicenter or multinational collaboration in the validation of promising therapies.

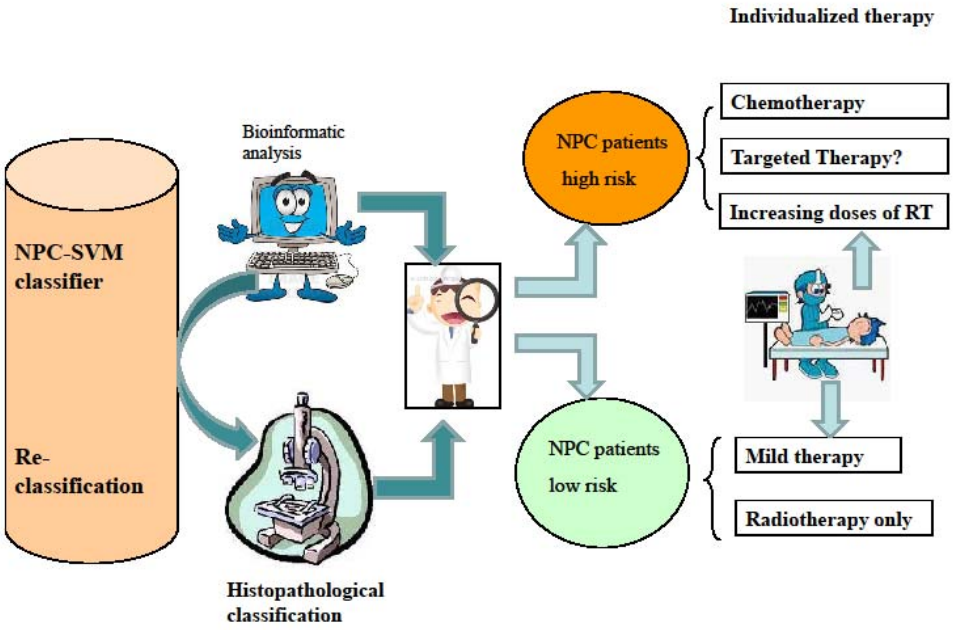


Figure 15. NPC reclassification and its implication in personalized medicine

ACKNOWLEDGEMENTS

The Master (Confucius) said, “Whenever I am with several people, there must be teachers for me among them”. I write this sentence here can only express a small portion of what I would like to convey to so many people, who have contributed to this thesis, directly or indirectly. I would particularly like to thank the following.

Foremost thanks go to Ingemar Ernberg, my main supervisor and head of the research group, for welcoming me to his research group and providing me with exceptional Ph.D. training; for sharing his scientific intellect and enthusiasm and for contributing to my personal and scientific development; for his kindness to my family and my lovely daughter JiaJia; for his friendship with China and his great contribution to scientific cooperation between the Karolinska Institutet and universities and laboratories in China.

Thanks also to Li-Fu Hu, my co-supervisor, for many important comments; for having significantly improved my scientific writing; for introducing me to Stockholm; for support and guidance throughout my Ph.D. work; and more importantly, for our friendship.

Thanks to Yi-Xin Zeng, my supervisor for the Chinese PhD degree, and co-supervisor for the Karolinska Institutet PhD, for your support with my daily work and applications for scientific funds.

Many thanks to those in Ingemar’s group: Fu Chen, Jie-Zhi Zhou, Gösta Winberg, Anna Berg, Anita Westman, Helena Lönnqvist, Ludmilla Matskova, Xiang Ningzhang.

Thanks to Zi-Ming Du, Hai-Yun Wang, Jian-Xing Zhang, and all the other PhD students who have shared in intellectual and practical matters.

Thanks to all past and present colleagues at the MTC, Karolinska Institutet, and at the Sun Yat-Sen University Cancer Center (SYUCC), for intellectual contributions and friendship.

I have many dear friends who mean a lot to me, and I would like to thank Wen-Lin Huang from SYSUCC, Maria Maccui, Yi-Hai Cao, and Anders Zetterberg from Karolinska Institutet for being there.

I thank my wife, Dr. Yu-Hong Li, and my daughter, JiaJia, for supporting me with their love, fun, energy, and warmth, and for successfully improving my multi-tasking talents.

I also thank my parents for providing a warm and loving childhood and for their faith in me. They sincerely brought me up believing that I could do everything I wanted to, and this thesis is the result of that.

I would also like to thank:
WHO International Agency for Research on Cancer (IARC) Fellowship; the National Nature Science Foundation Committee of China; National High Technology Research and Development Program of China (863 Program); the Chinese State Key Basic Research Project; the Swedish Cancer Society; and the Swedish Research Council for funding the study.

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