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ARTICLE

Effect of decalcification protocols on immunohistochemistry and molecular analyses of bone samples

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Abstract

Diagnosis of osteocartilaginous pathologies depends on morphological examination and immunohistochemical and molecular biology analyses. Decalcification is required before tissue processing, but available protocols often lead to altered proteins and nucleic acids, and thus compromise the diagnosis. The objective of this study was to compare the effect of different methods of decalcification on histomolecular analyses required for diagnosis and to recommend an optimal protocol for processing these samples in routine practice. We prospectively submitted 35 tissue samples to different decalcification procedures with hydrochloric acid, formic acid, and EDTA, in short, overnight and long cycles for 1 to >10 cycles. Preservation of protein integrity was examined by immunohistochemistry, and quality of nucleic acids was estimated after extraction (DNA and RNA concentrations, 260/280 ratios, PCR cycle thresholds), analysis of DNA mutations (high-resolution melting) or amplifications (PCR, in situ hybridization), and detection of fusion transcripts (RT-PCR, in situ hybridization). Hydrochloric acid- and long-term formic acid-based decalcification induced false-negative results on immunohistochemistry and molecular analysis. EDTA and short-term formic acid-based decalcification (<5 cycles of 6 h each) did not alter antigenicity and allowed for detection of gene mutations, amplifications or even fusion transcripts. EDTA showed superiority for in situ hybridization techniques. According to these results and our institutional experience, we propose recommendations for decalcification of bone samples, from biopsies to surgical specimens.

Introduction

Decalcification is required for processing bone tissue in routine diagnostic practice. Control of this step is crucial because it may have detrimental consequences for

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establishing the diagnosis. Decalcifying agents may alter proteins and nucleic acids and thus render morphology and immunohistochemistry or molecular biology results biased. These alterations represent a pitfall particularly in these samples increasingly requiring immunohistochemistry and molecular analyses [1].

Agents commonly used for decalcification are acids of varying ionic strength or chelating agents. Acids that ionize and solubilize calcium ions include strong inorganic acids, such as hydrochloric or nitric acid, and weaker organic acids such as formic or phosphoric acid. Chelating agents such as ethylenediaminetetraacetic acid (EDTA) link to calcium ions and form an insoluble complex. Deleterious effects are less serious with EDTA solutions because of their neutral pH, but their routine use is limited because of the need for several successive bath changes to achieve complete decalcification.

Some studies demonstrated significant effects of decalcification on immunohistochemistry and molecular biology results, particularly with breast carcinoma tissue and bone-marrow biopsies [2–5]. However, to our knowledge, no study has compared the impact of decalcifying agents on all techniques such as immunohistochemistry and molecular analyses used for establishing the diagnosis of human bone pathologies.

In a preliminary study of 8-gauge standardized-diameter biopsies of femoral head tissue decalcified with hydrochloric acid, formic acid or EDTA, we found that formalin-fixed paraffin-embedded (FFPE) sections contained less DNA when decalcified with hydrochloric acid than with formic acid or EDTA (unpublished data). This finding suggests a variable impact of decalcifying agents on human tissues and underlines the need to adapt the protocol to subsequent analyses. We also observed that optimal decalcification time for morphological analysis was 2–2.5 times higher for formic acid, and 8–16 times higher for EDTA compared with hydrochloric acid.

The objective of this study was to compare the effect of different decalcifying solutions (hydrochloric acid, formic acid, and EDTA) and decalcification protocols (various number and duration of cycles) on immunohistochemistry, molecular and in situ hybridization analyses of bone and soft tissue samples. We propose recommendations to optimize decalcification for routine processing of bone tissue samples.

Material and methods

Material acquisition and tissue preparation

The present study is a multicentric prospective study performed in five centers with an expertise in bone lesions including four French hospitals (Lille, Marseille, Tours, Paris-Cochin) and one Belgian hospital (Bruxelles) between November 2015 and February 2017. The study was performed in agreement with the requirements for the use of biological material proposed by our institutional ethics guidelines. We used a total of 35 specimens in two sets of samples (Fig. 1).

The first set included 25 specimens from French centers (21 surgically removed specimens and four curettages). Immunohistochemistry and molecular analysis were performed in this set. This sample consisted of ten surgical resection specimens of primitive bone tumors (four osteosarcomas including two conventional high-grade, one raised in fibrous dysplasia and one parosteal; four conventional chondrosarcomas, one Ewing sarcoma, one undifferentiated

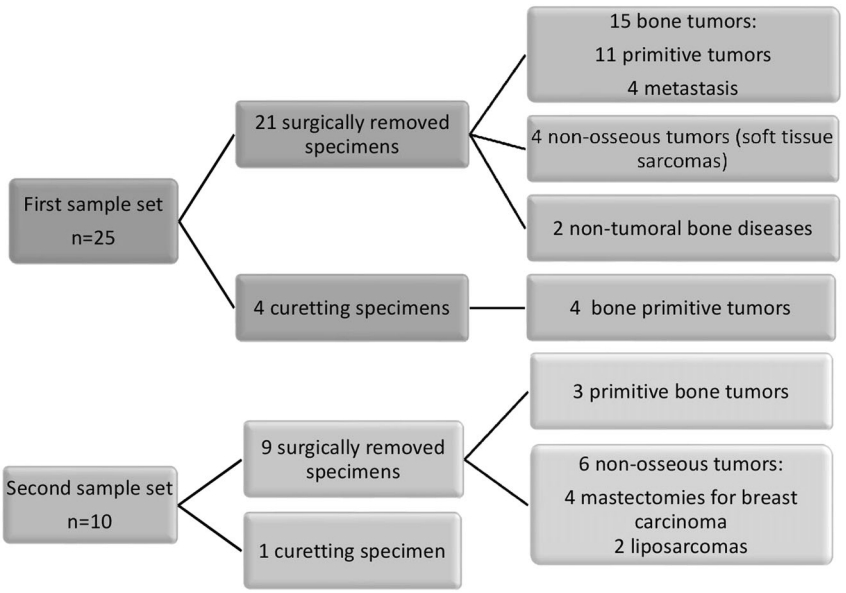

Fig. 1 Distribution of the 35 specimens in the two sample sets. Both sets underwent morphological analysis and immunohistochemistry; DNA and RNA analyses in the first set and in situ hybridization in the second.

Table 1 Content and pH provided by manufacturers of commercial decalcifying agents.

	Decalc	DC2	DC3	DC1	TBD2	EDTA
Manufacturer	Histolab, Gothenburg, Sweden	VWR, Radnor, PA, USA	VWR, Radnor, PA, USA	VWR,Radnor, PA, USA	Thermo Fisher Scientific, Waltham, MA, USA	Promega, Madison, WI, USA
Content	Hydrochloric acid 10–20%	Hydrochloric acid 10–25%	Hydrochloric acid 5–10% Alcohols, C12–14, ethoxylated, propoxylated <1% EDTA disodium salt <0.1%	Formic acid 5–15% Formaldehyde 5–10%	Water 77–80% Formic acid 21–23% Fluorad >1% Sodium citrate >1% Polyvinyl pyrrolidone >1%	EDTA 0.5 M
pH	<1	<1	<1	1.3–2.7	2.3–2.4	8

sarcoma raised in fibrous dysplasia), four resections for carcinoma metastasis, two resections for nontumoral pathology (osteonecrosis and osteoarthritis), four surgical resection specimens of soft tissue sarcomas (one malignant peripheral nerve sheath tumor, one synovial sarcoma, one epithelioid sarcoma, one low-grade fibromyxoid sarcoma), four curettages for primitive bone tumors (two giant cell tumors of the bone, one atypical cartilaginous tumor/grade one conventional chondrosarcoma, one fibrous dysplasia), and one surgical biopsy of primitive bone tumor (one Ewing sarcoma).

The second set of samples collected independently in the Belgian center included ten additional specimens: four bone tumors (three Ewing sarcomas surgically removed and one curettage for breast carcinoma metastasis), and six non-osseous surgically removed specimens (four mastectomies for breast carcinoma, two well-differentiated liposarcomas). In situ hybridization analyses were performed in this set.

For both sets, surgical specimens were adequately fixed in 4% buffered formaldehyde according to routine procedures. Each specimen was sampled in equal-sized fragments, and then decalcified.

As mentioned above, this decalcification procedure was also applied, although not necessary, to a subgroup of soft tissue lesions as part of this study. Decalcification agents used were hydrochloric acid (Decalc from Histolab, Gothenburg, Sweden; DC2 and DC3 from VWR, Radnor, PA, USA), formic acid (DC1 from VWR; TBD2 from Thermo Fisher Scientific, Waltham, MA, USA), and EDTA (Promega, Madison, WI, USA) (compositions provided by the manufacturers are summarized in Table 1).

For each sample, one to several cycles were used until complete decalcification. For hydrochloric acid, one cycle corresponded to 4 h of decalcification. For EDTA, one cycle corresponded to 8 h of decalcification. For formic acid, one short cycle corresponded to 6 h of decalcification; one overnight cycle corresponded to 12 h of decalcification; and one long cycle corresponded to continuous 24 h decalcification.

Table 2 Antibodies used for immunohistochemistry.

Antibody [clone]	Manufacturer
PAX8	Zytomed Systems, Berlin, Germany
P63 [4A4]	Biocare Medical, The Hague, The Netherlands
Ki-67 [SP6]	Diagomics, Berlin, Germany
Ki-67 [Mib-1]	Dako Ltd, Cambridge, UK
INI1 [BAF47]	BD Biosciences, San Jose, CA, USA
MDM2 [IF2]	Zymed Laboratories, San Francisco, CA, USA
S100	Dako Ltd, Cambridge, UK
EMA [E29]	Dako Ltd, Cambridge, UK
CK7 [OV.TL12]	Dako Ltd, Cambridge, UK
MUC4 [8G7]	Santa Cruz Biotechnology, Santa Cruz, CA, USA
HER2 [4B5]	Ventana Medical Systems, Illkirch Cedex, France
Estrogen receptors [SP1]	Ventana Medical Systems, Illkirch Cedex, France

In each case, decalcification was followed by washing in running tap water and a new fixation in buffered formaldehyde.

Immunohistochemistry method and interpretation

Immunohistochemistry antibodies are listed in Table 2. For both sample sets, immunohistochemistry was independently performed in each hospital according to the manufacturer's instructions. For each sample, the staining intensity was semiquantitatively scored as 0 (no signal), 1 (weak), 2 (moderate), and 3 (intense signal). Mean ± SD values were calculated for each decalcifying condition.

Molecular analysis (DNA, RNA)

All DNA and RNA analysis of the first sample set were centralized in the same laboratory (Platform of Somatic

Tumor Molecular Genetics, Tours) except for RNA sequencing which was performed by Institut Bergonié, Bordeaux.

DNA or RNA extraction

Genomic DNA and RNA were isolated from FFPE tissue samples by using a Maxwell 16 Instrument (Promega, Madison, WI, USA) with the Maxwell 16 FFPE Plus LEV DNA purification kit and the Maxwell 16 LEV RNA FFPE kit (AS 1135 and AS 1260, Promega) according to the manufacturer's instructions. Concentration and purity (260/280 nm ratio) of DNA or RNA were determined by using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Results were expressed as mean ± SD.

High-resolution melting (HRM)

HRM screening for *IDH1*, *IDH2*, and *GNAS* was performed on a LightCycler 480 II (Roche, Boulogne-Billancourt, France) using the LightCycler 480 High Resolution Melting Master Kit (Roche). Each reaction contained 10 µl of 2X Master Mix, 2.4 µl of 25 mM MgCl2, 3.6 µl of H2O, 1 µl of each 10 µM primer (Invitrogen, Waltham, MA, USA), and 2 µl of DNA (30 ng) in a total volume of 20 µl (see Table 3 for primers). The final product sizes were 56 bp for *IDH1*, 87 bp for *IDH2,* and 92 bp for *GNAS*. The cycling conditions were 95 °C for 5 min, followed by 50 cycles at 95 °C for 15 s, 55 °C (*IDH1/IDH2*) or 67 °C (*GNAS*) for 15 s, and 72 °C for 20 s. The melting conditions included a cycle at 95 °C for 1 min, 40 °C for 1 min, and 65 °C for 2 s, followed by an increase in temperature from 65 to 95 °C at 1 °C/s. All samples were tested in duplicate. Data were analyzed by using LightCycler 480 SW1.5 software. The normalized melting curves were established for each sample, and samples were compared with sample controls in a deduced difference plot. Significant deviations from the horizontal line relative to the wild-type control curve indicated sequence changes within the analyzed amplicon. The samples with distinct melting curves as compared with the wild-type allele were recorded as potentially positive for a mutation, and pyrosequencing was performed.

Pyrosequencing

PCR was performed using the PyroMark PCR Kit (Qiagen, Hilden, Germany): 30 ng of DNA was added to 20 µl of a reaction mix containing 2.5 µl of CoralLoad 10×, 12.5 µl of master mix, and 10 pmol of each primer (see Table 3 for primer and sequences). The PCR conditions were 15 min at

Table 3 Primers and sequences for high-resolution melting, pyrosequencing, PCR, and RT-PCR.

Gene	HRM	
IDH1	IDH1-HRM-F: 5′-TGG ATG GGT AAA ACC TAT CAT CA-3′ IDH1-HRM-R: 5′-GAC TTA CTT GAT CCC CAT AAG CA-3′	
IDH2	IDH2-HRM-F: 5′-AAC ATC CCA CGC CTA GTC CCT-3′ IDH2-HRM-R: 5′-CTC TCC ACC CTG GCC TAC CT-3′	
GNAS	GNAS-HRM-F: 5′-TCC ATT GAC CTC AAT TTT GTT TCA G-3′ GNAS-HRM-R: 5′-AAG TTG ACT TTG TCC ACC TGG AAC T-3′	
Gene	Pyrosequencing	Sequence to analyze
IDH1	IDH1-PY-F-bio: 5′-TGG ATG GGT AAA ACC TAT CAT CA-3′ IDH1-PY-R: 5′-GAC TTA CTT GAT CCC CAT AAG CA-3′ IDH1-PY-S: 5′-TGA TCC CCA TAA GCA T-3′	GACNACCTAT GAHGACCTAT
IDH2	IDH2-PY-F: 5′-AAC ATC CCA GCG CTA GTC CCT-3′ IDH2-PY-R-bio: 5′-CTC TCC ACC CTG GCC TAC CT-3′ IDH2-PY-S: 5′-AGC CCA TCA CCA TTG-3′	GCAGBCACG GCANGCACG
GNAS	GNAS-PY-F: 5′-TGT TTC AGG ACC TGC TTC G-3′ GNAS-PY-R-bio: 5′-ACC TGG AAC TTG GTC TCA AAG AT-3′ GNAS-PY-S: 5′-AGG ACC TGC TTC GCT-3′	GCYRTGTCCT
Gene	PCR	
MDM2	MDM2-PCR-F: 5′-CCG GAT GAT CGC AGG TG-3′ MDM2-PCR-R: 5′-AAA AGC TGA GTG AAC CTG CCC-3′	
ALB	ALB-PCR-F: 5′-TGA AAC ATA CGT TCC CAA AGA GTT T-3′ ALB-PCR-R: 5′-CTC TCC TTC TCA GAA AGT GTG CAT AT-3′	
B2M	B2M-PCR-F: 5′-TGA CTT TGT CAC AGC CCA AGA TA-3′ B2M-PCR-R: 5′-AAT CCA AAT GCG GCA TCT TC-3′	

95 °C followed by 20 s at 94 °C, 30 s at 53 °C and 20 s at 72 °C for 42 cycles, and 5 min at 72 °C. Then 10 µl of PCR product were added to a DNA immobilization mix containing 1 µl of streptavidin beads (GE Healthcare, Chicago, IL, USA), 40 µl of PyroMark binding buffer (Qiagen) and 29 µl of H2O on a 24-well plate. After sealing, the plate was agitated for 10 min at 1400 rpm on a plate mixer. By using a PyroMark workstation, single-stranded DNA was added to a PyroMark Q24 plate in 25 µl of sequencing primer (8 pmol) in an annealing buffer. After 2 min at 80 °C, the plate was kept at room temperature for 20 min before processing the pyrosequencing reaction. Pyrosequencing involved use of PyroMark Q24 (Qiagen) and results were analyzed by using PyroMark sw 2.0.6 software (Qiagen).

Real-time quantitative PCR (DNA)

Real-time quantitative PCR was used for detecting *MDM2* gene amplification with LightCycler 480 II (Roche) and the Sybr Green Master Kit (Roche). *ALB* expression was used as a reference. Each reaction was performed with 50 ng of DNA. Primer sequences are in Table 3. The cycling conditions were 95 °C for 5 min, followed by 45 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. All samples were tested in duplicate. The relative *MDM2* level was determined as a ratio to *ALB* level by using LightCycler 480 SW1.5 software. We considered that all ratio >2.5 indicated *MDM2* amplification.

Reverse transcription and real-time quantitative PCR (RNA)

Reverse transcription followed by real-time quantitative qPCR analysis were performed using the GoTaq Probe 2-Step RT-qPCR System kit (Promega) according to the manufacturer's instructions. Quantitative PCR involved primers targeting *B2M* as a reference (see Table 3 for primers). Amplifications were performed in a final volume of 20 µl containing 2 µl of cDNA. The final product size was 87 bp. Results were expressed as cycle threshold (Ct) values.

DNA sequencing

DNAs obtained from FFPE samples were first amplified by qPCR and compared with a standard DNA to obtain a DCt. According to the manufacturer's recommendations (Truseq FFPE DNA Library Prep QC Kit, Illumina), only samples with a DCt ≤6 can be used for library preparation. Libraries were then generated by a consortium product from Illumina, INCa Solid Tumor Panel V1, and sequenced on a MiSeq instrument (Illumina). The bioinformatics analyses were processed with a homemade pipeline (SARDINe). For each sample, the analysis focused on the total number of reads, the percentage of reads with a Q30 quality score > 75%, and interpretable samples requiring region of interest (exon) covered at 100% with a 600× depth read.

RNA sequencing

Total RNA was extracted from FFPE tissues using TRIzol reagent (Invitrogen) following manufacturer recommendations. Quantity and quality of total RNA were evaluated using NanoDrop (Thermo Fisher Scientific) and Tape Station with Hs RNA Screen Tape (Agilent, Santa Clara, CA, USA). Libraries were prepared with an input of 100, 40, or 20 ng of total RNA depending on the quality of RNA as assessed by the fraction of RNA fragments above 200 nucleotides ("DV200") using TruSeq RNA Exome Library Prep Kit (Illumina, San Diego, CA, USA). Libraries were pooled by group of 12 samples. Paired-end sequencing was performed using the NextSeq 500/550 High Output V2 kit (150 cycles) on Illumina NextSeq 500 platform (Illumina). The read length was 75 bp. Transcript fusions were identified with the following algorithms: DeFuse, FusionMap, and StarFusion. For each sample, the analysis focused on the total number of reads, the number of reads covering the fusion, and the detection of a fusion transcript.

In situ hybridization

All in situ hybridization analyses were performed in the Cliniques Universitaires Saint-Luc of Brussels, Belgium.

Fluorescence in situ hybridization (FISH)

FISH on interphase nuclei from paraffin-embedded 4 µm sections was performed using the commercial probe for *EWING*, Vysis EWSR1 Break Apart FISH Probe Kit (1/30, Abbott, Chicago, IL, USA) and *MDM2*, Poseidon Repeat Free MDM2 (12q15) and SE12 (½, Leica, Heerbrugg, Switzerland). After dewaxing, the slides were immersed first in 0.2N HCl for 20 min, then in a target retrieval pretreatment solution (Dako Ltd, Cambridge, UK) for 20 min at 95 °C. Samples were digested with pepsin (Dako Ltd) for 3 min at 37 °C, washed with a saline-sodium citrate buffer (2 × SSC) for 2 × 5 min at room temperature, then dehydrated by immersing in progressive 70%, 85%, and 100% ethanol for 1 min each at room temperature. An amount of 10 µl probe mixture was added to specimens and heated at 90 °C for denaturation. Slides were then incubated at 37 °C overnight in a humidified chamber. After hybridization, they were washed in posthybridization wash buffer (50% formamide solution) (Acros Organics, Thermo Fisher Scientific), counterstained with DAPI (10 µl), and coverslipped. All slides were maintained in the dark until microscopic examination. Hybridization signals were visualized with a fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany), and images were

captured by using a CCD camera. Two different observers counted 50 nuclei that showed both green and orange signals. The percentages of green, orange, and fused yellow signals were calculated. Nuclei with an incomplete set of signals were omitted from the score.

A positive score was interpreted when at least 10% of the nuclei showed a break-apart signal.

Chromogenic in situ hybridization (CISH)

The HER2 Dual ISH DNA Probe Cocktail Assay (Ventana Medical Systems, Inc., Illkirch Cedex, France) was used to determine *HER2* gene status by enumeration of the ratio of the *HER2* gene to centromeric probe chromosome 17 (*HER2*/CEP17). *HER2* and chromosome 17 probes were detected by using two-color CISH in the same tissues after staining on a Ventana BenchMark XT automated slide stainer.

Statistical analysis

All statistical analyses involved use of GraphPad Prism v5.0 (GraphPad Software, La Jolla, CA, USA). Nonparametric Mann–Whitney test was used for comparison of nonpaired samples for two groups. Comparison of paired tissue samples involved nonparametric Wilcoxon test. P values < 0.05 were considered significant.

Results

Immunohistochemistry

Overall, 11 samples from the first set and two from the second set were analyzed by immunohistochemistry with nuclear antibodies (directed against PAX8, P63, Ki-67, INI1, MDM2, estrogen receptors) and membranous or cytoplasmic antibodies (directed against S100 protein, EMA, CK7, MUC4, HER2). Mean immunohistochemistry scores were similar with formic acid (2.2 ± 0.9), EDTA (2.8 ± 0.4), and nondecalcified samples (2.7 ± 0.6) (Fig. 2). By contrast, the score was lower, but not significantly, with hydrochloric acid than with controls (0.88 ± 1.2 vs. 2.7 ± 0.6, $P = 0.06$), and was significantly lower than with formic acid ($P = 0.04$) and EDTA ($P = 0.009$). Scores were similar for the two formic acid solutions (TBD2 vs. DC1) when different durations of cycles were applied (data not shown).

Staining intensities differed according to cellular localization of antigen targets. The nuclear expression of PAX8, P63, Ki-67 (Fig. 3), MDM2, and estrogen receptors was lost after decalcification with hydrochloric acid versus formic acid and EDTA. Among membranous and cytoplasmic markers, EMA expression was preserved (Fig. 3), as was

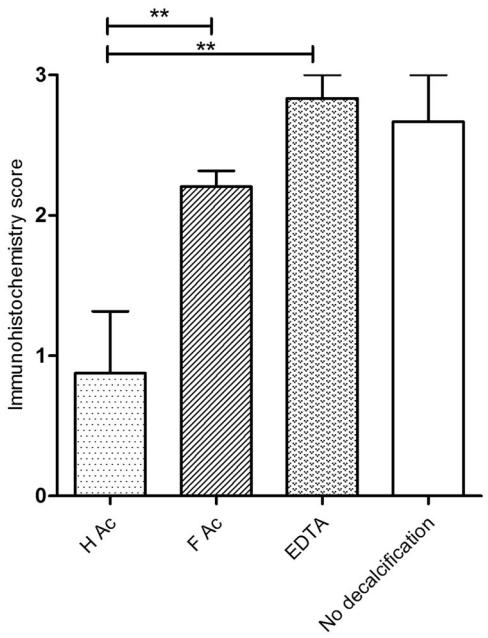

Fig. 2 Immunohistochemistry scores by decalcification protocols. Data are mean ± SD. **$P < 0.01$. H Ac hydrochloric acid, F Ac formic acid.

S100 protein, CK7, and MUC4 expression, whereas HER2 expression was lost with hydrochloric acid. For one sample, the expression of S100 protein was lost after decalcification with formic acid solutions (TBD2 and DC1) (long cycles, respectively 8 and 9 days) but was conserved with other protocols (hydrochloric acid included).

Molecular analysis

DNA and RNA concentration and purity

DNA concentration (in ng/µl) was higher in nondecalcified samples (237.4 ± 57.6) than in all decalcified tissues, reaching 20.4 ± 5.3 for hydrochloric acid ($P = 0.0003$), 71.6 ± 8.8 for formic acid (pooled results $P = 0.0009$), and 96.7 ± 35.6 for EDTA ($P = 0.009$) (Fig. 4a). Formic acid did not produce significant differences depending on cycle duration (short, overnight or long cycle). DNA concentrations were lower with hydrochloric acid than all pooled formic acid results ($P = 0.006$) or with short formic acid treatment alone ($P = 0.0095$). After pooling all results of cycle durations, DNA concentrations after decalcification with TBD2 and DC1 were comparable (77.2 ± 13.6 and 65.8 ± 11.2, $P = 0.85$) (data not shown).

The 260/280 ratio, evaluating DNA purity, was 1.7 ± 0.07 for nondecalcified samples and was lower with hydrochloric acid (1.3 ± 0.2, $P = 0.0009$), formic acid (pooled results, 1.6 ± 0.17, $P = 0.001$), and EDTA (1.6 ± 0.13, $P = 0.017$) (Fig. 4b). DNA purity was lower with hydrochloric acid than formic acid (pooled results, $P <$

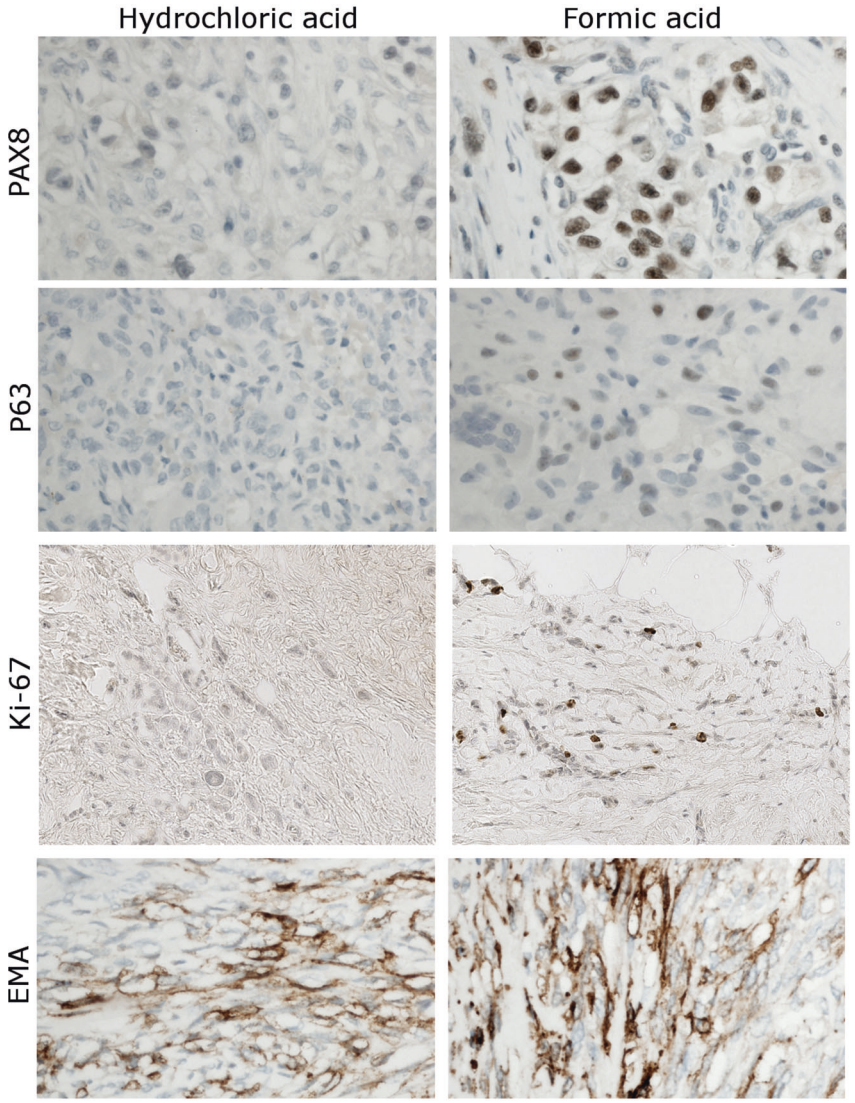

Fig. 3 Immunohistochemistry. Immunohistochemistry of PAX8, p63, Ki-67, and EMA in bone samples decalcified with hydrochloric acid (left column) or formic acid (right column).

0.0001). Ratios did not differ between TBD2 and DC1 ($P = 0.08$) (data not shown). DNA purity was lower with long cycles (1.48 ± 0.19) than short cycles (1.58 ± 0.14, $P = 0.012$) and overnight cycles (1.6 ± 0.17, $P = 0.028$). DNA purity decreased with increasing numbers of cycles, whatever the decalcifying solution used, but results were only significant for formic acid (Fig. 4c). The mean ratio with formic acid was 1.63 ± 0.1 with short decalcification (<5 cycles) and decreased to 1.5 ± 0.2 with 5–10 cycles ($P = 0.0007$) and 1.4 ± 0.18 with >10 cycles ($P < 0.0001$). With <5 cycles, the ratio was higher with formic acid than hydrochloric acid (1.63 ± 0.1 vs. 1.4 ± 0.2, $P = 0.0273$) but did not differ from EDTA (1.63 ± 0.1 vs. 1.63 ± 0.13, $P = 0.82$). For 5–10 cycles, no difference was seen between formic acid and EDTA (1.5 ± 0.2 vs. 1.54 ± 0.14, $P = 0.58$). More than ten cycles of formic acid was as deleterious as <5 cycles of hydrochloric acid (1.4 ± 0.18 vs. 1.39 ± 0.19, $P = 0.95$).

RNA concentration (in ng/µl) was higher in non-decalcified samples (27.3 ± 16.5) than with hydrochloric acid (1.2 ± 1.8, $P = 0.0155$) but did not significantly differ from pooled results for formic acid (21.2 ± 30.8, $P = 0.28$) and EDTA (10.7 ± 13.9, $P = 0.22$) (Fig. 4d). RNA concentration was also higher with EDTA than hydrochloric acid ($P = 0.01$). With formic acid, RNA concentration did not differ among short, ON or long cycles. In contrast, RNA concentration was significantly higher with both pooled results of formic acid and short-cycle formic acid than hydrochloric acid ($P < 0.0001$ and $P = 0.0028$). RNA concentrations were comparable in samples with TBD2 and DC1 (16.9 ± 20.9 and 24.8 ± 36.9, $P = 0.28$; data not shown).

For RNA purity, the 260/280 ratio was higher for non-decalcified samples (1.8 ± 0.2) than with hydrochloric acid (1 ± 0.2, $P = 0.012$), whereas no difference was observed with pooled results of formic acid (1.6 ± 0.22, $P = 0.05$) or

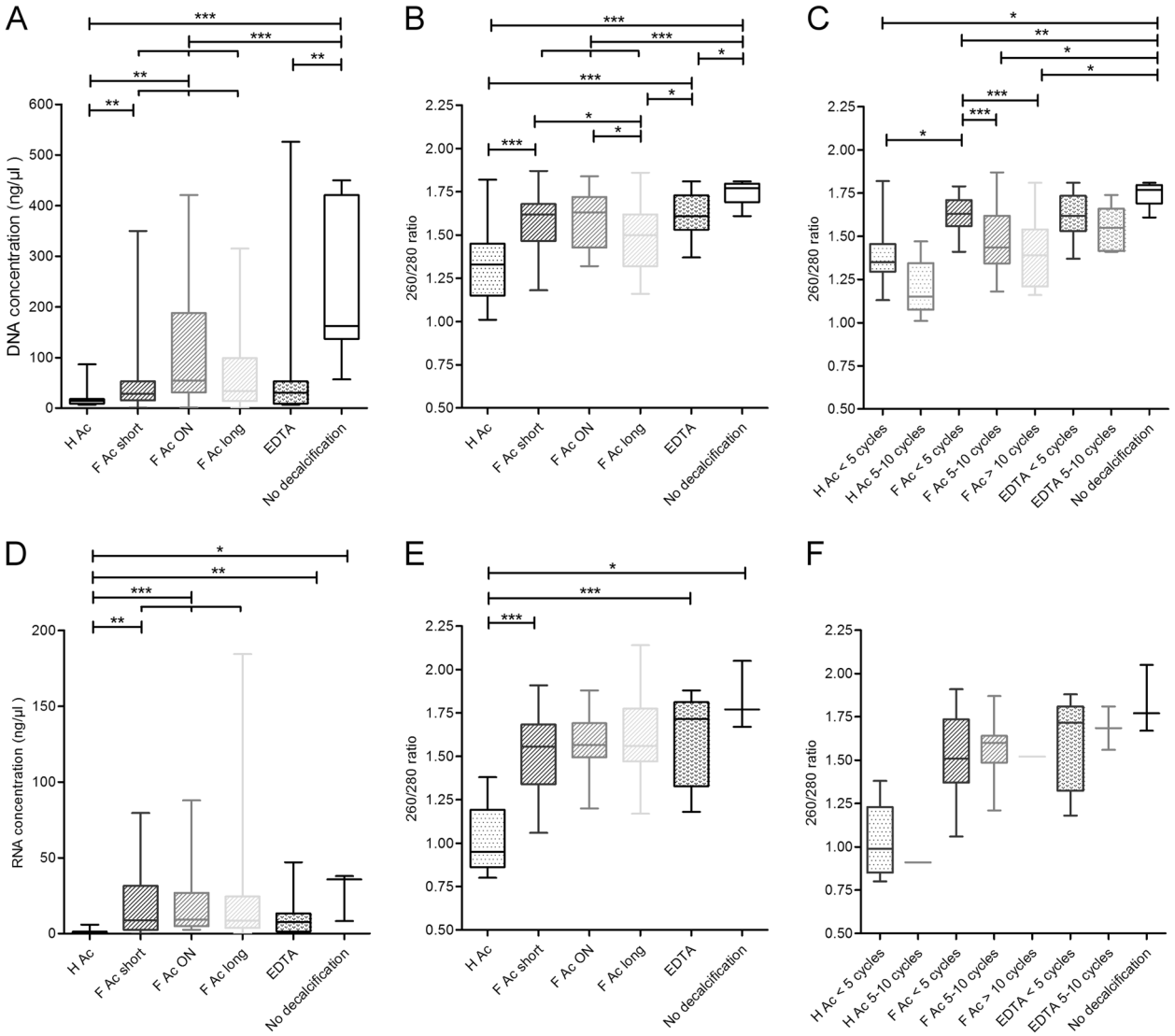

Fig. 4 DNA and RNA concentration and purity. DNA (**a**) and RNA (**d**) concentrations and DNA (**b**, **c**) and RNA (**e**, **f**) 260/280 ratios by decalcification duration (**b**, **e**) and number of cycles (**c**, **f**). Horizontal bars are mean, box edges are ranges, and whiskers are SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. H Ac hydrochloric acid, F Ac formic acid.

EDTA (1.6 ± 0.25, $P = 0.4$) (Fig. 4e). The 260/280 ratio was lower with hydrochloric acid than with EDTA ($P = 0.0005$) and short-cycle formic acid (1.53 ± 0.25 $P = 0.0003$). RNA purity with formic acid did not differ according to different cycle durations (short, ON or long) or number of cycles (Fig. 6e, f). We did not observe significant differences between formic acid <5 cycles and EDTA <5 cycles (1.54 ± 0.24 vs. 1.63 ± 0.27, $P = 0.52$).

DNA and RNA integrity

We used quantitative PCR analysis of *IDH1* after DNA extraction in 22 samples, and *B2M* after RNA extraction and reverse transcription in eight samples with the first sample set (Fig. 5). Cycle thresholds (Ct) for *IDH1* were lower in nondecalcified samples (27.9 ± 1.4) than with hydrochloric acid (40.3 ± 2.2, $P = 0.0003$) and pooled results of formic acid (34.1 ± 4.4, $P < 0.0001$) but no significant difference was observed with EDTA (28.8 ± 1.1, $P = 0.14$) (Fig. 5a). *IDH1* Ct were also lower with EDTA than hydrochloric acid ($P < 0.0001$) and pooled results of formic acid ($P < 0.0001$), and lower with formic acid than hydrochloric acid ($P < 0.0001$). *IDH1* Ct increased with cycle duration (short, ON or long), with a significant difference between short and long cycles (32.5 ± 3.1 vs. 36.5 ± 5.2, $p = 0.0002$). Decalcification with TBD2 induced lower Ct compared to DC1 (33.3 ± 4.2 vs. 34.9 ± 4.5, $p = 0.03$; data not shown). *IDH1* Ct increased with cycle number for formic acid: 31.4 ± 2 for <5 cycles, 36.5 ± 3.3 for 5–10 cycles ($P < 0.0001$) and 39.6 ± 4.8 for >10 cycles ($P <$

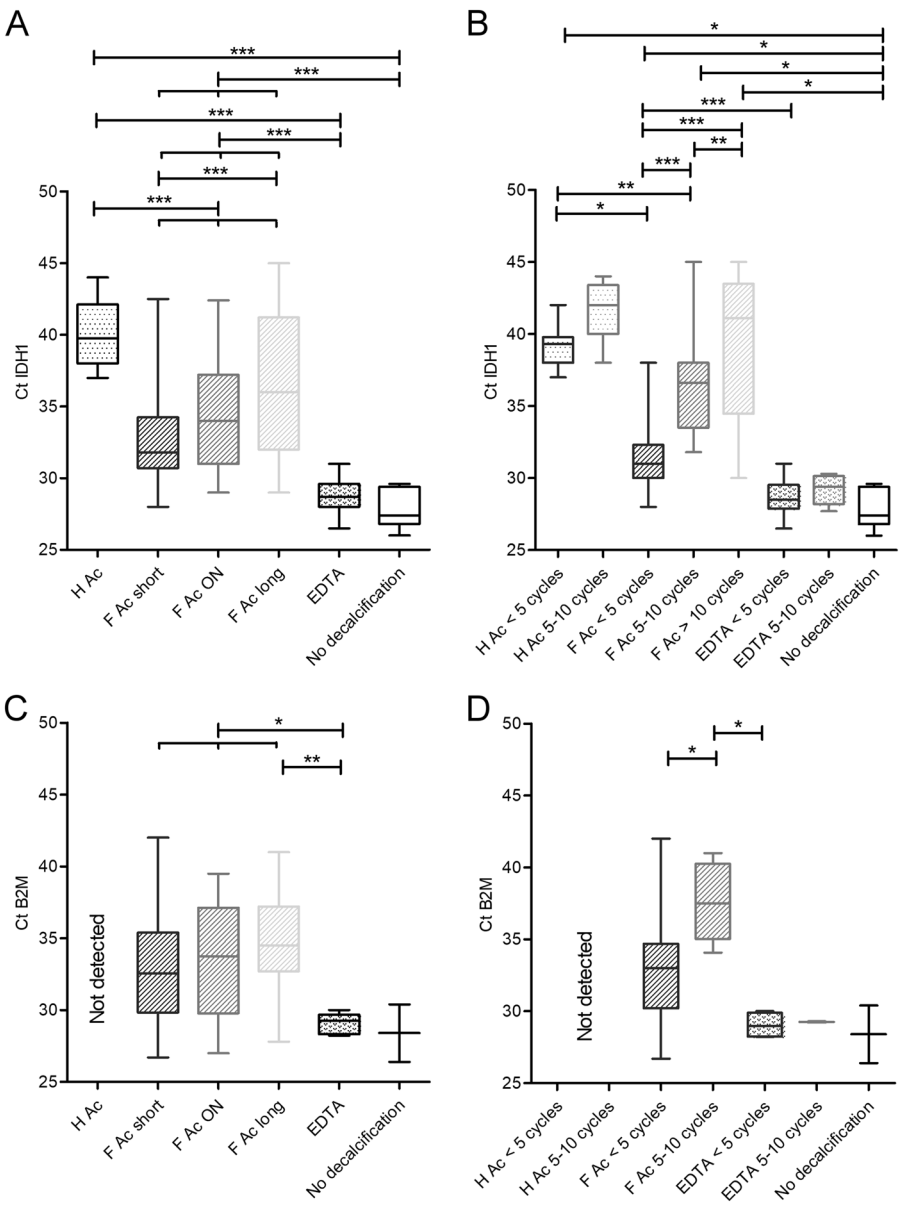

Fig. 5 DNA and RNA integrity. DNA IDH1 (**a**, **b**) and RNA B2M cycle thresholds (**c**, **d**) by decalcification duration (**a**, **c**) and number of cycles (**b**, **d**). Horizontal bars are mean, box edges are ranges, and whiskers are SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. H Ac hydrochloric acid, F Ac formic acid.

0.0001) (Fig. 5b). Samples with formic acid >10 cycles and hydrochloric acid <5 cycles did not differ (39.6 ± 4.8 vs. 39.1 ± 1.5, $P = 0.37$).

RT-PCR failed to detect *B2M* amplification in any sample decalcified with hydrochloric acid (Fig. 5c). Ct level was lower for nondecalcified samples (28.4 ± 2) than pooled results of formic acid (33.9 ± 0.8) and EDTA (29.1 ± 0.3), but the number of samples was too low for statistical analysis (Fig. 5c). Ct did not differ with TBD2 and DC1 (35.4 ± 4.9 vs. 32.8 ± 3.2, $P = 0.14$; data not shown), and was lower with formic acid <5 cycles than 5–10 cycles (32.9 ± 4 vs. 37.6 ± 2.7, $P = 0.03$), but results were similar between formic acid <5 cycles and EDTA <5 cycles (32.9 ± 4 and 29.04 ± 0.9, $P = 0.06$) (Fig. 5d).

Detection of mutations, gene amplification, and transcript fusion

Expected *IDH1* and *IDH2* mutations were searched in four chondrosarcoma samples and *GNAS* mutation in one sample of fibrous dysplasia, osteosarcoma, and undifferentiated sarcoma, both raised in fibrous dysplasia. One example of *IDH2* mutation is represented in Fig. 6a. In 3 out of 4 chondrosarcoma samples (75%), no *IDH* mutation was detected in samples decalcified with hydrochloric acid, but the mutation was found in samples decalcified with formic acid or EDTA during one cycle. Similar results were observed for *GNAS* mutation in samples of fibrous dysplasia, osteosarcoma, and sarcoma raised in fibrous dysplasia decalcified during six

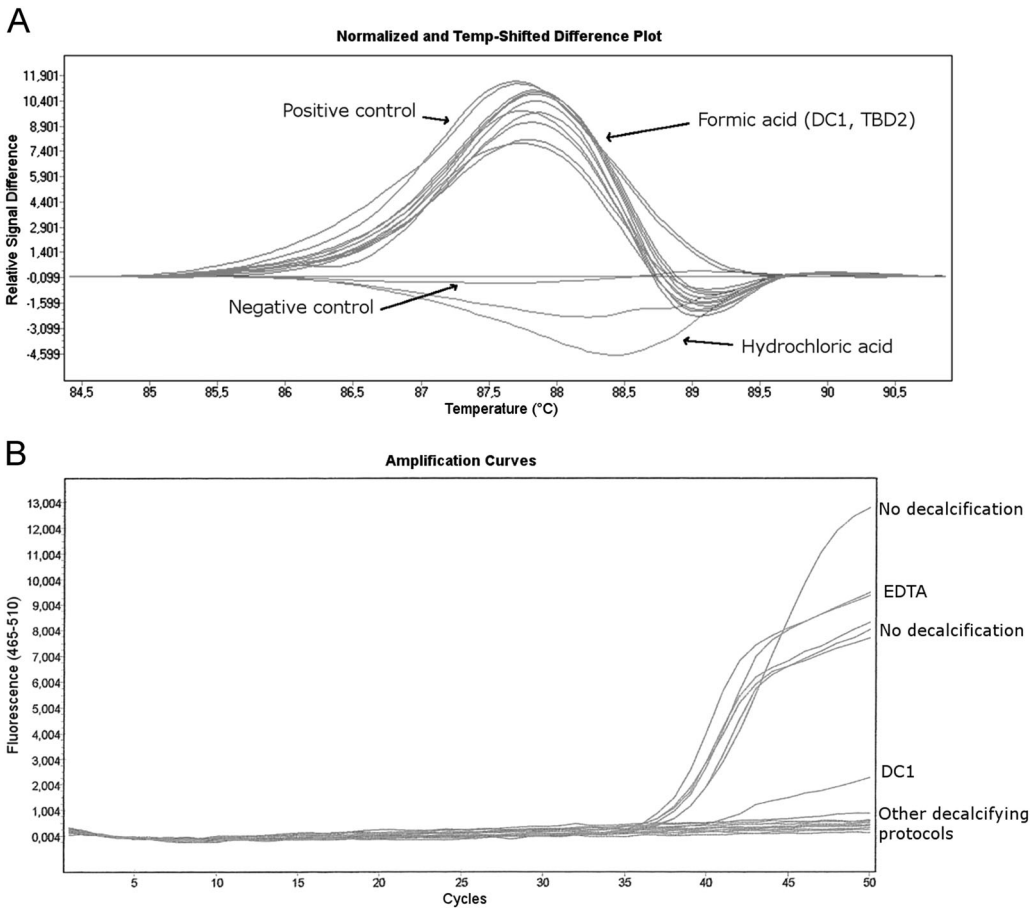

Fig. 6 Detection of mutations and transcript fusion. Detection of IDH2 mutation in one chondrosarcoma sample by high-resolution melting (**a**) and fusion transcript SS18-SSX1 in one synovial sarcoma sample by RT-qPCR (**b**).

cycles or less. In one parosteal osteosarcoma sample, *MDM2* amplification was detected in samples decalcified with TBD2 whatever the number of cycles (short, ON, long), but not with DC1 or hydrochloric acid (data not shown).

In one synovial sarcoma sample, the fusion transcript *SS18-SSX1* was detected in samples decalcified with EDTA or with DC1 used in one short cycle, but the signal was weak. With all other protocols tested (DC1 ON, long cycle; TBD2; hydrochloric acid—one cycle each) qPCR failed to detect this transcript (Fig. 6b).

DNA and RNA next-generation sequencing

We performed DNA next-generation sequencing in samples obtained from two cases of bone carcinoma metastasis decalcified with hydrochloric acid, formic acid (DC1 and TBD2; short, overnight and long cycle) used in short cycle and EDTA.

We first performed a qPCR for qualification of samples. Generation of libraries requiring DNA samples with a DCt ≤6, none of the samples analyzed could be qualified in theory. PCR failed to detect amplification in any sample decalcified with hydrochloric acid and formic acid >5 cycles. For seven samples, an amplification curve was obtained: three samples decalcified with formic acid <5 cycles (DCt comprised between 12.5 and 13.2), and four samples decalcified with EDTA 1–6 cycles (DCt comprised between 7.5 and 10.6). Despite recommendations issued by the manufacturer, libraries were generated and analyzed by NGS for these seven latter DNA samples. The total number of reads was between 24,518 (TBD2 1 cycle) and 564,749 (EDTA 3 cycles) and the three libraries with the highest number of total reads were obtained from two samples decalcified with EDTA <5 cycles and one sample decalcified with TBD2 3 cycles. The percentage of reads with Q30 quality score was between 67.5% (TBD2 1 cycle) and 95.4% (EDTA 3 cycles), and the libraries with the highest percentage of Q30 were obtained from three samples decalcified with EDTA <5 cycles. The percentage of region of interest considered as interpretable was comprised between 0% (TBD2 1 cycle) and 100% (EDTA 3 and 4 cycles) and the libraries with the highest percentage were also obtained from these three same samples decalcified with EDTA <5 cycles.

Whole RNA sequencing was performed in samples obtained from one case of synovial sarcoma: two samples were not decalcified; others were decalcified with 1 cycle of hydrochloric acid, formic acid (DC1 and TBD2; short, overnight and long cycle) and EDTA.

RNA extraction failed only for the sample decalcified with hydrochloric acid. Regarding the other conditions, the yields of extraction were good with concentrations of total RNA varying from 507 ng/µl (DC1 long cycle) to 1683 (no decalcification). After decalcification, higher yields of RNA extraction were obtained with the samples decalcified with EDTA (1471 ng/µl), and with DC1 used in short cycle or overnight (924 and 1130 ng/µl, respectively). Concomitantly, the DV200 values were beyond 30% with these three conditions of decalcification (Supplementary Table S1).

Regarding the library preparation, the targeted yield of 200 ng of RNA was obtained with the nondecalcified samples and the sample decalcified with DC1 used in short cycle. After sequencing, the decalcified samples which yielded a number of sequencing reads beyond 50 millions included those decalcified with EDTA, TBD2 overnight, and DC1 used in short cycle.

Overall, the number of sequencing reads varied from 14 millions (DC1 used in long cycle) to 191 millions (nondecalcified sample), and the sample decalcified with EDTA provided the highest number of sequencing reads (129 millions). The fusion algorithms detected the *SS18–SSX2* fusion in virtually all samples with the exception of the sample decalcified with DC1 overnight.

In situ hybridization

In situ hybridization was used in nondecalcified samples and in samples decalcified with hydrochloric or formic acid. *MDM2* FISH analysis was performed in three samples of dedifferentiated liposarcoma. The signal completely disappeared with hydrochloric acid and formic acid, whatever the protocol, but was preserved in nondecalcified samples. *EWSR1* FISH analysis was performed in four samples of Ewing sarcoma. The signal was preserved in nondecalcified samples and in samples decalcified with EDTA, whereas it was altered or even undetected after decalcification with 1 and 5 cycles of formic acid (data not shown).

For CISH analysis of five primary breast carcinoma samples, a focal signal was observed in three cases with formic acid, but *HER2* signals and CEP17 were undetected with hydrochloric acid (data not shown).

Discussion

Decalcification of bone samples remains necessary for routine diagnosis activity. To our knowledge, our study is the first to evaluate the effects of different decalcifying agents and decalcification protocols on protein and nucleic acid integrity in a large cohort of tissue samples in order to optimize decalcification technique procedures. Hydrochloric acid has the advantage of limiting the duration of the decalcification process, but is known to affect morphology and damage both proteins and nucleic acids. Indeed, staining modifications were observed in samples treated with hydrochloric acid in our study, but they were too subtle to compromise the diagnosis (data not shown). By contrast, we obtained false-negative results on immunohistochemistry and in situ hybridization. DNA and RNA purity were decreased, which therefore affects the detection of expected molecular modifications (mutations and translocations).

As reported in previous studies, we observed altered antigenicity after decalcification with hydrochloric acid, but it was preserved with EDTA or formic acid [2, 5, 6]. Moreover, most false-negative results occurred for nuclear antigens (PAX8, P63, MDM2, Ki-67), which suggests that membranous and cytoplasmic antigens were more resistant to strong acid-based decalcification. These observations align with previous results. In breast cancer, for which decalcification is necessary in case of bone metastases, samples decalcified with strong acid showed greater decline in nuclear antigens (estrogen receptor, progesterone receptor, P53 and Ki-67) than membranous antigens [3, 4]. In another study of rat salivary glands, endothelial growth factor (EGF) and EGF receptor cytoplasmic staining was not affected by strong acid-based decalcification as compared with EDTA and nondecalcified samples [7].

Our findings of altered DNA and RNA integrity with hydrochloric acid agreed with published data. Singh et al. observed a decrease in DNA and RNA yield as well as increased cycle thresholds in various tissue samples decalcified with a strong acid [8]. Similar results were observed in bone-marrow trephine biopsies decalcified under the same conditions [9]. In our study, we detected no gene mutations or amplifications (*GNAS*, *IDH,* and *MDM2*) in 75% of samples decalcified with hydrochloric acid as early as one cycle of decalcification. Shin et al. observed comparable results, reporting a lower detection rate of *GNAS* mutation in fibrous dysplasia samples decalcified with hydrochloric acid (65.7% in nondecalcified specimens vs. 9.6% in decalcified specimens) [10]. Alteration of nucleic acids secondary to strong acid decalcification did not allow the use of these samples for DNA or RNA sequencing. Strong acid decalcification did not allow for detecting in situ hybridization signals, whatever the target studied. Given all these deleterious effects, strong acid used to decalcify samples is therefore not suitable for immunohistochemistry, in situ hybridization or molecular analyses [1].

With formic acid, short-cycle decalcification preserved proteins but induced nucleic acid alterations as compared

with nondecalcified samples. Nevertheless, these alterations were not deleterious for diagnosis because we still detected protein expression and gene mutations. When we compared two different commercial solutions (TBD2 from Thermo fisher Scientific and DC1 from VWR), most of the results were comparable, except for detection of fusion transcripts. One short cycle of DC1 but not TBD2 allowed for detecting fusion transcript by RT-qPCR, which suggests that either the concentration of formic acid or other components of TBD2 may affect nucleic acid integrity. However, these results observed only in a small number of cases need to be verified in a larger cohort.

The integrity of proteins and nucleic acids depended on decalcification duration. For one sample decalcified in long-term treatment during 8 days, S100 protein expression was lost, which suggests that these conditions are not suitable for immunohistochemistry. For nucleic acid integrity (260/280 ratio for DNA, *IDH1* and *B2M* Ct), samples decalcified with formic acid for 5–10 cycles showed alterations. These alterations were comparable to those observed with hydrochloric acid when formic acid-based decalcification was longer than 10 cycles or with long-term cycling. Similarly, long-term decalcification with formic acid had a negative impact on mutations, gene amplifications, fusion transcript detection, and DNA sequencing. For RNA, short-term formic acid-based decalcification (1 cycle) allowed to detect fusion transcript by next-generation sequencing regardless of duration of cycle (except for one condition: DC1 overnight). Moreover, for RNA, we detected no difference in 260/280 ratio between lengths or numbers of cycles, but the number of samples was too restricted to obtain conclusive results. To our knowledge, the significance of the number of cycles used for formic acid decalcification has never been pointed out because previous results were always restricted to short decalcification duration [2, 5, 8]. This factor must be taken into account and we recommend decalcification <5 cycles with formic acid for immunohistochemistry and molecular analysis. Our results suggest that formic acid is not suitable for translocation detection or amplification FISH analysis as previously reported [5]. CISH seems more resistant because amplification could be detected in 3 of 5 breast cancer samples, but signals were altered and heterogeneous. Our results suggest that use of EDTA is more relevant for samples dedicated to in situ hybridization techniques.

With EDTA, protein and morphology were preserved. As with formic acid, slight nucleic acid alterations were observed as compared with nondecalcified conditions, without consequences for diagnosis. As previously reported, no difference in nucleic acid properties was observed between EDTA and short-length formic acid decalcification [8]. However, decalcification with EDTA provided better results for DNA and RNA next-generation sequencing and for in situ hybridization techniques compared with formic acid, suggesting that this chelating agent should be privileged for samples dedicated to these techniques. Nevertheless, decalcification with EDTA requires longer time and may be more difficult to use routinely [8, 11]. In our series, the number of cases for which the decalcification was incomplete was limited but more frequent with EDTA (1–4 cycles of 8 h for 4 samples) than formic acid (1–6 cycles for two samples). Moreover, EDTA remains difficult to use with surgical specimens because the solution is rapidly saturated and needs to be changed frequently. Consequently, if EDTA (without the addition of strong acid) is privileged, particularly for in situ hybridization techniques, it should be used in small-sized samples, like percutaneous fine-needle biopsies, which can be easily decalcified. Based on our preliminary study performed in 8-gauge biopsies, we recommend a 24–48 h decalcification with EDTA to obtain a satisfactory level of decalcification in a small tissue sample of 3–4 mm diameter.

According to these multisite results and our institutional experience, we propose recommendations for decalcification of both resection and biopsy bone samples (Table 4). In any case, adequate formalin fixation before decalcification and daily periodic control of the process are necessary. When immunohistochemistry and molecular analyses are required, nondecalcified samples (frozen and/or FFPE) should be used. When this is not possible, the pathologist should favor formic acid used in short-term cycles (<6 h) for <5 cycles, over hydrochloric acid. Pure EDTA (without strong acid) may be an alternative for fine-needle biopsies

Table 4 Recommendations for routine decalcification of bone samples.

- General principles
  - Adequate fixation before decalcification
  - Daily control of decalcification progress
- Decalcification protocols by sample type
  - Fine-needle percutaneous biopsy: formic acid for 1.5–3 h (depending on size and consistency)—alternative: pure EDTA for 24–48 h (depending on size and consistency)
  - Curettage and surgical open biopsy:
    - A nondecalcified sample should be performed when possible,
    - Decalcification with formic acid for one cycle of 6 h (cycle repeated if decalcification is not complete, for up to 5 cycles)
    - An additional small-sized sample decalcified with EDTA for 24–48 h can be eventually also performed, particularly if in situ hybridization studies are considered.
  - Surgical specimens for tumoral pathology:
    - One or two samples (dedicated to immunohistochemistry and molecular analyses): if nondecalcified samples are not possible, formic acid for one cycle of 6 h (cycle repeated if decalcification is not complete, within the limit of 5 cycles) and/or EDTA for one cycle of 24–48 h in small-sized samples
    - Remaining tissue: hydrochloric acid for 4 h (cycle repeated until complete decalcification)
  - Surgical specimens for nontumoral pathology: hydrochloric acid for 4 h (cycle repeated until complete decalcification)

and small-sized samples collected from curettage or surgical biopsies (particularly for samples dedicated to in situ hybridization analyses).

Our recommendations for bone-sample processing are as follows:

(1) Percutaneous fine-needle biopsies must be decalcified with formic acid for 1.5–3 h depending on the size and consistency of the sample, or with EDTA for 24–48 h depending on the size and consistency of the sample.
(2) Curettages and surgical open biopsies must be decalcified with formic acid during at least one complete cycle of 6 h (cycle repeated until complete decalcification); in addition, and particularly for in situ hybridization techniques, a small-sized sample can be decalcified with EDTA for 24–48 h.
(3) For surgical specimens in a context of tumoral pathology, sawing of the bone specimen often allows access to the tumor to obtain one or two samples, when possible, which can be fixed without any decalcification. When this is not possible, these samples must be decalcified with formic acid for at least one cycle of 6 h for immunohistochemistry and molecular analyses. As for curettages or surgical open biopsies, an additional small-sized sample can eventually also be decalcified with EDTA for 24–48 h in order to perform in situ hybridization techniques.
(4) The remaining tissue can be decalcified with hydrochloric acid.
(5) Surgical specimens in a nontumoral context may be decalcified with hydrochloric acid with 4 h cycles until complete decalcification.

To conclude, decalcification is required for processing bone tumor samples but is responsible for irreversible alterations of protein and nucleic acid, and false-negative results, particularly for in situ hybridization and molecular biology. Thus, pathologists must be very careful with negative results obtained from samples after decalcification, whatever the decalcifying agent used. Nevertheless, our results show that EDTA and short-term formic acid-based decalcification can be used without prejudicial effects on ancillary techniques and most molecular analyses.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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