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Early detection of ovarian carcinoma by proteome profiling based on magnetic bead separation and matrix-assisted laser desorption/ionization time of flight mass spectrometry

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In this study, affinity purification with magnetic beads and matrix-assisted laser desorption/ionization time of flight mass spectrometry analysis was used to screen candidate biomarkers of ovarian carcinoma. Mass spectrometry compiled from serum of 30 cases of ovarian carcinomas was compared with that from serum of 10 cases of benign group and13 cases of normal group. Interesting peaks were further sieved from ascites and cystic fluid. Peptides were deduced by searching in http://us.expasy.org/tools/tagident.html and were detected by immunohistochemistry and enzyme-linked immunosorbent assay. Results showed that two peaks of 2881 and 2897Da elevated in the serum, malignant ascite and cyst fluid of cancer group when compared with normal and benign groups were deduced as gastrin -releasing peptide which was higher in the malignant tissues and serum than other two groups. It was concluded that gastrin-releasing peptide in serum might be a potential marker for ovarian carcinoma.

Key words: Gastrin-releasing peptide, matrix-assisted laser desorption/ionization time of flight mass spectrometry, magnetic bead, and ovarian carcinoma.

INTRODUCTION

In many countries, ovarian carcinoma (OC) is the most lethal gynecological cancer (Permuth-Wey and Sellers, 2009). It often eludes the clinician, due to its insidious localization in the pelvis and lacking of symptoms and signs during early stages (stage I/II). This is why nearly 70% patients are not diagnosed with OC until the advanced stages (stage III /IV) (Permuth-Wey and Sellers, 2009). The five-year overall survival of advanced stage cases is not more than 20%, compared to the 90% survival rate of early stage cases (Tung et al., 2008). Therefore, increasing the number of women diagnosed in early stage should have a direct effect on the mortality and economics of this cancer.

Serum CA125 integrated with transvaginal sonography can only detect about 25% of the OC in the early stage (Tung et al., 2008). Laparoscopy can identify nearly 100% of the OC within the early stage, but the high cost and invasive properties tremendously impede its feasibility in clinical practice (Tung et al., 2008).

The identification of cancer biomarkers facilitates early detecting, better monitoring of tumor progression and even targeting therapy (Tung et al., 2008; Jackson et al., 2007). Traditional strategies like immuning animals with tumor cells was once used to track down the biomarker of cancers, but the high cost and labor consumption limit its application in practice(Fields and Chevlen, 2006). Although 2-dimensional gel electrophoresis and multi-dimensional liquid chromatography after mass spectro-

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metry (MS) analysis seemed a somewhat economical, these techniques were also laborious and time-consuming (Coombes, 2005). Recently, the discovery of biomarkers in body fluids has been advanced by the introduction of MS based screening methods such as Matrix-assisted Laser Desorption/ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) and a protein chip coupled with Surface enhanced Laser Desorption/ Inionation Time of Flight Mass Spectrometry (SELDI-TOF-MS) (Coombes, 2005). The first clinical investigations using SELDI-TOF-MS in different cancer types revealed high diagnostic sensitivities and specificities (Coombes, 2005).

SELDI-TOF-MS has been developed to facilitate protein profiling of complex biological mixtures with high efficacy discovery of cancer biomarkers in serum or plasma (Zhang et al., 2006; Whiteley et al., 2009). However, different patterns for the same type of cancers have been identified by different individual groups using the same types of biological specimens and analytical platforms of SELDI-TOF-MS. These discrepancies could be attributed to poorly define analytical protocols, which were not established with reproducibility (Whiteley et al., 2009; Keith et al., 2005; Kristina et al., 2009). Therefore, the reproducibility of the proteomic profiling approach is yet to be established (Keith et al., 2005). Based on mag-netic beads integrated with MALDI-TOF-MS analyzing, serum peptides were fractionated and concentrated on surface-modified targets with specific protein-capture properties by Josep et al. (2006). In their study, 106 serum samples from patients with advanced prostate, bladder and breast cancers were profiled. By way of this method, they have showed that peptides generated from the serum of cancer patients could be used for the detec-tion and classification of prostate, bladder and breast cancers (Josep et al., 2006). The data of Josep's study indicated that the method they used could afford a more sensitive analysis than other mass-spectrometry based approaches. Magnetic bead purification for the analysis of low-abundance proteins in body fluids facilitates the identification of potential new biomarkers with MALDI-TOF-MS (Sven et al., 2005; Cheng et al., 2005) and the hydrophobic surface functionalities can significantly enhance the reproducibility of the experiment (Gobom et al., 2003).

However, the proteins and peptides in the serum came from all over the body, which made the specificity and sensitivity of these serum biomarkers insufficient to capture most of the patients in early stage from the large scale of women without any symptoms. Thus, exploring highly specific and sensitive biomarkers of ovarian carcinoma has been an important mission confronted by many researchers. The aim of our study was to research candidate serum biomarkers for early diagnosis of OC based on the magnetic bead separation and MALDI-TOF-MS analysis.

MATERIALS AND METHODS

Materials and sample preparation

A total of 30 consecutive patients with OC (11 in early stages and 19 in advanced stage), 10 with benign ovarian tumors and 13 age-matching patients without cancer and tumor collected from the Department of Gynecology, The First and Third Affiliated Hospitals of Zhengzhou University, from January 2008 to December 2008, were recruited in this study. Three milliliter of serum and tissues from each patient were collected before surgery. Ascites and cyst fluid samples were further obtained from five patients in the 19 patients with OC at advanced stages (stage III or IV) during the operation and all the samples were kept from blood contamination. All of the serum specimens were centrifuged at 3000 g for five minutes within 2 h after collection. All samples were divided into dichotomized parts (one for proteomic profiling and the other for ELISA detecting) before stored at -70°C until use and were assayed within 1 year of frozen storage. All of the tissues matching with the serum were collected at the beginning of the operation and were disposed by paraffin after being fixed with 10% formalin. Normal ovarian tissues came from the patients with hysteroptosis who wanted to resect their ovary by transvaginal hysterectomy. A total of 63 samples were selected as the training set. Another 8 cases of OC, 5 cases of benign ovarian tumor and 5 cases of normal groups were also collected from the serum by the same method and were conducted as the testing set. Additional 10 cases of tissues and 10 cases of unmatched serum of benign patients were also collected and handled by the same method for immuno-histochemistry (IHC) and enzyme-linked immunosorbent assay (ELISA) detect. All the necessary informed consents were obtained. The average age of patients at the time of surgery was 53 ± 10.6 years (range from 30 to 71 years). Pathological parameters were carefully recorded. The clinical staging was based on the FIGO (2000) stage protocol. Radiotherapy, chemotherapy or immunotherapy was not implemented on all patients before surgery. All of the participants were identified with normal renal function and without any compli-cations. The study was approved by the committee at Zhengzhou University of Medical Science for Ethical Review of Research involving Human Subjects.

Proteomic analysis to profile plasma proteins

Samples were thawed and the proteomic fractionation of the samples was performed with ClinProt purification reagent sets from Bruker Daltonics. Chemically coated magnetic beads (particle size <1 m), defined as hydrophobic surface functionalities [Magnetic bead-hydrophobic interaction chromatography (MBWCX)] were used for separation of peptides. According to the recommendation in the manu-facturer's protocol, 5 L of the serum, 10 L of the ascite and 2.5 L of the cyst fluid samples were diluted respectively with 10 L of binding solution and added to the bead slurry (5 L) in a 200 L poly-propylene tube. After thoroughly mixing, the binding incubation time took 1 min to get the eluted solution. Samples were purified through three steps-binding, three washings and elution according to the manufactures' protocol. Various solutions were prepared and spotted for MALDI-TOF-MS by mixing 1 L sample with 1 L matrix solution containing 2 g L^{-1} -cyano-4-hydroxycinnamic acid in ethanol- acetone (2:1 by volume) on the AnchorChipTM target (Bruker Daltonics). As for the proteome analysis, the parameters of liner MALDI-TOF-MS (Autoflex; Bruker Daltonics) were: ion source 1, 20 kV; ion source 2, 18 kV; lens, 7.5 kV; pulsed ion extraction, 210 ns; nitrogen pressure, 2000 mbar. Mass calibration was performed with the calibration mixture

of peptides and proteins in a mass range of 1000 – 10000 Da. Ionization was achieved by irradiation with a nitro-gen laser (= 337 nm) operating at 20 Hz. The matrix suppression mode was gating and a high gating factor with signal suppression was up to 700 Da. Mass spectra were detected in linear positive mode. Eight MALDI preparations (MALDI spots) from each magnetic bead fraction were measured. For each MALDI spot, 400 spectra were acquired (50 laser shots at eight different spot positions). To increase the sensitivity of detection, excess matrix with six shots at a laser power of 45% were removed before data acquisition at 25%. The m/z was also used to dispose the primitive spectra by base line cutting down, correcting and normalization. Top Hat Baseline and Savitsky Golay smoothing were used to dispose and smooth the baseline. The proteomic peaks were detected and quantified by an algorithm that takes the maximal peak area of every denoised, baseline-corrected and calibrated mass spectrum into account. All signals with a signal-to-noise (S/N) ratio >5 in a mass range of 1000 - 10000 Da were recorded with the ClinProt^{TM} software (Version 2.2; Bruker Daltonics) for proteome pattern recognition.

Deduction and verification of the early detected protein of ovarian carcinoma

From the serum samples, peaks of cancer group (early stage and advanced stage), benign group and control group were compared. The peaks, which were elevated in the serum, ascite and cyst fluid of cancer group simultaneously, were obtained at last. Consecutively, the peptides matched with the interesting m/z peaks were deduced by imputing the peptide mass in the http: // us.expasy.org / tools / tagident. Html, searching and referring to previous studies.

Immunohistochemistry

A total of 30 matched tissues of OC (11 in early stage, 19 in advanced stage), 20 tissues of benign ovarian tumor (10 with matched serum and 10 without) and 13 tissues of normal ovary were evaluated for GRP expression by IHC. Immunostaining was performed with the anti-bodies of the GRP (rabbit polyclonal, MAB35841 R&D Co, American) and IgG fluorescence Secondary Antibodies (supported by Yueyan Biotech Co. Shanghai). According to the manufacture's protocol, a standard streptavidin-biotin-peroxidase method was used. A semi- quantitative scoring criterion was used to evaluate the IHC staining. The evaluation of IHC grade was performed by referring the study of Watari et al. (2009). A staining index was obtained from the sum of scores of the percentage of positive cells (0: <20% positive staining; 1: 20 - 80%; 2: >80%) and staining intensity (0: negative staining; 1: weak; 2: moderate; 3: strong). Scores varying from 0 to 6 were obtained by multiplying the two variables. Immunostaining of all slides was evaluated blindly without any knowledge of the clinical data. Scores 0 - 1 were defined as low expression (including negative expression), score 2 as moderate and 3 as high expression.

Enzyme-linked immunosorbent assay

The serum samples of 30 cases with OC (11 in early stage and 19 in advanced stage), 20 cases with benign ovarian tumor (10 with matched tissue and 10 without) and 13 with normal ovaries were used to assess the serum level of GRP by gastrin-releasing peptide ELISA kit (QC01-1, R&D Co, American). The reactions were done by following the manufacture's protocol. Briefly, five wells were set on the coated ELISA plate and 100 L of standard sample were added to the first well. Then the standard dilution was used to dilute the standard sample gradually to the concentration of 9, 6, 3, 1.5 and 0.75 pmol L^{-1} at each of the five wells. 40 L sample dilution and 10 L testing sample were added sequentially to each of the 63 testing wells. After incubated for 30 min in 37°C, the liquids were discarded and washed for five times in 30 s and dried by patting. 50 L of ELISA reagent were added to each well except the blank well. After incubated for 30 min at 37°C and five washing, 50 L of color reagent A and B were added to each well. After being gently mixed and incubated for 15 min at 37°C, the reaction of the solution was stopped by adding a 50 L stop solution to each well. Fifteen minutes later, the optical density (OD) value was measured at 450 nm by taking the blank well as zero. Standard curve was drawn by taking the standard concentration as horizontal and the OD value as vertical. The corresponding con-centration of testing samples was calculated according to the standard sample curve.

Statistics

The build-in ClinProt^{TM} software (Bruker Daltonics Company) was used to compile the peaks from all samples. A linear support vector machine (SVM) classifier was used to distinguish between the different groups of data. Diagnostic value of each peak was estimated by Kruskal-Wallis test. The combinations of the selected peaks were analyzed by the leave-one-out cross-validation. The results of IHC and ELISA were analyzed by ^{2} test and variance analysis (q-test). Spearman correlations analysis was used to get the relevance of the IHC and ELISA in cancer group. The 95% credibility interval of ELISA results of every group was calculated. Pearson correlation coefficient were used to analyze the corre-lationship of serum level of candidated peaks and suspected peptide. These data analysis were completed by analysis software SPSS13.0.

RESULTS

The peaks elevated in both early stage and advanced stage

MALDI-TOF -MS was used to profile the mass spectral patterns of serum samples from 13 patients without tumor, 10 patients with benign ovarian tumor and 30 patients with ovarian carcinoma (11 of early stage and 19 of advanced stage) . After normalizing the peak and modifying the S/N >5 by the ClinProtools, we analyzed the peaks across the spectra coming from the samples by the built-in software and found that there were no different peaks between benign and normal groups (Figure 1). Eight elevated peaks significantly discriminating the early stage OC group from the normal group were 2104, 4438, 4120, 4627, 8775, 4468, 2883 and 2899 Da of the mean molecular masses ($P < 0.05$) (Figure 2a). Nine significantly different peaks were detected between the advanced stage OC group and control group were 4019, 2881, 4266, 4466, 8935, 3278, 2897, 6050 and 6068 Da of the mean masses ($P < 0.05$) (Figure 2a). As seen in Figure 2a, the inten-sities of these peaks in OC samples were all stronger than in the normal group. By the same way, seven peaks

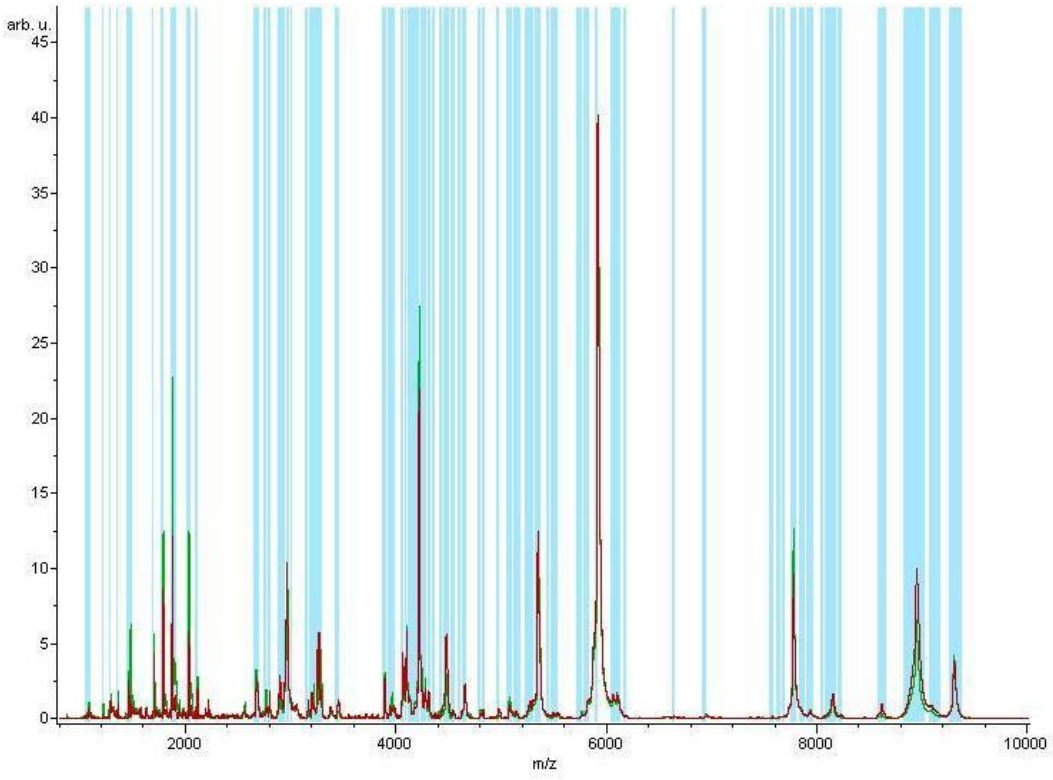

Figure 1. The average spectrums of the two groups (benign and normal).

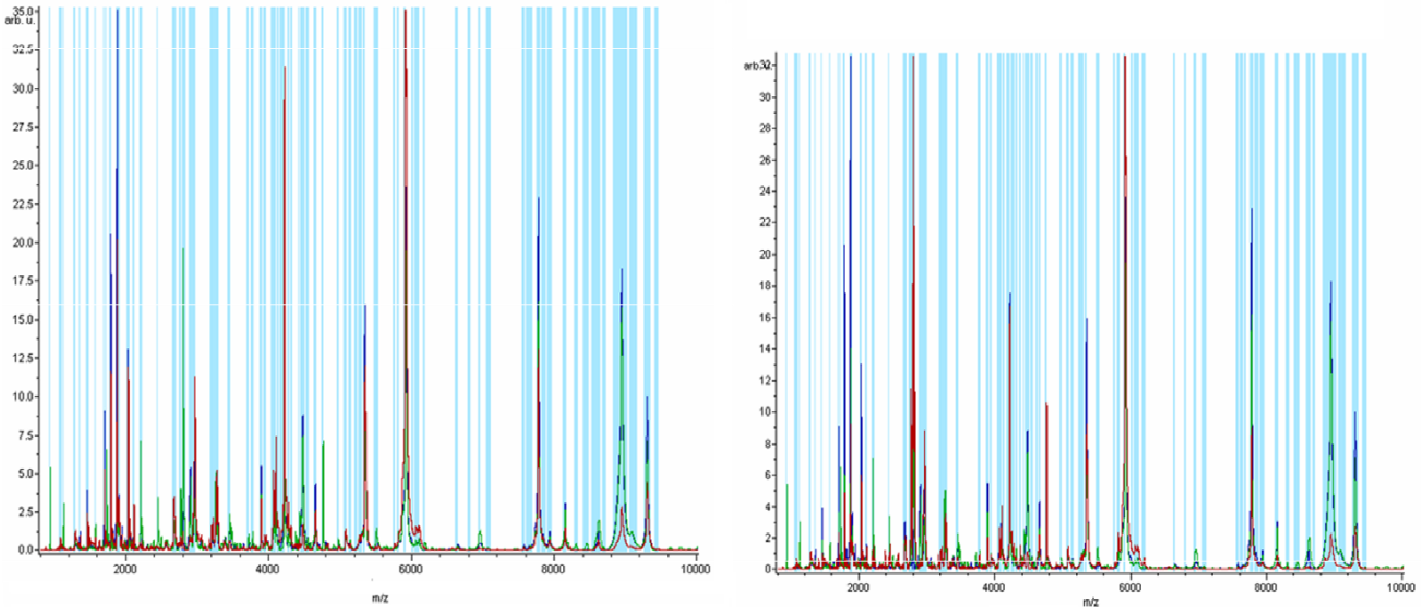

Figure 2. The average spectrums of the three groups (cancer, benign and normal). The average spectrums of the early stage, advanced stage and normal groups in A and the early stage, advanced stage and benign groups in B are all displayed with blue marked picked peaks. The green is early stage, the blue is advanced stage. The red is normal in A and benign in B.

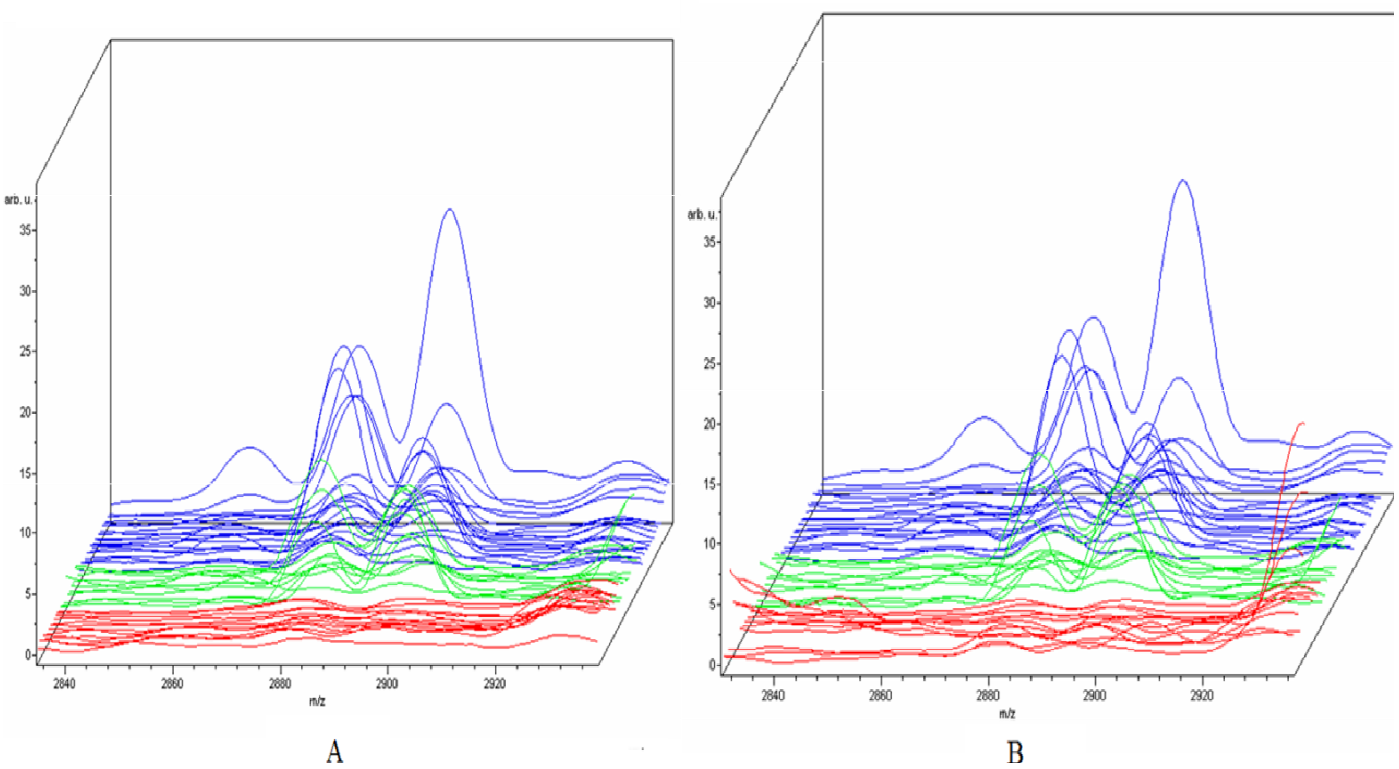

Figure 3. The stack view of the areas of peaks 2881 and 2897 Da in all the spectra of the three groups (cancer, benign and normal).

significantly elevated in early stage of OC when compared with the benign group were 8163, 4437, 8773, 2937, 2883, 2898 and 3470 Da (Figure 2b) ($P < 0.05$). There were also eight elevated peaks in advanced stage when compared with the benign group as 2881, 5804, 8603, 1863, 2897, 6049, 6068 Da (Figure 2b) ($P < 0.05$). The common elevated peaks in early and advanced stage of OC compared with benign tumor and normal group were 2881 and 2897 Da (Figures 3A and B).

The average spectrums of the two groups (benign and normal) are displayed with blue marked picked peaks. The x-axis represents the m/z value. The y-axis represents the peak intensity in arbitrary units. The green is benign group and the red is normal group. There was no difference between the two groups.

The stack view displays the areas of peaks 2881 and 2897 Da in all the spectra of the loaded early stage, advanced stage and normal groups in Figure 3A and early stage, advanced stage and benign groups in Figure 3B. The x-axis represents the m/z value, the y-axis represents the peak intensity in arbitrary units and the z-axis represents the loading order. The green is early stage, the blue is advanced stage. The red is normal in Figure 3A and benign in Figure 3B. On the 2881, 2897 Da peaks, spectrograms in all samples of early and advanced stage show significantly higher abundance than the normal and benign groups in Figures 3A and B.

The peaks elevated in serum, ascite and cyst fluid of cancer group

Between the two sets of data, two common peaks of 2881 and 2897 Da were summarized and elevated in ovarian carcinomas both at early and advanced stages compared with the benign tumor and normal groups (Table 1). It was further found interestingly that the two peaks were also elevated in the ascite and cyst fluid samples of four patients with OC in advanced stage (Figures 4A - D).

Figures 4A - D were all indicated by flexanalysis 3.0 and show the peptide peaks intensity on 2881 and 2897 Da in the ascite and cyst fluid of the four patients, respectively. The x-axis represents the m/z value and the y-axis represents the peak intensity.

The differential pattern of ovarian carcinoma versus benign and health control

The peaks at 2881 and 2897 Da were highly expressed in ovarian carcinoma group and weakly expressed in the benign and normal groups. The descriptive statistics of

Table 1. The statistics of 2881 and 2897 Da peaks in serum for screening ovarian carcinoma from benign group and normal group.

m/z	Intensity in OC group (mean ± SD)	Intensity in benign group (mean ± SD)	Intensity in normal group (mean ± SD)	P
2881	69.69 ± 49.81	24.68 ± 11.92	19.87 ± 2.51	0.0000026
2897	77.75 ± 50.56	26.41 ± 5.65	20.62 ± 2.86	< 0.000001

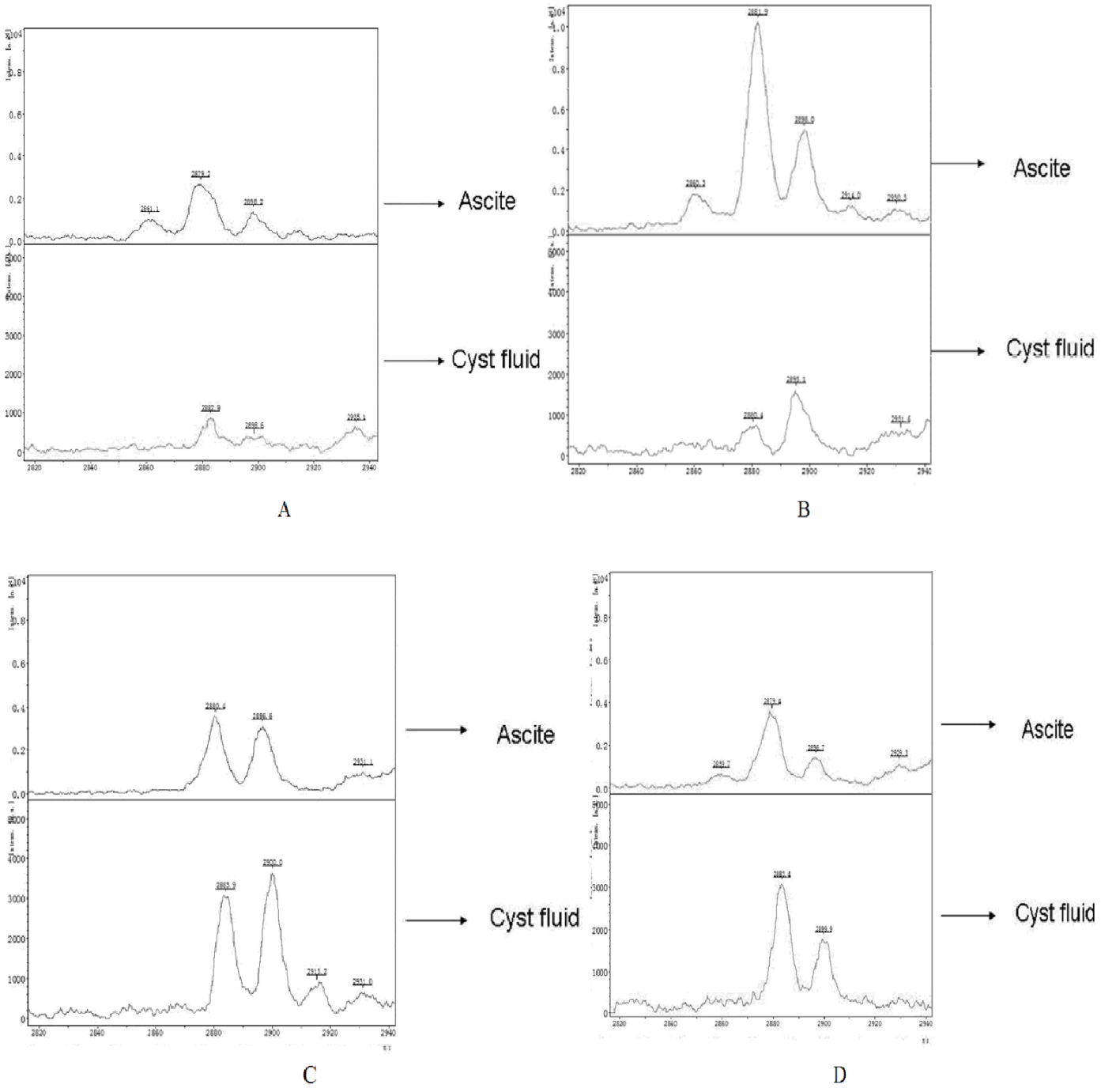

Figure 4. The common elevated peaks 2881 and 2897 Da in ascites and cyst fluid of 4 patients with OC.

Table 2. The predicted results of the pattern of 2881Da peak combined with 2897Da peak distinguishing ovarian carcinoma group from benign and normal groups.

	Training		Leave-one-out		Blind test	
	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)
OC vs normal group	100	100	100	100	100	87.5
OC vs benign group	90	93.33	80	93.33	80	87.5

these two peaks were shown in Table 1. The pattern of two combined peaks had a specificity of 100% and a sensitivity of 100%, as evaluated by leave-one-out cross validation. The remaining 18 serum samples were analyzed in the second day to test this pattern as a blind test set (Table 2) . Diagnosis was correctly made in 7 out of 8 patients with OC and in 5 out of 5 patients with normal ovaries by analyzing the blind serum. The specificity and sensitivity of the blind test were 100 and 87.5%, respectively. The diagnostic significance of the combination of the 2881 and 2897 Da peaks were visualized by the standard deviation comparing Figure and the sample distribution Figure (Figures 5A - B).

In Figures 5A and B, the red was cancer group, the green was benign group and the blue was the normal group. From the figure, it can be seen that the level of the 2881 (Figure 5B) and 2897 (Figure 5A) were all higher than in other groups.

In Figure 5C, the red was cancer group, the green was benign group and the blue was the normal group. From the figure, it can be seen that the combination of 2881 and 2897 Da peaks can easily differentiate the three groups.

Immunohistochemistry and enzyme-linked immunosorbent assay

To deduce the interesting peptides, we searched the masses of 2881 and 2897 Da in the http://us.expasy.org/tools/tagident data bank (Mw range was 2%) (Table 3). Among the previous research results, gastrin-releasing peptide (GRP) was the only peptide relevant to cancer among the nine kinds of searched peptides and thought as the probable matching peptide of 2881 and 2897 Da peaks.

Subsequent IHC and ELISA were used to further test the expression status of GRP in tissues and serum. It was shown that GRP was mainly localized in the cytoplasm and representative results of immuno-histochemistry were shown in Figure 6. The positive expression of GRP in tissues of OC were 72.73% within early stage and 78.95% within advanced stage, more higher than the 30% of the benign tumor. The expressions of GRP in the normal group were all low to negative expression (13/13) (^{2} = 24.599, P = 0.00).

The higher serum level of GRP in OC (15.85 ± 4.40 pmol L^{-1} and 16.98 ± 3.81 pmol L^{-1} in early and advanced stage) than benign (5.66 ± 3.88pmol L^{-1}) and normal groups (5.19 ± 3.02 pmol L^{-1}) was verified by ELISA ($P < 0.05$, by q – test) (Table 4). Serum level of GRP had 90.91% of specificity and 72.73% of sensitivity when detecting the early stage of OC. There was a high correlation between level of gastrin-releasing peptide in tissue and serum ($r = 0.809$, $P = 0.000$). The correlations of the serum level of 2881, 2897 Da peaks and serum level of GRP in three groups were all high (Table 5).

Figure 6A - C shows the immuno staining of GRP in cancer tissue (a), benign tissue (b) and normal tissue (c). Over expression of GRP was seen in cancer, but not in benign and normal. Magnifications were 10 × 20 and 10 × 40 (lower right corner) in each picture.

DISCUSSION

One challenge of the research on early detection of ovarian carcinoma is that most screening tests lacking adequate sensitivity and might cause considerable confusions (Ian and Usha, 2004). This was because most markers were developed from clinically diagnosed and often advanced stage which caused the markers not to sensitively screen the patients in early stage as well as advanced stage (Ian and Usha, 2004; Chen et al., 2009). The author first found that the protein profiling of the serum in the early and advanced stages of OC were different when compared with the benign and the normal groups. The data of this study illustrated that the biological characteristics of the ovarian carcinoma in early stage and advanced stage were partly different. Furthermore, the peptides elevated both in early stage and advanced stage could be valuable for detection of OC in any stage. In the previous studies (Ott et al., 2003; Gortzak-Uzan et al., 2008), diagnostic markers had been screened from ascites and cyst fluids. Some studies (Ott et al., 2003; Zhang et al., 2009) showed that calgranulins in cystic fluid and serum from patients with OC could be a valuable biomarker for early detection of OC by 2-DE. But the reproducibility of 2-DE has recently been considered lower than the MALDI-TOF-MS combined with magnetic

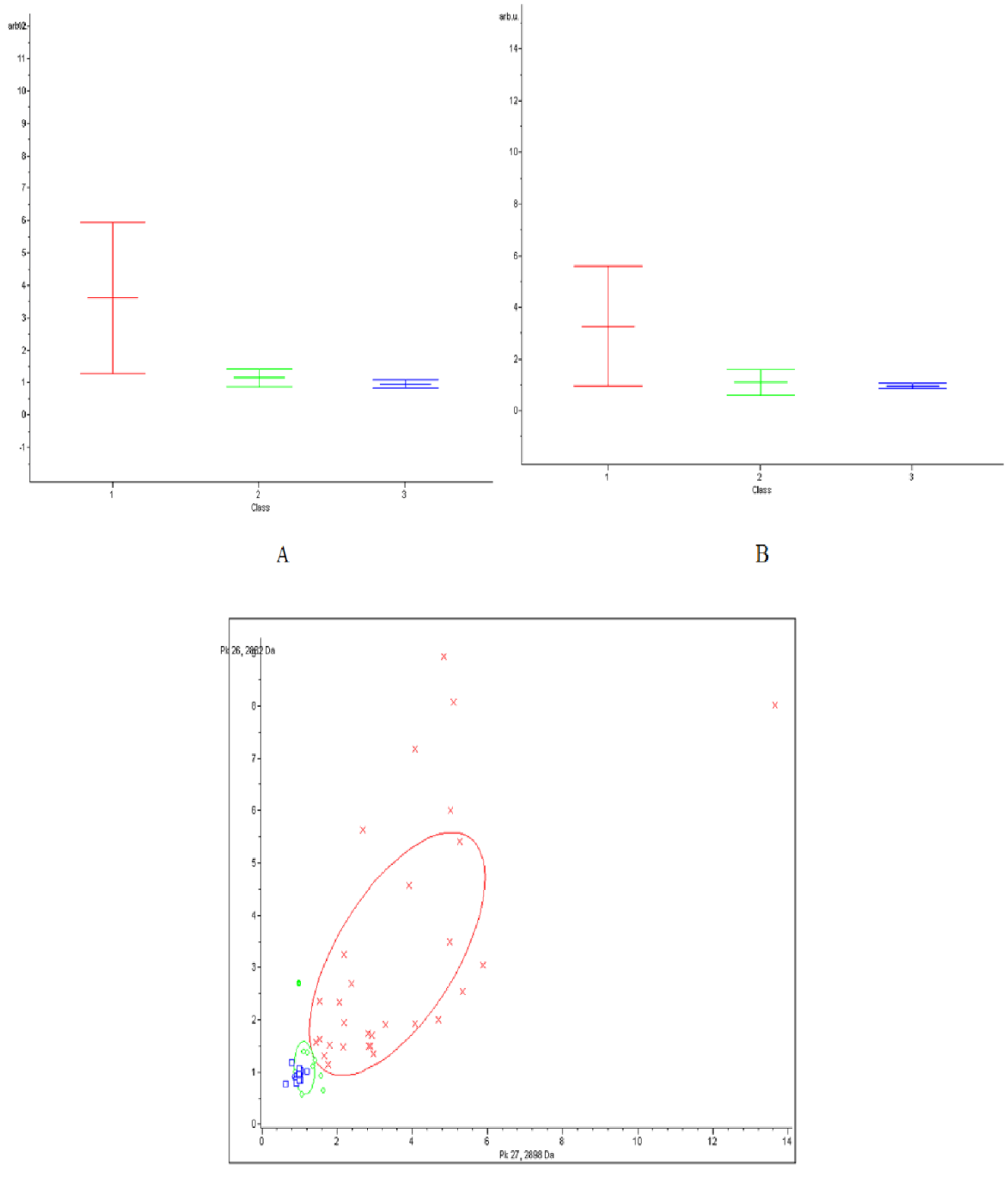

Figure 5. The means and standard deviations comparing figure of the three groups (A and B); the sample distribution figure of the three groups (C).

Table 3. Results of the matching peptides of 2881 and 2897Da peaks by searching in http://us.expasy.org/tools/tagident.html data bank and the references relative with elevating in cancer.

2881 Da			2897 Da		
Matching peptide	**Mw**	**References relative with elevating in cancer**	**Matching peptide**	**Mw**	**References relative with elevating in cancer**
Orexin-B	2900	0	Orexin-B	2900	0
Gastrin-releasing peptide	2860	128	Gastrin-releasing peptide	2860	128
BNP(4-30)	2800	0	BNP(4-30)	2800	0
BNP(5-31)	2885	0	BNP(5-31)	2885	0
BNP(1-28)	2903	0	BNP(1-28)	2903	
BNP(3-29)	2832	0	Putative uncharacterized protein c14orf144	2908	0
Putative uncharacterized protein c14or f144	2908	0	P-beta	2876	0
P-beta	2876	0	Insulin-like peptide INSL6A chain(Potential)	2887	0
Insulin-like peptide INSL6A chain(Potential)	2887	0			

Table 4. Serum GRP level by ELISA test in three groups ($P < 0.05$, q –test).

Groups	Concentration ($\bar{\chi}$ ±S pmol L^{-1})	95% credibility interval
Normal	5.19 ± 3.02	3.37- 7.02
Benign	5.66 ± 3.88	4.08- 7.24
Early stage	15.85 ± 4.4*, **	12.89- 18.81
Advanced stage	16.98 ± 3.81*, **	15.14- 18.82

Compared with the control group, * represent $P < 0.05$, with a significant difference; compared with the benign group, **represent for $P < 0.05$, with a significant difference; $P < 0.05$, q-test.

Table 5. Correlation of 2881 and 2897 Da (m/z) peaks and GRP levels in serum.

	GRP level (pg/ml), correlation coefficient(P)		
	Normal	**Benign**	**Cancer**
2881Da m/z	r = 0.92, P< 0.0001	r = 0.91, P < 0.0001	r = 0.96, P < 0.0001
2897Da m/z	r = 0.88, P < 0.0001	r = 0.95, P < 0.0001	r = 0.97, P < 0.0001

beads profiling (Latterich et al., 2008; McDowell et al., 2009). Another study (Gortzak-Uzan et al., 2008) screened what is in the ascites of OC patients and got 2500 proteins from OC patients by proteomic. After referring to the microarray and proteomics data from the previous studies in serum, 80 kinds of interesting proteins to diagnose OC in early stages were obtained in that study. But the proteins in ascites should be further screened in the serum in order to be more clinically useful. Different from the previous studies, we compared the results of early and advanced stages of OC VS control groups separately. The 2881 and 2897 Da peaks found elevated both in early stage and advanced stage ($P = 0.0000026$; $P < 0.000001$) were thought to be the most potential early diagnostic markers of OC. Authors' discovery supported the idea that profiling the mass spectral patterns of proteins or peptides in serum samples in early and advanced stages of OC patients separately should be more helpful to the early detection of patients with OC than not profiling separately.

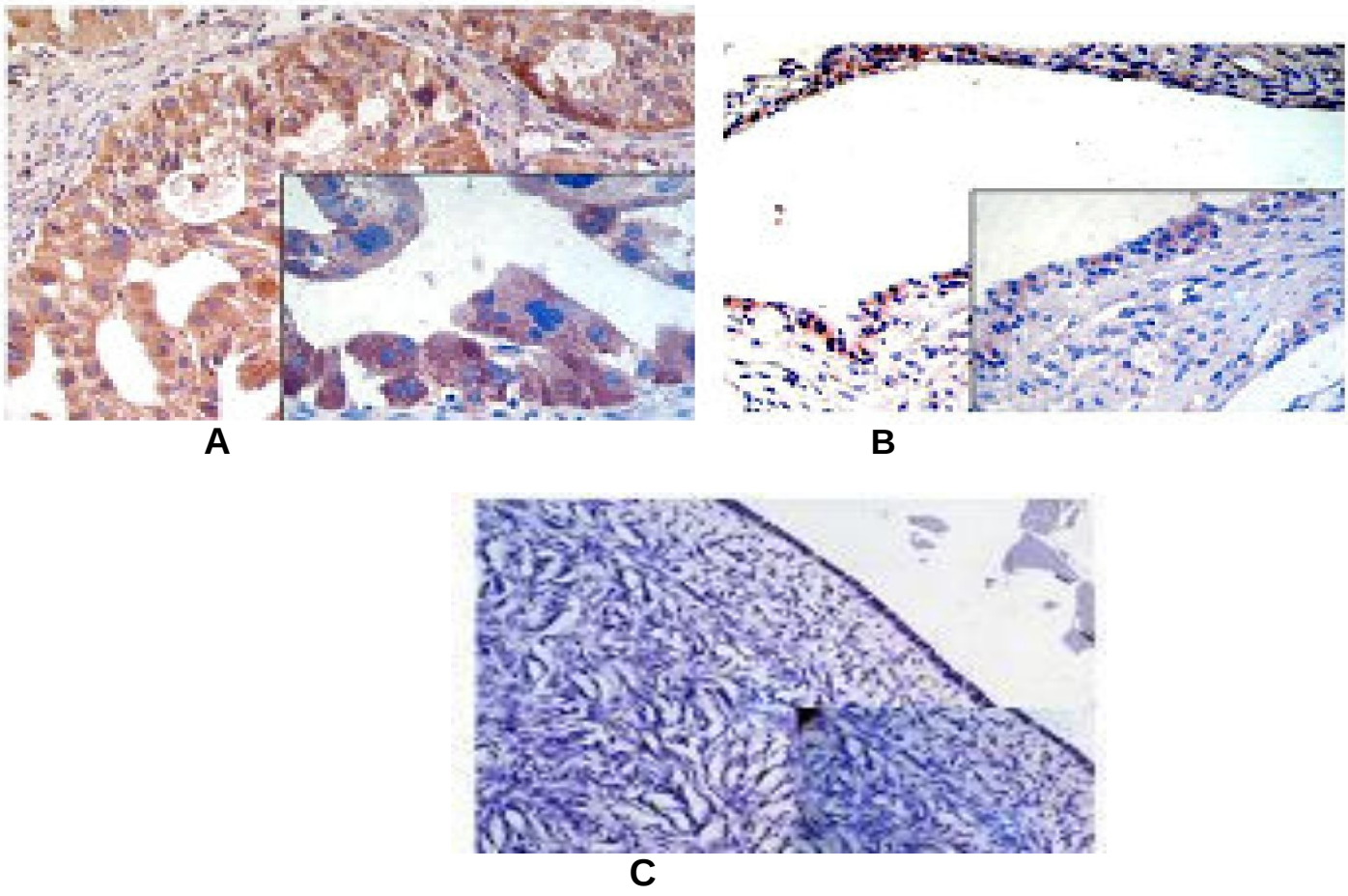

Figure 6. Immuno staining of GRP in three groups (cancer, benign AND normal).

However, the peptides in the serum come from all over the body and have poor specificity. To improve this condition, the mass spectral pattern in the ascite and cyst fluid was also profiled based on the idea that the cancer cells might secrete some peptides into the serum, ascite and cyst fluid simultaneously during the biological process. Therefore, we further found that the 2881 and 2897 Da peaks elevated in serum, ascite and cyst fluid had a specificity of 100% and sensitivity of 87.5% when patients with OC from the normal group were diagnosed and a specificity of 80% and sensitivity of 87.5% when patients with OC from the benign group were diagnosed. Compared with previous studies, the pattern of 2881 combined with 2897 Da in the present study possessed superiority and reliability in early detecting of ovarian carcinoma.

According to the results searched from the http://us.expasy.org/tools/tagident data bank, and referring to the previous studies, GRP was thought as the possible peptide matching with 2881 and 2897 Da. We considered that the peptide matching with the 2881 and 2897 Da peaks might be the same by referring to other study (Moore et al., 2006), in which they thought that it was the ingredient on the matrix that caused the two peaks for one matching peptide. The subsequent results of IHC and ELISA and the high correlation of the serum level of 2881, 2897 Da peaks and GRP accounted for our deduction. Gastrin-releasing peptide is an autocrine growth factor in neuroblastomas and esophageal squamous cell cancer (Kim et al., 2002; Fang et al., 2004). Previously, GRP has been found as a mitogen in digestive system cancers like gastric and colon cancers (Fang et al., 2004). Recently, GRP and its receptors (GRPR) have been verified over-expressed in many other cancers and have been shown to act as a potent mitogen for cancer cells (Fang et al., 2004; Patel and Baldwin, 2006; Ghosh et al., 2010). Our study first veri-fied that GRP was over-expressed in OC tissue than in tissues of benign and normal groups (^{2} = 24.599, P = 0.00) which indicated that GRP might play a role in the tumerigenesis of OC. However, there was no difference in the expression of GRP in early and advanced stage of OC

which indicated that GRP might not be an index of the development of OC. That is to say, GRP might be involved in the carcinogeniesis but not the development of OC. The significant difference of GRP in serum between cancer group and benign and normal groups by ELISA, suggested that over expressed GRP in serum might be useful to hunt down patients with OC in early stage well from benign and normal group. Because GRP has been found to be elevated in other cancers and its instability in serum, the diagnostic value of GRP in ovarian carcinoma needs further study by a larger sample size and the stable pro-GRP should be used to substitute for GRP in early detection of ovarian carcinoma.

In summary, we first used the MALDI-TOF -MS integrating with magnetic beads analysis to screen the peptide profile in the serum, ascite and cyst fluid of patients with OC. By way of this method, we first found that 2881 and 2897 Da peaks were over expressed in serum of OC. We also provided a new idea of dividing the OC group into early and advanced groups to further investigate the variation of peptides profile in serum. Because of this promising searching mode, expanding sample scales will make the cancer type and other pathologic data more distinguishable. To be clinically useful, urine samples should be recruited to further provide a non-invasive biomarker. The proteomic of the early stage in OC allowed us to elucidate the true condition of the early variations of OC from normal ovaries. Gastrin-releasing peptide might be the matching peptide of the two peaks and a biomarker for early diagnosis of ovarian carcinoma.

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