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ORIGINAL PAPER

The C-terminal fragment of the immunoproteasome PA28S (Reg alpha) as an early diagnosis and tumor-relapse biomarker: evidence from mass spectrometry profiling

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Abstract This study reports on the C-terminal fragment of the 11S proteasome activator complex (PA28 or Reg alpha), a novel ovarian-specific biomarker of early and late stages of ovarian cancer (OVC) relapse, in patient biopsies after chemotherapy. A total of 179 tissue samples were analyzed: 8 stage I, 55 stage III–IV, 10 relapsed serous carcinomas, 25 mucinous carcinomas and 12 borderline and 68 benign ovarian tissue samples. This fragment was detected by MALDI mass spectrometry profiling in conjunction with a novel extraction method using hexafluoroisopropanol (1,1,1,3,3,3-hexafluoro-2-propanol; HFIP) solvents for protein solubilization and by immunohistochemistry using a specific antibody directed against the C-terminal fragment of PA28. Due to its specific cellular localization, this fragment is a suitable candidate for early OVC diagnosis, patient prognosis and follow-up during therapy and discriminating borderline cancers. Statistical analyses performed for this marker at different OVC stages reflect a prevalence of 77.66 ± 8.77 % (with a correlation coefficient value $p < 0.001$ of 0.601 between OVC and benign tissue). This marker presents a prevalence of 88 % in the case of tumor relapse and is detected at 80.5 % in stage I and 81.25 % ± 1.06 in stage III–IV of OVC. The correlation value for the different OVC stages is $p < 0.001$ of 0.998. Taken together, this report constitutes the first evidence of a novel OVC-specific marker.

Keywords Ovarian cancer · MALDI imaging · Biomarker · Relapse · Diagnostic · Mass spectrometry

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Introduction

Since the concept of directly identifying molecules on tissues using MALDI ion sources was proposed at the end of the 1990s (Caprioli et al. 1997), MALDI imaging mass spectrometry has been used in many clinical applications (Cornett et al. 2006; El Ayed et al. 2010; Lemaire et al. 2006a, b; Pevsner et al. 2009; Schwamborn et al. 2007; Schwartz et al. 2004; Seeley and Caprioli 2008). A decade of developments in instrumentation and chemistry has been required to achieve optimal extraction, detection, and spatial resolution when mapping compounds at the tissue level (Franck et al. 2009a, b; Lemaire et al. 2006a, b; 2007a; van Remoortere et al. 2010). These developments, combined with using the appropriate data-processing tools, gave rise to the discovery of biomarkers for diverse pathologies (Bonnel et al. 2011; Djidja et al. 2010; McCombie et al. 2005; Stauber et al. 2008), of which cancer is the most studied. MALDI mass spectrometry imaging (MALDI-MSI) technology makes it possible to study the molecular profiles of the benign and malignant portions of a solid tumor. This technique is particularly

interesting for screening biomarkers in stage I cancer biopsies, a stage when the disease is still treatable and the 5-year survival rate for managed patients is yet high.

In the USA, ovarian cancer (OVC) is the sixth most prevalent cancer among women and the second most prevalent gynecologic cancer (after endometrial cancer). A total of 21,550 new cases and 14,600 deaths were reported in 2009 (Khalil et al. 2010). Cancer antigen 125 (CA-125: MUC16) is the most commonly used of the known biomarkers. CA-125 has a sensitivity of 80 % and a specificity of 97 % in epithelial cancers (stage III or IV). However, its sensitivity is approximately 30 % in stage I cancers because it is associated with several physiological phenomena and is also detectable in benign circumstances (Lambaudie et al. 2006). Therefore, CA-125 is useful for monitoring disease progression, but it cannot be used as a biomarker to screen for early-stage disease in large cohorts (Lambaudie et al. 2006).

Our group has previously found that the C-terminal fragment of the 11S proteasome activator (PA28, Reg alpha) was specifically found in cancerous ovarian biopsies and not in benign tissues (Lemaire et al. 2005; 2007b) (Table 1). Using 25 ovarian carcinomas (stage III and IV) and 23 benign ovarian tissues analyzed by MALDI-TOF-MS, this new biomarker was detected with a high prevalence (80 %) and has been fully characterized using MALDI-MS and nano-ESI trypsin peptides profiling. The full identification was performed by nano-ESI-QTOF in MS/MS mode. This marker has an m/z ratio of 9,744 and corresponds to 84 amino acid residues from the 11S proteasome activator complex named PA28 or Reg alpha. This marker was validated using MALDI imaging, by classical immunocytochemistry with an antibody against the C-terminal portion of the protein, by specific MALDI with an anti-Reg alpha-tagged antibody and by Western blot analyses. The results confirmed the epithelial localization of this fragment, with a nuclear localization in benign epithelial cells and a cytoplasmic localization in malignant cells (Lemaire et al. 2005, 2007b; Stauber et al. 2006). More recently, 20 ovarian carcinomas (stages I, III and IV) and 10 borderline and 20 benign ovarian tissue samples were directly analyzed by automatic profiling mass spectrometry subjected to hierarchical clustering, using unsupervised principal component analysis and characterized using a tissue bottom-up strategy after on-tissue digestion and shotgun sequencing by nano-LC–IT-MS in MS/MS mode (El Ayed et al. 2010; Franck et al. 2010). In all of the cases, the C-terminal fragment of Reg alpha was detected and identified. We also confirmed that this peptide is a marker for the immunosuppressive events that occur during disease progression (Franck et al. 2009a; Longuespée et al. 2012).

Table 1 This cohort was established 6 years ago through collaboration between FABMS and Hospital Jeanne de Flandre, CHRU Lille and consisted of 179 specimens

Pathology type	MALDI-MSI, identification, validation	MALDI profiling	Immunohistochemistry
Serous cancer			
Stage I	5	8	8
Stage III–IV	35	55*	28**
Relapse	5	10	5
Mucinous cancer			
Stage III–IV	10	25	15
Borderline	10	12	10
Benign	43	68*	19**

The sample collection was performed with institutional review board approval (CCP Nord Ouest IV, CP 10/05 then CP 10/12). All of the human tissues were resected by a surgeon affiliated with FABMS, preserved immediately in isopentane, placed in nitrogen, and stored at −80 °C until analysis. The data from this cohort have been previously published (El Ayed et al. 2010; Franck et al. 2009a; Lemaire et al. 2005, 2007b; Stauber et al. 2006)

* 25 OVC versus 23 benign were identified previously

Based on previous data showing that the Reg-alpha C-terminal fragment can be detected in the late stages (III–IV) of OVC and can discriminate among borderline tissues, we investigated the properties of this marker after chemotherapy for early-stage OVC. Advanced OVC treatment involves explorative celioscopic surgery followed by neoadjuvant treatment with paclitaxel and platinum or with carboplatin and paclitaxel (Lhomme et al. 2009) if carcinosis is visible during the explorative celioscopic surgery. Radical surgery is then performed if the chemotherapeutic treatment efficiently removes the entire genital tract. The 5-year survival rate remains poor at approximately 40 % (Kehoe 2008; Tanner et al. 2012). The responders will relapse approximately 18 months after completing first-line therapy and will require further systemic therapy. The median survival of patients with recurrent OVC ranges from 12 to 24 months. The traditional clinical measures of relapse include disease progression (usually defined as a 25 % or greater increase in tumor size), appearance of new lesions and death. In this context, it is important to have an easily executed, highly reliable procedure. We investigated the expression of our proposed marker in the cancerous regions of interest by comparing the molecular profiles of small cancerous tissue areas with those of benign areas from the same tissue section. Extracting the marker compounds from the regions of interest was challenging due to the small size of the cancerous or benign regions.

To access a small polar protein with an m/z ratio of 9,744, we previously developed a sample preparation method using hexafluoroisopropanol (1,1,1,3,3,3-hexafluoro-2-propanol; HFIP) solvent, which is optimal for polar or high-mass proteins, for protein solubilization (El

Ayed et al. 2010; Franck et al. 2010). This technique has been preferred over classical methods (Redeby et al. 2004, 2006). Using this method, the Reg-alpha C-terminal fragment was detected in early-stage (stage I) serous OVC and in patients who present with OVC recurrence after chemotherapy. Therefore, the fragment can be considered a marker for tumor relapse. This work provides evidence that Reg alpha is a novel OVC biomarker and that MALDI-MSI tissue profiling is useful for early-stage OVC diagnosis and for evaluating the prognosis of OVC patients.

Materials and methods

Materials

Sinapinic acid (SA), trifluoroacetic acid (TFA), and HFIP were obtained from Sigma-Aldrich and used without further purification. Water, acetonitrile, methanol, ethanol, and acetone were provided by Biosolve. The anti-Reg-alpha polyclonal antibodies were purchased from Invitrogen. The secondary antibodies for fluorescence microscopy were provided by Jackson, and the mounting medium was obtained from Vectashield.

Samples

The tissues (179 samples, Table 1) were obtained with informed consent and institutional review board approval (CCP Nord Ouest IV, CP 10/12) from patients undergoing ovarian tumor resection at the Hospital Jeanne de Flandre. The patient information collected included age, treatment received before and after surgery, extent of surgery, current status (i.e., alive, alive with progressive disease, deceased, and cause of death), and survival from the time of the original pathological diagnosis. The samples were collected during the surgery, immediately frozen in −50 °C isopentane, and stored at –80 °C until analysis. Typically, 10-µm sections were cut using a cryostat and thaw-mounted on flat electrically conductive sample slices (indium tin oxide). The histopathological diagnoses were performed by a pathologist (O.K.) who was blinded to the original clinical diagnosis using subsequent H&E-stained sections.

Tissue preparation

The frozen 10-µm ovarian sections were sliced on a cryostat and immediately transferred onto conductive indium tin oxide (ITO) glass (Bruker Daltonics, Wissenbourg, France). The tissues were dried using a desiccator, and the sections were washed for 1 min in an acetone solution, 15 s in a 70 % ethanol solution, 15 s in a 95 % ethanol solution, and 1 min in chloroform.

Microscopy methods

Hematoxylin–eosin–safranin staining

For the hematoxylin–eosin–safranin (HES) staining, the sections were heated for 5 min and stained with hematoxylin for 3 min. The sections were rinsed with water prior to being bathed twice in a solution containing 156 mL of 95 % EtOH, 44 mL of H_2O and 80 µL of HCl. The sections were rinsed in H_2O, a solution of 0.48 mL of 35 % NH_4OH and 200 mL of H_2O, and H_2O for an additional 5 min each. The sections were then rinsed in 80 % ethanol, stained with eosin for 10 s, and washed twice with 95 % ethanol and twice in 100 % ethanol. Finally, the sections were stained with safranin (10 g/L in 100 % EtOH) for 6 min and washed twice in 100 % EtOH and once in xylene for 1 min.

Fluorescence microscopy

The sectioned tissues were pre-incubated with a PBS buffer containing 1 % BSA, 0.05 % Triton, 1 % ovalbumin, and 1 % normal goat serum (NGS) for 30 min and then incubated with a specific rabbit polyclonal anti-C-terminal Reg-alpha antibody that was diluted to 1/100 with a PBS solution containing 1 % BSA, 0.05 % Triton X-100, 1 % NGS, and 1 % ovalbumin (AB solution). A total of 500 µL of the antibody solution was added to each tissue section, and the sections were incubated overnight at 4 °C. After washing three times with PBS, the samples were incubated for 1 h at room temperature with goat anti-rabbit goat antibody (Invitrogen) conjugated to Alexa Fluor 488 (1:2,000 in the AB solution), rinsed with PBS, and mounted with Glycergel (Sigma Life Science, USA). The samples without primary antibody were used as negative controls. The slides were maintained in the dark at 4 °C until observation and analysis with an inverted Leica DM IRE 2 microscope. The light exposition was 100 ms.

Immunohistochemistry

The immunohistochemistry (IHC) was performed on paraffin-embedded ovarian tissues using a standard peroxidase-based staining method. The paraffin-embedded 4-µm tissue sections were cut on a microtome and dried for 12 h at 60 °C, dewaxed with 100 % xylene for 5 min, and rehydrated in brief successive baths of 100 % alcohol, 95 % alcohol, and distilled water. The tissue sections were stored in distilled water until the following steps were performed. The endogenous peroxidase activity was quenched with 10 % H_2O_2 for 5 min. The tissue sections were incubated with the primary anti-Reg alpha C-term antibody (Zymed Laboratories, Invitrogen, ref. 3-2400) at a dilution of 1/50 in TBS (50 mM, pH 7.4) for 1 h at room temperature. The tissue sections were washed with TBS

and successively incubated with the secondary antibody (biotin–streptavidin peroxidase, horseradish peroxidase (HRP) conjugate) and the chromogen (3,3′-diaminobenzidine) following the manufacturer's protocol (streptavidin-HRP, Southern Biotechnology and Associates, Inc.). The nuclei were counterstained with hematoxylin.

Mass spectroscopy methods

HFIP extraction method

To extract compounds from the small-area stage I tissue biopsies, a hydrophobic solvent was used following the procedure previously described by our group (Franck et al. 2010; van Remoortere et al. 2010). A total of 10 mg of SA was dissolved in 1 mL of HFIP. A total of 3 μL of the solution was manually dropped onto the region of interest in 6 0.5-μL droplets. After this deposition, 3 μL of 10 mg/mL SA in 1 % ACN/aqueous TFA (7:3) was added.

The same procedure was used to extract compounds from the large-area stage III and IV tissue biopsies, except that a total of 10 μL of SA in HFIP was dropped onto the tissue section in 5 2-μL droplets. A total of 10 μL of SA in 0.1 % ACN/TFA 7:3 was added.

Tissue profiling using MALDI mass spectrometry

The molecular profiling was performed on an UltraFlex II MALDI-TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a micro-channel plate (MCP) detector. The instrument was equipped with a Smartbeam™ laser and controlled by FlexControl 3.0 (Build 158) software (Bruker Daltonics, Bremen, Germany). For the stage I tissue biopsies, the raw spectra were compared using the FlexAnalysis 3.0 software (Bruker Daltonics, Bremen, Germany). The spectra from the stage III and stage IV biopsies were processed with the FlexAnalysis 3.0 software (Bruker Daltonics, Bremen, Germany) using the convex hull baseline subtraction method. The spectra were recorded in positive ion, linear time-of-flight mode. For these experiments, the laser offset was typically set to 30 %, the laser range was set to 20 %, the laser fluence was set to 50 %, and the laser focus was set to small.

Results

Using MALDI-MSI followed by classical proteomic methods, we previously identified from 25 OVC (stage III and IV) and 23 benign ovarian tissues, the C-terminal Reg-alpha fragment marker (Table 1). This marker has been validated by Western blot (9 OVC (stage III and IV) versus 16 benign ovarian tissues) (Lemaire et al. 2007b). A new strategy to investigate this marker at different OVC stages and grades has been developed (Table 1). We investigated the expression of this marker in the cancerous regions of interest by comparing the molecular profiles of small cancerous tissue areas with normal areas from the same tissue section. Extracting marker compounds in the regions of interest was challenging due to the small size of the cancerous and benign regions. Therefore, we developed a procedure that improved compound extraction from small regions of interest by using the HFIP extraction buffer (El Ayed et al. 2010; Franck et al. 2010). HFIP is applied directly to the tissue to improve polar protein extraction. The mass spectra of the classical MALDI mass spectrometry imaging and the profiling procedure using only SA without HFIP on the tissue extractions demonstrate that the C-terminal Reg-alpha fragment with an m/z ratio of 9,744 is either not detected or not clearly distinguishable from the chemical spectral background (Fig. 1). The spectral differences observed when detecting this fragment in a 5-mm cancerous region from stage I OVC serous tissue (Fig. 2) using the HFIP extraction (Fig. 1a) or classical matrix deposition procedure (Fig. 1b) confirmed this hypothesis. The ion at 9,744 can be easily detected in small tissue fragments using the HFIP procedure (Fig. 1a), whereas only a small SA peak is detected without HFIP (Fig. 1b).

The C-terminal Reg-alpha fragment is a specific OVC-diagnosis marker

We first validated our on-tissue extraction procedure using the late-stage OVC samples. Under these conditions, 30 stage IIIc and IV serous OVCs versus 45 benign were analyzed by MALDI-MS profiling after on-tissue extraction (Fig. 2a). The mass spectra focused on the ion at 9,744, which corresponds to the C-terminal Reg-alpha fragment and was present in all of the ten stage IIIc or IV serous adenocarcinomas (ADK, Fig. 2a). We validated these results on 12 patients using immunohistochemistry (IHC) (Figs. 3, 4). Among these 12 patients, 7 OVC were diagnosed (2 of which were on the same specimen or on the same cut) and 2 demonstrate a well-differentiated morphology (one benign or typically benign and malignant morphologies). Moreover, among the seven ovarian cancers, there was an endometrioid carcinoma of which a large area of the specimen was found to be an undifferentiated carcinoma. The two sections amount to two different ovarian cancers on the same ovary. A total of 15 samples were tested: 8 malignant and 7 benign (Figs. 3, 4). The signal was consistently balanced, with a cytoplasmic localization in the cancer cases (87.5 % were balanced, $n = 7/8$ with cytoplasmic labeling specifically in the endometrioid carcinomas) and a predominantly nuclear localization in the benign tumors (96 % nuclear labeling) (Table 2).

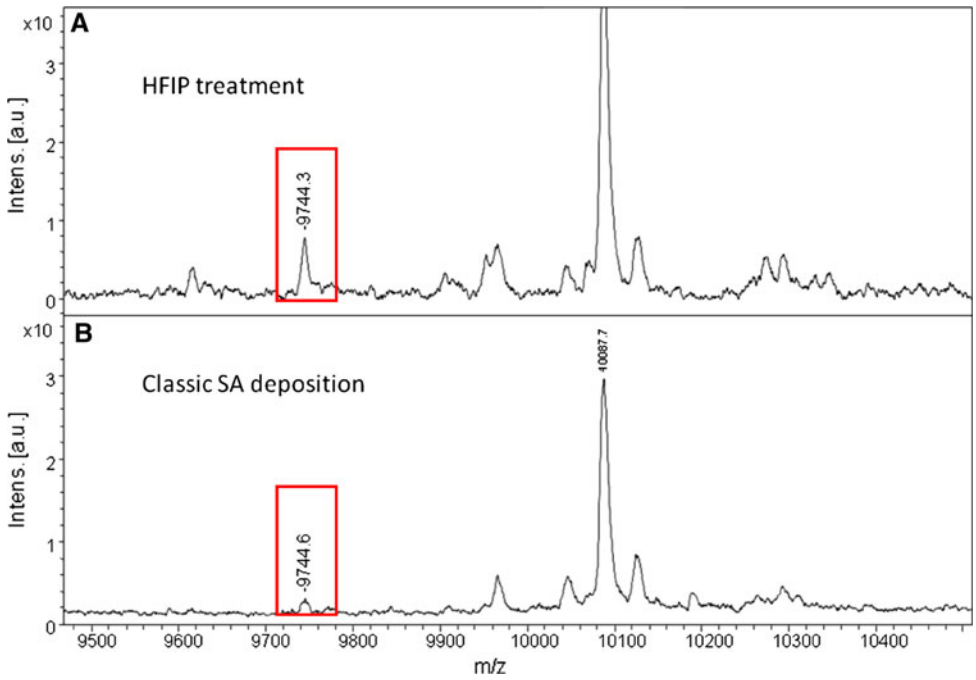

Fig. 1 The spectral differences observed between the classical and HFIP protein extraction methods from small tissue sections. **a** The profiling spectrum obtained from a 4-mm cancerous region of a stage I serous cancerous tissue using the HFIP procedure; **b** the spectrum generated with the classical drop-of-SA method. With the classical drop method, the C-terminal Reg-alpha fragment is not clearly distinguishable from the background. In contrast, this fragment is readily apparent using the HFIP procedure

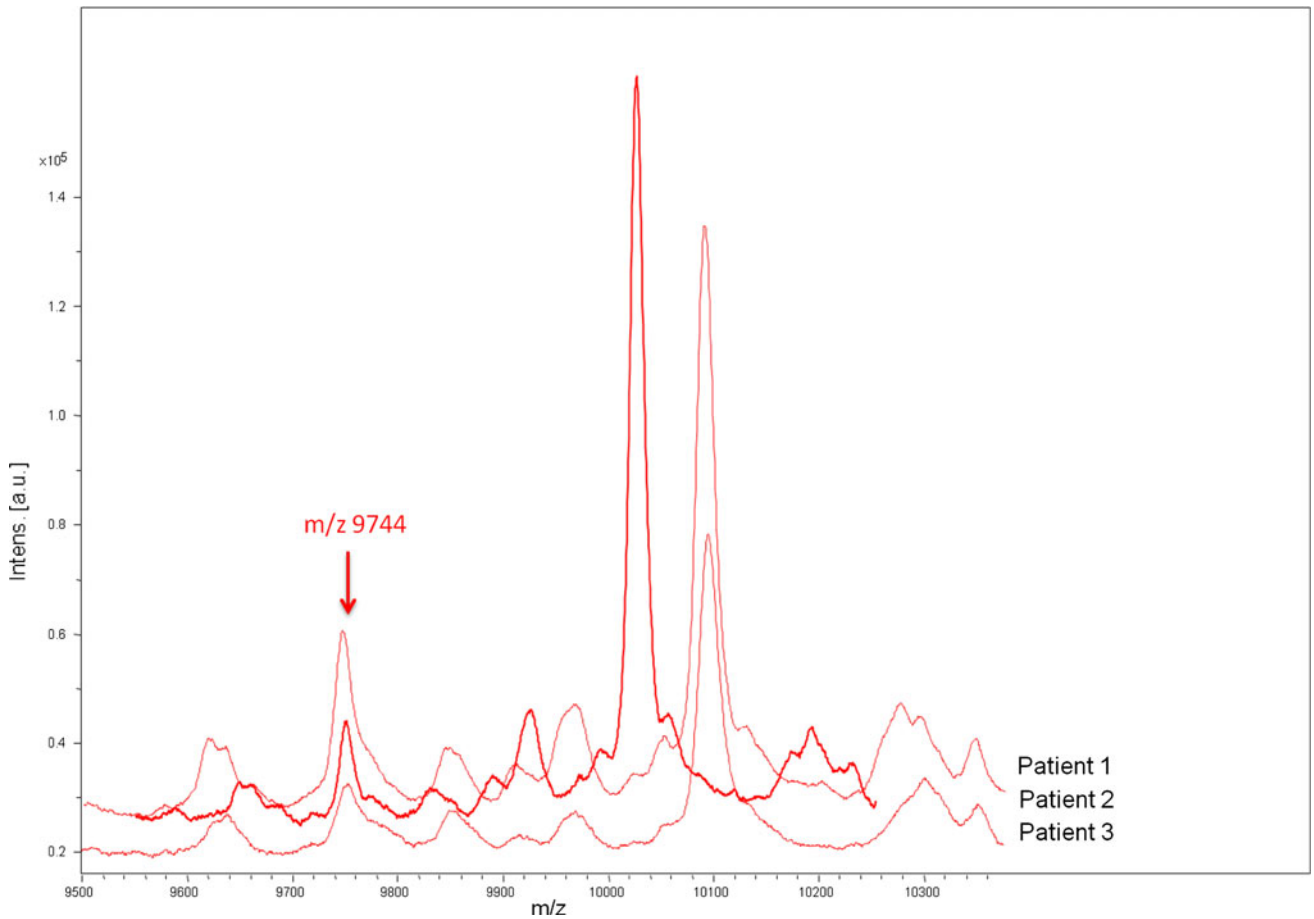

Fig. 2 MALDI-MS spectra of the HFIP extracts from the carcinoma regions of three different stage III–IV patients. The ion with an m/z ratio of 9,774 corresponds to the C-terminal Reg-alpha fragment, which is found in each patient and indicated by an *arrow*

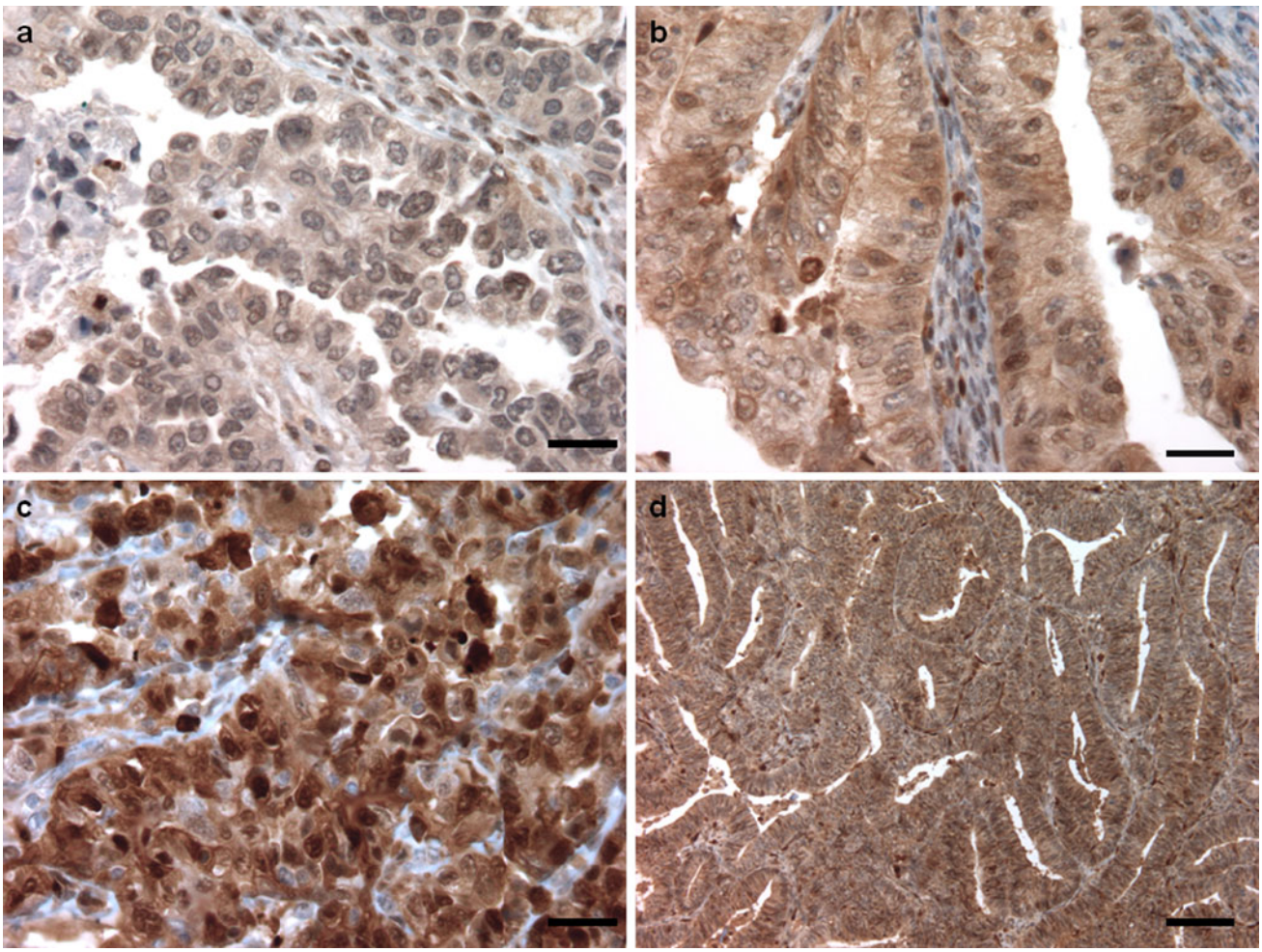

Fig. 3 Immunohistochemical studies using a polyclonal antibody directed against the C-terminal portion of the Reg-alpha fragment. This study was performed on 12 different samples (see Table 2). This set of pictures represents carcinoma tissues. **a** Papillary clear-cell carcinoma (*scale bar* 50 μm). **b** Epithelial cells of immunolabeled serous carcinoma (*scale bar* 50 μm). **c** Epithelial cells of immunolabeled serous poorly differentiated carcinoma (*scale bar* 50 μm). **d** Endometrioid carcinoma (*scale bar* 50 μm)

The difference in cellular localization based on the benign or malignant character of the epithelial samples is illustrated in Figs. 3 and 4. Moreover, in these surface epithelial–stromal tumors types, both epithelial and stroma cells are labeled in IHC with the anti-C-terminal Reg-alpha antibody. It is now possible to discriminate through the localization of the labeling (nuclear vs. cytoplasmic), these tumors which are a class of ovarian neoplasms that may be benign or malignant. It has to be noted that in this group of tumors serum CA-125 is often elevated but is only 50 % accurate, so it is not a useful tumor marker to assess the progress of treatment. We have now a novel marker, Reg alpha, which completes the diagnosis. Statistical analyses performed for this marker at different OVC stages reflect a prevalence of 77.66 ± 8.77 (with a correlation coefficient value of $p < 0.001$ of 0.601 between OVC and benign tissue) (Table 3).

Considering this strict difference, we then investigated the presence of C-terminal Reg-alpha fragment in the borderline tissues (Fig. 5). Ten tissues were investigated in IHC. The borderline cysts (Fig. 5) revealed strong labeling in the cytoplasm and nucleus, which indicated a malignant diagnosis for these samples. The HFIP-based MALDI profiling analyses for the 12 samples (Fig. 6) confirm the presence of the marker in the malignant portion, and its absence in the healthy portion of the ovary (Fig. 6) confirmed the specificity and usefulness of Reg alpha for diagnosing borderline tumors. Thus, the presence of the Reg-alpha C-terminal fragment with an m/z ratio of 9,744 confirms the malignant character of the tissue.

The C-terminal Reg-alpha fragment is a specific OVC-relapse diagnosis marker

Treatment for advanced OVC involves an explorative celioscopic surgery followed by a neoadjuvant treatment

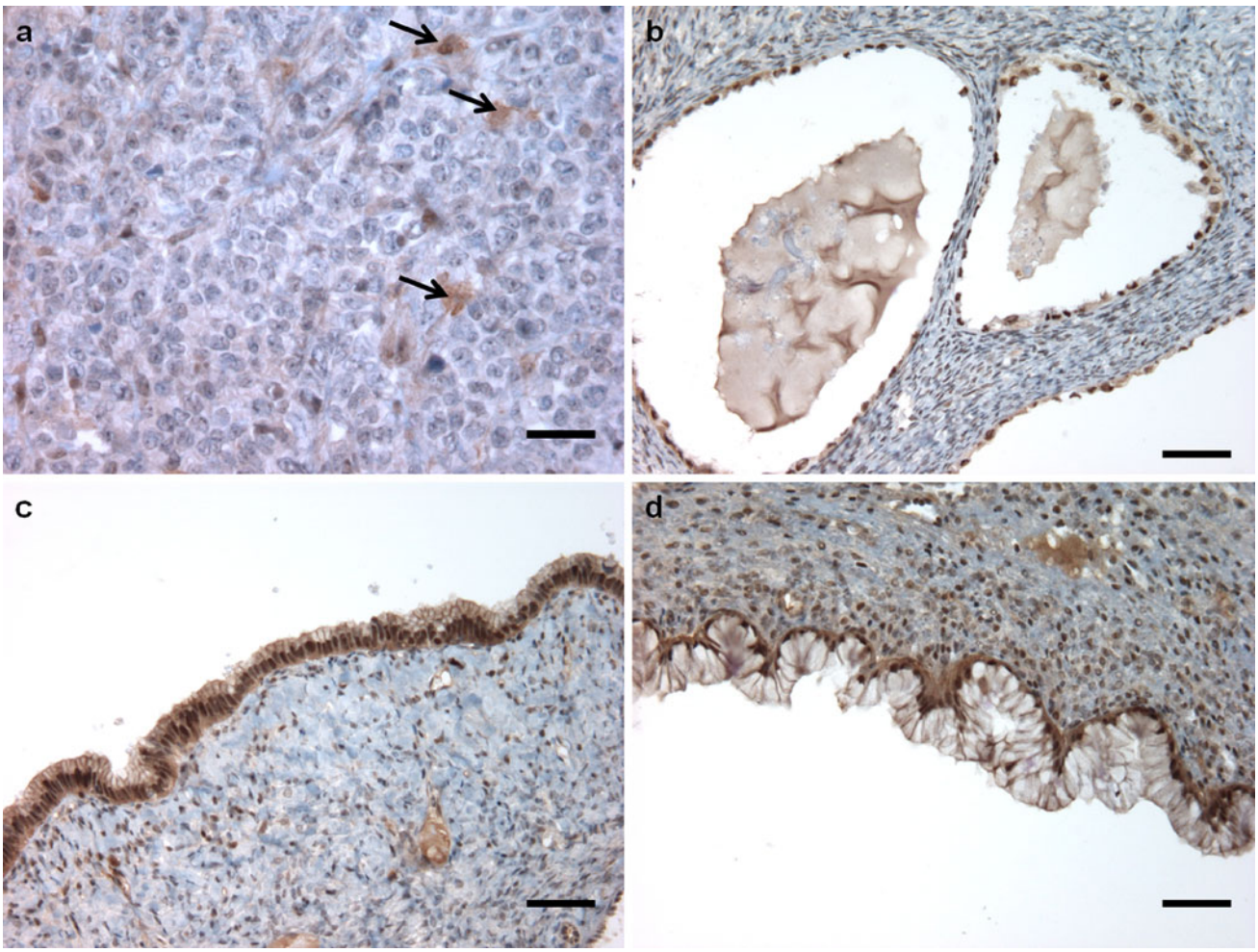

Fig. 4 Immunohistochemical studies using a polyclonal antibody directed against the C-terminal portion of Reg alpha. This study was performed on 12 different samples (see Table 2). This set of pictures represents carcinoma and benign tissues. **a** Epithelial cytoplasmic labeling of the undifferentiated contingent on the endometrioid carcinoma (*scale bar* 50 μm). **b** Mucinous invasive carcinoma (*scale bar* 200 μm). **c** Serous cystadenomas (benign ovary tissue; *scale bar* 50 μm). **d** Mucinous cystadenocarcinoma (benign ovary tissue; *scale bar* 50 μm)

with paclitaxel and platinum, or carboplatin with taxol (Lhomme et al. 2009) if carcinosis is visible during the explorative celioscopic surgery. The patients for whom this chemotherapy was effective had their reproductive organs (i.e., ovaries, fallopian tubes, and uterus; Table 4) completely removed at least 10 months after the initial diagnosis. The 5-year survival remains poor at about 40 % (Kehoe 2008; Tanner et al. 2012). The responders will relapse within approximately 18 months after completing first-line therapy and require further systemic therapy. The median survival of patients with recurrent ovarian cancer ranges from 12 to 24 months. Traditional clinical measures of relapse include disease progression, usually defined as a 25 % or greater increase in tumor size, appearance of new lesions, or death.

In this context, ten patients were surgically explored by celioscopy. The carcinosis samples from the first surgery or the ovarian samples from the second surgery were analyzed for tumor recurrence by tracking the C-terminal Reg-alpha fragment using MALDI-MS profiling (Fig. 7). The C-terminal Reg-alpha fragment was present in the mass spectra of these patients. In patient 97, for example, the chemotherapeutic treatment was completely ineffective. In this patient, all of the samples taken during the exploratory celioscopy (the second surgery) were malignant, and the C-terminal Reg-alpha fragment was found in all of the biopsies. For patients 66 (Fig. 7 see inset 66) and 81, the relative efficacy of the chemotherapy allowed the surgeon to perform the radical surgery; however, the patients are still under surveillance. A biopsy from patient 75 that was collected 7 months after the treatment also presented a small peak with an m/z ratio of 9,744, confirming that the tumor was still present and that relapse might be beginning (Fig. 7; see inset 75). Consequently, this patient received

Table 2 The immunohistochemistry results from 12 patients

	Patients	Pathologist diagnosis	Epithelial labeling intensity	Cellular localization of the labeling	Stroma reaction
Ovarian cancer	1	ADK	Strong	Cytoplasmic and nuclear	Low and nuclear
	2	Papillary carcinoma	Strong	Cytoplasmic and nuclear	Low and nuclear
	3	Mucinous ADK	Strong	Cytoplasmic and nuclear	Low and nuclear
	4	Serous ADK less differentiated	Strong	Cytoplasmic and nuclear	Low and nuclear
	5	ADK less differentiated invasive	Strong	Cytoplasmic and nuclear	Low and nuclear
	6	Borderline cyst	Strong	Cytoplasmic and nuclear	Low and nuclear
	7	Endometrioid ADK	Strong	Cytoplasmic and nuclear	Low and nuclear
	8	Endometrioid ADK	Strong	Cytoplasmic and nuclear	Low and nuclear
Benign ovarian tumors	1	Serous cystadenocarcinoma	Strong	Nuclear	Low and nuclear
	2	Adenocarcinoma borderline	Strong	Nuclear	Low and nuclear
	3	Benign part of serous ADK	Low	Nuclear	Low and nuclear
	4	Serous cystadenocarcinoma	Low	Nuclear	Low and nuclear
	5	Leiomyoma	Low	Nuclear	Low and nuclear
	6	Mucinous cystadenocarcinoma	Strong	Nuclear	Low and nuclear
	7	Serous cyst	Strong	Nuclear	Low and nuclear

The immunohistochemistry used an antibody directed against the C-terminal fragment of the 11s proteasome activator complex (PA28, Reg alpha)

Table 3 Statistical analyses of detection of Reg-alpha marker in the different cases

Pathology type	Statistical prevalence of the C-terminal fragment (%); (time detection/number of samples)
Serous cancer	
Stage I	66 %; (14/21)
Stage III–IV	80.5 %; (95/118)
Relapse	88 %; (22/25)
Mucinous cancer	
Stage III–IV	82 %; (41/50)
Borderline	71.8 %; (23/32)
Benign	20 %; (26/130)

The method used for statistics takes into account all techniques used for the detection of this maker. This includes MALDI profiling, IHC, Western blot, nano-LC–IT-MS, and MALDI-MSI. Considering the technique used, this marker is detected at 73 % in IHC, 89 % in MALDI profiling, and 77 % in MALDI-MSI combined with Western. MALDI profiling seems to be the best technology to detect this marker. The variation intra-essay of the detection of this marker is 12 % between OVC and benign tissues

radical surgery and is now under surveillance. The positive control for the experiment was the detection of the C-terminal Reg-alpha fragment in the mass spectra of patients 67 and 79, who had BRCA1 genetic mutation and breast cancer. Pathology-based recidivism was observed in patient 66 (Fig. 7 see inset 66), confirming that the presence of the C-terminal Reg-alpha fragment could be used to evaluate pathological resurgence or persistence. This demonstrates that this marker (C-terminal fragment of Reg alpha) can detect tumor recurrence and can be used to monitor relapse and treatment efficacy. This marker presents a prevalence of 88 % in the case of tumor relapse (Table 4).

The C-terminal Reg-alpha fragment is a specific early-stage OVC-diagnosis marker

A good marker for disease screening must be found in the early stages of disease development. We obtained stage I surgical tissues from different types of OVCs. We then investigated the C-terminal Reg-alpha fragment in the cancerous regions of interest by comparing the molecular profiles of small areas of cancerous tissue with normal areas from the same tissue section. Due to the difficulty of diagnosis at the early stage of pathology, this study was performed on eight collected tissues. Figures 8 and 9 show the restricted size of the diseased areas from stage 1a and stage 1c serous OVC biopsies, respectively. The relative spectra acquired in each cancerous and benign tissue zone focus on the mass range of Reg alpha and confirm that it is present only in the OVC cells. The spectra clearly show that this marker is specifically localized in the cancerous regions of the OVC tissue and is absent in the benign tissue areas. The immunofluorescence data confirmed the localization of the C-terminal Reg-alpha fragment in the three cells of the acini (Fig. 8; see inset picture). Moreover, we confirmed the presence of Reg alpha in stage I tissues from the most frequent OVC-epithelial cancer types:

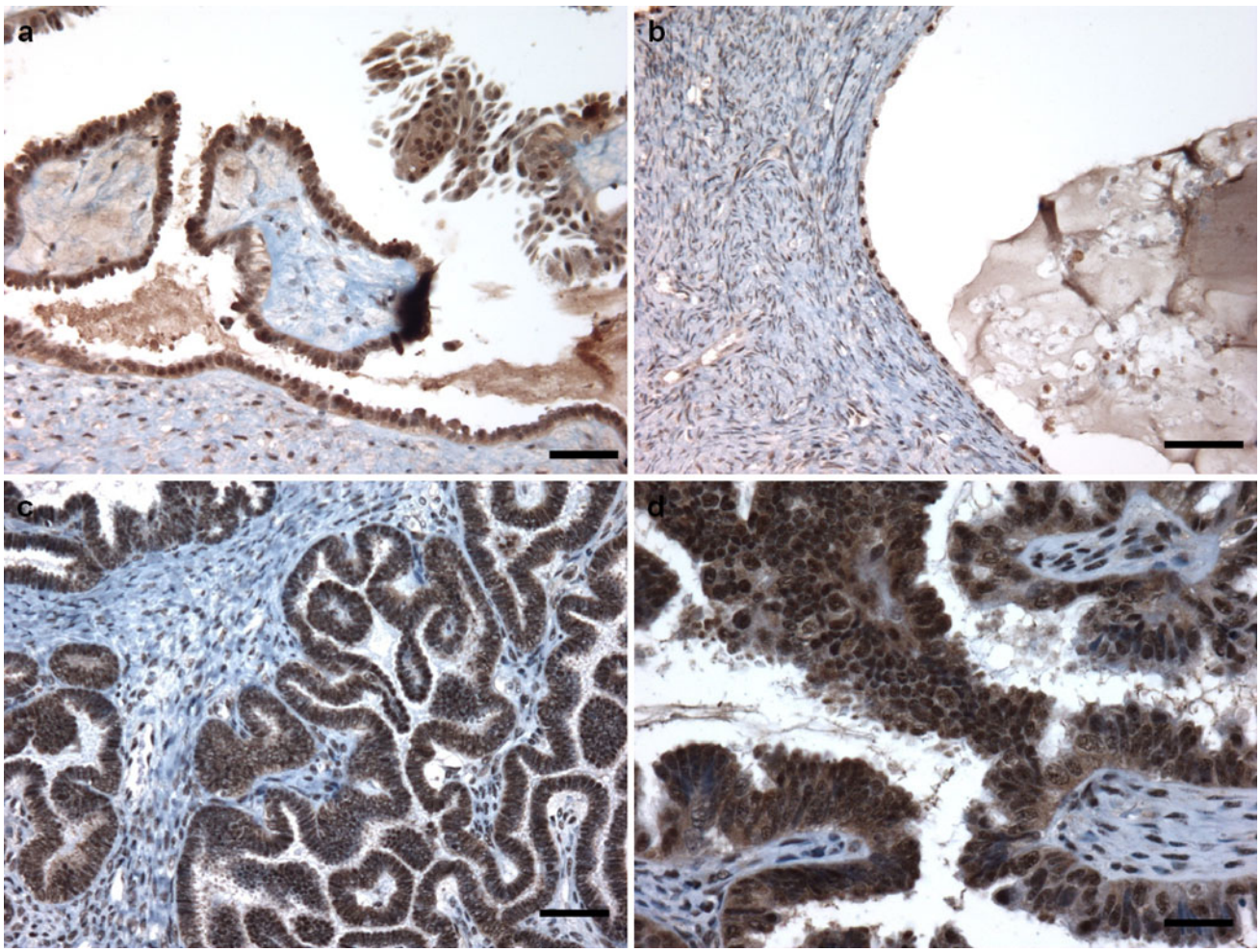

Fig. 5 Immunohistochemical studies using a polyclonal antibody directed against the C-terminal portion of Reg alpha. This study was performed on samples from 12 different patients (see Table 2). This set of pictures represents borderline tissues. **a** A borderline malignant cyst (*scale bar* 50 μm). **b** Borderline adenocarcinoma tissue (benign tissue; *scale bar* 200 μm). **c** Borderline malignant mucinous tissue (*scale bar* 200 μm). **d** Borderline malignant mucinous tissue (*scale bar* 50 μm)

endometrioid (Fig. 9), which represents 15 % of epithelial OVC cases; mucinous OVC (Fig. 10), which represents 5 % of OVC cases; and serous OVC (Fig. 2), which represents 80 % of OVC cases. We also found this marker in borderline mucinous tissue. Statistically, Reg alpha is detected at 81.25 % ± 1.06 in stage III–IV and 80.5 % in stage I of OVC (Table 3). The correlation coefficient for all OVC stages is $p < 0.001$ of 0.998.

Discussion

These data confirmed that our novel method was useful for rapid diagnosis using small pieces of tissue taken from extemporaneous biopsies. This procedure can be used for extemporaneous biopsies and will help pathologists provide a diagnosis. Indeed, the extemporaneous analysis of tissue sections usually relies on a simple visual evaluation of the HES section of the biopsy. The final pathological reports confirm the initial diagnosis using IHC for known pathological biomarkers, such as P53 and HER2/neu, and are only available 1 week later. This rapid and simple method can be used for fast tissue marker analysis that provides additional disease information to the pathologist and the surgeon for use in the extemporaneous analysis that will be followed by the IHC-based final report.

Therefore, the C-terminal Reg-alpha fragment biomarker has been detected in early- and late-stage OVC using MALDI mass spectrometry profiling with a specific HFIP extraction procedure. The presence of Reg alpha in certain borderline tissues illustrates its usefulness in cases that are difficult to diagnose. This marker can also be used to evaluate cancer treatment, and its presence in patients after chemotherapy and tumor relapse confirms that this

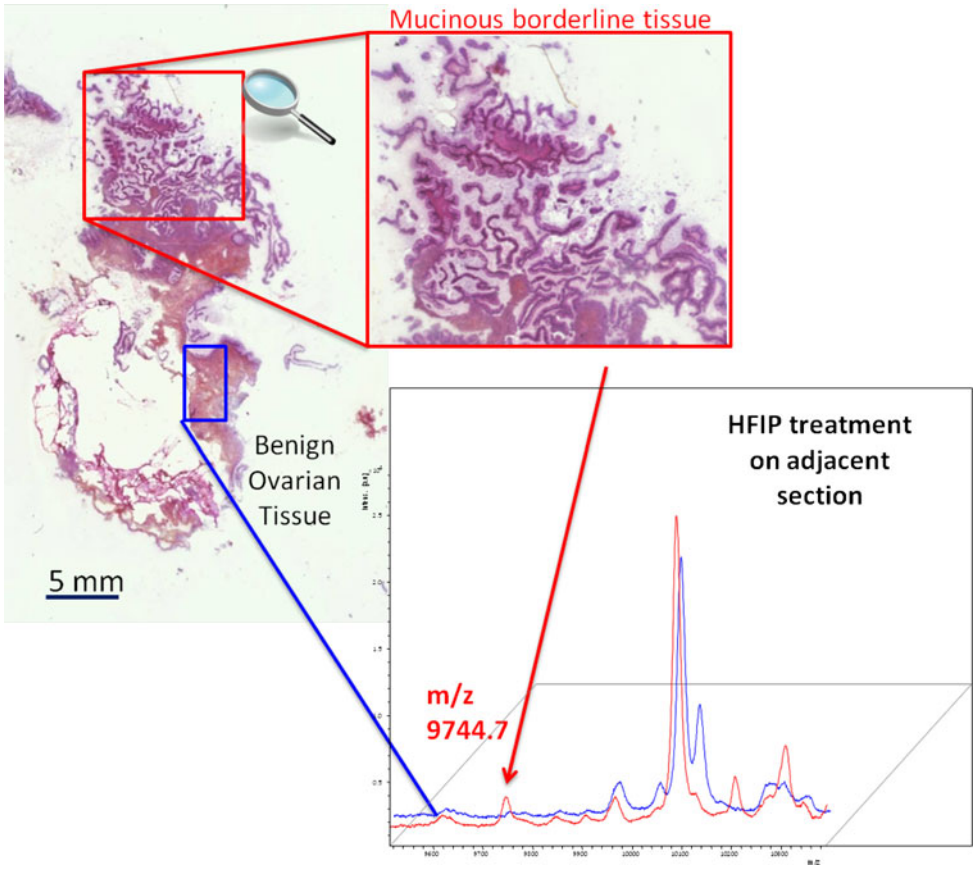

Fig. 6 MALDI-MS spectra of the HFIP extracts from a mucinous borderline region. The ion with an m/z of 9,774 ratio corresponds to the C-terminal Reg-alpha fragment and is indicated by an *arrow*. The HFIP extraction from the healthy region confirmed the absence of the ion at an m/z of 9,774, validating the specificity of the C-terminal fragment detection. These data demonstrate that this borderline tissue is malignant. *The inset* illustrates hematoxylin and eosin (H&E) staining of the tissue subjected to MALDI-MS profiling analyses (*scale bar* 5 mm)

Table 4 The characteristics of the patients from whom tissue was collected after neoadjuvant chemotherapy

Patient number	Patient age	Observations	Type of treatment	Time between the diagnosis and second surgery (months)
58	58		6 Cycles of carboplatin/taxol Complete surgery	4
65	61		6 Cycles of carboplatin/taxol Complete surgery	10
66	69		6 Cycles of carboplatin/taxol Complete surgery carboplatin/Caelyx	15
67	55	Mutation BRCA1	4 Cycles of carboplatin/taxol Complete surgery 2 Cycles of carboplatin/taxol	8
69	63		4 Cycles of carboplatin/taxol Complete surgery 2 Cycles of carboplatin/taxol	8
75	60		6 Cycles of carboplatin/taxol Complete surgery	7
79	59	Mutation BRCA1 and breast cancer associated	6 Cycles of carboplatin/taxol Radiotherapy and herceptin	1
81	48		6 Cycles of carboplatin/taxol Complete surgery	11
95	65		6 Cycles of carboplatin/taxol Taxol	12
97	59		6 Cycles of carboplatin/taxol Taxol	18

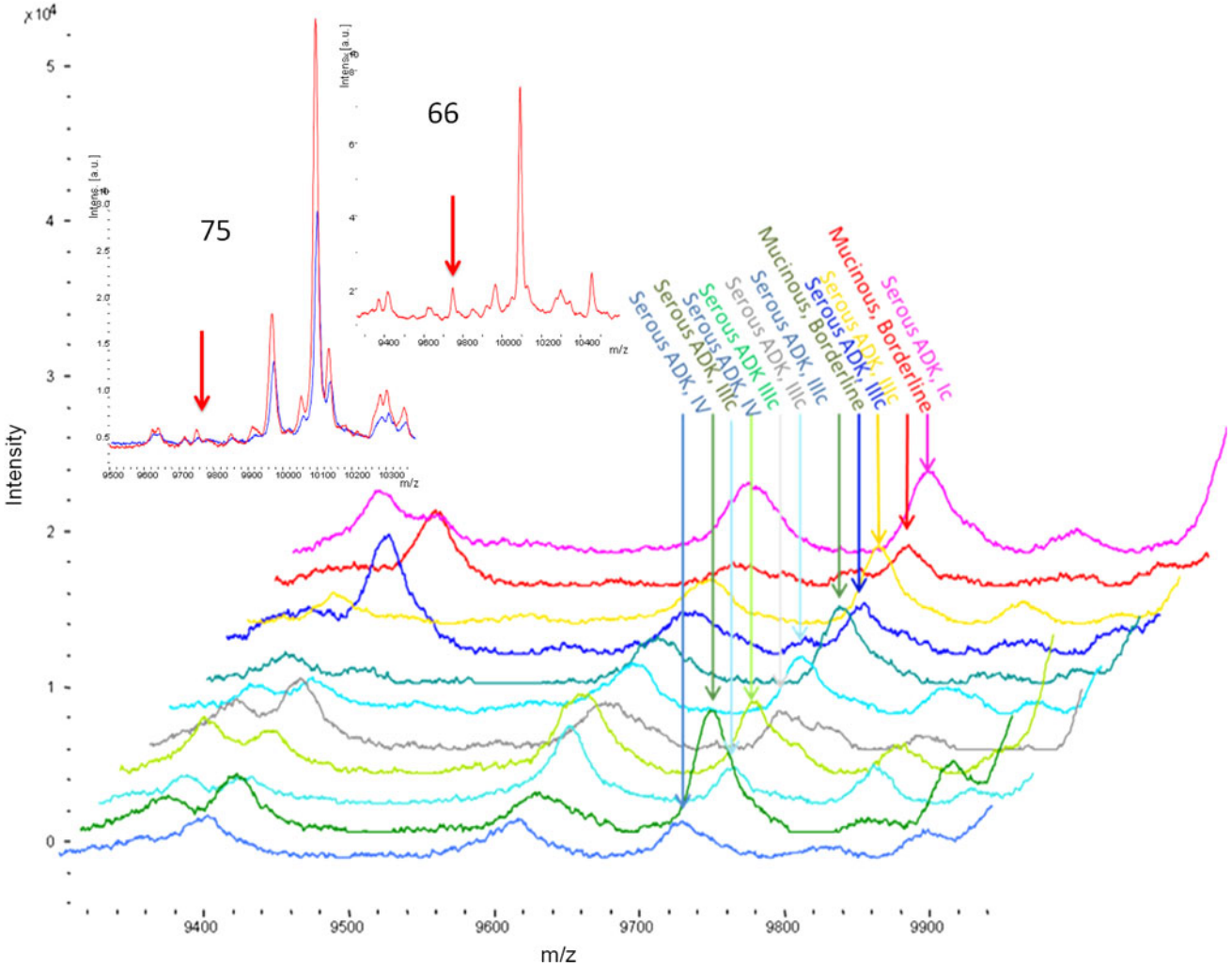

Fig. 7 MALDI-MS spectra of the HFIP extracts from the carcinoma regions of ten patients at different pathologic stages after neoadjuvant chemotherapy. Mass spectra analyses of HFIP extracts from stages I, IIIc, and IV serous tumors after neoadjuvant chemotherapy. The ion with an m/z ratio of 9,774 corresponds to the C-terminal Reg-alpha fragment, is found in each patient, and is indicated by an *arrow*. These data confirm that the C-terminal fragment of Reg alpha can be a good marker for the early diagnosis of tumor relapse. (*inset 75* a tissue analysis example from a patient subjected to 15 months of prior neoadjuvant chemotherapy with 6 cycles of carboplatin/taxol followed by carboplatin with Caelyx before complete surgery. The ion corresponding to the C-terminal Reg-alpha fragment (m/z 9,744) is detected. *Inset 66* a tissue analysis example from a patient subjected to 7 months of prior neoadjuvant chemotherapy with six cycles of carboplatin/taxol. The ion corresponding to the C-terminal Reg-alpha fragment (m/z 9,744) is detected)

protein fragment is a promising candidate for monitoring patient response during therapy. The data obtained by MALDI mass spectrometry profiling are consistent with the IHC data.

A specific antibody allows for discriminating malignant from benign cells and can be useful for diagnosing borderline tissues. In this context, we performed IHC on borderline tissues and confirmed the specificity of Reg alpha in tissue for which a specific diagnosis was difficult. The borderline OVC stage constitutes a precancerous lesion, confirming that the Reg-alpha C-terminal fragment is an early marker of ovarian tumors and can be used as a screening marker for this pathology. Our specific mass spectrometry protocol obtains molecular information in under 10 min, as compared to 2 h for IHC. This technology will give the pathologist a new diagnostic tool that may be particularly useful for extemporaneous biopsy diagnosis.

In ovarian cancer, the median interval to the first recurrence is 18–24 months. After recurrence, approximately 70 % of advanced-stage ovarian cancer cases relapse, and even in stage I or II patients, the relapse rate is 20–25 %. Half of the recurrences occur more than 12 months after the end of first-line therapy, and one-quarter of all recurrences occur within less than 6 months. A single carboplatin cycle has been a standard regimen for patients with platinum-sensitive disease. A phase II trial of

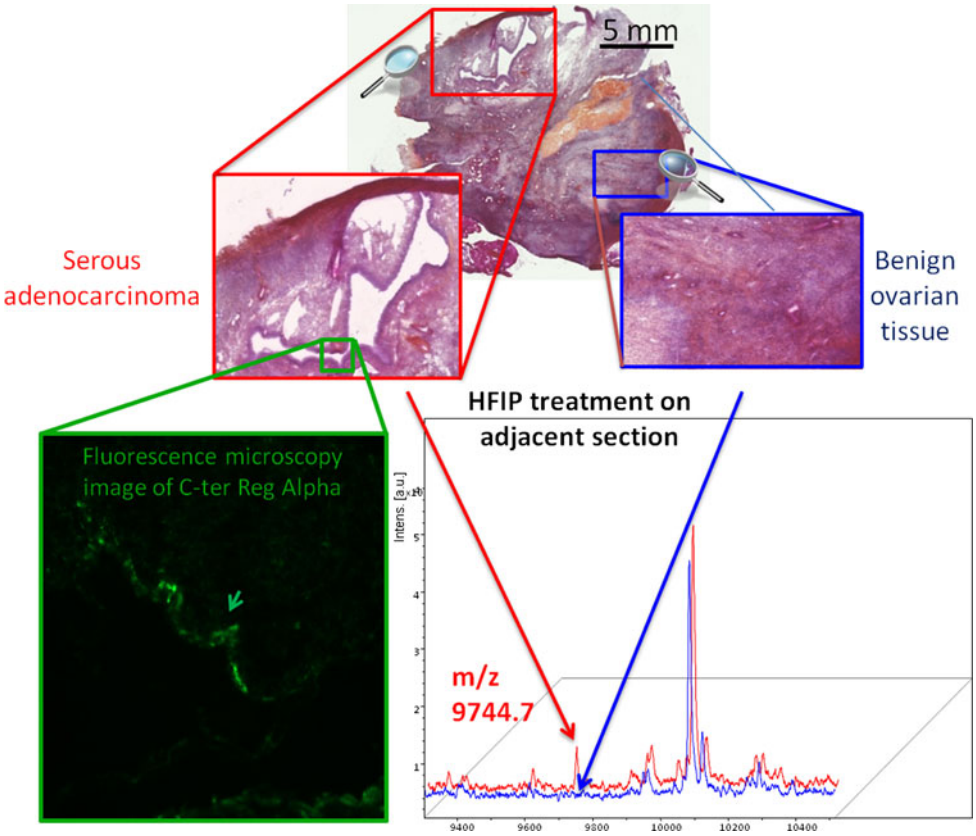

Fig. 8 MALDI-MS spectra of HFIP extracts from stage 1a serous OVC biopsies. The C-terminal Reg-alpha fragment (m/z 9,744) is detected in the acini cells. The *inset* shows fluorescent immunocytochemistry data using the anti-C-terminal Reg-alpha fragment and hematoxylin and eosin (H&E) staining of the tissue subjected to MALDI-MS profiling analyses (*scale bar* 5 mm)

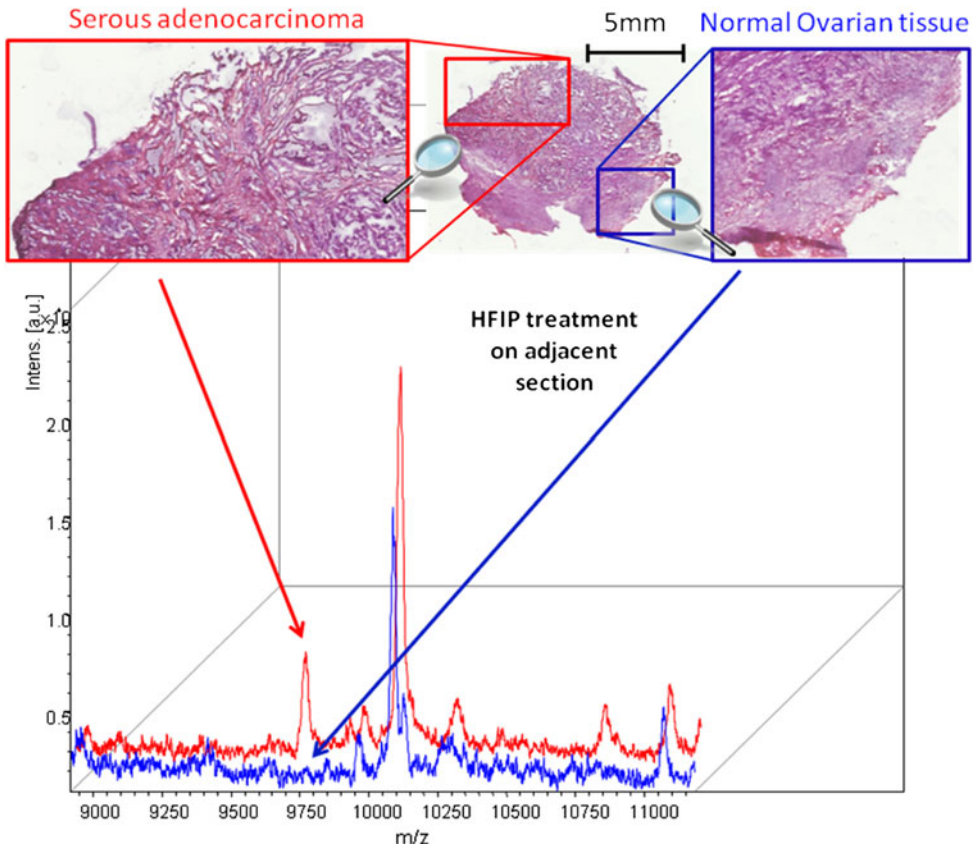

Fig. 9 MALDI-MS spectra of HFIP extracts from stage 1c serous OVC biopsies. The C-terminal Reg-alpha fragment (m/z 9,744) is detected in the OVC cells. The *inset* shows the hematoxylin and eosin (H&E) staining of the tissue subjected to MALDI-MS profiling analyses (*scale bar* 5 mm)

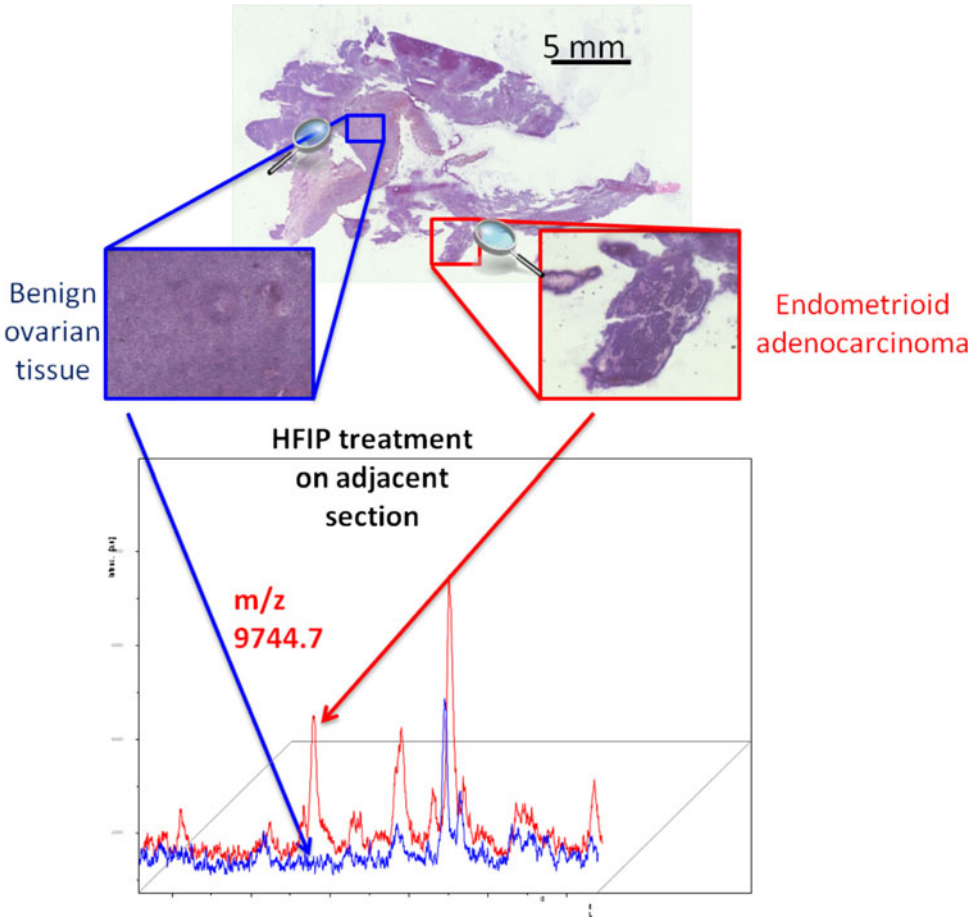

Fig. 10 MALDI-MS spectra of HFIP extracts from stage 1c endometrioid OVC biopsies or from healthy adjacent tissue. The C-terminal fragment of Reg-alpha (m/z 9,744) is detected in the tumor sample. *The inset* shows the hematoxylin and eosin (H&E) staining of the tissue subjected to MALDI-MS profiling analyses (*scale bar* 5 mm)

a paclitaxel and carboplatin combination for sensitive (6 months) patients is currently used after phase I (Ushijima 2010). Patients with persistent ovarian cancer have cancer cells that are detected after initial surgery and first-line chemotherapy. Persistent ovarian cancer is detected by elevated serum CA-125, abnormal X-rays and CT scans, or a biopsy performed during a second-look laparotomy. The standard course of the initial chemotherapy is approximately six cycles (or 4 months of treatment). It is thus important to have good tumor-relapse markers to evaluate whether the first-line treatment is sufficient or whether a second cycle of chemotherapy is necessary. In this context, the C-terminal Reg-alpha fragment is a good candidate for evaluating tumor treatment resistance. In fact, the marker is useful soon after treatment, because it can be detected 1 month after 6 cycles of carboplatin/Taxol followed by pharmacologic treatments (Table 3). This marker can also be used in conjunction with mucin-9, which we have previously identified in tumors (El Ayed et al. 2010; Franck et al. 2009a) and in patients' serum (Maines-Bandiera et al. 2010). Notably, the C-terminal Reg-alpha fragment has also been detected in ascites fluid (El Ayed, unpublished data). Therefore, these two markers can be used to track the therapeutic efficacy.

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