行政院國家科學委員會專題研究計畫　成果報告

鼻咽癌的抗藥機轉研究：抗藥基因、EB 病毒基因、化學激素、化學激素受體在腫瘤細胞的表現和相關性(3/3)

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中文摘要

關鍵詞：鼻咽癌、化學治療抗藥性、EB病毒基因、細胞凋亡基因、抗藥基因、Syndecan-1、細胞趨化激素受體

鼻咽癌是國人常見的頭頸部惡性腫瘤之一。對原發腫瘤作放射線是治療鼻咽癌最常用方式，但因多數鼻咽癌病例的臨床分期為第 III 期以上，因此常在放射線治療後有腫瘤復發或遠端轉移，這類病人的預後仍十分不好。對此類病患，化學藥物是治療的另一選擇；有時對末期鼻咽癌病患，合併放射線和化學治療是唯一的治療方法。化學藥物對原發、再發或轉移的鼻咽癌雖然在治療初期極為有效，但卻經常因腫瘤對化學治療藥物產生抗藥性而逐漸失敗。EB 病毒與鼻咽癌的密切相關性是眾所周知，某些 EB 病毒蛋白已知會影響細胞對外來壓力的反應，改變細胞週期進行，並影響細胞凋亡的現象。已知 EB 病毒之 LMP-1 會活化上皮細胞表現 EGFR，過去研究已証實鼻咽癌會表現 EGFR。近年來，對抗 EGFR 的藥物，在實驗室及臨床研究中均証實可以加強化學治療的效果。然而，此一治療策略尚未在鼻咽癌中嘗試。本計畫以原發、再發和轉移鼻咽癌腫瘤組織的切片，配合鼻咽癌細胞株的研究，作分子和免疫組織化學染色研究，希望能對鼻咽癌腫瘤細胞產生抗藥性的各種可能機轉做更深入的探討。

在第一年的研究中，本計畫探討原發、再發和轉移性鼻咽癌腫瘤中與細胞凋亡（apoptosis）相關基因（如 p53、bcl-2、bax、bcl-X、epidermal growth factor receptor [EGFR]）、EB 病毒之潛伏膜蛋白（Latent membrane protein-1，LMP-1）、抗藥基因（如 Multidrug Resistance-1, [MDR-1]、Glutathione-S-transferase-π [GST-π]、thymidylate synthase [TS]）的表現情形。結果顯示 EB 病毒的存在可能與鼻咽癌腫瘤的抗藥性無關，MDR-1 在多數鼻咽癌並不表現，但表現 MDR-1 的原發性鼻咽癌的預後比較差。

在第二年的研究中，本計畫原本預計以雷射細胞摘取技術（Laser Capture Microdissection, LCM）摘取冷凍組織切片中的腫瘤細胞，進行MDR-1，GST-π，TS，p53，bcl-2，bax，bcl-$X/_L$，bcl-$X/_S$，LMP-1，EGFR，HER-2 或其他HER family等基因的表現情形，但因以雷射細胞摘取技術自鼻咽癌石蠟組織切片摘取腫瘤細胞的方法，在技術上遇到困難，使實驗進度受到阻礙，無法順利完成。因此，轉而探討其他與腫瘤生長與轉移相關因子。

EB 病毒 LMP-1 會透過活化上皮生長因子受體的表現，對上皮細胞有很強的致腫瘤生成效果，並有促進腫瘤轉移的作用。Syndecan 為一群細胞表面的分子，可與許多不同的細胞表面作用分子相互反應，如細胞外介質分子和生長因子，經由這些作用而影響細胞的生長、分化與形狀表現。Syndecan-1（CD138）是 Syndecan 家族之一。許多上皮細胞產生惡性轉形時 Syndecan-1 的表現會減低，syndecan-1 表現減低與腫瘤細胞的分化程度有關，也可能影響腫瘤細胞的抗藥性。

本計畫的第二年研究就以探討 syndecan-1 在鼻咽癌腫瘤的表現，並探討 syndecan-1 表現與 EB 病毒基因表現，病人預後的關聯性研究。結果發現 86 例原發性鼻咽癌有 18 例（20.9%）表現 syndecan-1，37 例再發病例有 9 例（24.3%），16 例轉移病例有 7 例（43.8%）。以 Real time 反轉錄　－多　鏈連鎖反應法（reverse-transcriptase polymerase

chain reaction, RT-PCR)法證實 syndecan-1 在鼻咽癌的低表現度是因為 syndecan-1 基因的 mRNA 量下降所致。這些結果與病人臨床表現作統計分析，發現表現 syndecan-1 的原發性鼻咽癌病人的五年存活率較低，具有統計意義。LMP-1 與 syndecan-1 在鼻咽癌常同時表現，顯示 LMP-1 會影響 syndecan-1 的表現，進而影響鼻咽癌病人的預後。

本計畫的第三年研究是探討化學激素受體在鼻咽癌腫瘤的表現情形。首先，我們利用定量 RT-PCR（quantitative reverse-transcriptase polymerase chain reaction, qRT-PCR）篩選 18 種（CCR1-10, CXCR1-6, XCR1 和 XC3CR1）細胞激素受體基因在五株不同鼻咽癌細胞株（TW01, TW04, HONE1, BM1, AS1）的表現，發現 CXCR4、CXCR6、CCR7、CCR9 在鼻咽癌細胞株的表現量明顯增加，以流體細胞定量分析（flow cytometric analysis）證實這四種化學激素受體的蛋白質在鼻咽癌細胞株表現很強。進一步以細胞移動實驗探討這四種化學激素受體蛋白質的功能，發現它們都會使鼻咽癌腫瘤細胞移動加速。最後，我們以免疫組織化學染色法探討 CXCR4、CXCR6、CCR7 在鼻咽癌組織切片的表現。結果發現，大多數原發和再發的鼻咽癌都不表現這三種化學激素受體蛋白質，但是轉移的鼻咽癌腫瘤則大多數會表現這三種化學激素受體蛋白質，表示這三種化學激素受體的表現會影響鼻咽癌腫瘤細胞的生物行為。

英文摘要

Keywords：Nasopharyngeal carcinoma (NPC), Chemotherapeutic resistance, Apoptosis -related genes, Drug resistance genes, Epstein-Barr virus (EBV) genes, Syndecan-1, Chemokine receptors

Nasopharyngeal carcinoma (NPC) is an important endemic malignancy of Taiwan and is strongly associated with Epstein-Barr virus (EBV). Radiotherapy is the standard treatment for loco-regional NPC and results in a 5-year survival rate around 40% in Taiwan. The survival is influenced by the initial clinical stage; and the recurrence is predicted by the bulky nodal disease. For patients with recurrent or metastatic NPC, systemic chemotherapy is the most important modality of treatment although the result is still not good. Further NPC patients with recurrent or metastatic diseases respond particularly poor to chemotherapy as compared to chemo-naive loco-regional disease. In other words, with progression or metastases of the primary disease, NPC cells rapidly develop resistance to anti-cancer drugs. The mechanism underlying this phenomenon remains obscure. However, significantly increased number of EBV particles has been demonstrated in the metastatic NPC cells, and thus raises the possibility that virus may have played a role in this scenario. In this study, we explored the possible role of EBV-encoded viral genes and proteins, several drug resistance markers, extracellular matrix molecules, and chemokine receptors in the drug resistance of NPC tumor tissues.

First Year:

In the first year, we studied the expression of apoptosis-related proteins, including p53, bcl-2, bax, bcl-$X_{L/S}$, and c-myc, in NPC using immunohistochemical method on tumor tissues in 143 patients with primary NPC, 43 patients with recurrent NPC and 20 patients with metastatic NPC and correlated the results with clinical presentation and histopathological findings of these patients. The predominant expression of long or short fragment or both of bcl-$X_{L/S}$ was also studied in NPC by analyzing the mRNA of bcl-X using RT-PCR.

The results demonstrated that p53 protein expressed in 125 (87.4%) of 143 patients with NPC, 37 (86%) of 43 patients with recurrent NPC and 19 (95%) of 20 patients with metastatic NPC. The bcl-2 was expressed in 128 (89.5%) of patients with primary NPC, 40 (93%) of patients with recurrent NPC and all patients with metastatic NPC. Bax was expressed in 95.8% of primary NPC, all recurrent NPC and 90% of metastatic NPC. The bcl-$X_{L/S}$ was expressed in 95.1% of primary NPC, all cases of recurrent NPC and metastatic NPC. The c-myc was expressed in 90.9% of primary NPC, 95.3% of recurrent NPC and 95% of metastatic NPC.

We also studied the expression of several drug resistance genes (multidrug resistance

gene-1 [MDR-1], Glutathione-S-transferase-π [GST-π], and thymidylate synthase [TS]) in NPC by immunohistochemistry and correlated the results with the expression of LMP-1. The results showed that the expression of LMP-1 was not correlated with expression of drug resistance genes. The expression of MDR-1 was uncommon in NPC but its expression was correlated with poor prognosis of NPC patients.

In correlation with clinicopathological presentation, expression of LMP-1 did not related to any remarkable histological changes, including tumor cell anaplasia or pleomorphism or clinical significance. The expression of LMP-1 did not correspond with the amount of infiltrating lymphocytes in tumor tissue either. In contrast, the over-expression of p53 in NPC corresponded well with clinical presentation.

Second Year:

LMP-1 has been found to have potent tumorigenetic effect in epithelial cells through activated epidermal growth factor receptor (EGFR) expression and also can promote metastasis of tumor cells. Syndecans are a family of cell surface proteoglycans, which can interact with various effector molecules, such as extracellular matrix (ECM) molecules and growth factors and are shown to maintain the morphology of epithelium. Syndecan-1 (CD138) is a membrane of the syndecan family and it has been shown to mediate cell adhesion. Reduced syndecan-1 expression during malignant transformation of various epithelia and correlated with the histological differentiation grade of tumors are noted.

In the second year of this study, we performed the following works in the second year:

1. Detection of the expression of syndecan-1 in tumor tissues of primary, recurrent, and metastatic NPC by immunohistochemical method

Totally 86 primary, 37 recurrent, and 16 metastatic NPC samples were included. Of them, 18 (20.9%) primary, 9 (24.3%) recurrent, and 7 (43.7%) metastatic specimens were positive for staining of syndecan-1. Meanwhile, 37 (43.0%) primary, 18 (48.6%) recurrent, and 6 (37.5%) metastatic NPC samples showed positive for LMP-1 expression.

2. Detection the quantity of mRNA of syndecan-1 in primary and metastatic NPC by real time RT-PCR

After capturing tumor cells from frozen tissue sections of NPC specimens using LCM, RNA was extracted using RNA extraction kit followed by synthesis of cDNA and quantity of syndecan-1 RNA was detected by real time RT-PCR. The results revealed the quantity of mRNA of syndecan-1 was significantly lower than the housekeeping gene indicating low expression of syndecan-1 in transcription level in both primary and metastatic NPC. The mechanism of down regulation of *syndecan-1* gene in NPC was not known.

3. Clinicopathological correlation of expression of syndecan-1 and LMP-1

The expression of syndecan-1 in primary NPC samples was related with advanced clinical stage (P=0.025) and worse prognosis (P=0.01) but was not related to age and sex of

the patients and histological types of the tumor. The expression of LMP-1 was related with histological types (P=0.025) of primary NPC samples but was not related with to age, sex, clinical stage, or outcome of the patients. Co-expression of syndecan-1 and LMP-1 was noted in primary (P=0.05) but no statistic significance was noted between primary and recurrent or primary and metastatic NPC samples.

The above findings point to syndecan-1 was unusually expressed in NPC specimens and its expression was related to advanced clinical stag and worse prognosis of patients.

These results are published in the pages 1279-85, volume 37, 2006 of Human Pathology.

Third Year:

The interaction of chemokine receptors of tumor cells with their ligands on the endothelial cells of target organs plays an important role in determining patterns of metastases in several human tumors. Little information is available for the expression of chemokine receptors in NPC. In this study, we investigated the expression and biologic significance of chemokine receptor expressions in NPC.

At first, we screened the expression of 18 chemokine receptors (CCR1-10, CXCR1-6, XCR1, and CX3CR1) in five NPC cell lines (TW01, TW04, HONE1, BM1, AS1) using qRT-PCR, and then assessed the chemokine receptor pattern and function of the NPC cells with flow cytometric analysis and migration assay. Subsequently, fifty-two tumor tissues from 48 patients with NPC were evaluated for chemokine receptor expression by using immunohistochemical stain.

The NPC cell lines demonstrated possibly meaningful expression of mRNA or membranous protein of CCR7, CXCR4, and CXCR6. These three chemokine receptors were further examined in tumor tissues. The tumor tissues of primary and recurrent NPC were for the most part CXCR4-, CCR7- and CXCR6-negative (21/25, 84%); the remaining four specimens showed expression of only CXCR6. On the other hand, the metastatic tumors in regional lymph nodes and distant metastases were mostly positive for CXCR4, CXCR7, and CXCR6 (16/17, 94%), with the remaining single specimen only showing expression of CXCR4. The different expression of chemokine receptors in primary and metastatic NPC did not correlate with a history of radiotherapy or other clinicopathologic features.

This is the first report demonstrating chemokine receptor expression in NPC tissues. There were significant different chemokine receptor expressions between NPC tumor of the primary site and the metastatic sites

This part of work is appeared in the pages 363-73, volume 210, 2006 of the Journal of Pathology.

目　　錄

報告內容

Introduction

Nasopharyngeal carcinoma (NPC) is an important endemic malignancy of Taiwan and is strongly associated with Epstein-Barr virus (EBV). Radiotherapy is the standard treatment for loco-regional NPC and results in a 5-year survival rate around 40% in Taiwan [1]. The survival is influenced by the initial clinical stage; and the recurrence is predicted by the bulky nodal disease. Recently, efforts have been devoted to incorporating systemic chemotherapy with radiotherapy to treat high-risk loco-regional or metastatic diseases and have promising results [2-8].

For patients with recurrent or metastatic NPC, systemic chemotherapy is the most important modality of treatment although the results are still not good [9-11]. Cisplatin, cyclophosphamide, doxorubicin, vincristine, 5-fluorouracil (5-FU), and methotrexate are the major agents for treating NPC [1,3,12]. Either of these agents used singly or in combination, although with very high response rate in advanced NPC patients, consistently results in low 2-year survival rate [9-15]. Further NPC patients with recurrent or metastatic diseases respond particularly poor to chemotherapy as compared to chemo-naive loco-regional disease [16-19]. In other words, with progression or metastases of the primary disease, NPC cells rapidly develop resistance to anti-cancer drugs. The mechanism underlying this phenomenon remains obscure. However, significantly increased number of EBV particles has been demonstrated in the metastatic NPC cells, and thus raises the possibility that virus may have played a role in this scenario [20]. In this study, we explored the possible role of EBV-encoded viral genes and proteins, several drug resistance markers, and HER family in the drug resistance of NPC tumor tissues.

Objectives

In this three-year project, we mainly focused on the studies of the following two parts and these worked were accomplished in each year.

Part.1 <u>The Expression of Drug Resistance Genes, Apoptosis Related Proteins, and EBV Genes in NPC</u>:

Immunohistochemical method and laser capture microdissection (LCM) method were used for detecting the expression of MDR-1, MRP, GST-π, TS, p53, bcl-2, bax, bcl-$X/_L$, bcl-$X/_S$, and important EBV proteins, such as LMP-1, BHRF-1, Zebra, in tumor cells. The results were corrected and analyzed in correlating with clinical presentations and prognosis of the patients.

Part.2 The Role of Chemokines and Chemokine Receptor in Drug Resistance of NPC:

We used LCM method to dissecting tumor cells and infiltrating lymphocytes in and outside tumor tissue from frozen sections of NPC samples and analyzed the expression of chemokines/chemokine receptors in NPC. The results were correlated with the results of part 1 for exploring the role of chemokines/chemokine receptors in drug resistance of NPC.

First Year:

In our past study, we have been proved that all NPC cases from Taiwan contain EBV genome in the tumor cells but not in the infiltrating lymphocytes and certain EBV latent genes are expressed in NPC. The significance of the expressed EBV gene products on the biologic behavior of tumor cell is not clear. Also the clinical implication of EBV in the response of treatment and prognosis of NPC is not known.

Latent membrane protein-1 (LMP-1) is one of the EBV latent proteins that present in tumor cells of NPC. LMP-1 is a potent onco-protein and has been implicated for cellular transformation of B lymphocyte and also for inhibition of cell differentiation of human epithelial cell lines. P53 protein is a product of human tumor suppressor gene and has been proved to be over-expressed in many human malignancies. Mutation of the DNA encoded for p53 gene is responsible for the over-expression of p53 protein in most tumors and implication for the development of the tumor.

In the first year, we studied the expression of apoptosis-related proteins, including p53, bcl-2, bax, bcl-$X_{L/S}$, and c-myc, in NPC using immunohistochemical method on tumor tissues in 143 patients with primary NPC, 43 patients with recurrent NPC and 20 patients with metastatic NPC from Taiwan and correlated the results with clinical presentation and histopathological findings of these patients. The predominant expression of long or short fragment or both of bcl-$X_{L/S}$ was also studied in NPC by analyzing the mRNA of bcl-X using RT-PCR.

Second Year:

LMP-1 has been found to have potent tumorigenetic effect in epithelial cells through activated epidermal growth factor receptor (EGFR) expression and also can promote metastasis of tumor cells. Epithelial malignancy metastasis and tumor invasion is a multi-step process involving several crucial events: the loosening of intercellular junctions, attachment of tumor cells to the extracellular matrix (ECM), degradation of the ECM, the migration of tumor cells through the ECM, angiogenesis, the detachment of tumor cells, vascular permeation, the homing of tumor cells and trafficking of cancer cells through blood vessels, extravasations, organ-specific homing, and growth. Numerous molecules are involved in tumor invasion and metastasis, particularly heparin sulfate proteoglycans, which can interact with various effecter molecules, such as ECM molecules and growth factors, and

are modulators of cell growth and differentiation. Syndecans belongs to a family of cell surface proteoglycans that associate with the actin cytoskeleton to help maintain the morphology of epithelial sheets. Syndecan-1 (CD138) is a membrane of the syndecan family and it has been shown to mediate cell adhesion.

Syndecan-1, also known as CD138, is the most extensively studied member of the syndecan family. The extracellular domain of syndecan-1 binds both growth factors and extracellular matrix components, while its cytoplasmic portion interacts with cytoskeletal components. Syndecan-1 expresses mainly in epithelial cells and its expression is up-regulated during embryonic development. Syndecan-1 is thought to be involved in the processes of cell growth, differentiation, and adhesion and it acts as a co-receptor for fibroblast growth factors (FGF), a potent angiogenic growth factors involved in differentiation. The expression of syndecan-1 appears to be generally down-regulated in human carcinomas and in experimental cancer models, whereas transfectional expression of syndecan-1 in cultured cancer cells has been shown to inhibit their growth and other aspects of malignant behavior. Loss of expression of syndecan-1 in tumor cells leads to decreased intercellular cohesion, increased potential for tumor invasiveness, and metastatic spread. However, the expression of syndecan-1 is associated with poor prognosis in some malignancies, such as breast carcinoma.

In the second year, this study examined the expression of syndecan-1 and LMP-1 in primary, recurrent, and metastatic NPC specimens using immunohistochemical staining and analyzed the mRNA quantity of the *syndecan-1* gene using real-time RT-PCR in primary and metastatic NPC tumor tissues using LCM on frozen sections of NPC samples. The results correlated with histological types and clinical data, including age, sex, clinical stage, and outcome, in patients with primary NPC.

Third Year:

Metastasis of cancer cells is a highly organized, non-random, and organ-selective complex process involving multiple steps, including dissemination of metastasizing cells from the primary tumor, invasion of the surrounding tissue, intravasation and extravasation of the circulatory system, arrest, initiate angiogenesis, organ-specific homing, and growth while evading the immune system. The organ predilection of metastases depends on a variety of factors, such as the growth advantage, adhesion molecules and chemokine/chemokine receptor interaction. Interplay between those proposed mechanisms might operate to a different extent across various cancer types.

A complex network of chemokines and their receptors influences the development of primary tumors and metastases. New information about the biological role of chemokines in these processes is providing insights into host-tumor interactions, such as leukocyte infiltration and the site-specific spread of cancer cells. Recent studies have clearly

demonstrated the importance of chemokine receptor expression in breast cancer cells, melanoma cells and gastric carcinoma cells metastasizing to specific organs, such as the lymph node, bone marrow, liver and lung. Subsequently, CCR6-expressing colon, thyroid and ovarian carcinoma cells were reported to have a preference for metastasizing to the liver. In addition, CCR9 contributed to the localization of plasma cells to the small intestine; CXCR1- and CCR10-expressing melanoma cells could also home to endothelial cells and the skin, respectively. Whether specific chemokine(s)/chemokine receptor(s) are associated with metastasis of NPC to the lymph node, bone, liver or lungs is still unclear. In this study, we screened all known chemokine receptor expressions in five NPC cell lines (TW01, TW04, HONE1, BM1, AS1) using qRT-PCR, and then assessed the chemokine receptor pattern of NPC cell lines and fifty-two tumor tissues from 48 patients with NPC with flow cytometric analysis and immunohistochemical stain.

Literature Review

The Possible Role of EBV-encoded Viral Proteins in Drug Resistance of NPC

1.Roles of Viral Proteins in Drug Resistance of Cancers

The possible association of viruses and resistance to anti-cancer treatments was suggested by several clinical observations. In EBV-associated peripheral T-cell Lymphoma (PTCL), a clinicopathological study demonstrated that the presence of EBV in PTCL was associated with a more aggressive clinical course [1]. And, most importantly, the expression of MDR-1 and GST-π was significantly higher in EBV-positive PTCL [2].

2. Implication of EBV in Progression and Dug Resistance of NPC

Several evidences indicate that EBV might play a role in the progression of NPC. When both primary and metastatic NPC tissues were examined, the expression of EBER1 was much higher in metastatic lesions than the primary sites, indicating higher amount of EBV in the metastatic lesions [3]. In addition, elevated titers of antibodies to ZEBRA and EA, both are lytic-cycle proteins of EBV, preceded the development of recurrent or metastatic disease [4,5]. These evidences strongly suggest that the activation and proliferation of EBV is closely associated with progression of NPC.

In addition to possible direct transactivation of the promoters of drug resistance molecules, several EBV-encoded viral proteins, such as LMP-1, LMP-TW, BHRF-1, ZEBRA, are shown to affect cellular stress response, cell cycle control, and apoptosis regulation. These proteins are expressed in NPC tumor cells of various degrees. We hypothesize that some of these viral proteins might contribute to the clinical drug resistance of NPC. In

particular, the very poor response to chemotherapy of the metastatic NPC may be partially explained.

LMP-11: A multi-potential viral protein

Latent membrane protein-1 (LMP-1) has been extensively studied in many EBV-related malignancies because of its multiple biologic activities. It transforms rodent fibroblasts, changes the phenotypes of B-lymphocytes and prevents them from apoptosis by up-regulation of bcl-2. In epithelial cells, introduction of LMP-1 leads to the alteration of normal differentiation, expression of EGFR, and expression of several anti-apoptotic molecules [6-8].

This protean biologic activity of LMP-1 can be explained partly by its structural resemblance to TNF receptor family [9,10]. LMP-1 acts as a constitutively activated receptor, interacting with downstream signaling pathways, including NF-κB [8]. Recently, several reports have demonstrated that NF-κB plays a critical role in cellular response to anti-cancer treatments. In general, suppression of NF-κB pathway promotes apoptotic reaction of the cells to chemotherapeutic drugs, irradiation, and TNF [11-13].

BHRF-1 transcript: A bcl-2 homologue

BHRF-1 protein, a viral bcl-2 homologue, is another EBV-encoded viral protein which may have a role in cellular drug resistance [14]. BHRF-1 transcript is exclusively found in early phase of viral lytic cycle, and is responsible for the restrictive type of EBV-early antigen complex (EBV-EA) [15]. The sequence similarity and conserved binding domains suggest that BHRF-1 protein may regulate cell death and survival as bcl-2 family does [14,16]. When BHRF-1 transcript was expressed in cultured cells, it contributes to cellular resistance towards several apoptotic signals, including anti-cancer drugs [16-19]. Since BHRF-1 protein has been detected in NPC tissue, it is interesting to see whether it promotes the chemoresistance of NPC.

ZEBRA protein: A switch between latency and lytic phase

ZEBRA protein, encoded by BZLF-1 of EBV genome, acts as the switch of latency and lytic proliferation of EBV. ZEBRA shares several structural similarities with AP-1 family of transcriptional factors and it forms homodimers before binding to specific DNA sequence of viral genome, which is termed as ZEBRA-responsive element (ZRE) [20,21]. AP-1 binding site is important for cellular response to multiple signaling pathways. The similarity of ZRE and AP-1 binding site provides a possible base for the integration of viral and cellular gene regulation.

Since antibody titer to ZEBRA is particularly high in recurrent and metastatic NPC, a group of patients with poor response to chemotherapy, [5] a role of ZEBRA protein in the

progression and development of drug resistance is suspected.

3.Correlation of Expression of EBV Viral Proteins and Chemoresistance of NPC

There are only few articles discussing the correlation and importance of EBV-viral proteins on clinical drug resistance of NPC. The only one article, which evaluated the expression of LMP1 in NPC tissues and the treatment outcome of the patients, was hardly informative because of incompleteness of clinical data and very small sample size [22]. In the present proposal, we plan to re-address this question with a more systemic and sophisticated approach.

The Roles of Classical Drug Resistance Markers in NPC

Resistance to a broad spectrum of anti-cancer drugs is the major obstacle to effective treatment of cancer patients. Several classical molecular mechanisms have been described in the past two decades. These classical drug resistance mechanisms include: (1) to decrease the intracellular concentration of drugs by a drug pump; (2) to decrease the intracellular concentration of drugs by increasing drug metabolism; (3) to alter the drug target, interfering with the interaction of specific drug with its target [23]. On the other hand, the cytotoxic effect of anti-cancer drugs works through the process of programmed cell death after the binding of the drug to its target. These post-target-binding events may be important in the phenomenon of multi-drug resistance [23-25].

1.Classical Drug Resistance Mechanisms of Conventional Anticancer Drugs:

The first well-characterized specific drug resistance marker is Mutidrug Resistance-1 (MDR-1). MDR-1 encodes p-glycoprotein which is an membrane pump for many natural product drugs, including doxorubicin, vinca alkaloids, etoposide, and paclitaxel [26,27]. The presence of increased expression of MDR-1 in several types of tumor has been correlated with poor clinical response rate of chemotherapy and poor survival [27,28]. The MDR-1 reversing strategies were tested in several clinical trials.

Glutathione, as a coenzyme with a variety of enzymes, including glutathione-S-transferase (GST), plays a central role in metabolism or detoxification of drugs [29]. It is demonstrated that the cross-talk between intracellular glutathione-mediated detoxification and platinum accumulation is important in cisplatin resistance [30].

High expression of thymidylate synthase (TS), the target enzyme of 5-fluorouracil (5-FU), has been demonstrated to be associated with chemoresistance to 5-FU-based chemotherapy in colorectal cancers [31,32]. Our previous study on gastric cancers showed that the expression of TS, detected by immunohistochemistry, reliably predicted the poor response to 5-FU-based chemotherapy and short survival time of the patients [33].

2.Specific Drug Resistance Mechanisms of Novel Anticancer Drugs for NPC:

Several new drugs have been marketed in the last 10 years, such as paclitaxel, docetaxel, gemcitabine, and vinorelbine. Paclitaxel was found to be an active agent for NPC, with a single-agent response rate of 22%. The combination of paclitaxel with a platinum, either cisplatin or carboplatin, was subsequently reported by several groups as an attractive regimen for patients with advanced NPC. Regimens involving gemcitabine and vinorelbine are now undergoing.

Paclitaxel and vinorelbine are drugs affecting the balance of tubulin dynamics. The former promotes the assembly of microtubules from tubulin dimers; the latter acts in a totally different direction. Specific drug resistance mechanisms to these tubulin-acting agents were described [34]. Gemcitabine, 2'-2'-difluorodeoxycytidine, is a newly developed anti-metabolite with the structure resembling to that of cytosine arabinoside (ara-C). Gemcitabine, through the phosphorylation by intracellular deoxycytidine kinase (dCK), transforms into active form to inhibit DNA synthesis [35]. It has been demonstrated that lack of the activation enzyme (dCK) and over-expression of metabolizing enzyme (cytidine deaminase, CDA) may lead to drug resistance to gemcitabine [36,37].

3.Apoptosis-Related Mechanisms in Drug Resistance of NPC:

Most anticancer drugs induce apoptosis as a mechanism of cytotoxicity [38]. Failure to induce apoptosis is being recognized as an important category of multi-drug resistance [39,40]. TP53-dependent apoptosis is one of the major pathways [40].

TP53, a tumor suppressor gene, acts as a gatekeeper in cell cycles. It detects DNA damages and prevents the G1/S propagation, allowing either DNA repair or apoptosis [41]. An intact TP53 molecule is thus a pre-requisite for the cancer cells to go to apoptosis in response to anti-cancer drugs [42,43]. Over-expression of p53 due to mutated p53 has been shown to confer chemoresistance in various cancers [44,45].

Bcl-2 is an oncogenic protein that acts by inhibiting programmed cell death [46]. The expression of bcl-2 confers resistance to a variety of DNA-damaging agents. The expanding members of bcl-2 family fall into two opposing classes depending whether they induce or repress apoptosis [47,48]. The ratio of pro-apoptotic bcl-2 homologues (such as bax) and anti-apoptotic homologue (such as bcl-2) may decide whether the cells go to apoptosis in response to anti-cancer drugs.

Bax and bcl-X are also oncogenic proteins that involved in apoptosis. bax can promote apoptosis. The long transcript of bcl-X (bcl-$X/_L$) can protect cell from apoptosis while the short transcript (bcl-$X/_S$) has adverse effect. These two apoptosis-related genes have not been studied in NPC before.

Although the expression of p53 and bcl-2 in NPC was described before [48-50], whether these markers can predict the treatment results of systemic chemotherapy has never been

addressed.

Materials and Methods

First Year:

Patients and Tissues

Formalin-fixed paraffin-embedded biopsy specimens of 46 cases of NPC from pathologic file of National Taiwan University Hospital (NTUH) were collected and analyzed. Age, sex and initial clinical stage of these cases were evaluated too.

Histopathological Examination

All 46 cases of NPC were diagnosed by routine H & E sections and classified according to new WHO classification and the classification proposed by Hsu in 1987. The presence of specific histological features, such as evident cellular atypia, bizarre or multinucleated tumor giant cells and increased lymphoid infiltrate in tumor tissue resulting in a picture of lymphoepitheliomatous pattern were recorded.

Immunohistochemical Study

Immunohistochemical study of expression of LMP-1 and p53 were performed in all 46 cases of NPC. The procedure was briefly as: 5-6 um of paraffin sections were mounted onto microscopic glass slide treated with 3-aminopropyl-triethoxysilane (3-AES, Dako, Carpinteria, CA, USA). After deparaffinized and rehydrated, the sections were doing antigen retrieval in citrate buffer (0.01 M citric acid, pH 6.0) once in microwave oven. After cooled down and rinsed with Tris buffered saline (TBS), primary monoclonal antibody was added and incubated at RT for 20 min then washed with TBS three times, 3 min each. The sections were reacted with link antibody in the LsAB detection kit (Dako, Carpinteria, CA, USA) at RT for 10 min and washed with TBS again. Then the sections were incubated with labeled streptavidin at RT for 10 min and washed with TBS. The sections were developed with solution containing new fuchsin for 2-10 min. After washed with running water and counterstained with hematoxylin, the sections were dehydrated and mounted.

In LMP-1-positive cases double staining of both LMP-1 and p53 was performed immunohistochemically with detection of p53 first followed by detection of LMP-1. The detection of p53 was performed with detection kit labeled with peroxidase and developed with diaminobenzidine (DAB) and the LMP-1 was detected with streptavidin labeled with alkaline phosphate and colorized with new fuchsin. No counterstaining was done in these cases.

The primary monoclonal antibody for detection the expression of LMP-1 was CS1-4

(Dako, Carpinteria, CA, USA) and for the p53 was mouse anti-human p53 (Dako, Carpinteria, CA, USA). The red color in cytoplasm of tumor cells was read as LMP-1 positive and the red color in nuclei of cells were positive for p53. In double stain cases, the p53 was brown color and the LMP-1 was red. The positive control for LMP-1 was EBV-carrying B95-8 and for p53 was adenocarcinoma of colon.

Follow-up Data

These cases were followed up at least for 5 years and survival rate was estimated by Chi-square exact test.

Second Year:

Patients and Tissues

Totally, one hundred and thirty-nine formalin-fixed paraffin-embedded tissue samples from primary (n=86), recurrent (n=37), and metastatic NPC tumors (n=16) were obtained from the Department of Pathology, National Taiwan University Hospital (NTUH). Histopathologic classification of primary and recurrent NPC samples was based on the revised histological classification of tumors of upper respiratory tract and ear by the World Health Organization (WHO) in 1991. The staging system in this study was adapted from the UICC, 1997. The patients with primary NPC received radiotherapy and those with recurrent NPC received radiotherapy and/or chemotherapy for treatment. For patients with metastatic NPC, excision of metastatic lesion in neck, lung, or liver was performed. All these patients were followed up at NTUH.

Immunohistochemical Study

For immunohistochemical studies of expression of syndecan-1 and LMP-1 in NPC tumor cells, serial paraffin sections were used and cut in 6 μm in thickness. The paraffin sections were baked briefly at 60^{o}C followed by depaffinized and rehydrated using descending alcohol. After antigen retrieval with 0.1 M citrate buffer, pH 6.0, these sections were incubated with monoclonal antibody against syndecan-1 (CD138) (B-B4, Serotec, Oxford, UK) at dilution of 1:100 and LMP-1 (CS1-4, Dako, Carpinteria, CA, USA) at dilution of 1:50, respectively followed by adequate linked antibody (LsAB, Dako, Carpinteria, CA, USA) and colorized by diaminobenzidine (DAB) using a standard indirect avidin-biotin-peroxidase method. Then the sections were counterstained with Mayer's hematoxylin solution. The immunohistochemical staining results were arbitrarily classified into four scores dependent on the intensity of immunoreactivity: 0, negative staining; 1+, <10% tumor cells with positive immunostaining; 2+, 10%–50% tumor cells with positive immunostaining, and 3+, >50% tumor cells with positive immunostaining.

The plasma cells in chronic inflammation of skin were used as external positive control

and the internal control was plasma cells in stroma of tumor tissues. The staining pattern of syndecan-1 was membranous staining on epithelial cells and cytoplasmic stain in plasma cells. The reaction pattern of LMP-1 was cytoplasmic staining in tumor cells of NPC. The EBV-LMP1 positive and –negative controls included Raji, B95-8, and BJAB lymphoblastoid cell lines (LCL), respectively.

Quantify mRNA of Syndecan-1 in NPC tumor cells by Laser Capture Microdissection and Real-Time RT-PCR

Eight freshly frozen NPC samples, including 4 primary and 4 metastatic, were available for analyzing mRNA of *syndecan-1* gene in this study. Laser capture microdissection (LCM) method by Autopix (Archurus, CA, USA) was used for dissecting tumor cells from frozen sections of primary and metastatic NPC samples for studying the quantity of mRNA of *syndecan-1* gene in tumor cells. The primer sequences of *syndecan-1* gene in current study were published previously were sense: 5'-GAG GGC TGC TGA GGA TGG A-3' and antisense: 5'-ATT CTC CCC CGA GGT TTC AA-3'. The *hypoxanthine phosphoribosyltransferase (HPRT)* gene was used as internal control for real-time RT-PCR. The primer sequences of *HPRT* gene for real time RT-PCR in current study were sense: 5'-TGA CAC TGG CAA AAC AAT GCA-3' and antisense: 5'-GGT CCT TTT CAC CAG CAA GCT-3'.

Real-time RT-PCR was carried out on an ABI Prism 7700 (Perkin-Elmer-Applied Biosystems, Foster City, CA, USA), using SYBR-green as detection dye. Conditions for PCR included 50^{o}C for 2 min, 95^{o}C for 10 min, and 40 cycles of 95^{o}C for 15 sec (denaturation) and 60^{o}C for 1 min (annealing/extension). Relative mRNA amount of the target gene: internal control gene, HPRT, was calculated using the CT method as follows:

Relative expression = $2^{-\Delta CT}$; where CT = C_T (target gene)-C_T (HPRT).

Correlation Study of Expression of Syndecan-1 and LMP-1 and Clinical Data of Patients with Primary NPC

The results of expression of syndecan-1 and LMP-1 protein by immunohistochemistry and mRNA of *syndecan-1* gene by real time RT-PCR in patients with primary NPC were correlated with clinical presentation including age, sex, clinical stage, and outcome and histological classification of the studied patients. Statistic analysis using Chi-square method was done. The clinical stage and follow-up data of the patients studied were collected from the medical records of the Department of Registration, NTUH. In our series, 116 patients with primary NPC had enough clinical data available for staging analysis. For statistic analyses, only primary NPC patients were selected. Chi-square analysis was used for univariate statistic analysis.

Third Year:

Cell lines

Nasopharyngeal carcinoma cell lines TW01, TW04, and HONE1 were from primary tumors of Chinese patients with de novo NPC. Another two cell lines were from tumor cells in bone marrow (BM1) and lymph node (AS1) of Chinese patients with NPC metastasized to bone and lymph node, respectively. These cell lines were grown in Dulbecco's modified eagle's medium (DMEM) (Gibco Life Technologies, Maryland, USA), supplemented with 10% fetal calf serum (Gibco Life Technologies, Maryland, USA), 2 mM L-glutamine, and 100U/ml streptomycin/gentamycin, at 37^{o}C in 5% CO_2.

Extraction of RNA and RT-PCR

RNAs were isolated from various cell lines according to the manufacturer's recommendation (Trizol, Glbco Life Science). Briefly, total RNA was prepared from cell lines using zol B Reagent^{TM} (TEL-TEST, Texas, USA). Purified RNAs were then treated with RNase-free DNase before cDNA synthesis. All reverse transcriptase reactions were performed using Moloney murine leukemia virus reverse transcriptase (Epicentre, New Jersey, USA) with oligo-dT and random hexamer under standard conditions. The PCR amplification mixture for each reaction was 50 μl, which contained 5 μl cDNA, 5 μl 10X buffer, 2.5 mM $MgCl_2$, 200 μM dNTP, 0.4 μM each primer, and 2.5 U Ampli-Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA). Thermal cycling consisted of an initial conditioning for 10 min at 95^{o}C, followed by 35 or 39 cycles of 95^{o}C for 30sec, 58^{o}C for 1 min, and 72^{o}C for 1 min. PCR products were then size-fractionated on 2% agarose gel and visualized with ethidium bromide staining. Negative controls were omission of a target template and omission of reverse transcriptase. The sequences of primers designed for 18 different chemokine receptors were according to Nakayama's report.

Real-time RT-PCR

Primers for CXCR4, CXCR6, CCR7 and CCR9 were designed using the computer program Primer Express (Perkin-Elmer-Applied Biosystems, Foster City, CA, USA). Primers were synthesized by and purchased from Genemed Synthesis (South San Francisco, CA, USA). On the basis of the cDNA sequence (Genebank accession no. NM_003467, NM_006564, NM_001838 and AJ132337 for CXCR4, CXCR6, CCR7 and CCR9, respectively), the sequences of the primers used for real-time RT-PCR were as follows: 5'-TTC AGT GGC ATG CTC CTA CTT CT-3' and 5'-GCT GAG ACA GCC TGG ACG AT-3' for CCR7; 5'-CAT TGA CGC CTA TGC CAT GTT-3' and 5'-GGT GAC CTG GAA GCA GAT GTC-3' for CCR9; 5'-TGA CCG CTT CTA CCC CAA TG-3' and 5'-AGG ATA AGG CCA ACC ATG ATG T-3' for CXCR4; and 5'-GCC ATG ACC AGC TTT CAC TAC A-3' and 5'-TTA AGG CAG GCC CTC AGG TA-3' for CXCR6. The hypoxanthine

phosphoribosyltransferase (HPRT) gene was used as the internal control for real-time RT-PCR. The primer sequences were 5'-TGA CAC TGG CAA AAC AAT GCA-3' and 5'-GGT CCT TTT CAC CAG CAA GCT-3'. Real-time RT-PCR was carried out on an ABI Prism 7700 (Perkin-Elmer-Applied Biosystems, Foster City, CA, USA), using SYBR-green as the detection dye. Conditions for PCR included 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec (denaturation) and 60°C for 1 min (annealing/extension). Relative mRNA amount of the target gene to internal control gene, HPRT, was calculated using the ΔCT method as follows:

Relative expression = $2^{-\Delta CT}$; where $\Delta CT = C_T$ (target gene)-C_T (HPRT).

Flow Cytometric Analysis

NPC cell lines were detached from the culture dishes using 0.0005% EDTA in phosphate-buffered saline (PBS) and washed twice with PBS. Cells were resuspended in PBS at a concentration of $4x10^6$/ml. In order to evaluate the chemokine expression on cell membrane, each $2x10^5$ cells were incubated at 2-8°C for 40 min with the following murine monoclonal antibodies (MAbs), 25 μg each: anti-human CXCR4 (clone 44708.111), anti-human CXCR6 (Clone 56811.111), anti-human CCR7 (Clone 150503) or anti-CCR9 (clone 112509). All of the above antibodies were purchased from R&D Systems, Minneapolis, USA. The mouse primary antibodies were then detected by incubating at room temperature for 30 min with fluorescein isothiocyanate (FITC)-conjugated goat $(Fab')^2$ anti-mouse IgG (Serotec Ltd, UK). After being washed twice with PBS, the cells were resuspended and fixed in 1% (w/v) paraformaldehyde for analysis. A murine IgG antibody was used as the isotype control. Ten thousand cells from each sample were evaluated for fluorescence detection using FACScan (Becton Dickinson, San Jose, CA), and the data were analyzed with CellQuest software (Becton Dickinson, San Jose, CA).

In order to detect intracellular chemokine receptor protein by flow cytometry, permeabilized cells were prepared with 0.2% saponin reagent after 4% (w/v) paraformaldehyde fixation, and then went through the procedure stated above. These permeabilized cells were maintained in staining buffer containing 0.2% saponin to ensure complete membrane permeabilization throughout.

Tissue Samples and Immunohistochemical Study

Formalin fixed paraffin-embedded tissue samples of NPC tumors from either primary site or metastases sites were taken, pooled from the Department of Pathology, National Taiwan University Hospital (NTUH). Histopathological diagnosis was confirmed for each specimen by a pathologist. Histopathologic classification of these cases was done according to the revised histologic classification of tumors of upper respiratory tract and ear by the World Health Organization (WHO) in 1991.

Tissue samples cut to 6 mm thick were mounted on Silane-coating slides (Dako, carpinthia, CA, USA). After antigen retrieval, these sections were reacted with anti-human CXCR4, anti-human CXCR6, anti-human CCR7, or IgG isotype, respectively, by using a standard indirect avidin-biotin-peroxidase method. Color reaction was developed with diaminobenzidine (DAB) solution, and sections were counterstained with Mayer's hematoxylin solution. The specimens were evaluated independently in a blinded fashion without prior knowledge of the clinicopathology. The IHC results for chemokine receptors were arbitrarily classified into four scores dependent on the field of positive-immunoreactivity: 0, negative immunostaining; 1+, <10% tumor cells with positive immunostaining; 2+, 10 – 50% tumor cells with positive immunostaining; and 3+, >50% tumor cells with positive immunostaining.

Results

First Year:

Clinical Data

The 46 cases included 30 male and 16 female and age ranged from 19 to 76 years old with mean age of 48 years old. Most cases were stage III at initial diagnosis.

Histopathological Study

The 46 cases of NPC were classified according to new WHO classification as: 9 keratinizing squamous cell carcinoma (KSCC), 21 cases of differentiated non-keratinizing carcinoma and 16 cases of non-keratinizing carcinoma of undifferentiated type. In cases of KSCC, the tumor cells surrounding the keratinizing area were undifferentiated type which was similar to the tumor cells in non-keratinizing carcinomas.

At the mean time, all cases were classified with the classification that proposed by Hsu in 1987. According to this classification, the 46 cases were classified as: 9 cases of KSCC, 25 cases of type A and 11 cases of type B.

Immunohistochemical Study

The EBV-related LMP-1 was demonstrated in the cytoplasm of tumor cells of 18 of 46 cases (18/46. 39.3%). The LMP-1 negative tumor cells were also present within LMP-1 positive cases. The tumor nests composed of exclusively LMP-1 positive or negative cells and the LMP-1 positive and negative areas were completely separated. The appearance of the LMP-1 positive cells did not show any differences from those cells which were negative for LMP-1. The infiltrating lymphoid cells and lining mucosa of all cases were all negative for LMP-1.

The presence of p53 protein was noted in all 46 cases although the p53-positive cells were varying in different cases. In same sample the p53-containing tumor cells were randomly distributed throughout the tumor nests and the staining intensity was different from cell to cell.

Second Year:

Clinical Data

Totally 139 NPC samples were included in this study. Of them, 86 samples were primary NPC. The patients with primary NPC included 62 males and 24 females and age ranged from 18 years to 81 years. The histological classification of these primary NPC was classified as 7 keratinizing squamous cell carcinoma (KSCC) and 79 non-keratinizing carcinoma with 26 differentiated type and 53 undifferentiated type.

Expression of Syndecan-1 and EBV Latent Membrane Protein-1 (LMP-1)

Regarding the expression of syndecan-1 by immunohistochemical stain in formalin-fixed paraffin-embedded sections of 139 NPC specimens, 18 (20.9%) of 86 primary, 9 (24.3%) of 37 recurrent, and 7 (43.8%) of 16 metastatic specimens were positive for staining. The staining intensity of syndecan-1 in tumor cells was usually weaker than squamous cells of skin or plasma cells in the NPC specimens. In addition, the stromal tissue in primary and recurrent NPC specimens did not show any positive staining for syndecan-1 but two of the metastatic NPC samples showed positive staining in stromal tissue while the tumor tissue was negative.

About the expression of LMP-1 in NPC, 37 (43.0%) of 86 primary, 18 (48.6%) of 37 recurrent, and 6 (37.5%) of 16 metastatic specimens were positive for staining. The staining pattern in NPC tumor cells was cytoplasmic stippling but not membranous. The infiltrating lymphocytes in tumor and overlying epithelium in all samples were negative.

In LMP-1-positive group, 4 of 37 primary, 3 of 18 recurrent, and 3 of 6 metastatic NPC tissues had co-expression of syndecan-1 in tumor cells while in LMP-1-negative group, 14 of 49 primary, 6 of 19 recurrent, and 4 of 10 metastatic NPC samples expressed syndecan-1. In contrast, in syndecan-1-positive group, 4 of 18 primary, 3 of 9 recurrent, and 3 of 7 metastatic NPC specimens co-expressed LMP-1 in tumor cells and in syndecan-1-negative group, 33 of 68 primary, 15 of 28 recurrent, and 3 of 9 metastatic NPC samples expressed LMP-1.

The real time RT-PCR study of mRNA of *syndecan-1* gene revealed low amount of syndecan-1 mRNA in four primary NPC specimens and four metastatic NPC as compared with internal housekeeping gene, the *HPRT* gene, when the *HPRT* gene mRNA was detected as early as 28 cycles but the syndecan-1 mRNA was detected only since 35 to 37 cycles.

Clinicopathological Correlation of Expression of Syndecan-1 and LMP-1

Statistic significance of expression of syndecan-1 and LMP-1 was analyzed with the clinical data, including age, sex, clinical stage, and survival, and histological types of the NPC patients with primary tumor. Statistic significance was observed in NPC specimens expressing syndecan-1 with clinical stage and survival of patients. Expression of syndecan-1 was more often in patients at stage III of IV (p=0.025) and was also more in patients survived less than 5 years (p=0.01). The expression of syndecan-1 in NPC tumor specimens was not correlated with age, sex, and histological types of patients.

The expression of LMP-1 in primary NPC samples was not correlated with age, sex, clinical stage, and survival of the patients but it was correlated with histological type of NPC samples with less expression of LMP-1 in KSCC cases than in non-keratinizing carcinoma (p=0.025).

When did the statistic analysis of the expression of syndecan-1 and LMP-1 in tumor tissues of primary versus recurrent or primary versus metastatic NPC specimens, no significant difference was noted in either expression of syndecan-1 or LMP-1 in primary versus recurrent or primary versus metastatic NPC specimens.

About the co-expression of syndecan-1 and LMP-1 in NPC specimens, statistic significance was noted in primary NPC samples but was not in recurrent or metastatic specimens (p=0.05).

Third Year:

RT-PCR and real-time PCR analysis revealed that the NPC cell lines exhibited CCR7, CCR9, CXCR4 and CXCR6 messenger transcript

To determine expression of all known 18 chemokine receptors (CCR1–CCR10, CXCR1–CXCR6, XCR1, and CX3CR1) in NPC, five NPC cell lines (TW01, TW04, HONE1, AS1 and BM1) were analyzed for the presence of chemokine receptor mRNA by using the semi-quantitative RT-PCR method. We found that CCR1, CCR3, CCR4, CCR5, CCR8, CCR10, CXCR1, CXCR5, XCR1, and CX3CR1 were consistently negative even after 39 cycles of PCR in all studied cell lines (data not shown). In contrast, CCR7, CCR9, CXCR4, and CXCR6 showed substantial amounts of mRNA after 35 cycles of PCR in one or more NPC cell lines (data not shown). Quantitative real-time PCR technique verified the RT-PCR results and put a figure on the mRNA amount in those NPC cell lines. We found that all these NPC cell lines exhibited heterogeneous amounts of CCR7, CCR9, CXCR4, and CXCR6 mRNA. Apart from the AS1 cells, with abundant CXCR4 (0.17:1 relative to HPRT) and little CCR7 (10^{-4}:1 relative to HPRT) expression, the others demonstrated a similar trend for chemokine receptor expression as CCR9>CXCR6>CCR7>CXCR4.

Flow cytometric analysis and migration activity assay revealed that CCR7, CXCR4 and CXCR6 were meaningful in NPC cells

Although there were substantial amounts of mRNA in these NPC cell lines, whether these chemokine receptors expressed in cytoplasm or on cell membrane remained unknown. We determined these receptor patterns on the NPC cell lines by flow cytometry and found that the CXCR4 protein was expressed only on the cell membrane of AS1 cells; the CXCR6 protein was expressed on the cell surface of TW01, TW04, and BM1cells; and the CCR7 protein was expressed on the cell surface of TW04 and BM1 cells. Intracellular analysis of these proteins revealed that all the receptor proteins also existed in cytosol of the NPC cells. Surprisingly, CCR9 only existed in cytosol, but not on membrane, despite the high presence of mRNA, of NPC cells.

The corresponding chemokines for CCR7, CXCR4 and CXCR6 were CCL21, CXCL12, CXCL16, respectively. In order to evaluate the functional activity of these membrane chemokine receptors, we used the Transwell® system for migration assay. We coated the membranes of inserts with fibronectin to ensure attachment of NPC cell lines to the membrane. We found that CCL21, CXCL12 and CXCL16 could attract the NPC cell lines through the membrane, but only if these cells had corresponding chemokine receptors on surface membrane (Figure 3). That BM1 cells did not show a significant increased migration index might have been due to their great spontaneous migration.

Significant differences in CXCR4 expression between primary and metastatic tissues demonstrated that CXCR4 played an important role in metastasis

We examined the presence of CCR7, CXCR4, and CXCR6 proteins in fifty-two formalin-fixed paraffin embedded NPC tumor tissues from 48 NPC patients by using IHC. The patients ranged in age from 19 to 75 years (mean$\pm$SD, 47$\pm$13 years); there were 41 males and 7 females. Chemokine receptor-expressing tumor cells in breast cancer were used as positive controls. There were significant differences between these three chemokine receptor expressions between primary and metastatic NPC. Table 1 summarized the clinical status of the NPC patients when the tumor specimen was taken, the kind of specimen, and their chemokine receptor expressions. We found that nasopharyngeal (NP) specimens from patients with primary NPC (n=12) showed all negative immunoreactivity of CCR7, CXCR4 and CXCR6, except for those from patient 11, who had previously received chemotherapy. NP specimens from patients with regional metastatic NPC (n=13) also showed all negative, except for those from two patients (patients 18 and 20). All three exceptions demonstrated CXCR6-positive. On the contrary, nose specimens from two patients with regional metastasis (patient 26, 27) showed substantial CXCR4 expression. NP specimens from patients with liver metastasis (patients 29 and 30) showed heterogeneous expression of CXCR4, CCR7, and CXCR6. These results were similar to those results from several primary NPC cell lines, TW-01, TW-02 and HONE1, with heterogeneous expression of these three chemokine receptors. However, metastases specimens of regional and distant metastasis (neck mass,

axilla and inguinal lymph node, lung and liver) showed strong positives of all three-chemokine receptors (patients 32 to 37). Intriguingly, NP specimens from patients with brain invasion (patients 28 and 31) also showed CXCR4- and CCR7-positive to different extents. Regarding whether there was a history of radiotherapy or not, CXCR4 showed no significant differences in the primary site between radiotherapy-positive and –negative groups (2/7 vs. 1/22; p=0.269). CCR7 and CXCR6 exhibited the same trend. All metastases sites demonstrated all positive for three chemokine receptors whether there was a history of radiotherapy or not (patients 37-47). Among them, three patients had follow-up evaluations (patients 9, 40, and 41). The results of these three patients verified that the chemokine receptor expressions correlated with the existence of metastasis and the kind of metastases, despite the presence of absence of radiotherapy.

Discussion

First Year:

LMP-1 is one of the EBV latent proteins that present in tumor cells of NPC. LMP-1 is a potent onco-protein and has been implicated for cellular transformation of B lymphocyte and also for inhibition of cell differentiation of human epithelial cell lines. P53 protein is a product of human tumor suppressor gene and has been proved to be over-expressed in many human malignancies. Mutation of the DNA encoded for p53 gene is responsible for the over-expression of p53 protein in most tumors and implication for the development of the tumor. In the present study, we demonstrated the expression of LMP-1 and natural p53 protein in tumor tissues of NPC biopsy specimens and correlated the data with histopathological findings and clinical presentation of the tumor to clarify the significance of LMP and p53 protein in biological behavior of NPC.

The expression and biological significance of expression of MDR-1 and GST-π in patients with NPC are unclear. In this study, we found that weak cytoplasmic staining for MDR-1 was detected in tumor cells in 12.6% of patients with primary NPC and 32.6% of those with recurrent NPC. However, none of the 20 patients with metastatic NPC expressed MDR-1. The lower expression of MDR-1 in patients with primary NPC than in those with recurrent NPC has statistical significance (12.6% vs. 32.6%, p=0.0024). On the other hand, GST-π was detected in the cytoplasm of tumor cells of patients with NPC in 58% of those with primary, 69.8% of those with recurrent, and 65% of those with metastatic NPC. The patients with primary and recurrent NPC did not differ in GST-π expression. Our results suggested that drug resistance genes, particularly GST-π, were often expressed in patients with NPC. MDR-1 expression was more closely related to the relative chemosensitivity of primary NPC, however, more drug resistance was found in patients with recurrent tumor.

Moreover, we found that patients with NPC of the KSCC type that were often radioresistant had a significantly higher positive rate of MDR-1 expression (36.4%) than NK and UD types (16.7% and 7.8%, respectively; p=0.0170). However, the GST-π expression did not correlate with the histologic types of NPC. Neither the expression of MDR-1 nor GST-π correlated with tumor stage. Moreover, patients with NPC with MDR-1 expression that had more often stage II to IV disease had more unfavorable outcomes whereas patients with the expression of GST-π did not. The reason for the absence of MDR-1 protein expression in metastatic NPC is unknown.

Interestingly, the staining pattern of MDR-1 in tumor cells of NPC was mainly in the cytoplasm and not on the membrane as that seen in healthy hepatocytes. The reasons for this different staining character of MDR-1 in NPC tumor cells are not known.

A hypothesis has been proposed which suggested that GST might provide the cytotoxic agents with an S-conjugation necessary for the recognition and extrusion by the ATP-hydrolyzing transmembrane pump of P-glycoprotein. If this is true, simultaneous expression of GST-π and MDR-1 should confer to the cells not only a broader spectrum of resistance but also better protection against cytotoxic agents. This may facilitate selection of tumor cells that express both markers during the stress of combination chemotherapy. In our study, simultaneous expression of MDR-1 and GST-π was only detected in 13 (9.1%) of 143 patients with primary NPC, in 12 (27.9%) of 43 with recurrent NPC and in none with metastatic NPC. The expression of MDR-1 correlated with the expression of GST-π in patients with primary and recurrent but not in patients with metastatic NPC. The data show that 72.2% of patients with primary NPC and 85.7% with recurrent NPC expressed both MDR-1 and GST-π simultaneously and none of the patients with metastatic NPC did.

In a previous study, we have demonstrated that the poor prognosis in peripheral T cell lymphoma harboring EBV was related to the higher MDR-1 and GST-π expression than other EBV negative lymphoma which correlated with the expression of EBV latent membrane protein-1 (LMP1). We found EBV was virtually present in all tumor cells of all patients with NPC in Taiwan. However in the present study, the expression of MDR-1 and GST-π in NPC did not correlate with the expression of LMP-1 in patients with NPC. The results suggest that LMP-1 did not up-regulate the expression of either MDR-1 or GST-π in patients with primary NPC.

Second Year:

The significance of expression of syndecan-1 has been studied in many malignancies and inverse significance has been found in different tumors. For example, expression of syndecan-1 correlates with differentiation of tumor cells, low clinical stage, and favorable prognosis in carcinomas of head and neck region, esophagus, larynx, liver, lung, colon, and uterine cervix, while inverse results are noted in malignancies of breast, prostate, and thyroid.

Since expression of syndecan-1 has prognostic significance in squamous cell carcinoma of head and neck, in this study we studied the expression of syndecan-1, the CD138, and EBV-encoded LMP-1 in primary, recurrent, and metastatic NPC specimens by immunohistochemical stain and analyzed the quantity of mRNA of *syndecan-1* gene by real time RT-PCR. This is the first study about the expression of syndecan-1 and its clinicopathological significance in NPC.

The current results showed that expression of syndecan-1 in tumor cells of NPC was not common and in the expression level was low in most cases expressing syndecan-1. The results might correlate with poor differentiation or undifferentiated character of tumor cells of NPC because morphologically most NPC composed of poorly differentiated or anaplastic tumor cells. However when correlated the results of syndecan-1 expression and histologic types of primary NPC specimens, no significant difference was observed. Instead, the expression of syndecan-1 in NPC samples was statistically related with high clinical stage and poor outcome of the patients with primary NPC. These results were in contrast to squamous cell carcinoma of other head and neck regions in which syndecan-1 expression indicates a differentiated tumor and has more favor outcome.

To elucidate the possible mechanism of uncommon expression of syndecan-1 in NPC samples, we analyzed the quantity of mRNA of syndecan-1 gene by real time RT-PCR and the data supported the immunohistochemical results that the quantity of mRNA of *syndecan-1* gene was low in most NPC specimens as compared with amount of mRNA of housekeeping gene. We concluded the down regulation of syndecan-1 expression in primary and metastatic NPC was at the transcription level. The results were similar to previous report of Fujiya et al. and they find that down regulation of syndecan-1 in carcinomas of colon is due to hypermethylation of *syndecan-1* gene.

Recent experimental studies on the role of syndecan-1 in malignant transformation have shown that syndecan-1 expression is associated with the maintenance of epithelial morphology, anchorage-dependent growth and inhibition of invasiveness in vitro. The down expression of syndecan-1 in primary and recurrent NPC might make tumor cells having higher potential for early metastasis to regional lymph nodes or other visceral organs as noted in NPC patients clinically. However, no statistic significance was noted in the expression of syndecan-1 in primary and recurrent NPC or between primary and metastatic NPC although higher percentage of syndecan-1 expression in metastatic NPC samples. The reason for metastatic NPC samples had higher percentage of expression of syndecan-1 was not clear.

Induced expression of syndecan-1 in stromal tissue of different types of malignancy has been reported. The induced expression of syndecan-1 in stroma may stimulate growth of epithelial cells and also contribute to tumor cell invasion and development of metastasis. In our study, expression of syndecan-1 in stromal tissue was not found in primary or recurrent NPC specimens but two samples of metastatic NPC had syndecan-1 expression in stromal

cells and the tumor cells were negative.

EBV-encoded LMP-1 has been found to have potent tumorigenetic effect in epithelial cells and it can promote metastasis of tumor cells. Up regulation of matrix metalloprotease 9 (MMP9) correlates with metastatic potential in NPC and the up regulation of MMP9 in NPC is associated with expression of LMP-1. LMP-1 also can induce expression of interleukin-8 (IL-8) through nuclear factor kappa B (NF-κB) pathway resulting in angiogenesis in NPC tumor. In current study, expression of LMP-1 was detected in primary, recurrent, and metastatic NPC and the overall detection rate of LMP-1 in our NPC specimens was lower than previous reports which may be due to inadequate process of the tissue samples before immunohistochemical study.

The expression of LMP-1 in primary NPC specimens classified as KSCC was all negative while in 37 out 79 NPC samples classified as non-keratinizing carcinoma were positive. There was statistic significance in histological types of primary NPC and status of expression of LMP-1. When analyzed the expression of LMP-1 in primary NPC samples and clinical data, including age, sex, clinical stage, and outcome, of patients with primary NPC, no statistic significance was found; also no significant difference between the LMP-1 expression in primary to recurrent NPC or primary NPC to metastatic NPC.

Co-expression of syndecan-1 and LMP-1 was noted in this study indicated expression of syndecan-1 in NPC might induced by expression of LMP-1 in tumor cells and expression of both molecules resulting in angiogenesis and aggressive behavior of NPC tumor.

Third Year:

In this study, a comprehensive *in vitro* and *in vivo* survey for chemokine receptor expression revealed heterogeneous expression of CCR7, CXCR4 and CXCR6 in the primary sites of patients with distant metastasis and abundant expression of them in the metastases sites of patients with regional or distant metastasis. This difference was not related to previous radiotherapy treatment.

Previous investigations on the significance of chemokine/chemokine receptor interaction in NPC focused on intense infiltration of leukocytes in tumor tissue. They demonstrated that those infiltrating cells were mainly composed of T lymphocytes and macrophages, which expressed CCR2 and CCR5. Subsequently, interferon-γ (IFN-γ, secreted from the tumor-reactive T cells could further activate the accompanying tissue macrophages to express MIP-1α (a ligand of CCR5) and MCP-1 (a ligand of CCR2), which consequently recruited more T cells and macrophages into the tumor tissue. The LMP-1 could induce expression of matrix metalloproteinase 9 (MMP-9, an enzyme digesting extracellular matrix) and was supposed to be associated with metastasis of NPC. LMP-1 and Z transactivator (Zta) proteins could also up-regulate the expression and activity of MMP1 and confer the invasive properties of these metastatic cells.

This is the first article to evaluate the importance and significance of chemokine receptor expression in NPC. Our data revealed that NPC cells could synthesize several chemokine receptors, CCR7, CXCR4, or CXCR6, which then translocate to membranes. Lymph node, bone, liver and lung were the major metastasis sites for NPC. Several reports stated previously that CCL21, a ligand for CCR7, was highly expressed in lymph node; CXCL12, a ligand for CXCR4, was moderately expressed in lymph node, lung, liver and bone marrow; and CXCL16, a ligand for CXCR6, was highly expressed in lung and liver and moderately expressed in lymph node. Therefore, the expression of CCR7, CXCR4, or CXCR6 in NPC tumor cells might be the reason for their potential for metastasizing to lymph node, liver or lung. As to CCR9, although there was abundant CCR9 mRNA expression in these NPC cells, all CCR9 proteins remained in cytoplasm. These results were consistent with the finding that CCL25, a ligand of CCR9, existed mainly by epithelial cells of the small intestine, which is not a site NPC metastasizes to. Previous nude mouse-transplantation experiments of TW01 cell lines demonstrated that many small metastatic granules disseminated on the surface of the diaphragm and in the lung parenchyma, and only a few were in the liver. These results were consistent with our finding that TW01 cells had abundant CXCR6 expression on their membrane surface in our study.

Immunohistochemistry analysis for NPC biopsy from primary and metastatic lesions demonstrated significant differences in chemokine receptor expression between specimens from primary and metastases sites. Usually, primary NPC had one or more chemokine receptor expressions; however, metastatic NPC expressed multiple chemokine receptors, simultaneously. This difference did not correlate with a history of radiotherapy. We wonder if the substantial chemokine receptor expression in the metastatic tissue might be the result of the autocrine effect of chemokine/chemokine receptors at the distant tumor sites. CXCR4/CXCL12 and CCR7/CCL21 signaling pathways are widely known to determine significantly the destination of breast cancer and gastric carcinoma cells. Further studies showed that the aortic smooth muscle cell proliferated via Gi, phosphatidylinositol 3-kinase, Akt, IκB kinase and NF-κB signaling pathways. In this study, we demonstrated for the first time that CXCR6/CXCL16 signaling might play a crucial role in the migratory activity of tumor cells. Determination of whether the metastasis sites could also offer environments to activate CXCR4, CCR7 and CXCR6 expressions requires further evaluation.

Conclusion and Comments

In conclusion, we have studied the expression of apoptosis-related genes, latent genes of Epstein-Barr virus (EBV), drug resistance genes, syndecan-1 (CD138), and different chemokine receptors those possible involved in the mechanism of drug resistance in NPC

tumor cells. We concluded that:

1. The expression of MDR-1 was low in primary, recurrent, and metastatic NPC and the expression of GST-π was relatively more frequent than MDR-1 in NPC tumors. The expression of MDR-1, but not the GST-π, correlated with the histological types and outcomes of patients with primary NPC and the status of expression of MDR-1 may be a useful prognostic indicator for NPC. The expression of MDR-1 and GST-π did not correspond with the expression of LMP-1 in NPC tumor tissues.
2. Syndecan-1 was uncommonly expressed in NPC samples, its expression correlated with advanced clinical stage and poor outcome, and the down regulation of syndecan-1 in NPC specimens was at the transcription level. The syndecan-1 expression in NPC tumors was not correlated with age and sex of the patients and was also not correlated with histological types of NPC samples. The expression of syndecan-1 in primary NPC was correlated with expression of LMP-1.
3. Expression of CXCR4, CXCR6, and CCR7 was detected in NPC cell lines and they are functioning in tumor cells. However, by immunohistochemical studies, strong positive results were demonstrated for all three chemokine receptors for almost all of the regional and distant-metastasis specimens. The significance of the differences in positive expression for CCR7, CXCR4 and CXCR6 between primary tumors and metastases was $p<0.001$, $p<0.001$ and $p<0.002$, respectively. Finally, significant differences in CXCR4 expression were demonstrated comparing de novo and post-radiotherapy groups (1/22 vs. 5/8; $p<0.003$). It appears reasonable to conclude, therefore, that CCR7, CXCR4and CXCR6 expressions are expressed and active in human metastatic NPC, while CXCR4 expression is associated with radiotherapy history.

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計劃成果自評

In this 3-year study, we have made some progress in understanding the mechanism of drug resistance of NPC and published two articles in two outstanding pathology journals.

First, we studied the expression of apoptosis-related proteins including p53, bax, bcl-2, bcl-X, and c-myc in NPC. Unfortunately, the expression of these apoptosis-related proteins is not correlated with clinical outcome of the NPC patients. Also, we studied the expression of multidrug resistance gene-1 (MDR-1) of NPC and correlated its expression with the expression of EBV latent genes, clinical presentations, and outcome of the NPC patients. The results demonstrated the expression of MDR-1 is correlated with poor outcome of the patients.

Second, we studied the expression of syndecan-1 and EBV-LMP-1 in NPC and correlated these results with clinicopathological presentations of NPC patients. We presented part of our results in the 94th Annual Meeting of United States and Canadian Anatomical Pathologist Society (USCAP) at March 2005. The results are published in the Human Pathology of October issue 2006 (see Appendix 1).

Third, we screened and demonstrated the expression of certain chemokine receptors (CXCR4, CXCR6, and CCR7) in NPC and concluded their expression is functioning in NPC. The expression of chemokine receptors was mostly in metastatic NPC samples. We presented part of our results in the 97th Annual Meeting of American Association of Cancer Research (AACR) at April 2006. The results are published in the Journal of Pathology of November issue 2006 (see Appendix 2).

In overall, this project achieved the original goals of the proposal and have valuable information about the mechanism of drug resistance in NPC. Further study about drug resistance of NPC in molecular mechanisms is pending.

附 錄 一

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Expression of Syndecan-1 (CD138) in Nasopharyngeal Carcinoma (NPC) is Correlated with Advanced Stage and Poor Prognosis

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Key words: Syndecan-1; Nasopharyngeal carcinoma; Epstein-Barr virus

Abstract

Nasopharyngeal carcinoma (NPC) is an important Epstein-Barr virus- (EBV) associated head and neck malignancy in Taiwan. Syndecan-1 (CD138) is involved in growth, differentiation, invasiveness, and metastatic potential of certain tumors but its expression has never been studied in NPC. In this study, detection of expression of syndecan-1 protein and EBV-encoded latent membrane protein-1 (LMP-1) in primary, recurrent, and metastatic NPC specimens in paraffin sections was performed by immunohistochemistry. The quantity of syndecan-1 mRNA in tumor cells was investigated by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) using Laser Capture Microdissection. The results of immunohistochemical staining of syndecan-1 and LMP-1 correlated with clinicopathological features of NPC. Eighteen of 86 (20.9%) primary, 9 of 37 (24.3%) recurrent, and 15 of 34 (44.1%) metastatic NPC samples were positive for syndecan-1; and 37 (43.0%) primary, 18 (48.6%) recurrent, and 12 (35.3%) metastatic samples were positive for LMP-1 expression. Primary NPCs with syndecan-1 protein expression were more frequently associated with advanced clinical stages and worse 5-year survival rates than those without (P=0.015 and P=0.0021, respectively). Conversely, LMP-1 expression did not correlated with tumor stage or prognosis, but occurred more often in non-keratinizing carcinoma than keratinizing squamous cell carcinoma (Unpublished observation). Inverse expression of syndecan-1 and LMP-1 was noted in primary NPC specimens (total 4/18 *versus* 35/68, P=0.05). The real-time RT-PCR revealed low syndecan-1 mRNA levels in both

primary and metastatic NPC. In conclusion, protein expression of syndecan-1 in 21% of primary NPC was associated with advanced disease and poor prognosis and protein expression correlated with transcription levels.

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common cancers in Taiwan [1]. Most patients have stage III or more advanced disease at the time of diagnosis [1]. NPC can be cured by radiotherapy alone or combined chemoradiotherapy, but recurrent or distant metastasis is common and the outcome of patients with recurrent or metastatic NPC remains poor. Still, patient with primary tumors have an initial 5-year survival rate of approximately 60% to 70% [1,2].

NPC is an Epstein-Barr virus (EBV)- associated malignancy that expresses a limited number of latent EBV genes, including Epstein-Barr nuclear antigen-1 (EBNA-1), latent membrane protein-1 (LMP-1), latent membrane protein-2A (LMP-2A), Epstein-Barr virus-encoded RNAs (EBERs), and *Bam*HI-A rightward transcripts [3]. LMP-1 can induce the expression of epithelial growth factor receptor (EGFR) in epithelial cells [4]. Also, LMP-1 has been shown to possess potent tumorigenic effect and promote tumor cell metastasis [5,6].

Epithelial malignancy metastasis and tumor invasion is a multi-step process involving several crucial events: the loosening of intercellular junctions, attachment of tumor cells to the extracellular matrix (ECM), degradation of the ECM, the migration of tumor cells through the ECM, angiogenesis, the detachment of tumor cells, vascular permeation, the homing of tumor cells and trafficking of cancer cells through blood vessels, extravasations, organ-specific homing, and growth [7,8]. Numerous molecules are involved in tumor invasion and metastasis, particularly heparin sulfate proteoglycans [7,8], which can interact with various effecter molecules, such as ECM molecules and growth factors [9], and are modulators of cell growth and differentiation. Syndecans belongs to a family of cell surface proteoglycans that associate with the actin cytoskeleton to help maintain the morphology of epithelial sheets [10,11].

Syndecan-1, also known as CD138, is the most extensively studied member of the syndecan family. The extracellular domain of syndecan-1 binds both growth factors and extracellular matrix components, while its cytoplasmic portion interacts with cytoskeletal components [10]. Syndecan-1 expresses mainly in epithelial cells and its expression is up-regulated during embryonic development [12,13]. Syndecan-1 is thought to be involved in the processes of cell growth, differentiation, and adhesion and it acts as a co-receptor for fibroblast growth factors (FGFs), a potent angiogenic growth factors involved in differentiation [9,14-16]. The expression of syndecan-1 appears to be generally

down-regulated in human carcinomas and in experimental cancer models, whereas transfectional expression of syndecan-1 in cultured cancer cells has been shown to inhibit their growth and other aspects of malignant behavior [17]. Loss of expression of syndecan-1 in tumor cells leads to decreased intercellular cohesion, increased potential for tumor invasiveness, and metastatic spread [18]. However, the expression of syndecan-1 is associated with poor prognosis in some malignancies, such as breast carcinoma [19].

This study examined the expression of syndecan-1 and LMP-1 in primary, recurrent, and metastatic NPC specimens using immunohistochemical staining and analyzed the mRNA quantity of the *syndecan-1* gene using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in primary and metastatic NPC tumor tissues using Laser Capture Microdissection (LCM) on frozen sections of NPC samples. The results correlated with histological types and clinical data, including age, sex, clinical stage, and outcome, in patients with primary NPC. The inter-relationship between expression of syndecan-1 and LMP-1 in NPC was also clarified.

Materials and Methods

A. Pathological Samples

In total, 157 formalin-fixed paraffin-embedded tissue samples from primary (n=86), recurrent (n=37), and metastatic (n=34) NPC tumors were obtained from the Department of Pathology, National Taiwan University Hospital (NTUH). Histopathologic classification of primary and recurrent NPC samples was based on the "Pathology and Genetics of Head and Neck Tumours" by the World Health Organization (WHO) in 2005 [20]. The staging system in this study was adapted from the International Union Against Cancer (UICC) 1997 [21]. Patients with primary NPC received radiotherapy and those with recurrent NPC received radiotherapy and/or chemotherapy for treatment. For patients with metastatic NPC, metastatic lesions in the neck, lung, or liver were excised. All patients were followed up at NTUH.

B. Immunohistochemical Study

Serial paraffin sections were cut to 6-μm in thickness for immunohistochemical studies of the expression of syndecan-1 and LMP-1 in NPC tumor cells. The paraffin sections were baked briefly at 60^{o}C and next deparaffinized and rehydrated using descending alcohol. After antigen retrieval with a 0.01 M citrate buffer at pH 6.0, these sections were incubated with monoclonal antibody against syndecan-1 (CD138) (clone B-B4, Serotec, Oxford, UK) at a 1:100 dilution and LMP-1 (clone CS1-4, Dako, Carpinteria, CA, USA) at a 1:50 dilution, respectively, followed by adequate linked antibody (LsAB, Dako, Carpinteria, CA, USA). For detecting syndecan-1, the reaction was colorized by diaminobenzidine (DAB) using a standard indirect avidin-biotin-peroxidase complex and for LMP-1, the reaction was

colorized by new fuchsin using an indirect avidin-biotin-alkaline phosphatase method. Then the sections were counterstained with Mayer's hematoxylin solution. The immunohistochemical staining results were arbitrarily classified into 4 scores depending on the intensity of immunoreactivity: 0, negative staining; 1+, <10% tumor cells with positive immunostaining; 2+, 10%–50% tumor cells with positive immunostaining, and 3+, >50% tumor cells with positive immunostaining.

For syndecan-1, plasma cells in tissues with chronic inflammation were used as the external positive control and plasma cells in stroma and non-tumor squamous or respiratory cells in tumor tissues were used as the internal positive control. The staining pattern of syndecan-1 was membranous on these cells. The reaction pattern of LMP-1 was cytoplasmic in the NPC tumor cells. The EBV-LMP1-positive and -negative controls included B95-8 and BJAB lymphoblastoid cell lines (LCL), respectively.

C. Quantify Syndecan-1 mRNA by Laser Capture Microdissection and Real-Time RT-PCR

Eight freshly frozen NPC samples, including 4 primary and 4 metastatic, were available for analyzing the mRNA of *syndecan-1* gene in this study. Laser capture microdissection (LCM) by AutoPix^{TM} (Arcturus, Mountain View, CA, USA) was used to dissect tumor cells from primary and metastatic NPC-frozen sections in order to study the quantity of *syndecan-1* gene mRNA in the tumor cells. The primer sequences of the *syndecan-1* gene in the current study were published previously [22] and were sense: 5'-GAG GGC TGC TGA GGA TGG A-3' and antisense: 5'-ATT CTC CCC CGA GGT TTC AA-3'. The *hypoxanthine phosphoribosyl -transferase (HPRT)* gene was used as internal control. The primer sequences of the *HPRT* gene for real-time RT-PCR in the current study were sense: 5'-TGA CAC TGG CAA AAC AAT GCA-3' and antisense: 5'-GGT CCT TTT CAC CAG CAA GCT-3'.

Real-time RT-PCR was carried out on an ABI Prism 7700 (Perkin-Elmer/-Applied Biosystems, Foster City, CA, USA), using SYBR-green as detection dye. Conditions for PCR included 50^{o}C for 2 min, 95^{o}C for 10 min, and 40 cycles of 95^{o}C for 15 sec (denaturation) and 60^{o}C for 1 min (annealing/extension). Relative mRNA amount of the target gene: internal control gene (HPRT) was calculated using the CT method as follows:

Relative expression = $2^{-\Delta CT}$; where CT = C_T (target gene)-C_T (HPRT).

D. Correlation Study of Expression of Syndecan-1 and LMP-1 and Clinical Data of Patients with Primary NPC

The results of syndecan-1 and LMP-1 protein expression by immunohistochemistry and *syndecan-1* gene mRNA by real-time RT-PCR in patients with primary NPC were correlated with presenting clinical factors including age, sex, clinical stage, outcomes, and histological classification of the studied patients. The clinical stage and follow-up data of the patients

studied were collected from the medical records of the Department of Registration, NTUH. In our series, 70 patients with primary NPC had enough clinical data available for staging analysis. For statistical analyses, only primary NPC patients were selected. Univariate statistical analysis was performed using Chi-square tests. The survival curve of patients with positive and negative for syndecan-1 was estimated by Kaplan-Meier analysis.

Results

A. Clinical Data

In total, 157 NPC samples were included in this study. Of these, 86 samples were from primary NPC. Patients with primary NPC included 62 males and 24 females whose ages ranged from 18 to 81 years. These primary NPC patients were histologically classified as follows: 7 with keratinizing squamous cell carcinoma (KSCC) and 79 with non-keratinizing carcinoma, 26 were the differentiated type and 53 were the undifferentiated type.

B. Expression of Syndecan-1 and Latent Membrane Protein-1 (LMP-1)

In the studied cases, metaplastic squamous cells are strongly positive for syndecan-1 and is used as internal control. Regarding the expression of syndecan-1 by immunohistochemical staining in formalin-fixed paraffin-embedded sections of 157 NPC specimens, 18 (20.9%) of 86 primary, 9 (24.3%) of 37 recurrent, and 15 (44.1%) of 34 metastatic specimens stained positively. The staining intensity of syndecan-1 in tumor cells was usually weaker than squamous cells of skin or plasma cells in the NPC specimens. In addition, stromal tissue in primary and recurrent NPC specimens did not show any positive staining for syndecan-1 but 6 of the metastatic NPC samples showed positively staining stromal tissue, while the tumor tissue was negative in two of them.

Regarding the expression of LMP-1 in NPC, 37 (43.0%) of 86 primary, 18 (48.6%) of 37 recurrent, and 12 (35.3%) of 34 metastatic specimens stained positively. The staining pattern in NPC tumor cells was cytoplasmic stippling but was not membranous. The infiltrating lymphocytes in both tumor and overlying epithelium in all samples were negative.

In the LMP-1-positive group, 4 of 37 primary, 3 of 18 recurrent, and 5 of 12 metastatic NPC tissues had co-expression of syndecan-1 in tumor cells while in LMP-1-negative group, 14 of 49 primary, 6 of 19 recurrent, and 10 of 22 metastatic NPC samples expressed syndecan-1. In contrast, in the syndecan-1-positive group, 4 of 18 primary, 3 of 9 recurrent, and 5 of 15 metastatic NPC specimens co-expressed LMP-1 in tumor cells and in the syndecan-1-negative group, 33 of 68 primary, 15 of 28 recurrent, and 7 of 19 metastatic NPC samples expressed LMP-1.

The real-time RT-PCR study of the *syndecan-1* gene mRNA revealed a low amount of syndecan-1 mRNA in 4 primary NPC and 4 metastatic NPC specimens as compared with the

internal housekeeping (*HPRT*) gene. The *HPRT* gene mRNA was detected as early as 28 cycles, but the syndecan-1 mRNA was detected only after 35 to 37 cycles.

C. Clinicopathological Correlation of Expression of Syndecan-1 and LMP-1

The statistical significance of syndecan-1 and LMP-1 expression was analyzed in regards to the clinical data, including age, sex, clinical stage, survival, and the histological types of primary tumors in NPC patients. Statistical significance was observed in NPC specimens expressing syndecan-1 when correlated with clinical stage and survival of patients. Syndecan-1 was expressed more often in patients at stage III of IV (P=0.015) and in patients who survived less than 5 years (P=0.0021). The expression of syndecan-1 in NPC tumor specimens did not correlate with age, sex, and histological types of patients.

The expression of LMP-1 in primary NPC samples did not correlate with age, sex, clinical stage, and patient survival rates, but it did correlate with histological type of NPC samples with less expression of LMP-1 in KSCC cases than in non-keratinizing carcinoma.

Statistical analysis of syndecan-1 and LMP-1 expression in tumor tissues of primary versus recurrent or primary versus metastatic NPC specimens revealed considerable differences between primary and metastatic NPC, but no significant difference in syndecan-1 expression in primary versus recurrent NPC or LMP-1 expression in primary versus recurrent or primary versus metastatic NPC specimens.

Regarding the co-expression of syndecan-1 and LMP-1 in NPC specimens, statistical significance was noted in primary NPC samples but not in recurrent or metastatic specimens (P=0.05).

Discussion

The significance of syndecan-1 expression has been studied in many malignancies and inverse significance has been found in different tumors. For example, expression of syndecan-1 correlates with the differentiation of tumor cells, low clinical stage, and favorable prognosis in carcinomas of the head and neck regions [23], esophagus [24], larynx [25], liver [26], lung [27], colon [28], and uterine cervix [29]; while inverse results are noted in malignancies of the breast [19], prostate [30], and thyroid [31].

Since syndecan-1 expression has prognostic significance in squamous cell carcinoma of the head and neck [23], this study examined the expression of syndecan-1, CD138, and EBV-encoded latent membrane protein-1 (LMP-1) of Epstein-Barr virus (EBV) in primary, recurrent, and metastatic NPC specimens by immunohistochemical stain, analyzing the quantity of mRNA of *syndecan-1* gene via real-time RT-PCR. To the best of our knowledge, this is the first study of syndecan-1 expression and its clinicopathological significance in NPC.

The current results show that syndecan-1 expression in NPC tumor cells is uncommon and that the expression level is low in most tumors expressing syndecan-1. These results might correlate with poorly differentiated or undifferentiated NPC tumor cells because morphologically most NPC tumors are composed of poorly differentiated or anaplastic tumor cells [32]. However when correlated the results of syndecan-1 expression and histologic types of primary NPC specimens, no significant difference was observed. Instead and surprisingly, the expression of syndecan-1 in NPC samples was statistically related to high clinical stages and poor outcomes for patients with primary NPC. These results were in contrast to those of squamous cell carcinomas of other head and neck regions in which syndecan-1 expression indicates a better-differentiated tumor and a more favorable outcome [23].

Our data suggest that syndecan-1 expressed by NPC tumor cells may play a role in the progression of NPC. The reason for this inverse prognostic implication of syndecan-1 in NPC and other head and neck squamous cell carcinomas is not known but may contribute by the known role of syndecan-1 in tumor progression. Syndecan-1 is known to interact with heparin-binding growth factors (HBGFs) and fibroblast growth factors (FGFs), which are known angiogenic and mitogenic growth factors for many tumors [9,14-16]. It could be hypothesized that high syndecan-1 expression in NPC may confer a particularly important growth advantage by enhancing the response to the other growth factors [19].

To elucidate the possible mechanism of this uncommon syndecan-1 expression in NPC samples, we analyzed the quantity of mRNA of the syndecan-1 gene via real-time RT-PCR. These data support the immunohistochemical results showing that the quantity of *syndecan-1* gene mRNA was low in most NPC specimens when compared with the amount of mRNA of housekeeping gene. We concluded that this down-regulation of syndecan-1 expression in primary and metastatic NPC was at the transcription level. These results were similar to a previous report by Fujiya et al., who found that down-regulation of syndecan-1 in colon carcinomas is due to hypermethylation of the *syndecan-1* gene [28].

Recent experimental studies surrounding the role of syndecan-1 in malignant transformation have shown that syndecan-1 expression is associated with the maintenance of epithelial morphology, anchorage-dependent growth and inhibition of invasiveness in vitro [15,16]. The down-expression of syndecan-1 in primary and recurrent NPC might lead to tumor cells having a higher potential for early metastasis to regional lymph nodes or other visceral organs as noted in NPC patients clinically. However, no statistical significance was noted in the expression of syndecan-1 in primary and recurrent NPC or between primary and metastatic NPC, although there was a higher percentage of syndecan-1 expression in metastatic NPC samples. The reason for this higher syndecan-1 expression in metastatic NPC samples was not clear.

Induced expression of syndecan-1 in stromal tissue of different types of malignancy has

been reported [18,31]. The induced expression of syndecan-1 in stromal tissue might stimulate the growth of epithelial cells and also contribute to tumor cell invasion and development of metastasis [33,34]. In our study, expression of syndecan-1 in stromal tissue was not found in primary or recurrent NPC specimens but 6 samples of metastatic NPC had syndecan-1 expression in stromal cells and the tumor cells were negative in 2 cases.

EBV-encoded LMP-1 has been found to have potent tumorigenic effect in epithelial cells and it can promote the metastasis of NPC tumor cells [5,6]. Up-regulation of matrix metalloprotease-9 (MMP-9) correlates with metastatic potential in NPC and the up-regulation of MMP-9 in NPC is associated with expression of LMP-1 [5]. LMP-1 also can induce expression of interleukin-8 (IL-8) through the nuclear factor kappa B (NF-κB) pathway resulting in angiogenesis in NPC tumors [35]. In the current study, expression of LMP-1 was detected in primary, recurrent, and metastatic NPC and the overall detection rate of LMP-1 in our NPC specimens was lower than previous reports. This might be due to inadequate processing of the tissue samples before immunohistochemical study.

The expression of LMP-1 in all primary NPC specimens classified as KSCC was negative, while 37 out of 79 NPC samples classified as non-keratinizing carcinoma were positive. There was statistical significance when correlating the histological types of primary NPC and the status of LMP-1 expression. When analyzing the expression of LMP-1 in primary NPC samples and clinical data including age, sex, clinical stage, and outcomes of patients with primary NPC, no statistical significance was found. Additionally, there was no statistically significant difference between LMP-1 expression between primary and recurrent NPC or between primary and metastatic NPC.

In regarding the inter-relationship of expression of syndecan-1 and LMP-1 in NPC, co-expression of syndecan-1 and LMP-1 was noted in this study, which indicates that the expression of syndecan-1 in NPC might be induced by the expression of LMP-1 in tumor cells and that the expression of both molecules might result in angiogenesis and aggressive behavior of NPC tumors.

In conclusion, in the present work we studied two important molecules in tumorigenesis, angiogenesis, and metastatic ability in NPC tumor cells: syndecan-1 and EBV-encoded LMP-1. We found that syndecan-1 is uncommonly expressed in NPC samples; that its expression correlates with advanced clinical stages and poor outcomes; and that the down-regulation of syndecan-1 in NPC specimens is at the transcription level. Syndecan-1 expression in NPC tumors did not correlate with age and sex of the patients and did not correlate with the histological types of the NPC samples. A certain proportion of NPC specimens expressed LMP-1, and the KSCC samples were usually LMP-1 negative. The expression of LMP-1 in NPC samples correlated to the histological types of the primary NPC specimens, but had no statistically significant correlation with age, sex, clinical stage, or survival of patients with primary NPC. The expression of syndecan-1 in primary NPC

correlated to the expression of LMP-1.

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附 錄 二

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Chemokine receptor expression profiles in nasopharyngeal carcinoma and association with metastasis and radiotherapy

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Key words: chemokine receptors; nasopharyngeal carcinoma; metastasis; radiotherapy

Running title: Chemokine receptor expressions in nasopharyngeal carcinoma

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Abstract

Nasopharyngeal carcinoma (NPC) is an epithelial cancer which metastasizes predictably to cervical lymph nodes or distant organs. In order to assess whether the chemokine receptors of NPC cells play important roles in metastasis and are associated with radiotherapy history, the significance of various chemokine receptors (CCR1-10, CXCR1-6, XCR1 and CX3CR1) in NPC cell lines (TW01, TW04, HONE1, BM1 and AS1) and 52 NPC tumor biopsies from 48 NPC patients was evaluated via mRNA and cytometric analyses, chemotaxis and actin polymerization assays, and immunohistochemical staining. Quantitative real-time reverse transcription-PCR revealed substantial expressions of CCR7, CCR9, CXCR4 and CXCR6 mRNA in all of the NPC cell lines. Of these, however, only CCR7, CXCR4 and CXCR6 were functional in NPC cells. Negative immunoreactivity to CCR7, CXCR4 and CXCR6 was demonstrated for almost all nasopharyngeal (NP) specimens from patients with primary NPC (n=12) and analogs with regional metastatic NPC (n=13). However, expression of two or

three of these chemokine receptors was demonstrated in NP specimens of patients with liver metastasis. Strong positive results were demonstrated for all three of these chemokine receptors for almost all of the regional and distant-metastasis specimens. The significance of the differences in positive expression for CCR7, CXCR4 and CXCR6 between primary tumors and metastases was $p<0.001$, $p<0.001$ and $p<0.002$, respectively. This observation was further confirmed using laser capture microdissection of freshly frozen tumors from primary (n=5) and metastatic (n=8) NPC sites ($p=0.04$, 0.03 and 0.03 for CCR7, CXCR4 and CXCR6, respectively). Finally, significant differences in CXCR4 expression were demonstrated comparing de novo and post-radiotherapy groups (1/22 vs. 5/8; $p<0.003$). It appears reasonable to conclude, therefore, that CCR7, CXCR4 and CXCR6 expressions are expressed and active in human nasopharyngeal carcinoma metastases, while CXCR4 expression is associated with radiotherapy history.

Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial cancer. Unlike cancers of the oral cavity and oropharynx, metastatic nodal disease in NPC frequently appears in the posterior cervical triangle. Bilateral neck nodes are a site of neoplasm in 53% of patients. Bone, liver and extra-regional nodes are the most common sites of distant metastases [1].

Metastasis of cancer cells is a complex, highly organized, non-random and organ-selective process. It involves multiple steps, including dissemination of metastasizing cells from the primary tumor, invasion of the surrounding tissue, intravasation and extravastion of the circulatory system, evasion of the immune system, organ-specific targeting, initiation of angiogenesis, and growth advantage at the target organ [2,3]. The target organ for the metastasis depends on a variety of factors, such as growth advantage [4,5], the presence of adhesion molecules [6], and chemokine/chemokine receptor interaction [7]. It is proposed that interplay among these factors might vary across various cancer types.

A complex network of chemokines and their receptors influences the development of primary tumors and metastases [8-10]. Recent studies have clearly demonstrated the importance of chemokine receptor (CR) expression in metastasis to specific organs (i.e., lymph nodes, bone marrow, liver and lungs) by breast cancer [11], melanoma [12] and gastric carcinoma cells [13]. CCR6-expressing colon, thyroid and ovarian carcinoma cells are reportedly associated with hepatic metastasis [14]. It has been demonstrated that CCR9 contributes to the localization of plasma cells to the small intestine [15,16]. CXCR1 and CCR10-expressing melanoma cells may also target endothelial and dermal cells [17,18]. Evidence with respect to the association between specific chemokine(s)/chemokine receptor(s) and NPC metastasis is limited, except for two recent reports about CXCR4 [19,20]. In this study, therefore, we screened for expression of all known CRs in five NPC cell lines (TW01,

TW04, HONE1, BM1, AS1) using quantitative real-time (qRT-PCR), and then assessed the CR pattern of the NPC cell lines and a variety of tumor tissues samples from 48 NPC cases using flow cytometry analysis and immunohistochemical (IHC) staining.

Materials and Methods

Extraction of RNA from NPC Cell Lines and RT-PCR

NPC cell lines TW01, TW04 and, HONE1 were derived from primary nasopharyngeal tumors from Chinese patients with *de novo* NPC [21-23]. Another two NPC cell lines were derived from metastasized tumor cells in the bone marrow (BM1) and lymph nodes (AS1) of Chinese NPC patients [24]. These cell lines were a generous gift from Dr. Ann-Lii Cheng (Cancer Research Center, NTUH, Taiwan). Total RNAs were isolated from various cell lines using RNAzolTM B Reagent (Tel-Test, Friendswood, TX, USA) and then reverse-transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase (Epicentre, NJ, USA) with oligo-dT and random hexamer under standard conditions. The primer sequences and PCR conditions for RT-PCR of 18 kinds of chemokine receptors were as reported by Nakayama [25].

Quantitative Real-time RT-PCR (qRT-PCR)

The primers for qRT-PCR of CXCR4, CXCR6, CCR7 and CCR9, which were designed using Primer Express software (Perkin Elmer Applied Biosystems, Foster City, CA, USA), are shown in Table 1. QRT-PCR was carried out on an ABI Prism 7700 (Perkin Elmer Applied Biosystems) using SYBR green as the detection dye; conditions were 50^{o}C for 2 min, 95^{o}C for 10 min, and up to 40 cycles of 95^{o}C for 15 sec (denaturation) and 60^{o}C for 1 min (annealing/extension). The dissociation curve following the reaction confirmed the specificity of this quantitative assay. The amount of target gene mRNA relative to the internal control gene, HPRT, was calculated using the ΔCT method as follows:
Relative expression = $2^{-\Delta CT}$; where ΔCT = CT (target gene) - CT (HPRT).

Flow Cytometry Analysis

For surface CR detection, 2 x 10^5 NPC cells were incubated at 2-8^{o}C for 40 min with nonspecific isotype-matched controls and 10-25 μg/mL of each of the following murine monoclonal antibodies: anti-human CXCR4 (clone 44708.111); anti-human CXCR6 (clone 56811.111); and, anti-human CCR7 (clone 150503) or anti-CCR9 (clone 112509). All of the above antibodies were purchased from R&D Systems, Minneapolis, MN, USA. The mouse primary antibodies were then detected by incubating them at room temperature for 30 min with fluorescein isothiocyanate (FITC)-conjugated goat $(Fab')^2$ anti-mouse IgG (Serotec, UK). After being washed twice with PBS, the cells were resuspended and fixed in 1% (w/v)

paraformaldehyde for analysis. Ten thousand cells from each sample were evaluated for fluorescence detection using FACScan (Becton Dickinson, San Jose, CA), and the data were analyzed with CellQuest software (Becton Dickinson).

For intracellular CR detection, permeabilized NPC cells were prepared with 0.2% saponin reagent after 4% (w/v) paraformaldehyde fixation, and subjected to the above procedure. These permeabilized cells were maintained in staining buffer containing 0.2% saponin to ensure complete membrane permeabilization throughout.

Chemotaxis Assay

Chemotaxis assay was performed in 24-well cell-culture chambers using inserts with 8-μm pores (Cambridge, MA, 6.5-mm diameter), the membranes of which were pre-coated with 2.5-μg fibronectin. The upper chamber was seeded with 2 x 10^4 cells/well, and various chemokines were added to the lower chamber. After incubation for 24 hours, the migrated cells attracted by chemokines were attached beneath the membrane. After removing the cells in the upper well with Q-tips, the migrated cells were stained with Liu's stain and quantified by counting five random fields with a light microscope at low power (100X). All experiments were performed in triplicate. The migration index was calculated as follows:

Migration index = migrated $cells_{chemoattract}$/migrated $cells_{serum\text{-}free\ medium}$

Actin polymerization assay

Actin polymerization assay was performed according to the standard protocol [26,27]. Briefly, cells were incubated with 150 ng/mL CXCL16 and 100 ng/mL CXCL12 or with 25 ug/mL fibronectin as positive control. At the indicated time points, cells were fixed, permeabilized and stained in a solution containing 18% paraformaldehyde, 100 μg 1-P-lysophosphatidyl-choline (Sigma-Aldrich, St Louis, MO, USA) and FITC-labeled phalloidin (Molecular Probes, Eugene, OR, USA). Actin polymerization was analyzed by flow cytometry, with the mean fluorescence ratio of the sample before the addition of chemokine plotted for all time points. For confocal microscopy analysis, cells were pre-seeded and incubated with 150 ng/ml CXCL16 or assay buffer DMEM for 30 sec. Cells were then fixed, permeabilized, and stained with FITC-labeled phalloidin and 100 nM Hoechest (Sigma-Aldrich). Images were visualized with a Leica TCS SP2 confocal spectral microscope (Leica Microsystems, Heidelberg, GmbH).

Patients

From 1993 to 1997, formalin fixed paraffin-embedded NPC tissue samples from 48 NPC patients (Table 2) and freshly frozen tissue samples of NPC tumors from 12 additional NPC patients were collected at the National Taiwan University Hospital (NTUH). This study was approved by the institutional review board (IRB) of NTUH. Written informed consent

was obtained from all patients. Pathologists confirmed the histopathological diagnosis for each specimen. Histopathological classification in these cases was performed according to the revised histological classification for tumors of the upper respiratory tract and ear by the World Health Organization (WHO) in 1991.

Expression Study of Chemokine Receptors in NPC Tissues

All of the formalin-fixed-paraffin-embedded tissue samples of NPC tumors sliced to a thickness of 6 μm were used and mounted on Silane-coated slides (Dako, Carpinteria, CA, USA). After antigen retrieval by heating in a microwave for 5 min with citrate buffer (pH 6.0), these sections were then reacted with anti-human CXCR4, anti-human CXCR6, or anti-human CCR7 using a standard indirect avidin-biotin-peroxidase method. Chemokine receptor-expression breast cancer-tumor tissues were used as positive controls. The specificity of immunostaining was also confirmed by use of serial sections with nonimmune serum instead of the primary antibody as a negative control. The specimens were evaluated independently without prior knowledge of the clinicopathological information. The results of IHC for each chemokine receptor were arbitrarily classified according to score, based on the percentage of immunoreactive tumor cells: 0, negative immunostaining; and, 1+, 2+ and 3+ for <10%, 10–50% and >50% positive immunostaining, respectively.

Laser Capture Microdissection (LCM)

For quantitative analysis of mRNA of chemokine receptors in NPC, freshly frozen primary and metastatic NPC tumor samples were used. The frozen tumor tissues were cut to a thickness of 8 μm. The sections were dehydrated and stained with hematoxylin, and then the tumor cells were microdissected using the AutopixTM system (Arcturus Engineering, Mountain View, CA, USA) according to the manufacturer's instructions. The selected cells were transferred to the LCM transfer film (CapSure TF-100S transfer film carrier, optical-grade transparent plastic, 5-mm diameter; Arcturus Engineering).

The dissected specimens were placed directly into 50 μl of cell lysis buffer consisting of 10 mmol/L Tris-HCl (pH 7.4), 20 mmol/L EDTA, 0.5% SDS and 20 μL/mL Proteinase K (Wako, Osaka, Japan), and incubated at 45^{o}C for 30 min. Extraction of total RNA, cDNA synthesis and real-time PCR were then performed using the above protocols.

Statistical analysis

The statistical significance of the individual findings and their association indices were evaluated using the chi-squared test with Yates' correction. Probability (*P*) values less than 0.05 were considered significant.

Results

RT-PCR and qRT-PCR analysis revealed that NPC cell lines exhibited different amounts of CCR7, CCR9, CXCR4 and CXCR6 mRNA A panel of 18 known CRs (CCR1 – CCR10, CXCR1 – CXCR6, XCR1 and CX3CR1) were examined in five NPC cell lines (TW01, TW04, HONE1, AS1 and BM1) with semi-quantitative RT-PCR, and showed substantial amounts of mRNA of CCR7, CCR9, CXCR4, and CXCR6. Subsequent qRT-PCR revealed that all cell lines demonstrated a similar trend for CR expression of CCR9>CXCR6>CCR7>CXCR4, except AS1 cells, which showed abundant CXCR4 (0.17:1 relative to HPRT) and little CCR7 (10-4:1 relative to HPRT) expression.

Flow cytometry analysis, migration activity assay and actin polymerization assay reveal functional CCR7, CXCR4 and CXCR6 in NPC cells

Flow cytometry analysis showed: (i) the CXCR4 protein was expressed only on the cell membrane of AS1 cells (in keeping with their high mRNA content); (ii) the CXCR6 protein was expressed on the cell surface of TW01, TW04, as well as BM1 cells; and, (iii) the CCR7 protein was expressed on the cell surface of TW04 and BM1 cells. The NPC cells carrying CRs on membrane also had abundant same CRs in their cytoplasm. In contrast, CCR9 was only found in the cytosol but not on the membrane, despite the high mRNA levels of the latter in NPC cells.

Further Transwell® migration assay verified that CCL21 and CXCL12 could attract migration of NPC cells with surface CCR7 and CXCR4, respectively, just like other carcinoma cells. In addition, CXCL16 dose-dependently attracted migration of TW01 and TW04 cells (both CXCR6 positive) but the same results were not obtained with BM1 cells even if they expressed substantial CXCR6 on the membrane, which could be explained by the substantially higher spontaneous migration of BM1 cells, relative to TW01, TW04 and HONE 1 cells (136.3+5.3/LPF, 18.8+1.3/LPF, 28.8+3.9/LPF, 33.1+0.9/LPF, respectively). Further actin polymerization assay by flow cytometry and confocal microscopy revealed that 150 ng/ml CXCL16 induced a transient 1.7-fold increase in intracellular F-actin content in TW01 cells within 15 sec, and showed distinct pseudopodia formation and intense F-actin staining near the periphery of the TW01 cells treated with CXCL16, suggesting the morphological changes triggered by the interaction between CXCR6 and CXCL16 are prerequisites for TW01 cell mobility. Similar phenomena were observed when TW04 was exposed to CXCL16.

Significant differences in CXCR4, CXCR6 and CCR7 expression between primary and metastatic tissues; only CXCR4 expression is associated with radiotherapy history

The IHC staining of CCR7, CXCR4 and CXCR6 showed that these CRs were detected in both cytoplasm and membrane of metastatic tumor tissues, but not in primary NPC tumors.

Treatment status for NPC patients at the time of tumor-specimen collection, the associated site and chemokine receptor expressions are summarized in Table 2. We found that nasopharyngeal (NP) specimens from patients with primary NPC (n=12) all showed negative immunoreactivity to CCR7, CXCR4 and CXCR6, except for one case (patient 11), who had previously received chemotherapy treatment. Negative immunoreactivity was also demonstrated for all NP specimens from patients with regional metastatic NPC (n=15), except for those from four individuals (patients 18, 20 26, 27). All three exceptions were CXCR6-positive only. Heterogeneous expression of CXCR4, CCR7 and CXCR6 was demonstrated for NP specimens from liver metastasis cases (patients 29 and 30). These results were similar to those for the above-described primary NPC cell lines, TW-01, TW-04 and HONE1. However, specimens from regional and distant metastases (neck mass, axilla and inguinal lymph node, lung and liver) were strongly positive for all three CRs (patients 32-48), except for those from two subjects (patients 42 and 48). Intriguingly, NP specimens from brain-invasion cases (patients 28 and 31) were also CXCR4 and CCR7-positive to different extents. Three of these individuals also had sequential evaluations (patients 9, 40 and 41), which revealed associations between CR expression and the existence of metastasis as well as its type. Overall statistical analysis of the expression of these three CRs in NPC cases with metastasis demonstrated significant differences between tumors from primary and metastasized sites. Furthermore, significant differences were only demonstrated for CXCR4 comparing nasopharyngeal specimens of patients with or without radiotherapy history (5/8 vs. 1/22; $p<0.003$), while no relationship was demonstrated between CCR7 and CXCR6 and radiotherapy history (Table 2).

To further assess the RNA expression of CR in NPC tumor tissues, we evaluated 13 freshly-frozen NPC tumor samples from 12 additional NPC patients. Out of them, paired NP and metastases specimens were studied from one patient, and only NP or metastases specimen from the other patients. LCM coupled with qRT-PCR was used to compare the relative mRNA amount in NP specimens (n=5) and metastases (n=8) (as mean+SD), respectively, as follows: CCR7 to HPRT (internal control gene), 0.029+0.051 and 0.437+0.367 ($p=0.04$); CXCR4 to HPRT, 0.209+0.281 and 1.389+0.824 ($p=0.03$); CXCR6 to HPRT, 0.005+0.008 and 0.191+0.091 ($p=0.03$). The differences between the NP specimens and metastases were statistically significant for all comparisons, with these results consistent with the IHC finding. As to the only paired specimen all three CR mRNA showed substantial amount in metastases and few in NP specimen.

Discussion

As part of the manuscript preparation, two reports investigating the relationship between CXCR4 and NPC were considered. Wang et al., [19] found that high CXCR4 expression was

associated with poor overall survival, while Hu et al., [20] revealed that expression of functional CXCR4 is associated with the metastatic potential of human NPC cells. In this study, comprehensive *in vitro* and *in vivo* survey for chemokine receptor expression revealed that, in addition to CXCR4, CCR7 and CXCR6 were significant in NPC metastasis. Further, only CXCR4 expression was associated with radiotherapy history.

Our *in vitro* data reveal that NPC cells can express CCR7, CXCR4 and CXCR6 in both cytoplasm and cell membranes. It has been shown that: CCL21, a ligand for CCR7, is highly expressed in the lymph node [11]; CXCL12, a ligand for CXCR4, is moderately expressed in the lymph node, lung, liver and bone marrow [11]; and, CXCL16, a ligand for CXCR6, is highly expressed in the lung and liver [28,29] and moderately expressed in the lymph node [30]. Therefore, the expression of CCR7, CXCR4, or CXCR6 in NPC tumor cells may be responsible for the potential of NPC to metastasize to lymph node, bone, liver and lung [1]. However CCR9 proteins remain in the cytoplasm and are not expressed on the cell membrane; and this may be reason why NPC cells do not usually metastasize to intestine, which contains abundant CCL25, a ligand of CCR9 [15, 31]. Nude mouse-transplantation experiments with TW01 cell lines [21] demonstrated that many small metastatic granules are disseminated in the lung parenchyma, and a few in the liver. These results are consistent with our finding of only CXCR6 expression on the membrane surface of TW01 cells. Further, the CXCR6/CXCL16 signaling is recently indeed associated with liver-specific homing [32-35] and lung-specific homing [36,37]. Here, we also revealed the CRs expressed on NPC cell membrane were functional, as evidenced by the biologic consequences, including chemotaxis and actin polymerization induced by their cognate ligands. Accordingly, the circulating NPC cells with functional CR might arrive and proliferate at distant metastatic site where the respective ligands are expressed. Recently, Scala's group also reported similar mechanism that CXCR4 is expressed and active in human melanoma metastases [38].

Our in vivo data reveal that by using IHC analysis for tumor biopsies from NPC patients with metastasis, there was heterogeneous expression of CXCR4, CCR7 and CXCR6 in the primary tumor specimens of patients with regional or distant metastasis, and abundant expression of these CRs in the metastatic NPC of analogs with regional or distant metastasis. These CRs expression were detected in both cytoplasm and membrane in these metastases. Similar features were reported in tumor biopsies from breast cancer, colorectal cancer, melanoma and squamous cell carcinoma [38-41]. Two possibilities are suggested for the differential expression of CR between primary and metastatic NPC. One is that cancer cells with CR expression in primary NP tumor may be selected for distant metastasis, and the other is that environmental factors may up-regulate the CRs expression of the metastasizing cells [42-44]. The latter possibility has been supported by several studies using in vitro cell systems, which revealed a cytokine-mediated induction of CXCR4 expression [42, 45].

Another interesting finding from our IHC analysis of NPC tissues is that the CXCR4

expressions were significantly associated with positive radiotherapy history. Recently, several studies have proposed that cancer cells undergo a hypoxia-reoxygenation process after radiotherapy, which then leads to nuclear accumulation of hypoxia-induced factor-1α (HIF-1α) [46, 47], and subsequently to the regulation of HIF-1α-responsible genes such as CXCR4 and VEGF [48, 49]. Staller et al., also provided convincing evidence that CXCR4 is a HIF-1α-regulated gene, whose expression can be negatively regulated by the von-Hippel-Lindan (VHL) protein [50]. Therefore, the HIF-α1-CXCR4 pathway may regulate trafficking in and out of hypoxic tissue microenvironments and trigger a homing mechanism that enables the migrating cells to target specific organs [51]. These findings are consistent with our results of high CXCR4 expression by NPC tumor cells in post-radiotherapy patients. As CXCR4 is a prominent HIV-1 receptor, several inhibitors for it have been recently generated as potential therapies to block HIV-1 infection, and consequently may theoretically block direct interactions between CXCR4 and CXCL12. Hence, our finding may be useful in the future development of novel strategies for targeting hypoxic NPC tumors.

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Table 1. Primer sequences for quantitative real-time RT-PCR analysis and amplicon sizes

	Forward primer	Reverse primer	Amplicon (bp)	Genebank accession number
CCR7	5'-TTCAGTGGCATGCTCCTACTTCT-3'	5'-GCTGAGACAGCCTGGACGAT-3'	71	AY587876
CCR9	5'-CATTGACGCCTATGCCATGTT-3'	5'-GGTGACCTGGAAGCAGATGTC-3'	73	AJ132337
CXCR4	5'-TGACCGCTTCTACCCCAATG-3'	5'-AGGATAAGGCCAACCATGATGT-3'	72	AF348491
CXCR6	5'-GCCATGACCAGCTTTCACTACA-3'	5'-TTAAGGCAGGCCCTCAGGTA-3'	68	NM_006564
HPRT	5'-TGACACTGGCAAAACAATGCA-3'	5'-GGTCCTTTTCACCAGCAAGCT-3'	94	M31642

CCR7: CC chemokine receptor 7; CCR9: CC chemokine receptor 9; CXCR4: CXC chemokine receptor 4; CXCR6, CXC chemokine receptor 6; HPRT: hypoxanthine phosphoribosyl transferase

Table 2 Comparison of chemokine receptor expressions in primary, metastases, and post-radiotherapy tumors

	CCR7#		CXCR4#		CXCR6#	
	Positive	Negative	Positive	Negative	Positive	Negative
NP tumor (n=31)						
From NPC patients with metastasis (n=19)	3	16	6	13	6	13
From NPC patients without metastasis (n=12)	0	12	0	12	1	11
P value	0.410		0.089		0.286	
NPC patients with metastasis (n=36)						
NP tumor (n=19)	3	16	6	13	6	13
Metastases (n=17)	16	1	17	0	15	2
P value	<0.001*		<0.001*		<0.002*	
NP tumor (n=30)						
From NPC patients without radiotherapy (n=22)	1	21	1	21	3	19
From NPC patients after radiotherapy (n=8)	2	6	5	3	3	5
P value	0.335		<0.003*		0.353	

Chemokine receptor expressions evaluated as stated in Table 1; negative: 0; positive: 1+ ~ 3+.

* Significant difference between groups stated