Proteomic Analysis of Differently Archived Breast Cancer Tissues

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Abstract. *Currently, the most common practice of human breast tissue preservation is formalin fixation which ensures good quality for histopathological analyses but damages DNA, RNA, and proteins, impairing their usefulness for molecular analysis and biomarker investigations. We investigated the potential value of a non-toxic fixative for sparing proteins preserved in paraffin-embedded breast biopsies. Specimens were fixed in formalin-free fixative prior to paraffin embedding, and then processed for quality and quantity of protein conservation. Similar protein patterns were observed in formalin-free fixative and frozen tissues using mono- and bi-dimensional electrophoresis, as well as western blotting. Protein patterns assessed by mass spectrometric analysis were found to be identical for frozen and formalin-free-fixed tissues. Immunohistochemistry using various antibodies showed comparable results for both tissue storage methods. In conclusion, we believe that formalin-free fixative represents an easy-to-use alternative to formalin for archived tissue and for biomarker investigations, since it simultaneously protects both the histomorphology and the integrity of macromolecules.*

Human tissue is an important biological material for the discovery of novel disease biomarkers and the identification of novel therapeutic targets. Tissue banks represent a major biological resource for such studies. These banks are generally formed of formalin-fixed and paraffin-embedded (FFPE) tissue. Although formalin preserves the cellular and architectural morphological details suitable for anatomopathology, it facilitates the formation of protein-protein cross-links, rendering conventional proteomic studies difficult, although optimized extraction protocols for proteins from FFPE tissue are available (1-6). The use of frozen tissues could be an interesting alternative to their preservation in formaldehyde since the proteins are preserved in conditions close to those of fresh tissue. Unfortunately, the morphological details of cryopreserved tissues are usually not appropriately conserved, impairing histological diagnostic. In addition, sample acquisition and long-term storage is complicated and costly (7, 8).

With the recent interest in personalized medicine, including the kirsten retrovirus-associated DNA sequence (KRAS) and the epidermal growth factor receptor (EGFR) genotyping in adenocarcinoma lung cancer, there is a strong need for a new fixative that would allow molecular analyses together with a reliable classical histological diagnosis from the same sample. Recently, studies have proposed new fixative methods for preservation of tissue architecture, nucleic acids and proteins. Methacarn, a solution of methanol, chloroform and acid acetic, is a non cross-linking organic solvent used to maintain tissue morphology and to preserve nucleic acid and protein integrity (9-11). Morales *et al*. and Vincek *et al*. evaluated UMFIX, a mixture of methanol and polyethylene glycol, with properties similar to methacarn, as a relevant fixative (12-14). The PAXgene tissue system, based on a mixture of different alcohols, including methanol, acid acetic and a soluble organic compound has also been proposed (15, 16). However, although the use of these fixatives seems promising, the presence of methanol, a toxic compound, seriously affects their use in laboratories. FineFIX, a compound made with ethanol, has also been proposed for standard molecular analysis (9, 17). Finally, the HEPES-glutamic acid buffer-mediated organic solvent protection effect (HOPE) technique for tissue fixation has been shown to allow a wide variety of biochemical investigations, all enabling good preservation of the morphological structures of DNA, RNA and proteins (18-20). However, although potentially interesting for the field of proteomics, the ability of methacarn, UMFIX and FineFIX to maintain proteins close to their original state has been poorly investigated.

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In this study we evaluated the feasibility of proteomic investigations on formalin-free-fixed paraffin-embedded breast tissue using a comprehensive panel of proteomic methods. We demonstrate that this free-formalin fixative might be widely used as a tissue fixation system for the extensive proteomic studies of clinical breast tissue biopsies and biomarker identification.

Materials and Methods

Tissue samples. Four primary breast carcinoma biopsies were obtained by the Department of Pathology (Montpellier, France). One part of the tissue was fixed in 4% neutral buffered formaldehyde (NBF) for 24 hours at room temperature (RT), dehydrated, and paraffin embedded using a TissueTek VIP automated processor (Sakura Finetek, Torrance, CA, USA) according to the standard protocol used for diagnosis. The remaining tissue was divided into two samples. One sample was immediately snap-frozen in liquid nitrogen and stored at –80˚C. The other sample was fixed overnight at 4˚C in formalin-free fixative (CS100) (Alphelys, Plaisir, France) before paraffin embedding. FFPE tissue was conserved at RT, and formalin-free-fixed paraffin-embedded blocks were maintained at –20˚C.

Protein extraction. Three 5 μm-thick sections from FFPE and formalin-free tissues were deparaffinized with xylene. Tissue sectioning was performed in the same way for frozen samples. Proteins were extracted using 150 μl of lysis buffer [50 mM Tris-HCl buffer (pH 7.5) containing 7 M urea, 2 M thiourea, 2% 3-[(2-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate hydrate (CHAPS), 1% Mega, 1% n-octyl b-D-glucopyranoside (OGP) and 50 mM dithiothreitol (DTT)] added with protease inhibitors. Pellets were centrifuged at 15,000 ×*g* for 15 min at 4˚C, and supernatants were recovered for subsequent proteomic analysis.

Protein assay. Protein concentration was measured using the Micro BCA Protein Assay reagent Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's protocol. Protein concentrations were measured using 1 or 2 μl from the protein extracts. Protein concentration was expressed as μg/cm² of tissue. Three replicates were conducted, and the performance of protein extraction protocols was evaluated.

One- and two-dimensional electrophoresis (1- and 2-DE) analysis. For 1-DE, 10 μg of protein were precipitated and resuspended in loading buffer. Electrophoresis was conducted on a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel and silver staining was performed according the procedure of Shevchenko *et al*. (21). All 2-DE reagents and materials were purchased from GE Healthcare (Uppsala, Sweden). Nucleic acids, lipids, and salts were removed with 2-DE Clean-Up Kit. Proteins (150 μg) (GE Healthcare, Uppsala, Sweden) were solubilized in 350 μl of isoelectrofocusing medium, as described elsewhere (22). For the second dimension, the strips were loaded onto vertical 10-17% SDS polyacrylamide gradient gels prior to silver staining. Spot detection and gel alignment were performed with the Image Master 2D Platinum software (GE Healthcare, Uppsala, Sweden). For protein identification, spots were excised from 2-DE gels and digested with trypsin Gold (Promega, Madison, WI, USA). Digest products were completely dehydrated in a vacuum centrifuge and re-suspended in 10 μl of 2% formic acid, desalted using Zip Tip C18 (Millipore, Billerica, MA, USA), eluted with 10 μl acetonitrile-trifluoroacetic acid (50-0.1% ACN-TFA) and concentrated to 2 μl. Aliquots (0.5 μl) were mixed with the same volume of 10 mg/ml alpha-cyano-4-hydroxycinnamic acid in 50-0.1% ACN-TFA (LaserBio Labs, Sophia-Antipolis, France) before applying the samples to target plates and analyzing them with the matrix-assisted laser desorption ionization-tandem time-of-flight (MALDI-TOF/TOF) method using a 4800 plus MALDI TOF/TOF™ Analyzer (ABsciex, Foster City, CA, USA). Identification of proteins was performed using ProteinPilot software (version 2.0.1; ABSciex, Foster City, CA, USA) against the UniProt database (uniProtKB release 2009_09).

Western Blot. Thirty micrograms of protein extracts were loaded onto a 12% polyacrylamide separating gel. After protein transfer, polyvinylidene difluoride (PVDF) membranes were blocked and incubated overnight at 4˚C with several antibodies: mouse monoclonal anti-E-Cadherin (BD Biosciences, Franklin Lakes, NJ, USA), anti-estrogen receptor (ER) (Dako, Glostrup, Denmark) and rabbit anti-human epidermal growth factor receptor 2 (HER2) (antibodies-online, Aachen, Germany) at 1:2500, 1:1600 and 1:1000 dilution, respectively. The Peroxidase-conjugated secondary anti-mouse IgG (Jackson ImmunoResearch Laboratories, Suffolk, UK) or anti-rabbit IgG (Santa Cruz, Heidelberg, Germany) antibodies were diluted at 1:5000. The blots were developed using the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce Biotechnology, Rockford, IL, USA) and Hyperfilm ECL (GE Healthcare, Uppsala, Sweden).

Histology and immunohistochemistry. Four micrometer-thick sections of FFPE and free-formalin-fixed tissues were deparaffinized with xylene and rehydrated with several graded ethanols before staining with hematoxylin-eosin-saffron or immunohistostaining. Immunohistochemical analyses were performed by using a Dako autostainer (Dako, Glostrup, Denmark). According to the tested antibody [1:500 anti-E-Cadherin (BD Biosciences, Franklin Lakes, NJ, USA); 1:250 anti-HER2 (Novocastra reagent, A. Menarini Diagnostic, rungis, France); 1:250 anti-ER (Novocastra reagent, A. Menarini Diagnostic, rungis, France); 1:250 anti-progesterone receptor (PR) (Dako, Glostrup, Denmark)] and whenever needed, tissue sections were treated for 45 min at 95˚C with citrate or ethylenediaminetetraacetic acid for antigen retrieval. Slides were treated with a peroxidase inhibitor (Dako, Glostrup, Denmark) for 10 min to quench the endogenous peroxidase activity. The detection of the antibody binding was visualized with the streptavidin-biotin peroxidase complex (LSAB™ 2; Dako, Glostrup, Denmark) using diaminobenzidine as a chromogen. The sections were then counterstained with hematoxylin and assessed by a pathologist.

Results

To reliably extract high amounts of non-degraded, full-length, and immunoreactive proteins, including membrane proteins and low abundance proteins, a specific buffer extraction method was used. Quantitative comparisons of extracted proteins from formalin-free-fixed paraffin embedded, FFPE, and frozen tissues from four separate breast biopsies were performed (Figure 1A). Overall, the protein yield obtained from frozen tissue was found to be lower than the one of formalin-free-fixed tissue, whereas protein yields were highly less for the FFPE sample.

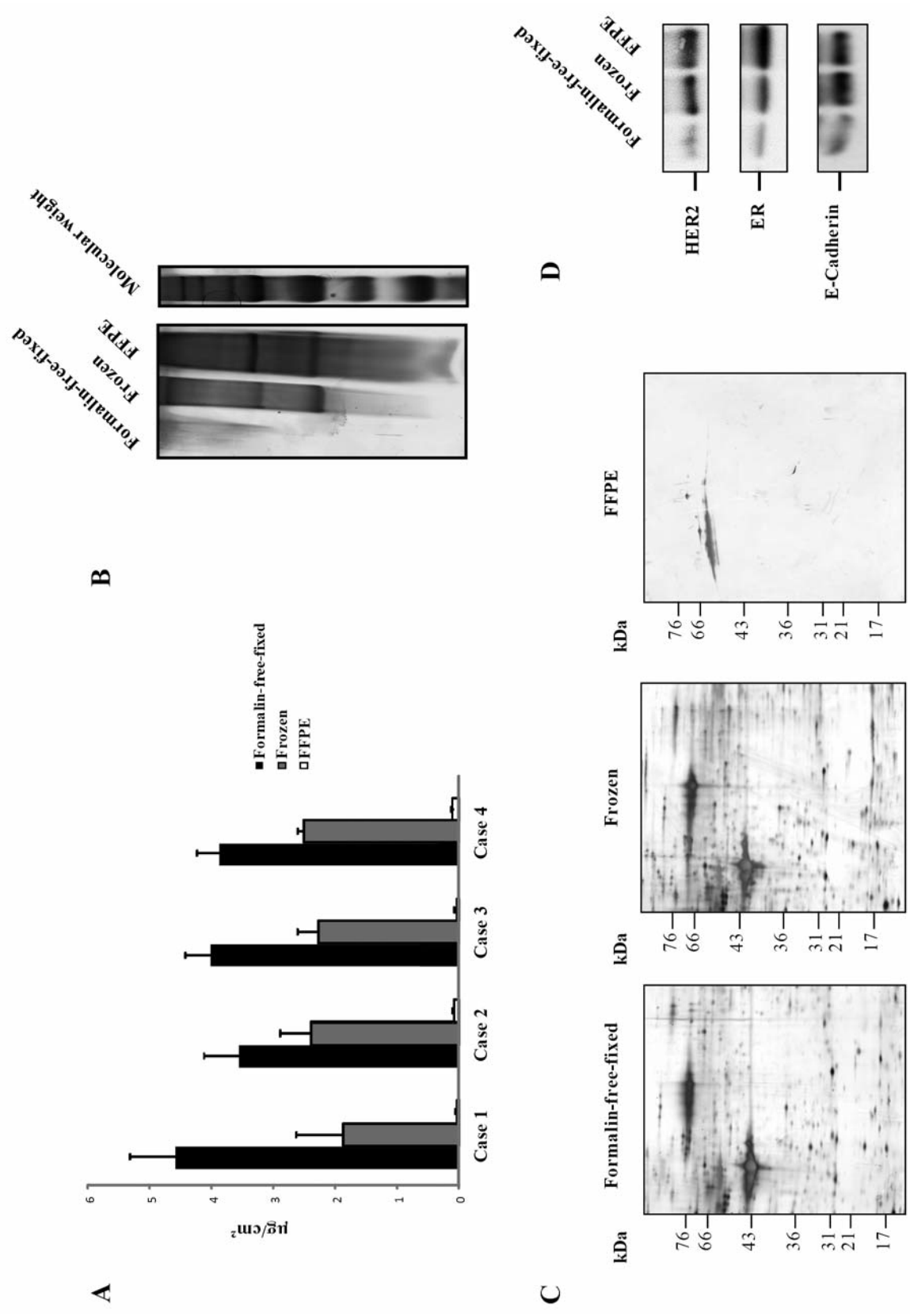

Figure 1. *A: Quantitative comparison of extracted protein yield from four different cases of formalin-free-fixed paraffin-embedded, frozen, and formalin-fixed paraffin-embedded (FFPE) breast tissue biopsies using the protein extraction protocol as described in the Materials and Methods. B: Sodium dodecyl sulfate polyacrylamide gel electrophoresis gels of extracted proteins from formalin-free-fixed, paraffin-embedded, FFPE, and frozen breast biopsies. C: Silver-stained two-dimensional electrophoresis maps obtained from formalin-free-fixed paraffin-embedded, frozen, and FFPE tissues. D: Western blot, performed using antibodies against human epidermal growth factor receptor (HER2), estrogen receptor (ER), and E-Cadherin for breast tissue biopsies.*

Table I. *List of five selected protein spots from two-dimensional electrophoresis analysis of frozen and formalin–free-fixed paraffin-embedded tissues and subjected to mass spectrometric analysis.*

		Frozen breast tissue			Formalin-free breast tissue		
	Accession no. name	Score	% Cov.	Nb pept >95% conf	Score	% Cov.	Nb pept >95% Cov
P06733	Alpha enolase	14.35	30.9	7	8.55	20.7	4
P04083	Annexin A1	8.59	20.5	4	9.3	23.7	5
Q9NZT1	Calmodulin-like protein 5	6.32	37.0	3	8.05	37.7	4
P21333	Filamin A	50.0	19.7	25	22.54	12.3	11
P09429	High-mobility group protein B1	8.0	30.2	4	8.0	29.3	4

% Cov., percentage of coverage; Nb pept.>95% conf, number of peptides identified at 95% confidence.

The overall protein extraction profile was then evaluated for tissue storage using 1-DE analysis and sensitive silver staining. Distinct bands were observed with both frozen and formalin-free-fixed samples, whereas no protein pattern was detected using formalin-fixed materials (Figure 1B). Protein profiles were then extensively analyzed using 2-DE gels and silver staining (Figure 1C). The FFPE protein extraction showed a degraded pattern, without any distinct spots, confirming the unsuitability of formalin-fixed tissue for 2-DE analysis. However, frozen tissue exhibited excellent protein quality and quantity, and formalin-free-fixed tissue revealed a very similar pattern. In addition, when compared to proteins extracted from frozen tissues, protein mass and pI from formalin-free-fixed tissues were not affected by the tissue-processing method, although some spots did appear to be slightly fuzzy. To evaluate formalin-free-fixed tissue for protein expression, proteins with different cellular localization were analyzed by western blot. Membranous E-Cadherin and HER2, and cytoplasmic/nuclear ER protein expression patterns and resolution were very similar in formalin-free-fixed and frozen tissues (Figure 1D). In agreement with our previous experiments, no signal was detected in formalin-fixed tissues. Finally, we were able to identify several protein spots extracted from formalin-free-fixed tissues separated onto 2-DE gel by mass spectrometry. Table I shows the proteomic parameters obtained from five of these spots using a MALDI-TOF/TOF method. Alpha enolase, annexin A1, calmodulin-like protein 5, filamin A and high-mobility group protein B1 were identified by mass spectrometric analysis and direct sequencing of tryptic peptides in both formalin-free-fixed and frozen samples.

Finally, we analyzed tissue morphology and immunohistochemical reactivity of breast tissue after fixation and paraffin-embedding using formalin and formalin-free fixatives. Formalin-free fixative clearly preserved tissue integrity compared to the reference fixative of formalin. We then performed immunohistochemistry to compare antigen integrity and accessibility in both formalin- and formalin-free fixed tissues. Formalin-free fixative required optimization of the immunostaining procedures (*i.e.* antibody concentration dilution), likely due to better antigen preservation and accessibility. Interestingly, immunoreactivities for various antibodies, including HER2, ER, and E-Cadherin were similar in formalin-free-fixed samples as compared to formalin-fixed tissues (Figure 2).

Discussion

Due to the widespread use of mammography as a breast cancer-screening tool, the percentage of infracentimetric lesions detected as early-stage breast cancer has increased tenfold in the past two decades, especially among women older than fifty years (23). Approximately, these lesions today account for 20-25% of all newly diagnosed cases of breast cancer (23). In order to diagnose early-stage breast cancer, core biopsies are performed and entirely fixed in formalin before paraffin-embedding for accurate diagnoses and staging of the lesion. In addition, in some cases of pre-malignant lesions, the result of a core biopsy prevents the need for surgery to take place. Unfortunately, such archiving procedures preclude molecular analysis on small breast lesions and hamper new biomarker identification. Indeed, formalin does not allow reproducible nucleic acid analysis, since it alters and fragments nucleic acids, and impairs efficient extraction and quality of both DNA and RNA. In addition, formalin is a toxic compound which is an irritant to the skin, eyes, nose and throat and can also cause severe complications, including squamous carcinoma of the nose and pharynx (24, 25). Furthermore, formalin leads to chemical reactions such as the formation of methylenic bridges between protein side chains (26). These changes account for the insolubility of many normally soluble proteins and for the lowering of the isoelectric point (26). Formalin also causes an impairment or blockage of reactive sites, causing changes in biological properties of proteins (loss or gain of antigenicity, loss of toxicity, or enzymatic activity) (27). Altogether, these characteristics render formalin fixation processes inappropriate for genomic and proteomic investigations. Frozen tissue,

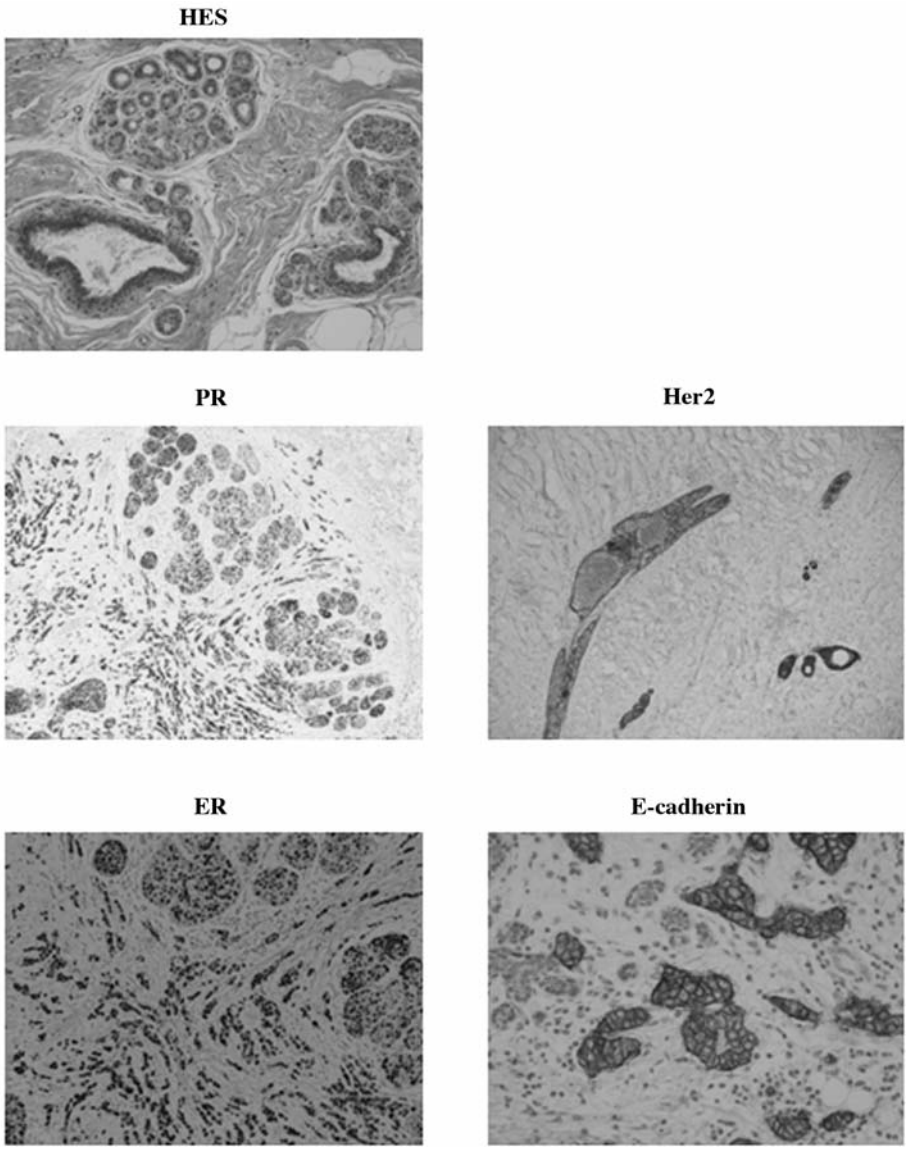

Figure 2. *Hematoxylin-eosin-saffron staining (HES) section of breast cancer biopsy fixed with formalin-free fixative and paraffin-embedded and representative images of immunohistochemical staining for human epidermal growth factor receptor (HER2), estrogen receptor (ER), progesterone receptor (PR) and E-Cadherin proteins in breast cancer biopsies are shown. Original magnification ×10 (ER, PR, HER2) and ×40 (E-Cadherin).*

another method used to conserve tissue, is generally reserved for molecular biology studies and is not compatible with clinical laboratory analysis. Several alternatives to formaldehyde or freezing are currently available (*e.g.* Excell+, Finefix, Glyo-Fixx, UMFix, Methacarn, PAXgene). They generally use flammable alcoholic fixatives which require the use of appropriate equipment. New fixative techniques which preserve both proteins and nucleic acid from embedded tissue and which are compatible with daily practice in anatomopathology laboratories are crucial. CS100, a promising new fixative, has great potential for concomitantly allowing morphological and molecular analyses to be performed on the same tissue sample. It allows the extraction of good quality DNA and RNA (28, 29). Several groups, including ours, have evaluated a very similar formalin-free fixative called RCL2 in colorectal and brain cancer tissues (22, 30, 31). Here, we showed that CS100 can also be used for biopsy for early-stage breast cancer investigations. Our 1- and 2-DE experiments, as well as the protein detection performed by western blot, demonstrated relevant conservation of proteins. Furthermore, immunohistochemical analyses showed that this formalin-free fixative allowed great accessibility of

antigenic sites. Mass spectrometric analysis confirmed that the proteins do not undergo changes which could affect their identification. All of these results allow us to propose the establishment of formalin-free-fixed tissue for the formation of tumor banks adapted to the study of proteomics. Components of formalin-free fixative have been demonstrated as being safe for human health and the environment in relation to European and American regulations. Moreover, the use of a formalin-free fixative makes it possible to work with the same tissue for molecular biological and histopathological diagnoses. Thus, such a fixative is compatible with molecular biology tools after classical cold fixation without the need to change current procedures significantly. In addition, formalin-free-fixed tissues can be kept at room temperature or –20˚C when a high quality is required. Consequently the budget required for storage is lower.

The ideal fixative should possess the combined advantages associated with formalin and freezing without their drawbacks. It should enable correct conservation of tissue morphology, allow reproducible results and ensure the stability of tissue characteristics for at least 10 years. Moreover, it should be compatible with studies of the genome, transcriptome and proteome and should present a minimized risk for users. Formalin-fixed tissue currently remains the most common method for tissue conservation, even if it does not fulfill all these criteria. Our study demonstrates that formalin-free fixative could be proposed as an outstanding solution to the establishment of new conservation methods of tissue samples in clinical settings. Importantly, this fixative is particularly suitable for precious or small tissues, such as biopsies, which are usually difficult to obtain for protein biomarker discovery.

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