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MICROBIAL AND EPIGENETIC FACTORS IN THE PATHOGENESIS OF NASOPHARYNGEAL CARCINOMA

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To my family

ABSTRACT

While uncommon in most of the world, nasopharyngeal carcinoma (NPC) shows an unusual geographic and ethnic distribution, being highly prevalent in Southern China and Southeast Asia. Genetic susceptibility, Epstein-Barr virus (EBV) infection and additional environmental exposures are well established risk factors for NPC in endemic areas. However, the detailed molecular mechanisms of NPC pathogenesis remain largely unknown. In this thesis, several novel pathogenic mechanisms of NPC development and progression are presented.

The interaction of EBV encoded latent membrane protein 2A (LMP2A) with cellular proteins promoting invasiveness of NPC cells is described in paper I. Spleen tyrosine kinase (Syk) interacts with integrin β4 subunit (ITGβ4) in epithelial cells through an ITAM-like motif, and concurrent LMP2A expression interferes with this interaction by competitive binding to Syk. Both Syk and LMP2A affect cell surface expression of ITGβ4. Particularly, ITGβ4 concentrates at cellular protrusions in LMP2A expressing cells, which may contribute to the migration property of NPC-cells.

Paper II and paper III focus on the epigenetic alteration of candidate tumor suppressor genes (TSGs) and their possible role in NPC tumorigenesis. Cadherin 4 (CDH4) and ubiquitin-conjugating enzyme 2L6 (UBE2L6) are downregulated due to promoter hypermehtylation in NPC. Both genes suppress the proliferation and colony formation of NPC-cells. CDH4 impedes cell migration and elicits cell communication; UBE2L6 induce apoptosis of NPC cells and counteracts degradation of adipocyte triglyceride lipase (ATGL) through ISG15-conjugation of valosin-containing protein (VCP). CDH4 and UBE2L6 could be involved in both initiation and progression of NPC. Suppression of UBE2L6 encoded protein UbcH8 correlated with poor outcome in NPC patients.

In paper IV, we compared the response of NPC and normal nasopharyngeal epithelial (NNE) cell lines to bacteria and bacterial cell wall components. Strong nuclear translocation of NF-κB and significant induction of proinflammatory factors IL6, IL8, IL1α and CXCL2 were observed in NNE cells, but not in NPC cells upon exposure to Gram-positive bacteria streptococci and peptidoglycan (PGN). We identified three different mechanisms by which the activation of NF-κB in NPC cells could be hampered. It could be trapped by an enhanced accumulation of cytoplasmic lipids. I-κB degradation could be impaired due to downregulation of UBE2L6. We also showed that overexpression of lysine-specific demethylase-1 (LSD1) blocked the transcriptional activation of proinflammatory genes. Together these mechanisms might contribute to decreased immune reactivity in NPC and thus affect tumor progression.

LIST OF SCIENTIFIC PAPERS

I. **Xiao-Ying Zhou#**, Liudmila Matskova#, Li-Sophie Z. Rathje#, Xue Xiao, Gerald Gish, Maria Werner, Ilya Ignatyev, Na-Na Yu, Wei-Lin Zhao, Fang-Yun Tian, Bo Hou, Zhe Zhang, Tony Pawson, Fu Chen, Ingemar Ernberg*, SYK interaction with ITGβ4 suppressed by Epstein-Barr Virus LMP2A modulates migration and invasion of Nasopharyngeal Carcinoma Cells. Oncogene. 2015 Aug 20;34(34):4491-4499. PMID: 25531330

II. Chun-Ping Du#, Ting-Ting Huang#, Di Sun, Ying-Xi Mo, Hai-Yan Feng, **Xiao-Ying Zhou**, Xue Xiao, Na-Na Yu, Bo Hou, Guang-Wu Huang, Ingemar Ernberg, Zhe Zhang*, CDH4 as a novel putative tumor suppressor gene epigenetically silenced by promoter hypermethylation in nasopharyngeal carcinoma. Cancer Lett. 2011 Oct 1; 309 (1):54-61. PMID: 21665361

III. **Xiao-Ying Zhou#**, Jia-Zhang Wei#, Fu Chen, Xue Xiao, Ting-Ting Huang, Qian He, Shu-Min Wang, Chun-Ping Du, Ying-Xi Mo, Long-De Lin, Ying Xie, Li-Li Wei, Ying Lan, Mairiko Murata, Guang-Wu Huang, Ingemar Ernberg*, Liudmila Matskova, Zhe Zhang*, Epigenetic downregulation of the ISG15-conjugating enzyme UbcH8 impairs lipolysis and correlates with poor prognosis in nasopharyngeal carcinoma. Oncotarget, in press.

IV. **Xiao-Ying Zhou**, Liudmila Matskova, Xue Xiao, Yu-Feng Chen, Feng He, Guang-Wu Huang, Zhe Zhang, Ingemar Ernberg*, Induction of inflammatory response in nasopharyngeal epithelial cells by microflora components is impaired in nasopharyngeal carcinoma cells. Manuscript.

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CONTENTS

LIST OF ABBREVIATIONS

5-aza-dC	5-aza-2'-deoxycytidine
ATGL	Adipocyte triglyceride lipase
CDH4	Calcium-dependent cell adhesion glycoprotein 4
ChIP	Chromatin immunoprecipitation
CXCL2	Chemokine (C-X-C motif) ligand 2
DNA	Deoxyribonucleic acid
DUBs	Deubiquitinating enzymes
EBV	Epstein-Barr virus
EMT	Epithelial-mesenchymal transition
GJIC	Gap junction-mediated-intercellular communication
HDACi	Histone deacetylase inhibitor
HDs	Hemidesmosomes
IL1α	Interleukin 1, alpha
IL6	Interleukin 6
IL8	Chemokine (C-X-C motif) ligand 8
ISG15	Interferon-stimulated gene 15
ITAM	Immunoreceptor tyrosine-based activation motif
ITGβ4	Integrin β4
I-κB	Inhibitor of nuclear factor of kappa B
LDs	Lipid droplets
LMP2A	Latent membrane protein 2A
LPS	Lipopolysaccharide
LSD1	Lysine-specific demethylase 1
MSP	Methylation specific PCR
NF-κB	Nuclear factor kappa B
NNE	Normal nasopharyngeal epithelium
NPC	Nasopharyngeal carcinoma
PCR	Polymerase chain reaction
PGN	Peptidoglycan
RNA	Ribonucleic acid
SH2 domain	Src homology 2 domain
Syk	Spleen tyrosine kinase
TLRs	Toll-like receptors
TSA	Trichostatin A
TSG	Tumor suppressor gene
UbcH8	Human ubiquitin-conjugating enzyme 8
UBE2L6	Ubiquitin-conjugating enzyme 2L6
UPS	Ubiquitin proteasome system
VCP	Valosin-containing protein

1 INTRODUCTION

Cancer constitutes a social challenge to the whole world. Some lifestyles are known to increase the risk of cancer (e.g. smoking, diet, lack of physical activity and fertility changes). Along with population growth and aging, the occurence of tumor is expected to increase globally, especially in underdeveloped countries [1].

The tumor formation is a multistep process of great complexity. After exposure to risk factors, normal tissues show abnormal histologies with mild hyperplasia, which is still reversible. The subsequent irreversible, severe hyperplasia means the onset of cancer, leading to aggresive growth. There are three levels of counteracting strategies. One can avoid the occurrence of cancer by reducing the exposure of individuals to diverse risk factors or increase their resistance to them. One can apply appropriate screening test for early detection and treatment of cancer. Finally, one can improve the outcome of cancer course [2]. All of these measures, designed to cure cancer, have to be based on the knowledge of tumor biology.

1.1 Brief introduction to tumor biology

According to the Hanahan–Weinberg concept, eleven hallmarks of cancer constitute essential factors for oncogenesis [3]. The overgrowth and growth distant from the primary tumor are the most essential factors for oncogenesis.

1.1.1 Over-growth

Normal cells can be converted to neoplastic growth out of control in almost every tissue of the human body. To maintain the tissue architecture and function, the number of normal cells are carefully controlled. Cancer cells aquire the capacity to sustain proliferation by conveying growth signals through cell surface receptors [4]. These growth factor receptors are activated and transduce mitogenic signals upon phosphorylation of cytoplasmic tyrosine domains. For example, the tyrosine kinase Src is needed for phosphorylation of the epidermal growth factor receptor (EGFR) signaling and is regarded as a proto-oncoprotein in lung cancer since it is upregulated there [5, 6]. EGFR stimulation leads to activation of the transcription factor erythroid-2-related factor 2 (Nrf2), promoting cell proliferation. On the other hand, Nrf2 could be continuousely activated by abolishing its cytoplasmic repressor, independent of EGFR signaling [7, 8]. In addition, tumor cells can produce and secrete growth factors by themselves and express the corresponding receptors, establishing an autocrine signaling loop, thus enhancing proliferation. For example, in pancreatic cancer, the activating integrin $\alpha 6\beta 4$ signals enhances transcription of EGFR ligands, as well as EGFR, resulting in enhanced cell malignant behavior [9].

Another feature of tumor is the ability to overcome the end-replication problem by elongating telomeric DNA and activate telomerase, thereby escaping cell senescence or crisis [10]. The human telomerase reverse transcriptase (hTERT) catalytic subunit, and the human telomerase

(hTR) RNA subunit, responsible for activating telomerase and telomere maintenance, are required for cellular immortalization and tumor progression [11, 12]. The cells with thus acquired lifespan accumulate genetic and epigenetic alterations. This may lead to malignant transformation. Further, increased hTERT expression is suggested to modulate the expression of numerous genes to accelerate cell cycle progression and activate mitogenic signaling [13].

Besides, tumor cells inactivate or silence the tumor suppressor genes (TSGs) involved in multiple cellular activities to prevent conversion to the tumorigenic phenotype [14, 15]. The loss of TSGs function, caused by either genetic mutations/deletions [16, 17], loss of heterozygosity (LOH) [18] or epigenetic alteration, including promoter hypermethylation, histone methylation/deacetylation, and micro-RNA [19-21], has been widely demonstrated in tumors. TSGs generally follow the "two-hit hypothesis" suggested by Knudson, which says that both alleles coding for a particular protein must be damaged to achieve the loss of function [22]. However, not all TSGs are undetectable in tumors, but gain new powers in promoting oncogenesis through a mutant variant, such as p53 [23].

Aposptosis is a cell suicidal program, which is essential in normal tissues to maintain tissue hemeostasis, such as the turnover process of epithelial cells. Cells sense the damaged genome and subsequently initiates the apoptosis program to eliminate the possible chance for abnormal cell growth [24]. There are two canonical apoptosis machineries, the death receptor- and mitochondria-dependent pathway [25]. Once being induced, the downstream executioner caspases such as caspase 3 and caspase 7, are cleaved and obtain capacity to kill cells [26, 27]. Tumor cells counterwork apoptosis by overexpression of anti-apoptotic proteins and inactivation of pro-apoptotic genes [28]. As a results, too much growth and too little death generate a massive tumor. "Autophagic cell death" is another aspect of programmed cell death [29]. Recent findings suggested dual roles of autophagy in tumorigenesis, either protecting cells from metabolic stress or prolonging tumor suvival [30, 31].

Human body defense itself against abnormal growth by tumor surveillance exerted by the immune system [32]. The suvival of tumor is often enhanced when immunity is suppressed. Tumor cells, due to ability to immune-edit themselves, may escape the attack from cytotoxic T lymphocyts [33] and promote induction of T regulatory cells [34].

It has been suggested that the evolution from normal to a malignant phenotype takes few years to decades. During this period, cells sustain proliferative signaling, enable replicative immortality, resist cell death and evade growth suppression and immune surveillance. These features are important in tumor initiation and massive growth.

1.1.2 Distanced growth

Approximately 90% of all deaths, caused by cancer, arise from the metastasis of primary tumors [35]. The out-growth of tumor is a multi-stage process, which include tumor invasion,

dissemination through the bloodstream or the lymphatic vessels and colonization of distant organs.

Most of the cancers originate from epithelial tissues. The abarrently adhesive and migratory capablilities of tumor cells endow the spread of primary tumors. Typical morphological changes occur during epithelial-mesenchymal transition (EMT). During the EMT process, cells progressively redistribute or downregulate their apical and basolateral epithelial-specific tight and adherence junction proteins, and re-express mesenchymal molecules [36, 37]. EMT is induced by several growth factors (e.g. transforming growth factor, epidermal growth factor, insulin-like growth factor and fibroblast growth factor) produced by tumor cells themselves or by tumor associated stromal cells, and also by elevating the proteolytic activity of matrix metalloproteases [38]. The subpopulation of tumor cells, known as cancer stem cells (CSCs) able to self-renew and regenerate the tumor, are shown to endow a hallmark of EMT-like properties and high aggressiveness [39, 40]. Rencently, it was proposed that the EMT program in CSCs and normal stem cells is different [41], suggesting that it might be possible to find specific targets for CSCs in cancer therapy.

Hypoxia is a feature of tumor microenvironment facilitating cancer metastasis [42-44]. The hypoxia-inducible factors (HIF-1α and HIF-2α), as transcription factors, drive the expression of an angiogenic factor VEGF-A [45, 46] and contribute to the formation of new blood vessels. Therefore, angionenesis not only supports primary tumor growth but also facilitates the metastatic dissemination of tumor cells. The amount of circulating tumor cells (CTCs) in the blood stream could potentially serve as a prognostic factor and help to optimize therapy in tumor patients [47, 48]. Besides, the induction of lymphangiongenesis also could be found in many kinds of tumor, stimulated by VEGF-C, VEGF-D and chronic inflammation [49-51].

The capillaries in circulatory system, as well as the molecues on the luminal surface of endothelial cells that are recognized by the adhensive molecules in cancer cells, offer a specialized docking site for disseminating cancer cells [52]. The subpopulation of CTCs with a certein gene set even obtain the capacity to home to particular organs [53-55]. However, only a minority of CTCs with CSC characteristics can form metastases, termed as "metastatic inefficiency" [56, 57]. The program of adaptation and colonization of distant sites by metastatic tumor cells remains unclear. The microenvironment at the distant site may be instrumental for this [58, 59].

1.1.3 Other aspects of tumor biology

1.1.3.1 Abnormal metabolism

Tumors show various metabolic abnormalities, requiring energy for their accelerated rate of proliferation. The Warburg effect is central in tumorigenesis of many cancers, characterized by a high rate of glycolysis and lactate production in tumors in the presence of sufficient oxygen [60]. Several genes are involved in this aerobic glycolytic process. For example, the activation of PI3K/Akt/mTOR system in tumor cells enhances the metabolic activities to

support cellular biosynthesis [61, 62]. The oncogenic transcription factor c-Myc has been shown to regulate glycolysis through the direct activation of lactate dehydrogenase [47] and genes associated with mitochondrial biogenesis [63-65]. Another well known transcription factor HIF-1 is involved in upregulation of genes encoding glucose transporter 1 (GLUT1) and activation of pyruvate dehydrogenasekinases (PDKs), thus limiting the entry of pyruvate into the tricarboxylicacidcycle acid (TCA) cycle [66-68]. On the other hand, the tumor suppressor p53 has been found to inhibit glycolysis by elevating superoxide and blocking autophagy [69].

A part of the pyruvate, generated by glycolysis is converted to acetyl-CoA (Ac-CoA) and enter into TCA cycle, where it is converted into citrates, required for the synthesis of fatty acids and cholesterol [70]. Under physiological conditions, fatty acids are mainly synthesized and stored in hepatocytes and adipocytes. Normal cells tend to uptake exogenous, circulating fatty acids. On the contrary, tumor cells are primarily dependent on endogenous fatty acid, which are stored in the form of lipid droplets (LDs) [45] or oxidated to supply energy [71, 72]. Fatty acid synthase (FASN), an enzyme catalyzing de novo fatty acid synthesis, mainly produces 16-carbon saturated fatty acid, palmitic acid, which is the main component of cell membrane [73]. FASN is found to be upreguated in various tumors (e.g. breast, prostate and colorectal carcinomas) [74-76], classified as an oncogene, leading to excessive lipogenesis [77, 78]. Some viruses like Hepatitis C virus and Epstein-Barr virus also modulate fatty acid metabolism by upregulating the translation of FASN [79, 80]. However, the mechanism involved is not clear yet.

Increased biosynthesis of fatty acids and cholesterol/triacylglycerides lead to LDs accumulation, which have been widely documented in tumors [81-84]. LDs are highly ordered intracellular structures, with a neutral lipid rich core, which is surrounded by a phospholipid monolayer, and decorated by proteins that play roles in the biology of LDs (Figure 1) [85]. CSCs, which show accumulation of LDs, retain their tumorigenic potential [84, 86]. The LDs may also serve as a source of inflammatory mediators [87, 88], and impact on the signaling transduction pathways [89, 90].

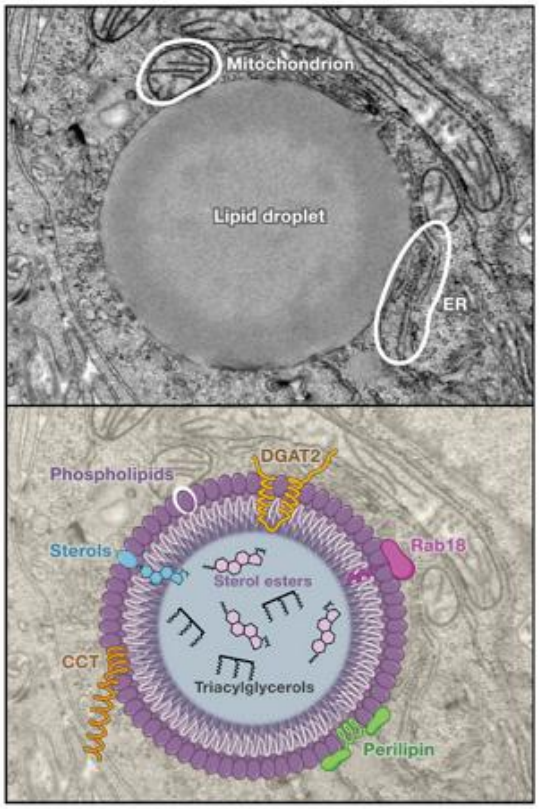

Figure 1 Anatomy of Lipid droplets.[85]

1.1.3.2 Chronic inflammation

Histologically, injured tissues and tumor tissues look similar, both containing inflammatory cells, endothelial cells and fibroblast cells. In many cases tumors seem like wounds that fail to heal (Figure 2). Chronic inflammation is a risk factor for cancer development [91, 92]. The recruited inflammatory cells not only produce reactive oxygen species (ROS), which leads to oxidative DNA damage, contributing to neoplastic initiation [93], but also release growth factors to foster tumor proliferation [94]. In addition, they secrete matrix metalloproteinases (MMPs) and VEGFs [95, 96], faciliating tumor metastasis and produce IL10 that limit the anti-tumour response by cytotoxic T cells [97].

Cancer also causes inflammation. The sustained activation of NF-κB, STAT3, or HIF-1α was found in tumor cells, mediating the expession and release of proinflammatory cytokines, chemokine and the inflammatory enzyme (e.g. TNF-α, IL6 and COX-2), which form a complex inflammatory tumor microenvironment and further support the progression of tumors [98].

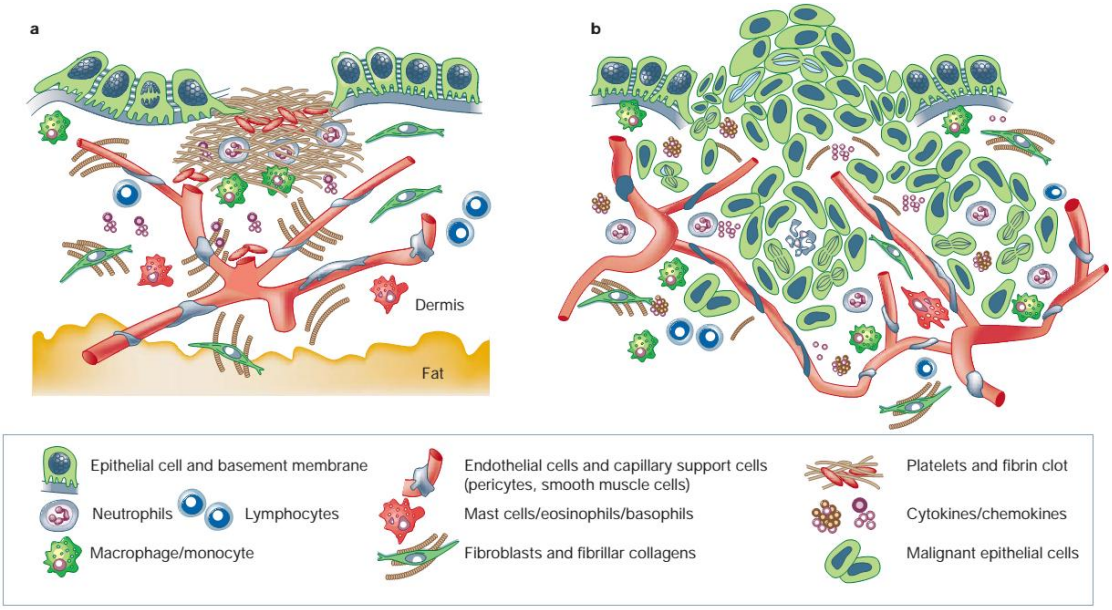

Figure 2 General view of wound healing vs. tumor tissues. [99]

1.1.3.3 Infections and tumor biology

In fact, over 15% of of cancers worldwide are associated with microbial infection [100], chiefly by virus infection. The most well documented oncogenic viruses are human papillomavirus (HPV), Epstein-Barr virus (EBV), Kaposi's Sarcoma Herpesvirus (KSHV), hepatitis B virus (HBV), hepatitis C virus (HCV), human T-cell leukemia virus (HTLV1). They cause diverse malignancies of people world-wide (Table 1) [101]. A key carcinogenesis strategy, utilized by these viruses is the evasion of apoptosis and immune surveillance to establish long-term infection. However, virus itself is not sufficient to develop tumors. Additional co-factors such as genetic mutations, inflammatory microenvironment, and the effect of environmental carcinogenes, co-operate to achieve malignant transformation [102].

Table 1. Human tumor viruses.

Virus	Human cancers
Human papillomavirus	Cervical, oral, and anogenital cancer
Epstein-Barr virus	Burkitt's lymphoma, nasopharyngeal carcinoma, lymphomas, Gastric carcinoma
Kaposi's Sarcoma Herpesvirus	Kaposi sarcoma, primary effusion lymphoma
Hepatitis B virus	Hepatocellular carcinoma
Hepatitis C virus	Hepatocellular carcinoma
Human T-cell leukemia virus	Adult T-cell leukemia

Modifed from Biochimica et Biophysica Acta, 1782 (2008):127–150.[101]

Chronic inflammation induced by bacteria also could contribute to tumor development. For example, helicobacter pylori infection is strongly associated with gastric carcinoma. The colonization by helicobacter pylori evokes the pro-inflammatory responses in gastric mucosa. Besides, its oncoprotein CagA has multiple ways to reprogram epithelial cells, for example,

by overactivating the cellular signaling transduction pathways and interfering with tumor suppressors [103].

1.2 Characteristics of nasopharyngeal carcinoma (NPC)

1.2.1 Epidemiology of NPC

An estimated 86,700 new cases of NPC and 50,800 deaths were registered in 2012 globally. Approximately 92% of new cases occurred in less developed countries [1]. NPC occurrence prevails in select geographic and ethnic populations. In Southern China (e.g. Guangdong and Guangxi provinces and Hong Kong) [104, 105] and Southeast Asia (e.g. Malaysia, Indonesia, Singapore) [105-107] the incidence rate of NPC is especially high (20 to 30/100,000 per year) [108]. Besides, North Africa (e.g. Tunisia and Algeria) [109, 110] and Arctic regions (e.g. Alaska, Greenland and North Canada) [111-113] show relatively high incidence. Immigrants from Southern China also have a higher risk of NPC as compared to the local western population [108]. Independent of race and/or ethnicity, men are two to three times more likely to develop NPC than women. In the low risk populations, NPC incidence increases with increasing age (Figure 3A). In contrast, the peak age of disease occurrence is between 50 to 59 years in endemic area and declines thereafter (Figure 3B) [114, 115], suggesting the exposure to carcinogens early in life

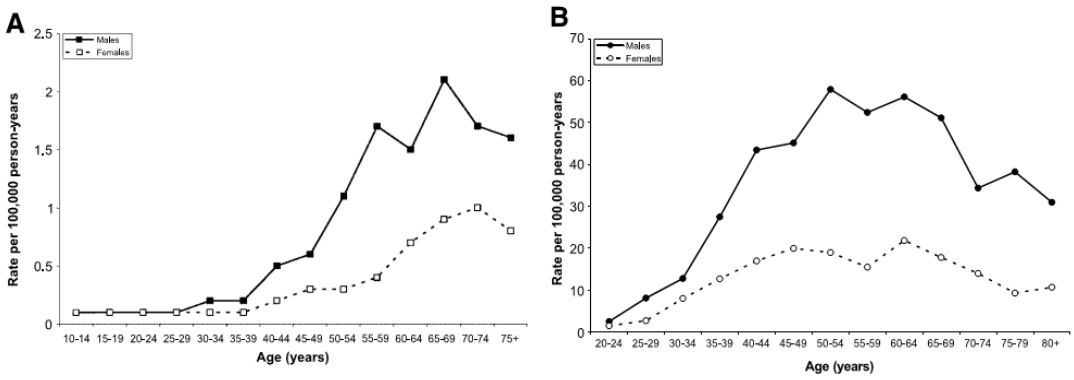

Figure 3 Age-specific incidence rates of NPC for white males and females in the U.S.A.(A) and males and females in Hong Kong (B). [115]

1.2.2 Histopathological classification of NPC

NPC is a cancer arising from the epithelium of nasopharynx. Histologically, NPC cells are regarded to be of squamous in origin, but with a high background of lymphoid cells. The world health organization (WHO) categorized NPC into three types: nonkeratinizing carcinoma, keratinizing squamous cell carcinoma and basaloid squamous cell carcinoma. Over 95% of the cases belong to the nonkeratinizing type, while less than 5% belong to the keratinizing squamous cell carcinoma type. Rare case may be diagnosed as basaloid squamous cell carcinoma type in endemic areas. In nonendemic Western countries like United States, keratinizing squamous cell carcinoma accounts for 25% of NPC cases [114].

1.2.3 Etiological factors of NPC

It has been suggested that the pathogenesis and development of NPC is a multistep process, however the molecular mechanism involved remains unclear. At present, the most widely accepted etiological factors for NPC are latent EBV infection, genetic susceptibility and carcinogen exposure at early age [108, 115].

1.2.3.1 Epstein-Barr virus infection

Epstein-Barr virus (EBV), or human herpes virus 4, was the first human tumor virus to be described. It ubiquitously infects more than 90% of the population in the world and establishes persistent asymptomatic infection with a specific tropism for B and epithelial cells [116]. EBV is transmitted via salivary contact. It preferentially infects B cells through binding to the surface CD21 receptor and/or human leukocyte antigen class II (HLA II) molecules [117, 118]. In normal healthy carriers, EBV persists in circulating memory B cells. In NPC, the EBV genome has been detected in the epithelial tumor cells but not in the infiltrating lymphoid cells [119]. However, it remains unclear how EBV enter epithelial cells which express extremely low level of CD21 [120]. Several possible mechanisms have been proposed, including direct cell-to-cell contact of the surface of epithelium with EBV-infected salivary B cells [121], the internalization of EBV mediated by polymeric immunoglobulin-A receptor(pIgR) of epithelium [122], and a virus-epithelial cell fusion triggered by integrin receptors [123-125]. Recently, a nonmuscle myosin heavy chain IIA (NMHC-IIA) protein of nasopharyngeal epithelial cells, was found to play an important role for efficiency of EBV infection [126].

EBV DNA could be detected in all NPC tumor cells, with monoclonal characteristics, which suggest a clonal expansion of a single EBV-infected progenitor cells [127]. The virus exists as an extra-chromosomal episome in NPC cells and it is not integrated into the host genome. EBV adopts a specific form of latent infection in NPC, in which only a few EBV encoded genes are expressed, including EBV nuclear antigen 1 (EBNA-1), latent membrane protein 1 (LMP1), LMP2, EBV-encoded RNAs (EBERs), and BamHI A rightward transcripts [128, 129]. Figure 4 summarizes the impacts of EBV latent genes on carcinogenesis of epithelial cell. Recent studies have showed that virions, produced during the EBV lytic phase, increase apoptosis of NPC cells and induce a powerful immune response toward the tumor cells [130, 131]. On the other hand, smoking is demonstrated to activate EBV infection, but this is still associated with increasing risk of NPC [132]. In support of this, it was shown that recurrent lytic EBV promotes genome instability and drives the progression of NPC cells to acquire a more malignant phenotype [133]. The role of lytic EBV infection in NPC carcinogenesis is still under debate.

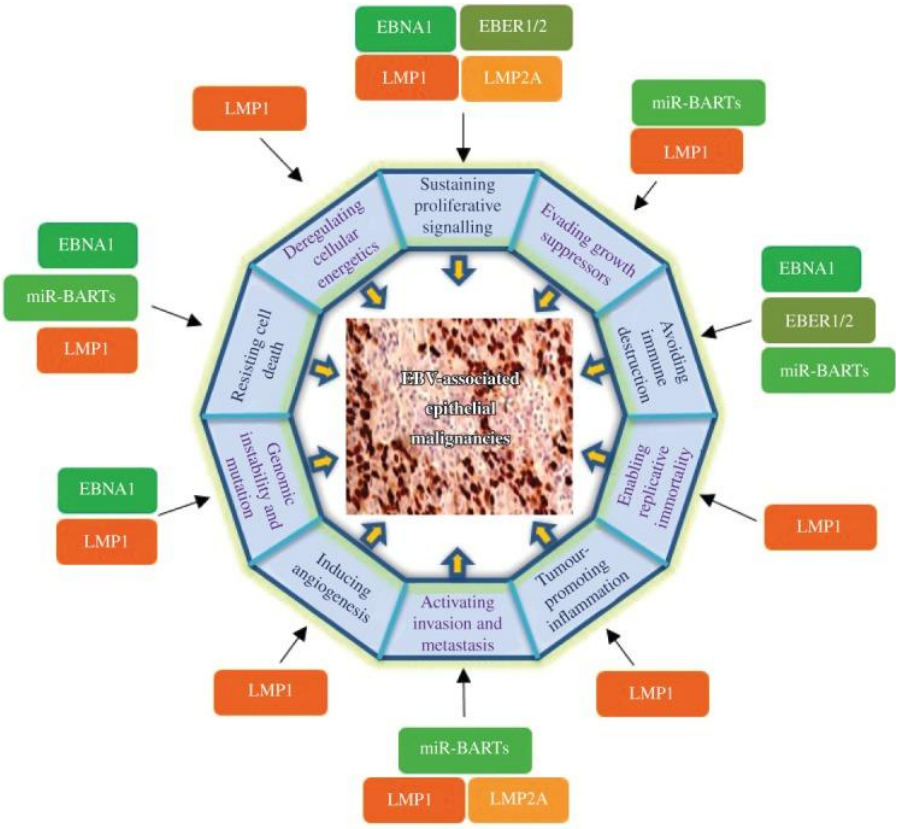

Figure 4 Epstein–Barr virus (EBV) latent genes target cancer hallmarks of epithelial malignancies. [134]

1.2.2.2 Genetic and epigenetic factors

Genetic susceptibility is considered as a determining risk factor for NPC development in endemic regions. Familial aggregation is a feature of NPC. Several genetic susceptibility loci, linked to NPC development in families, have been identified in chromosomes 3, 4 and 5 [135-137]. The increased risk of cancer in NPC families is not restricted to nasopharyngeal carcinoma, but extends to the other cancers, associated with different viruses [111]. The presentation of foreign antigens to the immune system is mediated by a human leukocyte antigen (HLA), leading to a cell lysis. Individuals, who inherited some types of HLA alleles, which reduce the efficiency of EBV presentation, as a particular case, may have a higher risk in developing NPC. The dysfunctional HLA alleles facilitate tumor evasion from the immune surveillance [138]. Recent genome-wide association studies (GWAS) of NPC patients and healthy controls identified the most significant single nucleotide polymorphisms (SNPs) in the HLA-A gene. Other, non-HLA, susceptibility loci were also identified, like those coding for the GABB receptor 1 (GABBR1), HLA-F, tumor necrosis factor (TNF) receptor superfamily 19 (TNFRSF19), myelodysplasia 1 and ecotropic viral insertion site 1 fusion proteins (MDS1-EVI1), cyclin-dependent kinase inhibitor 2A (CDKN2A), and CDKN2B [139, 140].

Apart from SNPs, chromosome rearrangements are associated with NPC development. The chromosome gains are frequently detected in chromosomes 3q, 11q, 12p. The most studied oncogenes e.g. Cyclin D1 (CCND1), transformed 3T3 cell double minute 2 (MDM2),

Ecotopic viral integration site 1 (EVI1), all suggested to be involved in NPC pathogenesis, were found at these loci [141-143]. The LOH is commonly found in chromosome 3p, 9p, 11q, 13q, and 14q [115]. Many putative TSGs have been described at chromosome 3p and 9p [144-147].

Accumulating data suggest that epigenetic alterations, including promoter hypermethylation and histone modifications, are the most important mechanisms for inactivation of TSGs [148, 149]. Hypermethylation of CpG islands at TSG promoters is much more frequently reported phenomenon in NPC than histone modification. Thus, promoter hypermethylation is the major mechanism for inactivation of TSGs in NPC. For example, silencing of CDKN2A gene expression due to promoter methylation is an important co-factor for malignant transformation of epithelial cells of nasopharynx [108]. Furthermore, EBV-associated cancers were shown to be influenced by epigenetic modifications. A distinct high methylation profile was observed in EBV-positive gastric cancer in contrast to EBV-negative gastric cancer [150]. In NPC, EBV infection epigenetically affect both the viral and cellular genomes [151, 152], which may relate to EBVs role in NPC carcinogenesis.

1.2.2.3 Environmental carcinogens

Epidemiological studies have proposed associations between several dietary and social practices, and an increased risk for NPC development. The most common and strong association has been established for salted fish consumption. In salt preserved food, nitrosamines, known as carcinogens in animals, is accumulated during the process of preservation. The use of traditional herbal medicines elevates the risk, as shown in some studies, may be due to a capacity to induce EBV lytic phase [115]. Cigarette smoking, smoking habits and alcohol consumption have weaker association with NPC, with inconsistencies between studies [114]. Another significant factor is chronic inflammation of respiratory tract [153, 154]. In addition, the imbalance of microflora composition might contribute to the inflammatory condition and production of pro-tumorigenic chemical compounds in nasopharynx [128, 155].

1.3 LMP2A, spleen tyrosine kinase (SYK) and integrin β4 (ITGβ4)

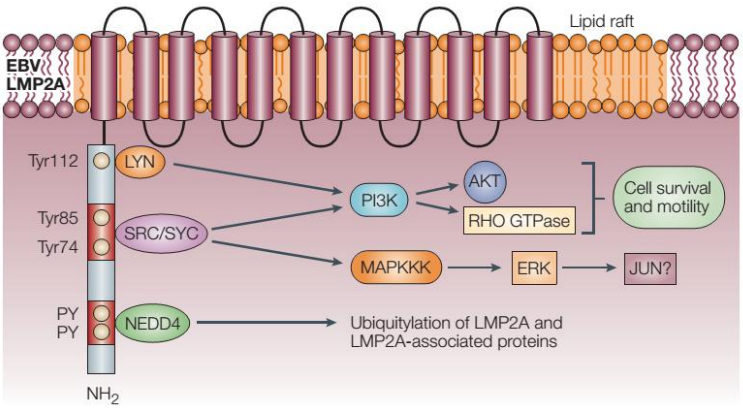

Figure 5 Structure and function of LMP2A.[156]

As shown in figure 5, LMP2A is a 12 transmembrane protein containing an 119 amino acid-long intracellular N-terminal tail and a C-terminal tail of 27 amino acids [157]. In the N-terminus, tyrosine residue (Tyr112) binds the LYN tyrosine kinase and mediates the constitutive phosphorylation of the other tyrosine residues [158]. Two tyrosine residues (Tyr74 and yr85) form an immunoreceptor tyrosine-based activation motif (ITAM), when phosphorylated, recruiting members of the SRC family of protein tyrosine kinases and the SYK tyrosine kinase. In B cells, LMP2A blocks the B cell antigen receptor (BCR) signaling transduction by sequestering these tyrosine kinases, initiating instead its own signals to enhance B cell survival and motility, and controlling viral latency [157, 159]. In addition, proline rich motifs, around Y60 and Y101 of LMP2A recruit NEDD4-like ubiquitin protein ligases, resulting in ubiquitin-dependent degradation of LMP2A and LMP2A associated proteins (e.g. LYN and Syk) which contribute to blocking BCR signaling [160]. LMP2A mRNA is consistently expressed in almost all NPCs, whereas at the protein level, it was detected in approximately 58% of the NPC tumors samples and mainly localized at the tumor invasive front [161, 162]. LMP2A-transfected epithelial cells exhibit increased proliferation, EMT, invasion, and migration through the activation of several signaling cascades, including Notch, ERK, Syk, PI3K/Akt, c-Jun and Wnt/-catenin [159, 163-165], and can induce a stem cell-like phenotype [162]. Furthermore, LMP2A limits the anti-viral response by targeting interferon receptors for degradation [166]. These biological functions of LMP2A support a potentially tumorigenic capacity of this viral protein.

The Syk tyrosine kinase possesses two N-terminal SH2 domains that engage phosphorylated tyrosine sites within the ITAMs of antigen receptor subunits, as a signaling effecter [167]. For a long time, Syk function was only studied in hematopoietic cells. It was shown to play a crucial role in the immunoreceptor signaling by influencing processes such as proliferation, differentiation and phagocytosis [168, 169]. Later, the Syk expression was identified in a variety of other cell types [170]. Besides, the SH2 domains of Syk can bind integrin cytoplasmic domains through ITAM containing adapter proteins or directly to participate in the integrin signalling [171-173]. In a number of tumors, loss of Syk expression by promoter hypermethylation has been frequently reported [174-177]. It was demonstrated that the low Syk expression contributes to genomic instability, increases cell proliferation and promotes invasion of cancer cells [178-180]. This resulted in classification of Syk as a tumor suppressor [181]. Overexpression of Syk has also been documented (e.g. in head and neck cancer including NPC, and lung cancer), which correlated with poor outcome in patients and was suggested as a therapeutic target [182-184].

Integrins, the heterodimeric cell surface receptors consisting of α and β subunits, belongs to a class of major membrane signaling molecules required for cell trafficking and for adhesion to other cell types and to constituents of the extracellular matrix. They also function as signal transducing receptors that can control intracellular pathways, can regulate cell survival, proliferation and cell fate [185, 186]. ITGβ4 has been implicated in two apparently contrasting processes, organizing stable adhesion of epithelial cell via hemidesmosomes [187] and promoting cell motility and invasion [188]. It has been shown

that LMP2A promotes cell migration through inducing membrane translocation or stabilization of ITGαV and ITGα6 [189, 190]. Interestingly, although ITGα6 and ITGβ4 work as a dimer, the regulation of each differs during tumorigenesis. ITGβ4 is downregulated by promoter methylation during EMT, upon the treatment by transforming growth factor-β (TGF-β) [191]. At the same time, ITGβ4 was regarded as an oncogene, linked to increased invasiveness and tumor progression [192-194]. In contrast to other integrins, ITGβ4 has a long cytoplasmic tail with several protein interaction domains [195-197]. Moreover, like the canonical ITAM-motif, the ITGβ4 consists of two Y-XX-L like motifs. The difference is that they are separated by a 13 amino acid long spacer instead of the canonical seven amino acids, and also they are surrounded by three additional conserved amino acids R-Y-X-X-L-T-S. However, the cytoplasmic tyrosine kinase interaction with the ITAM like motif in epithelial cells has not been reported. Besides, the role of ITGβ4 in epithelial cell migration remains to be further elucidated.

1.4 Cadherin 4 (CDH4)

Calcium-dependent cell adhesion glycoprotein family (cadherins) is a group of molecules mediating intercellular adhesion. They possess a crucial role in the process of cell aggregation during development [198]. Among them, Epithelial-cadherin (CDH1) has been extensively studied. CDH1 functions in normal epithelial cell architecture and tissue formation, as well as a TSG in a variety of tumors [36, 199, 200]. During EMT, CDH1 can be replaced by N-cadherin (CDH2), allowing tumor cells to form homotypic interactions with various types of mesenchymal cells, such as fibroblasts and endothelial cells, facilitating tumor invasion and distant metastasis [201]. In NPC, expression of CDH1 was reduced by promoter methylation [202], cellular or EBV encoded microRNA, onco-proteins and onco-signaling transduction [162, 203-206]. Other family members, such as CDH11, CDH13, protocadherins 8 (PCDH8) and PCDH10 have been reported to be TSGs in NPC [207-209]. The nuclear but not cytoplasmic expression of CDH2 correlated with poor outcome of NPC patients [210].

R-cadherin (cadherin 4, CDH4), located at the chromosome 20q13.33 and was firstly identified in mouse retina [211]. The expression of CDH4 has been detected in nerve system, muscle, kidney, pancreas, and gastrointestinal tract [212]. It is important for vascularization of the retinal epithelium and development of specific tissues (e.g. brain, kidney and muscle) [213-215]. Similar to CDH1, mutations in CDH4 decrease cell adhesion activity [216], and the loss of CDH4 expression facilitates cancer progression [217, 218], indicating a role as TSG. In addition, CDH4 not only can form homophilic dimers, but also interact with CDH2 [219]. A recent study reported an unusual cadherin switch in high grade gliomas, when CDH2 was replaced by CDH4 at the cell-cell junctions to release cells from contact inhibition [220]. This contradicts the role of CDH4 as a TSG.

1.5 Ubiquitin/ISG15-conjugating enzyme E2L 6 (UBE2L6)

The ubiquitin proteasome system (UPS) is responsible for the proteasome-mediated degradation of proteins, requiring ubiquitin-activating enzyme (E1), ubiquitin-conjugating

enzymes (E2) and ubiquitin ligases (E3) to fulfill ubiquitination of target proteins. Ubiquitin is activated by E1 in the presence of ATP, subsequently transferred to E2. Finally, E3 binds directly to target proteins and transfer the activated ubiquitin from the E2 to the lysine residues in the substrate [221, 222]. The other ubiquitin-like modifiers (e.g. SUMO, ISG15 and NEDD8) also have adopted similar catalytic procedures [223]. E2s depend on interactions with the E1 and E3 enzymes to obtain selective protein-protein interactions and thus connect covalent modification with specific activation. By doing so, E2s appear to play a critical role in determining the physiological consequences of ubiquitin and ubiquitin-like conjugation [224]. In addition, one of the E2s, human ubiquitin conjugating enzyme 10 (UbcH10), has been suggested to participate in the regulation of cell cycle progression, particularly in cancer development and progression [225, 226].

UbcH8, encoded by UBE2L6 gene, participates in both ubiquitinylation and ISG15ylation [227]. It was originally discovered as an E2 in ubiquitin-mediated degradation of P53 in HPV-infected cells [228], but additional UbcH8 substrates were found later such as cyclin-dependent kinase (Cdk) inhibitor p21, the replication licensing factor Cdt1, the histone 4 lysine 20 monomethyltransferase Set20 and PCNA. UbcH8 not only catalyzes the covalent attachment of ubiquitin to cellular substrates, but also influences the substrate specificity of the cognate E3 ubiquitin ligase [229].

The function of UbcH8 in cancer remains unclear. An upregulation of the UPS including UBE2L6 expression has been shown in inflammatory bowel disease in the colonic macrophages. This is necessary for macrophages to present antigens and support inflammation [230], which might promote inflammation as a tumor risk. Overexpression of UBE2L6 has been shown in esophageal squamous cell carcinoma [231]. On the other hand, histone deacetylase inhibitor (HDACi) treatment led to induction of UbcH8 in several human cell lines, enhancing the degradation of the Wilms tumor gene 1 (WT1) [232], suggesting that epigenetic silencing of UbcH8 contribute to overexpression of oncoproteins in a number of malignancies. Besides, the expression of UBE2L6 was elevated after cyclophosphamide treatment [233]. One can regard the elevated UBE2L6 as an antitumor response.

1.6 Microflora and tumor biology

The skin and cavity organs exposed to the external environment of the human body are populated with a large population of microorganisms, up to 100 trillion cells [234]. Advances in sequencing technology have increased our ability to characterize these microbial species.

It has been suggested that compositional changes in the microbiota could be associated with various cancer types, in turn closely tied to chronic inflammation, host genome and immune reactions [235]. Some bacterial species can directly damage DNA and alter host cellular processes. For example, Escherichia coli expressing colibactin promotes crosslinking of duplex DNA [236]; proteobacteria produced cytolethal distending toxin introduce breaks in double stranded DNA [237]; Fusobacterium nucleatum and Salmonella typhi produce proteins which dysregulate the β-catenin signaling pathway [238, 239]; and anaerobic

metabolites of Fusobacterium, butyric acid -a kind of short chain fatty acid-, belongs to the HDACi family and affects the host cells epigenetically [155]. The microflora is also involved in the regulation of host immune response. In addition, the microbiota composition in intestinal mucosa determines inflammatory reactions by regulating the balance of T effector- and T regulatory-cells [240]. Bacteria and its components can lead to NF-κB activation, which is critical in regulation of cancer-associated inflammation [241]; and facilitate tumor progression by activating STAT3 and c-Jun/JNK signaling pathways [242, 243]. The causal role of the microflora in carcinogenesis indicates a therapeutic potential for cancer prevention [244].

The characteristic bacterial community colonizing the mucosa of nasopharynx might trigger the chronic inflammation of the upper respiratory tract. Butyric acid can induce undifferentiated carcinoma in nude mice when synergizing with another tumor promoter, tetradecanoylphorbol acetate (TPA) [245]. It is a fair assumption that the microflora of nasopharynx participate in NPC tumorigenesis. Understanding the direct response of nasopharyngeal epithelial cells to bacteria may help to further elucidate the pathogenesis, including the role of inflammation and EBV infection, of NPC.

2 AIMS OF THIS STUDY

Our study covers three aspects of NPC pathogenesis:

1. Viral contribution to NPC pathogenesis. Being one of the important tumor promoting factors, EBV infection, especially, the latent viral transcripts and proteins, contributes to the multistep carcinogenic transformation of nasopharynx.

1.1 We demonstrated the interaction of cellular proteins Syk and ITGβ4, and investigated the role of this complex in invasion and migration of NPC cells. We demonstrated a viral interference with Syk-ITGβ4 complex. Namely, we demonstrated a competition of the EBV encoded protein LMP2A for Syk binding and the consequence of this for invasion and migration of NPC cells. (Paper I)

2. Epigenetic regulation of cellular gene expression in NPC. Promoter hypermethylation was found to be the major mechanism for silencing of critical TSGs in NPC. The silenced TSGs influence multiple cellular functions involved in initiation and progression of NPC. Novel candidate TSGs may shed more light on NPC tumorigenesis.

2.1 We investigated the transcriptional regulation and clinical significance of CDH4 in NPC. (Paper II)

2.2 We investigated the expression UBE2L6 and the role of its promoter hypermethylation in NPC. We evaluated the prognostic value of UBE2L6 expression for NPC progression and explored the UBE2L6 function (Paper III).

3. Whether the local microflora populating the mucosa of the nasopharynx, can contribute to the tumorigenic conversion of the host epithelial cells.

3.1 We set up a co-culture system of cell lines from nasopharyngeal epithelia with bacteria or bacterial cell wall components, aiming to investigate the response of epithelial cells and explore the possible mechanisms involved. (Paper IV)

3 RESULTS AND DISCUSSION

3.1 SYK interaction with ITGβ4 suppressed by Epstein-Barr virus LMP2A modulates migration and invasion of nasopharyngeal carcinoma cells

In this study, we investigated the molecular mechanism of LMP2A induced migration of epithelial cells, established from NPC. First, we showed by RT-PCR that LMP2A was expressed in NPC biopsies, while non detectable in normal nasopharyngeal epithelium (NNE). It was shown that LMP2A interacts with Syk in epithelial cells and ITGβ4 is involved in epithelial cell migration.

Both Syk and ITGβ4 were detected in NPC and NNE, with no significant difference at the gene and protein expression level. The LMP2A-Syk complex is formed through the ITAM motif of LMP2A and two SH2 domains of Syk [159]. ITGβ4 protein sequence also contains an ITAM like motif. Thus, ITGβ4 could interact with Syk in epithelial cells. Next, we compared ITGβ4-Syk complex formation in parental cells and in the stably LMP2A-positive TW03 and CNE2 cells in an immunoprecipitation assay. ITGβ4 co-precipitated with Syk in both cell lines. There was a clear decrease in ITGβ4-Syk complex formation in cells co-expressing LMP2A, compared with LMP2A-negative cells. LMP2A expression affected tyrosine phosphorylation of Syk bound to ITGβ4. It was significantly reduced in LMP2A-expressing CNE2 cells. We demonstrated that LMP2A competition with ITGβ4 for Syk binding depended on the TAM-like motif of ITGβ4. We employed a microscope immunofluorescence analysis of NPC cells and observed a conspicuous co-localization of ITGβ4 and pSyk in parental TW03, while in LMP2A-postive cells the co-localization between ITGβ4 and pSyk was markedly lower.

In the parental cells, some ITGβ4 localized to well-spread adhesion lamellae at the cell edges and to a basal area underneath the nuclei. The LMP2A-positive cells showed accumulation of ITGβ4 into distinct protruding and trailing edges. We found that Syk co-localized with ITGβ4 at the basis of the parental cell, while in the LMP2A-positive cell sits there were fewer ITGβ4-pSyk co-localisation spots and they showed primarily intracellular localization. We further demonstrated that in LMP2A positive cells ITGβ4 aggregates at the cellular edges. The same pattern of ITGβ4 localization was observed in cells where Syk expression was knocked down (KD). FACS analysis confirmed that LMP2A-expressing and KD Syk TW03 cells showed higher ITGβ4 expression at the cell surface. The redistribution of ITGβ4 in LMP2A-expressing cells from lamellae and probable hemidesmosomal (HD) adhesive structures to protruding edges, most likely reflects a higher turnover of ITGβ4 from the cellular adhesion structures, facilitating cellular migration.

We performed a canonical wound healing assay on a NPC-derived epithelial cells and demonstrated that LMP2A-positive cells as well as cells with a reduced Syk expression show an equally increased capacity to invade through the matrigel in Boyden chambers.

Our results suggest that expression of LMP2A correlates with increased cell surface localization of ITGβ4 in the NPC cells, which may be a consequence of the competitive binding of LMP2A to Syk. Deregulation, re-localization and activation of Syk caused by LMP2A increase cell surface expression of ITGβ4, and might thus contribute to the invasive and metastatic phenotype of NPC.

3.2 CDH4 as a novel putative tumor suppressor gene epigenetically silenced by promoter hypermethylation in nasopharyngeal carcinoma

Several cadherin family members have been identified to be TSGs in NPC based on analysis of promoter hypermethylation. We discovered that one more member -CDH4- also has this feature. It was readily detectable in normal nasopharyngeal epithelial cell line NP69. In contrast, the expression of CDH4 was weaker in NPC cell line TW03; and was almost absent in two NPC xenografts (C15, C17) and another four NPC cell lines (CNE1, CNE2, HONE1, C666-1). The overall expression level of CDH4 was downregulated in 15 primary NPC tissues compared to 12 samples from normal nasopharyngeal epithelium (NNE).

The CDH4 promoter was completely methylated in two NPC xenografts and 4 of 5 NPC cell lines (CNE1, CNE2, C666-1 and HONE1); TW03 cells showed partially methylated pattern; whereas it was not detected methylation of the CDH4 promoter in NP69 cells. We further evaluated the methylation status of the CDH4 promoter in 53 NPC primary tumors. Promoter hypermethylation was detected in 94.3% (50/53) of the tumors but in none of the 12 NNE samples. Our data suggest that promoter hypermethylation is associated with the inactivation of CDH4 in NPC. The demethylating agent 5-aza-dC restored expression of CDH4.

Moreover, we suggest that the CDH4 promoter hypermethylation is a general feature of NPC, since there was no significant association between CDH4 promoter hypermethylation and other clinico-pathological features such as age, sex, cancer staging or pathological subtype of NPC patients. The similar methylation frequency in NPC patients at early stages (I and II) and late stages (III and IV) indicates that epigenetic inactivation of CDH4 gene expression occured already at early essential stages of NPC carcinogenesis. The high (94.3%) frequency of CDH4 promoter methylation, may serve as a powerful diagnostic biomarker already at early stages of NPC.

To investigate a functionality of the CDH4 gene coded protein, we restored CDH4 in NPC cell line HONE1 and analysed cell proliferation, cloning formation and cell mobility. Reexpression of CDH4 gene increased the gap-junction formation between tumor cells, shown by a scrape-loading/dye-transfer assay. It further supported the potential function of CDH4 in decreasing the invasive and migratory capacity of NPC cells.

In conclusion, the expression of CDH4 was frequently inactivated in NPC due to promoter hypermethylation. The restored CDH4 expression suppressed the growth and movement of NPC cells, thus CDH4 could be classified as a TSG. We suggest that the CDH4 promoter

methylation analysis could be exploited for early detection of NPC, but maybe considered for NPC therapy since methylation modification is reversible.

3.3 Epigenetic downregulation of the ISG15–conjugating enzyme UbcH8 impairs lipolysis and correlates with poor prognosis in nasopharyngeal carcinoma

To identify genes down-regulated in NPC due to promoter hypermethylation we used a model system, cell lines established from NPC. We treated them with the demethylaing agent 5-aza-dC in combination with a histone deacetylation inhibitor (HDACi) Trichostatin A (TSA). We performed a cDNA microarray on two NPC cell lines, CNE2 and HONE1.

Among genes, which showed restored expression upon the treatment, a gene coding for ubiquitin conjugated enzyme E2L6 (UBE2L6), was activated (up to 7.8-fold) after treatment in both cell lines. We found that UBE2L6 could be restored in the NPC cells only upon 5-aza-dC treatment.

We went on to show in vivo and in vitro that silencing of UBE2L6 gene was specific indeed for tumor cells. While 12 normal NNE samples expressed an easily detectable level of UBE2L6 mRNA, the overall expression level of UBE2L6 was significantly lower in NPC tumor biopsies. The five NPC cell lines, CNE1, TW03, C666-1, HNE1 and HONE1 showed negligible UBE2L6 expression. It was undetectable only in one of the six NPC cell lines, CNE2.

Further, we showed that the impairement of UBE2L6 gene expression in NPC was reflected at protein level. The expression of UbcH8 protein (encoded by UBE2L6 gene) was found to be significantly lower in NPC cancer nests than in adjacent stromal tissues. Moreover, Univariate Kaplan-Meier survival analysis, based on the expression level of UbcH8, indicated that the disease-specific survival period was significantly shorter for patients with low UbcH8 expression than for patients with high UbcH8 expression. Therefore, UbcH8 may serve as a prognostic factor in NPC patients.

We did not find a correlation between degree of methylation of the UBE2L6 promoter and other factors like age, sex, cancer staging, lymph node metastasis or pathological subtypes (data not shown).

We went further to investigate the functional impact of UBE2L6 silencing. We showed that restoration of UBE2L6 expression inhibited NPC cell proliferation, clonal formation and that it induced apoptosis. These data support that UBE2L6 may function as a TSG in NPC.

Refering to the recently established association of UbcH8 with lipid metabolism [246], we analysed accumulation of lipid droplets [45] in NPC cell lines. While CNE2, HONE1 and C666-1 showed clear LDs accumulation, there was no visible LDs in non-malignant cells (NP69 and NP460), as demonstrated by staining of cells with lipid specific fluorescent dye Bodipy 493/503. Ectopic expression of UbcH8 in NPC cell lines conversely correlated with the amount of intracellular LDs. Moreover, we found that expression of adipose triglyceride

lipase (ATGL), inversely correlated with LDs. ATGL was stabilized by ectopic expression of UbcH8 in NPC cell lines (CNE2 and HONE1). To further confirm this correlation, we knocked down UbcH8 in a human embryonic kidney cell line 293, and found that ATGL protein was decreased. Moreover, we also demonstrated LDs accumulation in NPC tumor sections.

The ATPase valosin-containing protein (VCP) has been identified as an ISG15 modified protein [247] and it was shown to be responsible for the degradation of ATGL [248]. Inspired by this, we investigated whether UbcH8 could be involved in VCP-mediated ATGL protein turnover.

We showed significant upregulation of ISG15 in NPC tissues in a comparison to NNE. In addition, the exogenous–expression of UbcH8 induced ISG15-conjugated species formation in NPC cells, including the ISG15-VCP complex. Thus, our study shows that UbcH8 functions as an E2 enzyme which conjugates ISG15 to VCP, which in turn would inhibit ATGL degradation.

In summary, we show that UBE2L6 is inactivated by promoter hypermethylation in NPC, and subsequently causes LDs accumulation in the NPC cells due to increased inappropriate ATGL protein turnover. Our data indicate for the first time, that VCP ISG15ylation opposes a degradation promoting function of VCP. Importantly, the reduced expression of UbcH8 correlated with a poor outcome in patients with NPC. This and other studies suggest that UBE2L6 may be a TSG in NPC due to its ISG15-conjugating function.

3.4 Induction of inflammatory response in nasopharyngeal epithelial cells by microflora components is impaired in nasopharyngeal carcinoma cells

A systemic overview analysis of expressed genes was performed to map the response of normal nasopharyngeal epithelial cells to bacteria during short term exposure. We detected a strong response in the normal nasopharyngeal epithelial (NNE) cell NP69 upon peptidoglycan (PGN) and streptococcus exposure. The most significantly upregulated genes belongs to the proinflammatory cytokines and chemokines such as IL6, IL8, IL1α and CXCL2. The effect of PGN and that of streptococci on NNE cells was related, with around 30% of the upregulated genes overlapping and mainly included in toll-like receptor, Jak-Stat signaling and cytokine-cytokine receptor interaction pathways, as suggested by KEGG pathway analysis. In contrast, there was no crosstalk between genes activated by lipopolisaccharide (LPS) and PGN, respectively, suggesting that the effect of Gram-negative and -positive bacterial cell wall components differ.

Interestingly, the NPC cell line C666-1 was not much affected by streptococci with fewer genes upregulated compared to NP69. The upregulated genes in C666-1 upon streptococci treatment were rather associated with leukocyte transendothelial migration pathway, phosphatidylinositol signaling system, Wnt signaling pathway and so on, but not inflammatory related pathway. We further confirmed that PGN but not LPS significantly

stimulates the expression of inflammatory cytokines and chemokines in NNE cells (NP69 and NP460). In the NPC cell lines (HONE1, HK1 and C666-1) the inflammatory response was much lower, irrespective whether they were treated with LPS or PGN.

Toll-like receptor 2 (TLR2) and TLR4, essential for sensing Gram-positive and -negative bacteria in mammals, respectively, were found properly expressed at the surface of normal and NPC cell lines. Thus, the differential inflammatory response in NNE and NPC cells could not be explained by differences in TLR2- and TLR4-expression.

Still, the downstream molecule of TLRs pathway NF-κB (p65) was induced by PGN but not LPS resulting in nuclear translocation, indicating that the NF-κB signaling pathway can be activated by Gram-positive bacteria. In contrast, in NPC cells we found NF-κB to be constitutively present in the nuclei and there was no further translocation to the nuclei, neither upon LPS nor PGN exposure. Consistent with our microarray data, we found a profound inflammatory response in NNE cells induced by PGN and live streptococci, while these microbial factors were unable to increase the inflammatory response in NPC cells. Cytoplasmic retention of NF-κB may explain this phenomenon.

Previously we have noticed excessive LDs accumulation in NPC cells (**paper III**) and suggested one mechanistic explanation for this phenomenon. Now, we observe an increase of LD in normal cells in response to PGN. Apparently, LDs may be part of the transient inflammatory response, while NPC cells showed LDs at a steady state level. NF-κB entrapment in lipids in T cells was reported previously, which were also unable to respond to TNF-α exposure [249]. Lipid fractionation experiments demonstrated that in tumor cells NF-κB colocalized with lipids to a higher degree than in NNE cells resulting in repression of NF-κB. However, it remains unclear how lipids trap the cytoplasmic NF-κB.

As mentioned, the E2-enzyme UbcH8 is down regulated in NPC cells (**paper III**). Interestingly, VCP was recently reported to mediates I-κB degradation [250]. We observed that restoration of UbcH8 expression in HONE1 cells results in a decrease of I-κB expression. Moreover, re-expression of UbcH8 restored NF-κB nuclear shuttling in response to TNF-α treatment in HK1 cells.

Lysine-specific demethylase-1 (LSD1) is a H3K4me demethylase[251], which was observed to be highly expressed and efficiently transferred to the nuclei of tumor cells, in comparison to normal epithelial cells. Using ChIP assays with LSD1 antibody, we showed that LSD1 could bind directly to the promoter of proinflammatory genes IL6 and IL8 in C666-1 cells, and thus affect their accessibility. Furthermore, the LSD1 inhibitor upregulated the transcription of proinflammatory genes (IL6, IL8, IL1α and CXCL2), indicating that indeed, LSD1 exerts one of the controls on the inflammatory responses in NPC cells and impairs the targeting of the transcription of these proinflammatory genes by nuclear NF-κB.

In conclusion, our findings revealed three molecular mechanisms behind the impaired inflammatory response in NPC cells, which may shed light on the tumorigenic processes in nasopharynx.

4 MATERIALS AND METHODS

This chapter contains a brief discussion of main methods used in this study. For other methods and details see the individual papers.

4.1 Clinical samples

All of the NPC primary tumor biopsies were collected from newly diagnosed and untreated NPC patients, while normal nasopharyngeal epithelial tissues obtained during tonsillectomy were used as controls at the department of Otolaryngology Head and Neck Surgery, First Affiliated Hospital of Guangxi Medical University (Nanning, China), with informed consent from donors. The study was approved by the Ethical Review Committee of Guangxi Medical University, China (Date of Approval: 2009-07-07) and Karolinska Institutet, Sweden (Reference number: 00-302).

4.2 Cell lines

To study aspects of NPC experimentally in vitro is challenging due to the limitations of available cell lines. To date, most of the in vitro study of NPC are based on EBV-negative cell lines established 1-2 decades ago. Only a few cell lines maintain the EBV genome stably, such as C666-1 and the xenografts C15 and C17[252, 253]. Being identified by Leibniz-Institut DSMZ, it is clear that all of the NPC cell lines we used are derived from human and, that they comply with their original profile, but their definite origin from NPC cannot be verified. Consistent with others, we found that CNE-1 and CNE-2 cell lines carry both HPV and some identity-markers from HELA cells indicating that they may be hybrids, while some batches of HONE1, TW03 and HNE1 cells were cross-contaminated with CNE1 cells[254]. These cell lines are still feasible to study protein interactions and pathways biochemically, but any findings specific to NPC should be verified in tissue biopsies or in C666-1. Due to the high cost of maintaining xenografts, it is necessary to establish additional appropriate and qualified NPC cell lines in vitro for future NPC research. Non-malignant nasopharyngeal epithelial cell lines NP69 and NP460 were immortalized by oncogene SV40 large T and hTERT, respectively[255, 256]. Together with HK1 and C666-1, their profiles showed complete matches to the published data. ’

4.3 Migration and invasion studies

Wound healing and transwell assays are the most frequently applied in vitro approaches to investigate capacity of tumor migration and invasion. Under physiological conditions, epithelial cells form a monolayer. Cells start to migrate based on lateral movement under pathological conditions, such as wound healing. Wound healing assay is a feasible 2-dimentional model, simple to perform and low-cost. Generally, we recorded the width of scratch within 24 hours to minimize effects of growth rate.

Invasion is a feature of tumors, which can not be covered by the wound healing assay. Thus a 3-dimentional model, an invasion chamber system is employed. Matrigel was coated on the membrane inside the upper chamber, subsequently seeded with cell suspensions without fetal bovine serum (FBS), while medium with 10% FBS was added in the lower chamber. This can mimic the process of tumor cells degrading extra cellular matrix and penetrating into normal tissues around.

4.4 Assays to detect proteins

All of the methods we used for protein detection are based on the high specificity of recognition between antibody and antigen. Immunohistochemistry and immunofluorescent staining assays can determine the localization or quantification of target antigens. However, the specificity of the signal cannot intrinsically be verified. Western blot assay is commonly used to quantify protein expression levels, and particularly, to identify the specific protein recognized according to the molecular weight after separation in sodium dodecyl sulfate-Polyacrylamide (SDS-PAGE) gel electrophoresis. Signals were captured in a microscope by CCD camera (confocal microscope or imaging instruments), and then analyzed by Image J software. Cell surface proteins were recorded by flow cytometry with fluorescent antibodies, thus showing intensity of the signal. An effecient method to study the interaction of two proteins in intact cells is co-immunoprecipitation. Cells were lysed under non-denaturing conditions, retaining the protein-protein interactions. When precipitating a protein A, the in vivo binding to protein B could be detected. After denaturing the precipitated proteins, protein B was detected by Western blot. Thus, the interaction between the two was demonstrated.

4.5 cDNA microarray

cDNA microarray enables large scale analysis of gene expression at the mRNA level. Each chip is made up by short oligonucleotides or cDNA fragments as probes. The isolated mRNA was reverse-transcribed into cDNA, labeled with fluorescence and subsequently hybridized with the probes on the chip. Then, fluorescence intensity was scanned and calculated. cDNA microarrays are nowadays high-throughput, efficient and accurate assays. It provides a large amount of data. Further bioinformatic analysis is necessary to make any meaningful conclusions.

4.6 Methylation studies

Methylation-specific PCR (MSP) is a site-specific methylation detection technology, based on the principle that unmethylated cytosine in genomic DNA is converted into uracil upon treatment with sodium bisulfite, while methylated cytosine is unchanged. Therefore, in theory, the difference of methylation in e.g. CpG islands can be detected by PCR with methylation-sensitive and insensitive primers, each preferably covering three CpG sites. This method is currently widely used, showing high sensitivity and good economy. The treatment of

genomic DNA with bisulfite is a crucial step. Incomplete conversion may lead to false positives.

Bisulfite sequencing is considered the gold standard for DNA methylation analysis. In this thesis, we only sequenced cloned PCR products generated from bisulfite treated and PCR amplified DNA, using primers not covering CpG sites. The results reflect the detailed methylation status in a selected DNA fragment. This approach is reliable and precise, but time consuming due to both cloning and sequencing.

4.7 Co-culture of bacteria and epithelial cells

The α-haemolytic streptococci is a normal member of the human respiratory tract flora, but can be an opportunistic pathogen. It was maintained on blood agar plates, showing typical characteristics with a green hemolytic ring around each colonies. A streptococcus clone was expanded in brain heart infusion (BHI) broth, shaken at 200 rpm, 37℃ overnight. Subsequently, bacterial broth was diluted to 1:1000 into fresh BHI broth, and a growth curve was made by optical density (600 nm) at different time points. When the optical density (600 nm) equaled 1, the streptococci were at mid-logarithmic phase. The colony-forming units per milliliter (CFU/ml), equivalent to the bacterial concentration, was determined by counting viable colonies after inoculating different dilutions of bacteria on blood agar plates for overnight. At optical density (600 nm) 1, the concentration of α-haemolytic streptococci was around 2.5×10^9 CFU/ml.

The multiplicity of infection (MOI) refers to the number of bacterial per (eukaryotic) cell. This concept originates from infection of bacteria with bacteriophages. Most commonly MOIs from 2:1 to 500:1 were used for different purposes[257-260]. We optimized MOI for the purpose of our model. The morphology of epithelial cells was well retained at an MOI of 100:1 streptococci: cell after co-culturing for 2 hours. In order to make sure most of the cells were exposed to streptococci, we decided to use the maximum MOI in this study. Cells not inoculated with bacteria were used as negative controls.

4.8 Chromatin Immunoprecipitation (ChIP) assay

Based on the specificity of antigen-antibody reactions, ChIP assay was applied to investigate the in vivo binding of specific proteins to genomic DNA. The protein-DNA complexes was fixed in living cells. Then chromatin was randomly sheared into small fragments with lengths around 1000bp. Next, the complexes were precipitated by antibody that specifically binds the target protein, by which the interacting DNA fragments were enriched at the same time. This method detects specifically the binding of target proteins to specific DNA fragments, but also allows the analysis of modifications of DNA-binding proteins (such as acetylation, methylation, phosphorylation or ubiquitination) in relation to gene expression.

4.9 Statistical analysis

SPSS 11.5 statistical software was used for this study. The gene expression levels in primary tumors versus normal nasopharyngeal epithelium were analyzed by the Mann -Whitney's *U* test or two-tailed *t*-tests. Associations between methylated samples and clinicopathological features of NPC patients were analyzed by the Pearson chi-square test or Fisher's exact test. Survival curves were calculated by Kaplan-Meier method and differences were analyzed using the log-rank test. Results presented as mean±SD were analyzed by the two-tailed *t*-tests. A *P* value of less than or equal to 0.05 was considered a statistically significant.

5 CONCLUSION REMARKS

A unique feature of NPC is its strong association with EBV [261]. The interplay between host cell genetics and EBV infection contributes to the development and progression of NPC [262]. Epigenetic modifications, especially promoter methylation, is a major mechanism in inactivating TSGs in tumors [263], frequently being identified in NPC. Therefore, clarifying the host epigenetic alterations and the influence of EBV on cell signaling and host proteins will shed light in understanding the molecular pathogenesis of NPC and make possible to identify useful biomarkers and targets for diagnosis and therapy. In addition, the nasopharyngeal microflora, represents an early environmental exposure of the nasopharyngeal mucosa which continues throughout life. It has recently been mapped by 454 pyrosequencing [264, 265], also putting focus to its role in NPC pathogenesis. Identifying correlations of microbes with NPC-risk combined with elucidating the molecular mechanisms in tumorigenesis may provide new angles on prevention of NPC.

In this study, we have unveiled a new mechanism of increased cell motility due to LMP2A in NPC cells **(Paper I)**; identified two new TSGs which are epigenetically inactivated in NPC and demonstrated their possible biological functions **(Paper II and III)**; and explored some effects of bacteria and microbial components on a model in vitro system **(Paper IV)**. If possible, the findings in paper I and IV would need to be further investigated in vivo, but it is very hard to design doable in vivo experiments for this purpose. The findings in paper II and III would benefit from validation in larger number of cases.

Here are the main conclusions from our work:

Paper I: Syk docks to a TAM-like motif of ITGβ4. LMP2A contributes to the invasive and metastatic characteristics by binding to Syk in competition with ITGβ4, thus affecting cell surface expression of ITGβ4.

Paper II: CDH4 is a putative TSG in NPC which is inactivated by promoter methylation.

Paper III: UBE2L6 is downregulated by promoter methylation in NPC. Reduced expression of the encoded protein UbcH8 correlates with poor outcome in NPC patients. UBE2L6 seems to be a TSG in NPC. Its ISG15-conjugating function increases lipolysis.

Paper IV: Non-malignant nasopharyngeal epithelial cells respond to microbes with an inflammatory response after short term exposure, while NPC-derived cells did not show any induction of inflammation. We proposed three possible molecular mechanisms for impaired inflammatory response to microbial subcomponents in NPC cells. A decreased inflammatory activity may affect the immune response in NPC and thus contribute to the tumor progression.

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