Cell. Mol. Life Sci. 65 (2008) 1007 – 1012
1420-682X/08/071007-6
DOI 10.1007/s00018-008-7444-4

Cellular and Molecular Life Sciences

Visions & Reflections (Minireview)

Proteomics in nasopharyngeal carcinoma

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Received 25 September 2007; received after revision 14 December 2007; accepted 2 January 2008
Online First 24 January 2008

Keywords. Nasopharyngeal carcinoma, proteomics, diagnosis pattern, biomarker.

Introduction

Nasopharyngeal carcinoma (NPC) is a disease that has remarkable racial and geographic distribution [1]. This cancer is one of the most common cancers in Southeast Asia and southern China, while the disease is rare in most other parts of the world. In the year 2005, 85 248 new cases were registered worldwide, and more than 68 % of those were reported from China and Southeast Asia [2]. In China, NPC is prevalent in the southern part of China including Guangdong and other southern provinces (25 – 50/100 000) but less so among northern Chinese (3/100 000). Moreover, the incidence rate of NPC in southern China is nearly 100-fold higher than that in the Western world [3, 4]. The marked geographic and racial differences in incidence of NPC indicate that the development of this cancer must be related to special genetic and environmental factors.

Etiology of NPC

The etiology of NPC has been investigated for more than half a century, and it is generally considered that several factors were involved in the pathogenesis of NPC including genetic, virological, and environmental factors. HLA haplotypes were considered as a susceptibility genetic factor in NPC, especially HLA-A2 [5]. However, recent linkage analysis, on NPC pedigrees from the Guangdong and Hunan provinces in southern China, showed that two susceptibility loci on chromosomes 4p15.1-q12 and 3p21, but not on the MHC region, are involved in the pathogenesis of NPC [6, 7].

Environmental factors are important for the development of NPC, including the consumption of salt fish and other preserved foods that contain high level of *N*-nitroso compounds [8]. For example, *N,N′*-dinitrosopiperazine (DNP) can induce nasopharyngeal carcinoma in rats *in vivo* and neoplastic transformation of human embryonic nasopharyngeal epithelial cells *in vitro* [9, 10]. Childhood consumption of preserved diets is associated with a high risk of NPC, and ~ 30 % decrease in NPC incidence in Hong Kong may be attributed to the change of traditional lifestyle, particularly the avoidance of feeding young children with salted fish [11].

Epstein-Barr virus (EBV) infection is consistently associated with NPC, and is classified as a group I carcinogen by the International Agency for Research on Cancer (IARC) [12, 13]. In NPC cells, a type II latent infection of EBV, with an episome form virus and limited genes expression, was detected, and EBV infection was considered to play a critical role in transforming nasopharyngeal epithelial cells into invasive cancer [3, 14].

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Proteomics technologies

The term 'proteome' is a hybrid of "protein" and "genome", and it is defined as the protein complement of the genome. The process of studying the proteome became known as 'proteomics' [15]. Proteomic technologies and strategies have been extensively reviewed [16, 17]; therefore, we only summarize here the most relevant aspects of this rapid expanding field. There are two major strategies used for protein separation in proteomics: gel-dependent and gel-independent strategies [18]. In the gel-dependent strategy, two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry (MS) is commonly used to screen the "differential display" of proteins to compare their levels, and this has a potential application in a wide range of disease. In the gel-independent strategy, liquid chromatography (LC) or multi-dimensional LC replaces 2-DE to separate proteins or peptides, and these proteins or peptides are identified by MS. LC-tandem MS (MS/MS) allows more definitive identification and quantitative determination of protein compounds, and this method complements the gel-dependent strategy; 2-DE has the disadvantage that is very labor intensive and that it is difficult to separate hydrophobic membrane proteins by 2-DE [19, 20].

The surface-enhanced laser desorption/ionization time-of-flight MS (SELDI-TOF-MS) or SELDI ProteinChip® technology is a hybrid technology of retentate chromatography and matrix-assisted laser desorption/ionization (MALDI) TOF-MS, which allows protein capture, purification and analysis onto the ProteinChip surface and peaks in a mass spectrum (SELDI peaks) with unique mass-to-charge ratios (m/z) values detected by TOF-MS. After clustering the protein profiles, the characterized peaks are modeled into a diagnostic pattern and the patterns serve as biomarkers. Probably owing to the high-throughout nature, SELDI-TOF-MS has been commonly used in the discovery of biomarkers in the serum or plasma sample for various type of disease [21, 22].

Besides these "traditional" proteomics technologies, much effort has been made in the development of new proteomics technologies directed to providing systematic proteomic data, including quantitative expression profiles, activity profiles, modification profiles and interaction map. For example, quantitative proteomics strategies, such as differential in-gel electrophoresis (DIGE), isotope-coded affinity tag (ICAT) and stable isotopic labeling strategy, have been developed to precisely compare differences in protein quantity [17, 23, 24]. More advanced fragmentation methods in MS, such as electron capture dissociation (ECD) and electron transfer dissociation (ETD), and chip-based proteomics strategies including forward or reverse protein microarrays have been used in proteomic modification profiles [25–27].

Proteomics in NPC

Publications on the proteomics in NPC that can be searched from PubMed or ISI database are summarized and classified here into three research orientations: proteome map, biomarker discovery and proteome response to various stimuli.

Proteome map

The proteome map, or the database in which the proteins are identified, can help physiological proteomics studies and provide a list of cellular protein components. To understand NPC in a global view at the protein level, we established a 2-DE reference map of NPC tissues and identified 216 landmark spots using MALDI-TOF-MS and ESI-Q-TOF-MS, and constructed a nasopharyngeal carcinoma 2-DE/MS repository based on HUP-ML model with an open source XML database Xindice [28, 29]. To analyze the protein expression profile of the NPC cell line, we constructed the reference map of the NPC cell line CNE2. The 2-DE secreted patterns of the CNE2, stimulated or not by TGF-α, and eight non-redundant proteins were identified with MALDI-TOF-MS; these proteins were shown to be involved in invasion, metastasis, apoptosis and proliferation of cancer cells [30, 31].

Biomarker discovery

An early diagnosis is one of the promises that proteomics hopes to fulfill. Currently, two biomarker-screening strategies have been developed using the proteomics method: screening specific disease proteins and constructing diagnosis patterns, which also serve as biomarkers. NPC is often diagnosed late due to its deep location and vague symptoms. Thus, there is an urgent need to find sensitive biomarkers for an early diagnosis of NPC. Cho [32] reviewed the current biomarker research in NPC, and divided the biomarkers into seven types according to its different effect in NPC.

With proteomics technology, Wu et al. [33] analyzed secreted proteomes of two NPC cell lines, and identified three potential biomarkers (fibronectin, Mac-2 BP and PAI-1) that were highly expressed in the NPC biopsies and in the serum of NPC patients. Doustjalali et al. [34] identified ceruloplasmin as being highly expressed in the sera of NPC patients with 2-DE/MS technology, and, using ELISA, confirmed that ceruloplasmin was also highly expressed in

the NPC tissue and down-regulated in the patients after treatment for 6 months. In our laboratory, we found nine proteins that can selectively react with sera from NPC patients, among which cytokeratin 19 (CK19), Erb3 binding protein (EBP1), and Rho GDP dissociation inhibitor-beta (Rho-GDI-2) induced autoantibodies in more than 36.8% of NPC patients but not in healthy individuals, and can be used as potential biomarkers in NPC screening and diagnosis [35]. We compared highly malignant NPC tissues and poorly differentiated NPC cell line CNE2 with high reproducibility 2-DE/MS technologies, and found that overexpressions of nm23-H1, DJ1 and TIM1 in both the NPC tissues and the cell line may be correlated with the pathogenesis of poor differential nasopharyngeal squamous carcinoma [36].

In China, the pathological character of NPC is that WHO type III (undifferentiated carcinoma) accounts for more than 97% of cases, and this has a typical morphology with a prominent lymphoplasmacytic infiltration. To compare the differentially expressed proteins between nasopharyngeal carcinoma cells and normal nasopharyngeal epithelia cells, which normally account no more than 10% of nasopharynx mucosa, we used laser capture microdissection (LCM) technology to first acquire the purified nasopharyngeal carcinoma cells and normal nasopharyngeal epithelia cells, then identified 36 differentially expressed proteins, among which, stathmin, 14-3-3σ and annexin A1 were differentially expressed and validated this with tissue microarray (in press: Clinical Cancer Research). Stathmin and annexin A1 have been reported to be signal targets triggered by EBV latent membrane protein 1 in NPC cell line CNE1 using functional proteomics technology [37].

The diagnosis pattern is composed of several *m/z* peaks, screened from high-throughput SELDI-TOF-MS results, which can acquire very high sensitivity and specificity in cancer diagnosis. Cho et al. [38] showed that serum amyloid A (SAA) could be used as a potentially useful biomarker to monitor relapse of NPC by serum proteomic profiling using a ProteinChip. Using pre- and post-chemotherapy sera samples from NPC patients, Cho et al. [39] discovered 13 candidate biomarkers associated with different clinical parameters. Among the 13 SELDI peaks, two peaks (3593 and 7765 Da) were identified as a fragment of interalpha-trypsin inhibitor precursor and platelet factor-4 by MS/MS sequencing and/or immunoaffinity capture assay. Guo et al. [40] obtained a proteomic pattern from the serum of NPC patients using an artificial neural network (ANN) technology; this pattern comprised 11 distinct *m/z* peaks that could distinguish type A (ascending type) from type D (descending type) NPC with 100% accuracy in the training sets and 89.5% accuracy in the test sets. Chang et al. [41] developed a bead-based affinity-fractionated proteomic method to search potential plasma markers for NPC. The results showed that 12 *m/z* markers were different between cancer and control spectra, among which the combined markers (2020 and 4635 Da) were the best discriminators with high sensitivity (94%) and specificity (93%). Ho et al. [42] constructed a classification tree with 6 distinct *m/z* markers that were generated using Biomarker Pattern software based on the proteomics spectra of serum samples. Combining this classification tree with EBV nuclear antigen 1 (EBNA1 IgA) test, the diagnostic sensitivity and specificity were increased to 99% and 96%, respectively.

The NPC biomarkers currently identified by proteomics technologies are shown in Table 1. The protein markers identified in different laboratories do not overlap, although several proteins such as stathmin and annexin A1 identified by 2-DE/MS technology were reported in the biomarker list from different labs. Stathmin and annexin A1 have also been found in other cancers and identified as differentially expressed proteins in the NPC cell line CNE2 stimulated by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), which suggests that these two proteins need more strict validation as biomarkers of NPC [43–45]. The diagnosis patterns in NPC have achieved high accuracy and specificity in the published papers, but the SELDI peaks and diagnosis patterns do not overlap in different laboratories. This may be for several reasons: the NPC clinical sample itself has a geographic character, SELDI technology is still not sufficiently stable and reliable, and the data mining technology used is different in different labs. In summary, SELDI technology is a hopeful technology, but needs to be optimized to achieve more reproducible results using complex clinical samples and different data analysis methods.

Proteome response to various stimuli

The proteomics approach has also been used as a tool to explore the changes in the cellular signal network caused by RNAi, drugs, some chemical materials or other techniques. In NPC, p53 protein overexpression or accumulation occurs at high frequency, but p53 gene mutations are rare, and the mechanisms of inactivation and stabilization of p53 in NPC are not well known [46]. To detect p53 function-associated proteins, we identified several p53 interaction proteins *via* immunoprecipitation coupled to MS, which included HSP27, GRP75 and GRP78. [47]. To further understand the function of p53 protein in NPC, we established a p53 silence NPC cell line, CNE2sip53, and compared the proteomic changes between CNE2-

Table 1. Biomarkers in nasopharyngeal carcinoma identified by proteomics.

Biomarkers	Technology	Sample	Ref
CK19, EBP1, Rho-GDI-2	SERPA[a]	Tissues and paired sera	[37]
Stathmin, 14-3-3σ, Annexin A1	LCM-2D-MS/MS	Tissues	[b]
Ceruloplasmin	2D-MS	Sera	[36]
Fibronectin, Mac-2 BP, PAI-1	1D gel/MS-MS	Cell lines	[35]
Fragment of interalpha-trypsin inhibitor*[1] precursor, platelet factor-4*[2, d]	SELDI-TOF-MS Tandem MS/MS	Pre- and post-CT[c] paired sera	[39]
Serum amyloid A protein (11 800 Da) and 11 600 Da	SELDI-TOF-MS Tandem MS/MS	Sera	[38]
Complement C3f fragment (2020 Da) and 4635 Da	MALDI-TOF-MS	Sera	[41]
4053, 5885, 4072, 5798, 4209, 8689, 2382, 9357, 2221, 4230, and 5901 Da	SELDI-TOF-MS	Sera	[40]
6692, 6811, 6862, 7979, 9176 and 10272 Da	SELDI-TOF-MS	Sera	[42]

[a] Serologic proteome analysis.
[b] Accepted by Clin Cancer Res.
[c] chemotherapy.
[d] Pattern: 2083, 2509, 2756, 2950, 3963*[1], 6701, 7588, 7659, 7765*[2], 7843, 8372, 13510, and 14855 Da.

sip53 and the control cell line CNE2/pSUPER with 2-DE/MS. Among the differentially expressed proteins, HSP27 was also found to be associated with p53. The expression of p53 dramatically decreased when HSP27 was knocked down using RNAi [48].

TPA is a plant derivative, and is considered to be closely related with NPC as it can facilitate EBV infection of human primary epithelial cells and subsequent malignant transformation. Yao et al. [45] analyzed the proteomic changes caused by treating the NPC cell line CEN2 with TPA and identified six significantly and reproducibly changed proteins, including triosephosphate isomerase 1, stathmin, and 14-3-3σ. Epidermal growth factor receptor (EGFR) is frequently overexpressed in human tumors and correlated with a poorer clinical outcome, Chan and colleagues [49] used the EGFR-specific monoclonal antibody (cetuximab, C225) to block the EGFR signal pathway and observed the proteomic changes in the NPC cell lines HONE-1 and HK1 after treated with cetuximab. The results showed that down-regulation of gp96 and up-regulation of maspin and p97 coincided with changes in their mRNA levels. The G-quadruplex ligand 3,3′-diethyloxadicarbocyanine iodide (DODC) was reported to enhance the apoptotic potency through the inhibition of telomerase activity. Li et al. [50] analyzed the mitochondrial proteome changes of NPC-TW01 cell apoptosis that were induced by DODC, and found that DODC could induce p53 and an 18-kDa truncated Bax in mitochondria, which in turn potentiated the release of cytochrome c for activation of caspases.

Conclusions and prospects

In summary, current proteomics research in NPC has been reviewed. Screening for biomarkers in NPC forms an important part of NPC proteomics, although so far the biomarkers for NPC identified by proteomics have not been reproducible between different laboratories. To identify more reliable biomarkers for NPC, new strategies for NPC biomarker screening need to be adopted, including using quantitative proteomics technology, multi-center preliminary validation for sample cohort and standardization of specimen collection, handling, and choice of fractionation and analysis technologies.

The effort to construct a proteome map on NPC and monitor NPC proteome responses to various stimuli should be strengthened. At present, this effort can provide more detailed information about what is actually happening in NPC, but this is just the beginning, and the molecular mechanism underlying NPC remains far from being well understood. Integration of data obtained from different "omics" studies is necessary for us to gain a better understanding of the molecular basis of this complex disease. In the future, genomics, transcriptomics, proteomics and metabolomics in NPC will be integrated into systems biology to provide a full view to understand this cancer.

Acknowledgement. We are grateful to Ms. Esther Young for her editing. This work was supported by a grant from National 973 Project of China (2001CB510207), National Natural Science Foundation of China (30772401, 30240056, 30370642), and key research program from Science and Technology Committee of Hunan, China (02SSY2001-1), and key research program from Public Health Bureau of Hunan Province, China (Z02–04).

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