A Study of Serum Biomarkers Associated with Relapse of Cervical Cancer

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Abstract. *Background/Aim: To discover candidate protein biomarkers in the serum of patients with cervical cancer that differentiate between patients with relapse from those who are tumor-free after primary treatment with (platinum-based chemo-) radiation. Patients and Methods: Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) with cation exchange (CM10) and hydrophobic/reverse-phase (H50) was used to examine 44 serum samples from patients with advanced cervical cancer, primarily treated with (platinum-based chemo-) radiation. Results: Ten candidate biomarkers were identified in the serum of 34 patients. Six candidate markers were elevated in patients with no relapse and four were elevated in patients with relapse [p=0.007-0.11; area under the curve (AUC)=0.70-0.75]. Masses of candidate biomarkers ranged from 2,022 to 116,165 Da. Conclusion: Patients with relapse from primary advanced cervical cancer exhibit different serum protein expression profiles from those with no relapse.*

Despite the significant benefits achieved by screening programs for cervical cancer, the specific disease is still the leading cause of female cancer mortality worldwide (1, 2). In 2010, there were 428 new cases of cervical cancer (1.6% of all female cancer diagnoses) diagnosed in Sweden (3). The 5-year survival rate after surgery for patients with early-stage disease exceeds 90%, but is only 60-70% in patients with advanced-stage disease, despite the use of prognostic factors such as tumor staging, tumor size, histopathology and lymph node status, when selecting for patients who could benefit from radiation upfront (4-7). Thus, there is a need for improvement in targeting-therapy, as well as in our understanding over the mechanisms behind the disease.

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The evidence supporting the biological rationale of combining novel non-cytotoxic agents with chemoradiotherapy is strong, and drugs targeting different molecular pathways are under clinical development [such as epidermal growth factor receptor (EGFR) inhibitors, cyclooxygenase (COX)-2 inhibitors, hypoxia-targeted agents, etc.] (8). As yet, there is no single-biomarker that can predict which patients will experience disease relapse after primary radiation and which will remain in remission.

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) is a new technique used to identify biomarkers for cancer. The protein mixture from cancer cells is spotted on a surface modified with a chemical functionality. Some proteins in the sample bind to the surface, while the others are removed by a washing procedure. After washing the spotted sample, a new mixture of proteins is applied to the surface and allowed to crystallize with the sample peptides. Binding to the SELDI surface acts as a separation step and the subset of proteins that bind to the surface are then easier to analyze (9, 10).

Some of the previous studies using the SELDI-TOF-MS technique have investigated the role of protein biomarkers in distinguishing cervical cancer from healthy controls and in screening for early diagnosis of cervical cancer, while others have investigated the different proteomic profiles of cervical cancer in patients before and after neoadjuvant chemotherapy (11-13). These results are promising but have to be confirmed and validated in larger cohorts.

In the present study, we aimed at examining candidate protein biomarkers in the serum of patients with advanced-stage cervical cancer that could differentiate patients with relapse from those who are tumor-free after primary treatment with (platinum-based chemo-) radiation.

Materials and Methods

Study workflow. These steps were followed in the workflow process: i) The serum samples were fractionated to enrich for low-abundance proteins and to dilute high-abundance proteins; ii) chromatographic ProteinChip Arrays were used to capture different subsets of proteins using multiple array chemistries; iii) the SELDI ProteinChip System was used to detect retained proteins by TOF-MS; iv) data analyses

using univariate and multivariate statistics and quality control of candidate markers were finally carried out (Figure 1).

Patients. Serum samples from 44 consecutive patients with invasive cervical cancer, treated with (platinum-based chemo-) radiation at the Department of Gynecological Oncology, Örebro, Sweden between 1993 and 2006 were analyzed using the SELDI protein profiling. The patients are part of a larger consecutive selected cohort of a total of 131 patients treated with a combination of external pelvic irradiation in parallel with brachytherapy. External beam therapy was given with a four-field box technique with the upper border at L4-L5 level, the lateral borders 1-1.5-cm lateral of the linea terminalis, and the lower border, 1 cm below the obturator foramen. The dose per fraction was 2.0 Gy, given five days a week, and the total dose was 50 Gy (stages IB, IIA and early IIB) or 60 Gy (late IIB and III). The mean overall treatment time was 40 days. The brachytherapy was given in three or five fractions of 5 Gy by a high-dose rate (HDR) technique using an Ir-192 source. The absorbed doses and volumes were defined according to International Commission of Radiation Units and Measurements (ICRU) Report 38 (International Commission on Radiation Units and Measurements (ICRU). Dose and volume specification for reporting intracavitary therapy in gynecology. ICRU Report. Bethesda, MD: ICRU; 1985). A computer tomography-based 3-D dose planning system was used for external beam therapy. Concomitant single-agent cisplatin chemotherapy of 40 mg/m^2 every week was given in eight patients (23%), four patients of each group in this series. All patients were followed-up during the first five years by a physician specialized in Gynecological Oncology at the Department of Gynecological Oncology, Örebro.

Twenty-two of these patients were tumor-free after treatment and 22 patients had recurrent disease. The mean follow-up of patients alive was 91 months (range 60-131 months). The sites of recurrences were locoregional in nine cases and distant in eight cases. No significant differences were found in the background factors in the two groups (Table I).

The study was performed with informed consent from the patients as well as with permission from the local Ethics Committee in Uppsala, Sweden.

Serum fractionation and preparation of ProteinChip arrays. Serum (20 μl) was denatured and bound to anion exchange resin at pH 9 then treated with 30 μl of U9 denaturing buffer {9 M urea/2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)/50 mM Tris, pH 9} to reduce protein–protein interactions. U9-treated samples were applied to BioSepra Q Ceramic HyperD F resin (Pall Corporation, Port Washington, NY 11050, USA), which had been pre-equilibrated in 50 mM Tris, pH 9. The flow-through was collected, and bound proteins were eluted in a step-wise gradient by using buffers with pH values of 7, 5, 4, and 3, followed by an organic wash (33% isopropanol, 16.7% acetonitrile and 0.5% trifluoroacetic acid). The fractionation process yielded a total of four anion exchange fractions or pools. Fraction 1: pH 9 eluate, fraction 2: pH 7 eluate, fraction 3: pH 5 eluate, fraction 4: pH 4 eluate and fractions 5/6: pool of pH 3 and organic eluates. Fraction 2 was omitted due to high concentrations of hemoglobin in some samples. Two array types were used (CM 10 and H50) and each sample was profiled in duplicate and under eight unique conditions.

SELDI protein profiling. Each sample was profiled in duplicate during eight unique conditions using the four anion exchange fractions/pools. Duplicate 10-μl aliquots of each fraction were diluted with binding buffer and applied to H50 and CM10 ProteinChip arrays (Ciphergen Biosystems, Inc., 6611 Dumbarton Circle, Fremont, CA 94555, USA). The binding buffer was 10% acetonitrile and 0.1% trifluoroacetic acid for H50 arrays. Sinapinic acid was used as the energy-absorbing molecule. The fractionation and array preparation was fully-automated and performed by using a Biomek 2000 robot with an integrated Micromix 5 shaker (Beckman Coulter, Inc., 250 South Kraemer Boulevard, Brea, CA 92821-6232, USA). For quality control purposes, human reference serum samples and blind duplicates were included with the test serum samples. Hemolysis was noted as red coloration in some samples, and the alpha- and beta-chains of hemoglobin were quantified to determine the extent of this hemolysis.

Table I. *Characteristics of patients and tumor and treatment data*.

Factor*	No relapse (n=17)	Relapse (n=17)
Patient data		
Age (year)	66.1 (14.7)	67.8 (11.5)
BMI (kg/m^2)	26.4 (4.1)	29.7 (5.2)
Hb (mg/ml) 127 (15.2)	128 (15.2)	
AP (cm)	22.3 (3.2)	23.1 (3.2)
Tumor data		
Tumor size (mm)	40.8 (11.6)	45.0 (12.6)
Stage 1:2:3:4	7:7:2:1	1:12:3:1
SCC:AC	16:1	13:4
Grade 1:2:3	1:11:5	1:7:9
Diploid:non-diploid	7:8	8:8
MGS	16.3 (2.5)	15.8 (3.1)
Treatment data		
Total HDR dose (Gy)	28.2 (4.5)	29.0 (3.5)
Time in days (HDR)	27.1 (6.0)	25.8 (9.4)
Total EBRT dose (Gy)	54.1 (5.8)	53.8 (5.0)
Time in days (EBRT)	39.5 (4.7)	38.4 (5.4)
Concomitant chemotherapy	4/17	4/17

BMI=Body-mass index; MGS=malignancy grading score; HDR=high-dose- rate brachytherapy; EBRT=external-beam pelvic radiation therapy; AP=anterior-posterior diameter of the pelvic region; SCC=squamous cell carcinoma; AC=adenocarcinoma. *Mean values (standard deviation, SD) for continuous variables; number of cases in each group for non-continuous variables.

Statistics. Statistical analyses regarding SELDI protein profiling were performed by the Bio-Rad ProteinChip Data Manager software. Comparisons between patients with relapse and patients without relapse were performed using the Mann-Whitney test and area under the receiver operator characteristics (ROC) plot (AUC). Differences were considered statistically significant if $p<0.05$ and AUC >0.7. For analyses of clinical data *t*-test, chi-square test and the log-rank test were used (Statistica version 10, StatSoft Inc., Tulsa, USA).

Results

Clinical results. There were no significant differences concerning age, body-mass index (BMI), Hb and AP-diameter of the patients in the two groups. Tumor stage,

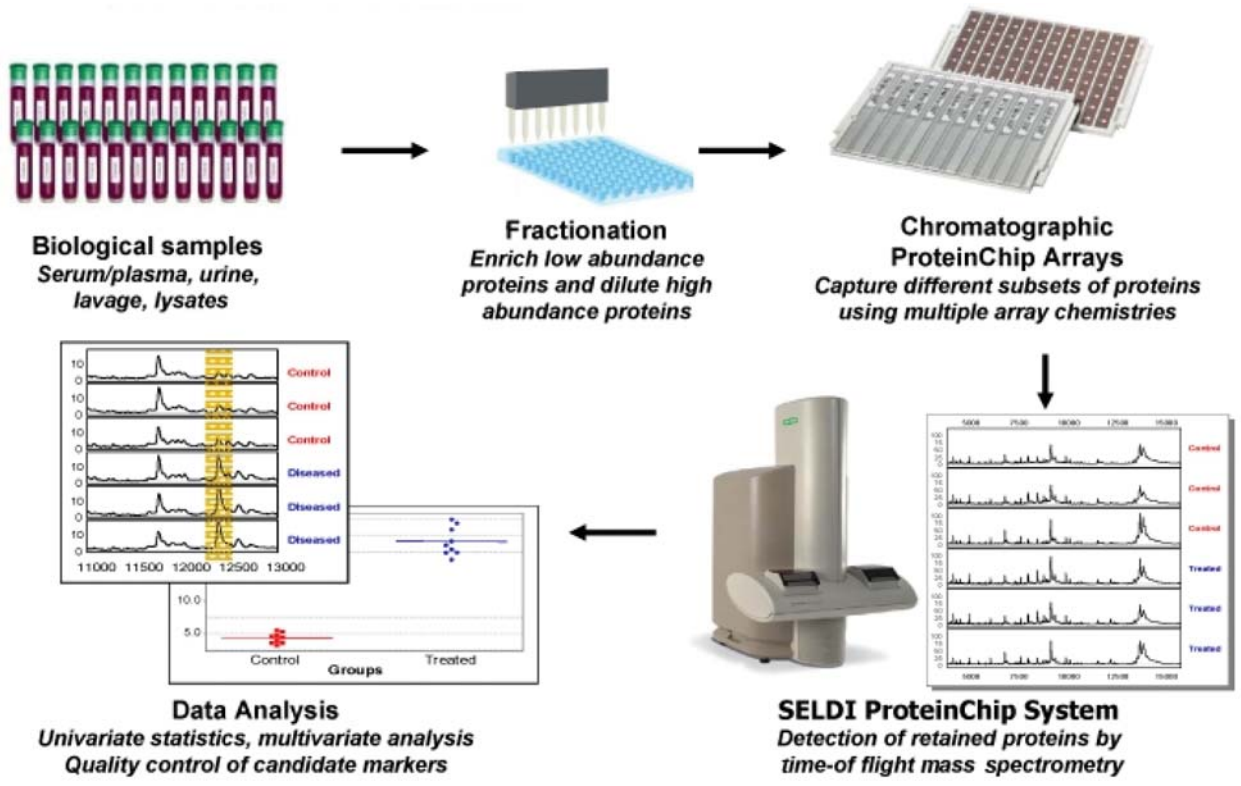


Figure 1. *The workflow of the biomarker discovery study.*

tumor size, type of histology, tumor grade, DNA-ploidy and the malignancy grading score were not significantly different in the two groups of tumors with and without relapse. Treatment-related data were fully comparable in the two groups (Table I). HPV and p53 status of the tumors were not complete and therefore were not included in the analyses. However, we observed a trend (numeric) of more recurrences in patients with adenocarcinoma (23%) *versus* patients with squamous cell carcinoma (6%). In the group with recurrent tumors (n=17) nine cases were locoregional and eight cases distant metastases. Median overall survival was 30.7 months [95% CI: 7.0-54.4 months] in the group with recurrences and 93.8 months [95% CI: 67.3-120.3 months] in the group without recurrences (log-rank test; p=0.00012).

Assay reproducibility. The reproducibility of the assay was estimated by using pooled human reference serum, which was applied in duplicate to CM10 and H50 ProteinChip arrays. The flow-through fraction 1, 5 and 6 on CM10 and H50 arrays yielded a median coefficient of variation (CV) of 13% and 9.8%, respectively. The detected masses ranged from 2.8 to 5.2 kDa in fraction 1 and 10-32 kDa for fractions 5 and 6.

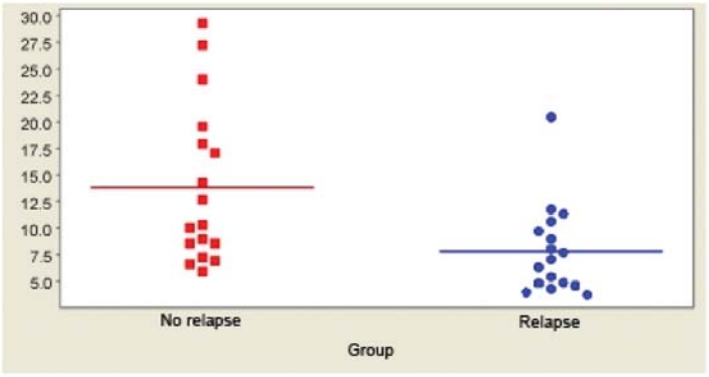
Table II. *Proteins up-regulated in patients with cervical cancer with and without relapse.*

Peak information			No relapse *vs*. relapse		
m/z	Fraction	array	*p*-value	AUC	Increased in
2022	F1	CM10	0.0069	0.77	No relapse
2689	F5/6	CM10	0.0138	0.75	No relapse
2742	F1	CM10	0.0559	0.70	No relapse
3159	F1	CM10	0.0220	0.73	Relapse
4643	F1	CM10	0.0167	0.73	Relapse
9949	F4	CM10	0.0125	0.77	No relapse
10226	F1	H50	0.0241	0.75	No relapse
40937	F4	CM10	0.1092	0.70	No relapse
49943	F5/6	H50	0.0183	0.73	Relapse
116165	F5/6	CM10	0.0138	0.75	Relapse

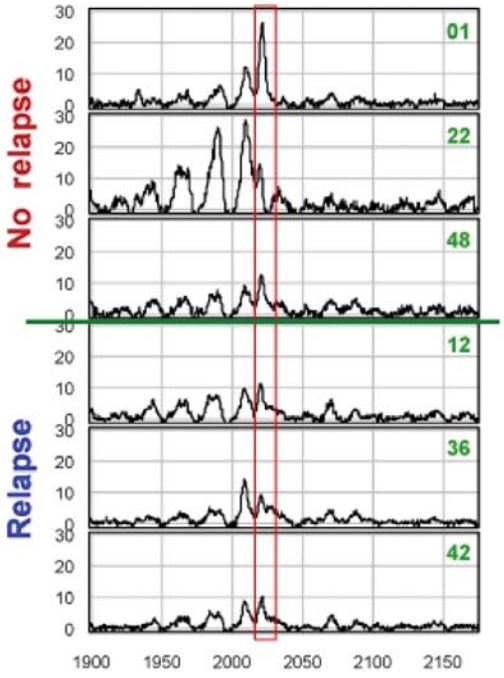
Candidate biomarkers. Out of 44 patients, results from 10 serum samples (six patients with no relapse and four patients with relapse) were excluded from statistical analysis due to high levels of hemolysis.

Using the Mann-Whitney test and AUC, a statistically significant differential peak was observed for 10 candidate biomarkers. Six candidate markers were elevated in patients

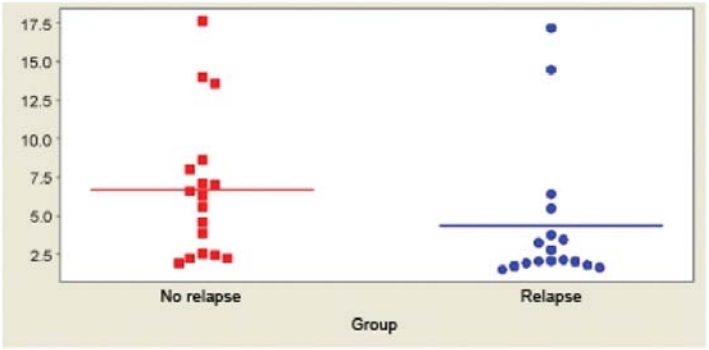
2022 m/z



Median of no relapse group is 47% higher than that of the relapse group



2689 m/z



Median of no relapse group is 194% higher than that of the relapse group

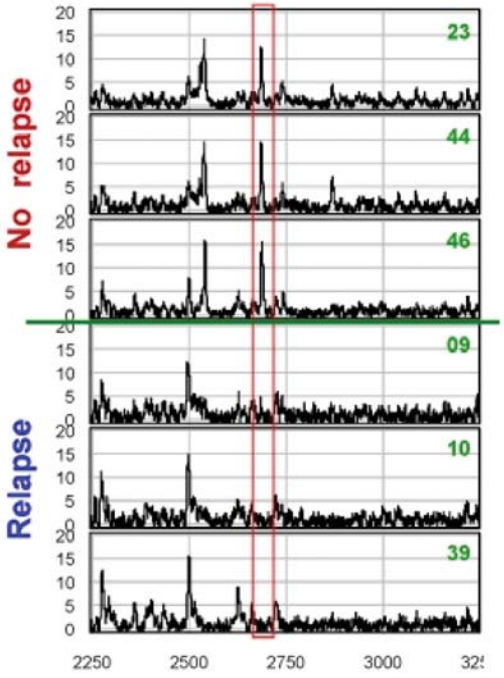
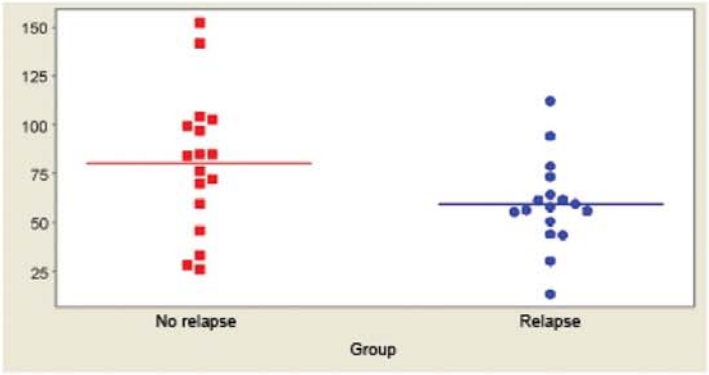
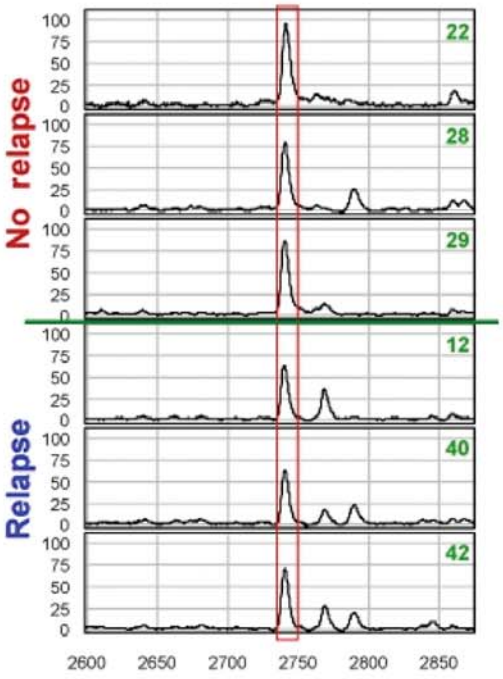


Figure 2. *Continued*

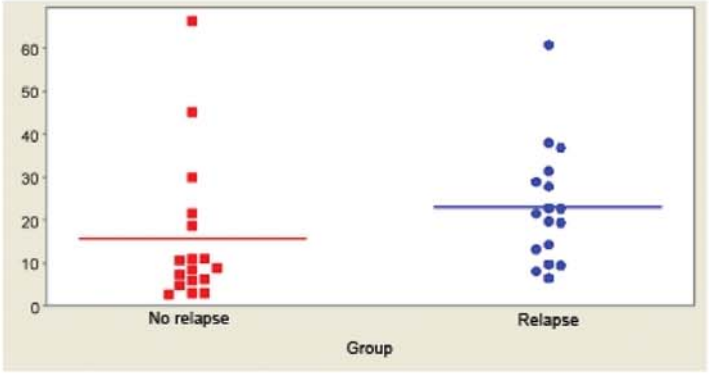
2742 m/z



Median of no relapse group is 45% higher than that of the relapse group



3159 m/z



Median of no relapse group is 140% higher than that of the relapse group

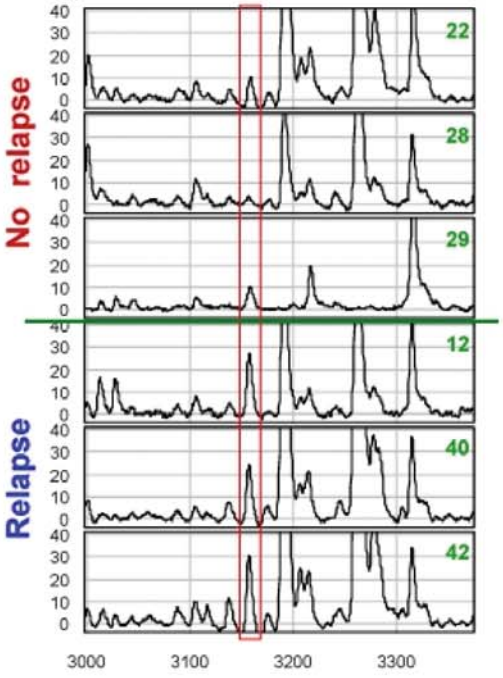
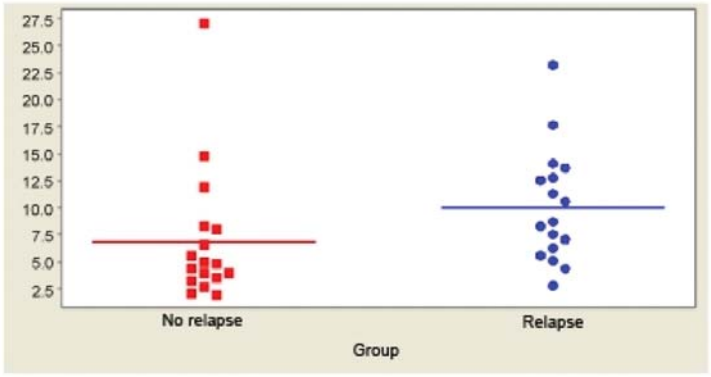
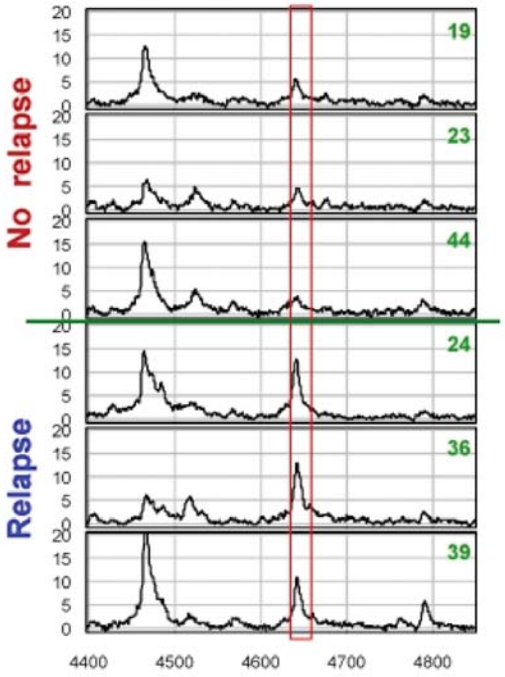


Figure 2. *Continued*

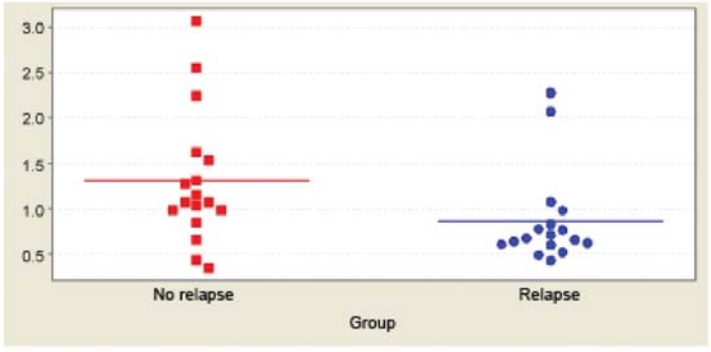
4643 m/z



Median of no relapse group is 80% higher than that of the relapse group



9949 m/z



Median of no relapse group is 59% higher than that of the relapse group

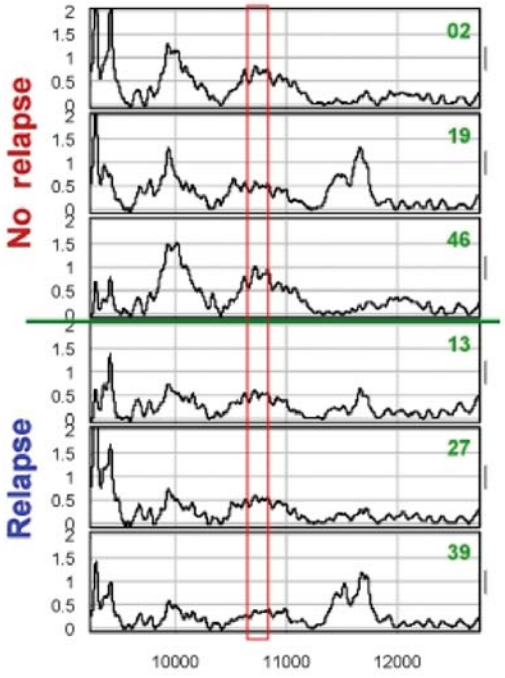
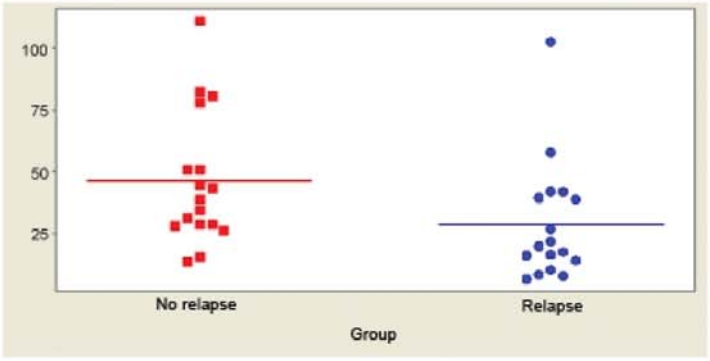
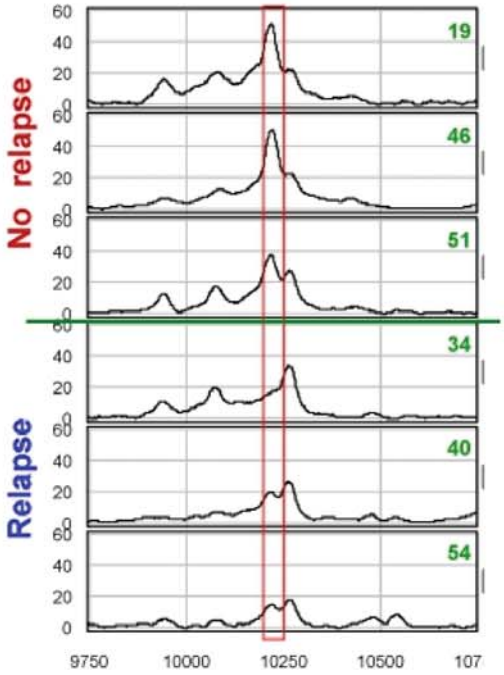


Figure 2. *Continued*

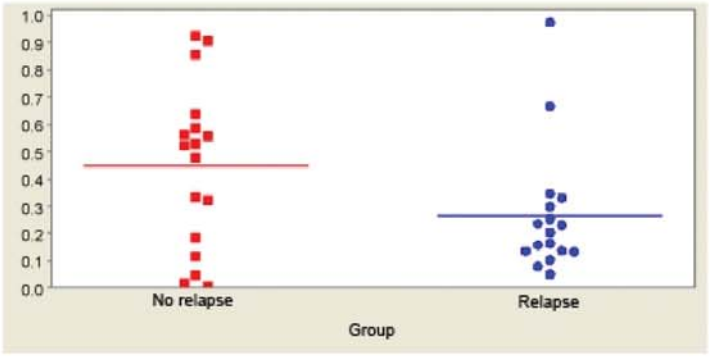
10226 m/z



Median of no relapse group is 95% higher than that of the relapse group



40937 m/z



Median of no relapse group is 156% higher than that of the relapse group

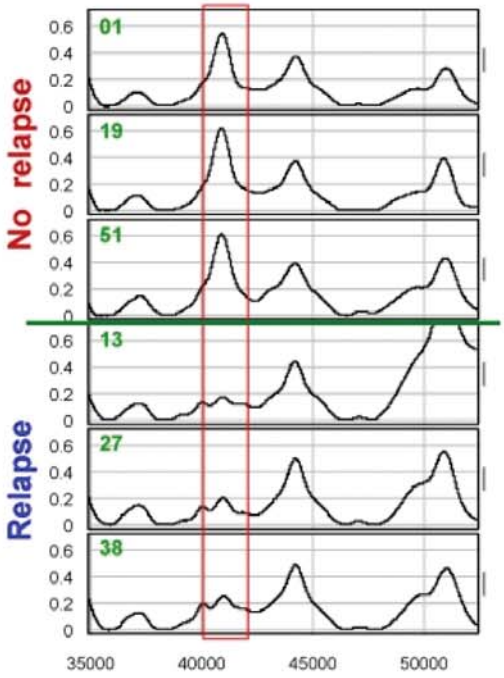
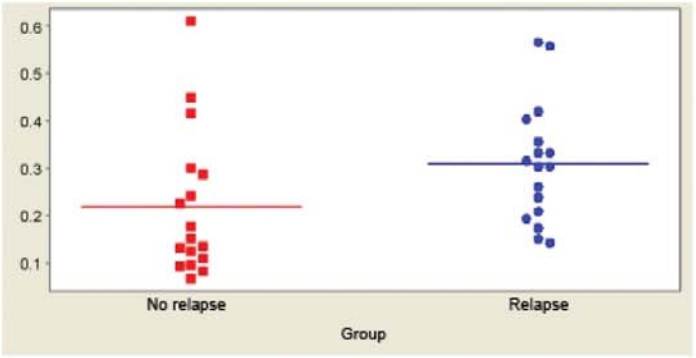
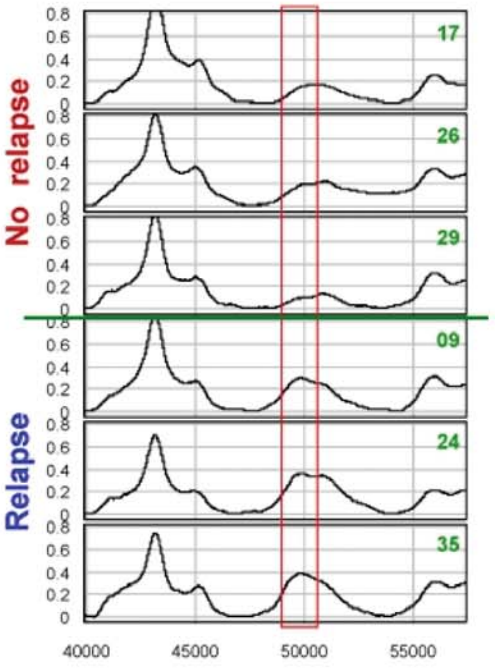


Figure 2. *Continued*

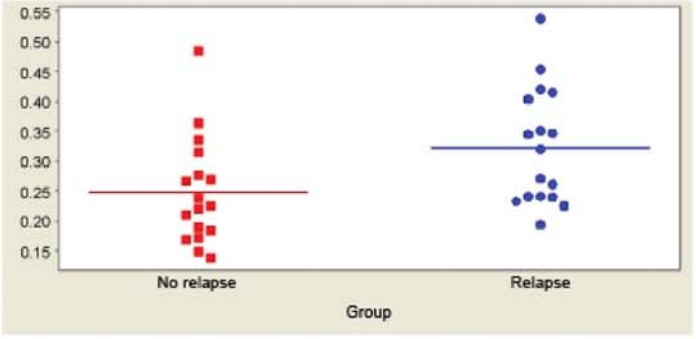
49943 m/z



Median of no relapse group is 102% higher than that of the relapse group



116165 m/z



Median of no relapse group is 43% higher than that of the relapse group

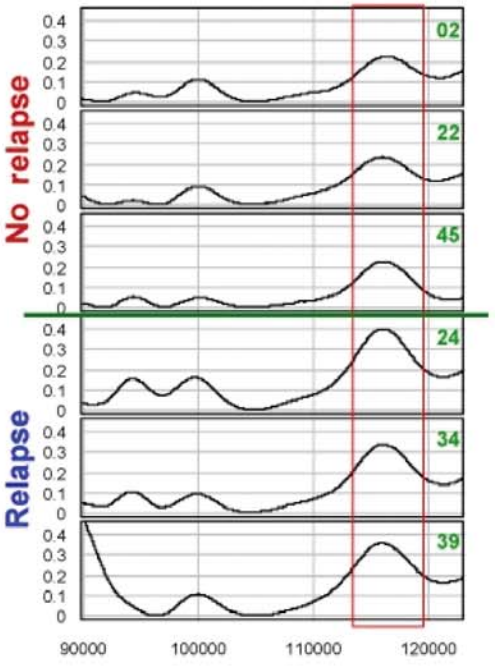


Figure 2. *The analyses show selected peaks exhibiting a more than 40% difference between the two patient groups.*

with no relapse and four were elevated in patients with relapse. Masses of candidate biomarkers ranged from 2,022 to 116,165 Da (Table II). Statistically significant peaks were further qualified based on percentage peak quality change, and consistency between conditions. Selected peaks exhibited a more than 40% difference between the two patient groups (Figure 2).

Discussion

Patients with cervical cancer may develop pelvic recurrence, distant metastases, or a combination of both. Several studies have reported a recurrence rate of 10-20%, following primary surgery or radiotherapy in women with early disease (stage IB-IIA), while patients with nodal metastases and/or locally advanced tumors have a 70% risk of relapse, where uncontrolled disease is the cause of death for most of these women (14, 15).

Metastases are thought to arise from clinically undetectable residual or micrometastatic disease, activated by different genes involved in several molecular processes such as angiogenesis, matrix degradation, cell-cycle regulation, oncogenic pathways, DNA repair mechanisms, adhesion, invasion, migration, cell proliferation and apoptosis. These processes are potentially affected by both surgical extirpation of the tumor, and by other treatments, such as platinum-based chemoradiation, and they play a role in tumor control (16).

Proteomic techniques have been extensively applied in tumor research, such as research for breast (17, 18), colorectal (19, 20) and ovarian cancer (21). To date, there has been limited research in cervical cancer patients for the study of serum protein expression profiles after treatment with radiation and chemotherapy, since most studies have generally only determined the different serum profiles between cancerous and normal tissues (11, 12). In a study by Liu *et al.,* proteomic profiles of cervical cancer were analyzed before and after neoadjuvant chemotherapy (NAC) with paclitaxel (T) and cisplatin (P) from six patients with locally-advanced cervical cancer. The results showed that the NAC therapy with TP down-regulated proteins involved in glycolysis and movement of the cytoskeleton, and up-regulated proteins involved in apoptosis (13).

In this study, we analyzed candidate protein biomarkers in serum from 44 consecutive patients with invasive cervical cancer, treated with (platinum-based chemo-) radiation in search of novel predictive biomarkers using SELDI-TOF-MS. Our results are summarized in Table II and indicate that the serum protein expression profiles differ between patients with relapse and those without. Six candidate markers were elevated in patients without relapse (namely m/z 2022, m/z 2689, m/z 2742, m/z 9949, m/z 10226 and m/z 40937) and four were elevated in patients with relapse (namely m/z 3159, m/z 4643, m/z 49943, m/z 116165). The ten candidate predictive markers detected in the current study also most likely correlate with the post-treatment tumor response, and therefore may relate to the tumor phenotype and its sensitivity to platinum-based chemoradiation.

In a study by Juan Carlos Higareda-Almaraz *et al*. (22) the proteomic patterns in six cervical cancer cell lines and one control line were studied by two-dimensional gel electrophoresis and MALDI analysis. Sixty-six proteins were identified as a "central core of cervical cancer" and these proteins could be divided into three groups. The first group (*e.g*. annexin-2, vimentin, vinculin, ezrin) was related to cell migration, adhesion, invasion and metastasis. The second group (*e.g*. GRP78, HSP71, HSP7C, HSP90B, GRP75) was associated with apoptosis, cell survival, proliferation and angiogenesis. The third group (*e.g*. glyceraldehyde 3 phosphate dehydrogenase, phosphoglycerate mutase 1, enolase A, triosephosphate isomerase, L-lactate dehydrogenase B) encompassed proteins involved in or associated with central metabolism.

In another two studies (23, 24) using two-dimension gel electrophoresis and MALDI-TOF mass spectrometry on cervical cancer tissue and normal tissue from cervix, protein expressions profiles were studied. In one of these studies (23) 35 proteins were detected (17 up-regulated and 18 down-regulated). Twelve of these proteins (*e.g*. anexin A2 and A5, keratin-19 and -20, HSP27, alpha-enolase, apolipoprotein a1) were previously known to be involved in tumors and 23 were newly-identified. In the second study (24) 99 proteins were identified and a differential protein pattern for squamous cell carcinoma of cervix was found for eukaryotic translation initiation factor 3-2β, neutrophil cytosolic factor 2 and annexin A6. Improved current diagnostics of cervical carcinoma was foreseen.

Hence, the investigation of serum proteome from cervical cancer patients by, *e.g*. anion-exchange fractionation, followed by SELDI-TOF-MS analysis, is promising also in the search for new predictive and prognostic factors. However, validation of our results by analysis of similar, prospectively collected study populations, as well as purifying and identifying the confirmed biomarkers is warranted to assess the true clinical applicability of the identified proteomic markers.

Acknowledgements

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