Proteomic Comparison of Nasopharyngeal Cancer Cell Lines C666-1 and NP69 Identifies Down-Regulation of Annexin II and $\beta_2$-Tubulin for Nasopharyngeal Carcinoma

Charles M. L. Chan, MPhil; S. C. Cesar Wong, PhD; Money Y. Y. Lam, MPhil; Edwin P. Hui, MD; John K. C. Chan, MD; Elena S. F. Lo, MSc; W. Cheuk, MD; Manson C. K. Wong, BSc; S. W. Tsao, PhD; Anthony T. C. Chan, MD

Nasopharyngeal carcinoma (NPC) is endemic in southern China, Southeast Asia, and North Africa, but it is rare in most parts of the world. The highest incidence of NPC is in the central region of the Guangdong Province in southern China and in Hong Kong (25–30 per 100,000 persons per year). Although patients with stages I and II disease have a high rate of cure with radiotherapy (RT) alone, the prognosis for those with locally advanced, non-metastatic stage III or IV disease and distant metastatic spread remains poor. The main reasons accountable for this poor outcome are increased toxicity of concurrent chemoradiotherapy and the resistance of chemoradiotherapy with time. Therefore, there is an urgent need to look for novel biologic agents that can potentiate the efficacy and overcome the resistance of chemoradiotherapy without exacerbating toxicity.

Recent improvements in 2-dimensional (2D) electrophoresis, image analysis, mass spectrometry, and the development of advanced bioinformatic databases and analysis software have enabled proteomics technology to identify disease-associated protein markers that are helpful in diagnosis or prognosis. Previously, this technology has been applied to analyze many cancers, such as prostate cancer, ovarian cancer, breast cancer, and lung cancer. There are still no data comparing the protein expression profiles between NPC and normal NP cells. In this study, we investigated the proteomic changes between an Epstein-Barr virus (EBV)–associated NPC cell line (C666-1) and a normal nasopharyngeal epithelial cell line (NP69). We chose EBV-harboring NPC cell line C666-1 because EBV is present in almost all NPCs and high EBV load is related to the course of disease. Therefore, the findings may aid in better understanding of the etiology and pathogenesis of NPC, and possibly help to discover some novel markers for early diagnosis, prognosis, and specific drug therapy.

Results.—Proteomic findings indicated that adenosine triphosphate synthase $\alpha$ chain was up-regulated, whereas annexin II, annexin V, $\beta_2$-tubulin, and profilin 1 were down-regulated. After confirming the results in agar-processed cell lines, annexin II and $\beta_2$-tubulin expression were found to be lower in tumor cells than in adjacent normal epithelial cells in 100% and 90% of the patients’ specimens, respectively. Finally, annexin II down-regulation was positively associated with lymph node metastasis, suggesting that it may be a prognostic factor in NPC.

Conclusions.—The results suggest that annexin II and $\beta_2$-tubulin down-regulation is important in NPC formation and may represent potential targets for further investigations.

(Arch Pathol Lab Med. 2008;132:675–683)
were scraped thoroughly with a scraper and allowed to lyse on land), pH 8.8, was then added to each culture dish. The cells Complete protease inhibitor (Roche Diagnostics, Basel, Switzerland) 0.6% pH 3 to 10 nonlinear-imobilized pH gradient buffer, 1 M urea, 4% CHAPS, 10mM dithiothreitol, 50mM dithiothreitol, 50mM dithiothreitol, pH 8.8) for 30 minutes with shaking at room temperature. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was then performed for 220 V-h in 4% to 12% Bis-Tris Criterion XT precast gels (Bio-Rad Laboratories). The proteins were fixed in the gel, and silver staining was performed using PlusOne Silver Staining kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instructions.

Agar Block Preparation for C666-1 and NP69 Cell Lines

The cells harvested after trypsinization were fixed in 7.5% buffered formalin for 18 hours followed by centrifuging at 1560g for 10 minutes. The supernatant was discarded, and 300 μl of 2% agar was added to the cell pellet. The mixture was then placed on ice to solidify the agar with the cells before processing to become paraffin-embedded blocks for in situ hybridization and immunocytochemical staining.

In Situ Hybridization

The aim was to confirm the EBV status in the C666-1 and NP69 cell lines. Four-micrometer sections from C666-1 and NP69 agar-processed cell lines were cut for EBV-encoded RNA (EBER) detection using EBER peptide nucleic acid probe/fluorescein (Y5200, DakoCytomation, Glostrup, Denmark). Pretreatment, hybridization, and stringent wash were performed according to the instructions of a peptide nucleic acid in situ hybridization detection kit (K5201, DakoCytomation). Posthybridization detection and counterstaining were performed in the Bond-max automated immunostainer (Vision BioSystems, Mount Waverley, Australia) using a rabbit F(ab') anti–fluorescein isothiocyanate/AP (1:50; DS101, DakoCytomation) for the localization of fluorescein isothiocyanate–labeled EBER peptide nucleic acid probe.

Cell Lysis and Preparation for 2D Electrophoresis

Duplicate experiments from cell lysis to 2D electrophoresis were performed. Cells were examined under a phase-contrast microscope to ensure that they were about 50% to 70% confluent before lysis began. The culture medium was discarded, and the cells were rinsed with ice-cold isotonic buffer (250mM sucrose, 10mM Tris-HCl, pH 7.2) 4 times on ice. Lysis buffer composed of 9M urea, 4% CHAPS, 10mM Tris-HCl, 50mM dithiothreitol, 0.6% pH 3 to 10 nonlinear-immobilized pH gradient buffer, 1× Complete protease inhibitor (Roche Diagnostics, Basel, Switzerland), pH 8.8, was then added to each culture dish. The cells were scraped thoroughly with a scraper and allowed to lyse on ice for 15 minutes. The cell lysates were collected and centrifuged at 10,000g for 20 minutes at 4°C in order to obtain the clear supernatant without insoluble cell debris. The protein concentration of each cell line was measured using an RC DC protein assay kit (Bio-Rad Laboratories, Hercules, Calif) according to the manufacturer's instructions. Cell lysates were then reduced with 50mM dithiothreitol for 2 hours at 23°C followed by alkylation with 150mM iodoacetamide at 23°C for an additional 2 hours, and the cell lysates of each cell line were ready for 2D electrophoresis.

2D Electrophoresis

Thirty micrograms of reduced and alkylated proteins from each cell line were made up to a volume of 200 μl with rehydration buffer (9M urea, 4% CHAPS, 10mM Tris-HCl, 50mM dithiothreitol, 0.6% pH 3 to 10 nonlinear immobilized pH gradient buffer, pH 8.8). Passive rehydration was performed, after which isoelectric focusing on 11-cm ReadyStrip immobilized pH gradient strips (Bio-Rad Laboratories) with a nonlinear pH range of 3 to 10 was performed for 20000 V-h. After isoelectric focusing, the immobilized pH gradient strips were incubated with equilibration buffer (6M urea, 375mM Tris-HCl, 20% glycerol, 2% sodium dodecyl sulfate, 2% dithiothreitol, pH 8.8) for 30 minutes with shaking at room temperature. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was then performed for 220 V-h in 4% to 12% Bis-Tris Criterion XT precast gels (Bio-Rad Laboratories). The proteins were fixed in the gel, and silver staining was performed using PlusOne Silver Staining kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instructions.

Image Analysis

The stained gels were scanned using Model GS-700 Imaging Densitometer (Bio-Rad Laboratories) and analyzed using PDQuest 2D analysis software (Bio-Rad Laboratories). Total intensity in valid spots was used as the normalization method in the analysis. Spots differing by 3-fold or more with an intraspot covariance less than 20% were considered as proteins with differential expression.

In-Gel Protein Digestion

Five differentially expressed protein spots with the highest fold changes (4.3–19.9) were selected for protein identification. The selected spots were excised from the gel and transferred to glass vials. After sodium dodecyl sulfate removal and destaining of silver, the gel pieces were incubated with a trypsin solution containing 25 μg/ml sequencing-grade trypsin (Promega Corp, Madison, Wis), 40mM ammonium bicarbonate, and 10% acetonitrile. Trypsin digestion was allowed to proceed at 37°C for 15 hours, and peptides were harvested with 50 μl of 1% trifluoroacetic acid solution twice.

Peptide Mass Fingerprinting Using Mass Spectrometry

Peptides were purified using ZipTip C18 microcolumns (Millipore Corp, Billerica, Mass) according to manufacturer's instructions and eluted in 2 μl solution containing 80% acetonitrile and 0.1% trifluoroacetic acid. The eluted peptides then were spotted onto a 400-spot Teflon-coated sample plate (Applied Biosystems, Foster City, Calif) with α-cyano-4-hydroxycinnamic acid as matrix using the “Sandwich Method.” Samples were irradiated with a laser using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Voyager DE-Pro, Applied Biosystems) in reflector mode with external calibration using Sequenzyme Peptide Mass Standards kit (Applied Biosystems), and mass spectra were acquired. Monoisotopic mass values from the spectra were submitted to the Aldente (http://www.expasy.org/tools/aldente/) and Mascot (http://www.matrixscience.com/) search engines for protein identification.

Tissue Samples

Formalin fixed, paraffin-embedded specimens of 40 undifferentiated NPCs with adjacent normal epithelium (2004–2005) were retrieved from the archives of the Department of Pathology of Queen Elizabeth Hospital (Hong Kong Special Administrative Region, China) for immunocytochemical staining. All cases were selected consecutively and histologically confirmed to be undifferentiated NPCs after reviewing by 2 pathologists (J.K.C.C. and W.C.). The clinical information of the patients is presented in the Table.

Antibodies

Mouse monoclonal anti–annexin II (anti-AII) antibody (03-4400, clone Z014, Zymed Laboratories, San Francisco, Calif); rabbit polyclonal anti–annexin V (anti-AV) antibody (3357-100, BioVision, Mountain View, Calif); mouse monoclonal anti-β3-tubulin antibody (NCL-TUB-B2, clone KNY-379, Vision Biosystems, Novocastra, Newcastle upon Tyne, United Kingdom); and mouse Annexin II and β3-Tubulin in Nasopharyngeal Carcinoma—Chan et al
### Patient Clinical Information*

<table>
<thead>
<tr>
<th>Patients With Undifferentiated NPC Cases†</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>31/40</td>
</tr>
<tr>
<td>Female</td>
<td>9/40</td>
</tr>
<tr>
<td><strong>Age, y</strong></td>
<td></td>
</tr>
<tr>
<td>Male, range</td>
<td>32–82</td>
</tr>
<tr>
<td>Female, range</td>
<td>37–65</td>
</tr>
<tr>
<td>Male, mean</td>
<td>55.5</td>
</tr>
<tr>
<td>Female, mean</td>
<td>52.7</td>
</tr>
<tr>
<td><strong>TNM staging</strong></td>
<td></td>
</tr>
<tr>
<td>T1N0M0</td>
<td>2/40</td>
</tr>
<tr>
<td>T1N1M0</td>
<td>2/40</td>
</tr>
<tr>
<td>T1N3M0</td>
<td>2/40</td>
</tr>
<tr>
<td>T2N0M0</td>
<td>6/40</td>
</tr>
<tr>
<td>T2N1M0</td>
<td>3/40</td>
</tr>
<tr>
<td>T2N2M0</td>
<td>5/40</td>
</tr>
<tr>
<td>T2N2M1</td>
<td>2/40</td>
</tr>
<tr>
<td>T3N0M0</td>
<td>2/40</td>
</tr>
<tr>
<td>T3N1M0</td>
<td>4/40</td>
</tr>
<tr>
<td>T3N2M0</td>
<td>2/40</td>
</tr>
<tr>
<td>T3N3M0</td>
<td>2/40</td>
</tr>
<tr>
<td>T3N0M1</td>
<td>2/40</td>
</tr>
<tr>
<td>T4N0M0</td>
<td>2/40</td>
</tr>
<tr>
<td>T4N1M0</td>
<td>2/40</td>
</tr>
<tr>
<td>N0M0</td>
<td>1/40</td>
</tr>
<tr>
<td>N3M0</td>
<td>1/40</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>22/37</td>
</tr>
<tr>
<td>CRT</td>
<td>15/37</td>
</tr>
<tr>
<td><strong>Follow-up</strong></td>
<td></td>
</tr>
<tr>
<td>NED</td>
<td>20/35</td>
</tr>
<tr>
<td>AWD</td>
<td>2/35</td>
</tr>
<tr>
<td>DUD</td>
<td>2/35</td>
</tr>
<tr>
<td>DOC</td>
<td>3/35</td>
</tr>
<tr>
<td>DOD</td>
<td>8/35</td>
</tr>
</tbody>
</table>

* Treatments for 3 patients and follow-up for 5 patients were unknown. NPC indicates nasopharyngeal carcinoma; NA, not available; RT, radiotherapy; CRT, chemoradiation; NED, no evidence of disease; AWD, alive with disease; DUD, died of unrelated disease; DOC, died of disease complications; and DOD, died of disease. † Values are number/total number, unless otherwise specified.

Monoclonal anti–profilin 1 antibody (308011, clone 2H11, Synaptic Systems GmbH, Gottingen, Germany) were used.

### Immunochemical Staining and Evaluation

Serial tissue sections (4 μm thick) were cut, and antigen retrieval was performed using Bond Epitope Retrieval Solution 2 on the Bond-Max automated immunostainer (Vision BioSystems) at 100°C for 25 minutes. Staining was performed according to a standard protocol in the immunostainer. Polymer detection system was selected to avoid the problem of nonspecific endogenous biotin staining. Benign prostate glandular epithelial cells, benign lung pneumocytes, colorectal tumor, and invasive breast tumor were used as positive controls for AII, AV, β₂-tubulin, and profilin 1 immunostaining, respectively. A positive control tissue was mounted on every test slide, and negative controls were performed by replacing the antibody with Tris-buffered saline. The stained slides were evaluated in 5 fields under a light microscope at ×400 magnification by 2 independent observers. All slides were scored semiquantitatively and expressed as an immunochemical (ICC) score by multiplying the percentage of positive cells and the staining intensity, as described previously. Staining intensity was scored as follows: 0, negative; 1, weak; 2, moderate; 3, strong; and 4, very strong. The ICC score ranged from 0 to 400.

### Statistical Analyses

The differences in ICC scores of AII, AV, β₂-tubulin, and profilin 1 between C666-1 and NP69 cell lines were studied using the Mann-Whitney test, whereas those between tumor and adjacent normal epithelial cells were studied using Wilcoxon matched-pairs test. GraphPad Prism software version 4.0 (GraphPad Software Inc, San Diego, Calif) was used for all statistical analyses, and *P* < .05 was considered significant.

### RESULTS

#### In Situ Hybridization for EBER Status

As expected, EBER was found in 95% of C666-1 cells, whereas no EBER signal was found in NP69 cells (Figure 1).

#### Identification of Differentially Expressed Proteins Between C666-1 and NP69 Cells by 2D Electrophoresis and Mass Spectrometry

A total of 38 spots were found to be up-regulated, whereas 82 spots were down-regulated in the C666-1 cell line compared with the NP69 cell line after 2D electrophoresis and image analysis. Among them, 1 up-regulated spot and 4 down-regulated spots were digested by trypsin, followed by protein identification using mass spectrometry. The identified up-regulated protein was aden...
Figure 2. A, Differential expressed proteins of adenosine triphosphate synthase α chain (ATPSα), annexin II, annexin V, β2-tubulin, and profilin 1 between C666-1 and NP69 cells after 2-dimensional electrophoresis in duplicate gels. Respective fold changes are indicated on the right. B, Search results for peptide mass fingerprinting using the Aldente (top) and Mascot (bottom) search engines.

Confirmation of Proteomic Findings Using Immunocytochemical Stainings in Paraffin-Embedded Sections of C666-1 and NP69 Cells

The localization of AII, AV, β2-tubulin, and profilin 1 proteins in C666-1 and NP69 cells found after immuno-

Annexin II and β2-Tubulin in Nasopharyngeal Carcinoma—Chan et al
staining matched with the expected results. AII was more strongly expressed in the cell membrane than in the cytoplasm, whereas AV and β-tubulin expression were found in the cytoplasm. Furthermore, profilin 1 expression was found in both the nucleus and cytoplasm. In general, there were more C666-1 cells than NP69 cells without AII, AV, β-tubulin, and profilin 1 staining (Figure 3, A through H). Moreover, the staining intensity of AII, β-tubulin, and profilin 1 was similar in both C666-1 and NP69 cells (Figure 3, A and B, E through H), whereas the staining intensity of AV was much decreased in C666-1 cells compared with NP69 cells (Figure 3, C and D). Therefore, the ICC scores of all 4 proteins calculated were lower in C666-1 cells relative to NP69 cells, and the differences in ICC scores of all 4 proteins between the 2 cell types were highly significant (Mann-Whitney test, \(P < .05\); Figure 4).

**All, AV, β-tubulin, and Profilin 1 Protein Expression in 40 Paraffin-Embedded Specimens of NPC Biopsies and Their Adjacent Normal Epithelia**

All was expressed more strongly in the membrane than in the cytoplasm of the adjacent normal epithelial cells, which had a much higher staining intensity than that of the tumor cells (Figure 5, A). In general, AV was expressed in any combination of 3 patterns in tumor cells. The first and second patterns were that some tumor cells lost the cytoplasmic staining, but complete and incomplete membrane staining remained, respectively. The third pattern was that some tumor cells showed total loss of both cytoplasmic and membrane staining. In summary, the ICC scores of AV were lower in tumor cells than adjacent normal epithelial cells in all 40 specimens (Figure 6, A), and the difference was highly significant (Wilcoxon matched-pairs test, \(P < .001\)). To investigate the clinical significance of all down-regulation in tumor cells, we analyzed the lymph node status in 15 cases, each with the highest and of AII down-regulation in tumor cells, we analyzed the pairs test, the difference was highly significant (Wilcoxon matched-scores test).

Profilin 1 expression was found in both the nuclei and cytoplasm of tumor and adjacent normal epithelial cells. The immunoreactivity of profilin 1, as shown by the ICC scores, was similar in both tumor and adjacent normal epithelial cells (Figure 6, D). On the other hand, profilin 1 was strongly expressed in lymphoid and stromal cells.

**COMMENT**

In this study, we analyzed the differential proteomic expression profiles between an EBV-associated NPC cell line and a normal NP cell line. One up-regulated and 4 down-regulated proteins were identified after mass spectrometry. The results were confirmed by ICC staining in both cell lines. After successful validation, their expression in patient samples was then measured using 40 pairs of undifferentiated NPCs together with their adjacent normal epithelia in the same specimens.

ATPSα is localized in the inner membrane of mitochondria and is a part of the complex V of oxidative phosphorylation that plays a key role in energy production. A single gene located on chromosome 18q12-q21 encodes ATPSα, which is sorted to mitochondria by a signal peptide. It has been shown that ATPSα is up-regulated in apoptosis, endotoxic shock, and the neurodegenerative process of Alzheimer disease. The up-regulation of ATPSα is mediated by calcium-binding inhibitor protein CaBI, which dissociates from the ATPSα molecule in the presence of high intracellular calcium. In this study, ICC staining to verify the result of ATPSα was not done because a specific antibody was not available. Nevertheless, this result should be reliable, because up-regulated ATPSα was consistently detected. Previous studies indicated that ATPSα is important in melanoma metastasis and recurrence and is up-regulated in liver cancer tissues and a breast cancer cell line. Nevertheless, the exact role of ATPSα in NPC requires further investigation, despite the fact that Fang et al. showed that the gain of 18q is one of the most frequently detected chromosomal abnormalities in NPC tissues.

All belongs to a family of calcium-dependent, phospholipid-binding proteins expressed in diverse tissues and cell types. Like all annexins, All contains a conserved protein core domain that comprises 4 repeated segments of about 70 amino acids each. Initially identified as an intracellular molecule, All also is involved in extracellular functions through interaction with matrix proteins and specific proteases to regulate plasminogen activation, cell migration, cell adhesion, cell proliferation, and cell differentiation. In this study, we are the first to show the down-regulation of All in NPC. Similar to our finding, All down-regulation is also important in prostate carcinogenesis, and up-regulation of All is correlated with poor prognosis in colorectal carcinoma, gastric carcinoma, and conventional renal cell carcinoma. Therefore, results from this study can provide evidence that All protein down-regulation is very important in NPC carcinogenesis, and follow-up studies should be carried out to determine the molecular mechanism of All down-regulation. A recent study indicates that All is up-regulated by redox regulation and plays an important role in oxidative stress-induced renal carcinogenesis and metastasis. Therefore, the findings of simultaneous ATPSα up-regulation and All down-regulation in this study are interesting because All was not up-regulated by oxidation when ATPSα was overexpressed during oxidative ATP synthe-
Figure 3. Immunostaining in paraffin-embedded sections of C666-1 and NP69 cells. Annexin II (AII) in C666-1 cells (A), AII in NP69 cells (B), annexin V (AV) in C666-1 cells (C), AV in NP69 cells (D), β-tubulin in C666-1 cells (E), β-tubulin in NP69 cells (F), profilin 1 in C666-1 cells (G), and profilin 1 in NP69 cells (H) (original magnifications x400).
Annexin II and αβ-Tubulin in Nasopharyngeal Carcinoma—Chan et al

Figure 4. Immunocytochemical (ICC) scores of annexin II, annexin V, β2-tubulin, and profilin 1 in C666-1 and NP69 cells.

Figure 5. Annexin II (A) and β2-tubulin (B) immunostaining in tumor and adjacent normal epithelial cells of undifferentiated nasopharyngeal carcinoma biopsies. T indicates tumor area; N, adjacent normal squamous epithelial cells (original magnifications ×400).

sis, and we hypothesize that the redox regulation of ATP6ε and AII may be a cancer-specific phenomenon. On the other hand, those patients with intense AII down-regulation showed a higher frequency of lymph node metastasis compared with those showing slight AII down-regulation, suggesting that AII down-regulation may be involved in tumor metastasis and, hence, a potential prognostic indicator for NPC.

AV is a 35- to 36-kDa, Ca2+-dependent, phospholipid-binding protein member of the annexin family and was first isolated from human placenta as a protein with anticoagulant activity. AV is found in various tissues, such as liver, spleen, lung, intestine, and placenta. Its role in carcinogenesis is still uncertain, as exogenous AV protein can inhibit lung carcinoma cell migration in vitro, whereas positive correlation between AV overexpression and gastrointestinal stromal tumor grade suggests that AV overexpression may contribute to tumor invasion and metastasis. Our results indicated that AV is not important in NPC cancer formation, as both the percentage of positive cells and staining intensity are similar in both tumor and adjacent normal epithelial cells. We speculate that the deviation from results on cell line studies is that the lower expression of AV in C666-1 in comparison to NP69 cells may not represent the true scenario in the tumor. However, this proteomic finding is true due to the fact that the same result was consistently obtained.

Tubulin is a 100-kDa αβ heterodimer with 7 genes encoding α-tubulin and β-tubulin isotypes, and the major difference between the 2 isotypes resides in the last 15 to 20 amino acids of the carboxy-termini. Mammalian microtubules are formed from a mixture of α-tubulin and β-tubulin isotype classes. Until now, 7 isotypes of β-tubulin have been shown to exist in mammals, termed β1, β2, β3, β4, β5, β6, and β7. However, the distribution and functions of different β-tubulins are still not clear. Previous reports indicated that β2-tubulin isotype was a potential marker for prostate adenocarcinoma. Our results of a decreased expression of β2-tubulin in tumor cells indicated that the abolition of the β2-tubulin network is important in NPC formation. This down-regulation is an uncommon event, as previous studies showed that β2-tubulin isotypes are up-regulated in many cancers, including nervous system neoplasm, pancreatic solid cystic carcinoma, basal cell carcinoma, non–small cell lung carcinoma, and ovarian carcinoma. To our knowledge, this is the first report of β2-tubulin down-regulation in NPC. Further study on a larger scale with relapsing NPC cases is warranted to determine the potential prognostic use of β2-tubulin and, similar to AII, molecular studies should also be carried out to explore its role in NPC formation.

Profilins are small (14- to 17-kDa) proteins found in vertebrates and invertebrates like protozoa, fungi, plants, and viruses. Profilins have important roles in the signal-dependent regulation of actin dynamics through polymerization for cell motility and other actin-linked processes and in various cellular processes, such as membrane trafficking, small-GTPase signaling, and nuclear activities. Most eukaryotes contain more than one profilin gene, and splicing may generate further isoforms. Among them, the most ubiquitously expressed isofrom is profilin 1. The latter is important in human cancer, since its overexpression has been shown to reduce the migration and suppress the tumorigenic phenotype of breast tumor cells. Therefore, profilin 1 may be a potential target for anticancer treatment in the future. Our finding of a similar profilin 1 staining in tumor and adjacent normal epithelial cells provides solid evidence that it is not important in the pathogenesis of NPC. This result is different from the recent studies suggesting that profilin 1 had a tumor sup-
pressor function in breast cancer,\textsuperscript{53} and down-regulation of this protein was also found in pancreatic cancer.\textsuperscript{54} In this study, profilin 1 staining was detected in both the nucleus and cytoplasm of the cell, which is in contrast to those reported by Janke et al.,\textsuperscript{53} whose profilin 1 staining was only found in the cytoplasm. Our observation should be accurate, because nuclear and cytoplasmic staining were consistently found in both cell lines and patient samples. As Janke et al.\textsuperscript{53} used the same clone of profilin 1 antibody (2H11) used in this study, we hypothesize that profilin 1 may have different expression patterns in different types of tissues.

Cell lines were used to detect differentially expressed proteins because of their ready availability, known medium, propagation conditions, and easy check for reproducibility.\textsuperscript{55} However, a disadvantage in using cell lines is that those cells were not representative of the tissue of origin because they have been maintained and propagated in vitro for extended time periods.\textsuperscript{55} Moreover, each cell line was derived from a few cells of a single patient sample, so that the cells are not truly representing the tumor characteristics.\textsuperscript{55} Therefore, validation of the cell line results in patient biopsies is definitely necessary and, based on this reasoning, 2 proteins were successfully detected and validated. However, the loss of AII- and $\beta_2$-tubulin–related chromosomal regions has not been found in previous molecular studies.\textsuperscript{56–58} and we hypothesize that the aberrations found for AII and $\beta_2$-tubulin do not lie at the chromosome level but may be due to gene mutation, altered transcription, and translation or protein stability.

In summary, this study is the first to show the down-regulation of the cytoskeletal proteins AII and $\beta_2$-tubulin in undifferentiated NPC biopsies. This is the first comprehensive study to compare the protein expression between an EBV-associated NPC cell line and a normal NP cell line. Although the NP69 cell line consists of 5 genetic alterations commonly found in NPC, it maintains many characteristics of normal NP cells and is a good model to study NPC pathogenesis.\textsuperscript{59} In view of the heterogeneous nature of NPC, we believe our current proteomic and ICC approach has provided valuable information in NPC development. We will further define their roles and potential use as therapeutic targets.

Supported in part by the Hong Kong Research Grants Council (Central Allocation Group Research Project, CA03/04.SC01), and The Direct Grant (Project Code: 2041119), The Chinese University of Hong Kong.

References
13. Emoto K, Sawada H, Yamada Y, et al. Annexin II overexpression is corre-


