RAPID COMMUNICATION

Microproteomics by liquid extraction surface analysis: Application to FFPE tissue to study the fimbria region of tubo-ovarian cancer

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Purpose: We have developed a new method for rapid analysis of a specific region on formalin fixed and paraffin embedded (FFPE) tissue sections. This method combines advantages of direct tissue MS analysis keeping histological information and conventional proteomics approaches for confident identification of proteins in complex sample.
Experimental design: After histological annotation, heat-induced antigen retrieval is performed on FFPE tissue. Using a chemical inkjet printer, trypsin is deposited on discrete regions of less than 1 mm^2. After protein digestion, a liquid extraction is performed to retrieve all the peptides. Data coming from identification of proteins in cancer and benign region are compared.
Results: In total, 3649 unique peptides were identified (with a peptide strict false discovery rate less than 1%) corresponding to 983 and 792 nonredundant protein groups identified in benign and cancer region, respectively. A total of 123 protein groups are found only in cancer region and 315 are specific to the benign part. From these data, it has been possible to obtain different important signaling pathways involved in cancer processes and some proteins already known as biomarkers.
Conclusions and clinical relevance: This new approach using a combination of localized on-tissue protein digestion and liquid microextraction followed by LC-MS/MS analysis is useful for advancing our understanding of cancer biology. It is a rapid and innovative technique that will contribute positively to clinical proteomics.

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To understand the molecular basis of diseases such as cancer, it is becoming crucial to enhance the diagnosis, prognosis, and treatment. In 2011, the incidence of cancer amounted to 365 500 people in France of whom 158 500 were women [1]. Some cancers such as ovarian cancer (OC) are difficult to detect at early stages due to the lack of clinical symptoms. However, OC represented the fifth cause of death with 3.2% of the women cancers. Ten per cent of OC are related to a hereditary context (e.g. mutations on BRCA 1/2 genes). Based on advanced histopathological techniques along with biomolecular studies, the 15 histotypes of ovarian carcinomas have been reclassified in two major histomolecular and prognostic subtypes [2]. Type I tumors encompass low-grade

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Abbreviations: FFPE, formalin fixed and paraffin embedded; **OC**, ovarian cancer; **PML**, promyelocytic leukemia; **RPL5**, ribosomal protein L5; **STIP1**, stress-induced phosphoprotein 1

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serous, mucinous, endometrioïd, and clear cell carcinomas. They share a progressive evolution with progressive alterations in the mitotic signaling pathways (specific for each subtype), but BRCA and p53 alterations are an exception and/or a late event in their evolution. These tumors have a longer progression time, and although they seem less sensitive to platinum-paclitaxel chemotherapy, their overall prognosis is globally better (40–50% of overall survival at 5 years). In contrast, type II tumors regroup high-grade serous, undifferentiated carcinomas, and carcinosarcomas. Their common characteristic is the constant alteration in DNA repair complexes (BRCA and p53 especially). These tumors share a rapid evolution and after, generally, a good response to chemotherapy, they recur rapidly. The survival of type II tumors rarely exceeds 30% at 5 years. Unfortunately type II tumors represent 80% of ovarian carcinomas explaining the high lethality of this disease. During the last 10 years, special attention has been paid to the pathological examination of operative specimens of early OC and prophylactic salpingo-oophorectomies performed in women at risk of OC due to an inherited mutation in BRCA genes. In this group of patient, an unusual rate of genuine cancer or at least cellular abnormalities were observed in the epithelium of fallopian tube, especially at its terminal end, the fimbria. A recent hypothesis claims that fallopian tube could be responsible for most OCs [3]. Most type I tumors arise from uterine cells that transit through the tubes and transform within ovarian inclusion or endometriotic cysts (endometrioïd, clear cell and some low-grade serous tumors). In contrast, type II tumors originate from transformed Müllerian cells of fallopian tube especially at the fimbria level. These transformed cells may deposit onto the mesothelial ovarian surface to give rise to a high-grade serous tumor (initially of tubal origin, the development on the ovarian surface masks the tubal origin (effect of the ovarian dominant mass)) or develop inside ovarian inclusion cysts to give rise to borderline serous, then low-grade carcinomas (which could exceptionally transform into high grade carcinomas). Focusing on these high-grade serous tumors, a thorough pathological reexamination following a specific protocol (Sectioning and Extensively Examining the FIMbria (SEE-FIM) [4]) showed that cellular abnormalities of this Müllerian epithelium are progressive from SCOUT (secretory cell outgrowth, lack of PAX2 expression [5]), STIL (serous transitional intraepithelial lesions, with p53 signature), TILT (tubal intraepithelial lesion in transition), STIC (serous tubal intraepithelial carcinoma, Ki67 marking), and finally invasive tubal/ovarian carcinoma. Accompanying these morphological transformations, immunohistochemistry detect biomolecular modifications such as loss of expression of PAX2, nuclear accumulation of TP53 (the "p53 signature") and Ki67, a marker of proliferation. These modifications are specifically observed by the pathologist and are often uneasy to detect. Thus, it seems interesting to detect whether these modifications have a substrate at a molecular level, in order to enhance the early detection, and to enable the pathologist to work with more specific markers and possibly to provide blood test to detect at a very early stage these aggressive tumors.

Novel and sensitive proteomics strategies such as MALDI-MS imaging (MSI) allow the precise localization of markers and interaction between the tumor and the direct environment [6]. Nevertheless, direct identification of biomarkers on tissue by in situ top-down or bottom-up strategies remains difficult and only allows access to a limited number of major proteins [7]. Contrariwise, shotgun proteomics is now established as the most powerful and widespread approach for the proteomics characterization of complex samples, such as FFPE tissue extracts. In this case, all proteins of the mixture are digested and the resulting peptides are separated by LC-MS/MS for identification. The main disadvantage is the loss of histological information due to the sample preparation. Here we present, for the first time on FFPE tissue, a new

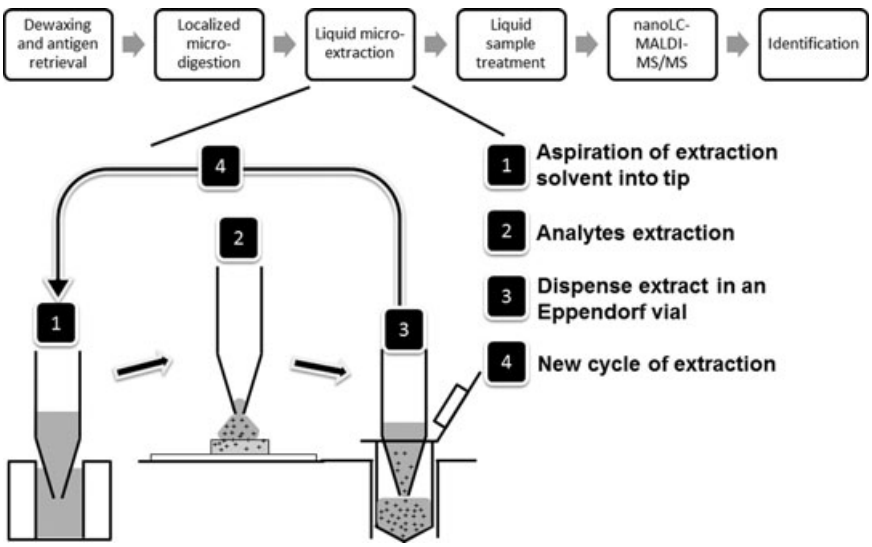

Figure 1. On tissue microextraction strategy for identification of biomolecules by liquid microjunction surface sampling. Microextraction consists following three steps: (1) solvent sampling (2) solvent dispensing on the tissue section though liquid microjunction and analyte extraction during liquid microjunction to the tissue (3) dispensing extract in a tube. Steps 1–4 can be reiterated several times to reach optimized extraction yields.

approach combining the fine correlation of location in tissue and the identification of proteins in various abundances.

A complete *Materials and Methods* description could be found in Supporting Information. Briefly, a chemical inkjet printer is used to perform on tissue microdigestion on selected region of the tissue. The discrete point of digestion could be defined from several hundred micrometers to millimeters to allow a precise selection of a region. In this case, the goal is to study a group of cells (around 2500) permitting to understand all the mechanism involved in the region such as communication or cellular organization. After on tissue microdigestion, the region is subjected to an extraction

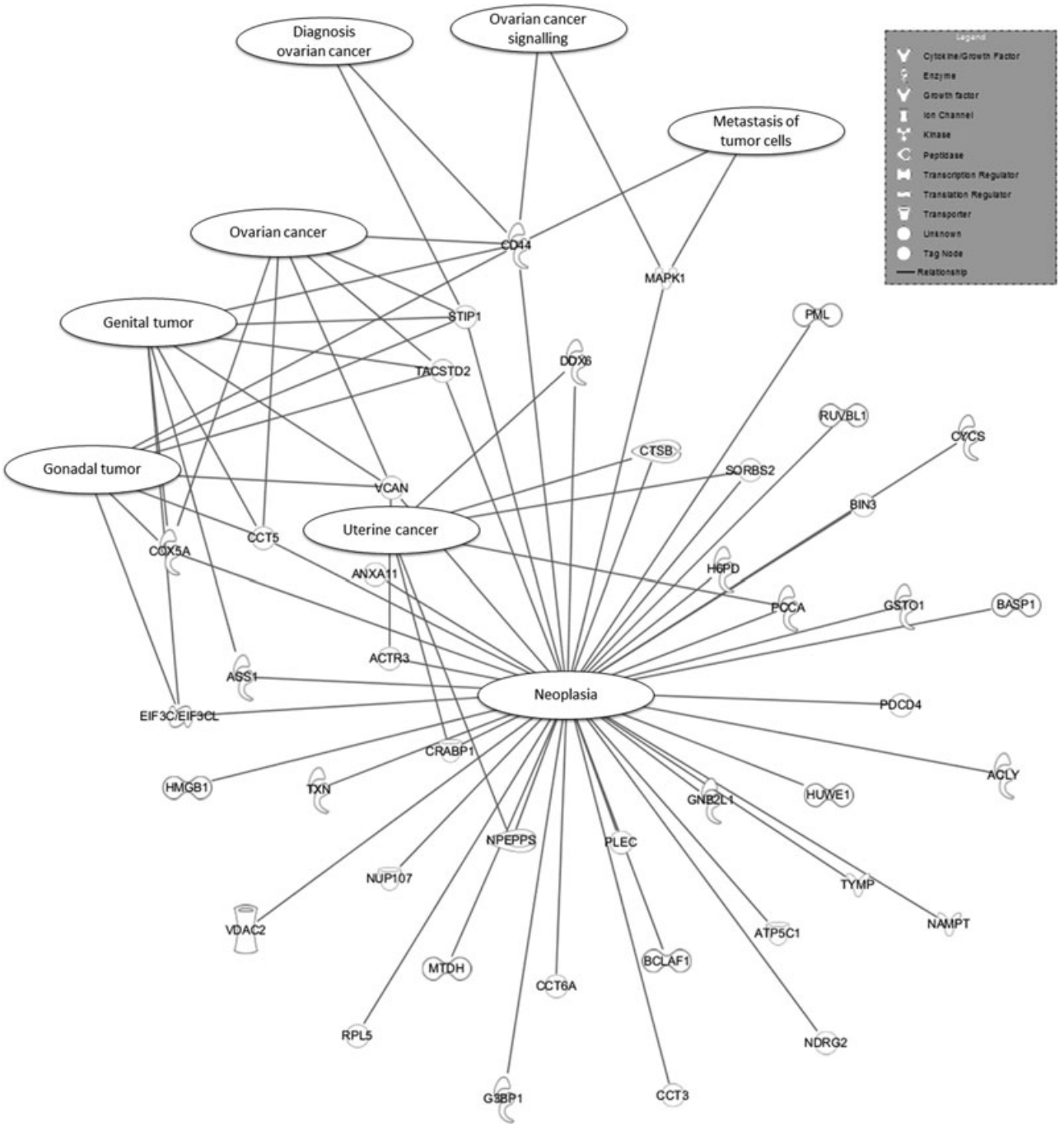

Figure 2. Major biological function and signaling pathway identified after analyzing proteomics results using IPA. Identified proteins were filtered according to proteins involved in neoplasia, uterine cancer, gonadal tumor, genital tumor, ovarian cancer (OC), diagnosis OC, OC signaling and metastasis of tumor cells.

by liquid microjunction (Fig. 1). Using appropriate solvent, tryptic peptides are extracted from the tissue and recovered in a tube to perform classical analysis. Due to the complexity of the sample, a step of separation is necessary prior to MS analysis and MS/MS identification.

This technique was successfully used on frozen tissue section previously [8], but it is the first time that it is applied on FFPE tissue. Formalin fixation followed by paraffin embedding is the most common technique used in the conservation of patient tissues. It is a long established standard procedure for the preservation of tissue architecture and long-term storage of samples. The major part of tissue bank mainly contains FFPE tissue, and represents a golden mine for those who aimed to the understanding of pathological processes and to the identification of new biomarkers.

In our study, all samples are of FFPE tissue coming from salpingo-oophorectomies of women with BRCA mutations or unexpected discovery of tumor during medical examination. All samples are examined following a protocol for SEE-FIM [9]. All tumors were Ki-67 positive and p53 positive.

After liquid microextraction and LC-MS/MS analysis, an average of 1109 nonredundant proteins are identified. Taking all in account, 792 proteins are identified in the cancer part and 983 in the benign part. Comparing the results from benign region versus cancer region, 123 proteins are specific to the cancer part (Supporting Information Table 1) and 315 are absent of the tumor part and only found in the benign region (Supporting Information Table 2).

By using different algorithms for analyzing proteins interaction network such as PINA [10] or STRING [11], interaction are found between proteins specific to cancer region and proteins already shown to be involved in the fallopian tube cancer (Supporting Information Table 3).

Four proteins interact with p53 namely HUWE1, MAPK1, ribosomal protein L5 (RPL5), and promyelocytic leukemia (PML). E3 ubiquitin-protein ligase, HUWE1 is a tyrosine phosphorylated nuclear protein that mediates a negative regulation of p53. The inactivation of HUWE1 stabilizes p53, induces p53-dependent apoptosis, and reduces cell proliferation [12]. MAPK1 (also named ERK2) is a member of the MAP kinase family involved in a wide variety of cellular processes such as proliferation, differentiation, development, and transcription regulation. Some results also suggest that ERK2 is one of the regulators of p53 protein accumulation, activation, and inhibition of p53-MDM2 association [13]. The 60S RPL5 has been shown to interact with MDM2 and inhibits MDM2-mediated p53 ubiquitylation and degradation [14]. The PML is a nuclear phosphoprotein containing a tripartite motif characteristic to the TRIM protein superfamily. PML could also physically interact with p53 [15].

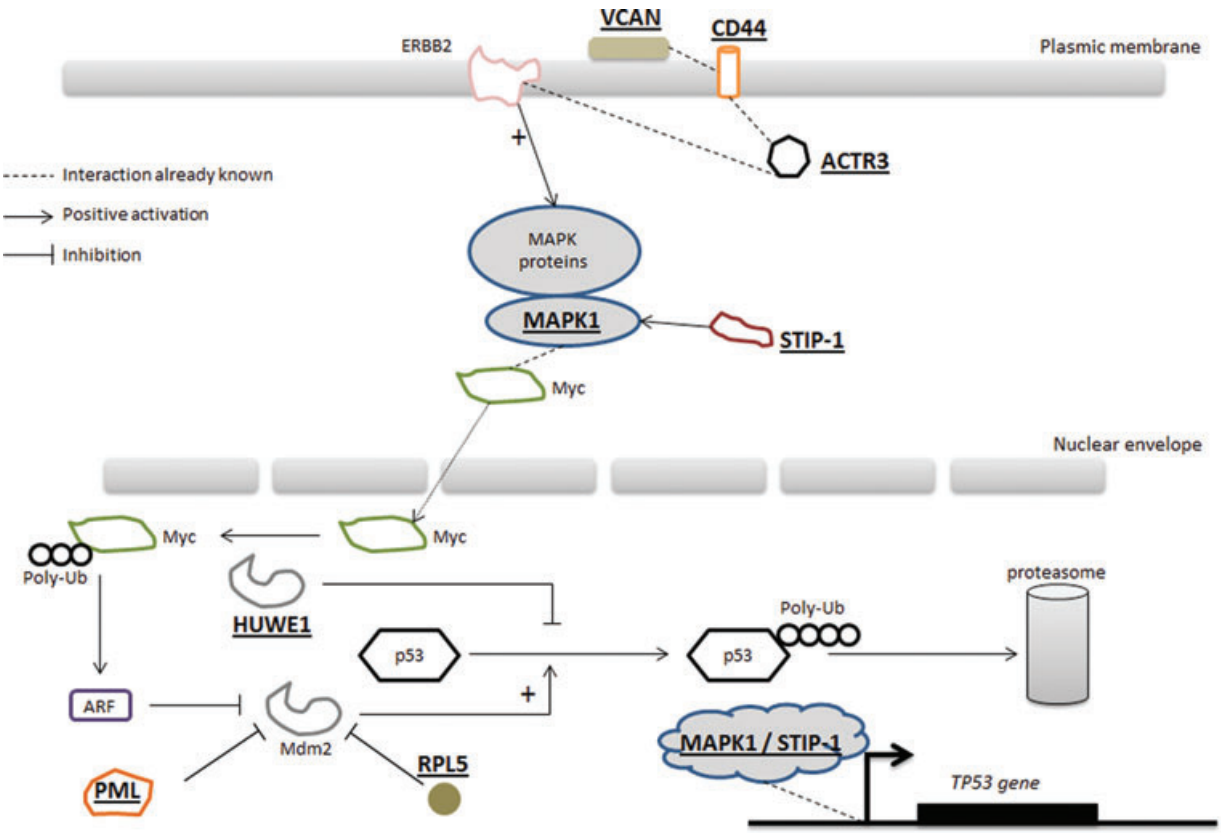

Figure 3. An example of possible interaction between proteins involved in fallopian tube cancer. Proteins identified by our technique are highlighted and underlined.

Clinical Relevance

Development of new techniques for characterization of proteins involved in cancer mechanisms has had a tremendous clinical impact. Most current technology allows either a molecular mapping of the tumor, such as MRI or MALDI-MSI, but with difficulties for direct proteins identification or a high-throughput identification of proteins from a complex mixture but with the loss of spatial information. To meet these needs, we have developed a new technique that combines the advantage of local on-tissue protein digestion and liquid microextraction followed by LC-MS/MS analysis. In addition to maintaining the histological information, localized digestion avoids dilution of the signal by the major abundant proteins and gives access to an abundance of information concerning medium or minor proteins. This new technique is therefore highly promising for growing our knowledge of cancer biology and thereby enhancing the diagnosis, prognosis, and treatment of cancer. As proof of this concept, we have successfully applied this method on FFPE tissue section of fallopian tube cancer. Most of the identified proteins were already shown in previous studies or are part of signaling pathways involved in tumorigenesis process and allow for better understanding of mechanisms involved in this disease. This technique proposes a rapid sample preparation making it easy for large-scale study.

Another important effector of the p53 pathway is MDM2. The MDM2 oncoprotein, an E3 ubiquitin ligase enzyme, plays a key role in negatively regulating p53 activity by either direct repression of p53 transactivation activity in the nucleus or promotion of p53 degradation in the cytoplasm by ubiquitination process. Like shown before, there are interactions between MDM2 and RPL5 that permit the activation of p53 by inhibiting the ubiquitinylation activity of MDM2 but also with PML to stabilize p53 by relocalization of MDM2 [16].

Some correlation could also be established between the proteins obtained in this study in cancers regions and proteins already demonstrated in both fallopian tube and OCs. Two proteins identified, ARP3 actin-related protein 3 homolog (ACTR3) and CD44, interact with ERBB2 protein that is able to enhance the MAPK signalization pathway and to increase the chances of the survival by inhibiting apoptosis. This complex is involved in many processes such as proliferation and invasion into OC cells [17].

One of proto-oncogene protein, myc, is linked with some proteins (MAPK1, PML, and HUWE1) identified to modulate its transcriptional activity and the beginning of tumorigenesis [18, 19].

Using the software IPA from Ingenuity Systems, all proteins specific to cancer regions have been analyzed (Fig. 2). Fifty-four genes are involved in cancer processes among which 43 are involved in neoplasia processes and 31 in carcinoma. If the hypothesis of origin of OC in the fallopian tube is considered, it is possible to demonstrated six proteins that have functions in OC namely cytochrome C oxidase subunit 5A (COX5A), chaperonin-containing TCP1 subunit 5 (CCT5), versican (VCAN), tumor-associated calcium signal transducer 2 (TACSTD2), stress-induced phosphoprotein 1 (STIP1), and CD44 molecules. CD44 and MAPK1 have been demonstrated as proteins involved in OC signaling pathway and also in metastasis of tumor cells function. Concerning STIP1, a recent study demonstrated a possible use as a secreted biomarker for the diagnosis of OC [20]. STIP1 is known as HSP-organizing protein that coordinates the functions of HSP70 and HSP90 in protein folding and also regulate TP53 expression. It is a marker of proliferation in OC cells line. CD44 is a receptor that interacts with hyaluronic acid as principal ligand. It is an indicator of good prognosis in epithelial OC patients and related to early-stage tumor [21]. VCAN is a large chondroitin sulfate proteoglycan of the extracellular matrix that interacts with hyaluronanic acid. An elevated level of VCAN has been reported in OC and is also associated with tumor progression and poor patient outcome [22]. As demonstrated, all these proteins may interact together (Fig. 3) in some pathways already known to be implicated in cancer process and cells migration.

Other interesting information could also be obtained by proteins only present in the benign region. The emerin protein (EMD) plays some role in gene regulation and signalization. In OC, this protein is lost and this change induces the nucleolar morphologic deformation [23]. The selenium-binding protein 1 (SELENBP1) has been identified and demonstrated like the most significant downregulated protein in OC cells. In addition, it may be relevant prognostic information of OC [24]. The synaptopodin-2 isoform a (SYNPO2, also named myopodin) is an actin-binding protein downregulated in OC [25] and the expression of which can induce suppression of tumor growth and metastasis in prostate cancer [26]. Another protein, the decorin (DCN), is a cellular proteoglycan of the small leucine-rich proteoglycan family and takes part in matrix assembly. The expression of this protein is not detected in OC cells and is proposed as a potential tumor suppressor gene [27].

An interesting thing to note is that some proteins could also reflect a start of change in the phenotype of cells in the neighborhood of the tumor for example in some samples the detection of the vitronectin. This protein present in the mesothelium could stimulate the migration of OC cells [28].

However, a study has shown that vitronectin seems to permit the transition from benign to malignant tissue [29].

All these result clearly show the possibility to use our new method to perform comparatives studies and clinical proteomics. The use of a strategy combining localized microdigestion and liquid microextraction on FFPE tissue coming from cancer tissue bank allow the detection of around 1000 proteins permitting the differentiation of benign and cancer region. A lot of proteins already shown to be involved in cancer processes and important pathways have been found by this way. By applying this method with a higher number of samples, it could allow a better understanding of mechanisms driving to the development of this particular type of tumor. The ultimate goal is to find correlation between the development of the STICs and high-grade OC. By this way, it will be possible to search biomarkers that can be used for early detection of these cancers before reaching the ovaries and thus improve the diagnosis and prognosis.

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