ORIGINAL

Patients with nasopharyngeal carcinoma demonstrate enhanced serum and tissue ceruloplasmin expression

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Abstract:The proteomics approach was adopted to study the simultaneous expression of serum proteins in patients with nasopharyngeal carcinoma (NPC). We have subjected unfractionated whole sera of ten newly diagnosed Malaysian Chinese patients with WHO type III NPC to two-dimensional gel electrophoresis (2-DE) and image analysis. The results obtained were then compared to that generated from sera of ten normal healthy controls of the same ethnic group and range of age. Our data demonstrated that the serum high abundance 2-DE protein profiles of NPC patients were generally similar to that of the controls, with exception of the ceruloplasmin (CPL) spots (identified by mass spectrometric analysis and MASCOT database search), which showed higher expression. The enhanced expression of CPL in the patients' sera was confirmed by competitive ELISA. Immunohistochemical analysis of nasopharyngeal lesions of NPC patients demonstrated moderate to strong positive CPL staining in the cytoplasm of cells at the regions of malignancy but only weak cytoplasmic staining at normal epithelial lining areas. When follow-up 2-DE and ELISA studies were performed on five of the NPC patients who responded positively to six months treatment, the difference in CPL expression was no longer significant. J. Med. Invest. 53 : 20-28, February, 2006

Keywords : *ceruloplasmin, nasopharyngeal carcinoma, serum proteomics*

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a rare malignant disease with worldwide age-adjusted incidence rates (per 100,000) of 1.66 among men and 0.64 among women (1). However, it is endemically found in Southern China (Guangdong province), Hong Kong and Singapore. According to the database of the International Agency for Research on Cancer, the highest rates in the world are in Hong Kong with incidence rates of 25 per 100,000 among male and 10 per 100,000 among female (1). Statistics are not available for the Guangdong Province in China, where the rates may be even higher. The rates of NPC are intermediate (2 to 6 per 100,000) in other areas of Southeast Asia, such as Malaysia, Vietnam, Thailand and Guam. In these countries NPC is also more common in men particularly of Chinese ancestry.

The World Health Organization (WHO) classification distinguishes three histopathological types of NPC based on the degree of differentiation. Different prevalent histologic types of NPC are found in endemic and nonendemic regions (2). Keratinizing squamous cell carcinoma is more common in the Western countries (75%) while in endemic areas such as Southern China, the undifferentiated carcinoma or WHO Type III NPC accounts for more than 97%.

NPC shows a remarkably high cure rate for early-stage disease, and early detection is critical to improve the overall prognosis of patients. However, the clinical

Received for publication May 2, 2005 ; accepted June 27, 2004.

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presenting features of NPC are often nonspecific, and examination of nasopharynx requires expertise and renders early detection difficult. Diagnosis of NPC is mainly made by biopsy of the nasopharyngeal mass (3). At present, the detection of IgA antibodies to EB-virus specific antigens is the only serological aid to the diagnosis of NPC (3-4), although the potential use of serum markers such as the circulating intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin, CYFRA 21-1, tissue polypeptide specific antigen and C-erb B2 has been proposed (5-9).

The proteomics approach may offer a paradigm shift in studies on the simultaneous expression of serum proteins in patients with cancer. Detection of selective or aberrantly expressed serum proteins in cancer patients may prompt investigations of their potential application as novel diagnostic or prognostic biomarkers. In the present study, we have subjected unfractionated whole sera of ten Malaysian Chinese patients with NPC and ten normal healthy controls of the same ethnic group and range of age to two-dimensional gel electrophoresis (2-DE) and image analysis. The data obtained were then compared to that generated by competitive ELISA as well as immunohistochemical studies of the patients' tissues.

MATERIALS AND METHODS

Serum samples.

Serum samples were obtained from ten Malaysian Chinese patients with NPC (n=7 males and n=3 females with ages ranging from 40-65) prior to treatment at the University of Malaya Medical Centre, Kuala Lumpur. All patients were confirmed histopathologically with undifferentiated carcinoma or WHO Type III NPC for either stage T1N1M0 or stage T2N1M0. Control sera were obtained from ten normal healthy Malaysian Chinese volunteers of comparable gender distribution and range of age. Serum samples were again collected from five of the NPC patients (n=3 males and n=2 females) six months after treatment, which involved radiotherapy and chemotherapy for 3 cycles. Samples obtained were with consent in accordance with approval granted by the Ethical Committee (Institutional Review Board) of the Medical Centre. All samples were kept at-20°C and subjected to similar treatment.

Two-dimensional gel electrophoresis.

2-DE was performed as previously described (10). Briefly, seven μl (450 μg protein) of unfractionated whole human serum samples were subjected to isoelectric focusing in rehydrated pre-cast immobilised dry strips pH 4-7, 11 cm (Amersham Biosciences, Uppsala, Sweden). For the second dimension, focused samples in the strips were subjected to electrophoresis using the 8-18% gradient polyacrylamide gel in the presence of sodium dodecyl sulphate. All samples were analysed in triplicate.

Silver staining.

The 2-DE gels were developed by silver staining as previously described by Heukeshoven and Dernick (11). For mass spectrometric analysis, gels were stained according to the method of Shevchenko (12).

MALDI-ToF Pro analysis.

Protein spots were initially identified by comparing resolved serum protein profiles with the SWISS ExPASy standard plasma protein reference (13). Confirmation of the spots of α_1-antitrypsin (AAT), α_1-B glycoprotein (ABG), α_2-HS glycoprotein (AHS), complement factor B (CFB), clusterin (CLU) and the β chain of haptoglobin (HAP) has been earlier described (10). Identification of ceruloplasmin (CPL) spots was similarly performed by using the Ettan MALDI-ToF Pro. In gel trypsin digestion was performed according to the method of Shevchenko (12). Mass analyses were performed by mixing 1 μl of extracted sample with equal volume of matrix solution consisting of 10 mg/ml alpha-cyano-4-hydroxy cinnamic acid in 0.5% TFA and 50% ACN. Only 0.3 μl of the solution was finally spotted onto the slide loader.

Database search.

The MASCOT programme (www.matrixscience.com) was used to search protein database. MASCOT uses peptide mass fingerprints (PMFs) to search database for matching peptides from known proteins. The following parameters were used in the searches : trypsin digest (one missed cleavage allowed), species : Homo sapiens, mass value : monoisotropic, peptide mass tolerance : $\pm$0.1 Da, peptide charge state : 1+ and NCBInr database. Identification was again confirmed using the Amersham Biosciences Ettan MALDI software.

Image analysis.

Protein spots were analyzed in terms of volume, performed by using the Molecular Analyst PDQuest densitometry software (Bio-Rad, Hercules, Calif., USA). The background was subtracted and analysis was restricted to ten clusters of protein spots with $M_r \geq 30{,}000$

distinctively separated by 2-DE, i.e., AAT, ABG, AHS, CFB, CLU, CPL, HAP and three unidentified proteins termed PR1, PR2 and PR3. Albumin, serum polypeptides having idiotypic and/or allotypic variations (such as the heavy and light chains of all isotypes of immunoglobulins and the α chains of haptoglobin) and the low M_r protein spots, the majority of which were not well resolved under the settings of our experiments, were not assessed. The percentage of volume contribution refers to the volume percentage of a protein taken against the total spot volume of all proteins including the unresolved peptides in each gel.

Competitive ELISA.

Competitive ELISA was performed as previously described (10). Plates were coated with human serum at 1 : 1000 dilution in 0.05 M carbonate-bicarbonate buffer pH 9.6 and blocked with 0.5% gelatin in PBS-Tween. After washing, plates were incubated with diluted sheep anti-human CPL (Code PC045 ; the Binding Site Limited, Birmingham, UK), in the absence and presence of sera of NPC patients and normal controls at 1 : 1000 dilution in PBS-Tween for 1.5 hr. Blanks were prepared by addition of PBS-Tween instead. Following extensive wash with PBS-Tween, the plates were further incubated with 200 μl of diluted horseradish peroxidase (HRP) conjugated donkey anti-sheep IgG (Code AP360;the Binding Site Limited, Birmingham, UK) per well for 1 hr. Enzyme activity was revealed with 0.5 mg/ml o-phenylenediamine dihydrochloride and 0.012% (v/v) H_2O_2 in 1 M sodium citrate buffer pH 5. Reactions were terminated by addition of 100 μl H_2SO_4 and absorbance values were read at 490 nm. The amount of CPL in the test sera is proportional to the % inhibition of substrate hydrolysis.

Immunohistochemical studies.

Sections (5 μm thick) from nasopharyngeal lesions of NPC patients (undifferentiated or WHO type III carcinoma) were subjected to immunohistochemical analysis as previously described (10), using sheep anti-human CPL (1:200 dilution) as the primary antiserum followed by detection with HRP-conjugated donkey anti-sheep IgG (1:100 dilution). Both antibodies were from the same batches of antisera that were used in ELISA. Negative control was obtained by omission of the primary antibody. Protein expression was scored as follows : negative if no staining was seen or if immunoreactivity was observed in less than 10% of tumor cells, and positive if more than 10% of tumor cells showed staining.

Statistical analysis.

All values are presented as mean $\pm$ SD (standard deviation). The Students T-test was used to analyze the significance of differences between normal subjects and patients. A p value of less than 0.05 was considered significant.

RESULTS

Separation of unfractionated whole sera of normal healthy individuals by 2-DE generated typical high resolution serum profiles comprising only the high abundance proteins. Under the conditions of our experiments, protein spots that are usually detected by silver staining include albumin, the heavy and light chains of IgA, IgG and IgM, α_1-antitrypsin, α_1-B glycoprotein, α_2-HS glycoprotein, complement factor B, clusterin and the β chain of haptoglobin and three other unidentified protein spot clusters (Fig. 1 a). Studies confirming the identities of the above known high abundance acute-phase proteins have been described recently (10).

When unfractionated serum samples from newly diagnosed patients with NPC (n=7 males and n=3 females) were subjected to 2-DE and silver staining under the same experimental conditions, comparable results were obtained for both the identified and unidentified proteins, with exception of a horizontal train cluster of protein spots which appeared at the 120 kDa region of the 2-DE gels. When subjected to 2-DE, serum glycoproteins are often resolved into several different isoform spots mainly due to the heterogeneity of their oligosaccharide chains. Fig. 1b demonstrates a typical representative unfractionated serum protein profile of patients with NPC. The 120 kDa protein spots cluster, which was not expressed in control serum 2-DE protein profiles, was found present in the serum 2-DE protein profiles of all NPC patients studied.

When a follow-up 2-DE study was performed on sera of five of the patients (3 males and 2 females) who responded positively to treatment after six months, the differential expression of the 120 kDa spots cluster was apparently normalized in all patients. Fig.1c demonstrates a typical representative of the unfractionated serum protein profiles of NPC patients subsequent to treatment.

Subjecting the 120 kDa gel spots cluster to trypsin in gel-digestion and mass spectrometry generated a MALDI-ToF Pro mass spectrum of tryptic peptides (Fig. 2). MASCOT database search of the peptide mass fingerprints obtained subsequently identified the up-

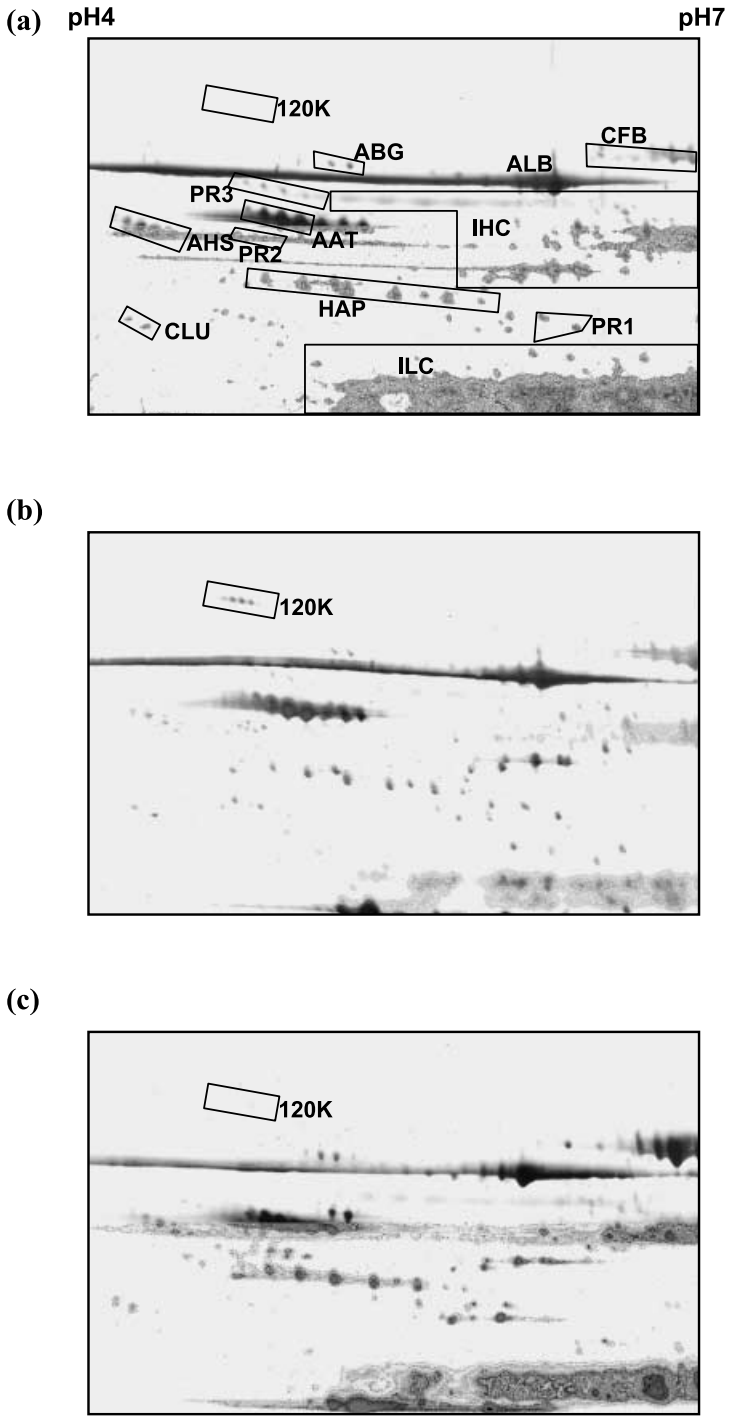


Fig.1. Typical2-DE serum protein profiles of normal healthy adult controls and patients with NPC. Serum samples of normal healthy controls (a) and patients with NPC prior (b) and subsequent (c) to treatment were subjected to 2-DE (pI4-7) and silver staining. Besides the detection of albumin (ALB), immunoglobulin α, γ and μ heavy chains (IHC) and immunoglobulin light chains (ILC), other high abundance protein spots resolved included α_1-antitrypsin (AAT), α_1-B glycoprotein (ABG), α_2-HS glycoprotein (AHS), complement factor B (CFB), clusterin (CLU), the β chain of haptoglobin (HAP) and three unidentified proteins termed PR1, PR2 and PR3. Comparable expression was observed for most of the resolved proteins, with exception of a120kDa protein spots cluster (120K), which was only detected in the serum protein profiles of NPC patients prior to treatment. Acid sides of all 2-DE gels are to the left and relative molecular mass declines from the top.

regulated 120 kDa protein spots as that of ceruloplasmin (CPL), with 21 matched peaks out of 42 peaks searched, 24 sequence percent coverage and a MASCOT score of 86 (MASCOT accession number gi/1620909).

2-DE is highly reproducible. Our previous report has indicated that image analysis performed on triplicate 2-DE gels of serum samples produced minimal relative standard deviation in percentage values of volume contribution of all serum protein spots analyzed (10). In the present study, image analysis performed on the silver-stained 2-DE serum protein profiles of ten NPC patients (prior to treatment) as compared to ten normal controls indicated significantly higher expression of CPL in patients (55-fold ; p=0.0001). However, comparable results were obtained for all the other high abundance serum proteins analyzed (Fig. 3). Differential expression of CPL, as opposed to controls, was observed in all ten NPC patients that were studied. There was no significant difference in the relative expression of CPL between control subjects and NPC patients who received six months of treatment.

For confirmation of the aberrantly expressed CPL in sera of the NPC patients, competitive ELISA was carried out by using antisera against CPL. Fig. 4 demonstrates the results of the competitive ELISA performed in the presence of sera of control subjects (n=7 males and n=3 females), NPC patients prior to treatment (n=7 males and n=3 females) and NPC patients subsequent to treatment (n=3 males and n=2 females). Higher levels of CPL (0.5-fold ; p=0.0001) were significantly detected in sera of non-treated NPC patients as compared to normal controls. There was no significant difference in the levels of CPL that were tested between treated NPC patients and control subjects.

Fig. 5 demonstrates results of the immunoperoxidase studies performed to compare the expression of CPL in the malignant areas of nasopharyngeal lesions of patients with undifferentiated carcinoma or WHO Type III NPC (n=3 males and n=3 females), which was earlier diagnosed on routine Hematoxylin & Eosin section staining, as opposed to their normal epithelial lining (squamous or respiratory). The expression of CPL was weakly positive throughout the cytoplasm of cells of the normal areas of all nasopharyngeal lesions but moderately to strongly positive in the malignant regions.

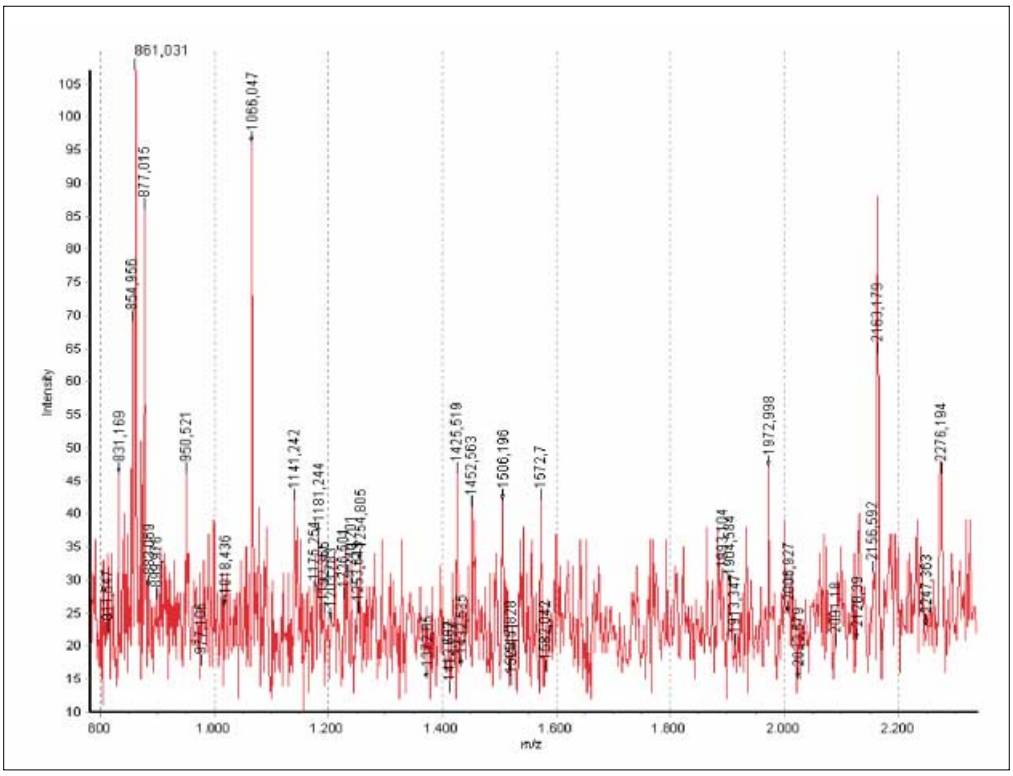


Fig. 2. MALDI-ToF Pro mass spectrum of tryptic peptides derived from the120kDa protein spots cluster. Identity of the120kDa spots cluster was determined by using the Ettan MALDI-ToF Pro mass spectrometry. Analysis was performed in reflectron mode. MASCOT database search of the peptide mass fingerprints obtained subsequently identified the 120 kDa protein spots as that of ceruloplasmin (MASCOT accession number gi/1620909),with 21 matched peaks(42 peaks searched), a MASCOT score of 86 and 24 sequence percent coverage.

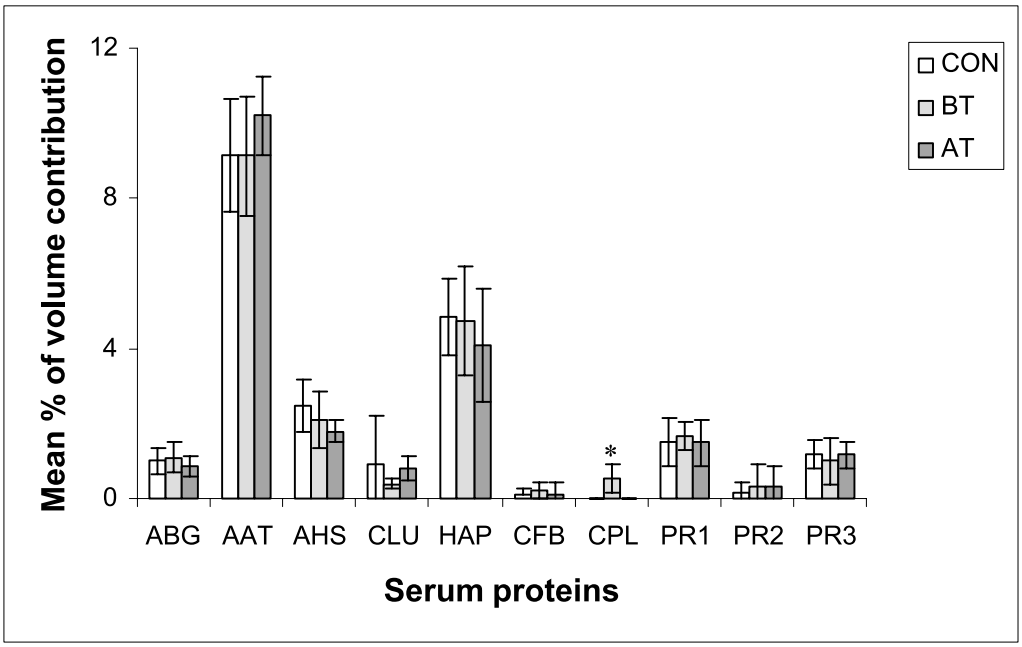


Fig.3. Mean percentage of volume contribution of 2-DE detectable serum proteins of patients with NPC. Volumes of protein spots were analyzed by Molecular analyst PDQuest densitometry software (Bio-Rad, Hercules, Calif., USA). AAT: α_1-antitrypsin, ABG: α_1-B glycoprotein, AHS: α_2-HS glycoprotein, CFB: complement factor B, CLU: clusterin, CPL: ceruloplasmin, HAP: haptoglobin (β chain), PR 1-3: unidentified proteins. CON, BT and AT refer to normal controls, newly diagnosed patients with NPC prior to treatment and NPC patients subsequent to treatment, respectively. Asterisk denotes value of statistical significant difference.

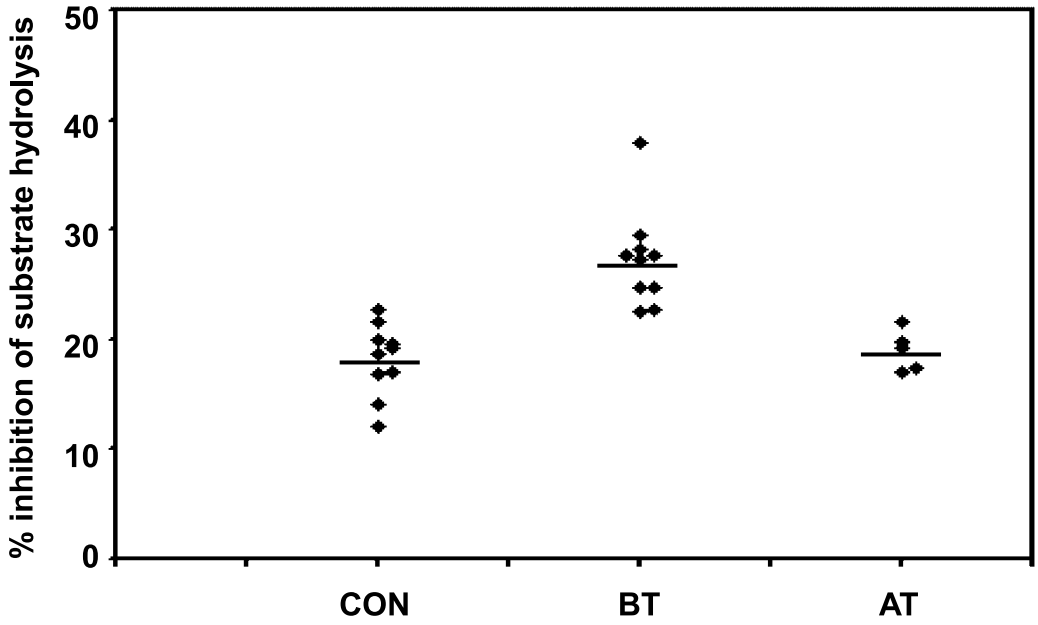
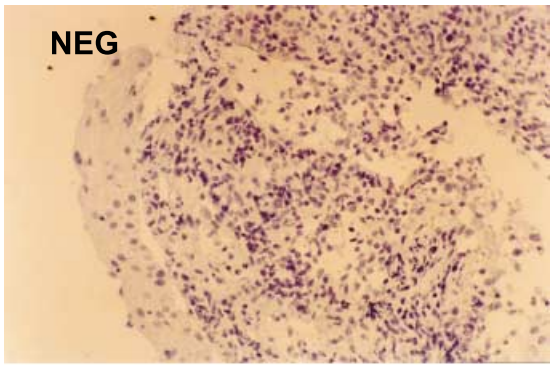


Fig.4. Analyses of serum ceruloplasmin expression by competitive ELISA. Competitive ELISA was performed using sheep anti-human ceruloplasmin as the primary antiserum, in the presence of sera of control subjects (CON ; n=7 males and n=3 females) and NPC patients before (BT; n=7 males and n=3 females) and after (AT; n=3 males and n=2 females) treatment. Analyses were performed in triplicate. The amount of ceruloplasmin in the test sera is proportional to the % inhibition of substrate hydrolysis.



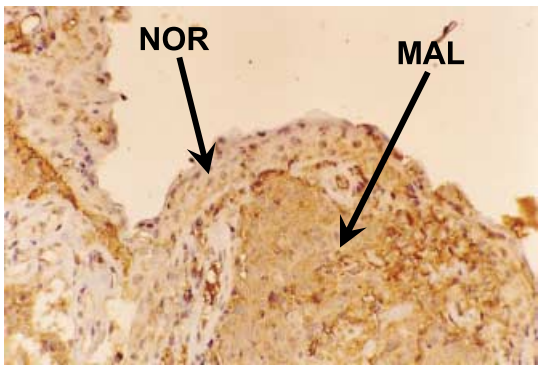


Fig.5. Immunohistochemical analysis of ceruloplasmin expression in nasopharyngeal lesions. Nasopharyngeal lesions from NPC patients were subjected to immunohistochemical analysis using antiserum to ceruloplasmin. Negative control (NEG) was obtained by omission of the primary antibody. NOR and MAL refer to the respective normal and malignant regions of the lesions as earlier diagnosed on routine Hematoxylin & Eosin section staining.

DISCUSSION

Whilst the higher levels of ceruloplasmin (CPL) in sera of patients with cancers of the kidney, urinary tract, gastrointestine, cervix, uterus and skin have been described (14-19), the serum levels of CPL have not been previously reported for patients with nasopharyngeal carcinoma (NPC). In the present study, the higher relative expression of CPL of more than fifty-fold was detected in the sera of all WHO type III NPC patients, whilst the expression of most of the other high abundance serum proteins resolved by 2-DE was comparable to that of the control sera. The sera of NPC patients generated typical protein profiles that were visually and analytically discernible for the enhanced CPL expression, and they were also different from our earlier reported 2-DE protein profiles of patients with breast cancer and fibrocystic disease of the breast (10).

The enhanced serum expression of CPL in patients with NPC was also confirmed by competitive ELISA although the magnitude of change was lower by approximately a hundred-fold compared to the 2-DE experiments. This is indicative of the gross higher sensitivity of 2-DE as compared to ELISA when used to differentiate the levels of CPL between patients and control subjects.

Alteration of the levels of CPL in the sera of NPC

patients may be attributed to the changes of its synthesis by the liver. CPL is an α_2-globulin which contains approximately 95% of the total serum copper (20). The synthesis of CPL is controlled by the amount of copper available to the liver. Like CPL, copper levels were also elevated in several cancers (21-26), but unlike CPL, the levels of copper in plasma are not regularly stable and may fluctuate in the presence of copper lowering agents like zinc or thiomolybdates (27, 28). Thiomolybdates and zinc lower the copper levels by inhibiting the absorption of copper into the bloodstream. However, an initial increase of plasma levels of copper may occur due to copper release from the liver.

CPL bound copper has been found to act as a molecular switch for activating proangiogenic factors, e. g., vascular endothelial growth factor, basic fibroblast growth factor, interleukin-1 and tumor necrosis factor alpha (29, 30). Tumors require an extensive network of capillaries to provide nutrients and oxygen. Thus, angiogenesis, which is reflected from the level of CPL (or copper) in plasma, is strongly implicated to support growth of tumors (31-34). The observed enhanced expression of CPL in the sera of NPC patients of the present study provides further support to this widely accepted notion.

Evidence for the direct association of malignancy with CPL is found from our immunohistochemical studies performed on nasopharyngeal lesions of the NPC patients. By using antiserum to CPL, moderate to strong positive CPL staining was distinctively detected in the cytoplasm of cells at the region of malignancy but only weak staining was observed at normal epithelial lining areas of the lesions. The selective enhanced expression of CPL at the malignant areas of the lesions as well as the previous report on the significant increase of microvessel density of NPC tissue as compared with those benign tumors of nasopharyngeal region and nasopharyngeal tissue without tumor (35) are indicative of the direct correlation of angiogenesis with malignancy. These results are also compatible with the earlier reported studies performed on human cerebral gliomas (36), as well as the follicular and papillary carcinomas of the thyroid (37, 38), in which various degrees of CPL staining were selectively detected in malignant tissues.

The progression of malignancy in NPC may also be reflected by the serum CPL levels. When follow-up 2-DE and ELISA studies were performed on sera of five of the NPC patients subsequent to treatment for six months, the differential expression of CPL was apparently normalized in all patients. Changes in the expression of CPL in the treated NPC patients may provide useful information for monitoring progression of malignancy since all the five NPC patients studied responded positively to treatment.

Taken together, the data of our 2-DE, ELISA and immunohistochemical studies performed on the small cohorts of NPC patients and normal subjects suggest the potential role of CPL as an additional serum biomarker for diagnosis and monitoring of NPC. In addition, there is also a possibility that the metastasis of tumor in NPC may be made stabilized by treatment with copper lowering agents due to the correlation of CPL with malignancy that is shown in the present study. Such copper deprivation treatment has already claimed considerable success in clinical trials performed on patients with advanced cancers (28, 39-41).

ACKNOWLEDGEMENTS

This work was supported by IRPA grant 06-02-03-1005 from the Ministry of Science, Technology and Innovation, Malaysia. The authors wished to thank Prof. Iekhsan Othman for use of the Ettan MALDI-ToF Pro at MAPAF.

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