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Utility of formalin-fixed, paraffin-embedded liver biopsy specimens for global proteomic analysis in nonalcoholic steatohepatitis

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Abstract

Purpose—To compare the proteomic profiles of formalin-fixed, paraffin-embedded (FFPE) liver biopsy material and matched frozen liver tissue from patients with nonalcoholic steatohepatitis (NASH).

Experimental design—A label-free mass spectrometry-based approach was used to profile global protein expression in FFPE and frozen liver biopsy specimens from five patients with NASH.

Results—Eight hundred and sixty proteins were identified with >75% confidence: 225 common proteins were identified in both the FFPE and frozen tissues, and an additional 142 and 493 proteins were identified in the FFPE and frozen tissues, respectively. Functional analyses revealed a general, nonspecific reduction in the number of proteins identified in FFPE tissue compared with frozen tissue. No bias toward proteins located in any specific subcellular compartments or implicated in any particular biological functions was observed. The relative abundance of several proteins with functions relating to the pathogenesis of NASH (peroxiredoxin-1, fatty acid binding protein 1, fatty acid synthase, vimentin, catalase, and glutathione *S*-transferase A1) was similar in FFPE and frozen liver tissues.

Conclusions and clinical relevance—FFPE liver biopsy material from NASH patients can be used for global proteomic analysis and biomarker identification studies, although a universal reduction in the number of identified proteins compared with frozen tissue is likely.

Keywords

Biomarkers; Formalin-fixed; Mass spectrometry; Nonalcoholic steatohepatitis; Paraffin-embedded

1 Introduction

Despite the increasing prevalence of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH), many questions remain regarding the pathogenesis and progression of these diseases. While noninvasive biomarkers are being aggressively investigated, liver biopsy remains the gold standard for definitive diagnosis and clinical management of NAFLD and NASH. Formalin-fixation and paraffin-embedding (FFPE) are common techniques used for preparing liver biopsy specimens for histopathological characterization. FFPE samples can be stored at ambient temperatures for many years, resulting in the formation of sizable tissue banks by many pathology laboratories. Although formalin-fixation of tissue induces cross-linking of proteins, many studies have now demonstrated that mass spectrometry (MS)-based proteomic techniques can be used for the identification of proteins in FFPE tissue [1-11]. In four of these studies, direct comparisons of protein expression in matched fresh and FFPE specimens were performed using mouse liver [2, 11], human glioblastoma tissue [4], or human colon adenoma biopsies [7]. Results from these studies have demonstrated impressive overlap of proteomics data obtained from FFPE and frozen tissues.

Banked FFPE liver specimens are an important resource for proteomics-based disease pathogenesis and biomarker discovery studies for NAFLD and NASH. Only one previous proteomics study has utilized frozen liver tissue from patients with NAFLD/NASH, and from that study two novel disease staging biomarkers were identified [12]. Importantly, there have been no studies directly comparing the proteomic signatures of matched FFPE and frozen human liver tissue from patients with fatty liver disease. Here, we utilized a label-free quantitative proteomics (LFQP) approach with the ability to resolve and identify hundreds of proteins in complex biological samples [13]. We previously used LFQP to explore changes in global serum protein expression in patients with simple steatosis, NASH, and NASH with advanced fibrosis compared with control subjects, and to identify potential protein biomarker panels capable of differentiating patient groups [14]. The purpose of this proof-of-concept study was to identify and compare the global proteome of FFPE and matched frozen human liver tissues from patients with NASH.

2 Materials and methods

2.1 Human subjects

Liver biopsies for proteomic studies were collected from NASH patients during bariatric surgery, after an overnight fast. Biopsies were collected as part of a prospective clinical research study that was reviewed and approved by the Institutional Review Board at Indiana University School of Medicine in accordance with the Helsinki Declaration of 1975. All volunteers provided written informed consent prior to participating in the study. At the time of surgery, liver biopsies were obtained using a Tru-Cut 14 gauge \times 15 cm needle, resulting in core biopsy specimens of 2–3 cm in length. One core biopsy specimen was immediately snap frozen in liquid nitrogen and stored at -80°C until analysis, and one core specimen was immediately placed in 10% buffered formalin and sent to the local Clarian Pathology clinical laboratory (Indianapolis, IN, USA) for routine processing, including paraffin-embedding, for subsequent clinical diagnosis. Histological analysis was carried out by an experienced hepatopathologist.

2.2 Sample preparation

Liver tissue blocks ranging in age from 23 to 25 months were used for the FFPE analyses. Four 10- μm tissue sections were cut from blocks, de-paraffinized with xylene, and rehydrated via a series of distilled water and graded ethanol solutions. The resulting liver biopsy material (\sim 30 000 cells) was collected by laser microdissection and processed

without any separation (centrifugation) steps with the Liquid Tissue[®] MS Protein Prep Kit (Expression Pathology, Rockville, MD, USA). Briefly, collected cells were suspended in liquid tissue buffer, heated to 95°C for 90 min, and cooled for 2 min on ice. Protein concentrations were determined by micro-bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Rockford, IL, USA), protein mixtures were subsequently digested with trypsin at 37°C overnight (16–18 h), and DTT was added to a concentration of 10 mM.

Frozen liver tissue samples were homogenized in lysis buffer (20 mM NH₄HCO₃ containing 1 × protease inhibitors, 8 M urea, and 10 mM DTT) using a Dounce tissue homogenizer and three freeze–thaw cycles. Protein concentrations were measured by Bradford assay [15]. Protein extracts were reduced and alkylated with DTT, iodoacetamide, triethylphosphine, and iodoethanol [16]. Protein mixtures were digested with trypsin at 37°C overnight (16–18 h) and filtered through 0.45-μm spin filters prior to injection onto the HPLC system.

2.3 LC/MS-MS

The stability of the HPLC and MS instruments was monitored by adding a constant amount of internal standard, chicken lysozyme, to each sample for the assessment of technical variations. All samples were randomized for injection to reduce systematic bias. Tryptic peptides (20 μg) were injected onto an Agilent 1100 nano-HPLC system (Agilent Technologies, Santa Clara, CA, USA) with a C18 capillary column. Peptides were eluted with a linear gradient from 5 to 45% ACN over 120 min at a flow rate of 500 nL/min. Effluent was electrosprayed into a LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Data were collected in the ‘Triple Play’ mode (MS scan, Zoom scan, and MS/MS scan) and subsequently filtered/analyzed by a proprietary algorithm [17].

2.4 Protein identification (ID)

The X!Tandem [18] and SEQUEST [19] algorithms were used for database searches against the International Protein Index (IPI) human database (European Bioinformatics Institute, 2005) and the nonredundant *Homo sapiens* database (National Center for Biotechnology Information, 2005). Briefly, each algorithm compared the observed peptide MS/MS spectrum and theoretically derived spectrums from the database to assign quality scores that were combined with other predictors in a proprietary algorithm to assign an overall score (ID confidence) to each peptide. Proteins were classified from priority 1 (highest ID confidence) to priority 4 (lowest ID confidence), which is dependent on the number of unique amino acid sequences identified and the ID confidence of those peptides. The ‘peptide ID confidence’ [the ID quality of the amino acid sequence(s)] of the ‘best peptide’ (the peptide with the highest ID confidence) was used to assign the proteins to a ‘high’ (90–100% confidence), ‘moderate’ (75–89% confidence), or ‘low’ (<75% confidence) category, and all ‘low’ ID confidence proteins were discarded. ‘High’ category proteins were considered priority 1 if multiple (≥ 2) unique sequences with 90–100% ID confidence were identified. Otherwise, they were ranked as priority 2. ‘Moderate’ category proteins were considered priority 3 if multiple (≥ 2) unique sequences with 75–89% ID confidence were identified. Otherwise, they were ranked as priority 4. Only proteins detected across all five FFPE and all five frozen liver tissue samples were reported (i.e. no proteins were detected in <5 of the FFPE or frozen liver tissue samples). In addition, all MS data were closely scrutinized and any proteins identified more than once (i.e. redundant proteins) were manually eliminated from the data set.

2.5 Protein quantification

Six proteins identified as priority 1 in both FFPE and frozen liver tissues, and with biological functions relevant to the pathogenesis of NASH, were selected for comparison of relative abundance. Quantification of selected proteins was carried out as previously

described [17]. Briefly, files were acquired from the MS instrument and extracted ion chromatograms were manually aligned for selected peptides. After alignment, the area-under-the-curve (AUC) for each individually aligned peaked was measured, normalized, and compared for relative abundance. Quantile normalization was used to normalize the data [20], and normalization was done on a \log_2 scale (one unit difference = twofold change). Relative abundance of each protein was expressed as the mean protein intensity \pm standard error (\log_2) calculated from all five samples in each group (FFPE or frozen liver tissue).

2.6 Pathway analysis and protein classification

Identified proteins were further compared using Ingenuity Pathway Analysis software (<https://analysis.ingenuity.com>). Comparison of proteins identified in FFPE or frozen tissue was carried out based on subcellular localization and biological function (molecular and physiological/disease-based).

3 Results

3.1 Patient characteristics

Demographics and clinical characteristics of the study patients are shown in Supporting Information Table 1. Histopathological analysis and scoring of liver biopsies resulted in a diagnosis of NASH for all five subjects included in the study. Elevated body mass index (BMI) and alterations in the fasting lipid profile were largely consistent with what would be expected in a bariatric cohort, although increases in liver biochemistry parameters were not observed.

3.2 Proteomic profiling

Representative chromatograms produced from MS analysis of FFPE and frozen liver tissues from Subject 1 are shown in Fig. 1, and the results from the comparative global proteomic analyses are summarized in Table 1. In frozen liver tissue, 718 total proteins were identified: 290 priority 1, 213 priority 2, 7 priority 3, and 208 priority 4. The number of proteins identified in matched FFPE tissue was reduced by 51% overall, with approximately equal reductions across all priority categories (367 total proteins were identified: 148 as priority 1, 120 as priority 2, 3 as priority 3, and 96 as priority 4). In addition, the number of peptides (5+, 4, 3, 2, or 1) used for protein identification of the different protein priority groups is included in Table 1.

Overall, a total of 860 proteins were identified: 225 in both frozen and FFPE tissues, 142 in FFPE tissue only, and 493 in frozen tissue only. Therefore, overlap among proteins identified in both tissue types was 26% when protein identification quality (protein priority) was not taken into account. As shown in Table 2, when the 225 common proteins identified in both the FFPE and frozen tissues were further analyzed according to protein priority, overlap among proteins was increased in the priority 1 group (43%), but overlap in the lower priority groups was not improved. This was attributable to many common proteins ($n = 66$) that were identified in both tissue types, but that fell into different protein priority categories.

3.3 Functional analyses

Proteins identified in FFPE and frozen liver tissues were compared on the basis of subcellular localization (Fig. 2A). The number of proteins identified in the FFPE tissue was reduced in all cellular compartments, and no bias toward a specific location was observed. In addition, proteins were characterized based on biological function, including physiological/disease-based (Fig. 2B) and molecular-based functions (Fig. 2C). Again, the number of proteins identified in FFPE tissue was decreased in most categories with no

obvious tendencies toward specific functions. It is important to note that identification of proteins with molecular and physiological functions implicated in cellular structure and integrity (including cellular development/assembly/organization/morphology, tissue morphology, and connective tissue development/function) was not disproportionately reduced in the FFPE tissue despite ~2 years of storage at ambient temperatures.

3.4 Comparison of protein expression

As shown in Table 3, six proteins identified as priority 1 in both FFPE and frozen liver tissues, and with biological functions related to the pathogenesis of NASH (including antioxidant, fatty acid synthesis/metabolism, and structural proteins), were selected for comparison of relative abundance [mean protein intensity \pm standard error (\log_2)]. Average protein expression in the two tissue types was very similar and did not differ by more than 3% for any individual protein.

4 Discussion

In this study, we demonstrate for the first time that archived liver biopsy material from patients with NASH can be used for global proteomic analysis. We identified hundreds of proteins in FFPE liver tissue, and many of these proteins were also identified in matched frozen liver biopsies from the same individual. Several priority 1 proteins with functions involved in the pathogenesis of NASH were expressed at similar levels in FFPE and frozen liver tissues. Our study highlights the potential for future retrospective biomarker identification and NASH pathogenesis-based studies using banked FFPE liver tissue specimens and powerful methods for protein extraction and detection.

Overall, fewer proteins were identified in the FFPE compared with the frozen tissue. However, comparison of proteins based on cellular locations or biological functions revealed no specific reductions in any protein groups. Importantly, the number of proteins involved in cellular structure and integrity, which could be affected by long-term storage at ambient temperatures, was not disproportionately reduced in FFPE liver tissue. Due to the large differences in retention times for unique peptides (as depicted in the chromatograms shown in Fig. 1), direct comparisons of expression of individual proteins between matched FFPE and frozen tissues could not be carried out with high confidence. However, the average relative abundance of a subset of several priority 1 proteins with biological functions previously implicated in NASH was nearly identical in FFPE and frozen tissue samples (Table 3).

The overlap of priority 1 proteins identified in both FFPE and frozen liver tissues was 43%, which is slightly lower than that reported in previous studies. For example, in matched FFPE and frozen human brain tissues and colon adenoma biopsies, an overlap of 83 and 67% was shown by Guo et al. and Sprung et al., respectively [4, 7]. Using FFPE and frozen mouse liver tissues, Hood et al. reported that 684 and 776 unique proteins were identified, respectively, in each tissue type, although the degree of overlap of common proteins was not described [2]. Finally, Ostasiewicz et al. also compared FFPE and frozen mouse liver tissues and found that 91% of proteins were identified in both tissues [11]. A potential explanation for this difference may lie in the sample preparation techniques, as the FFPE tissue blocks and frozen liver tissue were processed using slightly different protocols prior to HPLC and MS analysis. However, this is unlikely and a more plausible explanation is the highly complex nature and large number of proteins expressed in human liver tissue, which limits the utility of high-resolution separation techniques prior to peptide identification with MS. In future studies, subcellular and/or multidimensional fractionation of frozen liver tissue prior to peptide separation can be used to overcome these analytical challenges.

Several fundamental questions regarding suitability of FFPE tissue for proteomic analysis due to modification of proteins by formalin and paraffin and long-term storage have been addressed. For example, cross-linking of lysine residues by formalin was reported to modestly reduce detection of C-terminal lysine peptides in two studies [2, 7], but this effect was not observed in another study using a different FFPE sample preparation technique [11]. Furthermore, it has been demonstrated that fixation periods of up to 5 days (in 10% buffered formalin) and storage of tissue blocks at ambient temperatures for up to 10 years have no significant impact on proteomic analysis of FFPE tissue [7, 11]. Despite these findings, it should be noted that the FFPE liver tissue blocks used in the present study were relatively fresh (23–25 months of age), and it remains to be determined if longer storage periods would result in liver tissue degradation and decreased data integrity. In addition to the global proteome, Ostasiewicz et al. recently demonstrated that post-translational modifications, including phosphorylation and *N*-glycosylation sites, are also preserved and detectable in FFPE material using MS-based proteomic methods [11]. These novel findings highlight an entirely new level of future proteomic investigation using FFPE biosamples.

In conclusion, this proof-of-concept study demonstrates the feasibility of using FFPE liver biopsies from patients with NASH for global proteomic analysis. Future use of advanced sample preparation and proteomic techniques for the study of FFPE liver biopsies from patients across the spectrum of NAFLD will provide important information for biomarker discovery and exploration of disease pathogenesis.

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Abbreviations

FFPE	formalin-fixed, paraffin-embedded
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis

Clinical Relevance

Despite the increasing prevalence of NAFLD and NASH, the pathogenesis of these common liver conditions is not well understood. Although noninvasive serum biomarkers are being aggressively investigated, liver biopsy remains the gold standard for definitive diagnosis and clinical management of NAFLD and NASH. FF and PE are common techniques used for preparing liver biopsy specimens for histopathological characterization. FFPE samples can be stored at ambient temperatures for many years, resulting in the formation of sizable tissue banks by many pathology laboratories. Although formalin-fixation of tissue induces cross-linking of proteins, several studies have now demonstrated that MS-based proteomic techniques can be used for the identification of proteins in FFPE tissue. This study demonstrates for the first time that FFPE liver biopsies from patients with NASH can be used for global proteomic analysis, despite an overall reduction in the number of proteins identified compared with matched frozen liver tissue. Results from this proof-of-concept study using FFPE liver biopsy material may have implications for future biomarker discovery studies using liver tissue from not only patients with NASH but also patients with other common liver diseases such as hepatitis B, C, and alcoholic liver disease.

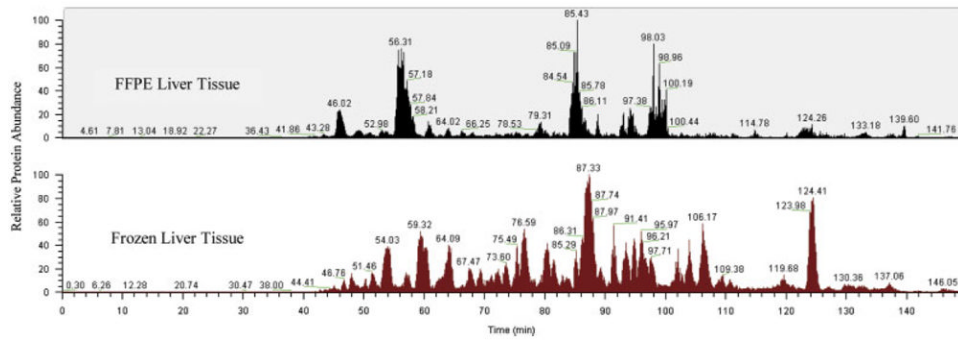


Figure 1. Representative chromatograms from formalin-fixed, paraffin-embedded (FFPE) and frozen liver tissues. Mass spectrometry data obtained from FFPE liver tissue (top panel) and matching frozen liver tissue (bottom panel) from Subject 1 are shown. Note the overall increase in the number of peptides identified in the chromatogram derived from frozen liver tissue.

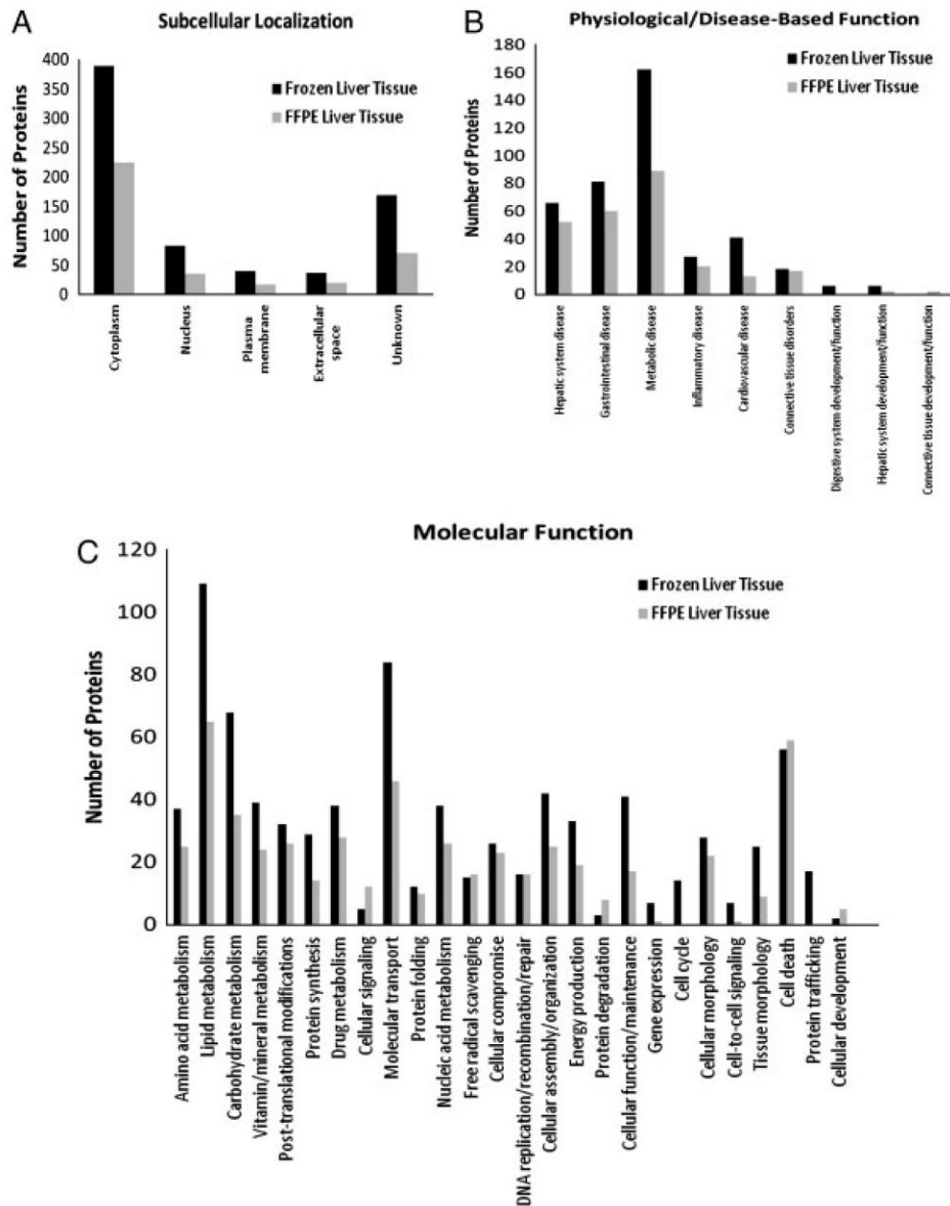


Figure 2. Comparison of the subcellular localizations and biological functions of proteins identified in frozen and formalin-fixed, paraffin-embedded (FFPE) liver tissues. Ingenuity Pathway Analysis software was used to classify cellular compartments (A), physiological/disease-based functions (B), and molecular functions (C) of proteins identified in the frozen and FFPE liver tissue global proteomic analyses.

Table 1

Summary of identified proteins

	Frozen liver tissue		FFPE liver tissue	
	<i>n</i> = 718 proteins identified	Number of peptides (number of proteins identified)	<i>n</i> = 367 proteins identified	Number of peptides (number of proteins identified)
Priority 1	290	5+ (140) 4 (32) 3 (43) 2 (75)	148	5+ (54) 4 (20) 3 (24) 2 (50)
Priority 2	213	1 (213)	120	1 (120)
Priority 3	7	5+ (0) 4 (1) 3 (0) 2 (6)	3	5+ (0) 4 (0) 3 (0) 2 (3)
Priority 4	208	1 (208)	96	1 (96)

FFPE, formalin-fixed, paraffin-embedded. Two hundred and twenty-five common proteins were identified in both the frozen and FFPE liver tissue analyses.

Table 2

Overlap of identified proteins

		FFPE liver tissue					
		Priority 1	Priority 2	Priority 3	Priority 4	Total	
Frozen liver tissue	Priority 1	131	42	0	5	178	
	Priority 2	8	22	0	5	35	
	Priority 3	0	0	0	0	0	
	Priority 4	2	4	0	6	12	
	Total	141	68	0	16	225	

FFPE, formalin-fixed, paraffin-embedded. Data are presented as the number of proteins identified in both types of liver tissue (overlapping proteins) according to priority categories (for example, 131 proteins were identified as priority 1 proteins in both the frozen and FFPE liver tissues, 42 proteins were identified as priority 1 proteins in the frozen liver tissue but as priority 2 proteins in the FFPE tissue, 5 proteins were identified as priority 1 proteins in the frozen liver tissue but as priority 4 proteins in the FFPE tissue, etc.).

Table 3

Relative abundance of selected priority 1 proteins

	Frozen liver tissue	FFPE liver tissue	Protein function
Peroxiredoxin-1	17.2±0.03	17.2±0.06	Antioxidant enzyme that reduces hydrogen peroxide and other reactive oxygen species
Fatty acid binding protein 1	16.6±0.07	16.1±0.01	Carrier/membrane transporter for fatty acids and other lipophilic molecules
Fatty acid synthase	16.2±0.02	16.3±0.10	Multimeric enzyme that plays a key role in synthesis of fatty acids
Vimentin	16.9±0.07	16.4±0.08	Component of intermediate filaments that comprise the cellular cytoskeleton
Catalase	16.7±0.04	16.3±0.04	Antioxidant enzyme that catalyzes the decomposition of hydrogen peroxide
Glutathione <i>S</i> -transferase A1	16.5±0.04	16.6±0.09	Antioxidant enzyme that catalyzes the detoxification of electrophilic compounds by conjugation with glutathione

FFPE, formalin-fixed, paraffin-embedded. Data are presented as relative protein expression [mean protein intensity ± standard error (log₂)] for all samples in each group (*n* = 5).