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BIOMARKERS IN NASOPHARYNGEAL CARCINOMA

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To my family

ABSTRACT

Nasopharyngeal carcinoma (NPC) is one of the most common malignancies in certain areas of Southern China, Southeastern Asia and Northern Africa. Currently, evaluation of NPC prognosis is mainly based on the tumor-node-metastasis (TNM) staging system. However, NPC patients with the same clinical stage often present different clinical courses, suggesting that the TNM staging is insufficient to predict prognosis of this disease. Therefore, it is important to find molecular biomarkers, which can help clinicians to identify NPC patients with worse prognosis and develop therapeutic interventions in NPC patients.

This thesis presents the identification and investigation of mechanism of several novel markers in NPC. In the first paper, Caveolin-1 (Cav-1), a major structural component of caveolae, and CD147 (also known as extracellular matrix metalloproteinase inducer, EMMPRIN), a glycoprotein, were found to be overexpressed in NPC. Both Cav-1 and CD147 expression levels correlated significantly with metastasis and poor prognosis of NPC patients. Further studies revealed that Cav-1 and CD147 enhance NPC cell migration, which is associated with MMP-3 and MMP-11 (active) secretion.

The role of microRNA-155 (miR-155) is associated with oncogenesis of several human tumors. In the second paper, miR-155 was found to be upregulated in NPC cell lines and clinical samples. EBV encoded LMP1 and LMP2A could further enhance the expression of miR-155 in NPC CNE1 and TW03 cells. JMJD1A was identified as a direct target of miR-155 in NPC. Downregulation of JMJD1A was significantly correlated with N stage of the TNM classification, a lower five-year survival rate, and a lower five-year disease-free survival rate of NPC patients.

Spleen tyrosine kinase (Syk) is a nonreceptor tyrosine kinase and often aberrantly expressed in human cancers. In the third paper, high expression of Syk was detected in 24% of NPC cases. High expression of Syk, resulted partly from LMP2A expression in NPC, is associated with tumor recurrence and poor prognosis of NPC patients.

Human chromosome 3 (Chr. 3) contains clusters of tumor suppressor genes (TSGs) involved in many cancer types. In the fourth paper, using Not I Chr. 3 microarray, ten candidate TSGs were found in NPC. Among them, the CpG island in the promoter region of Wntless-type Mouse mammary tumor virus integration site family, member 7A (WNT7a) and the intron 1 region of Integrin α 9 (ITGA9) were confirmed to be hypermethylated in NPC by bisulfite sequencing and methylation specific PCR. Demethylating agent 5-aza-2'-deoxycytidine (5-aza-CdR) treatment could restore the expression of WNT7a and ITGA9 in NPC cell lines. Furthermore, WNT7a and ITGA9 were downregulated in NPC clinical samples. As both proteins execute significant functions related to the tumor cell biology, the potential of WNT7a and ITGA9 as diagnosis or therapeutic targets for NPC should be considered.

LIST OF PUBLICATIONS

Articles included in this thesis:

1. **Zi-Ming Du**, Chun-Fang Hu, Qiong Shao, Ma-Yan Huang, Chang-Wei Kou, Xiao-Feng Zhu, Yi-Xin Zeng, and 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*: 125, 1832-1841 (2009). PMID: 19582878
2. **Zi-Ming Du**, Li-Fu Hu, Hai-Yun Wang, Li-Xu Yan, Yi-Xin Zeng, Jian-Yong Shao*, Ingemar Ernberg*. Upregulation of MiR-155 in Nasopharyngeal Carcinoma is partly driven by LMP1 and LMP2A and downregulates a Negative Prognostic Marker JMJD1A. *PLoS ONE*. 2011 Apr 26;6(4):e19137. PMID: 21541331
3. **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
4. Imran Nawaz#, **Zi-Ming Du**#, Ilya Ignatyev#, Tatiana V Pavlova, Vladimir Kashuba, Eugene R Zabarovsky, Ingemar Ernberg, Li-Fu Hu*. WNT7a and ITGA9 Gene are Hypermethylated and Downregulated in Nasopharyngeal Carcinoma. (manuscript)

Related publications:

1. 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
2. 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*. An Eight-signature Classifier for Prediction of Nasopharyngeal Carcinoma Survival. *J Clinl Oncol*. 2011 Dec 1;29(34):4516-25. PMID: 22025164

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LIST OF ABBREVIATIONS

NPC	Nasopharyngeal carcinoma
EBV	Epstein-Barr virus
TNM	Tumor- node-metastasis
WHO	World health organization
Cav-1	Caveolin-1
EMMPRIN/CD147	Extracellular matrix metalloproteinase inducer
MMP3	Matrix metalloproteinase 3
MMP11	Matrix metalloproteinase 11
miR-155	microRNA-155
LMP1	Latent membrane protein 1
LMP2A	Latent membrane protein 2A
BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor1
JMJD1A	Jumonji domain 1A
Syk	Spleen tyrosine kinase
WNT7a	Wingless-type mouse mammary tumor virus integration site family, member 7A
ITGA9	Integrin alpha-9
TSG	Tumor suppressor gene
3' UTR	3' Untranslated region
RISC	RNA-Induced Silencing Complex
Chr. 3	Chromosome 3
MSP	Methylation specific PCR
5-aza-CdR	5-aza-2'-deoxycytidine
NIM	Not I microarray
CGH	Comparative genomic hybridization
SNPs	Single-nucleotide polymorphisms
LOH	Loss of heterozygosity
ITAM	Immunoreceptor tyrosine-based activation motif
EBNA	Epstein-Barr virus nuclear antigen 1
EBERs	Epstein-Barr virus -encoded small RNAs
VCA	Viral capsid antigen
EA	Early antigen
BARF1	BamHI-A rightward frame 1
BALF1	BamHI-A leftward frame 1
NF- κ B	Nuclear factor kappa B
HIF-1	Hypoxia-inducible factor 1
siRNA	Small interference RNA
FFPE	Formalin-fixed, paraffin-embedded
SCCHN	Squamous cell carcinomas of the head and neck

1 INTRODUCTION

1.1 GENERAL CHARACTERISTICS OF NPC

1.1.1 Anatomy and histopathology

Nasopharyngeal carcinoma (NPC) is a tumor arising from the mucosal epithelium covering the nasopharyngeal surface, most often within the lateral nasopharyngeal recess or fossa of Rosenmüller (the space behind the nose). The histological classification system of the World Health Organization (WHO), which was most recently revised in 1991, defines NPC as either keratinizing squamous cell carcinoma (Type 1) or non-keratinizing carcinoma (Type 2); the latter is further subdivided into non-keratinizing differentiated carcinoma (Type 2a) and non-keratinizing undifferentiated carcinoma (Type 2b) (**Table 1**)¹. The most common form, non-keratinizing undifferentiated carcinoma, shows a close to 100% prevalence of Epstein-Barr virus (EBV) in the tumor cells².

Table 1 Histological subtypes of NPC classified by World Health Organization

Histological subtypes	Characteristics	WHO Types
Keratinizing squamous cell carcinoma	Highly differentiated, epithelial growth patterns, keratin filaments	Type 1 (I)
Non-keratinizing differentiated carcinoma	Retaining epithelial cell shape and growth pattern	Type 2a (II)
Non-keratinizing undifferentiated carcinoma	Lack of distinctive epithelial growth pattern	Type 2b (III)

1.1.2 Epidemiology and etiology

NPC is an uncommon disease in most countries of the world, and its age-adjusted incidence for male and female is less than one per 100,000 persons³. However NPC is one of the most common malignancies in certain areas of Southern China, Southeastern Asia and Northern Africa⁴⁻⁵. The highest incidence of 25-40 per 100,000 persons was reported in the Sihui area of Guangdong province, Southern China⁶. The incidence rate among Chinese people born in North America is lower than in those born in Southern China, but still remains considerable higher than the local⁷⁻⁸. These findings suggest that genetic, ethnic and environmental factors play a role in the etiology of NPC. Familial NPC has been linked to susceptibility loci on chromosome 3p 21⁹, 4p15.1-q12¹⁰ and 5p 13¹¹. Epstein-Barr virus (EBV) infection has been verified to be strongly associated with NPC¹²⁻¹³. Moreover, consumption of salted fish and other preserved foods containing volatile nitrosamines, especially during childhood, is important carcinogenic factor for NPC¹⁴⁻¹⁵.

1.1.3 Epstein-Barr virus and NPC

EBV is a prototype gamma herpes virus which infects more than 90% of the world's adult population¹⁶. Humans are the only natural host for EBV. Primary infection with EBV normally occurs in early childhood and is usually asymptomatic in most countries. However, in some individuals, EBV is implicated in the pathogenesis of several human malignancies including Burkitt's lymphoma¹⁷, Hodgkin's lymphoma¹⁸, gastric carcinoma¹⁹ and NPC²⁰. EBV infection in these tumors is characterized by the expression of viral genes expressed during latent infection, in particular, Epstein-Barr virus nuclear antigen 1 (EBNA1), Latent membrane protein 1 (LMP1), Latent membrane protein 2 (LMP2), BamHI-A rightward frame 1 (BARF1), BamHI-A leftward frame 1 (BALF1) and Epstein-Barr virus -encoded small RNAs (EBERs)²¹⁻²⁴ (**Table 2**).

Table 2 Expression of EBV latent genes in human diseases

Latency types	EBV genes expressed	Disease associated
Type I	EBNA1, EBERs	Burkitt's lymphoma
Type II	EBNA1, LMP1, LMP2, EBERs, BARF1	NPC, Hodgkin's disease, peripheral T cell lymphoma
Type III	EBNA1, EBNA2, EBNA3, LMP1, LMP2, EBERs	lymphoproliferative disease, X-linked lymphoproliferative disease, infectious mononucleosis

LMP1 and LMP2A are not expressed at a significant level in all NPCs, and the level of expression varies in different studies depending on material and methods used for detection. LMP1, which was reported to be expressed in as much as approximately 65% of NPC patients²⁵, is the first EBV latent gene found to be able to transform cell lines and alter the phenotype of cells due to its oncogenic potential²⁶. In human epithelial cells, LMP1 alters many functional properties that are involved in tumor progression and invasion²⁷. LMP2A, which is expressed in around 45% of NPC patients²⁸, was reported to induce primary epithelial cell migration and invasion²⁹. More importantly, Guasparri et al³⁰ supported that EBV LMP2A protein could affect LMP1-mediated Nuclear factor kappa B (NF- κ B) signaling and survival of lymphoma cells. LMP1 and LMP2A may partly affect the same pathway, also including transcriptional regulation of miRNAs. Moreover, BARF1³¹⁻³³ and BALF1³⁴ have been found to be expressed in NPC biopsies, suggesting that they play a role in the pathogenesis of NPC. EBERs are the most abundant EBV viral transcripts and are used to detect EBV-infected cells in tissues by in situ hybridization. EBERs could contribute to oncogenesis by modulating innate immunity in patients with NPC³²

1.1.4 Clinical pathological characteristic and TNM staging

NPC has a dominant clinico-pathological behavior of loco-regional recurrence and metastasis, which differs from that of other types of head and neck cancers³⁵. Currently,

tumor-node-metastasis (TNM) staging of NPC is based on clinical and radiologic examination. There are various ways of classifying NPC. At present, the American Joint Committee on Cancer Staging and End Result Reporting / International Union Against Cancer (AJC/UICC) system is preferred in Europe and America³⁶. The Chinese NPC staging system of 1992 (**Sidebar 1**) has incorporated prognostic significance, and according to this staging criteria, the 5-year survival rates of NPC patients in China for stages I, II, III and IV are 89.7%, 75.9%, 51.3%, and 22.2%, respectively³⁷. The Chinese 1992 NPC staging system is considered satisfactory and has been widely used in China³⁷⁻³⁹.

Sidebar 1 The characteristics of the 1992 NPC staging system

The staging system is characterized according to the following model: T, primary tumor: T1, limited to the nasopharynx; T2, involvement of the nasal cavity, oropharynx, soft palate, anterior cervical vertebrae soft tissue, and parapharyngeal space extension before the SO line (the SO line is between the styloid process and the midpoint on the posterior edge of the great occipital foramen); T3, extension over the SO line, involvement of the anterior or posterior cranial nerves alone, the base of the skull, the pterygoprocess zone, and the pterygopalatine fossa; T4, involvement of both anterior and posterior cranial nerves, parabasal sinus, cavernous sinus, orbit, infratemporal fossa, and direct invasion of the first or second cervical vertebrae; N, regional lymph node involvement: N0, no enlarged lymph nodes; N1, greatest dimension of upper neck lymph node <4 cm, movable; N2, lower neck lymph node or greatest lymph node dimension between 4 and 7 cm; N3, supraclavicular lymph node, lymph node greatest dimension >7 cm, fixed, or skin infiltration (the border between the upper neck and the lower neck is the inferior margin of the cricoid cartilage); M, distant metastasis: M0, absence of distant metastasis; M1, presence of distant metastasis; staging: Stage I, T1N0M0; Stage II, T2N0-N1M0, T0-T2N1M0; Stage III, T3N0-N2M0, T0-T3N2M0; Stage IVa, T4N0-N3M0, T0-T4N3M0; Stage IVb, M1.

1.1.5 Treatment and prognosis

NPC is a relatively radiosensitive tumor and therefore radiotherapy remains the standard treatment for almost all NPC patients. The fields for targeting of radiotherapy to the primary tumor and the surrounding anatomical structures at risk of tumor invasion were defined clinically by radiographic imaging. Although more than 70% overall survival rates can be expected for stages I and II, treatment outcomes are unsatisfactory for advanced-stage NPC when radiotherapy is offered alone⁴⁰⁻⁴³. Utilization of radiochemotherapy, which is combined modality therapy using chemotherapy and radiotherapy, is an important strategy for improving tumor control of advanced NPC to achieve improvements in survival⁴⁴⁻⁵⁰. Although NPC is radiosensitive, the average five-year survival rate remains between 50-60%. Regional lymph node and distant metastasis and loco-regional recurrence are the two major reasons resulting in failure of treatment for this cancer^{39, 51}.

1.2 HALLMARKS OF NPC

Inspired by the seminal reviews by Douglas Hanahan and Robert A. Weinberg in 2000 and 2011 describing general features of all cancers⁵²⁻⁵³, we have undertaken to describe the tumor biology of NPC following their suggested hallmarks.

1.2.1 Sustaining proliferative signaling

Cancer cells can acquire the capability to sustain proliferative signaling through numerous different routes. High expression of growth factor ligands themselves, elevating the levels of receptor proteins displayed at the cancer cell surface and structural alterations in receptor molecules could provide cancer cells with signals sustaining proliferation (**Figure 1**)⁵³. Epidermal growth factor receptor (EGFR), which could be induced by LMP1⁵⁴, was overexpressed in NPC⁵⁵ and could stimulate cell proliferation. Moreover, Met proto-oncogene (c-Met)⁵⁶, Insulin-like growth factor-1 receptor (IGF-1R)⁵⁷, Teratocarcinoma-derived growth factor 1 (TDGF1, also named cripto-1)⁵⁸ are capable of transmitting mitogenic signals to the NPC cells.

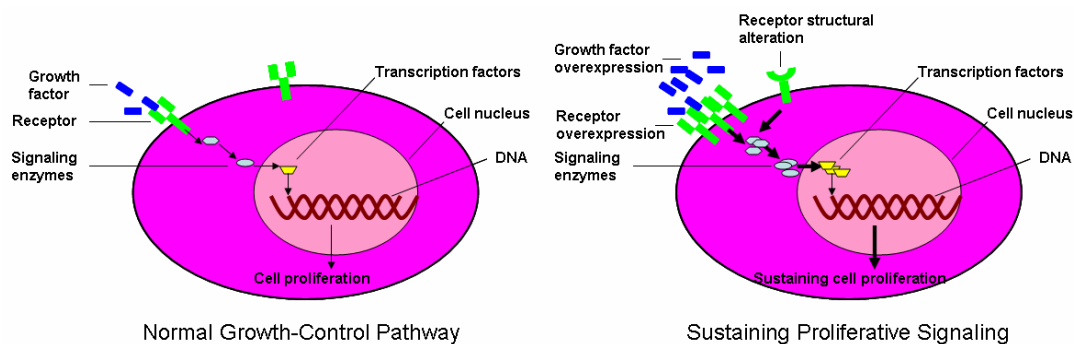


Figure 1 Growth-control pathway in normal cells and subversion of proliferative signaling in cancer cells.

1.2.2 Evading growth suppressors

Tumor suppressor gene (TSG) could negatively regulate cell proliferation, namely operate in various ways to limit cell growth and proliferation (**Figure 2**). In cancer cells, TSGs are usually silenced by genetic alteration (mutation and deletion)⁵⁹ or epigenetic alteration (methylation and histone modification)⁶⁰. MicroRNA let-7⁶¹, miR-26a⁶² and miR-218⁶³ could suppress NPC cell proliferation and cell growth through downregulating c-Myc, EZH2 and survivin respectively, and their expression was downregulated in NPC. Several TSGs, such as Ras association (RalGDS/AF-6) domain family member 1 (RASSF1 or RASSF1A)⁶⁴, The Wnt inhibitory factor-1 (WIF-1)⁶⁵, Fibulin-2 (FBLN2)⁶⁶, cadherin 4 (CDH4)⁶⁷, Tissue factor pathway inhibitor 2 (TFPI-2)⁶⁸, Zinc finger protein 382 (ZNF382)⁶⁹ were epigenetically silenced by promoter hypermethylation in NPC. Moreover, many TSGs have been discovered in NPC, including Coiled-coil domain containing 19 (CCDC19)⁷⁰, Thy-1 cell surface antigen

(THY1)⁷¹, Ubiquitin carboxyl-terminal esterase L1 (UCHL1)⁷² and stratifin (SFN or 14-3-3sigma)⁷³.

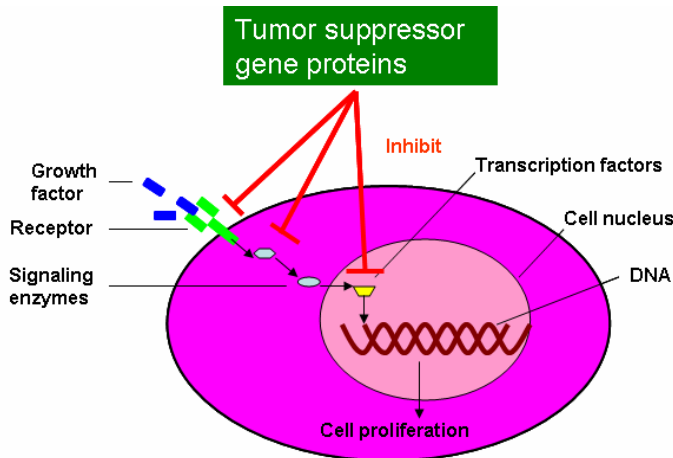


Figure 2 Tumor suppressor genes inhibit cell growth.

1.2.3 Resisting cell death

Programmed cell death by apoptosis serves as a natural barrier to cancer development and cancer cells evolved a number of strategies to limit or circumvent apoptosis. EBV infection has been shown to provide anti-apoptotic functions in NPC. Firstly EBV encoded LMP1⁷⁴⁻⁷⁶ and Epstein-Barr nuclear antigen 1 (EBNA1)⁷⁷ were reported to inhibit apoptosis and contribute to the survival of NPC. Furthermore, EBV encoded miR-BART5 could target PUMA to promote host cell survival⁷⁸. More and more anti-apoptotic and pro-apoptotic factors have been identified in NPC. Bax inhibitor-1 (Bi-1)⁷⁹, Hepatocyte growth factor (HGF)⁸⁰, B-cell CLL/lymphoma 2 (BCL2)⁸⁰, Survivin⁷⁶, Claudin-1 (CLDN7)⁸¹, CASP8 and FADD-like apoptosis regulator (CFLAR or FLIP)⁸² and Cellular inhibitor of apoptosis protein 2 (c-IAP2)⁸³ were overexpressed in NPC and mediated apoptosis-resistance in NPC. However many proapoptotic factors, such as Caspase-8^{84 85}, Zinc finger protein ZNF382⁶⁹, CKLF-like MARVEL transmembrane domain containing 3 (CMTM3)⁸⁶ and Prostate apoptosis response-4 (Par-4)^{75,87}, were found to be downregulated in NPC.

1.2.4 Inducing angiogenesis

Like normal tissues, tumors require provision of nutrients and oxygen as well as an ability to evacuate metabolic wastes and carbon dioxide. Hence angiogenesis is very important during tumor development⁸⁸ (**Figure 3**). EBV has been verified to regulate angiogenesis in NPC⁸⁹. EBV encoded LMP1 enhanced angiogenesis in NPC by induction of interleukin-8 (IL-8)⁹⁰ and cyclooxygenase-2 (COX-2)⁹¹. Furthermore, EBV-encoded EBNA1 could also promote angiogenesis in NPC⁹². In addition, Vascular endothelial growth factor (VEGF)⁹³, Macrophage migration inhibitory factor (MMIF)⁹⁴, Hepatocyte growth factor (HGF)⁹⁵ could induce angiogenesis in NPC. However matrix metalloproteinase-19 (MMP19)⁹⁶, Cysteine-rich intestinal protein 2

(CRIP2)⁹⁷ and Fibulin-2 (FBLN2)⁶⁶ had anti-angiogenic role and were downregulated in NPC.

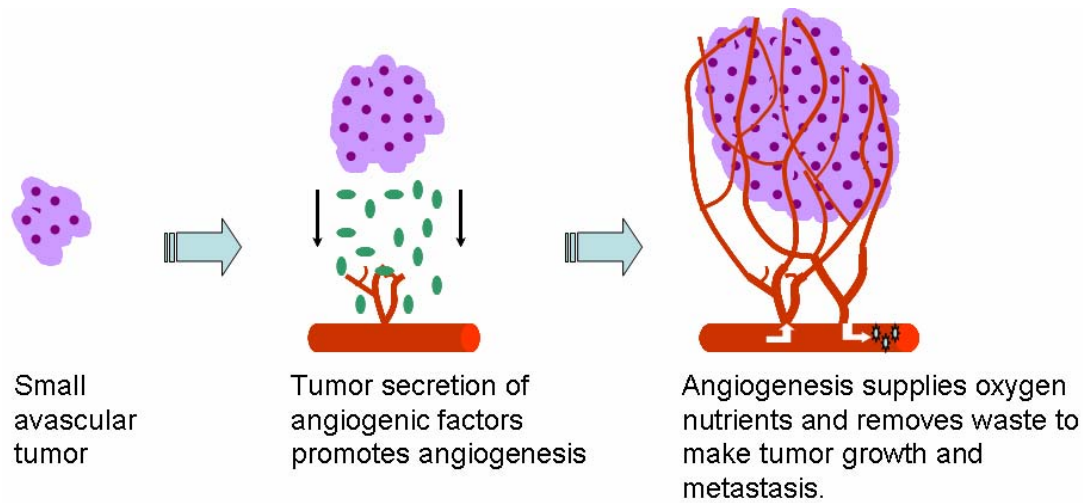


Figure 3 The angiogenic switch is necessary for tumor growth and metastasis. (Adapted from “Tribute to Judah Folkman, Father of Angiogenesis” from OncTalk. <http://onctalk.com/2008/01/22/folkman-tribute/>)

1.2.5 Activating invasion and metastasis

The multistep process of invasion and metastasis has been schematized as a sequence of discrete steps, often termed the invasion-metastasis cascade⁹⁸. This process includes several steps, beginning with local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic and hematogenous systems, followed by escape of cancer cells from the lumina of such vessels into the parenchyma of distant tissues (extravasation), the formation of small nodules of cancer cells (micrometastases), and finally the growth of micrometastatic lesions into macroscopic tumors, this last step being termed “colonization.”^{53,99}

(Figure 4). EBV encoded LMP1 could enhance NPC cell migration and invasion by induction of Snail¹⁰⁰, Decoy receptor 3¹⁰¹, matrix metalloproteinase (MMP-1)¹⁰², matrix metalloproteinase 9 (MMP9)²⁷ and MicroRNA-10b¹⁰³. EBV encoded LMP2A could induce an epithelial-mesenchymal transition in NPC and also contribute to invasion and metastasis of NPC¹⁰⁴. Several genes such as Fibroblast growth factor 8 (FGF8b)¹⁰⁵, VEGF¹⁰⁶, Centromere protein F (CENP-F)¹⁰⁷, Transient receptor potential melastatin 7 (TRPM7)¹⁰⁸, Hepatocyte growth factor (HGF)¹⁰⁹, Bcl-2 and MMP2¹¹⁰, Caveolin-1 and CD147³⁸, Cathepsin L¹¹¹, Chemokine (C-X-C motif) receptor 4 (CXCR4)¹¹² were found to be overexpressed in NPC and correlated with invasion and metastasis in NPC. On other hand, E-cadherin¹¹³, Non-metastatic cells 1, protein (NM23A) expressed in (NME1)¹¹⁴, Toll-like receptor 3 (TLR3)¹¹⁵, NPC-associated gene 6 (NGX6)¹¹⁶, Fibulin-3¹¹⁷, Phosphatidylethanolamine binding protein 1 (RKIP)¹¹⁸, 14-3-3 sigma¹¹⁹ were downregulated in NPC and had the ability to suppress cell migration and invasion in NPC cells.

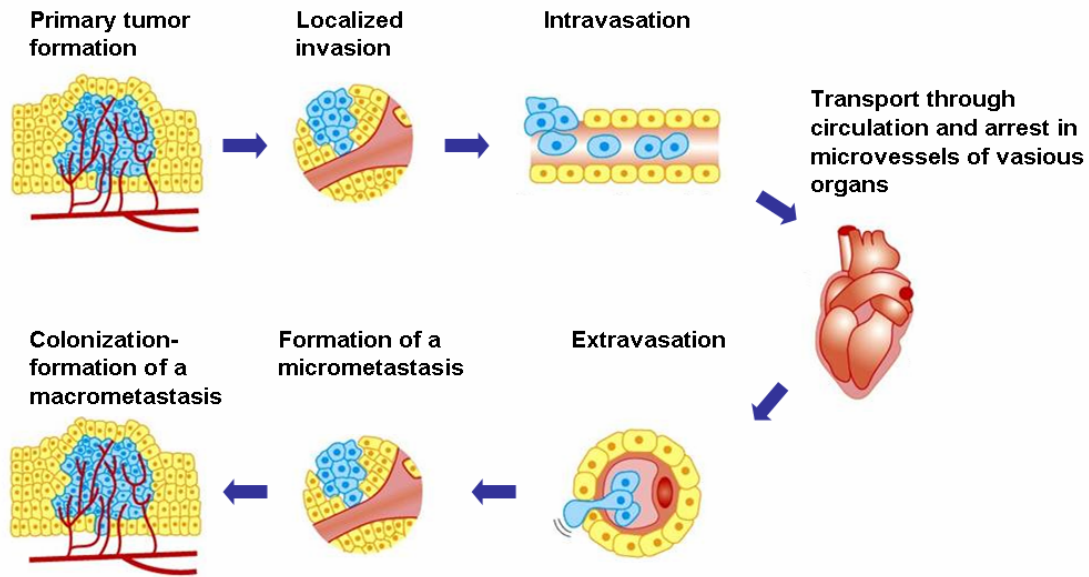


Figure 4 The invasion-metastasis cascade. (Adapted from ⁹⁹)

1.2.6 Enabling replicative immortality

Cancer cells require unlimited replicative potential in order to generate macroscopic tumors. Telomeres protecting the ends of chromosomes are centrally involved in the capability for unlimited proliferation¹²⁰. Telomerase, the specialized DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA, was usually expressed in many human cancer cells¹²¹⁻¹²³. Telomerase activity is frequently found in metaplastic and malignant human nasopharyngeal tissues¹²⁴ and may be useful as a diagnostic marker in the detection of tumor cells in recurrent NPC, but not in primary NPC¹²⁵. EBV encoded LMP1 could activate telomerase through c-myc¹²⁶ and led to immortalize primary cultures of nasopharyngeal epithelial cells¹²⁷. However LMP2A was shown to inhibit telomerase activity in epithelial cells¹²⁸. Protein kinase C has been shown to be involved in the regulation of telomerase activity by direct or indirect phosphorylation of telomerase proteins *in vivo* in NPC^{129 130}. Furthermore, BMI1 polycomb ring finger oncogene (Bmi-1) could immortalize primary human nasopharyngeal epithelial cells, and was a novel molecular marker of NPC progression¹³¹.

1.2.7 Genome instability and mutation

A large number of CGH (Comparative genomic hybridization)-based studies on NPC lead to the identification of consistent gain at chromosome 1q, 3q, 8q, 12 and loss at 3p, 9p, 11q, 14q¹³²⁻¹³³. Furthermore, using array based CGH, frequent amplifications were detected for several oncogene loci, including MYCL1 at 1p34.3 (66.7%), TERC at 3q26.3 (46.7%), ESR at 6q25.1 (46.7%), and PIK3CA at 3q26.3 (40%)¹³⁴. The most frequent LOHs (losses of heterozygosity) were observed at chromosome 3p, 9p, and 14q in NPC, which is in agreement with the CGH based findings¹³⁵⁻¹³⁸. PIK3CA gene amplification is frequent in NPC and occurs in the advanced stage¹³⁹. High frequency

somatic mutations in RASSF1A is seen ¹⁴⁰. Moreover, mitochondrial DNA somatic mutations are frequent in NPC ¹⁴¹.

1.2.8 Other hallmarks and characteristics

The role of defective immunological monitoring of tumors seem to be validated by the striking increases of certain cancers in immunocompromised individuals ¹⁴². EBV encoded EBERS induced the expression of insulin-like growth factor 1 (IGF-I) in NPC cells and modulated the innate immunity system ¹⁴³. Inhibition of NF- κ B by EBV encoded EBNA1 is employed by viruses as an immune evasion strategy which is also closely linked to oncogenesis during persistent viral infection ¹⁴⁴. The imbalances of T(reg) and effector T cell phenotypes and down-regulation of signal-transducing molecules in TILs, play important roles in suppression of immune response and immune evasion of NPC ¹⁴⁵.

Tumor associated inflammatory response had an unanticipated effect of enhancing tumorigenesis and progress by supplying bioactive molecules to the tumor microenvironment. Tumor necrosis factor alpha (TNF α) is an inflammatory cytokine that is present in the microenvironment of many tumors and is known to promote tumor progression. Blood level of TNF-alpha in NPC was higher than that of healthy controls ¹⁴⁶. Furthermore, TNF α -inducible protein 2 (TNFAIP2), which was induced by TNF α , promoted migration in NPC and its expression predicted distant metastasis of NPC patients ¹⁴⁷. Moreover, the increase of programmed death-1-expressing intratumoral CD8 T cells predicts a poor prognosis for NPC. ¹⁴⁸

Moreover, cancer cells could reprogram their glucose metabolism and their energy production by glycolysis (**Figure 5**) ¹⁴⁹. HIF1a had the capability to upregulate glycolysis in cancer cells ¹⁵⁰. HIF1a was found to be overexpressed in NPC ¹⁵¹, which suggested that NPC cells also reprogrammed energy metabolism.

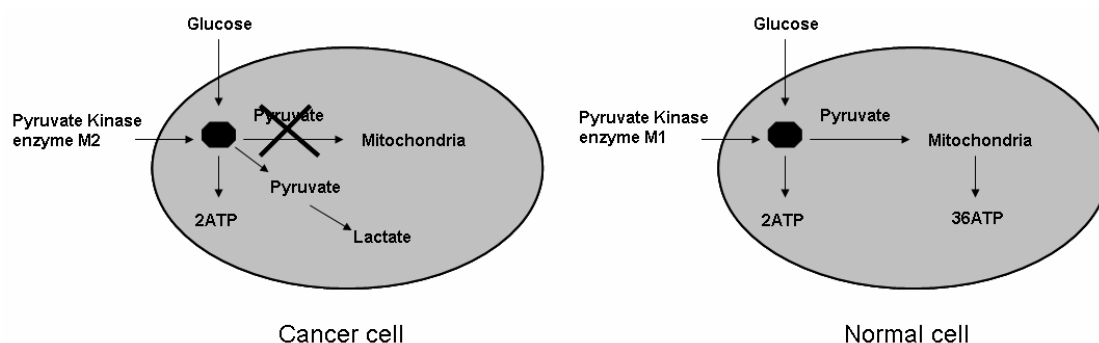


Figure 5 Tumor cells mainly generate energy by glycolysis, while the oxidative phosphorylation is switched off (the Warburg effect).

1.3 BIOMARKERS FOR NPC

1.3.1 EBV markers

EBV has been verified to correlate with the etiology of NPC, and EBV-specific antibody-based assays such as serum titers of IgA antibodies to viral capsid antigen (VCA), early antigen (EA), nuclear antigen (EBNA) and DNase assay have been commonly used for at least twenty years as standard markers for screening and monitoring the disease¹⁵². Serological levels of VCA/IgA and EA/IgA were significantly associated with increased risks for NPC, and VCA/IgA had better predictive performance for NPC incidence than EA/IgA¹⁵³. Some studies suggested that combination of EBV EA and EBNA recombinant antigens has high sensitivity and acceptable specificity and accuracy in early diagnosis and mass screening of NPC patients¹⁵⁴⁻¹⁵⁵. Antibodies targeting the BARP1 protein could be used as a new diagnostic tool to identify NPC patients³³. Recently, one paper reported that pretreatment serologic anti-enzyme rate (AER) of EBV DNase-specific neutralizing antibody could serve as an independent prognostic marker complementing TNM stage in NPC¹⁵⁶. EBV viral load measurement in serum or plasma has been shown to be useful for diagnosis and monitoring in NPC patients. It can reflect tumor stage, is predictive for overall survival, correlated with the residual tumor load after treatment and accurately predict recurrence and prognosis¹⁵⁷⁻¹⁶¹. Hence cell-free EBV DNA could become one of the potential diagnostic and prognostic molecular markers for NPC.

1.3.2 Epigenetic markers

DNA methylation of TSGs is widely known to be an early event in carcinogenesis and methylation analysis in the promoter of TSGs may serve as a complementary marker for identifying early cases. Epigenetic changes are frequently found in NPC and the high detection rate in body fluids suggests its potential application in non-invasive screening of NPC or detection of residual carcinoma after treatment¹⁶². Hutajulu et al¹⁶³ found that combined analysis of five methylation markers (RASSF1A, p16, WIF1, CHFR and RIZ1) in brushings shows good discrimination between NPC and non-NPC with a detection rate of 98% in a high risk population. 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 NPC¹⁶⁴.

1.3.3 Genomic markers

Several genomic markers in NPC have been identified as possible candidates for risk prediction. Familial NPC has been linked to genetic predispositions such as leukocyte

antigen (HLA) genotypes¹⁶⁵⁻¹⁶⁶ and susceptibility loci on chromosome 3p 21⁹, 4p15.1-q12¹⁰ and 5p 13¹¹. Recently, a genome-wide association study was performed using 464,328 autosomal single-nucleotide polymorphisms (SNPs) in 1,583 NPCs and 1,894 controls, and three new susceptibility loci, including TNFRSF19 on 13q12, MDS1-EVI1 on 3q26 and the CDKN2A-CDKN2B gene cluster on 9p21 were identified¹⁶⁶. Moreover, the allele of Cav-1 T29107A was found to be protective against the development of NPC and may be a novel useful genomic marker for early screening and prediction of NPC¹⁶⁷. The endothelin A receptor (EDNRA) / H323H polymorphism was a novel and independent prognostic marker for patients with locoregionally advanced NPC¹⁶⁸. MDM2 SNP309 T/G polymorphism¹⁶⁹ and Tumor necrosis factor alpha 308G/A polymorphism¹⁷⁰ could be considered a risk marker for the development of NPC. Moreover, the FAS-670 G allele¹⁷¹ and MCP-1 SNP-2518¹⁷² have been suggested as a genetic marker for risk of distant metastasis of NPC patients.

1.3.4 Protein and microRNA markers

Many aberrantly expressed cellular genes have been found in NPC cancer cells and patients' serum, and many studies indicated that the expression pattern of some particular genes could be the biomarkers for NPC diagnosis and prognosis prediction. Wang et al¹⁷³ developed a new NPC-SVM classifier, which integrated patient sex and the protein expression level of seven genes, including EBV encoded LMP1, CD147, caveolin-1, phospho-P70S6 kinase, matrix metalloproteinase 11 (MMP11), survivin, and secreted protein acidic and rich in cysteine (SPARC). The NPC-SVM classifier distinguished patients with NPC into low- and high-risk groups with significant differences in 5-year DSS in the evaluated patients (87% v 37.7%; $P < .001$) in the validation cohort. Moreover, the patients with NPC with higher levels of interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), macrophage inflammatory protein (MIP)-3 α , and EBV DNA in plasma had worse prognosis for overall survival¹⁷⁴. Until now, useful miRNA markers have not yet been identified in either NPC tumor tissue or the NPC patient serum.

1.3.5 Other biomarkers

Metabolic profiling revealed that kynurenine, N-acetylglucosaminylamine, N-acetylglucosamine and hydroxyphenylpyruvate are increased in NPC patient sera. After radiotherapy, these four metabolites decreased gradually, tended to normalize, and were associated with rate of tumor reduction. The results revealed that kynurenine, N-acetylglucosaminylamine, N-acetylglucosamine and hydroxyphenylpyruvate are potential markers for NPC diagnosis¹⁷⁵.

2 AIMS OF THE STUDY

Currently, NPC prognosis evaluation and the choice of treatment are mainly based on the tumor-node-metastasis (TNM) staging system. However, NPC patients with the same clinical stage often present with different clinical courses, suggesting that the TNM stage is insufficient to provide satisfactory prediction of prognosis. Therefore, it is important to find molecular biomarkers, which can help clinicians to identify high risk NPC patient, thus improving prognosis prediction, and therapeutic interventions in NPC patients.

To identify novel prognostic markers for NPC, we have studied the following aspects:

1. Investigating the expression pattern and clinical significance of Caveolin-1 and CD147 in NPC, and their possible functional mechanism.
- 2 Exploring the expression pattern of miR-155 and its possible mechanism of action in NPC, identifying targets and evaluating prognostic value in NPC
- 3 Investigating the expression of Syk and evaluating its prognostic value in NPC
- 4 Identifying putative TSGs in Chr.3 in NPC, and their possible regulatory mechanism in NPC

3 MATERIALS AND METHODS

3.1 METHODS FOR BIOMARKER EVALUATION

To evaluate the clinical value of certain biomarkers, immunohistochemistry was performed in NPC clinical samples, and then the prognostic value was analyzed by Kaplan-Meier analysis and log-rank tests. The detailed information and protocol of these methods are described in **Paper I, II and III**.

Clinical Samples

For this retrospective study, archival formalin-fixed, paraffin-embedded (FFPE) tissue specimens from primary NPC patients with clinical information were obtained from Sun Yat-Sen University Cancer Center (Guangzhou, China). All NPC samples in our study were obtained before treatment with standard curative radiotherapy, with or without chemotherapy. The study was approved by the Research Ethics Committee of Sun Yat-Sen University Cancer Center, Guangzhou, China (Reference number: YP-2009175) and Karolinska Institutet, Stockholm, Sweden (Reference number: 00-302).

Immunohistochemistry

Immunohistochemistry is applicable for the detection of protein expression in tissue sections with specific antibodies, which can provide both location and intensity of positive signal. The immunohistochemistry results were evaluated and scored by senior pathologists without knowledge of the clinicopathological outcomes of the patients. Then the NPC patients were divided into high expression group and low expression group according to the composite pathology score which each patient was given.

Statistical analysis

SPSS12.0 software was used to statistically evaluate the clinical biomarker data. The association between biomarker expression and clinicopathological parameters were assessed using a Chi-Square test. Kaplan-Meier analysis and log-rank tests were used to assess the survival rate and to compare the difference in survival curves. Differences were considered significant when $p < 0.05$.

3.2 METHODS FOR FUNCTIONAL STUDIES OF BIOMARKERS

To study the function of the certain biomarkers, gene transfection technology was used in NPC cell lines to knockdown or overexpress protein. Transwell migration assay was performed to evaluate the migration ability of the cells. All the detailed methods used can be found in section “Materials and methods” of **Paper I**.

Cell lines

Human NPC cell lines CNE1 and CNE2 (EBV negative, from Cancer Center, Sun Yat-sen University, China) were used for gene transfection.

Transfection

Transfection is the process of delivery of genetic material (DNA or RNA) into cells, such as human cancer cells. In the present study, Lipofectamine 2000 (Invitrogen, USA) was used to transfer specific small interference RNA (siRNA) or vectors for overexpression of proteins to NPC cell lines transiently. Since the genetic material (DNA or RNA) introduced in the transfection process is usually not inserted into the genome of host cells, the introduced DNA is lost after one or few rounds of mitosis. Hence different time points after transfection have been checked and the most efficient time point was chosen.

Transwell migration assay

Transwell migration assay is employed to evaluate the migration ability of cells *in vitro* using 24-well transwell units (Corning, USA) with a 8 μm pore size polycarbonate filter. Cell suspensions without FCS were placed in the upper chamber, and medium with 10% FCS was added to the bottom chamber. After overnight incubation, the top cells in the upper chamber were removed while the cells in the lower were fixed and stained with 4,6-diamidino-2-phenylindole (DAPI; 5 $\mu\text{g}/\text{ml}$) to visualize nuclei. The number of migrating cells was counted under a fluorescence microscope

3.3 METHODS FOR STUDIES OF REGULATION BY MIRNAS

To identify a direct target of a certain miRNA, software prediction was used and then luciferase reporter assay was performed for further validation. All the detailed methods used can be found in section “Materials and methods” of **Paper II**.

Prediction miRNAs target analysis

Since miRNAs bind to the 3' UTR of their target mRNAs with imperfect base pairing, leading to mRNA degradation or translational inhibition (**Figure 6**), many algorithms have been developed to predict the putative targets of miRNAs. In the present study, the putative targets of a certain miRNA were analyzed as follows¹⁷⁶: Firstly, the analysis was done by using four algorithms, miRanda¹⁷⁷, TargetScan¹⁷⁸, PicTar¹⁷⁹ and miRBase¹⁸⁰ respectively. Since any of the four approaches generates an unpredictable number of false positives, the putative targets, commonly predicted by at least three of the algorithms, were chosen.

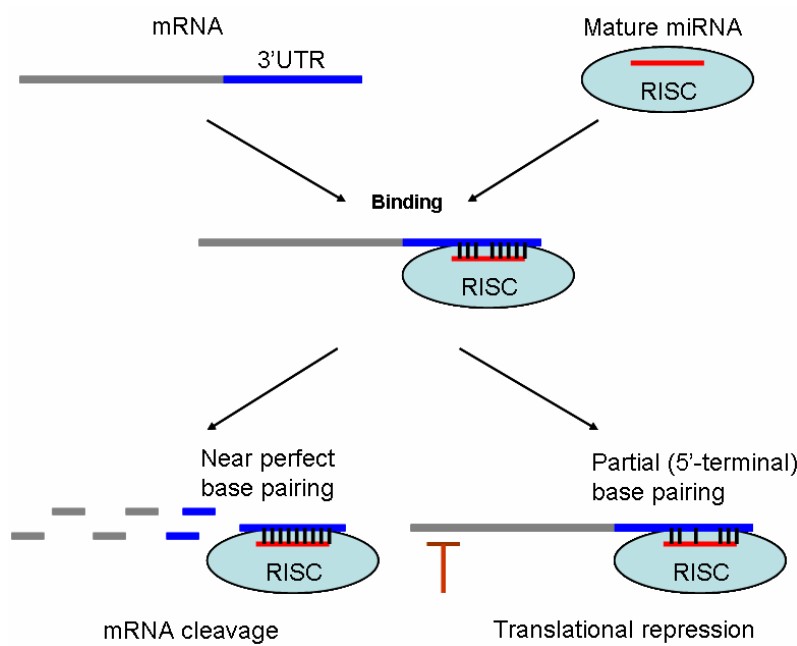


Figure 6 miRNA binds to the 3'UTR of its target mRNA with imperfect base pairing, leading to mRNA degradation or translational inhibition. RISC, RNA-Induced Silencing Complex; 3'UTR, 3'un-translated regions.

Luciferase reporter assay

To validate the direct target of a certain miRNA, luciferase reporter assay was performed. Firstly, the 3' UTR sequences containing the putative miRNA target sites were inserted by cloning downstream from the luciferase reading frame in the plasmid pMIR-report-Vector (**Figure 7A**). Luciferase reporter assays were performed by transiently transfecting HEK 293T cells with pMIR-reporter-vector containing the 3'UTR of the putative targets, together with miRNA mimic and pCMV-Renilla (internal control). Firefly and Renilla luciferase activities were measured consecutively by using Dual Luciferase Assay (**Figure 7B**).

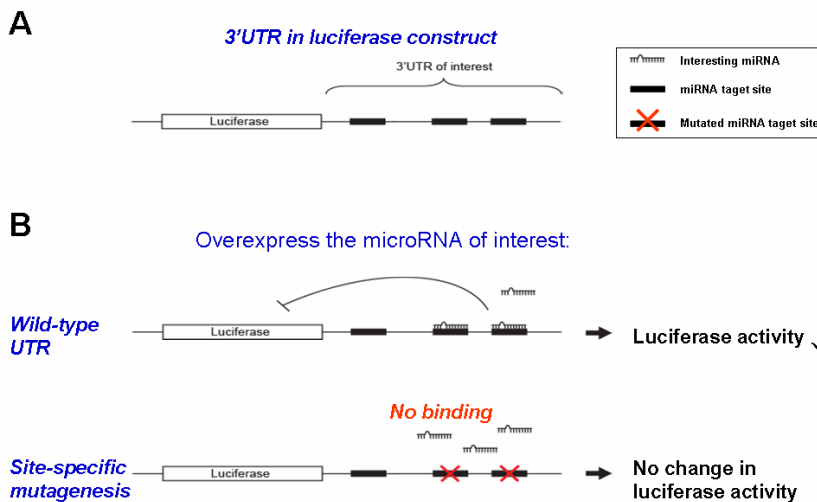


Figure 7 Luciferase reporter assay. (A) The 3' UTR sequences containing the putative miRNA target sites were cloned into the downstream from the luciferase reading frame in the plasmid. (B)

A result was positive when the miRNA could repress the luciferase activity in the wild type 3'UTR construct, but could not in the site-specific mutagenesis construct.

3.4 METHODS FOR STUDIES OF EPIGENETIC REGULATION

To investigate whether epigenetic modification might be involved in regulation of putative biomarkers, i.e. to identify epigenetically silenced biomarkers (tumor suppressor genes), Not I microarrays were performed to screen methylated or deleted genes in chromosome 3 in NPC and then Bisulfite sequencing and methylation specific PCR (MSP) were performed for further validation. All the detailed methods used can be found in section “Materials and methods” of **Paper IV**.

Chr. 3 specific NotI microarrays

Loss of heterozygosity (LOH) and cytogenetic studies have shown that frequent changes in Chromosome 3 (Chr. 3) is common in many tumors including NPC, harboring many TSGs^{137-138, 181}. Only a small fraction (0.1-0.05%) of the human genome containing NotI digested fragments and NotI sites are almost exclusively located in CpG islands and are closely associated with functional genes. Hence Chr. 3 specific NotI microarray, covering 188 genes/ putative TSGs, was developed in Eugene Zabarovskys group to search methylated or deleted genes in Chr. 3¹⁸². The main objective is to prepare and to use Not I microarrays (NMA, i.e. glass microarrays with attached NotI DNA fragments) for comparison of normal and tumor cells at genomic level. The main idea of the approach is that NotI enzyme cuts only unmethylated CpG pairs inside the recognition site of the enzyme (5'-GCGGCCGC-3') (**Figure 8**). This new type of microarrays opens new possibilities for large scale study of methylation/deletion patterns in normal and pathological cells

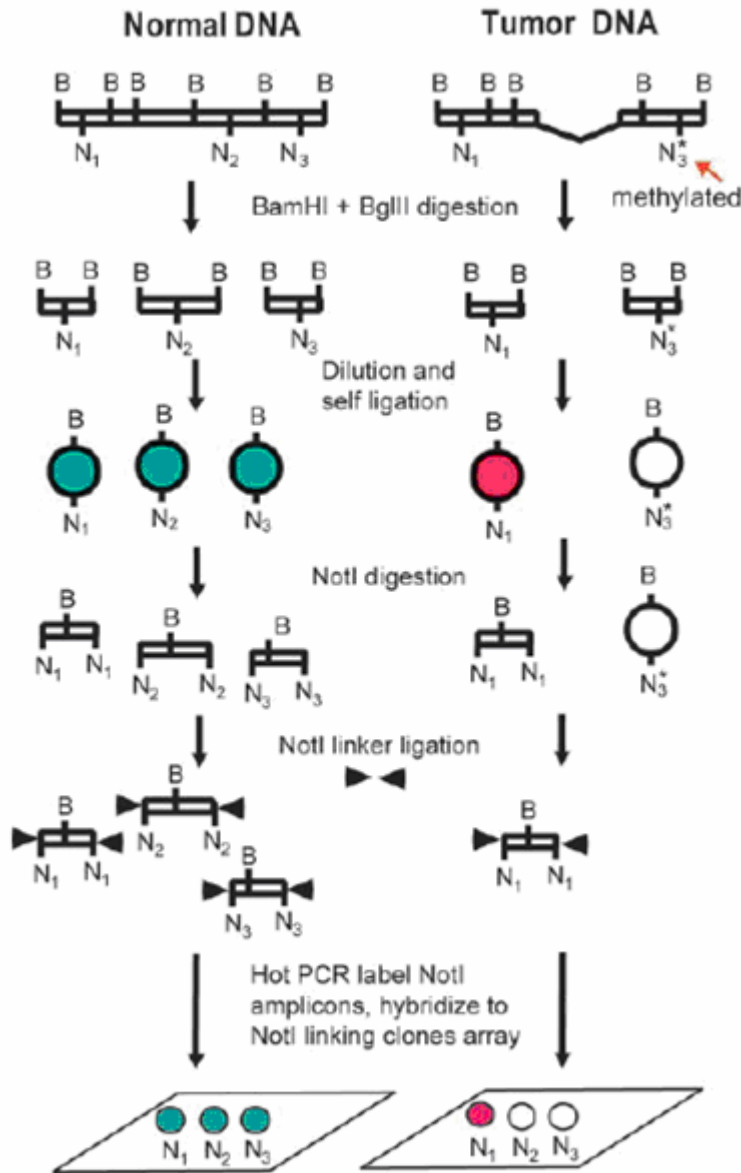


Figure 8 Schematic representation how NotI microarrays are employed to identify methylated or deleted sequences (The main idea of using NR for NotI microarrays). N and B represent Not I and BamH I / Bgl II sites, respectively. Methylated NotI sites are indicated by an asterisk. NR: NotI-representation. (Adapted from¹⁸²)

Bisulfite sequencing

Bisulfite sequencing is the golden standard to detect methylation status of CpGs in a site-specific manner. Bisulfite treatment of DNA template converts cytosine residues to uracil, but has no effect on 5-methylcytosine residual (**Figure 9**). Hence bisulfite treatment could incorporate specific changes in the DNA sequence and this provides information at the single nucleotide level regarding the DNA methylation state. However the major challenge for bisulfite sequencing technique is incomplete

conversion. If the conversion is incomplete, it will increase the possibilities of false positive result.

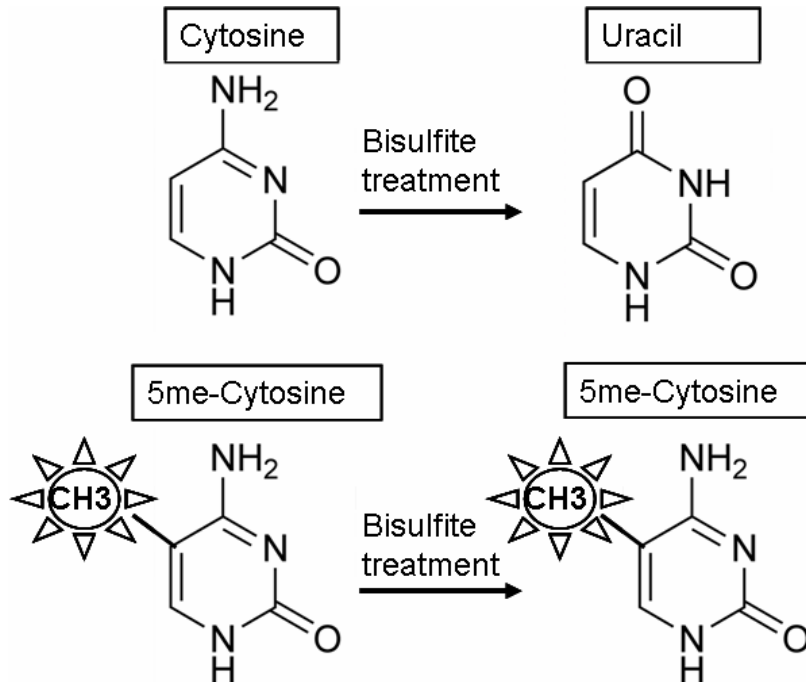


Figure 9 Bisulfite-mediated conversion of cytosine to uracil.

Methylation specific PCR (MSP)

Methylation specific PCR (MSP) is the most widely and conveniently used technique for studying the methylation status of CpG islands. Cytosines in CpG islands in promoter region of many tumor suppressor genes are usually methylated in human cancers, while they are usually unmethylated in normal tissues. The differences between methylated and unmethylated alleles that arise from sodium bisulfite treatment are the basis of MSP, and the primers for MSP are designed according to these differences to distinguish the different methylation status. MSP is the simplest technique with relatively low cost to investigate methylation status of CpG islands. Because the DNA template is converted and partially degraded after bisulfite treatment, optimizing the MSP reaction condition is critical to get the positive result.

4 RESULTS AND DISCUSSION

4.1 UPREGULATION OF CAVEOLIN-1 AND CD147 EXPRESSION IN NPC ENHANCED TUMOR CELL MIGRATION AND CORRELATED WITH POOR PROGNOSIS OF THE PATIENTS (PAPER I)

Expression of Cav-1 and CD147 and their associations with clinico- pathological parameters in NPC

Cav-1 is a major structural component of caveolae, which are involved in several cellular functions, including vesicle trafficking, cholesterol homeostasis and signal transduction¹⁸³⁻¹⁸⁴. Reduced Cav-1 expression has been reported in ovarian cancer¹⁸⁵, breast cancer¹⁸⁶ and lung cancer¹⁸⁷. On the other hand, Cav-1 overexpression has been observed in bladder cancer¹⁸⁸, prostate cancer¹⁸⁹ and esophageal squamous cell carcinoma (ESCC)¹⁹⁰. Extracellular matrix metalloproteinase inducer (EMMPRIN), also named CD147, is a glycoprotein that belongs to the immunoglobulin superfamily¹⁹¹. Due to serendipitous observation, we thought the expression patterns of Cav-1 and CD147 in NPC was of interest. In the present study, Cav-1 and CD147 were found to be upregulated in NPC cell lines as well as in NPC clinical samples.

We found that Cav-1 was expressed at a high level in 96 cases (49.5%) of NPC, which associated significantly with local recurrence ($p = 0.038$) and metastasis ($p = 0.025$) of NPC. No significant association between Cav-1 expression and age, gender and TNM stage of the patients was observed in this study. Overexpression of CD147 was observed in 117 cases (59.4%) of NPC, which was associated significantly with metastasis of the disease ($p = 0.017$). No significant association between CD147 expression and age, gender, clinical staging and recurrence of the disease was observed in this study.

Cav-1 overexpression was reported previously to be correlated with the metastasis of several malignancies, including prostate cancer¹⁸⁹, ESCC¹⁹⁰, breast cancer¹⁹² and lung cancer¹⁹³. In the present study, we revealed that Cav-1 overexpression in NPC tumor cells was significantly correlated with recurrence ($p = 0.038$) and metastasis ($p = 0.025$) of the disease. Overexpression of CD147 in the tumor cells has been reported to be correlated with metastasis of breast cancer¹⁹⁴, and oral squamous cell carcinoma¹⁹⁵; and with poor prognosis of patients with ESCC¹⁹⁶, breast cancer¹⁹⁴, serous ovarian cancer¹⁹⁷ and gastric cancer¹⁹⁸. We found that CD147 overexpression in NPC tumor cells was significantly correlated with metastasis ($p = 0.017$) of the disease.

Combined Cav-1 and CD147 overexpression predicts poor survival of the NPC patients

Cav-1 overexpression in tumor cells also has been reported to be correlated with poor prognosis in patients with ESCC¹⁹⁰, renal clear cell carcinoma¹⁹⁹, prostate cancer²⁰⁰, lung cancer²⁰¹, and pancreatic ductal adenocarcinoma²⁰². In the present study, we revealed that Cav-1 overexpression in NPC tumor cells was significantly correlated with poor prognosis in NPC patients. Several recent studies have demonstrated that overexpression of CD147 is correlated with poor prognosis in human cancers, including ESCC¹⁹⁶, breast carcinoma¹⁹⁴, serous ovarian carcinoma¹⁹⁷ and gastric carcinoma¹⁹⁸. We found that CD147 overexpression in NPC tumor cells was significantly correlated with poor prognosis in NPC patients. In addition, we found that NPC patients with overexpression of both Cav-1 and CD147 in tumor cells had a significantly worse prognosis and a significantly lower five-year overall survival rate relative to NPC patients with low expression of Cav-1 and / or CD147 ($p = 0.001$).

Cav-1 and CD147 increase the migration ability of CNE1 and CNE2 cells through increasing the secretion of MMP-3 and MMP-11 (active)

Recent evidence suggests a central role for Cav-1 in the regulation of cellular invasion and metastasis²⁰³⁻²⁰⁴. CD147 is enriched on the plasma membrane of tumor cells and triggers the production or release of MMPs in surrounding mesenchymal cells and tumor cells²⁰⁵⁻²⁰⁶. In this study, Cav-1 and CD147 overexpression were found to be associated with metastasis in NPC patients. Transwell migration assays further revealed that loss of Cav-1 and CD147 expression inhibited CNE1 and CNE2 cell migration ability, whereas Cav-1 and CD147 overexpression promoted cell migration ability. Taken together, our results are consistent with previous studies of Cav-1 and CD147 expression in other malignancies, and indicate that these two genes may play a key role in the invasion and metastasis of NPC.

How do Cav-1 and CD147 regulate the invasion and metastasis of NPC? Growing evidence suggests a close 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 fibroblasts²⁰⁷, 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 altered¹⁸⁶. In contrast, Cav-1 can induce MMP-11 secretion and the invasive potential of a murine hepatocarcinoma cell line²⁰⁸. 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 cells and tumor cells^{205-206, 209-211}. In the present study, we found that the suppression of Cav-1 and CD147 expression led to decrease MMP-3 and MMP-11 (active) secretion in CNE1 and CNE2 cells, whereas overexpression of Cav-1 in CNE1 and CNE2 cells promoted MMP-3 and MMP-11 (active) secretion. Furthermore, suppression of CD147 expression in Cav-1 overexpressing CNE1 cells could also lead to decrease of MMP-3 and MMP-11 (active) secretion. Taken together, these results indicate that Cav-1 and CD147 overexpression in NPC tumors can promote tumor cell migration by stimulating MMP-3 and MMP-11 (active) secretion in NPC tumor cells,

and Cav-1 regulates MMP-3 and MMP-11 (active) secretion partially, maybe directly, through CD147 in NPC tumor cells.

4.2 UPREGULATION OF MIR-155 IN NPC IS PARTLY DRIVEN BY LMP1 AND LMP2A AND DOWNREGULATES A NEGATIVE PROGNOSTIC MARKER JMJD1A (PAPER II)

EBV LMP1 and LMP2A further enhance miR-155 expression in NPC

MiR-155 was upregulated in several human tumors, such as lymphoma²¹²⁻²¹³, thyroid carcinoma²¹⁴, breast cancer²¹⁵⁻²¹⁶, colon cancer²¹⁵, cervical cancer²¹⁷, pancreatic cancer²¹⁸, and lung cancer²¹⁹. Furthermore, elevated expression of miR-155 was associated with poor prognosis of pancreatic cancer²¹⁸ and lung cancer²¹⁹. Recently, Chen et al used a stem-loop real-time-PCR method to quantify the expression levels of 270 human miRNAs in 13 NPC samples and 9 adjacent normal tissues. They identified 35 miRNAs whose expression levels were significantly altered in NPC samples, including upregulation of miR-155²²⁰. In the present study, we reported that miR-155 expression was enhanced in NPC.

Several studies have demonstrated that EBV could induce miR-155 expression in B cells and cell lines which in turn modulates EBV-regulated pathways²²¹⁻²²³. LMP1²²²⁻²²⁵ and EBNA2²²² were responsible for the upregulation of miR-155 after EBV infection of B-lymphocytes, while LMP2A did not influence miR-155 expression^{223, 225}. Furthermore LMP1 was demonstrated to trans-activate miR-155 transcription through the NF-kappaB and AP1 pathways^{223, 225}. In contrast, EBV did not induce the expression of miR155 in HEK 293 and Hela cells²²². We found that both LMP1 and LMP2A could induce the miR-155 expression in NPC CNE1 and TW03 cells. To our knowledge, this is the first report that LMP2A could induce miR-155 expression in NPC. Guasparri et al reported that EBV LMP2A protein could affect LMP1-mediated NF-κB signaling and survival of lymphoma cells, hence LMP2A might increase miR-155 expression through NF- kappaB pathway³⁰.

In addition, LMP1 was reported to induce the expression of miR-146a in B-lymphocytes²²⁴. However, in our study, we found that neither LMP1 nor LMP2A could induce the miR-146a expression in NPC cells. These differences might be due to different tumor cell type backgrounds.

LMP1 and LMP2A were expressed in approximately 65% and 45% NPC patients, respectively^{25, 28}, and miR-155 was also found to be upregulated in many human tumors, which were not related to EBV²¹⁴⁻²¹⁹. Furthermore, in our study, both CNE1 and TW03 were EBV negative NPC derived cell lines, the expression of miR-155 in these two cell lines was higher than that in the immortalized nasopharyngeal epithelial cell line NP69. Hence there are also other factors than EBV genes which might upregulate miR-155

expression in NPC. TGF-beta (Transforming Growth Factor – beta) was verified to induce miR-155 expression by increased promoter activity due to SMAD4 (SMAD family member 5)²²⁶. Elevated serum levels of TGF-beta1 was also found in NPC patients²²⁷. Hence TGF-beta and SMAD4 pathways might also contributed to miR-155 overexpression in NPC.

Identification of JMJD1A as a direct target of miR-155 in NPC

At present, many direct targets of miR-155 have been identified and show oncogenic features of miR-155. MiR-155 promoted the proliferation of breast cancer cells through down-regulation of SOCS1 (Suppressor of cytokine signaling 1)²²⁸ and FOXO3a (forkhead box O3)²²⁹. MiR-155 has also been reported to be involved in the development of lymphoma by targeting SMAD5 (SMAD family member 5)²³⁰ and SHIP1 (inositol polyphosphate-5-phosphatase)²³¹. MiR-155 could promote pancreatic tumor development through downregulation of TP53INP1 (Tumor protein p53 induced nuclear protein 1)²³². Moreover, some other genes implicated in differentiation, inflammation and transcriptional regulation, were direct targets of miR-155, including HIF-1 (Hypoxia-inducible factor 1)²³³⁻²³⁴, BACH1 (BTB and CNC homology 1, basic leucine zipper transcription factor1)^{223, 233}, HIVEP2 (Human immunodeficiency virus type I enhancer binding protein 2)²²³, IKKε (Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase ε)²³⁵, and so on. In our study, we found that miR-155 could repress endogenous JMJD1A and BACH1 protein expression in NP69 cells. Luciferase reporter assay was performed to identify both JMJD1A and BACH1 as direct targets of miR-155 in NPC cells. This is the first report that JMJD1A is a direct target of miR-155. Furthermore, JMJD1A and BACH1 are downregulated in NPC cell lines and NPC tumor tissues.

Downregulation of JMJD1A predicts poor survival in NPC

The expression of JMJD1A and BACH1 was detected by immunostaining in 185 NPC cases. Low expression of JMJD1A was observed in 113 (61%), and was associated significantly with N-stage ($p = 0.023$). No significant association was seen between JMJD1A expression and age, gender, T stage, TNM stage, recurrence or metastasis. In addition, no significant association was seen between BACH1 expression and age, gender, T stage, N stage, TNM stage, recurrence or metastasis.

The five-year overall survival rate was 61.3% for patients with low JMJD1A expression ($n = 113$), and 77.2% for patients with high JMJD1A expression ($n = 72$), which was a significant difference ($p = 0.021$). The five-year overall survival rate was 66.8% for patients with low BACH1 expression ($n = 94$), and 68.2% for patients with high BACH1 expression ($n = 91$), which was not significant ($p = 0.759$). Furthermore, the five-year disease-free survival rate was 57.0% for NPC patients with low levels of JMJD1A expression ($n = 113$), and 68.7% for those with high levels of JMJD1A expression ($n = 72$), and this difference in the disease-free survival rate was significant

($p = 0.049$). No significant difference was seen in the disease-free survival rate of NPC patients, with or without BACH1 overexpression ($p = 0.895$).

Adam et al.²³⁶ demonstrated that loss of JMJD1A is sufficient to reduce tumor growth of renal cell carcinoma and colon carcinoma in vivo, suggesting that function of JMJD1A in different cells and tissues depend on cell microenvironment. Hence, the function of JMJD1A and BACH1 in NPC deserve for further attention.

4.3 ELEVATED SYK EXPRESSION CORRELATES WITH LMP2A EXPRESSION IN NPC AND PREDICTS POOR PROGNOSIS (PAPER III)

High expression of Syk correlates with clinicopathological parameters and predicts poor survival in NPC

Spleen tyrosine kinase (Syk) is a nonreceptor tyrosine kinase that contains tandem NH2 terminal Src homology 2 domains, multiple tyrosine phosphorylation sites, and a COOH-terminal tyrosine kinase domain²³⁷. Moreover, Syk promoter hypermethylation leads to gene silencing and reduced Syk expression correlates with tumor metastasis and poor prognosis in breast cancer²³⁸, pancreatic adenocarcinoma²³⁹, hepatocellular carcinoma²⁴⁰, bladder carcinoma²⁴¹, and gastric carcinoma²⁴². In contrast, overexpression of Syk is associated with recurrence and poor prognosis of squamous cell carcinomas of the head and neck (SCCHN) patients²⁴³. In addition, overexpression of Syk has also been observed in peripheral T-cell lymphomas²⁴⁴⁻²⁴⁵, chronic lymphocytic leukemia²⁴⁶, and B-lineage acute lymphoblastic leukemia²⁴⁷.

In this study, we showed that high expression of Syk was significantly associated with T-stage and local recurrence of NPC. Our data also indicated that NPC patients with high expression levels of Syk had shorter survival time, shorter disease free time, and that Syk was an independent prognosis factor in NPC. These results are consistent with the study of another head and neck cancer, SCCHN, in which, Sutima et al.²⁴³ reported that overexpression of Syk was associated with recurrence and poor prognosis of the patients.

Syk has been reported to be involved in tumor metastasis in many human cancers, either suppressing or enhancing metastasis. Reduced expression of Syk could promote cell migration in breast cancer²³⁸, while overexpression of Syk could enhance cell migration in SCCHN cells²⁴³. However, in our study, no significant correlation was found between Syk expression and tumor metastasis. It would have been highly interesting to compare Syk expression in paired samples of primary tumors and metastasis, but such clinical material with sufficient number of samples has not been

made available as yet (biopsies are rarely removed by surgery). Taken together, our results indicate that Syk may play a role in local NPC recurrence, and correlates with poor prognosis of NPC patients, suggesting that Syk could be used clinically as one of several biomarkers for NPC prognosis.

LMP2A induces Syk expression in NPC

It has been demonstrated that Syk could bind to the ITAM motif in EBV encoded LMP2A in B cells and epithelial cells²⁴⁸⁻²⁴⁹. Moreover, Syk interacts selectively in immune cells with proteins containing a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) through its distinct tandem Src homology 2 domain²⁵⁰. LMP2A, which is detected in the majority of NPC, contains an ITAM²⁵¹. In our study, we could confirm the interaction and co-localization of LMP2A with Syk as demonstrated by co-IP and IF in NPC cell lines. Interestingly, our results of ISH and IF indicated that LMP2A could enhance Syk expression in two NPC-cell lines SUNE1 and CNE2 cells, and LMP2A high expression positively correlated with Syk high expression in NPC clinical samples. However, LMP2A has been reported to promote the degradation of Syk in B cells by ubiquitination²⁵²⁻²⁵³. The increased expression of Syk in LMP2A positive NPC might be a compensation for a shorter half life of Syk when LMP2A binds and affects Syk degradation. LMP2A was reported to induce cell migration and invasion in epithelial cells.²⁹ Lu et al²⁴⁹ further demonstrated that endogenous Syk was activated in epithelial cells in the presence of LMP2A, and that Syk was required for LMP2A-triggered cell migration in epithelial cells. Taken together, these data suggested that LMP2A expression partly contributes to Syk high expression in NPC, and that Syk and LMP2A may have co-operative roles in NPC development. The precise relationship between Syk and LMP2A and the mechanism by which LMP2A induces Syk in NPC deserve further experimental studies.

4.4 WNT7A AND ITGA9 WERE HYPERMETHYLATED AND DOWNREGULATED IN NPC (PAPER IV)

Screening for potentially methylated genes in NPC using NotI micrarrays

Loss of heterozygosity (LOH) and cytogenetic studies have shown that frequent changes in Chromosome 3 (Chr. 3) is common in many tumors including NPC, harboring many TSGs¹⁸¹. To screen the potentially methylated or deleted genes in chr. 3 in NPC, Not I micrarrays were performed on genomic DNA from three NPC biopsies (T9, T10 and T18), two normal nasopharyngeal epithelia tissues (N1 and N2), three NPC cell lines (CNE1, TW03 and C666-1) and one normal nasopharyngeal epithelium derived cell line NP69. Ten genes (WNT7a, FGD5, ITGA9, RBSP3,

ALDH1L1, NUDT16P, ZIC4, EPHB3, BCL6 and FGF12) were identified as showing reduced signal suggesting methylation or deletion. These genes were selected for further validation by MSP and Q-PCR. Among these, we selected two interesting genes WNT7a and ITGA9, for further study.

WNT7a and ITGA9 were hypermethylated in NPC

The methylation status of the CpG rich region in the WNT7a gene promoter was investigated by bisulfite sequencing in four NPC biopsies (T1, T2, T5 and T6) and one normal nasopharyngeal epithelia biopsy (N10). Twenty-three CpG sites were included in this region. The WNT7a promoter was partially methylated in the four NPC samples, whereas there was almost no of methylation in the control sample. Similarly, methylation status of CpG island in ITGA9 gene intron 1 region was investigated in the same samples. Fourteen CpG sites were included in this region. The CpG island in ITGA9 gene intron 1 region was partially methylated in four NPC samples, whereas it was totally free of methylation in the normal sample.

To investigate whether 5-aza-CdR could restore WNT7a and ITGA9 expression, NPC CNE1 and TW03 cell lines were treated with 10 μ M of the demethylating agent 5-aza-2'-deoxycytidine (5-aza-CdR) for 96 h. QPCR was performed to detect WNT7a and ITGA9 expression in 5-aza-CdR treated and untreated NPC cell lines. Both WNT7a and ITGA9 expression were found to be restored after 5-aza-CdR treatment.

Downregulation of WNT7a and ITGA9 were found in NPC

QPCR was performed to detect WNT7a and ITGA9 expression in the three clinical NPC samples (T2, T3 and T4) as well as in four normal epithelial tissues (N2, N4, N5 and N7). The average expression levels of WNT7a and ITGA9 in NPC tumors (9.4 ± 7.7 fold; 4.0 ± 2.6 fold) were downregulated, compared with the expression in normal epithelial tissues (28.9 ± 18.9 fold; 19.9 ± 10.2 fold). Moreover, immunostaining was performed to evaluate the expression of WNT7a and ITGA9 in three cases of NPC tumor cells and adjacent normal nasopharyngeal epithelium. Strong expression of WNT7a and ITGA9 were observed in the membrane and cytoplasm of normal adjacent nasopharyngeal epithelium cells, while weak expression of WNT7a and ITGA9 were observed in NPC tumor cells.

In Non-Small Cell Lung Cancer (NSCLC) cells, the re-expression of WNT-7a and signaling through Fzd-9 are associated with increased differentiation and thus represent a tumor suppressor pathway²⁵⁴. It has been demonstrated that WNT7a is a positive regulator of E-cadherin expression in lung cancer cells²⁵⁵ and E-cadherin is also downregulated in NPC²⁵⁶. Pharmacologic reversal of WNT7a losses could be achieved with the use of inhibitors of GSK3 β , HDACs, or in some cases only with combined treatment²⁵⁵.

ITGA9 is abundantly expressed in lung cancers in general and small cell lung cancers (SCLC) in particular. Its aberrant upregulation in the SCLC samples, both cell lines and primary tumors has been suggested to play a role in metastasis and in the progression to more malignant phenotypes²⁵⁷. This is in contrast to the downregulation of ITGA9 in NPC and in human papilloma virus associated head and neck squamous cell carcinoma²⁵⁸.

In conclusion, we found that WNT7a and ITGA9 are methylated and downregulated in Chinese NPC patients. As both proteins execute significant functions related to the tumor cell biology, in depth functional studies has to be pursued to understand the functional role of WNT7a and ITGA9 downregulation in the development of NPC. With such studies these two genes might be useful for improved diagnosis and treatment in the future.

5 CONCLUDING REMARKS

Nowadays, several biomarkers in serum, saliva and tissue have been identified for NPC screening, early diagnosis and prognosis prediction. Using serological biomarkers is a common and convenient way to screen and monitor NPC, such as EBV-specific antibodies in serum for early diagnosis and mass screening of NPC patients¹⁵⁴⁻¹⁵⁵, and EBV viral load in serum for diagnosis and monitoring recurrence in NPC patients¹⁵⁷⁻¹⁶¹ and Cell-free circulating methylated gene promoter DNA in assisting in screening of potentially local or regional recurrent NPC¹⁶⁴. Using epigenetic biomarkers in saliva and body fluids is a non-invasive way to screen and detect residual carcinoma after treatment of NPC¹⁶². Genomic biomarkers could be used for screening the high risk population through NPC specific susceptibility loci⁹⁻¹¹. Protein biomarkers in tissues could be used for diagnosis and prognosis prediction of NPC¹⁷³.

In this study, we have tried to identify the new protein biomarkers and epigenetic biomarkers for NPC early diagnosis and prognosis prediction. Several approaches to identify biomarkers of significant clinical and functional interest have been explored. We have investigated some genes with considerable impact in other tumors (**Paper I and III**), genes regulated by miRNAs (**Paper II**) and epigenetically deregulated genes (**Paper IV**). In paper I, II and III, four protein biomarkers have been identified for prognosis prediction of NPC, and a larger scale of NPC samples are needed for further validation. In paper IV, two putative epigenetic biomarkers have been found, and further study is needed to evaluate their potentials for early diagnosis of NPC using either saliva or blood.

These are my main conclusions from the work:

1. Cav-1 and CD147 overexpression predict poor NPC prognosis and enhanced tumor cell migration, which is associated with MMP-3 and MMP-11 (active) secretion.
2. Upregulation of miR-155 in NPC is partly driven by LMP1 and LMP2A, and results in downregulation of JMJD1A, which is associated with N stage and poor prognosis of NPC patients.
3. High expression of Syk, which is resulted partly from LMP2A expression in NPC, is associated with tumor recurrence and poor prognosis of NPC patients
4. Downregulation of WNT7A and ITGA9 in NPC is partly due to promoter hypermethylation. The potential of WNT7A and ITGA9 as diagnosis or therapeutic targets for NPC should be further investigated

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