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Peptide profiling in epithelial tumor plasma by the emerging proteomic techniques

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This paper is dedicated to the memory of Pete Tornatore and John Guardiola.

Abstract

The plasma peptide component (PPC) from ten melanoma (Mel), breast cancer (BC) and healthy individuals was examined by a combination of RP-HPLC, surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) and tandem mass spectrometry. A three peak pattern (2023, 2039, 2053.5 *m/z*) was primarily observed in melanoma. Two peaks (2236.1 and of 2356.3 *m/z*) were found only in BC samples. Fibrinogen alpha and inter-α-trypsin inhibitor heavy chain H4 fragments were absent in both tumor samples.

Keywords: Melanoma; Breast cancer; Plasma peptide component; SELDI-TOF MS

1. Introduction

The molecular pathogenesis of tumors remains a mystery. Specifically the critical genetic changes responsible for tumor progression are poorly characterized and the heterogeneity of the clinical course of the disease is not well understood [1,2]. As a consequence tumors continue to be an unpredictable disease.

Incorporating proteomic technology into tumor research is a valuable strategy for identifying novel-disease related peptides/proteins. It has been previously reported that serum and/or plasma protein abnormalities are most commonly found in association with disease processes and are potential targets for treatment [3–5]. In this context, the characterization of the human plasma proteome may facilitate both basic research and clinical diagnosis. However, the classical strategies for proteome analysis of the human plasma are based on the combination of 2D-PAGE and tandem mass spectrometry which fail to resolve most proteins less than 10 kDa [6]. To this end, the analysis of plasma peptide component has a critical and complementary role in defining the human plasma proteome [7]. In addition, the identification and the characterization of the peptides provide end point markers to examine changes in protein metabolism, processing, expression and physiology. Little work has been presented concerning the identification of abnormal peptides appearing in the plasma of patients bearing cutaneous melanoma (Mel) [8] and breast cancer.

In this study, we undertook a search for characteristic differences inherent in the human plasma peptide

Abbreviations: SELDI-TOF MS, surface enhanced laser desorption/ionization time-of-flight mass spectrometry; CHCA, α-cyano-4-hydroxy-cinnamic acid; ACTH, adrenocorticotropic hormone; RT, retention time; CNC, composite normal control; PPC, plasma peptide component

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component (PPC) accompanying melanoma using comparative peptide analysis. We examined the PPC of 10 patients bearing cutaneous melanoma and 10 healthy donors.

In order to identify changes in plasma peptide profiles we examined the peptide plasma samples initially as pools. The pools from the healthy (composite normal control, CNC) and from melanoma filtrates were fractionated by RP-HPLC and the resulting peaks were analyzed by surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) [9]. We were able to identify common peptides in healthy and patient individuals as well as peptides unique to physiological and pathological conditions.

In order to evaluate the clinical usefulness of plasma peptide alterations observed, we analyzed individual peptide filtrates from each melanoma patient and healthy donor. Furthermore, a group of ten breast cancer PPC samples was also examined to validate the specificity of biomarkers identified.

The peptide profile differences observed between normal and disease states were subsequently examined by microsequencing, using collision quadrupole time-of-flight (Qq-TOF) MS/MS mass spectrometry [10,11] to obtain their primary sequence. Although a pilot study, this approach may provide a powerful tool for the detection of melanoma and breast cancer.

2. Materials and methods

2.1. Materials

Gold chips and the calibration standards for the SELDI-TOF mass spectrometer were purchased from Ciphergen Biosystems Inc. (Fremont, CA). α-Cyano-4-hydroxycinnamic acid (CHCA) was obtained from Sigma (St. Louis, MO). All other chemicals used were of the highest purity available.

2.2. Plasma samples

Plasma samples from patients and healthy controls were collected in the presence of 0.6% sodium citrate as anticoagulant, centrifuged and recovered plasma samples were stored at −80 °C until used. All the melanoma patients and the ethnically matched unrelated controls were of Italian origin, from the southern regions of the country. Patients, 11 males and 9 females, with a mean age of 52.5 (range 22–79 years) were enrolled at Istituto Nazionale Tumori in Naples. All included subjects gave written informed consent for participation in this study.

2.3. SELDI-TOF MS analysis

The spectra were externally calibrated using the $[M + H]^+$ ion peaks of the Arg-8 cleaved vasopressin at *m/z* 1084.24, somatostatin at *m/z* 1637.90, β-chain bovine insulin at *m/z* 3495.94, human recombinant insulin at *m/z* 5807.65 and BKHV hirudin at *m/z* 7033.61 for peptide analysis. All mass spectra were recorded in the positive-ion mode using a Ciphergen PBS IIC ProteinChip® Array reader, a linear laser desorption/ionization-time-of-flight mass spectrometer equipped with time-lag focusing [9]. Prior to SELDI-TOF MS analysis, 0.5 μl of pre-fractionated plasma sample was applied to the chip surface and allowed to dry. One microliter of 0.5% aqueous trifluoroacetic acid was then applied followed by 0.5 μl of matrix (saturated CHCA in 50% acetonitrile containing 0.1% trifluoroacetic acid) and let dry. Raw data were analyzed using the computer software provided by the manufacturer and are reported as average masses.

2.4. Reverse phase HPLC

Each plasma sample was loaded to a final protein concentration of 1 mg/ml with phosphate buffer containing 150 mM NaCl and a 200 μl aliquot was diluted 1–5 in 50 mM ammonium bicarbonate pH 7.8 and ultrafiltered by using Microcon filter device (Millipore, Bedford, MA) with a 30 kDa cut-off according to manufacturer specifications. All the plasma filtrates deriving from melanoma, breast cancer patient samples and all from healthy donors were then pooled resulting in three groups. All pools (melanoma, breast cancer and healthy) were then lyophilized in a Speed-Vac centrifuge (Savant, Holbrook, NY). They were resuspended in 100 μl of 0.12% aqueous trifluoroacetic acid, 0.05% ammonium hydroxide, and a 50 μl aliquot from each group was separated on 100 mm × 2.1 mm BetaBasic-18 column (5 μm, 150 Å pore size, Keystone Scientific, Bellefonte, PA) employing a Beckman system Gold HPLC (Fullerton, CA) composed of a model 168 Diode Array Detector and a Model 126 pump module. A linear gradient from 10% to 75% B solvent over 30 min, with 5 min 10% B initial condition (where solvent A was 0.12% aqueous trifluoroacetic acid, 0.05% ammonium hydroxide and solvent B was 0.1% trifluoroacetic acid, 0.05% ammonium hydroxide in acetonitrile) was developed at a flow rate of 0.2 mL/min. All peaks were collected manually and further analyzed by SELDI-TOF MS employing the same method described above. Also whole sections of the gradient were collected for the analysis. The data were analyzed using Ciphergen Express software. The baseline was subtracted and the spectra were normalized against total ion current.

2.5. SELDI/Qq-TOF tandem mass spectrometry

Tandem mass spectrometric peptide sequencing was accomplished using an Applied Biosystems/Sciex QStar™ triple quadrupole time-of-flight instrument (Toronto, Canada) equipped with a Ciphergen SELDI ProteinChip PCI 1000™ Interface [9–11]. The instrument was calibrated externally using an acquired MS/MS spectrum of ACTH

fragment (18–39) peptide at *m/z* 2465.2, where four fragment ions and the parent were used as calibration points. All mass spectra were acquired in positive-ion mode with a collisional gas pressure of approximately 100 mTorr. MS/MS data were acquired using collisional energies following the approximate rule of 50 eV/kDa parent peptide mass. Matrix conditions were identical to SELDI-TOF analysis described previously. Raw data were analyzed using the instrument's Analyst® software.

3. Results

3.1. RP-HPLC and SELDI-TOF MS plasma peptide profiles in melanoma patients and healthy donors

In order to investigate differences in the plasma peptide component in melanoma versus healthy donors, we initially examined the plasma from pooled samples. We prefractionated the peptide component by ultra filtration on Cen-

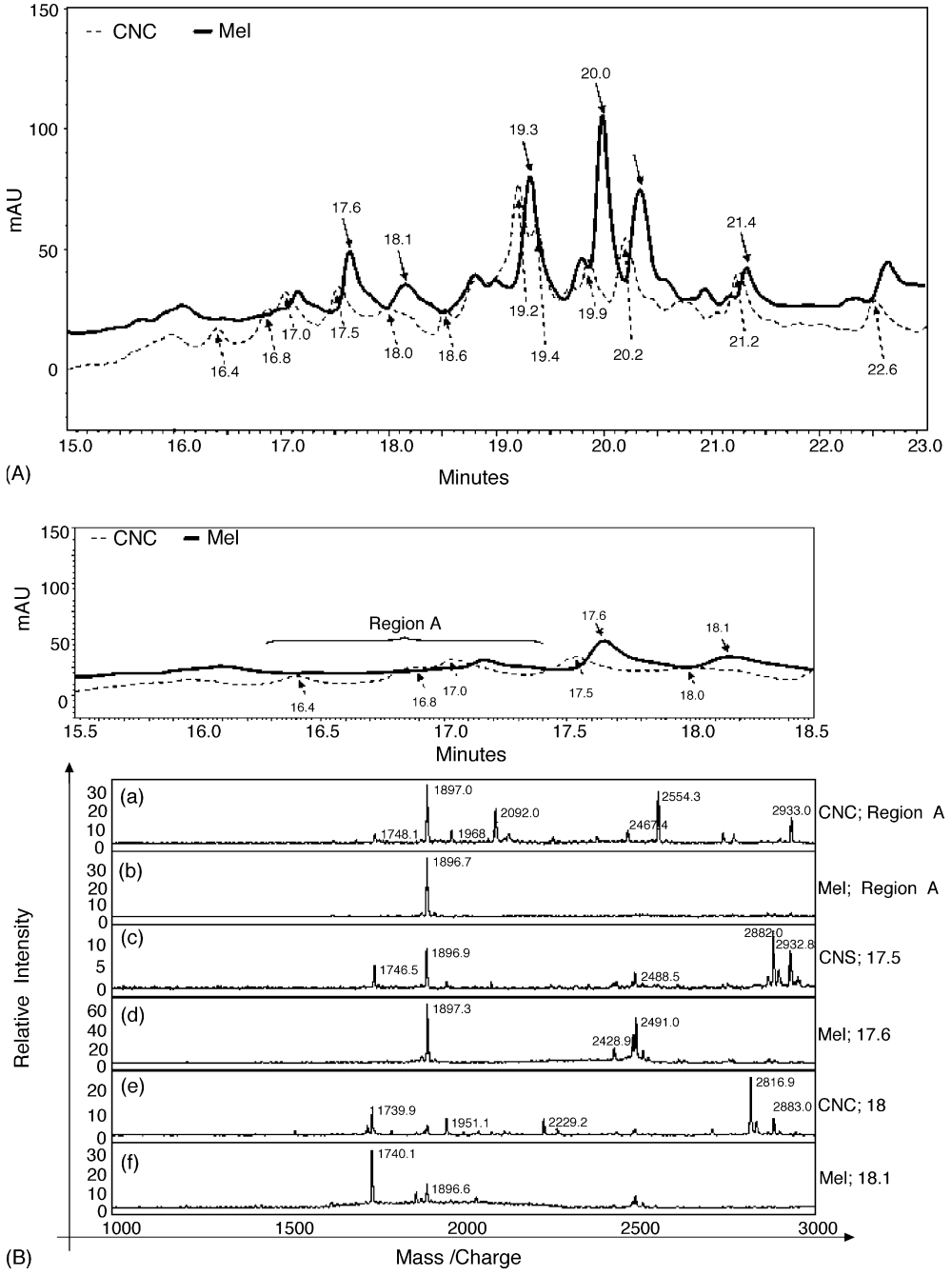

Fig. 1. HPLC profiles of PPC from melanoma and healthy samples. Plasma filtrates from melanoma, Mel (—) and from healthy individuals, CNC (- - -) were loaded on a BetaBasic-18 HPLC column (A). The trace indicated the absorbance at 220 nm. The retention time (RT) of the eluted species were reported on the top and on the bottom of the chromatogram for melanoma and for healthy samples, respectively. The fractions, obtained by BetaBasic-18 HPLC, were analyzed by SELDI-TOF MS, as described in the text. The mass/charge (*m/z*) values of each detected species were reported (B, C).

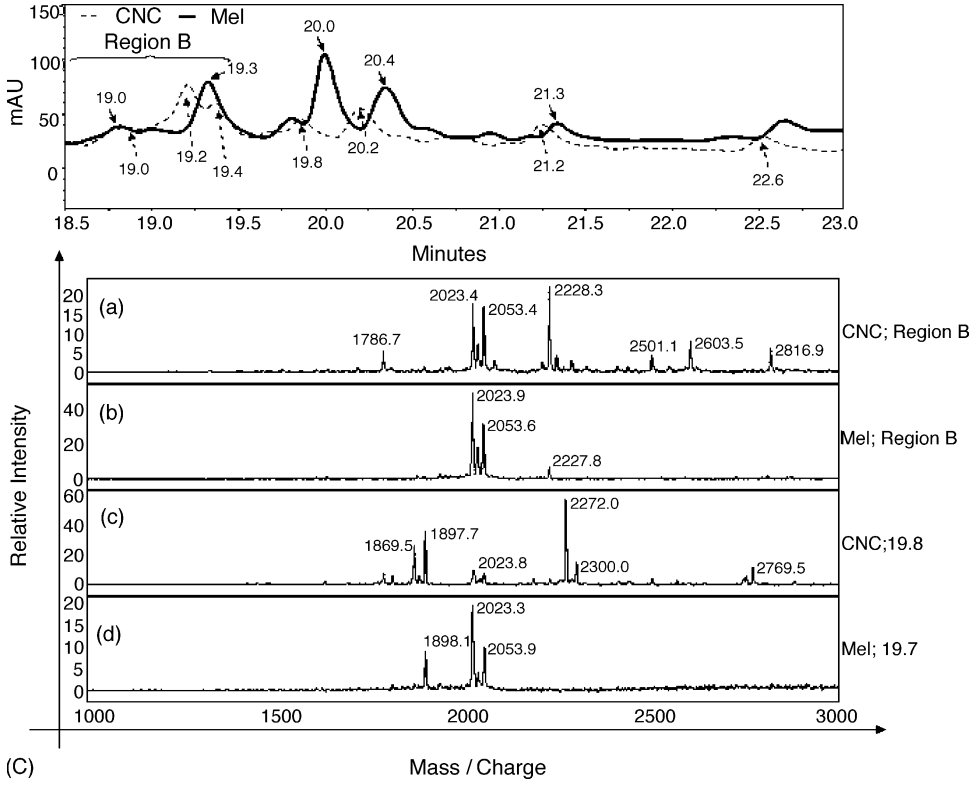

Fig. 1. (*Continued*).

tricon membranes with a 30,000 Da cut-off, as described in Section 2, and all the resulting filtrates were pooled generating the two groups: normal (CNC) and melanoma (Mel).

Both pooled groups were examined by RP-HPLC on a BetaBasic-18 column and their profiles were compared. We decided to focus our attention on the region in the chromatogram with retention times (RT) between 15 and 23 min, as shown in Fig. 1A. In particular, we observed both overlapping and unique peaks as well as variations in the relative abundance of some common peaks.

To further examine the differences observed from RP-HPLC analysis, we utilized SELDI-TOF MS to analyze the resulting HPLC fractions. In particular, we pooled all the collected fractions between 16.2 and 17.4 min and all fractions between 18.5 and 19.5 min separately as Regions A and B, respectively. Pooling of these fractions was done on the basis of the average peak height in order to get fractions with similar peptide content and to simplify the SELDI-TOF MS analysis. An aliquot from all fractions was spotted on a gold chip and analyzed. We focused on the analysis of the peptides with *m/z* from 1000 to 3000, in order to obtain tandem mass spectrometry sequence information. Significantly different spectra were observed for CNC and Mel groups (Fig. 1B), especially those fractions with similar retention times. Among the major components, molecular species with *m/z* values of 1897 and 1896.7 (Fig. 1B panels a and b) were seen. The other molecular species found in both groups were *m/z* 1739.9 (Fig. 1B, panel e) and 1740.1 (Fig. 1B, panel f), 1896.6 (Fig. 1B, panels e, f), 2023.4 (Fig. 1C, panel a) and 2023.9 (Fig. 1C, panel b), 2053.4 (Fig. 1C, panel a) and 2053.6 (Fig. 1C, panel b), 2228.3 and 2227.8 (Fig. 1C, panels a and b). No melanoma specific molecular species were identified using this approach and peptides at *m/z* 1746.5, 2554.3, 2816.9, 2882.0, 2272.0 were observed only in the CNC group.

3.2. Plasma peptide composition in breast cancer patients

Our analysis of the PPC from melanoma patients and healthy donors reveals differences in their respective peptide profiles. In order to determine the specificity of the peptide profile observed in melanoma, we decided to analyze the PPC from the plasma of individuals bearing a different epithelial cancer. Therefore, 10 plasma samples from breast cancer (BC) patients were subjected to the same procedure used for melanoma and normal samples. Fig. 2 shows that the HPLC profile from BC was different from CNC. The peptide fractions were analyzed by SELDI-TOF MS (Fig. 2, panels a–j). We found molecular species with *m/z* values of 2236.1 (Fig. 2, panel b) and 2356.3 (Fig. 2, panel d) only in BC fractions.

3.3. PPC in individual melanoma and healthy plasma

In order to validate the peptide profile previously observed from the pooled samples, we analyzed the PPC from

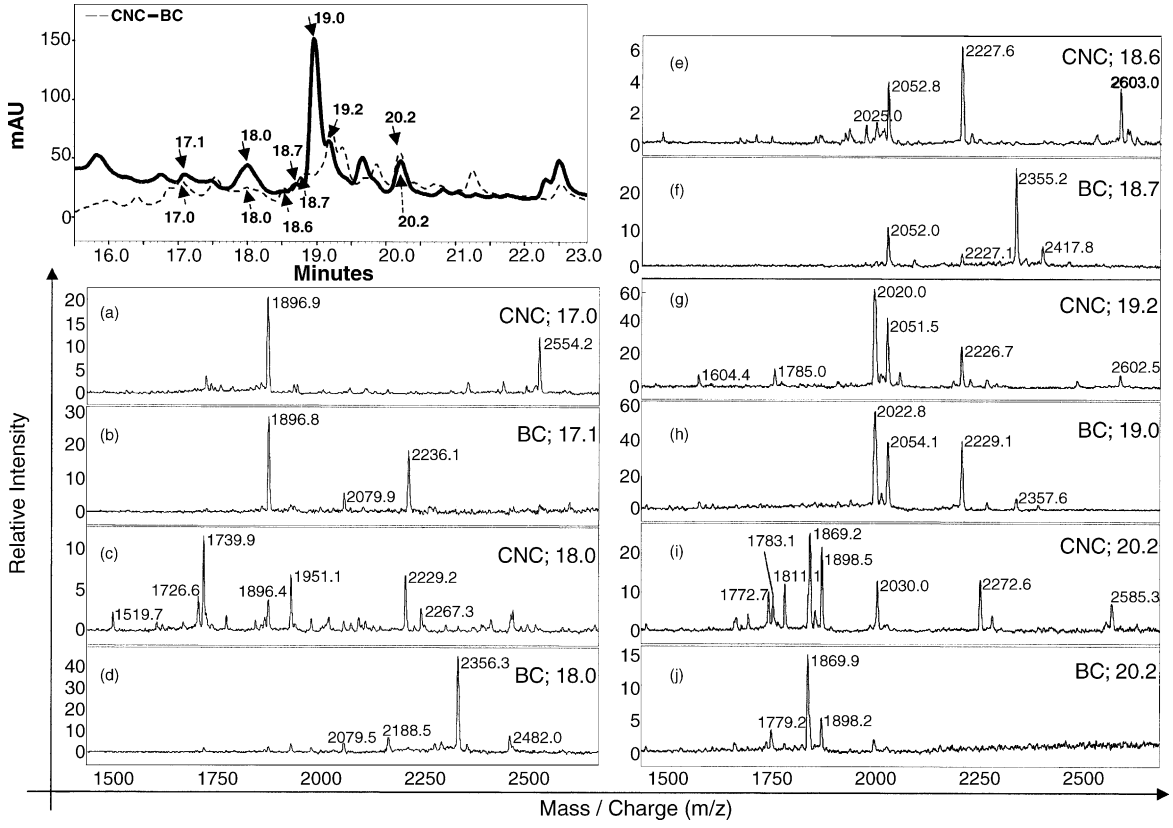

Fig. 2. HPLC profiles and SELDI-TOF MS spectra of PPC from breast cancer and healthy samples. Plasma peptides from breast cancer, BC (—) and from healthy individuals, CNC (- - -) were loaded on a BetaBasic-18 HPLC column. The retention time (RT) of the eluted species were reported on the top and on the bottom of the chromatogram for BC and for healthy samples, respectively; SELDI-TOF MS of each fraction were reported in panels a–j.

melanoma and healthy individuals by a combination of RP-HPLC and SELDI-TOF MS.

SELDI-TOF MS analysis of the molecular species eluted in Region A, from each healthy and melanoma individual is presented in Fig. 3A panel a and b, respectively. A comparative analysis revealed the absence of numerous molecular species in the range from *m/z* 2500 to 3000 in the melanoma PPC and a modest increase in relative intensity of the molecular species at *m/z* value of 1897.4. However, considering that the *p*-value is 0.4, the relevance of this observation will require the examination of a larger group of samples.

The molecular species eluted in Region B from each healthy (N) and melanoma (M) individual were also analyzed as shown in Fig. 3B and C, respectively. There were many differences in the peptide pattern from healthy compared to melanoma PPC. We observed a specific pattern of three molecular species, with *m/z* values of 2023, 2039, 2053.5 primarily in melanoma samples. In particular, the data analysis using Ciphergen Express software showed *p*-values for the 2023, 2039, 2053.5 molecular species of 0.087, 0.007 and 0.003, respectively (Fig. 3D). Thus, we demonstrated a pattern of three peaks for Region B in individual melanoma samples.

3.4. Peptide sequencing from melanoma and healthy donor plasma

In order to identify the different molecular species observed by SELDI-TOF MS in each group analyzed, we subjected some of the molecular species to Q-star MS/MS mass spectrometry [11].

In particular, the peaks at *m/z* 1897.0 and 1896.7 (Fig. 1B, panels a and b) observed in healthy and melanoma HPLC fraction samples respectively were examined. The obtained collision-induced dissociation (CID) spectra of both species revealed a peptide sequence derived from the Complement C4 precursor, amino acid residues 1337–1352 (Table 1). Sequence analysis of the peptide peaks at *m/z* values of 1739.9 (Fig. 1B, panel e) and 1740.1 (Fig. 1B, panel f) identified the same C4 precursor peptide missing the C-terminal arginine.

The peptide peaks unique to normal samples were also subjected to tandem mass spectrometry. In particular, the species at *m/z* value of 2554.3, 1951.1, 1786.7, 2272.0 and at *m/z* 1746.5 were examined. The CID spectra of the first species (2554.3) provided the sequence that corresponded to a peptide from fibrinogen alpha amino acid residues 578–600. The species at *m/z* 1951.1 was identified as a peptide derived

from fibrinogen beta chain precursor. The CID spectra of the species at *m/z* of 1786.7 revealed the sequence for a potentially active peptide deriving from inter-α-trypsin inhibitor heavy chain H4 precursor, ITH4. The peptide at *m/z* 2272.0 corresponded to a longer form of the same peptide deriving from the ITH4 protein (Table 1). The CID spectrum obtained for the molecular species at *m/z* 1746.5 did not give us sufficient information for its identification.

The molecular species at *m/z* 1869.5 common to both healthy and melanoma samples (Fig. 1B, panels c and d) was

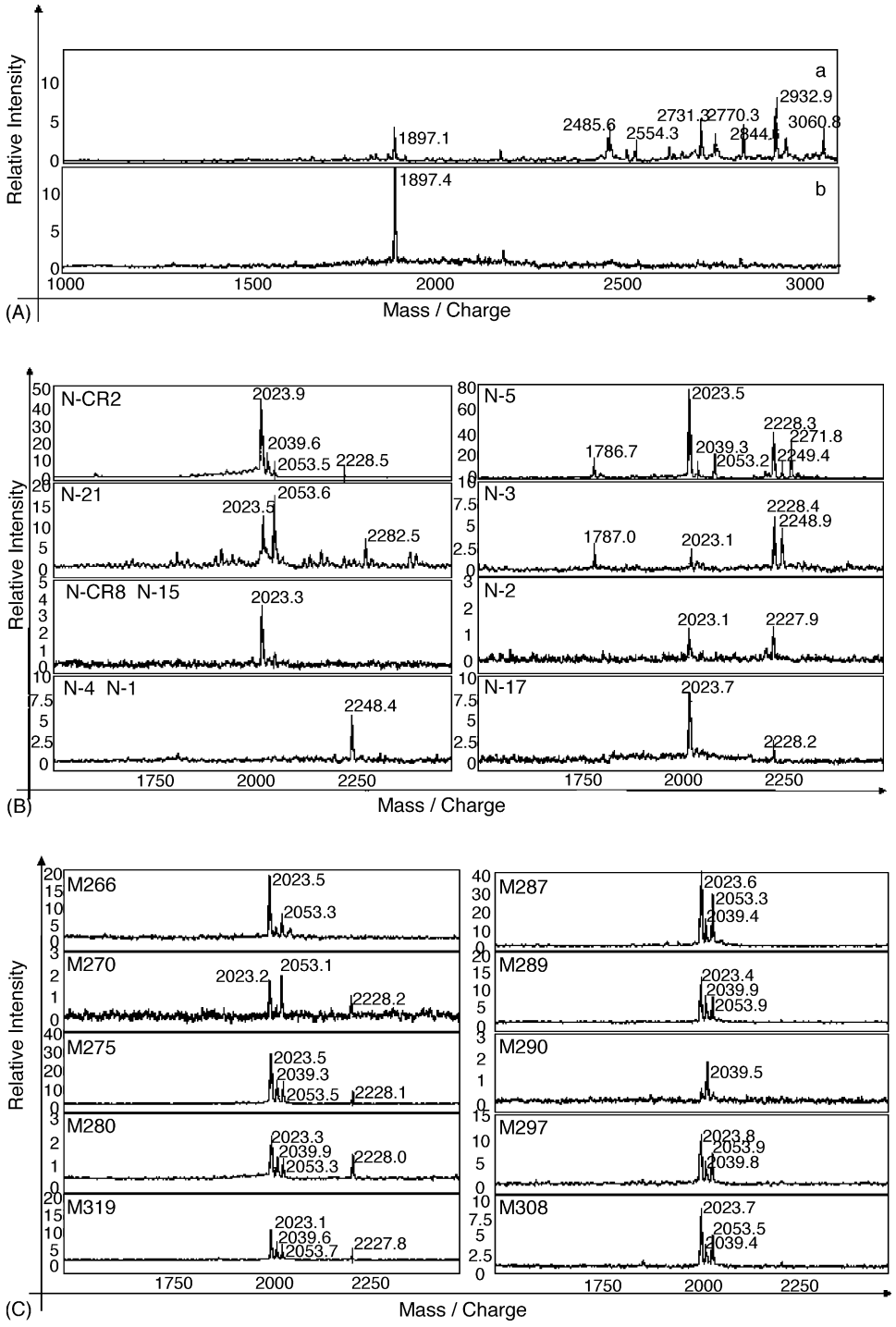

Fig. 3. SELDI-TOF MS spectra of PPC from melanoma and healthy individual plasma. The fractions corresponding to Regions A and B, obtained by BetaBasic-18 HPLC of each plasma from melanoma and healthy individuals, were analyzed by SELDI-TOF MS. (A) is a representative SELDI profile from healthy (a) and melanoma (b) individuals for HPLC Region A. SELDI analysis of HPLC Region B is presented in B and C from healthy individuals (B) and melanoma individuals (C), respectively. Each melanoma and healthy individual was indicated with a M and N (followed by a number), respectively. (D) is a 'box- and whiskers' plot for marker candidates, obtained from the data analysis using Ciphergen Express software. The *m/z* and *p*-value are reported.

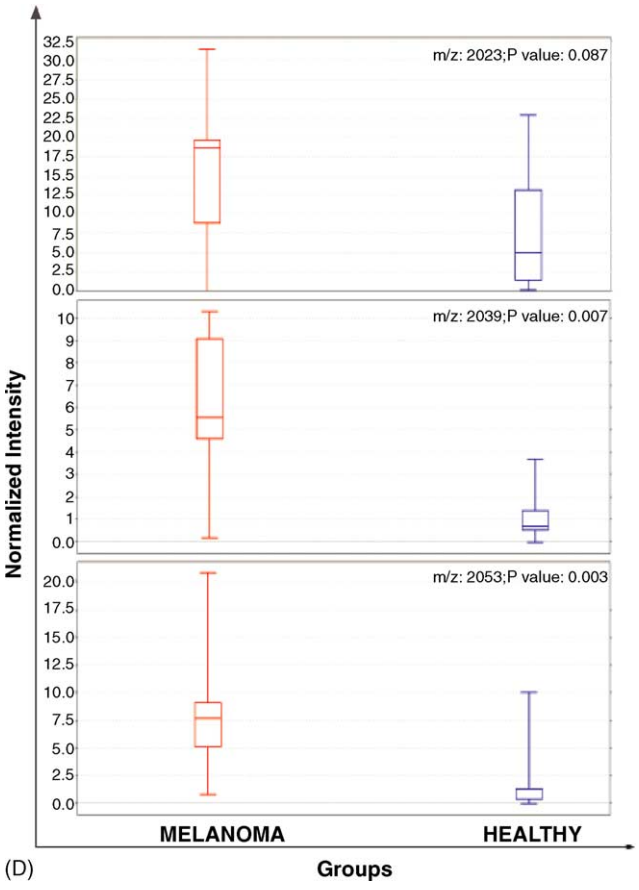

Fig. 3. (*Continued*).

identified as a 15 amino acid peptide containing the amino acid residues 1307–1322 of Complement C3.

4. Discussion

We analyzed the PPC from individuals bearing melanoma and breast cancer and demonstrated characteristic alterations of their composition versus healthy donors. We focused our analysis on the peptide composition of plasma which cannot be studied by classical proteomic procedures based on 2D-PAGE and mass spectrometry since low molecular masses as well as low and high p*I* proteins are not effectively resolved by PAGE [6]. In addition, the peptides in blood are the products of specific processes occurring in the organism and their analysis may contribute to the understanding of the altered molecular mechanisms which accompany diseases such as melanoma and breast cancer.

From the pooled samples, we observed degradation products of fibrinogen alpha chain and beta chain only in normal controls. Fibrinogen is a multidomain protein, consisting of three pairs of nonidentical polypeptide chains Aα, Bβ and γ, forming two identical subunits [12]. It plays a key role in several biological processes such as blood clotting, fibrinolysis, cellular and matrix interactions, inflammation, wound healing and neoplasia [13]. In particular, we demonstrated the presence of C-terminal degradation products from the Aα chain in normal plasma and the absence in melanoma and breast cancer plasma. As previously reported [14], the C-terminal degradation products from the Aα chain of the fibrinogen are indicative of mild plasmin-like activity, which is commonly seen in plasma from healthy individuals and absent in some defibrinating patients, especially those with disseminated cancer [15–17].

We demonstrated the presence in healthy plasma of fragments deriving from the inter-α-trypsin inhibitor heavy chain H4 protein (ITH4). It is also known as serum glycoprotein 120 (sgp120) and plasma kallikrein-sensitive glycoprotein (PK-120) and it belongs to a group of related plasma protease inhibitors [18–20]. Its physiological function is not known. We found two peptides corresponding to amino acid residues 667–687. These fall in the region of the reported potentially active peptide (residue 662–688) of the 35 kDa domain of the ITH4. This finding could be indicative of a possible different post-translational modification of this protein in melanoma patients.

The analysis of the PPC from breast cancer patients provided several interesting components. The presence of the peptides with *m/z* values of 2236.1 and 2356.3 was observed specifically in breast cancer patients. While not definitive, the peptides might be useful as potential markers for breast cancer detection.

The analysis of the individual samples proved to be informative. We observed a wide variety of molecular species in healthy plasma compared to the set of three seen in melanoma

Table 1
Sequences of the peptides from PPCs by MS/MS analysis

Mass observed (*m/z*) healthy P./melanoma P.	HPLC fraction/retention time (min)	Sequence identified by MS/MS	SwissProt number	Protein name
1739.9/1740.1	18/18.1	NGFKSHALQLNNRQI	p01028	Complement C4 precursor
1786.7/not detected	Region B	GLPGPPDVPDHAAYHPF	Q14624	Inter-α-trypsin inhibitor heavy chain H4 precursor
1869.5/1869.3	20.2/20.0	SSKITHRIHWESASLL	p01024	Human complement C3
1897.0/1896.7	Region A	NGFKSHALQLNNRQIR	p01028	Complement C4 precursor
1951.1/not detected	18	REEAPSLRPAPPPISGGGY	p02675	Fibrinogen beta chain precursor
2272.0/not detected	19.8	SRQLGLPGPPDVPDHAAYHPF	Q14624	Inter-α-trypsin inhibitor heavy chain H4 precursor
2554.3/not detected	Region A	SSSYSKQFTSSTSYNRGDSTEES	p02671	Fibrinogen alpha chain

plasma, by SELDI-TOF analysis. Although preliminary, the combination of 1) the presence of the three molecular species pattern, 2) the absence of the C-terminal degradation products from the Aα chain of fibrinogen and 3) the absence of the peptide (662–688) of the 35 kDa domain of the ITH4 in melanoma may prove to be a multi-biomarker diagnostic tool.

We initially examined a pool of plasma samples from healthy individuals and melanoma patients in order to investigate differences in its peptide component. Individual plasma samples were also analyzed and it would appear that pooling samples had no adverse effect. Although a preliminary study, we were able to observe different peptide profiles and to characterize several specific peptides. Further research involving larger numbers of samples will be required to validate these findings and discover other factors that define their role in disease status.

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