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PROTEOME STUDY OF NASOPHARYNGEAL CARCINOMA

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School of Chemical and Biomedical Engineering

A thesis submitted to Nanyang Technological University
in partial fulfillment of the requirement for the degree of **Doctor of Philosophy**

2012

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Sincerely dedicated to my parents

Feng Sherong & Luo Xia

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Abstract

Nasopharyngeal carcinoma (NPC) is one of the sneakiest cancer frequently occurred in Southeast Asia and southern China. Due to the vague symptoms and difficulty of physical examination, NPC urgently needs efficient approaches to improve its diagnosis, treatments and understanding of the disease. In our study we employ a high-throughput system, iTRAQ-coupled 2D LC-MS/MS, to analyse the protein profiles of patients' serum and NPC cell lines in response to Epstein-Barr virus (EBV) infection, which is considered as a major factor causing NPC. In the clinical study of patients' sera, 13 proteins showed significant changes compared with the healthy control. Changed proteins in NPC patients were classified and analysed according to their functions and served as potential biomarker candidates for NPC prognosis. In order to study the influence of EBV infection upon the NPC cell line, a comparative protein profile of NPC cells in response to EBV infection was established by using iTRAQ-coupled 2D LC-MS/MS system. 12 proteins were found to be significantly up-regulated in EBV infected NPC cells. By protein network analysis, a novel pathway was proposed associated with NF- κ B signaling pathway and p53 signaling pathway. In the process of NPC cell line proteome study, an up-regulated potential biomarker candidate, voltage dependent anion-selective channel protein 1 (VDAC1), was further explored because of its importance located in the outer membrane of mitochondrial for controlling apoptotic signals. Pro-apoptotic signals Ca^{2+} and cytochrome C were detected in response to EBV infection. Upon virus infection, cytoplasmic Ca^{2+} was decreased while cytochrome C was increased. In order to study whether the changes of pro-apoptotic signals was regulated through VDAC1, we used siRNA to inhibit VDAC1's expression. After inhibition, cytochrome C was back to the

same level as that of non-infected cells, indicating cytochrome C release was regulated through VDAC1 during EBV infection. These findings proved that iTRAQ-coupled 2D LC-MS/MS was an efficient approach to study protein profile of nasopharyngeal carcinoma in both clinical serum samples and cell lines. The changes of pro-apoptotic signals Ca^{2+} and cytochrome C upon EBV infection by using siRNA to inhibit the VDAC1 demonstrate new mechanism of virus infected apoptosis in cancer cells. In summary, these results might provide valuable information to elucidate NPC mechanisms and improve the management of nasopharyngeal carcinoma.

Abbreviation

NPC: nasopharyngeal carcinoma; EBV: Epstein-Barr virus; iTRAQ: isobaric tag for relative and absolute quantification; ELISA: enzyme linked immunosorbent assay; RNAi: RNA interference; VCA: virus capsid protein; Ea-D: EBV early antigen; EBNA: EBV nuclear antigen; VDAC1: voltage-dependent anion-selective channel protein 1; Hip-70: Hsc-70 interacting protein; 4F2hc: 4F2 cell-surface antigen heavy chain; Keratin-75: Keratin, type II cytoskeletal 75; TB8: Tubulin beta-8 chain B; LDH-B: L-lactate dehydrogenase B chain; TIM: Triosephosphate isomerase; HMG-1: High mobility group protein B1

Publications

The studies performed for this thesis have results included in publications of scientific journals which are listed below:

1 Feng XS, Zhang JH, Chen WN, Ching CB. Proteome profiling of Epstein-Barr virus infected nasopharyngeal carcinoma cell line: Identification of potential biomarkers by comparative iTRAQ-coupled 2D LC/MS-MS analysis. *Journal of Proteomics*. 2011; 74:567-76.

2 Feng XS, Chowbay B, Chen WN, Ching CB. ITRAQ-coupled 2-D LC-MS/MS Analysis of Differentially Expressed Serum Proteins in Nasopharyngeal Carcinoma Clinical Samples: Potential in Biomarker Discovery. *Journal of Medical Imaging and Health informatics* 2011; 1: 177-83.

3 Feng XS, Chen WN, Ching CB. EBV decreases the release of cytoplasmic Ca^{2+} and up-regulates cytochrome C in the NPC cell line through VDAC1 regulations. *Cell Biology International* 2012; Accepted.

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Chapter 1 Introduction

1.1 Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is a squamous-cell carcinoma that frequently occurred in several regions. The neoplasm happens in the epithelial lining of the nasopharynx, which is often observed at the pharyngeal recess posteromedial to the medial crura of the Eustachian tube [1].

NPC is an endemic carcinoma with high regional occurrence. Most patients were from Southeast Asia and southern China. Some are from northern Africa and Alaska. Alaska and Guangdong province in China are the most frequently occurred regions. The incidence of having NPC in people of North America is lower than that of people born in southern China, where immigration from China to North America makes the incidence of NPC of these regions correlated [2-3]. These findings shows NPC distribution was correlated with genetic, ethnic and environmental factors.

1.1.1 Pathology

When nasopharyngeal epithelial cells were transformed into tumor cells with malignancy, they appear to be polygonal and syncytial. The tumor cells were often found to be mixed with lymphoid cells in the nasopharynx, which triggered the study of “lymphoepithelioma” [4].

The most related pathogen of NPC was called Epstein-Barr virus (EBV) as it was constantly and frequently detected in patients with nasopharyngeal carcinoma. Both EBV DNA and RNA were found in nasopharyngeal epithelial tumor cells, where EBV-encoded RNA was not detected in the

neighborhood of them except their intermingled lymphoid cells. Before becoming malignant, nasopharyngeal epithelial cells with lesions were also found to take EBV DNA, which indicates the infection of EBV could be a major reason to cause carcinogenesis [5].

In 1978, WHO proposed the histological classification of nasopharyngeal carcinoma, when NPC was characterized into three groups: type I – typical keratinizing squamous-cell carcinomas; Type II – non-keratinising squamous carcinomas and type III undifferentiated carcinomas. The histological distribution of three types of NPC differs between North America (Type I – 25%; Type II – 12%; Type III – 63%) and southern China (Type I- 2%; Type II – 3% and type III – 95%). Due to the mixed histological pattern from nasopharyngeal carcinomas biopsies, the WHO updated the carcinoma as squamous-cell carcinomas and non-keratinising carcinomas. And non-keratinising carcinomas were sub-grouped into differentiated and undifferentiated carcinomas. Undifferentiated carcinomas have a higher distant metastasis incidence than undifferentiated carcinomas which have better control records with treatments [6].

1.1.2 Patients classification by symptoms

The symptoms of having NPC include one the four following categories: (A) Appearance of tumour mass accompanied with the epistaxis, nasal obstruction and discharge; (B) Dysfunction of eustachian tube as the tumour extend to paranasopharyngeal space. Patients have tinnitus and deafness; (C) With further extension of the tumor, patients feel pain from heads and faces. Sometimes patients feel diplopia and numbness. Because skull-base erosion and palsy of cranial nerves happen; (D) Upper neck masses. And because of the vague symptoms and difficulty of

examinations, most patients having NPC are diagnosed during late stages (stage III and IV).

Patients with above physical symptoms should consider clinical examination immediately. An EBV serological test is good choice to complement endoscopic examination and biopsy. High suspicion of nasopharyngeal carcinoma given by EBV test should be followed by CT or MRI, even if no physical symptoms were discovered. A biopsy taken from the lesion is the most validated diagnosis by visualizing in the nasopharynx with cross-sectional imaging.

1.1.3 Staging system

There are two major staging systems that are used to classify NPC in different regions. One is the American Joint Committee on Cancer Staging and End Result Reporting/International Union Against Cancer (AJC/UICC) system commonly used in Europe and America [7]. Another is Ho's system preferred in Asia [8-9]. In order to improve the management of NPC, a revised staging system was developed from many centers and institutions considering skull-base erosion, cranial nerves, primary tumour extension to paranasopharyngeal space and the conditions of cervical nodes [10-12]. This revised staging system was published in 1997 [12]. The old T1 and T2 stages were incorporated into the new T1 stage. The new T2 stage was when tumours extended to the nasal fossa, oropharynx or paranasopharyngeal space. The new T3 stage was when tumours extended to the skull base or other paranasal sinuses. The new T4 stage included tumours that extended to the infratemporal fossa, hypopharynx, orbit and cranium/cranial nerves. Considering cervical nodal staging, the new N1 system describes the unilateral nodes where N2 to bilateral nodal disease without considering the size, number and the anatomical location of the nodes.

The New N3 stage referred to lymph nodes larger than 6cm (N3a) and extended to the supraclavicular fossa (N3b) [13]. The new staging system has improved the sensitivity and accuracy when predicting the patients' survival [14-15].

1.1.4 Prognosis and imaging systems

Determining the stage of NPC using the revised new system was the most essential factor during NPC prognosis. In spite of staging system, there are many other methodologies regarding NPC prognosis. The factors include the conditions of neck nodes, gender, age, the existence of cranial nerve palsy and ear symptoms. Based on the different patterns of NPC, cross-stage categories were induced as follows: (1) T1-2N0-1 (relatively good treatment outcome); (2) T3-4N0-1 (mainly local failure); (3) T1-2N2-3 (mainly regional and distant failure); (4) T3-4N2-3 (local, regional and distant failure). These categories help doctors to adjust their diagnosis according to different failure patterns [16].

Normal clinical examination including endoscopic detection can assess the extent of NPC development in relatively shallow spaces such as nasal fossae and oropharynx. As for deep assessment such as skull-base erosion and intracranial spread were hard to achieve without revolved imaging technologies. Cross-sectional imaging was one of the most important technologies on clinical prognosis. CT was especially good at identifying paranasopharyngeal and intracranial extension of NPC. CT was also capable of detect perineural spread through the foramen oval without skull-base erosion [17].

Complementing CT, MRI is more suitable to display both superficial and deep nasopharyngeal soft tissue. It has higher sensitivity for detection of retropharyngeal and deep cervical nodal metastases [18]. For the bone details correlated with NPC development, CT should be employed when MRI cannot provide satisfactory data on the base of the skull [19]. For marrow infiltration by tumours with increase risk of distant metastases, MRI was more reliable because CT can only apply to infiltration associated with bone erosion [20].

As for distant metastases, both CT and MRI were not applicable with conventional radiographs. Bone scan, liver scintigraphy, abdominal ultrasonography and marrow biopsy were also studied and showed no significance for distant metastases [21]. The introduction of positron emission tomography (PET) in to this type of detection in other malignancies was established. When it could be used on NPC and its effect is still unknown.

After treatment, MRI is more applicable to detect tumour recurrence and postradiation complications than CT. However, both method have low sensitivity [22], since recurrent signals from NPC is various and hard to interpret [23]. CT is applied when detecting bone regeneration after treatments. And PET has been reported to perform higher sensitivity when detecting residual and recurrent tumours in the nasopharynx [24].

1.1.5 NPC treatment

During NPC treatment, similar with other cancers, radiotherapy is the standard treatment. However, due to the location of the tumour at the base of skull closely surrounded by

dose-limiting organs, radiotherapy was facing complicated limitations for NPC treatments, though it is still the most commonly used treatments.

Among radiotherapy approaches for NPC, doctors tends to begin phase I treatment using large lateral opposing faciocervical fields with matching lower anterior cervical field for lower neck lymphatics. A dose of 65-75Gy is normally adapted to suppress primary tumours. 65-70Gy is suitable to the involved neck nodes. Prophylactic treatment needs a dose around 50-60 Gy at a node-negative neck. This radiotherapy has suppressed tumours with higher successful rate in T1 and T2 than in T3 and T4 [16, 25-28]. 2D planning and 3D conformal radiotherapy and intensity-modulated radiotherapy were also employed for treatment with certain limitations [29-30].

Chemotherapy combined radiotherapy has been proved to improve NPC treatment in the past twenty years. The chemotherapy was mainly classified into three categories: using neoadjuvant, using concurrent and using adjuvant chemotherapy combined radiotherapy. The study in 1997 was proved for the first time that chemotherapy coupled radiotherapy improved overall survival than using solely radiotherapy [31]. Along with other groups study, it is now generally accepted that chemotherapy along with radiotherapy is suitable for NPC treatments, although conflicts rise concerning problems during local control and distant metastases. Among these three basic approaches (neoadjuvant, concurrent and adjuvant chemotherapy), concurrent chemoradiotherapy is considered the most effective method, while distant metastases still cause large amount of treatment failure. The survival rate of stage IV patients remains low.

1.2 Biomarkers discovery and progress in nasopharyngeal carcinoma

Factors influencing NPC infection include race, EBV infection, consumption of food with volatile nitrosamines and respiratory environment [32]. polymorphism of a nitrosamine metabolizing gene, CYP2A6, was considered as a potential biomarker's trait for NPC because of its crucial role in NPC susceptibility [33]. In Guangdong province of China, an endemic region with high rate of NPC occurrence, genetic polymorphism of CYP2F1 gene was popular in NPC patients. Accordingly the operations dealing with multiple genetic polymorphisms were found to be important factor [34]. Scientists also discovered another gene called XRCC1 responsible for excision repair of DNA. People assumed that two nucleotide polymorphisms of XRCC1 coding 194 Arg-Trp and 399 Arg-Gln were associated with NPC development and smokers tend to have these genetic defects. Another gene Cyclin D1, a key controller of the cell cycle, has been reported with associated altered activity to influence cancer development. The proportion of GG Cyclin D1 genotype related NPC cases takes 15% in Portuguese NPC patients. And this information provided biological profile for NPC occurrence in Portugal population [35].

1.2.1 Biomarkers for NPC diagnose and progression of cancer

NPC is a complex disease caused by environment factors, virus infections and host genes in a series of carcinogenesis process. In the DNA level, defects of genes can become a marker in early stages of NPC carcinogenesis. Aggravation of genomic alterations appears to be a less-powerful approach for prognosis of cancer [36]. For understanding the putative changes in NPC genes, evolutionary tree was used to compare genomic information in NPC issues. It has been found that Chromosome 12 has the symptoms of losing 3p for both tree models and this discovery demonstrate that this gene lose were important early markers in NPC progression. The reporter

also listed what specific genes are marked [37].

Scientists make use of bioinformatics to analyze the nuclear gene difference in the expression and the location of NPC-related proteins. EGFP/UBAP1 were kinds of proteins expressing in nuclear, especially displaying on the nuclear envelope. Their expression difference may be related with NPC progression [38]. And a locus on 3p21 was identified as a linker to NPC in a linkage analysis [39]. It has been discovered that a tumor suppressor gene RASSF1A is frequently silenced by promoter hypermethylation in NPC. Detected by high-density oligonucleotide array, the RASS highly regulates the expression of activin BE and Id2 in NPC. Id2 is repressed by RASSF1A and regulated by activin BE overexpression. The data demonstrate that a new reasonable RASSF1A pathway where both activin BE and Id2 were addressed [40].

Although most genes related with NPC carcinogenesis is up-regulated, a new gene name KIAA1173 has been found down-regulated in NPC patients [41]. And it was reported that novel cell cycles has been found in NPC development [42] and plasma osteopontin was up-regulated in patients with undifferentiated NPC [43].

In NPC histology, diagnosis is determined by traditional biopsy of nasopharyngeal mass. A common used imaging tool for staging judgment of NPC is fused positron emission tomography/computed tomography. However, NPC is a kind of sneaky cancer for its deep location and vague symptoms [44]. By observing the NPC cellular events, it has been found that DNA diploidy occurred prior to the development of premalignant to malignant head and neck

squamous cell carcinomas. And integral defects of cell-free circulating RNA was found to be related with NPC [45].

It was also reported that methylation of certain gene (e.g., CDH13 [46]) could lead to different NPC cell performance. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis combined with mass spectrometry, Protein level of changes in NPC was also explored including discovery of fibonectin, Mac-2 binding protein and plasminogen activator inhibitor 1 as potential markers. Western-Blot was employed to validate the results [47].

1.2.2 NPC biomarkers for targeted therapies

Targeted therapies have been employed from radiotherapy to molecular treatment. Radiotherapy dose and field margins are depend on location and size of the primary tumor and lymph nodes [48], [49]. In clinical trials, new types of treatment are being used, including biological treatments and intensity-modulated treatments. Advanced immunologic therapy and combined chemotherapy facilitate to control NPC development.

In the DNA level, researchers have been investigated novel ways for targeted therapy. Using oligonucleotide microarray analysis, THY1 showed continuous down-regulated expression in the tumor segregants. And its location is near a previously defined 11q22-23 NPC critical region. In the lymph node metastatic NPC, THY1 was proved to be capable of suppressing tumour [50]. A chromosome 3p has been demonstrated its ability to suppress tumor growth in vivo. Taking use of reverse transcription polymerase chain reaction (RT-PCR), a candidate gene as a suppressor of

NPC was identified. It was named BLU/ZMYND10 located in the 3p21.3 region. And it was down-regulated significantly in NPC cell lines and biopsies [51]. Gene DLC-1 was identified as a suppressor and it's down-regulated. The methylation-specific PCR indicated that DLC-1 might be a gene related with NPC tumor suppression by aberrant promoter methylation and gene deletion [52].

It was reported that a novel gene was responsible for NPC suppression called GADD25G by observing its changes in environmental stress [53]. A gene named BRD7 belonging to bromodomain was found to be related with NPC suppression. Overexpression of BRD7 might inhibit NPC cell growth by regulating some important molecules in its pathway. It was also mentioned that the nuclear address of BRD7 was crucial for expression of cell cycle related molecules and regulating cell functions [54]. A certain gene named MAD2bB found by Cheung's group was detected to be suppressed in tumor development by observing DNA damaging process. The results indicated that inactivation of MAD2B could lead NPC cell more sensitive towards DNA-damaging anticancer drugs [55].

It has been reported that, in NPC cells, Wnt signaling pathway was activated and WIF-1 was silenced. Through methylation-specific PCR and sequencing analysis, scientists observed abnormal Wnt signaling as a common phenomenon in NPC cells with WIF-1 being silenced [56]. A human cancer epithelial marker was found in involvement of the gene product in response to DNA damage. Over-expression of 14-3-36 in NPC cells reduces the tumor volume in nude mice. The discovering provided us a new way in DNA level for NPC treatment [57].

RNA interference (RNAi) has been applied popular in recent years. And it has been also used in studying NPC development. Bcl-xL gene has been proved to be related with cancer cell growth. Silencing bcl-xL gene with siRNA reduced the proliferation of NPC cells and induce cell cycle arrest at G1 phase, which enlighten us for gene therapy of NPC cells [58]. Pathway analysis by microarrays revealed that increase of NF- κ B and survivin decrease the possibility of tumor cell apoptosis. Changes in integrin and Wnt/ β -catenin signaling lead to excessive proliferating. RNAi technology let us assure that surviving takes as an important therapeutic target for NPC [59].

1.2.3 Biomarkers discovery towards treatment response

The effects of treatment towards NPC are always hampered when tumors are growing larger and stepping into advanced stage. Cervical lymph nodes also disturb the therapy [60], [61]. It was found that a decrease of Ki67 or DNA aneuploid lead to low sensitivity towards chemotherapy, liability to metastasis and a poor diagnosis. Thus this Ki67 and DNA ploidy can be used as biomarkers for chemo-sensitivity and NPC prognosis [62]. Zhao's group reported the vascular endothelial growth factor changes in response to radiotherapy. Patients with high serum VEGF level demonstrate poor diagnosis [63]. In the process of tumor cell spreading, Endothelin-1 is an important vasoactive peptide as an angiogenic growth factor induced by hypoxia. Study is of endothelin-1 may help us figure out novel ways preventing distant failure in NPC patients with late stages [64].

A potential serum biomarker was identified named Ceruloplasmin (CPL) by mass spectrometry

and MASCOT database search. Enzyme-linked immunosorbent assay (ELISA) was used to test the enhanced expression of CPL in NPC patients' serum. However, CPL level does not change much if patients show positive response to treatments [65].

For evaluating the relapse of NPC, two isoforms of serum amyloid A (SAA) were identified as potential biomarkers. In the recurrence of NPC, the SAA content showed upregulation while it was dramatically decreased after salvage chemotherapy [66]. Further examinations were conducted for evaluating the biomarker changes due to different drug combinations. It was found that an inter- α -trypsin inhibitor precursor and platelet factor-4 shows potentiality for NPC biomarkers. These biomarkers discovered may provide useful information for radio-and chemotherapy of NPC [67].

1.2.4 Molecular biomarkers for Epstein-Barr virus-infected NPC

EBV infects large amount of human beings, especially in endemic regions. EBV belongs to human gamma-herpesvirus family. A causal relationship between EBV and NPC was discovered in many cases. EBV encoded genes have been founded in the host immune system and in the process of signaling pathways. The vast presence of EBV genome in almost all NPC tissues renders it possible to use EBV gene as markers for NPC prognosis. EBV antibodies were clinically used to detect EBV coded proteins associated with gene mapping to facilitate NPC prognosis and monitoring.

Viability of immunoglobulin A (IgA) and immunoglobulin G (IgG) antibodies responses to various EBV antigen stimuli allows us to improve early diagnosis of NPC with additional serological assays.

EBNA-1 is coded as the viral nuclear antigen expressed in NPC. It is a primary target during T-cell based immunotherapy [68]. Two synthetic peptides representing immunodominant epitopes of EBNA-1 and viral capsid antigen VCA-p18 were employed in a one-step sandwich ELISA to specifically detect EBV induced IgA and IgG antibodies in NPC patients [69]. The ability of VCA-IgA and VCA-IgG was compared during biomarker study. VCA-IgA showed better performance, although its sensitive was hampered in the fluorescence reaction [70].

Circulating EBV DNA was often identified as forms of DNA fragments rather than intact virions. By quantitative size analysis, Chan and Lo reported that 80% of these DNA fragments were shorter than 180 bp [71]. Comparing between virus DNA in NPC patients' plasma from peripheral blood cell and cancer cells, plasma EBV DNA derived from the cancer cells has higher sensitivity and reliability than that of peripheral blood cell. Thus plasma DNA from NPC cell lines has greater potential for clinical evaluation in the molecular level [72]. Since plasma EBV DNA could reflect the progress of tumors growth, the change of its signal can help us manage NPC diagnosis and treatments [73]. Plasma EBV DNA concentration can be employed in monitoring NPC multiagency. The incidence of EBV DNA appearance in response to different treatments in patients with distant metastasis were much higher than those with continuous remission and local relapse [74]. The abundance of plasma EBV DNA was proved to be correlated with the presence of NPC. Thus these findings elevate the role of cell-free EBV DNA quantification in NPC prognosis [75].

Besides studying NPC patients with malignancy, patients treated with chemotherapy were also detected to harbor EBV DNA in the serum. For example, EBER-1 DNA could become a useful

adjunctive surrogate marker in response to chemotherapy due to its special appearance in the serum of NPC patients with distant metastasis or advanced local recurrence [76]. Altered expression of EBER and several other genes were found to be related with NPC progression using tissue microarray analysis. Upon EBV infection, protein p53 was overexpressed while p16 and p27 proteins were decreased in the process of nasopharyngeal epithelial carcinogenesis [77]. The EBV oncogene BARF1 was also found popular in NPC patients. Structural study focusing on the NPC related proteins were conducted. The secreted BARF1 glycoprotein was analyzed by X-ray crystallography. The structure was found to have high similarity with CD80 or B7-1, a co-stimulatory molecule present on antigen presenting cells, where evolution of BARF1 began [78]. Measurement of EBV DNA Abundance and BARF1 mRNA amount in simple nasopharyngeal brushings allowed non-invasive NPC diagnosis. Due to the simplicity of non-invasive biopsies, the procedure might have potential for scanning monitoring large serological NPC samples as an approach of validation [79].

The consequence of EBV infection towards the STAT3 and NF- κ B signaling pathways in nasopharyngeal epithelial cells was studied. The upregulation of their downstream targets (c-Myc, bcl-xL, IL-6, LIF, SOCS-1, SOCS-3, VEGF and COX-2) was also detected. EBV latent infection suppresses p38-MAPK functions without activating PKR cascade. The evidences support that EBV latent infection implement aberrant cellular functions in various pathways to prevent attacks from the immune system and facilitate cancer development [80]. After comparing the amount of latent EBV genes in NPC cells with normal nasopharyngeal tissue samples, it was concluded that key proteins responsible for apoptosis (bcl-2 related protein A1 and Fas apoptotic inhibitory

molecule), cell cycle checkpoints (AKIP, SCYL1 and NIN) and metastasis (matrix metalloproteinase 1) were closely dysregulated in EBV infected NPC cell [81].

LMP-1 is coded as the membrane protein of EBV and studied in NPC cell response to arsenic trioxide (As₂O₃). The telomere is elongated by LMP-1 mediated with As₂O₃ activities [82]. Researchers studied As₂O₃'s impact on cells and proved its involvement in inhibiting LMP-1 expression, triggering apoptosis and altering of cell cycles growth. NPC cells with LMP1 were more sensitive to As₂O₃ stimulus than LMP-1-negative NPC cells [83]. Functional study of As₂O₃ revealed that NPC cell malignancy might be reduced by inducing As₂O₃ through inhibiting MMP-9 expression. And the downregulation of LMP-1 were found to be a reasonable explanation to elucidate the progress against malignancy in As₂O₃ treated NPC cells [84].

In order to inhibit LMP-1's expression, RNAi was found to be effective to reduce proliferation on NPC cells. RNAi may become an powerful approach to study the functions of LMP-1 in NPC carcinogenesis and serve as novel therapeutic strategy against EBV infections [85]. A recombinant adeno-associated virus type 2 vector carrying siRNA was employed to inhibit EBV LMP-1 in the EBV-infected human NPC C666-1 cells. Results indicated that chronic suppression of EBV-encoded LMP-1 in vivo was an effective method to prevent NPC metastasis [86].

Molecular approaches including above polymorphism, evolutionary tree, bioinformatics in terms of linkage analysis and microarray, RT-PCR, methylation-specific PCR, RNAi and etc. These are promising approaches for biomarker research. However, the effectiveness and sensitivity of

molecular biomarkers remains improving due to several limitations. Genes needed to be transcript and translational to become functional proteins facilitated with many other co-factors and elements. Single alteration of DNA mutation or methylation may not necessarily cause cancer because defects of certain genes will be only expressed by associated activators and facilitators. A person could remain healthy when mutations in his or her genes are deactivated. The second limitation is the low specificity where several mutations of DNA are found in different diseases thus we are not able to determine whether certain genes are specific biomarkers for one disease. What's more, the status of some molecular biomarkers reported in literatures is transient and might cause inconsistency of experimental results, especially on the mRNA level. Due to the variety of approaches for discovering these molecular biomarkers, collection of molecular samples should be standardized so that the comparisons can be made based on the standard benchmark for each collection method.

1.3 Epstein-Barr Virus

The Epstein-Barr virus (EBV) is also named human herpesvirus 4 (HHV-4). It belongs to the herpes virus family, which includes herpes simplex virus 1 and 2. As one of the most common viruses in humans, EBV infects more than 95% of all human adults on earth and most infections persist for life [87]. Fortunately, most of the populations carrying EBV appear no sign of diseases unless triggered by certain stimuli either from outside of the body or from gene defects. Several cancers were found to associated with EBV including Hodgkin's lymphoma, Burkitt's lymphoma, nasopharyngeal carcinoma and central nervous system lymphomas associated with HIV [88-90].

It has reported that EBV is also related with autoimmune disease such as dermatomyositis, systemic lupus erythematosus, rheumatoid arthritis, Sjogren's syndrome and multiple sclerosis [91-93].

Upon infection, most people gain adaptive immunity. Infants will be vulnerable to EBV once maternal antibody protection vanishes. Similar with adults, many children carrying EBV show no signs of infection, since no symptoms appear to distinguish them from healthy children. When EBV infection happens during teenage years, there is a chance to cause infectious mononucleosis.

1.3.1 EBV history

Epstein-Barr virus was firstly discovered and documented by M. Anthony Epstein and Yvonne Barr. The story began from reporting Burkitt's lymphoma in a lecture reported by Denis Parsons Burkitt, a surgeon practicing in Uganda in 1961. In 1963, a biopsy from Uganda was sent to Middlesex Hospital for culturing and storage. In 1964, Epstein, Bert Achong identified the virus particles and published the results in the lancet. At the same time, cell lines carrying virus particles were delivered to the Children's Hospital of Philadelphia, where Werner and Gertrude developed serological markers from virus infections. In 1967, antibodies against EBV were developed by a technician in the laboratory.

1.3.2 EBV virology

Once infected into the cell successfully, EBV expresses its gene through two ways of cycles: Lytic cycle and latent cycle. The lytic cycle was triggered to produce infectious virions after expressing

several viral proteins including gp350 and gp110 [94]. EBV virions are budded out of the infected cells without necessarily leading to host cell lysis. The latent cycle does not result in producing virions. During this cycle, several proteins were produced including Epstein-Barr nuclear antigen (EBNA)-1, EBNA-2, EBNA3A, EBNA3B, EBNA3C, EBNA-leader protein (EBNA-LP), latent membrane proteins (LMP)-1, LMP-2A and LMP-2B and the Epstein-Barr encoded RNAs (EBERs). Besides, EBV also produces more than twenty microRNAs during latently infected cells. And the present snoRNA is detected during lytic cycle [95].

1.4 Study on the relationship between nasopharyngeal carcinoma and Epstein-Barr virus

Due to vast appearance of EBV gene and proteins in the cells and serum of the NPC patients, relationship between EBV and NPC has been took attention and studies for years. Both EBV DNA and latent membrane proteins were studied. It has found that the proliferated viral DNA was homogeneous with the original viral DNA, suggesting that the tumors are proliferated from the original cell infected with EBV [103]. Within the tumor cells, EBV genes were found to have high existence in early dysplastic lesions. Regarding the virus proteins, latent membrane protein 1 and 2 has been reported for their significant influence on host gene expression and cellular functions. And these two proteins are associated with malignancy of the tumors. The EBV mostly infects the B lymphocytes and more evidences have been discovered supporting the transforming infection into the epithelial cells, which could serve as a development of tumor malignancy. However, epithelial infection is much less efficient than that towards B lymphocytes.

1.4.1 Presents of EBV gene and protein in NPC cells

In the malignant epithelial cells, EBV DNA and EBV nuclear antigen complex (EBNA) were detected by hybridization kinetic analysis [104-105]. Compared with Burkitt's lymphoma, EBV DNA existed in almost all the samples from both high and low occurrence areas while not all of Burkitt's lymphoma patients carried EBV [106]. The WHO characterizes NPC into three classes: keratinizing squamous cell carcinoma (type 1); non-keratinizing carcinoma (type 2) and undifferentiated carcinoma (type 3). Among these three types of NPC, type 1 was found to contain EBV with least occurrence, since this type of NPC only appear less than 1% in the endemic areas of high incidence. Thus type 1 NPC wasn't drew full attention from the researchers. As the cloning probe technology becoming popular, EBV genomes have been detected in type 1 NPC in several cases while no viral genome was found in squamous NPC by traditional hybridization *in vivo*. During the study of NPC in Malaysia, EBV DNA was detected by Southern blots. And through in situ hybridization and immunohistochemistry, EBER RNAs and LMP1 were also detected in NPC cells [107].

1.4.2 Latent EBV infection in NPC

Unlike in permissive infection, EBV DNA was contained in an extrachromosomal episome. In permissive infection, the episome is transformed into intermediates and cleaved into linear DNA which is required during virions formation [108-109]. The evidence supporting EBV's latent infection was the detection of EBV episomes in NPC cells. By sequencing the linear EBV, people found that a tandemly repeated 500bp DNA (TR) existed in all the terminus of the virus DNA [110]. By observing the number of TR, it was found that every copy of episomes was identical. This discovery suggests that the tumor cells were identical clones of the original cell infected with

EBV. In some cases, ladder arrays of the EBV DNA fragments were detected suggesting the virus may reactivate from the latent infection and process the further production [111]. The occasional reactivation may provoke an elevated immune response. In addition, multiple copies of episomal EBV DNA were detected by pulse field electrophoresis, indicating a possible contribution to the tumor development of NPC [112].

1.4.3 Premalignancy in NPC

Unlike other types of tumors, NPC has poor early manifestations of malignancy such as dysplasia which is also termed as carcinoma *in situ*. During one study, only 11 samples were found to have positive atypical hyperplasia among over 5000 biopsies [113]. In the dysplasia-positive samples, LMP1 and EBERs were detected. EBNA1, LMP1 and LMP2A were detected in all of the premalignant lesions. All samples with dysplasia had a common restriction site at the end EBV episome through Southern blots. These evidences indicated that the premalignant NPC cells contain EBV. It was also proposed that genetic changes would affect viral infections before latent infections happen accompanied with expression of LMP1 and LMP2. Prior to EBV infection, a loss of a tumor suppressor p16 was found in NPC cells. This finding supported that NPC malignancy may result from a combination consequence of EBV infection and host gene defects.

1.4.4 EBV expression in NPC

Several studies have confirmed that EBV infection can express the small nuclear RNAs (EBERs), EBNA1, LMP1, LMP2 and transcripts from the BamH1 restriction site [114-116]. From the expression on the protein level, EBNA1 was detected and LMP1 was also detected in around 50%

of tumor cells through antigen-antibody specific binding tests, though these antibodies cannot recognize all the LMP1 [117-118]. LMP2 was not detected from the biopsies yet under current detection technology. It is highly likely that LMP2 was under the limit of detection and being undetectable. From the molecular perspective, several reasons that lead to the different expression in EBV infected cells have been identified. One element is that, in EBV infected cells, different promoter was used. A promoter in BamH1 F/Q was used during latent expression program while a promoter in BamH1 C was used activated by the expression of EBNA2 [119-121]. In lymphoid cells, LMP1 expression was controlled by its promoter combined with EBNA2 and EBNA-LP. But in epithelial cells, the exact promoter controlling LMP1 was unknown. We only knew that a larger mRNA transcripts LMP1 [122-123]. And the mRNA was regulated by the promoter controlled SP1 and STAT3 [124]. It has been reported that this promoter was more active in epithelial cells with STAT3 was continuously expressed [124]. In addition, a series of mRNA were identified from the BamH1 region in NPC cells from the cDNA libraries [123, 125]. The cDNA contains open reading frame (ORF) formed from identified exons. However, the pathways during the transcription and translation were still unknown due to the vague information discovered until now.

1.4.5 NPC management concerning EBV strain variation

Due to the patterns of EBV infected NPC and its malignancy, people rise concern about whether the distinct pattern results from different strains of EBV. In China, the EBV strain was found to have lost the BamH1 restriction site within W1' and I1' fragments. And the Chinese strain has also no XhoI restriction enzyme polymorphism in the frame of LMP1 gene, where the polymorphism

was popular in Alaska and Caucasian American regions. The Mediterranean Europe and Africa also lose the XhoI restriction site in their endemic places. Analysis showed that the Chinese EBV strain was type 1 strain while the Alaskan samples were EBV type 2. However, sequencing of LMP1 in different endemic regions indicated that variation of LMP1 was independent of EBV type [126]. The LMP1 sequences differ in the region of a repeated 11 amino acid fragment. In some cases, an insertion of 5aa was detected while some samples lose several amino acids between 343 to 352 of the B95B LMP1. The elevated voice about deletion strains supported that the deleted variant may be more popular in NPC tumor cells. The results from two studies indicated that isolation of LMP1 from NPC leads to higher chance of activating NFkB [127-128].

1.5 Proteome study on nasopharyngeal carcinoma

The term “proteomics” means the study of proteins [129]. Until now, several major aspects have been used as proteomics strategy. The first strategy is based on gel electrophoresis technology. Now it's often 2-dimensional and coupled with mass spectrometry (MS). This technology identifies different proteins at the same time, thus applicable for broad disease diagnose. However, in order to quantify each protein, 2-D gel electrophoresis is not sufficient to provide such information. To achieve quantification of proteins, scientists make use of liquid chromatography (LC) combined with mass spectrometry (MS). LC-MS/MS technology enables scientists to identify and quantify proteins with high efficiency.

Another technology is surface-enhanced laser desorption/ionization time-of-flight MS (SELDI-TOF-MS) or SELDI protein chip technology. These technologies allow us to identify, purify

and analyze proteins by judging the peaks in mass spectrum with unique mass-to-charge ratios (m/z). These technologies implement high-throughput screening of proteins in vast disease and have been used broadly in scientific institutions and hospitals [6, 7].

Besides above-listed technologies, novel strategies have been developed. For completing 2-D gel electrophoresis, differential in-gel electrophoresis (DIGE), isotope-coded affinity tag (ICAT) and isobaric tag for relative and absolute quantification (iTRAQ) have been taken as more scientifically advanced technologies. Electron capture dissociation (ECD), electron transfer dissociation (ETD) and chip-based proteomics strategies all help people get more reliable proteomics profiles [8-10].

1.5.1 Brief introduction of proteomics on clinical application

As mentioned earlier, proteomics provide us scientific and reliable strategy in such a way that most proteins within a cell or serum can be elucidated. And the proteins information gives us numerous profiles for disease diagnostics and treatments. Clinical institutions have been using this technology on various diseases, especially on carcinoma management. Scientists also develop advanced methods making clinical proteomics more effective and powerful.

1.5.2 Proteomics technologies

Proteomics technologies are considered powerful tools for protein analysis. Normally it's constituted of 3 major steps: a. sample preparation; b. protein separation; c. identification of proteins and post-translational modification. Each type of proteomics in these three aspects will

be evaluated here and the advantages of choosing LC-MS/MS approach will be elucidated.

The major steps begin from protein preparation. It requires proteins be solubilized in appropriate condition from tissue or blood [130]. Detergents, chaotropic and reducing agents must be used on a case-to-case manner since each type of protein has its own properties. Interactions between proteins and other agents such as lipid, peptide or themselves must be considered in order to minimize the side-effects during preparation.

Proteins sometimes share similar properties but functionalize in different ways. The sub-proteomics deal with these proteins with similar physical or chemical properties. Classification of them requires good understanding of biochemical characteristics and cellular compartment (e.g., mitochondria, sarcoplasmic reticulum) functions.

After protein preparation, they will be separated. The common method for doing this is two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2-D PAGE) [131], [132]. In the first dimension, protein mixtures are separated by isoelectric focusing due to isoelectric point. Then these proteins are proceeding resolved in the second dimension according to molecular weight in SDS-PAGE. Then protein can be stained by protein binding dyes for following analysis. However, 2D-PAGE encountered two problems including narrow span of protein range and low-throughput analysis. And 2-D PAGE is not necessary if other advanced strategy (e.g., HPLC) is powerful enough to execute protein separation.

The third step goes to protein identification. Mass spectrometry is one of the best choices for doing this because of its high-throughput capabilities and high analytical sensitivity [133]. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS is one of such friendly tools. Protein separated by 2-D PAGE, one dimensional SDS-PAGE, or other methods are digested into peptides. Because mass-to-charge ratio of peptide fragments is detected, the system can generate a peptide mass fingerprint from the sample. Compared with the existing database, the system is able to figure out proteins of interests effectively and efficiently.

Surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) is a modified system from MALDI-TOF. And it's capable to analyze higher molecular weight proteins (>100kDa), while normal mass spectrometry can only reach to 30-300Da peptides [134]. The advantage of SELDI lies in its potential to separate complex proteins more easily and less sample preparation procedures. Its advantage is due to the use of chromatographic chip surface technology (ProteinChip) to capture and separate proteins prior to identification [135]. However, SELDI-TOF MS is not suitable for analyze proteins in serum because high abundant proteins peak overwhelm low abundant proteins. And information about proteins with small amount may be lost in this way.

For completing the defects of mentioned problem, tandem mass spectrometry (MS/MS) provides amino acid sequence information of peptides from parent protein because another MS system is preceded. In this way peptide mass information determined from MALDI-TOF or SELDI-TOF is combined with peptide sequence information from MS/MS to provide an accurate protein

profiles from samples.

1.5.3 Clinical proteomics

Clinical proteomics is to apply proteomics onto disease diagnostics and treatments. Reports in the area of clinical proteomics have been increasing rapidly since 2004, especially in the field of biomarker discovery.

Blood in our bodies contains large amount of protein easily accessible. Besides erythrocytes, leukocytes, haematoblast within plasma and serum, blood also contains other components from other tissues of body, either by leakage or injections. Thus the difficulty of blood analysis lies in identifying proteins with large span of concentrations. For example, albumin is present in the milligrams per milliliter range while cytokine exists in the picograms per milliliter range [136]. This dramatic difference of protein concentration requires higher sensitivity of proteomics analysis.

Serum, derived from centrifugation of clotted plasma, provide rich information of proteomics. It contains around 70mg/ml protein and other small molecules. Up to date, there have been about 10000 proteins found in serum and their distribution is very imbalance. 97% of total protein in serum is high-abundant proteins such as albumin, immunoglobulin, haptoglobin and transferrin. Albumin comprises 55% of all serum proteins and immunoglobulin takes about 15% of them [136], while 3% contains various types of small-abundant proteins [137], [138]. To increase the possibility of finding new biomarkers, scientists already figure out ways to block side-effects from

large-abundant proteins [139]. And we also need to avoid over doing it because the more step we are taken in separation, the higher possibility we are losing our interest proteins.

Protein analysis is always implemented by mass spectrometry, which is high-throughput and highly sensitive. Generally two strategies have been employed for clinical application. One is identifying novel biomarkers for a specific disease and the other is to evaluate a patient's state through existed biomarkers.

Biomarker discovery has been evolved from a single marker to multi-marker systems. In the first place, people discover single protein changes in patients or drug-induced protein difference. However, due to the large amount of proteins affected by numerous factors, this approach is hampered by its accuracy and sensitivity [140]. Complementing its disadvantages, scientists developed multi-maker systems to better access patients' diseased state. The group of Rai developed three biomarker candidates that could be used to differentiate ovarian cancer patients from healthy individuals. Their group also tested the biomarker changes towards tumor antigen named CA125 [140]. The results showed that the three biomarkers changes more violently when treated with CA 125 and thus increase its sensitivity. And Zhang's group provided better performance by multiple-maker system [141]. These results demonstrate that multiple-biomarker system shows good potential for clinical application.

1.5.4 Today's issues on clinical proteomics

No matter discovering biomarkers or using them to examine a patient's diseased state, a good

understanding of resources is important. Scientists must be clear that steps before proteomics studies are crucial factors influencing test results. Marshall's group highlights the importance of operations prior to analytical steps during proteomic studies [142]. They treated blood samples with myocardial infarction. Through drawing samples at time intervals, rather than normal pathophysiological process, they found that, within two hours, the protein profile changes, showing a different present pattern at the same room temperature. They also discovered that the activity of proteases in serum and plasma remain same when concentration of protease inhibitor changes. This example demonstrates that a pre-analysis step is so important that it can either disrupt the results or make our better understanding the data.

Another important factor that may mislead the result is biological variation. Healthy people and patients with certain disease takes different proteomics condition in different age, gender, race, living condition, disease stage and even psychological status [143]. And these in-situ factors influence the proteomics analysis all the time. Thus a better understanding of our research subject is crucial for giving more scientific and reliable results.

1.5.5 Proteomics in nasopharyngeal carcinoma

Helped by those advanced proteomics technologies, scientists are able to monitor protein profile during NPC management.

A. Proteome map

The proteome map is the database in which proteins are identified in NPC patients. By using XML

database Xindice (URL: <http://xml.apache.org/xindice>), people have built more than 200 spots after MALDI-TOF-MS, ESI-Q-TOF-MS and 2-DE/MS analysis [144], [145]. NPC cell line is also established as CNE2 and these proteins are highly involved during infection, metastasis and apoptosis of tumor cells.

B. Biomarker for NPC diagnose

NPC is often lately discovered because of its deep location and vague symptoms. Thus biomarker discovery appears to be very important strategy for early-stage diagnose. By using proteomics technology, scientists have identified nearly 10 types of biomarkers in NPC patients [146]. Each protein is involved in different stages of cancer cells and some of them have been given reasonable proposed roles during cell development [14-16].

C. Proteomics of NPC cells responding to various stimuli

Researchers have been searching tools to control and cure NPC patients. And proteomics study is a great way to evaluate stimuli and develop effective treatments. RNA interference (RNAi) has been emerged as an advanced technology for treating disease at RNA translation and expression level. People have found that a specifically increased protein in NPC patients could be knocked down by RNAi silencing [147]. Some proteins were identified as functional ones during NPC development. For example, TPA has been discovered as a component to facilitate EBV infection to human epithelial cells and malignant transformation. Yao et al. [148] treated NPC cells with TPA and found six changed proteins as biomarkers. And other proteins or chemicals also have been discovered as functional treatment for NPC patients such as Epidermal growth factor receptor

(EGFR) [149] and G-quadruplex ligand 3,3'-diethyloxadycarbocyanine iodide (DODC) [150] by proteomics study of NPC. It's truly realized that proteomics study can be used as an effective way for discovering biomarkers of NPC, which is urgently important for NPC diagnose.

1.6 Pathways in nasopharyngeal carcinoma cells in response to EBV infection

Studying the pathways in NPC cell lines help us to understand the mechanisms from the non-malignant stage to malignant stages. And it is also important to know the pathways of NPC cells in response to EBV infection, since EBV was vastly discovered in NPC cells. During the study of NPC proliferations, the following critical signals were found to involved in tumorigenesis: Wnt pathway [151], NF- κ B pathway [154], [155] and β -catenin pathway [153]. Aberrant apoptosis was also discovered in NPC cells with up-regulated functional proteins. Unique pathways were generated where tumor suppressors were lost in NPC. In the process of tumorigenesis, aberrant cell cycle and cell adhesion also happen because of the correspondingly altered proteins.

Wnt pathway, NF- κ B pathway, β -catenin pathway, and aberrant apoptosis are key pathways involved in this study. Databases discussing these pathways are many. All of them can be accessed from a pathway resource list named pathguide (URL: <http://www.pathguide.org>). The key databases we used in our study are those widely used and published in other literatures. One database we used is Kyoto Encyclopedia of Genes and Genomes (KEGG) which focuses on gene interactions in many kinds of species and diseases (URL: <http://www.genome.jp/kegg>). Another used to study the protein network of virus infected NPC cell line is VirHostnet platform: a combination of pathways databases focusing on protein-protein interactions: Biomolecular

Interaction Network Database (BIND), the Human Protein Reference Database (HPRD) and the Molecular Interaction Database (MINT) (URL: <http://pbildb1.univ-lyon1.fr/virhostnet/login.php>).

1.6.1 Aberrant proliferation pathways

It has been found that aberrant Wnt pathway is present in NPC cells. In normal epithelial cell, the Wnt proteins bind to the frizzles family receptors and activates an intracellular cascade to inhibit the glycogen synthase kinase 3β (GSK- 3β), where abnormal Wnt signaling activates Akt and phosphorylates GSK- 3β [151]. β -catenin is accumulated due to the phosphorylation of GSK- 3β . β -catenin plays important role in the Wnt pathway. EBV infection has been proved to up-regulate β -catenin and caused nuclear localization mediated by the increased GSK- 3β [152]. While Akt was up-regulated in both NPC and Hodgkin's lymphoma, only in NPC cells phosphorylated GSK- 3β and β -catenin were up-regulated [153], which indicated the specific impact of EBV towards the Wnt pathway via changing phosphorylated GSK- 3β and β -catenin. NF- κ B was essential in controlling the cell growth and modulating inflammation. In NPC cells, NF- κ B signaling is activated by binding LMP1 with tumor necrosis factor receptor-associated factors (TRAFs) [154]. Interestingly, another protein coded by EBV named LMP2A down-regulates NF- κ B [155]. The tumor development is affected by the contradictory contribution of LMP1 and LMP2A during EBV infection. The details about how these two proteins interacting with each other remain unclear.

1.6.2 NPC apoptosis

Cancer cells undergo aberrant apoptosis which results in unstoppable proliferations. In NPC cells,

three antiapoptotic factors were found to be up-regulated: bcl-2, survivin and telomerase. In the EBV infected epithelial NPC cells, bcl2 is up-regulated independent of LMP1, since inhibition of LMP1 does not affect bcl-2 expression [156-157]. The findings suggest the important role of bcl-2 in EBV infection during NPC management. Survivin is famous for its function for inhibiting apoptosis by mediating with microtubules [158-160]. In NPC, survivin binds to cyclin-dependent kinase 4 (cdk4) and replaces inhibitory protein p21 and p16. In such arrangement cdk4 can trigger the transcription of S phase proteins [161]. NPC patients with low concentration of survivin have less chance to occur malignancy [162]. Thus up-regulated survivin expression was an important element resulting in aberrant apoptosis. In NPC cells, high activity of telomerase was also detected. One of the most important factors associated with telomerase is termed hTERT, which was inhibited by EBV coded LMP2A and decreased telomerase activity, though the detail inhibition was still unclear. In addition, PI3 kinases (PI3K), a family of kinases involved in many cellular activities was activated as a consequence of interacting with LMP1, inactivated phosphatase and tensin homolog [163]. And MAP kinase JNK and ERK that regulate gene expressions are up-regulated by LMP1 (108,109). What's more, epidermal growth factor receptor (EGFR) is acting more as a transcriptional factor than merely as a signal for proliferations [164-165].

1.6.3 Changes of tumor suppressors in EBV infected NPC

Several tumor suppressors were lost and malfunctioned in NPC cell. P53 is an important tumor suppressor responsible for cell cycle arrest. In head and neck cancers, p53 was down-regulated due to mutations [166]. On contrary, p53 is up-regulated in NPC cells correlated with increased

LMP1 expression [167-168]. And these up-regulated p53 are majorly wild-type, since mutations were rarely detected [157]. It is also possible that the increased p53 was the result from responding to EBV infection. But the mechanism within was still unclear. In cancer disease, another tumor suppressor named p16 is also important to suppress tumor generation [169]. In NPC cells, p16 was decreased caused by gene hypermethylation and activation of DNA methyltransferase caused by LMP1-induced c-Jun/JunB heterodimer was also a possible reason [170]. And LMP1 was also detected for deactivating p16 by inducing the accumulation of E2F4/5 and Ets2 required for p16 normal activity. P27 is a cyclin-dependent kinase inhibitor responsible for inhibiting cell progression [171]. Low level of p27 in NPC cells indicated that most of the p27 were phosphorylated. Therefore progression can proceed and develop into tumors. The dysregulation and malfunction of tumor suppressors make us understand the NPC development more deeply, while detail mechanisms remain being explored.

1.6.4 Dysregulation of proteins in NPC cell cycles

Similar with other types of cancer cells, NPC cells have irregular cell cycles mediated by LMP1. Regulated by p16, cyclin D1 is responsible for cell progression in G1 phase [172]. In NPC cells, cyclin D1 can be activated by LMP1 induced intranuclear accumulation of EGFR [173], resulting in high level of cyclin D1. The cyclin E/cdk2 complex controls cell progression into S phase and initiation of DNA synthesis. It is also related with p27 phosphorylation and degradation. Up-regulations of cyclin E/cdk2 lead to fast entry of S phase as well as increasing chromosomal instability [174]. C-myc regulates G1/S phase proteins including p27, thus controlling the cell proliferation and the entry of G1 phase [175]. C-myc was up-regulated in most cancers. In NPC

cells, both up and down-regulation were discovered with different performance between epithelial NPC and B lymphocyte cells [176-177]. The dysregulation of C-myc was also shown to be related with LMP1. Poorly differentiated NPC contains more down-regulated c-myc while well-differentiated NPC harbors increased c-myc. During study of chromosome condensation in NPC, checkpoint with forkhead-associated and ring finger domains (CHFR) were found to be up-regulated while it was decreased in most of the other cancers [178-179]. The abnormal behavior of CHFR in NPC was related with p16, RASSF1A and CKIs, where the tumor suppressing was inhibited [179-180].

1.6.5 Dysregulation of proteins in NPC cell adhesion

During cancer cell management, adhesion was also aberrant from the healthy cells. In NPC, E-cadherin and matrix metallopeptidases (MMPs) are detected to be dysregulated. E-cadherin is responsible for cell communication in normal cells and inhibiting metastasis in tumor cells. E-cadherin was down-regulated in NPC cells resulting higher chances of metastasis. And cytoplasmic β -catenin was down-regulated, which is cooperating with E-cadherin during cell adhesion maintenance [181]. MMPs are collagenases (type IV) which are up-regulated in many cancers including NPC. Because the family of MMPs is increased by LMP1, this rises up the elevating importance of MMPs, especially MMP1, since MMP1 acts as the initiator of the following MMPs' actions. Other functions of MMPs were also discovered, suggesting the interesting roles during management of NPC progression and metastasis [182-184].

1.7 VDAC1 and its role in EBV infected NPC cells

Research over the years has been recognized the importance of mitochondria due to its vast

regulation of cellular events. Besides generating energy for cells, the mitochondrion mediates apoptosis by controlling various signals. On the outer membrane of the mitochondria, one protein named voltage-dependent anion-selective channel protein (VDAC) functions at the gatekeeper for entry and exit of metabolites. VDAC proteins serve as the convergence point mediating apoptotic signals across mitochondria. Understanding VDAC proteins was crucial for us to study apoptosis and improve the cancer diagnosis and treatments, including NPC management.

1.7.1 Brief introduction of VDAC protein family and VDAC1

Voltage dependent anion-selective channel proteins VDACs are a family containing 3 isoforms: VDAC1, VDAC2 and VDAC3. The VDAC1 was the most abundant isoform and also the one that was studied for the last two decades. It was found in HeLa cells that the concentration of VDAC1 was 10 times fold of VDAC2 and 100 times fold of VDAC3 by real-time PCR [185]. From the perspective of their size, VDAC1 was slightly smaller than VDAC2 and VDAC3 was the smallest isoform according to their mobility in electrophoresis [186-187]. The evolution of VDACs happened million years ago and people have traced that VDAC3 was the oldest protein, which was 76 million years earlier before converged into VDAC1/2 [188-189]. The structure of VDAC1 and channel activity was studied, associated with its interacting proteins and molecules.

1.7.2 Channel activity of VDAC1

As a membrane proteins controlling flux of metabolites, VDAC1 conducts its function by channel activities. The pore structure of VDAC1 allows it to transfer molecules and small proteins across

the membrane. Several methods were employed by researchers to study the channel activity of VDAC1. The most common method is reconstitution of the pore into the planar lipid bilayers (PLB). The activities of the channel are recorded by detecting the ion flow across the bilayer. The resolution of this system is high enough to study only one channel, thus providing information on the molecular level. The conducting ability of VDAC1 was detected and indicating different stable conformations facilitating ion transference [187, 190-191]. The ion selectivity was controlled by the environmental voltage. A low and stable voltage makes VDAC1 open while an increasing voltage will initiate the close state [192-193]. The sub-state with different pore conductivity and selectivity occurs when potentials are high [194].

1.7.3 VDAC1 structures

The 3D structure of VDAC1 has been proposed in 2008 by three methods including resonance spectrometry (NMR) and NMR combined X-ray crystallography [195-197]. Since the amino acids of VDAC1 were very conservative in all mammalian cells, their structure share high level of similarity [198]. The general structure of VDAC1 is a barrel composed of 19 β -strands and an N-terminal helix folded into the pore interior. Analysis of VDAC1 shows that exterior sides were composed of hydrophobic β -barrels while in inside consists of hydrophilic residues [199]. The VDAC1 prefers transporting anions than cations. Several models explaining the gating property of VDAC1 were also proposed [197, 200-202]. Different oligomerization states of VDAC1 enable it to interaction with different proteins and ligands [203-205]. Further studies expecting to explain the detail interaction between VDAC1 and pro-apoptotic and anti-apoptotic proteins need to be conducted.

1.7.4 The role of VDAC1 in cell growth and apoptosis

VDAC1's important role in cell growth was studied by shRNA in T-Rex-293 cells. The inhibited expression of VDAC1 leads to reduced ATP and decrease in cell growth [206]. In most apoptotic cells, VDAC1 was found to be over expressed in response to various stimuli [207-210]. The mechanisms of VDAC1 overexpression include increasing of mitochondrial permeability, shifting between monomeric and oligomeric states, interaction with ANT to form and activate PTP complex and enhancement of ROS production [205, 207, 211-212].

1.7.5 VDAC1 and cytochrome C release

Pro-apoptotic signals including cytochrome C (Cyto C), calcium and other molecules are important to trigger apoptosis. Cytochrome C functions as the electron shuttle in the respiratory chain located in the inner membrane of mitochondria. Several models explain the possible mechanisms of VDAC1 release through VDAC1. One study proposed that increase of mitochondrial membrane permeability resulted swelling of membrane and increase of Cyto C release [213]. The other study suggested a permeability transition pore (PTP) complex was formed before release pro-apoptotic proteins [214-217]. Another mechanism was proposed when detecting the role of Bax, a pro-apoptotic protein. It suggested Bax formed selective large channels for Cyto C release [218-220]. Several other studies also reveal the possible initiation of Cyto C release were related with Bax and Bak oligomerization, VDAC1 hetero-oligomerization, mitochondrial apoptosis-induced channel (MAC) and ceramides [221-230].

1.7.6 VDAC1 and calcium release

Ca^{2+} has been proved to involve in modulating enzymes in TCA cycle, fatty acid oxidation, amino acid catabolism and ATPase and ANT metabolism [231]. Mitochondria also modulate intra-mitochondrial Ca^{2+} release through inducing Ca^{2+} - dependent permeability transition pore (PTP) [232-233]. The functions of Ca^{2+} impact VDAC activity through a novel mechanism during controlling mitochondrial outer membrane permeability [234]. And the contact sites between VDAC1 and Ca^{2+} was found by using azido ruthenium (AzRu), a photoreactive reagent specifically interacting with Ca^{2+} binding proteins [235].

1.7.7 VDAC1 related viruses

Many viruses produce proteins that interacting with mitochondria and VDAC1 thus impacting on apoptosis [236-240]. One example is HIV-1 which can induce mitochondrial apoptosis through several proposed mechanisms [241]. Influenza A virus coded protein PB1-F2 also targets mitochondria and interacts with VDAC1 [242]. Hepatitis B virus (HBV) encodes HBx proteins interacting with VDAC1 and results in Cyto C release [203, 205, 243-244]. However, few studies reveal the EBV's interacting with VDAC1, which is one of the targets during our research in order to elucidate the VDAC1's role in NPC cells in response to EBV infection.

Chapter 2 : ITRAQ-coupled 2-D LC-MS/MS analysis of differentially expressed serum proteins in nasopharyngeal carcinoma clinical samples: Potential in biomarker discovery

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Summary

Nasopharyngeal carcinoma (NPC) is one of the sneakiest cancers due to its vague symptoms. Efficient and precise early diagnoses remain advanced exploration. To identify novel biomarkers for NPC management, 30 patients' serum were analyzed by iTRAQ-coupled 2D LC-MS/MS system. Protein profiles in more than 25 out of 30 patients (>83%) showed similar changes. 13 proteins were identified as potential biomarkers for their significant difference versus healthy controls. Compared with healthy controls, Alpha-1-antitrypsin, Ig gamma-1 chain C region, Apolipoprotein B-100, Alpha-1-acid glycoprotein 1, Inter-alpha-trypsin inhibitor heavy chain H4, Antithrombin III, Plasma protease C1 inhibitor and haptoglobin were up-regulated for at least 39% increase in NPC patients, while Ig gamma-2 chain C region, Inter-alpha-trypsin heavy chain H2, alpha-2-macroglobulin, apolipoprotein A-I and histidine-rich glycoprotein were down-regulated for more than 20%. These proteins with significant difference in NPC patients may act as potential biomarkers. Coupled with their physiological functions, these potential biomarkers could contribute to provide reliable pathways and improve management during NPC diagnosis and treatments.

2.1 Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common cancers in Southeast Asia and Southern China. It occurs less frequently in the western world [179, 245-246]. Male patients are more likely to develop NPC and their occurrence rate is nearly twice as high as that in female individuals. Nasopharyngeal carcinoma starts to develop when tumor cells arise from the epithelium of the nasopharynx. Due to the vague clinical symptoms, NPC patients are mostly detected in the late stages of the disease with a unilateral neck mass, which is the enlarged painless cervical lymphonodes. The difficulty of early stage diagnosis and nasopharyngeal examination by traditional methodology make the precise and efficient diagnosis critically important.

In NPC histology, current diagnosis is made by biopsy of the nasopharyngeal mass. Physical examination is typically a biopsy of the lesion and optical tests require computed tomography (CT) and magnetic resonance imaging (MRI). A valuable imaging tool for staging diagnosis of NPC is the fused positron emission tomography/computed tomography. However, NPC is a type of cancer hard to detect by its deep location and vague symptoms [44]. Genetic analysis reveals that genetic defects of HLA-A2, HLA-B17 AND HLA-Bw26 double the risk of NPC by 30% [247]. On the other hand, Epstein Barr virus (EBV) has been reported as an important etiology factor in NPC. EBV nuclear antigen has been found in infected nasopharyngeal epithelial cells, suggesting its relationship with tumor development. DNA diploidy and integrity of cell-free circulating RNA has also been found to complement NPC early stage diagnosis [248]. Environmental factor is the most unnoticed crucial element for causing NPC people with nasal infections, poor hygiene, poor ventilation of the nasopharynx and consumption of salt-preserved foods containing high level

N-nitroso compound [249]. However, specificity and efficiency are bottlenecks that limit the application of DNA/RNA and environmental biomarkers. Identification of specific biomarkers by a sensitive technology would be useful to overcome the above-described limitations.

Proteomics study has been widely used for decades to study the protein profile in cancer diagnosis and treatment. And the “ace” of proteomics emerged as high-throughput, specific and cost effective. Traditional strategy is based on 2D-gel electrophoresis technology coupled with mass spectrometry (MS). This technology identifies different proteins at the same time, thus applicable for broad disease diagnosis. However, in order to quantify each protein, 2-D gel electrophoresis is not sufficient to provide accurate information. To achieve quantification of proteins, scientists make use of liquid chromatography (LC) combined with MS. LC-MS/MS technology overcomes the limit of 2D gel electrophoresis and enables scientists to identify and quantify proteins with high efficiency. Up to date, several proteins have been identified and used as biomarkers for their significant differences in NPC patients by LC-MS/MS proteomics technologies [250-252]. However, the numbers of biomarkers from serum were few and more sensitive and specific candidates are needed to provide better diagnostic information for NPC management.

In the current study, we applied a novel method - isobaric tag for relative and absolute quantification (iTRAQ) of proteins for NPC early diagnosis. The iTRAQ labeling has been proven to enhance the analytical accuracy and precision, and it has been successfully applied to establish reliable protein profiles in ibuprofen treated neuron cells, atenolol treated vascular smooth

muscle cells and HepG2 cells transfected with HBV genome [253-256]. We reported the identification of 13 novel proteins as potential biomarkers with significant up or down regulation in NPC patients using iTRAQ labeled 2D LC-MS/MS analysis.

2.2 Material and methods

2.2.1 Serum samples collection and patients' information

Serum samples of NPC patients and healthy controls were collected from National Cancer Centre of Singapore with the support of Dr. Balram and his lab members. The study group consists of 30 patients with NPC and 10 healthy controls randomly distributed in Singapore. Patients were randomly chosen from those diagnosed with NPC and their information was described (Table 2-1). This study was approved by Clinical Trial Committee at National Cancer Centre of Singapore. The collection of serum samples are as follows: 5ml blood sample of each patient was taken and each of them was transferred into a sterile empty tube without any anticoagulant. The tubes stood still for 30 minutes allowing the blood to be clotted. After that samples were centrifuged at 20°C, 1500g for 10min. Then serum was extracted quickly and frozen in -80°C for further assay.

S/N	Sub- jects ID	Ethnicity	G e n d e r	Height	Wt	BSA	Age	State of Disease	Sites of meta- stasis	ECOG perfor-- -mance status
				(cm)	(kg)	(m2)	(yrs)			
1	SCH 001	Chinese	M	178	55.6	1.66	34	III	0	1
2	ATC 002	Chinese	M	172	43.7	1.44	39	III	0	1
3	TKC 003	Chinese	M	162	53.8	1.56	54	IVC	1	1

4	TKT 004	Chinese	M	155	50.4	1.47	55	IVC	1	1
5	NBH 005	Chinese	M	175	70.4	1.85	67	IVB	0	1
6	LBJ 006	Chinese	M	163	66	1.73	51	IVB	0	1
7	TTS 007	Chinese	M	172	64.7	1.76	45	IVC	1	0
8	KFKJ 008	Chinese	F	162	48	1.47	34	IIB	0	0
9	SBS 009	Malay	M	158	49.8	1.48	43	III	0	1
10	NKH 010	Chinese	M	177	67.3	1.82	31	IVA	0	1
11	TGC 011	Chinese	M	164	58	1.63	37	III	0	1
12	LKT 013	Chinese	M	167	63.2	1.71	41	IVA	0	0
13	THC 014	Chinese	M	169	52.4	1.57	50	IVA	0	1
14	TTB 015	Chinese	M	164	63.8	1.7	40	IVC	1	1
15	KJF 016	Chinese	M	161	53.8	1.55	50	IIB	0	1
16	MIB M 017	Malay	M	171	53	1.59	25	IVC	1	1
17	LKA 018	Chinese	M	166	56.7	1.62	52	IIB	0	0
18	TBL 021	Chinese	F	145	45.9	1.36	49	IVC	1	1
19	CTB 022	Chinese	F	153	45.3	1.39	47	IIB	0	1
20	LCL 023	Chinese	M	174	72	1.87	48	IVA	0	1
21	CMF 024	Chinese	M	155	48.6	1.45	44	III	0	1
22	LEK 025	Chinese	M	172	39	1.37	52	IVA	0	2
23	TCM 026	Chinese	M	167	60.5	1.68	60	III	0	1
24	BCS 027	Chinese	M	174	91.9	2.11	52	IVC	1	0

25	TYK 028	Chinese	M	170	71	1.83	43	IVB	0	1
26	TCH 029	Chinese	M	165	81	1.93	37	IIB	0	0
27	CWT 030	Chinese	M	170	52.6	1.58	45	IVC	1	1
28	OTL 033	Chinese	M	151	55	1.52	57	IVB	0	0
29	HW M 034	Chinese	M	167	59	1.65	45	III	0	1
30	HLK 035	Chinese	M	170	46.7	1.49	43	IVA	0	1

Table 2-1: NPC patients' information of serum samples

Patients' information of serum samples was shown in terms of ethnicity, gender, height, weight, body surface area (BSA), age, state of disease, sites of metastasis and performance status of the patients standardized from Eastern Cooperative Oncology Group (ECOG).

2.2.2 Protein precipitation and labeling with iTRAQ reagents

Protein concentration of each sample was determined by 2-D Quant Kit (GE Healthcare). For each sample, serum volume (10 μ l) equal to 100 microgram was precipitated by adding four volumes of cold acetone. Mixtures were incubated at -20°C for 2 hours. Followed by iTRAQ protocol (Applied Biosystems), proteins in sera were dissolved, denatured and cysteine blocked. Then 20 μ l of 0.25 μ g/ μ l sequence grade modified trypsin (Promega) was used to digest each protein solution at 37°C overnight before labeling with iTRAQ reagents.

Patients' sera proteins were labeled individually with iTRAQ reagents 115, 116 and 117 and healthy controls were labeled with iTRAQ reagent 114. After labeling, 4 labeled samples containing 3 NPC and 1 healthy sera were mixed together into 1 fresh tube for further analysis.

Thus 10 batches of serum proteins labeled with iTRAQ reagents were prepared for 2D LC-MS/MS

analysis. The workflow of iTRAQ labeling was summarized in figure 2-1.

Figure 2-1: Workflow of iTRAQ reagent methodology. The standard method in iTRAQ labeling for NPC samples and controls was illustrated. The samples treated with these steps were then analyzed by LC-MS/MS technique.

2.2.3 Online 2-D LC-MS/MS analysis

After proteins are digested into separate labeled peptides, samples are ready to go to LC separation. In the first dimension, 5µl of the combined peptide mixture was injected onto a strong cation exchange column (0.32 x 50mm, 5µm). A reversed phase column was employed as second dimension and these two enrichment columns trapped the peptides. Buffer D is

constituted of series of increasing concentration of KCl salt solution as 10, 20, 30, 40, 50, 60, 80, 100, 300, and 500 mM used to elute the retained peptides in a stepwise manner[255]. Each KCl solution was used as a 100 min run. In the running process, some of the peptides bind to the SCX column during the flow. Peptides passing SCX column were trapped in the ZORBAX 300SB-C18 enrichment column I (0.3x5 mm, 5 μ m) and washed by buffer A (5% acetonitrile, 0.1% formic acid) at rate of 0.5 mL/min to remove the excess reagents. Two enrichment columns were alternatively switched into the solvent path of the nano pump by a 10-port valve. The previous pathway is considered as position 1. In the following run (100mM KCl), the 10-port switching valve switched to position 2, A total of 5 μ L of 10 mM KCl solution was injected into the SCX column to elute retained peptides to column II which was washed isocratically using the loading buffer for 100 min at 0.5 mL/min for removing excess reagent. The column I which trapped the unbound peptides in the first run was switched into the solvent path of the nanopump. Peptides were eluted using the buffer B (0.1% formic acid) and the buffer C (95% acetonitrile, 0.1% formic acid) with a nanoflow gradient starting with 5% of the buffer C and increasing up to 80% of the same buffer C over 100 min at a flow rate of 500 nL/min. This gradient wash is to make sure most of the peptides can be eluted out and further separation was achieve in analytical Zorbax 300SB C-18 reversed-phase column (75 μ m x 50 mm, 3.5 μ m), which separate peptides according to their hydrophobic-hydrophilic properties.

The LC was used to separate peptides and MS to identify protein by its unique m/z ratio. In the MS detection, the HP1200 LC system (Agilent Technologies) was interfaced with a QSTAR XL (Applied Biosystems-MDS Sciex) mass spectrometry. The ionization method was by electrospray

ionization. Survey scans were acquired from m/z 300-1500 with up to two precursors selected for MS/MS from m/z 100-2000 using dynamic exclusion, and the rolling collision energy was used to promote fragmentation. Finally, detection was performed in software for every 100-mins run coming from LC.

2.2.4 Data analysis and interpretation

The fold changes are determined by the relative concentration of a target protein in NPC patients compared with the healthy control. The relative protein concentration is calculated by the sum-up of peptides values which are labeled with iTRAQ. Due to unique m/z ratio, peptide identifications were performed using ProteinPilot^{TM} Software 2.0 packages (Applied Biosystems, Software Revision 50816). Each MS/MS spectrum was searched within the Uniprot protein database, and proteins were accepted if ProtScore value is more than 2.0, which gives the confidence value of 99%. The database allowed for iTRAQ reagent labels at N-terminal residues, internal K and Y residues, and the methylmethanethiosulfate-labeled cysteine as fixed modification, plus one missed cleavage. The analysis for the iTRAQ experiments was performed with ProteinPilot 2.0. The cutoff for the confidence settings was 75, and the tolerance settings for peptide identification in ProteinPilot searches were 0.15 Da for MS and 0.1 Da for MS/MS. ProteinPilot pooled data from all the series of runs of increasing concentration of KCL in one experiment. All identifications were manually inspected to minimize machine-related errors. Relative quantification of proteins in the case of iTRAQ was performed on the MS/MS scans and was the ratio of the areas under the peaks at 114, 115, 116, and 117 Da which were the masses of the tags that correspond to the iTRAQ reagents. The relative amount of a peptide in each

sample was calculated by dividing the peak areas observed at 115, 116, and 117 m/z by that observed at 114 m/z . The calculated peak area ratios were corrected for overlapping isotopic contributions, and were used to estimate the relative abundances of a particular peptide. For proteins with two or more qualified peptide matches, three average peak area ratios (designated as 115/114, 116/114, and 117/114) were calculated using the peak area ratios of the peptides originating from the same protein. To account for small differences in protein loading, these ratios have been normalized using the overall ratios for all proteins in the sample, as recommended by Applied Biosystems. In the study, protein quantification data with relative expression of >1.2 or <0.8 were considered as significant difference from control.

2.2.5 Statistical analysis

We consider these proteins as potential biomarkers for further analysis under the following criteria: Unused protein score was more than two (above 99% confidence); At least two peptides were identified to achieve a high confidence ($>99\%$); and Student's t-tests were employed and acceptable p-values for biomarkers (<0.05) fulfill the quantification requirement.

2.3 Results

2.3.1 Identification of potential serum biomarkers

According to LC-MS/MS analysis and ProteinPilot 2.0 scanning, each serum sample showed unique protein profile. Among these proteins, 13 proteins were selected as potential biomarkers for future NPC management based on the fact that more than 25 out of 30 patients shared a similar protein profile. And these proteins' relative concentration was either >1.2 or <0.8 ,

showing significant difference compared with healthy subjects. Compared with the reported data, most of the proteins identified in our study were unique in their cellular functions.

Under the criteria mentioned, 9 proteins were identified as up-regulated biomarkers for their relatively higher concentration (>1.2 fold) in NPC patients compared with healthy controls, while 4 proteins were identified as down-regulated biomarkers.

Both up-regulated and down-regulated biomarkers were summarized (Table 2-2) provided with sequence coverage, average concentration changing fold, number of distinct peptides detected and protein score.

Biomarker candidates		Sequence coverage (%)	Protein ratio (NPC/control)	P value	Peptides	Protein score
Signal proteins						
P01009	Alpha-1-antitrypsin	45.61	2.02	0.034	22	37.04
P01857	Ig gamma-1 chain C region	53.51	3.32	0.001	20	26.76
P01859	Ig gamma-2 chain C region	39.83	0.51	6.26E-08	16	12.11
P04114	Apolipoprotein B-100	3.37	1.4	0.005	10	21.8
P02763	Alpha-1-acid glycoprotein 1	29.85	2.32	3.68E-04	6	10.2
Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	4.44	1.4	1.75E-04	3	7.42
P19823	Inter-alpha-trypsin inhibitor heavy chain H2	6.413	0.76	7.00E-03	3	7.55
P01008	Antithrombin III	7.83	3.34	2.59E-04	3	6.58
P05155	Plasma protease C1 inhibitor	11.66	1.54	1.62E-04	3	7.01
P01023	Alpha-2-macroglobulin	28.14	0.53	7.73E-10	27	53.11
Binding proteins						
P00738	Haptoglobin	35.22	1.39	1.20E-02	12	21.41
P02647	Apolipoprotein A-I	45.75	0.57	2.17E-08	14	25.64

P04196	Histidine-rich glycoprotein	10.52	0.74	7.97E-07	3	6.52

Table 2-2: Proteins identified as potential biomarker candidates

2.3.2 Signaling proteins

Most biomarkers identified as signaling proteins were discussed as follows:

- Alpha-1-antitrypsin. Also known as Alpha-1 protease inhibitor, its targets include elastase, plasmin and thrombin. This protein was found to have an increased concentration in NPC patients by 2.01 fold.

- Constant region of immunoglobulin (Ig). The Ig gamma-1 chain C region and Ig gamma-2 chain C region were found to be contrastingly regulated in our study. The former was up-regulated in NPC patients for 3.32 fold while Ig gamma-2 chain C region down-regulated to 0.51 fold.

- Apolipoprotein B-100. The function of this protein is to act as a recognition signal for the cellular binding and internalization of LDL particles by the apoB/E receptor [257-258]. It was found to be up-regulated by 0.4 fold in the NPC patients selected in this study.

- Alpha-1-acid glycoprotein 1. This protein appears to function in modulating the activity of the immune system during acute-phase reactions. It was found to be up-regulated by 2.32 fold in the NPC patients selected in this study.

- Inter-alpha-trypsin inhibitor heavy chain H4 and H2. Both proteins are signal proteins. The

inter-alpha-trypsin inhibitor heavy chain H4 was found to be up-regulated by 1.40 fold in our study. Significantly, our results were consistent with its reported role as an NPC biomarker [259]. On the other hand, the inter-alpha-trypsin inhibitor heavy chain H2 was found to be down-regulated in our study.

- Antithrombin III. This protein is one of the most important serine protease inhibitor in plasma and it was found to be up-regulated by 2.34 fold in the NPC patients selected in our study.

- Plasma protease C1 inhibitor. This protein controls the activation of C1 complex where it forms a proteolytically inactive stoichiometric complex with C1r or C1s protease [260]. It was found to be up-regulated by 1.54 fold in the NPC patients selected in our study.

- Alpha-2-macroglobulin. This protein is able to inhibit all four classes of proteinases by a unique “trapping” system [261] and was found to be down-regulated (0.53 fold) in the NPC patients selected in our study.

2.3.3 Binding proteins

Other proteins with binding activities in cellular events were found to have altered levels in the NPC patients selected in our study and their potential roles were discussed as below:

- Haptoglobin. It has been reported that haptoglobin combines with free hemoglobin in blood plasma, preventing loss of iron through the kidneys and preventing the kidneys from damage by

hemoglobin [262]. It was found to be up-regulated by 1.39 fold in the NPC patients selected in our study.

- Apolipoprotein A-I. This protein has been implicated in the reverse transport of cholesterol from tissues to the liver for excretion by promoting cholesterol efflux from tissues and by acting as a cofactor for the lecithin cholesterol acyltransferase [263]. It was found to be down-regulated in the NPC patients in our study.

- Histidine-rich glycoprotein. This protein binds heme, dyes and divalent metal ions. It also inhibits rosette formation and interacts with heparin, thrombospondin and lysine-binding site of plasminogen [264-265]. It was found to be down-regulated by 0.76 fold in the NPC patients selected in our study.

2.3.4 MS/MS spectra of representative peptides

To elucidate the process of biomarker's identification, two representative MS/MS spectra were selected (Fig. 2-2). Firstly, the high confidence peptides were displayed in one dimension in one spectrum. Second spectrum provided a zoom-in picture, showing different performance of iTRAQ labeled serum samples. The NPC patients were labeled with iTRAQ reagents from 115 to 117, while the healthy control was labeled with reagent 114. The whole protein concentrations were calculated after summarizing all detected spectra information under provided criteria.

Fragmentation Evidence

Panel A

Fragmentation Evidence

Panel B

Figure 2-2: Alpha-1-antitrypsin MS/MS spectrum of a representative peptide. Panel A was a representative peptide's mass spectrum of this biomarker. Each peak represented one residue by detecting its unique m/z ratio. Figure B showed the signal intensity of iTRAQ labeled residues from 114 to 117.

2.3.5 Classification of potential biomarkers with physiological functions

Most NPC patients also suffer malfunctions in other tissues. Thus investigating the connection between NPC and other physiological events was of relevance in assessing the value of our identified proteins as NPC specific biomarkers.

As reported by other investigators, alpha-1-antitrypsin is involved in other related diseases. It can protect the lower respiratory tract against proteolytic destruction by human leukocyte elastase (HLE) [266-267]. A deficiency of this protein has been found in chronic obstructive pulmonary disease with high occurrence. Malfunction of alpha-1-antitrypsin may decrease coagulation time leading to bleeding diathesis [268]. Patients with NPC have relatively weak reconstruction systems probably due to damage related to cancer cells. And the weakness is presented in blood coagulation. Thus a high level of Alpha-1-antitrypsin may represent a low efficiency of platelets work in blood and indirectly indicating that the patients might have potential to have NPC.

Haptoglobin has been reported to suppress amyloid formation through interacting with prefibrillar protein species [269]. Apolipoproteins bind to lipids and serve as co-enzymes that regulate the metabolism of lipoproteins and their uptake in tissues [270-271]. Changes of concentration of apolipoproteins in plasma of NPC patients may reflect the performance of lipid delivery system.

Similarly to haptoglobin, Alpha-1-acid glycoprotein is an acute-phase plasma alpha-globulin glycoprotein. Thus certain diseases, infection, injury and inflammation can lead to the level changes in an abnormal way. It has been reported that alpha-1-acid glycoprotein is involved in infection of human immunodeficiency virus protease inhibitor clearance in HIV patients [272].

Inter-alpha-trypsin inhibitor heavy chain H4 and H2 are alpha-trypsin related inhibitor giving sensitive response against certain disease and surgical trauma. Inter-alpha-trypsin inhibitor heavy chain H2 also act as a carrier of hyaluronan (chief components of extra-cellular matrix) in serum

or as a binding protein between hyaluronan and other matrix protein, including those on cell surfaces in tissues to regulate the localization, synthesis and degradation of hyaluronan which are essential to cells undergoing biological processes [273].

Antithrombin-III, as the most important serine protease inhibitor in plasma, can regulate blood coagulation and bind heparin, a tumor suppressor. Antithrombin-III's activity is greatly increased when heparin exist [274]. The significant up-regulation (3.34 fold) of antithrombin-III may indicate its high interaction with herapin during tumor growth.

Histidine-rich glycoprotein also interacts with heparin to increase its inhibitory activity as reported. The down-regulation of Histidine-rich glycoprotein indicates that this protein might act as an opposite way in interacting with heparin. Studying its down-regulated performance in serum comparing with the up-regulated performance of Antithrombin-III would help us better understand the action of heparin in NPC development. Plasma protease C1 was found to inhibit chymotrpsin, kallikrein and FXIIa which involved in blood coagulation [260]. It has been reported that both Alpha-2-macroglobulin and Haptoglobin suppress Amyloid formation by interaction with prefibrillar protein species [269]. And two isoforms of serum amyloid A protein were identified as useful biomarkers to monitor replapse of NPC [275]. Since alpha-2-macroglobulin suppress Amyloid formation, which occurred as a useful biomarker in NPC, low level of Alpha-2-macroglobulin coincide with elevated Amyloid concentration in NPC patients. And Alpha-2-macroglobulin is hereby firstly reported as a low level NPC biomarker. Classification of identified biomarkers with physiological functions was provided (Table. 2-3)

Physiological functions	Biomarkers	Regulation	Protein ratio (NPC/control)
Blood			
coagulation	Alpha-1-antitrypsin	Up-regulated	2.02
	Antithrombin-III	Up-regulated	3.34
	Histidine-rich glycoprotein	Down-regulated	0.74
	Plasma protease C1 inhibitor	Up-regulated	1.54
Amyloid formation	Haptoglobin	Down-regulated	1.39
	Alpha-2-macroglobin	Down-regulated	0.53
	Apolipoprotein A-I	Down-regulated	0.57
	Inter-alpha-trypsin inhibitor heavy chain H4	Up-regulated	1.4
Immuno-genesis	Ig gamma-1 chain C region	Up-regulated	3.32
	Alpha-1-acid glycoprotein	Up-regulated	2.32
	Ig gamma-2 chain C region	Down-regulated	0.51
Lipid-transport system	Apolipoprotein B-100	Up-regulated	1.4
	Apolipoprotein A-I	Down-regulated	0.57
Hyaluronan carrier	Inter-alpha-trypsin inhibitor heavy chain H2	Down-regulated	0.76

Table 2-3: Classification of potential biomarkers with physiological functions

2.4 Discussions

Among the 13 biomarkers identified in our study, 3 pairs of proteins shared the similar structure while performing opposite regulations as NPC potential biomarkers. For example, Ig gamma-1 chain C region and Ig gamma-2 chain C region both belong to immunoglobulin with similar heavy

chain. But Ig gamma-1 chain C region has higher concentration than control and thus noted as high-level biomarker while Ig gamma-2 chain C region was down-regulated. The distinct concentration of these two supposed similar biomarkers raises our interest for investigation on their structure differences. The same occurrence appeared in the other two pairs of biomarkers. Table 2-4 shows three pairs of NPC biomarkers sharing similar structure but performing differently.

Up-regulated NPC Biomarkers	Down-regulated NPC Biomarkers
Ig gamma-1 chain C region	Ig gamma-2 chain C region
Inter-alpha-trypsin inhibitor heavy chain H4	Inter-alpha-trypsin inhibitor heavy chain H2
Apolipoprotein B-100	Apolipoprotein A-I

Table 2-4: NPC biomarkers sharing similar structures but performing in opposite direction

Ig gamma-1 chain C region and Ig gamma-2 chain C region are proteins coded by IGHG1 and IGHG2 gene respectively. IgG, a “flag” antibody in human body, is around 150kD composed of 2 identical heavy chain of about 50kD and 2 identical light chains of about 25kDa. They are sub-classified from IgG1 to IgG4 in human. After blasting Ig gamma-1 chain C region and Ig gamma-2 chain C region, these two proteins share 90% protein identity which means the 10% structural difference leads their distinct ratio in NPC patients. It has been reported that the most significant difference of structure lies in the hinge region of two proteins. IGHG are divided into 3 hinges: upper hinge; middle hinge and lower hinge. The structures of two intact monoclonal antibodies IgG1 and IgG2 by X-ray diffraction analysis reveals the structures of the two proteins

with hinge polypeptides. An IgG1, whose antigen is the drug phenobarbital, assumed a completely different conformation when compared with an IgG2a specific for canine lymphoma cells. In both IgGs, the Fc segment is obliquely disposed with respect to the plane of the Fabs, making an angle of 128 degrees in the IgG2a, and 107 degrees in the IgG1. Hinge angles of the IgG1 are notably different at 78 degrees and 123 degrees and unique as well from IgG2a values of 66 degrees and 113 degrees. Elbow angles within the IgG1 Fabs are the same at 155 degrees, but non-identical in IgG2a where they took on values of 143 degrees and 159 degrees. The IgG2a has an angle of 172 degrees between Fabs so that it exhibits a "distorted T" shape, whereas the angle in the IgG1 is a much more acute 115 degrees producing a "distorted Y" [276]. The reported conformation difference of these two proteins provides us more information to study their functions in NPC management.

Apolipoprotein B-100 is a glycoprotein consisted by 4536 amino acid residues with a 550kDa molecular mass. Its structure was studied and modeled by employing small angle neutron scattering with advanced shape reconstruction algorithms [277]. It has been predicted that apolipoprotein B-100 is composed of distinct domains connected by flexible regions. The protein adopts a curved shape with a central cavity. When apolipoprotein B-100 is dissociated with lipid, its structure may expand and its secondary structure is widely preserved. Apolipoprotein A-I is a much smaller hydrophobic protein with two 11 and eight 22 residue tandem repeats. This protein consists of exclusively amphipathic α -helices and its conformation undergoes changes when combined with other molecules. The relationship between its structure and function has been studied by focusing on a central α -helix pair with a particular charge distribution. It has been

proposed that, during cholesterol desorption, the active domain would be an intermolecular α -helix bundle constituted by two central helix pairs [278].

Both inter-alpha trypsin inhibitor H4 and H2 belong to the human blood plasma protein family. They share the similar domains: VWFA, VWFC and VWFD named after the von Willebrand factor (vWF). The VWFA domain has a 170-200 residues and the 3D structure is VWFA domain 1 was exposed (PDB: 1AUQ; 208 aa: Asp 498-Thr 705) by X-ray diffraction. This domain is a central β -sheet surrounded by α -helices and its VWFA domain 1 is the key binding site for glycoprotein Ib (GPIb). The VWFA domains also bind to collagen and heparin. Inter-alpha trypsin inhibitor H1-H4 all contain VWFA domain. VWFC domain is associated with large protein complex including the oligomerisation of vWF. The VWFD domain is around 350 residues. It has been reported that inter-alpha trypsin H2 are bound through glycosaminoglycan bridges to bikunin (which contains two Kunitz-type protease inhibitor domains), but H4 lacks the signal sequence for bikunin assembling. This different might provide us new clues that why this two inter-alpha trypsin inhibitor H4 and H2 perform oppositely in NPC patients' protein profile.

In conclusion, a number of serum proteins with changes in their levels have been identified by iTRAQ-coupled LC-MS/MS analysis in NPC patients selected in our study. Further validation would be needed to assess their potential as biomarkers for better NPC prognosis and management.

Chapter 3 : Proteome profiling of the Epstein-Barr virus infected nasopharyngeal carcinoma cell line: a potential biomarker discovery by comparative iTRAQ-coupled 2D LC/MS-MS analysis

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Summary

Epstein-Barr virus (EBV) has been shown to highly involve in the development of nasopharyngeal carcinoma (NPC), a squamous-cell carcinoma with high-occurrence in Southeast Asia and southern China. However, the underlying relationship of EBV and NPC squamous cell remains obscure. In this study, we employ a comparative iTRAQ-coupled 2D LC-MS/MS system to analyze the protein profile of NPC cell line upon EBV infection. Based on the proteome data and western blot validation, 12 proteins were significantly up-regulated and associated with signal transduction, cytoskeleton formation, metabolic pathways and DNA bindings. The interactions among NPC and EBV proteins were further analyzed and protein networks were established. Based on the functions of differentially expressed proteins, a metabolic pathway was proposed to elucidate their relationship in cytoskeleton formation, cell proliferation and apoptosis. Our results indicate a new proteome platform to analyze EBV's role in NPC squamous cell line. And these differentially expressed proteins may be studied as potential biomarkers for NPC diagnostics and treatments.

3.1 Introductions

Nasopharyngeal carcinoma (NPC) is a squamous-cell carcinoma with high-occurrence in Southeast Asia and southern China. Male patients are inclined to have higher rate of malignancy than that of female and man's rate of occurrence is between 15 and 50 per 100000 [279]. The tumor is regularly observed at the pharyngeal recess posteromedial facing the eustachian tube opening in the nasopharynx. Because of its vague symptoms in early stage and difficulty of physical examination, the patients are often diagnosed in late stages. The etiology of NPC includes genetic analysis, environmental factors and virological infections. Genetic analysis revealed that malfunction of chromosomes 3 and 9 would inactivate tumor suppressor genes such as *p14*, *p15* and *p16* [280-283]. Mutations of genes in p53 pathways led to metastasis of NPC [180, 284]. Environmental factor is the most unnoticed crucial element for causing NPC. People with nasal infections, poor hygiene, and poor ventilation of the nasopharynx and consumption of salt-preserved foods containing high level N-nitroso compound are more likely to suffer from NPC [285]. In perspective of virus infection, genes from Epstein-Barr virus (EBV) were commonly detected in most NPC cells [286].

EBV, a member of the herpesvirus family, is one of the most common viruses. More than 90% of the world's populations carry EBV with either a life-long silence infection or involving in certain diseases such as Burkitt's lymphoma, Hodgkin's disease and Nasopharyngeal carcinoma. Viral DNA has been consistently detected in circulating B cells and malignant epithelial cells [287]. By studying the B-cell infection by EBV, possible routes have been proposed, showing important

roles of EBV during its infection into B-cells [288]. However, the mechanisms of EBV infection into squamous epithelial cell remain largely unexplored.

In order to manage NPC diagnostics and treatment, key indicators were investigated on both molecular and cellular levels known as biomarker discoveries. Virus genes could be used as molecular markers. EBV DNA was found in the plasma of 96% of NPC patients by real-time PCR and advanced-stage NPC patients contain higher percentage of EBV DNA than early stage patients [289]. And this virus gene has been proven to monitor cells' response towards stimulus such as radiotherapy and chemotherapy [290]. On the level of cellular proteins, "Proteomics" was employed as an efficient strategy for discovering potential biomarkers. There have been 10 types of biomarkers discovered in NPC patients by MALDI-TOF-MS, ESI-Q-TOF-MS and 2-DE/MS analysis [291-293]. Most of proteomics study of NPC focus on serum protein and B-cell infection. However, the comparative protein profile of NPC squamous cells infected with EBV is still unclear.

In this study, we employ an iTRAQ-coupled 2D LC-MS/MS analysis to detect the comparative protein profile of NPC squamous cell line during EBV infection. Among 250 proteins detected, a list of proteins altered their expression after infection. 12 proteins was found to be up-regulated and involved in cytoskeleton formation, cell proliferation and apoptosis. Followed by Western blot validation, possible pathways were proposed according to their interactions during EBV infection. These up-regulated proteins could possibly serve as potential biomarkers to study the NPC-EBV interactions.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Urea, CHAPS, sodium dodecyl sulfate polyacrylamide (SDS), acetone, Tris, acetonitrile ($\geq 99\%$) and TEMED were all purchased from Sigma-Aldrich, Singapore. Acrylamide, Rainbow Markers RPN755/756, Ammonium Persulfate (AP) were purchased from Bio-Rad Laboratories, Singapore. ITRAQ^{TM} Reagent Multi-Plex Kit was from Applied Biosystems, Singapore. Phosphate buffered saline (PBS) and trypsin were from Invitrogen, Singapore. SuperSignal West Pico Stable Peroxide Solution and SuperSignal West Pico Luminal/Enhancer Solution were from Research Instrument. KD-90 X-ray Developer and KF X-ray Fixer were purchased from Konica, Japan.

3.2.2 Cells and viruses

The human nasopharyngeal carcinoma squamous epithelial cell line (ATCC: HTB-43) and The normal epithelial cell line (ATCC: CRL-2007) were cultured at 37°C with controlled 5% CO₂ in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum and 1% anti-mycotic. The virus (ATCC: VR-1492) used was the human Epstein-Barr virus shed in the supernate of persistently infected and transformed B95-8 cells (ATCC: CRL-1612).

3.2.3 In vitro EBV infections and immunofluorescence assay

Prior to in vitro infection with EBV, the cells were grown to confluency at 37°C in a 6-well plate and incubated at 4°C for 24 hours, which increased the efficiency of infection [294]. The virus was then recovered from liquid nitrogen stock and diluted into 9-fold volume of culture medium. Both NPC cell line and normal epithelial cell line were treated with trypsin and seeded with EBV solution. Mock NPC cells and normal epithelial cells treated with non-EBV solution served as

controls. The mixture of cells and virus were then incubated at room temperature for 1 h, centrifuged at 2000rpm for 2min and resuspended into culture medium. After 2 days of incubation at 37°C, the infected cells were ready for immunofluorescence assay.

In order to confirm the infection, immunofluorescence (IF) assay was employed as described by Reedman and Klein [295]. We used 3 different primary antibodies against EBV early antigen (EBV Ea-D: sc-58121, Santa Cruz), virus capsid antigen (VCA: sc-80774, Santa Cruz) and EBV nuclear antigen (EBNA-1: sc-57719, Santa Cruz) respectively followed by secondary antibody: Alex Fluor 488 goat anti-mouse IgG (A11001 Invitrogen, Singapore). The IF signals were examined by FITC excitation of an Olympus 1X71 microscopy. Using the same dilution ratio of antibodies, we chose the antibody that provided the strongest signal as our target antibody to test the infection results.

3.2.4 Protein isolation, digestion and labeled with iTRAQ reagents

3 batches of infected cells and controls were collected. Batch I was collected when cells grew into confluency after virus infection verified. Batch II was collected 48h after regrouping batch I confluent cells. Batch III was collected 48h after regrouping batch II confluent cells. Three batches of confluent cells were treated by trypsin and lysed with 300µl cell lysis solution containing 8M urea, 4% (w/v) CHAPS and 0.05% SDS (w/v) on ice with regular vortex for 20min. Then the mixture was centrifuged at 15,000×g for 1 hour at 4°C and the supernatant was removed. Protein was quantified by the 2-D Quant Kit (GE healthcare) and BSA was used as a control for standard curve plotting [296]. For each sample, around 100µg protein was

precipitated with 4 volume of cold acetone at -20°C for 2h. After that, as described in the iTRAQ protocol (Applied Biosystems), proteins were dissolved, denatured and cysteines blocked. Then samples were digested with 20µl 0.25µg/µl sequence grade modified trypsin (Promega) solution at 37°C for 12h. The virus-free NPC sample was labeled with iTRAQ tag 114 and the infected NPC sample was labeled with tag 115 respectively. The labeled samples were then mixed together prior to on-line 2D LC-MS/MS analysis. Three independent experiments were applied on extracted proteins with the same labeling sequence: Virus-free NPC: 114; Infected NPC: 115.

3.2.5 On-line 2D LC-MS/MS analysis

The analysis was performed on combination of an Agilent 1200 nanoflow LC system (Agilent Technologies) and a 6530 Q-TOF mass spectrometer (Agilent Technologies). In the first dimension 3 µl of the combined peptide mixture was loaded onto the PolySulfoethyl A SCX column (0.3 × 50 mm, 5 µm) and was eluted stepwise by injecting salt plugs of 10 different molar concentrations of 10, 20, 30, 40, 50, 60, 80, 100, 300, and 500 mM KCl solution [255]. The sequentially eluted peptides from the SCX column were trapped onto the enrichment HPLC chip and further eluted with buffer A (0.1% formic acid) and buffer B (a nanoflow gradient of 5–80% acetonitrile plus 0.1% formic acid) at a flow rate of 300 nl/min. For MS/MS analysis, survey scans were acquired from m/z 300 to 1500 with up to two precursors selected for MS/MS from m/z 100 to 2000 using dynamic exclusion, and the rolling collision energy was used to facilitate promoting fragmentation.

3.2.6 Data analysis and interpretations

We employed Spectrum Mill MS Proteomics Workbench (Agilent Technologies, Software Revision A.03.03.084 SR4) to identify proteins and to quantify their relative abundance. Each MS/MS spectrum was searched for species of Homo sapiens in the UniProt_sprot_20100123 database. The searches were run using the following parameters: fixed modification of methylmethanethiosulfate-labeled cysteine, fixed iTRAQ modification of free amine in the amino terminus and lysine. Other parameters such as tryptic cleavage specificity, precursor ion mass accuracy, and fragment ion mass accuracy were built as functions of Spectrum Mill software. The protein profile results were filtered with a protein score greater than 11 and peptides score of at least 6, which gives a confidence value of more than 99%. Relative quantification of proteins in the case of iTRAQ was performed on the MS/MS scans and displayed as the ratio of the areas under the peaks at 114, 115Da, which were the masses of the tags that correspond to the iTRAQ reagents. The relative amount of a peptide in each sample was calculated by the ratio of the peak areas observed at 115.1 m/z over that of 114.1 m/z. A raw MS/MS data was generated including a representative peptide information and intensity of 114. 1 m/z and 115.1 m/z shown in two panels. Sequence coverage was calculated as a result of the number of amino acids observed divided by the protein amino acid length. Standard deviation (std) was calculated by analyzing protein ratios between the infected NPC cells and non-infected cells rather than peptide ratios. It is a statistic that has been created for reporting errors in mean ratios and expressing the interval for an estimated ratio (the estimated ratio is in the range of (mean ratio - std) to (mean ratio + std)). The following criteria were required to consider a protein for further statistical analysis: more than two unique peptides with high confidence (95%) had to be identified, and the-fold differences of integral proteins had to be greater than 1.1.

3.2.7 Western blot analysis

Once cells were grown into confluency, they were treated with trypsin and lysed by 300µl cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150mM NaCl and 1% Triton-100) , vortexed and sonicated until the solutions became transparent. Protein inhibitor was added to prevent protein degradation. After being boiled at 98°C for 2min, protein samples were separated by running 12% SDS-PAGE. Proteins were transferred onto nitrocellulose membrane (GE Hybond, USA) detected by specific antibodies respectively with appropriate dilution ranging from 1:200 to 1:10000 according to the original concentrations. Enhanced Chemiluminescence (ECL) was employed by SuperSignal West Pico Chemiluminescent reagents and blots were exposed onto developed X-ray films, which was scanned by Bio-Rad's GS-800 calibrated densitometer and analyzed by Bio-Rad's QualityOne software. The specific antibodies used for detection were as follows: (1) Primary antibody mouse anti-β-actin (AC-74, Sigma) in 1: 10,000 dilution; (2) Primary antibody mouse anti-VDAC1 (sc-58649, Santa Cruz) in 1:200 dilution; (3) Primary antibody mouse anti-S-100A2 (sc-58844, Santa Cruz) in 1:200 dilution; (4) Primary antibody goat anti-Hip (sc-6158, Santa Cruz) in 1:200 dilution; (5) Secondary antibody, HRP-conjugated anti-mouse IgG (#31430, Pierce) in 1:5000 dilution; (6) Secondary antibody, Donkey anti-goat IgG-HRP (sc-2020, Santa Cruz) in 1:5000 dilution.

3.2.8 Protein network analysis

In order to elucidate the protein-protein interactions among host and virus proteins, a proteome-wide virus-host interaction network platform was employed to analyze the protein

networks in EBV infected NPC cells as described by Vincent Navratil, *et al*[297]. A series of databases were included during protein network analysis such as Biomolecular Interaction Network Database (BIND), the Human Protein Reference Database (HPRD) and the Molecular Interaction Database (MINT). Each differentially expressed protein was analyzed and integrated into the network to elucidate the interactions among proteins of infected NPC cells and proteins of virus. Novel proteins which were not included in the VirHostNet platform were identified to enrich the current database and understanding towards NPC-EBV interactions. Interactions were displayed using VirHostNet Visualisator provided by VirHostNet.

3.2.9 Statistical analysis

Data were displayed as mean $\pm$ std from 3 independent experiments. Student's t-test was employed to test the deviation between different batches. Values were only considered to be statistically significant when *p*-values were less than 0.05.

3.3 Results and discussions

3.3.1 Confirmation of EBV infection in NPC cells by IF

After infection for 48h, infected NPC cells, mock-infected epithelial cells and virus-free NPC cells were grown into the same density. They were fixed in 2% formaldehyde solution on glass chambers and blocked by 0.1% BSA serum respectively. 3 primary antibodies against EBV antigens were tested in the first place conjugated with secondary antibody. IF results (Fig. 3-1) showed that, for infected NPC cells, anti-EBV early antigen gave strongest signals compared with other two primary antibodies. And very weak signal (background) of mock-infected epithelial

cells indicated normal epithelial cells cannot be infected by EBV through described protocols.

Anti-EBV early antigen was then employed to confirm the infection of NPC cells (Fig. 3-2). The results showed that infected NPC cells provided stronger signal, indicating the virions had began to replicate after infection for 48h. The negative signals of mock-infected epithelial cells and virus-free normal epithelial cells indicated that infection of EBV on normal epithelial cells were not applicable through previous methods.

Figure 3-1: Antibody binding ability test. We used 3 antibodies (virus capsid protein (VCA), EBV early antigen (Ea-D) and EBV nuclear antigen (EBNA-1)) to test which antibody gives strongest signal. The results showed EBV early antigen (Ea-D) is the best antibody for immunofluorescence assay.

Virus-free NPC

Infected NPC with Ea-D

Virus-free epithelial

Mock-infected epithelial

Figure 3-2: EBV infection Test. We used Anti-EBV-early antigen to test infection. The first two pictures showed that EBV was successfully infected into NPC cells. The last two pictures showed that normal epithelial cells could not be infected by EBV through the described protocol.

3.3.2 Protein profile by iTRAQ-coupled 2D LC-MS/MS analysis

A total of 250 proteins were detected by iTRAQ-coupled 2D LC-MS/MS analysis. Identification and quantification of proteins was performed as previously described. A representative raw MS/MS data of Triosephosphate isomerase was shown in figure 3-3. Under mentioned criteria, 12 proteins were identified as significant changed proteins with confidence more than 99% (Table

3-1). All differentially expressed proteins were up-regulated compared with control, showing a higher expression level of proteins in EBV infected cells (Fig. 3-4). According to proteins' functions in cellular events, they were classified into 4 functions: Signal transduction, cytoskeleton formation, metabolic enzymes and DNA binding. Most of the altered proteins participate in cytoskeleton formation, cell proliferation and apoptosis, which will be described later.

Accession number	Protein name	Sequence coverage (%)	Average Infected/C ontrol mean $\pm$ SD	Peptides	Protein score	Function
P21796	VDAC1	16	4.47 $\pm$ 1.47	4	33.91	Signal transductions
P29034	S100-A2	30	2.01 $\pm$ 0.24	6	53.22	Signal transductions
P50502	Hip-70	7	1.36 $\pm$ 0.09	55	17.13	Signal transductions
P62988	Ubiquitin	82	2.38 $\pm$ 0.47	2	77.6	Signal transductions
Q56UQ5	TPT1-like protein	14	1.40 $\pm$ 0.15	14	13.52	Signal transductions
P08195	4F2hc	8	1.80 $\pm$ 0.49	4	36.17	Signal transductions
O95678	Keratin-75	4	1.72 $\pm$ 0.39	3	37.76	Cytoskeleton formation
A6NNZ2	TB8	4	2.45 $\pm$ 0.27	2	17.35	Cytoskeleton formation
P63167	Dynein light chain 1	40	1.74 $\pm$ 0.49	2	15.1	Cytoskeleton formation
P07195	LDH-B	20	1.59 $\pm$ 0.17	5	51.69	Metabolic enzyme
P60174	TIM	25	1.87 $\pm$ 0.06	4	49.11	Metabolic enzyme
P09429	HMG-1	35	1.33 $\pm$ 0.22	5	51.21	DNA binding

Table 3-1: Differentially expressed protein profile after EBV infection

Abbreviations: VDAC1: voltage-dependent anion-selective channel protein 1; Hip-70: Hsc-70 interacting protein; 4F2hc: 4F2 cell-surface antigen heavy chain; Keratin-75: Keratin, type II cytoskeletal 75; TB8: Tubulin beta-8 chain B; LDH-B: L-lactate dehydrogenase B chain TIM: Triosephosphate isomerase; HMG-1: High mobility group protein B1

Fragmentation Evidence

Fragmentation Evidence

Figure 3-3: Triosephosphate isomerase MS/MS spectrum of a representative peptide. Panel A was a representative peptide's mass spectrum of this biomarker. Each peak represented one residue by detecting its unique m/z ratio. Figure B showed the signal intensity of iTRAQ labeled residues from 114 to 115.

Figure 3-4: The differential expression of proteins. Figure 3-A, B & C shows the differentially expressed proteins of Batch I, II & III respectively. Batch I experiment was the comparison between the infected NPC cells and non-infected cells (control) collected when cells were confluent after the verified infection. 48h after regrouping batch I confluent cells, batch II cells were collected. Batch III cells were collected 48h after regrouping batch II confluent cells. The two columns of each group represent proteins from EBV infected NPC cells (right) and virus-free NPC cells as controls (left) respectively. The protein concentrations of controls were normalized to 1. The fold changes of the proteins between the infected cells and controls were shown. In the figure 3-D, mean values of 3 batches were shown and each arrow bar represents the standard deviation of the detected protein.

3.3.3 Validation of altered protein expression by Western blot

In order to validate the differentially expressed protein identified by iTRAQ labeled LC-MS/MS system, protein VDAC1, S100-A2 and Hip-70 were selected for Western blot analysis (Fig. 3-5). Equal amounts of cell lysates from EBV infected NPC cells and virus-free NPC cells were tested with antibodies to VDAC1, S100-A2 and Hip-70 respectively. The results indicated that VDAC1, S100-A2 and Hip-70 were all up-regulated in EBV infected cells by different extend. These data validated the iTRAQ-coupled 2D LC-MS/MS analysis and further confirmed the up-regulation of proteins in EBV infected cells.

Western Blot for NPC/EBV Proteomics

Figure 3-5: Validation of selected proteins expression by Western blot. NPC/EBV represented the EBV infected NPC cells and the virus-free NPC cells were used as controls. β -actin was the internal control with a size of 42.0kDa.

3.3.4 NPC-EBV protein network analysis

After employing the VirHostNet platform onto differentially expressed proteins, 7 proteins were identified in the database including VDAC1, S100-A2, Hip-70, Dynein light chain 1, HMG-1, LDH-B and TIM, while 5 proteins remain unclassified for protein interactions. 278 proteins (including differentially expressed proteins in virus infected NPC cells) from *Homo sapiens* were identified to have directed interactions with selected proteins and 8 (also include altered proteins) proteins were found to involve in host-virus interactions (data not shown). Protein EBNA-LP and EBNA-1 were nuclear proteins found in EBV, which showed directed interactions with host protein LDHB and Dynein respectively (Fig. 3-6C). A representative host-host interaction for protein VDAC1 was shown (Fig. 3-6A&B). 73 proteins were directed interacted VDAC1 and 24 of them interact with virus (here means all the viruses included in VirHostNet database) proteins. 21 proteins were interacted with Hip-70. And each potential biomarker showed significant interaction graphs in

terms of proteins numbers and classes in both host and virus. The unidentified potential biomarkers of infected NPC cells indicate that more interacting proteins or other molecules in response to EBV infection remain discovering.

B

C

Figure 3-6: Target protein networks. (A&B) Two representative host-host interaction graphs: VDAC1 (A) and Hip-70 (B) interacting proteins by VirHostNet Visualisator. ST13 is the alternative name of Hip-70. The left applet showed sources of proteins and the right applet displayed the protein interactions. Only direct interacted proteins were displayed. Cellular proteins were shown by blue nodes. Blue

nodes with red edges represent host proteins directed interacted with virus proteins.

(C) Host-virus interactions of target proteins. Host proteins were noticed with blue nodes and virus proteins were shown by red nodes. Proteins interacted with them were displayed by other colors.

3.4 Proposed metabolic pathways during EBV infection

3.4.1 Differentially expressed proteins in p53 signaling pathway

Protein p53 was considered as an important tumor suppressor in preventing cancer. Our results support that VDAC1, HMG1 and ubiquitin were highly interacted with p53 proteins reported by other groups. A minimum 3-fold up-regulation of VDAC1 support its interaction with p53 proteins during metabolic stress reported by Eydelnant IA , *et al* [298]. VDAC1 was also proposed to prevent apoptosis via inhibiting release of cytochrome C mediated by hexokinase I & II (HKI & HKII) during EBV infection [299]. Up-regulation of VDAC1 indicated there may have a similar interaction with Bax & Bak to BCL-2 during EBV infection by studying mechanisms of p53-mediated mitochondrial membrane permeabilization [300]. HMG1 was reported as an activator of p53 during Apoptotic Response to anti-metabolite drugs [301]. An up-regulation of 1.33 fold for HMG1 in NPC cells during EBV infection further proved its role during p53 activation. HMG1 was also investigated for its importance in cytoskeleton formation during studying migration of maturing dendritic cells [302], which was correlated with our data. Protein p53 was degraded by ubiquitination thus an up-regulation of ubiquitin deactivate the p53 signaling pathway [303]. The up-regulated ubiquitin in our results suggested similar role of ubiquitin in NPC cells. In addition, that the inhibition of forkhead transcriptional factor (FoxO1), a trigger protein on apoptosis, was mediated by ubiquitin was confirmed by up-regulation of ubiquitin [304]. In EBV infected NPC cells, up to 1.45-fold changes of Hip-70 indicated its inhibition towards

FoxO1 during preventing apoptosis of infected NPC cells supported by Fang Li *et al* when studying smooth muscle cell proliferation and survival through ubiquitin-mediated degradation of FoxO1 [305].

3.4.2 Differentially expressed proteins in NF- κ B signaling pathway

Proteins NF- κ B was a family of proteins that controls DNA transcription. Incorrect regulation of NF- κ B was lined to harmful stimuli such as virus infections. Yuyeon Jung *et al* reported that dynein light chain LC8 could negatively regulates NF- κ B through the Redox-dependent Interaction with I κ B α [306]. The up-regulation of NF- κ B in EBV infected NPC cells suggested dynein may act similarly in NPC cells. Tubulin was up-regulated for around 2.7 fold supported its correlation with NF- κ B during virus infection, which was previously studied in human breast cancer cells [307]. S100-A2, reported as a putative tumor suppressor, regulates cell migration of squamous cancer cell lines in vitro [308]. Up-regulation of S100-A2 in infected NPC cells further approved its role in cytoskeleton formation. Identification of S100A2 as a target of the Delta Np63 oncogenic pathway indicated that S100A2 was a downstream mediator of DeltaNp63 [306]. Our up-regulated results supported the correlation among S100-A2, DeltaNp63 and p53. It has been reported that removal of translation controlled tumor protein inhibited proliferation in colon adenocarcinoma cells [309]. The up-regulated protein level of TPT-1 like protein indicated it could possibly also increase the cell proliferation in EBV infected NPC cells. In summary, p53 and NF- κ B signaling pathways were combined together by interconnection of differentially expressed proteins in NPC cells by EBV infection. Accordingly, A possible metabolic pathway was proposed (Fig. 3-7).

Figure 3-7: A proposed NPC metabolic pathway by EBV infection. Proteins involved in p53 and NF-κB signaling pathways were interconnected. Up-regulated proteins were shown as red and bold. Meanings of lines and arrows were explained within the figure.

3.5 Conclusions

In summary, 12 proteins were differentially expressed in NPC cells influenced by EBV infections and most of proteins were newly discovered to link with EBV infections. Our results suggested that iTRAQ-coupled 2D LC-MS/MS analysis was an effective method to analyzed NPC cells by virus infection. Protein network and pathway analysis revealed that there were deeper interactions among NPC intracellular proteins and EBV proteins. Some altered proteins such as VDAC1, HMGB1 and ubiquitin were found to be highly involved in p53 signaling pathway. Dynein, tubulin and S100-A2 was interacted in NF-κB pathways. Influenced by EBV infection, all of the

differentially expressed proteins were found to be involved in cytoskeleton formation, cell proliferation and apoptosis and a possible pathway was proposed based on their interactions. Our integrated results may provide valuable information for managing NPC and EBV infection. And these differentially expressed proteins could possibly be targeted as potential biomarkers for NPC diagnostics and treatments.

Chapter 4 : EBV decreases the release of cytoplasmic Ca²⁺ and increases cytochrome C in the NPC cell line through VDAC1 regulations

(This part was accepted by Cell Biology International 2012)

Summary

Epstein-Barr virus (EBV) was considered as a major factor that causes nasopharyngeal carcinoma (NPC). Apoptosis and pro-apoptotic signals have been studied for decades. However, few researchers have extended the prevailing view of EBV to its impact on NPC in perspective of apoptosis. One of the important proteins named voltage dependent anion-selective protein 1 (VDAC1) on the mitochondrial outer membrane controls the pro-apoptotic signals in mammalian cells. The impact of EBV infection towards VDAC1 and related apoptotic signals remains unclear. In order to study the VDAC1's role in EBV infected NPC cells, we employ siRNA inhibition to analyze the release of Ca²⁺ and Cytochrome C signals, as they are important pro-apoptotic signals in the cytoplasm. The results show decrease of Ca²⁺ release and up-regulation of Cytochrome C (Cyto C) upon EBV infection. After siRNA transfection, the dysregulation of Cyto C was neutralized, which gives the evidence that the level of Cyto C's release in virus infected NPC cells was as same as that of non-infected NPC cells. This result indicates that EBV infection changes the cytoplasmic level of Cyto C through regulating VDAC1. In summary, this study firstly reports EBV can change the release of Ca²⁺ and Cyto C in the cytoplasm of NPC cells and these changes were mediated by interacting with VDAC1.

4.1 Introduction

Voltage-dependent anion channel proteins, also known as VDAC, are the most important proteins in the outer membrane of the mitochondria, where lots of cellular events take places. VDAC proteins have been proved to be involved in apoptosis as the gatekeeper of mitochondria metabolites. In the programmed cell death, various proteins and molecules are activated and released through mitochondria membranes. Defects of key proteins controlling apoptosis were proved to induce cancers. VDAC proteins have three isoforms in mammalian cells. VDAC1 is the most abundant one compared with the other two isoforms VDAC2 and VDAC3 and VDAC1 was studied most extensively out of the three isoforms [310]. As the controller on the convergence point on the outer membrane of mitochondria, VDAC1 has been studied for decades due to its powerful channel activity. The most common method to study the channel activity is the reconstitution of the VDAC1 pore into a planar lipid bilayer [311]. During the mitochondria-mediated apoptosis, VDAC1 controls many pro-apoptotic signals such as Ca^{2+} , cytochrome C (Cyto C) and reactive oxygen species (ROS)[312-314]. Cyto C is located in the inner membrane of the mitochondria and serves as an electron shuttle in the respiratory chain. Several models were proposed to study the process of releasing Cyto C by VDAC1 [214-215, 217, 315-316] , most of which were from electrochemical perspectives. Ca^{2+} is an important molecule that modulates key enzymes involved in TCA cycle, fatty acid oxidation, amino acid catabolism, the F1 ATPase and ANT [231]. VDAC mediate Ca^{2+} transportation from inner membrane to outer membrane of the mitochondria [217, 317-318]. Several models were proposed to suggest the VDAC- Ca^{2+} binding site [198, 319]. The relationship among VDAC1, Cyto C and Ca^{2+} needs to be

vastly explored due to the complexity of cellular events and mitochondria structure.

Nasopharyngeal carcinoma (NPC) is one of the most common types of cancers frequently occurred in Southeast Asia and southern China [179, 245-246]. The tumor cells often arise from the epithelium of the nasopharynx, where vague symptoms are hard to detect due to the difficulty of physical examination in early stages. NPC is caused by mainly three factors: Epstein-Barr virus (EBV) chronic infection, environmental stimuli and host gene defects. Because EBV gene was found to exist in 96% of NPC patients, this virus has been taken attention due to its significant correlation with NPC [292]. Many researchers studied the impact of EBV infection on B-cells on the level of immunochemistry [291]. Some studies were done on the relationship of NPC with EBV infection, where several potential biomarkers were discovered for prognosis and possible pathways were proposed [40-41, 47]. However, the mechanism of NPC occurrence in response to EBV infection remains unclear. Our previous study has shown that VDAC1 protein was significantly up-regulated in EBV infected NPC cell lines iTRAQ-labeled 2D LC-MS/MS analysis [320]. And clinical samples from NPC patients were also found to have up-regulation of VDAC1 in the serum [321]. These evidences triggered our interest to further investigate VDAC1's role in NPC cells during EBV infection.

In this study, we employ siRNA to inhibit VDAC1 expression for both EBV infected and non-infected NPC cell lines. Then we detected the changes of Ca^{2+} and Cyto C in the cytoplasm before and after VDAC1 inhibition. Before siRNA transfection, comparing the EBV infected and non-infected NPC cells, Ca^{2+} was found to be down-regulated in infected cells and Cyto C was

up-regulated. After siRNA transfection, Cyto C concentration was back to the same level in both cell lines. These finding suggests that EBV infection has a significant impact on pro-apoptotic signals Cyto C and Ca^{2+} . Giving evidence of changes of Cyto C in response to siRNA inhibition, we conclude that Cyto C is up-regulated in NPC cells in response to EBV's infection through VDAC1 mediation.

4.2 Material and methods

4.2.1 Cell culture and virus infection in vitro

The cell lines we used were human nasopharyngeal carcinoma squamous epithelial cell line (ATCC: HTB-43) and the normal epithelial cell line (ATCC: CRL-2007). Both cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum and 1% anti-mycotic at 37°C with controlled 5% CO_2 . The virus (ATCC: VR-1492) used was the human Epstein-Bar virus shed in the supernate of persistently infected and transformed B95-8 cells (ATCC: CRL-1612). Virus infection was employed as previous study described [320]. Cells were stored in liquid nitrogen at -80°C and recovered with heat shock at 37°C prior to further harvest.

4.2.2 Blocking of VDAC1 by siRNA transfection

Two sequences of siRNA to inhibit VDAC1 expression were ordered to perform the gene transfection (Mirus, Singapore). The TransIT-TKO® Transfection Reagent was used (Mirus, Singapore). Cells were cultured into a 24-well plate in 0.5ml complete growth medium/well until they reached the concentration of 3×10^5 per well before collection. siRNA complex was prepared immediately before transfection. Prior to transfection, medium volume was adjusted to 0.25ml of

the original medium. Then siRNA complex were added drop-wise to target wells and gently mixed (25nm final concentration per well). Transfected cells and controls were cultured for 48h before real-time RT-PCR validation.

4.2.3 RNA extraction and real-time RT-PCR

The sequence of the VDAC1 gene was obtained from NCBI nucleotide database (GI: 14250131; GB: BC008482.1). Primers were carefully designed to produce 150-200 base pair PCR products in order to reduce the non-specific binding of SYBR Green. Primers information was provided in table 4-1.

Protein	Primer Sequences	Primer ID	(1 st BASE)
Name			
VDAC1	Sense: 5'-ACTCACCTGAATGGGACTTT-3'	935171-1	
(human)	Antisense: 5'-ATCACATCCACCTTCTCCAC-3'	935172-1	
β -actin	Sense: 5'-CTTAGTTGCGTTACACCCTTTC-3'	182622-1	
(human)	Antisense: 5'-ACCTTCACCGTTCCAGTTTT-3'	182623-1	

Table 4-1: Primer sequences for real-time RT-PCR. Primers were used for real-time RT-PCR using SYBR Green. The amount of VDAC1 mRNA was calculated relative to the expression of β -actin. The primers were synthesized from 1st BASE, Singapore provided with product ID.

Total RNAs were isolated from both of the transfected cells and non-transfected cells by using the RNeasy Mini kit (QIAGEN, USA) according to the manufacturer's protocol. The concentration and the quality of RNA were acquired by detecting OD₂₆₀ and the absorbance ratio of OD₂₆₀ and OD₂₈₀.

Real-time RT-PCR was employed to quantify the amount of VDAC1 mRNAs in the RNAi transfected cells and controls using iScript One-step RT-PCR kit (Bio-Rad, USA). An IQ5 multicolor real-time PCR detection system was used to analyze the mRNA amount after incubation under the following thermal cycles: The first strand cDNA reverse transcription: 50°C 10min; Reverse transcriptase inactivation: 95°C 5min; 40 cycles of PCR and detection: 95°C 10s and 60°C 30s; The dissociation analysis was carried out by detecting fluorescence signals for 1°C increase each cycle from 55 to 95°C.

Amplification analysis, experimental report melting curve analysis and threshold cycle number were obtained by IQ5 optical system software 2.0 with the formatted Microsoft Excel data (Bio-Rad, USA). The fold changes were calculated as follows: Sample Δ Ct = Ct_{sample} - Ct _{β -actin}; $\Delta \Delta$ Ct = Sample Δ Ct - Control Δ Ct; The fold changes of sample/control = $2^{-\Delta \Delta \text{Ct}}$. Three independent experiments were performed with duplicate calculations.

4.2.4 Cytoplasmic Ca²⁺ Assay

Calcium level in the cytoplasm of the cell was detected as one of the important pro-apoptotic signals by using Fluo-4 NW Calcium Assay Kit (Molecular Probes, Singapore). NPC cells and virus

infected cells (Vir) were harvested in a 96-well plate. 50000 cells were grown in each well before calcium assay. Three independent batches of experiment were carried out with around 50000 cells per well in 96-well plate to make sure the comparison on the same level. Reagents were prepared within 6 hours before the test. Medium was removed from the well in order to eliminate sources of baseline fluorescence, particularly esterase activity. After quickly and carefully adding the 100µl loading solution into each well, the plate was incubated at 37°C for 30min then an additional 30min at room temperature. After that, cells were ready for the assay. Relative fluorescence was measured with excitation at 494nm and emission at 516nm.

4.2.5 Cytochrome C ELISA Test

The amount changes of Cytochrome C was detected in NPC cells, virus infected NPC cells (Vir), VDAC1 siRNA transfected cells (NPC/T) and siRNA transfected NPC cells infected with EBV (Vir/T) using enzyme linked immunosorbent assay (ELISA) according the protocol (Invitrogen, Singapore). Cells cultured in a 24-well plate were grown to 3×10^5 per well. Then cells were treated with trypsin and centrifuged at 2000 rpm for 2min at room temperature. Then cells were lysed in the lysis solution on ice for 30min with vortexing at 10min intervals. After that, cells were centrifuged at 13000rpm for 10min at 4°C to get the required lysate.

Cytochrome C standard was serially diluted from 5ng/ml concentration and labeled as 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.0078ng/ml Cyto C. Standard curve was obtained as figure 3. Samples prepared for ELISA were diluted 1:8 in the standard diluents buffer. 100µl of standards, controls and samples were incubated for 2 hours at room temperature with originally seeded primary

antibody (Anti-Cytochrome C). After aspirating and washing 4 times, 100µl Biotin Conjugate was added into each well and incubated for 1 hour at room temperature. After aspirating and washing 4 times, samples were incubated with 100µl Streptavidin-HRP Working Solution for 30min. Then wells were through another 4 times of aspirating and washing before incubated with 100µl stabilized chromogen for 30min per well. Finally, each well was added 100µl stop solution and read at 450nm. Three batches of experiment were performed independently and Cytochrome C concentrations were calculated. Optical density of each sample was based on the lysates of the same total cell amount.

4.2.6 Statistical Analysis

In our study student's t-test were employed to 3 independent experiments in each section. Data was displayed as mean $\pm$ std as calculated from different batches. Values were only considered as significant when *p*-values were less than 0.05.

4.3 Results

4.3.1 siRNA Transfection Validated by Real-time RT-PCR

After VDAC1 expression was blocked using siRNA transfection. We employed real-time RT-PCR to detect the mRNA abundance in response to siRNA transfection. Using NPC cells as the control, the fold changes of VDAC1 mRNA were displayed in figure 4-1. The results showed that around 80% of the VDAC1 mRNA was inhibited via siRNA transfection for both NPC cells and EBV infected NPC cells. Comparing between NPC cells (NPC) and EBV infected cells (Vir) , the amount of VDAC1 mRNA was much higher in virus infected cells, which is consistent with the results of previous

proteomics study [320]. The total RNA was isolated and used as the template for real-time RT-PCR. Three independent experiments were analyzed to have significant changes (p -value<0.05). The results indicate that VDAC1 expression was successfully inhibited via siRNA transfection in NPC cells and EBV infected NPC cells.

Figure 4-1: Real-time RT-PCR validation for VDAC1 siRNA transfection.

The dark blue column (NPC) was VDAC1 mRNA level in NPC cells used as the control. The light blue column (NPC/T) represents VDAC1 mRNA by siRNA inhibition in NPC cells. The dark red column (Vir) represents the VDAC1 mRNA in EBV infected NPC cells before siRNA inhibition. The light red (Vir/T) represents the VDAC1 mRNA in EBV infected NPC cells after siRNA inhibition. The arrow bar represents the standard deviation of VDAC1 mRNA during three independent experiments.

4.3.2 EBV infection decrease the Ca^{2+} release into the cytoplasm

As one of the important pro-apoptotic signals, Ca^{2+} abundance was tested in order to study the

changes of Ca^{2+} in NPC cells in response to EBV infection. Ca^{2+} abundance was displayed by relative fluorescence with excitation at 494nm and emission at 516nm. Table 4-2 showed the optical density of the fluorescence for NPC cells, virus infected NPC cells and the control. Three independent batches of experiments were tested. All of the batches showed that Ca^{2+} abundance in the cytoplasm was decreased in response to EBV infection (Figure 4-2A and Figure 4-2B). This result indicates that EBV can affects NPC cells in perspective of down-regulating the Ca^{2+} release in the cytoplasm.

OD	Batch 1	Batch 2	Batch 3	Ave	Act Ca^{2+}	Std
NPC	2808	2951	2861	2873	1288	72
Vir	2535	2533	2654	2574	989	69
Control	1622	1535	1597	1585		

Table 4-2: Optical density of the Ca^{2+} fluorescence in NPC and EBV infected cells

The entries in the centre of the table represent optical density (OD) of Ca^{2+} fluorescence in NPC cell line (NPC) and EBV infected NPC cell line (Vir). The control used was the complete medium without cells. The actual Ca^{2+} OD was calculated as the result of sample OD minus control OD. Std represents the standard deviation of three independent batches' results.

Figure 4-2A: Ca²⁺ assay in response to EBV infection.

Three independent batches were tested for their Ca²⁺ abundance. Relative fluorescence was measured with excitation at 494nm emission at 516nm. For each batch, the blue column represents optical density of Ca²⁺ in NPC cells (NPC) on the left. The red column (middle) represents Ca²⁺ optical density in EBV infected cells (Vir). The green column on the right is the background OD as the control.

Figure 4-2B: Ca²⁺ assay in response to EBV infection: Summary.

Mean values of 3 batches were shown. Blue column (left) represents the average OD of Ca²⁺ in NPC

cells while the red column (right) represents the average OD of Ca^{2+} in EBV infected NPC cells. Each arrow bar represents the standard deviation of the detected Ca^{2+} abundance.

4.3.3 EBV up-regulated the Cyto C release in the cytoplasm through VDAC1 by ELISA

First, Cyto C standard curve was drawn by measuring the optical density of absorption at 450nm with the Cyto C standard diluted from 5ng/ml to 0.0078ng/ml (Figure 4-3). Three independent batches showed similar trends of Cyto C changes in response to virus infection and VDAC1 inhibition (Table 4-3 and figure 4-4). Before VDAC1 blocking, by comparing the Cyto C concentration between the NPC cells and EBV infected cells, cells with virus had higher concentration of Cyto C, which means EBV infection increase the level of Cyto C in the cytoplasm. After VDAC1 blocking, Cyto C was down-regulated to the same level in both NPC cells and virus infected cells, which indicates the direct control of Cyto C release by VDAC1.

OD	Batch 1	Batch 2	Batch 3	Ave	Std	Cyto C (ng/ml)
NPC	1.541	1.502	1.408	1.484	0.068	17.536
Vir	1.717	1.603	1.662	1.661	0.057	19.752
NPC/T	0.822	0.967	0.989	0.926	0.091	10.584
Vir/T	1.032	0.928	0.935	0.965	0.058	11.072

Table 4-3: Cyto C ELISA assay

The central entries are optical density (OD) of Cyto C in ELISA. The NPC represents non-infected NPC cell line. Vir represents EBV infected NPC cell line. NPC/T represents NPC cell line whose VDAC1 expression was inhibited by siRNA. Vir/T represents EBV infected NPC cell line inhibited by VDAC1

siRNA. Std was calculated as a result of analyzing three independent batches. The real Cyto C concentration (the last column) was calculated from the standard curve considering sample dilution.

Figure 4-3: Standard curve of cytochrome C.

Serial dilution of Cyto C was done as 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.0078ng/ml. Absorption was measured at 450nm.

Figure 4-4: Cyto C detection by ELISA.

Figure 4-4 (A, B & C) were three batches' results of independent experiments. In each batch, Cyto C level was displayed by its optical density of absorption at 450nm. The cell names were abbreviated as follows: NPC - nasopharyngeal carcinoma (NPC) epithelial cell line without virus; Vir – NPC cell line infected with EBV; NPC/T: NPC cell line by VDAC1 siRNA inhibition; Vir/T: EBV infected NPC cell line by VDAC1 siRNA inhibition. Figure 4 (D) was the summary of three independent batches. Each arrow bar represents the standard deviation of three independent batches. Cyto C level was measured as optical density of absorption at 450nm. The *p*-value of Cyto C's concentraion in each type of cell is as follows: NPC: 0.0033; Vir: 0.0012; NPC/T: 0.1467; Vir/T: 0.2032.

4.4 Discussion

VDAC1's important role has been recognized in mammalian cells due to its significance in regulating the metabolism across the mitochondrial membrane. For nasopharyngeal carcinoma, several groups including us proved that VDAC1 was up-regulated in cancer cells in response to EBV infection. However, few researches were done to study the relationship of VDAC1's functions and EBV, though some groups revealed VDAC1's relationship with HIV, Influenza A virus and Hepatitis B virus [237, 241-242, 322-323]. Upon observing the Ca^{2+} and Cyto C changes in EBV infected NPC cells, the new findings triggered our interest to study whether the change of pro-apoptotic signals during EBV infection were related with the changes of VDAC1. However, due to the complexity of cellular events, details concerning the mitochondrial involved apoptosis and structure-based studies were not done. Ca^{2+} didn't show significant difference upon siRNA transfection, indicating the cytoplasmic Ca^{2+} concentration was not majorly controlled by VDAC1 (data not shown). The comprehensive pathways proving the relationship among Ca^{2+} , Cyto C, VDAC and EBV remains unclear.

Several other groups have studied the silencing of VDAC1 could efficiently prevent apoptosis by different stimuli [324-325]. It was also reported that overexpression of VDAC1 from different species could induce programmed cell death [207-210, 326]. In our study, apoptosis didn't happen in EBV infected NPC cells, giving evidence that cells were still proliferating as tumor cells. Down-regulation of Ca^{2+} by EBV infection indicated that EBV might prevent apoptosis by decreasing Ca^{2+} release into the cytoplasm. Although more Cyto C release were found to be related with overexpression of VDAC1, those weren't sufficient to trigger the apoptosis in NPC cell line without specific stimuli.

Upon siRNA inhibition, down-regulation of Ca^{2+} and Cyto C in both EBV infected cells and non-infected NPC cells further indicated the signals' interactions with VDAC1, which was consistent with other groups' studies in other cells lines [327]. This finding gives rise to the potential application of pro-apoptotic signals Ca^{2+} and Cyto C in the prognosis of NPC. Furthermore, the findings that Cyto C release was up-regulated in response to EBV infection was mediated by VDAC1 indicates the significance of VDAC1 during EBV infection. These finding provides us new information when managing the nasopharyngeal carcinoma and EBV infection.

4.5 Conclusions

Our results here reveal VDAC1's role in regulating Ca^{2+} and Cytochrome C release in response to EBV infection in the NPC cell line. During Cytoplasmic Ca^{2+} assay, Ca^{2+} was found to be down-regulated in EBV infected cells. In EBV infected NPC cells, Cyto C released into the cytoplasm was found to be up-regulated before siRNA inhibition. After siRNA transfection, the

increase was neutralized and the concentration of Cyto C w in virus infected NPC cells returned to the same level as those found in the non-infected NPC cells. The changes of Cyto C concentration in the cytoplasm in response to EBV infection by siRNA inhibition indicates that, upon EBV infection, Cyto C release was up-regulated in NPC cells mediated by VDAC1. Our study firstly report the pro-apoptotic signal Ca^{2+} and Cyto C's release was changed by EBV infection. And the changes were mediated through VDAC1's regulation. These findings extend our view of studying the mechanisms during EBV infection in NPC cells and could contribute as novel clues when improving the diagnosis and treatments for nasopharyngeal carcinoma.

Chapter 5 : Conclusions and future works

5.1 Conclusions

During detecting the protein profile of sera from NPC patients and NPC cell lines, significant changes on the protein level demonstrate that iTRAQ-coupled 2D LC-MS/MS system is an efficient and powerful approach to investigate nasopharyngeal carcinoma. According to the results of both clinical and cell line samples, we have successfully built the novel protein profile of NPC serum and identified the potential biomarkers. In addition, in order to study the impact of Epstein-Barr virus towards NPC cell, comparative protein profile was constructed using the iTRAQ-coupled 2D LC-MS/MS system. Affiliated with protein functions and net work analysis, a new proteome platform was established to study the protein changes in NPC cell line in response to EBV infection. And based on the potential biomarker candidates and their relationship, a novel pathway was proposed in NPC cell line. The results of proteomics in NPC cell line show a significant up-regulation of Voltage-dependent anion channel protein 1, which functional as an important gatekeeper in the outer membrane of mitochondria and influence the apoptosis by controlling various metabolites. When comparing the level of the pro-apoptotic signals-cytochrome C and Ca^{2+} between the NPC cells and EBV infected NPC cells, we found that both were significantly influenced by EBV infection. According to the changes of VDAC1 in EBV infected NPC cells, we manage to study whether EBV impact the cytochrome C and Ca^{2+} through regulating VDAC1. By siRNA inhibition, the results show that the virus indeed up-regulates the cytochrome C release through increasing VDAC1 expression, while Ca^{2+} is not solely controlled

by VDAC1, since the Ca^{2+} level doesn't change much after inhibiting VDAC1. In conclusion, our study successfully established two novel platforms of proteome profile for nasopharyngeal carcinoma: serum NPC patients versus healthy control and NPC cell lines in response to EBV infection. Based on protein functions, reasonable new pathways were proposed. In addition, one biomarker candidate-VDAC1 was further studied for its role in EBV infected NPC cells. We firstly discovered that EBV impacts NPC cells and changes Ca^{2+} and cytochrome C release. And the cytochrome C release was regulated by VDAC1 in EBV infection. These new findings provide us information to find novel potential biomarkers and understand the mechanism during nasopharyngeal carcinoma diagnosis and treatments. The results also help us to deeply understand the influence of Epstein-Barr virus infection towards NPC, which may improve the management to this type of cancer.

5.1.1 Establishment of a novel protein profile from NPC patients' sera

Facilitated by the efficient iTRAQ-coupled 2D LC-MS/MS system, we have discovered 13 proteins as potential biomarkers, since they showed similar changes with significance in 25 out of 30 patients. Alpha-1-antitrypsin, Ig gamma-1 chain C region, Apolipoprotein B-100, Alpha-1-acid glycoprotein 1, Inter-alpha-trypsin inhibitor heavy chain H4, Antithrombin III, Plasma protease C1 inhibitor and haptoglobin were up-regulated for at least 39% increase in NPC patients, while Ig gamma-2 chain C region, Inter-alpha-trypsin heavy chain H2, alpha-2-macroglobulin, apolipoprotein A-I and histidine-rich glycoprotein were down-regulated for more than 20%. The results show that 10 candidates are signal proteins and 3 are binding proteins. Functional analysis indicates that these changed proteins are related with blood coagulation, amyloid

formation, immunogenesis, lipid transportation and hyaluronan carrying. Interesting phenomena was also discovered. Three pairs of proteins shows opposite performance of regulation while sharing similar structures. This finding may provide new clues in perspective of structure during the study of NPC biomarkers.

5.1.2 Establishment of a novel protein profile in NPC cell lines in response to EBV infections

We compared the protein level of the squamous NPC cell line in response to EBV infection and established the comparative protein profile using iTRAQ-coupled 2D LC-MS/MS system. Validated with western blot, 12 proteins were found to be significantly up-regulated in EBV infected NPC cells compared with non-infected NPC cells. Functional analysis indicates these changed proteins are associated with signal transduction, cytoskeleton formation, metabolic pathways and DNA bindings. A protein network was also built by analyzing the relationship of host and virus proteins. Dysregulated proteins are in the path of p53 signaling pathway including VDAC1, HMGB1 and ubiquitin. Dynein, tubulin and S100-A2 are involved in NF- κ B signaling pathway. Thus a novel pathway was proposed based on their interactions. The newly discovered protein changes in EBV infected NPC cell lines reveals useful information to find potential biomarkers.

5.1.3 EBV decreases the release of cytoplasmic Ca²⁺ and up-regulates cytochrome C in the NPC cell line through VDAC1 regulations

As a result of previous study, VDAC1 was one of the most important protein up-regulated in EBV infected NPC cells. We investigate the impact of EBV towards pro-apoptotic signals including

cytoplasmic Ca^{2+} and cytochrome C release. Upon EBV infection into NPC cells, Ca^{2+} was found to be down-regulated while cytochrome C was up-regulated compared with virus-free NPC cells. After siRNA inhibition of VDAC1, cytochrome C in virus infected cells was back to the same level of virus-free NPC cells. This result indicates that EBV influences the cytochrome C release by interacting with VDAC1. And cytoplasmic Ca^{2+} was not purely controlled by VDAC1 since siRNA inhibition has no significant influence on Ca^{2+} concentration. Combined with proteomics results, we firstly find that EBV changes pro-apoptotic singals cytochrome C and Ca^{2+} release in NPC cells. And EBV infection increases cytochrome C through increasing VDAC1 protein. This finding supports the VDAC1's role in apoptosis and provides us deeper understanding of EBV's impact on nasopharyngeal carcinoma.

5.2 Future works

Based on current study, the future work succeeding the proteomics of NPC and EBV infection will include the further exploration of potential biomarkers and their relationships with EBV, study of individual biomarkers on the important pathways such as the NF- κ B pathway as well as the structure analysis and experiment aiming to explain the opposite performance of proteins sharing similar structures in the clinical study of NPC.

5.2.1 Focus study of the essential proteins as potential biomarkers in response to EBV infection

The model of study VDAC1 in EBV infected NPC cells can also apply to other proteins, especially those key biomarkers on the important pathways. Inhibition of them by siRNA or other reagents with similar knock-down function is very useful to study the proteins role during virus infection.

The first step is to find which downstream molecules or proteins are controlled by certain proteins in the process of cell growth, proliferation, apoptosis or even malignancy. Then we can detect the signal changes upon virus infection and observe whether the signal can be changed. Once finding significant changes of downstream signals, siRNA or other knock-down technologies can be used to block the protein's expression. In such a way we can determine whether the potential biomarker change its signals upon the influence of the virus. Most of the proteins can employ such approach to study the deep relationship among virus, proteins and downstream signals in cancer management.

5.2.2 Mechanism study of changed proteins on the NF- κ B pathway and the p53 pathway in NPC cells in response to drug treatments

Although proteins were identified as potential biomarkers for nasopharyngeal carcinoma, the detail map of each protein's role is unclear. As important signaling pathways controlling cancer cell development, the NF- κ B pathway and the p53 pathway should be paid full attention. We will block the key proteins during these pathways and determine the effects of these blocking. For instance, we will use DHMEQ, a molecular inhibitor, to block the NF- κ B pathway by combining with NF- κ B proteins. The benefits of doing this include a deeper understanding of protein performance in the key pathways as well as specificity assurance test for potential biomarkers. If one protein's changes can reveal the stage of the patient specifically considering drug's effects, that biomarker will be highly likely to be used on clinical trials.

5.2.3 Study of oppositely performing proteins sharing similar structures in the serum proteomics of NPC

It was interestingly noted that 3 pairs of proteins perform in opposite direction while sharing similar structures in the clinical study of NPC. For example, Ig gamma-1 chain C region and Ig gamma-2 chain C region both belong to immunoglobulin with similar heavy chain. But Ig gamma-1 chain C region has higher concentration than control and thus noted as up-regulated biomarker while Ig gamma-2 chain C region was down-regulated. The distinct concentration of these two supposed similar biomarkers raises our interest for investigation on their structure differences. Some structural analysis has been done by other group to purely demonstrate their 3D structures. However, the reason leading to the described phenomena was still unknown. We plan to modify or block the active sites of these 3 pairs of proteins by residue modification and detect the according changes. If pairs of proteins are regulated to the same level after modification, then we can determine what kind of molecules or proteins interact with these sites. Those reagents interacting with them may have chance to become novel drug for curing nasopharyngeal carcinoma.

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Appendix 1: A representative protein profile of NPC serum patients No.7-No.9

Unused	Total	%Cov	%Cov(50)	%Cov(95)	Accession	Name	Species
1	126.94	126.94	90.48	80.95	79.8	sp P02768 Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2	HUMAN
2	62.28	62.28	53.76	39.27	31.45	sp P01024 Complement C3 OS=Homo sapiens GN=C3 PE=1 SV=2	HUMAN
3	62	62	47.29	35.62	33.45	sp P01023 Alpha-2-macroglobulin OS=Homo sapiens GN=A2M PE=1 SV=1	HUMAN
4	42.75	42.75	63.88	54.55	54.55	sp P01009 Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=	HUMAN
5	42.32	42.32	57.16	43.84	39.11	sp P02787 Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=2	HUMAN
6	30.63	30.63	31.19	16.34	15.83	sp P0C0L5 Complement C4-B OS=Homo sapiens GN=C4B PE=1 SV=1	HUMAN
7	30	30	64.85	58.18	58.18	sp P01857 Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=	HUMAN
8	28.13	28.13	54.58	44.6	40.12	sp P02675 Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 SV=2	HUMAN
9	26.54	26.54	32.91	23.79	22.52	sp P02671 Fibrinogen alpha chain OS=Homo sapiens GN=FGA PE=1 SV=2	HUMAN
10	24.96	24.96	71.91	43.45	40.07	sp P02647 Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1	HUMAN
11	21.3	21.3	37.53	29.36	25.61	sp P02679 Fibrinogen gamma chain OS=Homo sapiens GN=FGG PE=1 SV=	HUMAN
12	18.23	18.23	45.81	33	31.03	sp P00738 Haptoglobin OS=Homo sapiens GN=HP PE=1 SV=1	HUMAN
13	17.57	17.57	16.26	3.748	3.156	sp P04114 Apolipoprotein B-100 OS=Homo sapiens GN=APOB PE=1 SV=1	HUMAN
14	13.8	13.8	48.73	34.28	32.29	sp P01876 Ig alpha-1 chain C region OS=Homo sapiens GN=IGHA1 PE=1 SV	HUMAN
15	13.42	13.42	27.23	12.11	12.11	sp P00450 Ceruloplasmin OS=Homo sapiens GN=CP PE=1 SV=1	HUMAN
16	13.29	21.29	46.63	38.34	38.34	sp P01859 Ig gamma-2 chain C region OS=Homo sapiens GN=IGHG2 PE=1 SV=	HUMAN
17	13.22	13.22	48.76	36.82	36.82	sp P02763 Alpha-1-acid glycoprotein 1 OS=Homo sapiens GN=ORM1 PE=1 SV=	HUMAN
18	12.86	12.86	40.04	28.14	16.67	sp P02790 Hemopexin OS=Homo sapiens GN=HPX PE=1 SV=2	HUMAN
19	11.74	11.74	26.99	18.36	18.36	sp P01871 Ig mu chain C region OS=Homo sapiens GN=IGHM PE=1 SV=3	HUMAN
20	11	11	19.68	7.634	7.634	sp Q14624 Inter-alpha-trypsin inhibitor heavy chain H4 OS=Homo sapiens	HUMAN
21	10.08	10.08	34.04	21.99	17.73	sp P01011 Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA3 PE	HUMAN
22	10	10	80.19	80.19	80.19	sp P01834 Ig kappa chain C region OS=Homo sapiens GN=IGK PE=1 SV=1	HUMAN
23	8.54	8.54	39.39	15.56	14.14	sp P04217 Alpha-1B-glycoprotein OS=Homo sapiens GN=A1BG PE=1 SV=3	HUMAN
24	8.09	8.09	65.71	65.71	65.71	sp P01842 Ig lambda chain C regions OS=Homo sapiens GN=IGLC1 PE=1 SV	HUMAN
25	8	8	30.3	14.9	14.9	sp P06727 Apolipoprotein A-IV OS=Homo sapiens GN=APOA4 PE=1 SV=3	HUMAN
26	7.7	7.7	23.2	14	14	sp P05155 Plasma protease C1 inhibitor OS=Homo sapiens GN=SERPING1	HUMAN
27	6.92	6.92	13.74	5.759	4.319	sp P00751 Complement factor B OS=Homo sapiens GN=CFB PE=1 SV=2	HUMAN
28	6.33	6.33	10.02	2.724	1.76	sp P02751 Fibrinectin OS=Homo sapiens GN=FN1 PE=1 SV=3	HUMAN
29	6	6	23.92	4.31	4.31	sp P01008 Anthrombin-III OS=Homo sapiens GN=SERPIN1 PE=1 SV=1	HUMAN
30	5	7	39.3	27.36	22.89	sp P19652 Alpha-1-acid glycoprotein 2 OS=Homo sapiens GN=ORM2 PE=1 SV=	HUMAN
31	5	5	13.92	8.439	6.751	sp P02774 Vitamin D-binding protein OS=Homo sapiens GN=GC PE=1 SV=	HUMAN
32	4.8	4.8	42	42	42	sp P02652 Apolipoprotein A-II OS=Homo sapiens GN=SERPIN1 PE=1 SV=1	HUMAN
33	4.54	4.54	54.1	22.95	22.95	sp P02735 Serum amyloid A protein OS=Homo sapiens GN=SAA1 PE=1 SV=	HUMAN
34	4.29	4.29	19.57	6.056	6.056	sp P01042 Kininogen-1 OS=Homo sapiens GN=KNG1 PE=1 SV=2	HUMAN
35	4.27	4.27	15.93	5.062	5.062	sp P00747 Plasminogen OS=Homo sapiens GN=PLG PE=1 SV=2	HUMAN
36	4.27	4.27	38.1	14.29	14.29	sp P68871 Hemoglobin subunit beta OS=Homo sapiens GN=HBB PE=1 SV=	HUMAN
37	4.14	4.14	56.48	31.48	31.48	sp P01610 Ig kappa chain V-1 region WFA OS=Homo sapiens PE=2 SV=1	HUMAN
38	4.09	4.09	13.58	18.05	18.05	sp P06313 Ig kappa chain V-1 region J1 OS=Homo sapiens PE=2 SV=1	HUMAN
39	4.04	4.04	14.86	6.857	6.857	sp P04196 Hemoglobin subunit alpha OS=Homo sapiens GN=HBA1 PE=1 SV	HUMAN
40	4.02	4.02	61.27	31.69	31.69	sp P69905 Hemoglobin subunit alpha OS=Homo sapiens GN=HBA1 PE=1 SV	HUMAN
41	4	4	19.03	3.7	3.7	sp P19823 Inter-alpha-trypsin inhibitor heavy chain H2 OS=Homo sapiens	HUMAN
42	4	4	56.44	31.68	31.68	sp P02655 Apolipoprotein C-II OS=Homo sapiens GN=APOC2 PE=1 SV=1	HUMAN
43	4	4	48.98	25.17	25.17	sp P02766 Transferrin OS=Homo sapiens GN=TTR PE=1 SV=1	HUMAN
44	3.6	3.6	6.58	2.843	1.543	sp P08603 Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4	HUMAN
45	3.4	3.4	12.55	5.021	5.021	sp P04004 Vitronectin OS=Homo sapiens GN=VTN PE=1 SV=1	HUMAN
46	2.92	21.97	49.07	42.44	37.4	sp P01860 Ig gamma-3 chain C region OS=Homo sapiens GN=IGHG3 PE=1 SV=	HUMAN
48	2.41	2.41	19.42	4.638	4.348	sp P02749 Beta-2-glycoprotein 1 OS=Homo sapiens GN=APOL1 PE=1 SV=3	HUMAN
49	2.35	2.35	27.52	14.17	5.45	sp P02765 Alpha-2-HS-glycoprotein OS=Homo sapiens GN=AHSG PE=1 SV=	HUMAN
50	2.03	2.03	5.466	3.055	3.055	sp P00734 Prothrombin OS=Homo sapiens GN=F2 PE=1 SV=2	HUMAN

Appendix 1: Continue

Peptides(95%)	115:114	PVal 115:114	116:114	PVal 116:114	117:114	PVal 117:114
89	0.149969	4.44E-16	0.258226007	3.60E-12	0.39445731	3.92E-09
30	1.282331	0.179339498	1.127197981	0.787042797	1.599557996	0.002728884
31	0.529663	0.078067273	0.591561615	0.327905297	0.625172675	0.232873499
25	3.191538	0.000184849	1.432188034	0.046969641	1.940886021	0.009275003
24	0.349945	5.82E-06	0.428548515	5.68E-06	0.586138189	0.002269478
14	2.488857	0.001101763	1.584892988	0.002058415	1.158776999	0.194661006
21	1.995262	0.004493729	2.443430901	0.145017505	3.564511061	0.014008
15	0.515229	0.01551142	0.40926069	0.008282223	0.534564376	0.040715721
14	0.879022	0.555822194	0.679203629	0.290880889	0.731139123	0.4734492
14	0.570164	0.01414987	0.465586096	0.01881741	0.765596628	0.195496395
12	0.937562	0.794828773	0.586138189	0.105672702	0.698232412	0.320133299
11	3.280953	0.003256248	1.541700006	0.207863897	1.584892988	0.284341186
9	1.247383	0.439215809	1.958845019	0.01536694	1.106624007	0.649829328
9	1.629296	0.042037182	1.318256974	0.231959194	1.096477985	0.245536804
7	1.342765	0.703085005	0.779830098	0.713152587	1.584892988	0.381944597
13	0.501187	0.409123808	0.155596599	0.1684798	0.401790798	0.295354694
7	5.19996	0.004120419	1.706081986	0.443635404	4.742420197	0.005077472
5	1.037528	0.627756476	0.580764413	0.085682161	0.744732022	0.468378514
6	1.086426	0.690968692	0.704693079	0.615839303	1.599557996	0.174272805
6	1.584893	0.072226003	1.29419601	0.121089503	1.51356101	0.078501754
4	1.870682	0.301037014	1.445440054	0.3654055	1.342764974	0.687651396
8	0.954993	0.927510083	0.515228629	0.856516123	1.258924961	0.286234885
4	1.127198	0.602052271	0.946237087	0.629731119	1	0.94465518
4	1.803018	0.602851629	1.393157005	0.469278991	2.167704105	0.237936601
4	0.887156	0.412412703	1.018591046	0.84416151	0.928966403	0.638865173
4	1.342765	0.494519114	1.28233099	0.252232313	1.342764974	0.358570009
3	1.44544	0.152525693	1.16949904	0.440417796	1.599557996	0.090219267
3	0.642688	0.258203089	1.629295945	0.209020704	1.380383968	0.327076703
3	8.87156	0.036159322	6.426877022	0.038349699	6.729765892	0.04006096
3	2.910717	0.339425713	1.19124198	0.71768719	2.606153011	0.357335508
2	0.801678	0.462008804	0.794328213	0.451559812	0.772680581	0.39980039
3	0.824138	0.496592402	0.586138189	0.298424691	0.779830098	0.541172087
2	2.85759	0.130163506	1.355188966	0.306171298	1.659587026	0.217906103
2	0.83946	0.738875329	0.831763685	0.732564926	0.895364821	0.839456677
2	0.912011	0.613756478	0.895364821	0.694027781	0.972747207	0.943624377
2	0.625173	0.449054211	1.247382998	0.63182658	0.363078088	0.326056987
2	0.83946	0.546139896	0.794328213	0.431304812	0.920449615	0.787012577
2	0.704693	0.523757279	1.485936046	0.392650008	1.127197981	0.813852429
2	0.903649	0.7284078	0.946237087	0.977785528	0.717794299	0.567398012
2	0.855067	0.757519484	1.29419601	0.61362958	0.685488224	0.503883183
2	0.83946	0.814217687	0.928966403	0.993956327	0.862978518	0.819299519
2	1.5417	0.194438204	1.819700956	0.157802299	1.247382998	0.305718094
3	0.239883	0.171351701	0.369828194	0.240101293	0.313328594	0.208031401
1	1.056818	0.907616615	1.037528038	0.951862395	1.028015971	0.918602228
2	0.83946	0.741078019	1.018591046	0.956280887	0.972747207	0.963223875
13	1.419057	0.525258183	1.19124198	0.715800583	1.584892988	0.437912285
1	0.831764	0.736959219	0.474242002	0.310955286	0.619441092	0.444385886
2	0.787046	0.587856472	0.928966403	0.860974908	0.990831971	0.99651581
1	1.018591	0.94974649	0.613762021	0.433300406	0.704693079	0.540715098