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Original paper

Optimal DNA quality preservation process for comprehensive genomic testing of pediatric clinical autopsy formalin-fixed, paraffin-embedded tissues

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Clinical autopsies are performed to reveal the process of the disease that caused patient death and validate the diagnosis and treatment decisions. In pediatric clinical autopsy, the feedback provided to bereaved families has a considerable social impact; however, pediatric diseases are diverse, which makes it difficult to elucidate them. Therefore, it is necessary to employ molecular biology techniques in addition to conventional methods. Formalin-fixed, paraffin-embedded (FFPE) tissues are routinely prepared. However, clinical autopsy FFPE tissue processing is not standardized, and it is unclear whether DNA from such tissues can be used for comprehensive genomic analysis.
In this study, we evaluated the DNA quality of FFPE tissues from 15 recent autopsy cases at a single-center children's hospital using quantitative polymerase chain reaction [PCR (Q129/Q41)] and nanoelectrophoresis (DNA integrity number (DIN)). Good quality DNA was obtained from every organ type excluding bone marrow within 6 days of formalin fixation. Prolonged proteinase K digestion (48 h > 24 h > 1 h) and thicker tissue sections (10 μm > 1 μm) improved Q129/Q41; however, 24 h fixed FFPE tissues showed better DNA quality.
We propose an optimal and feasible workflow for storing short-term fixed FFPE tissues as DNA-preserved FFPE tissues for future comprehensive genomic searches.

Key words: clinical autopsy, pediatric autopsy, molecular autopsy, FFPE, DNA quality.

Introduction

Clinical autopsies (hospital autopsies, pathological autopsies) are performed by pathologists, at the request of clinicians and with consent of the patient's family, to reveal the disease process that led to a patient's death. Different from a medicolegal autopsy, where the medical history is unknown and the purpose is to determine the cause of death, a detailed medical history can be compared to the findings of a clinical autopsy to resolve questions that clinicians may have had during the course of the disease and validate the diagnosis and treatment decisions [1].

Clinical autopsies in children are similar to those in adults; however, the feedback provided to bereaved families has a significant impact. Knowing the correct cause of the disease that led to mortality and its inheritability can help predict the disease and prevent its occurrence in other family members; it is thus also useful for family planning. According to Japanese statistics, even though the number of pediatric clinical autopsies is small, their rate is high. Specifically,

the clinical autopsy rate relative to overall death in 2018 was only 0.74%, but it was 14.4% for newborns and 8.1% for children aged 0–14 years old excluding stillbirths and newborns [2–4]. Thus, each pediatric clinical autopsy case has a great social impact.

Unlike those in adults, diseases in children are diverse and include many rare diseases, making it more difficult to elucidate the process of the disease, resulting in patient death. Furthermore, in some pediatric autopsy cases, tissues are physically difficult to retrieve, such as cases with organs that are extremely small or have maceration; moreover, in cases of stillbirth there is little clinical information or morphological changes. In these cases, to meet the high feedback expectations of clinicians and patients' families, it is necessary to go beyond conventional morphological observation methods and employ new molecular biology techniques.

In recent years, several molecular autopsy reports using next generation sequencing (NGS) have been published. In forensic medicine, a systematic review analysis showed that approximately 30% of sudden unexpected pediatric deaths were resolved after molecular autopsy [5]. Studies have also reported the analysis of stillbirth or early infant death through whole genome/exome sequencing [6–10]. Therefore, there is a need to incorporate molecular biology techniques into clinical autopsy analyses [11], especially in pediatric cases. In the future, in addition to whole genome sequencing, it might also be desirable to conduct epigenetic analysis or gene expression analysis of organs that show abnormal autopsy findings.

In pathological diagnosis, formalin-fixed, paraffin-embedded (FFPE) tissues are routinely prepared to obtain histological findings; moreover, DNA and RNA extracted from FFPE tissues can be used for molecular analyses. As the use of FFPE tissues to identify genomic alterations in cancer has spread, FFPE processing conditions that degrade DNA quality have come to light, and optimal workflows have been proposed and disseminated [12–15]. However, these workflows are currently limited to FFPE tissues obtained by surgical resection or biopsy. DNA in postmortem FFPE tissues is generally known to be poorly preserved [16–18] and, therefore, not applicable for use in further molecular biology research. Some studies have discussed DNA recovery from FFPE tissues obtained from medicolegal autopsies, but the tissue sampling method for such autopsies is not the same as that for clinical autopsies [19, 20]. Very few reports have evaluated the DNA quality of clinical autopsy FFPE tissues: Funabashi *et al*. compared DNA extraction methods for archived FFPE samples, but they only selected "well-preserved" samples [21]. There have been no previous studies that have assessed the DNA quality of routinely processed clinical autopsy FFPE tissues.

In this study, DNA was extracted from FFPE tissues of 15 clinical autopsy cases at a single-center children's hospital, over approximately one year, from 2020 to 2021. DNA quality was evaluated using quantitative PCR (Q129/Q41) and DNA integrity number. The effects of formalin fixation duration, postmortem interval, and organ type on the DNA extraction workflow were assessed, and ways to improve DNA quality were examined. Finally, an optimal and feasible workflow was proposed to enable the future incorporation of molecular biology techniques into pediatric clinical autopsy analyses.

Material and methods

Formalin-fixed, paraffin-embedded tissues were obtained from 15 patients, including 13 males and 2 females, who received clinical autopsies over approximately one year (2020–2021) at Saitama Children's Medical Center, Saitama, Japan. Patient information is shown in Table I. The patients were aged 1 day to 7 years (median age, 77 days), and their postmortem intervals ranged 6–64 hours (median postmortem interval, 13 h). Their organs were removed and immersed in 20% neutral buffered formalin (FUJIFILM Wako, Japan) during autopsy. Large organs were cut into smaller slices to promote formalin fixation. The number of fixation days ranged 2–104 (median, 29 days). The formalin-fixed organs and organ slices were then cut small enough to fit cassettes and embedded in paraffin according to standard pathological protocols. The process for bone marrow tissue varied slightly for decalcification, in that it was immersed in a mixture of 10% formic acid solution and 10% nonbuffered formalin for one day before paraffin embedding. Formalin-fixed, paraffin-embedded blocks were then stored in the dark at room temperature; storage times ranged 9–17 months. For the three most recent autopsy cases (No. 13–15 in Table I), one surgical liver specimen sampled during autopsy was frozen at –80°C and another was fixed in 10% neutral buffered formalin for 24 h before paraffin embedding. These three cases were used for a comparative study of the influences of tissue section thickness and prolonged proteinase K digestion on DNA quality. For control specimens, cultured A673 and SK-ES-1 cells (both purchased from American Type Culture Collection), human lymphocytes from a healthy donor, and three FFPE tissues of lung cancers surgically removed from patients who had previously undergone successful whole genomic sequencing were used. This study was conducted in accordance with the Declaration of Helsinki. The ethical committee of the Saitama Children's Medical Center approved this study (approval number: 2021-01-017), and consent was obtained from the patients' families using the opt-out method.

Table I. List of autopsied patients

No.	Age	Sex	Postmortem interval (h)	Fixation days	Pathological diagnosis	24 h-fixed FFPE and frozen tissue
1	5 m	M	20	2	Dilated cardiomyopathy	
2	6 m	M	19	9	Asplenia syndrome with complex cardiac malformations	
3	1 d	M	21	6	Pulmonary hypoplasia	
4	7 y	F	17	16	Immunodeficiency caused by STAT3 mutation	
5	5 m	M	7	26	VACTERL association, postoperative status of biliary atresia	
6	30 d	M	64	3	Mitochondrial disease, suspected	
7	3 m	M	21	35	Hypoplastic left heart syndrome	
8	2 m	M	9	104	Asplenia syndrome with complex cardiac malformations	
9	3 d	F	14	66	Hydrops fetalis, right ventricle hypoplasia	
10	2 m	M	8	32	Dilated cardiomyopathy	
11	3 y	M	6	29	18 trisomy, hepatoblastoma	
12	11 d	M	10	77	Hypoplastic left heart syndrome	
13	1 d	M	11	53	21 trisomy, neonatal leukemia	✓
14	1 y	M	13	33	Hypertrophic cardiomyopathy	✓
15	16 d	M	6.5	20	Pulmonary hypertension, abnormality of pulmonary capillaries	✓
Median	77 d		13	29		

FFPE – formalin-fixed, paraffin-embedded
For three cases, No. 13–15, 24 h-fixed FFPE and frozen liver tissues were obtained during autopsy in addition to tissues collected for normal processing.

DNA extraction and quantification

DNA extraction was performed using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) for FFPE tissues, the QIAamp DNA Mini Kit (Qiagen) for frozen tissues, and the DNeasy Blood & Tissue Kit (Qiagen) for cultured cells, according to the manufacturer's protocols. Each FFPE tissue block was cut into 5 μm thick scroll sections, and 2 to 10 sections were put into centrifuge tubes, to obtain a tissue volume between 2 and 5 mm^3. Xylene was used for deparaffinization following the manufacturer's protocol. RNase A (Qiagen) was used for excluding copurified RNA. For the comparative study on the influences of tissue section thickness and prolonged proteinase K digestion on DNA quality, FFPE blocks were sliced to thicknesses of 1 μm (20 sections) and 10 μm (2 sections), and proteinase K digestion was prolonged from 1 h (manufacturer's recommendation) to 24 h and 48 h. DNA concentration and purity were measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA). DNA yields were calculated using DNA amounts and tissue volumes of FFPE blocks. Tissue volumes were calculated using section thickness and surface area measured using whole slide images of hematoxylin and eosin stained sections, obtained using the NanoZoomer-RS slide scanner with NDP.view2 software (Hamamatsu Photonics, Japan).

DNA quality assessment

We used both the KAPA Human Genomic DNA Quantification and QC kit (Cat. KK4960, KAPA Biosystems, USA) and the 2200 TapeStation system (Agilent Technologies, USA) for evaluating DNA quality. The KAPA Human Genomic DNA Quantification and QC kit includes standard DNA samples of known concentrations and three kinds of primers with amplicon sizes of 41 bp, 129 bp, and 305 bp. The DNA of target samples was amplified using these primers in the LightCycler 96 thermal cycler (Roche, Switzerland); target sample DNA concentrations were then calculated using standard curves that had been plotted for the three primers in advance. For assessing DNA quality, the DNA concentration measured using the 129 bp-amplicon standard curve was divided by that measured using the 41 bp-amplicon standard curve (Q129/Q41). Similarly, Q305/Q41 was calculated. The values of these Q ratios for high-quality DNA are around 1, whereas they are closer to 0 for low-quality DNA. In the 2200 TapeStation system, an automated electrophoresis system

employing Genomic DNA Screen Tape and Genomic DNA Reagents (Agilent Technologies), DNA integrity number (DIN) values, ranging 1–10, are automatically calculated. A high DIN indicates intact DNA, and a low DIN indicates degraded DNA. There are no manufacturer-set thresholds for DNA quality for either of the abovementioned methods, which thus need to be determined empirically in the context of samples and workflows.

Statistical analysis

Data analysis and visualization were conducted using the Microsoft Excel 2019 software. Statistical analysis was performed using the IBM SPSS Statistics 28.0 software (IBM, USA). Pearson's correlation coefficient was used to evaluate the correlation between Q129/Q41 and DIN. ANOVA with Tukey HSD test, one-way repeated measures ANOVA with Mauchly sphericity test, and the paired t-test were used to assess the impacts of proteinase K digestion prolongation and thickness of tissue section on DNA quality.

Results

Determination of thresholds for Q129/Q41 and DIN

The Q129/Q41 and DIN values obtained from all samples in this study are plotted in Figure 1. These

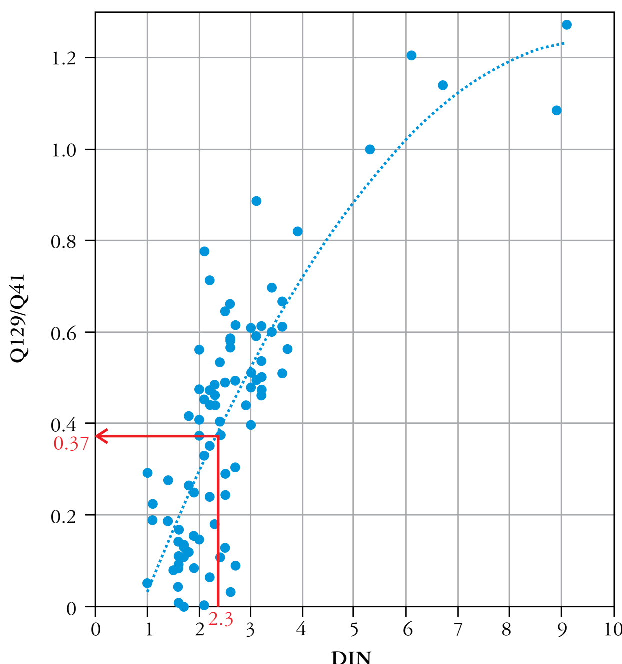

Fig. 1. Determination of Q129/Q41 and DNA integrity number thresholds

DIN – DNA integrity number
The scatterplot of DIN and Q129/Q41 values for all samples (including FFPE samples, frozen samples, and positive control samples, n = 86) is presented. The values are well correlated ($p < 0.001$), with a Pearson correlation coefficient of 0.795. Setting the DIN threshold at 2.3, the reference value for Q129/Q41 was determined to be 0.37 using the following regression curve: $y = -0.016x^2 + 0.3098^x - 0.2628$

values were highly correlated, as indicated by the Pearson's correlation coefficient of 0.795 ($p < 0.001$). The thresholds for Q129/Q41 and DIN are not indicated by the respective kit manufacturer protocols. A variety of threshold values have been reported, based on whether extracted DNA could be converted into a functional NGS library [12, 13, 22]. Because our study was limited to assessing DNA quality by our ethical committee, we applied the threshold value of 2.3 for DIN, following the guideline of the Japanese Society of Pathology for assessing postmortem colon and liver FFPE tissues [13]. Based on this DIN threshold and the regression curve presented in Figure 1, we set 0.37 as the threshold for Q129/Q41 in this study.

Effects of formalin fixation duration and postmortem interval on DNA quality

We examined the effects of formalin fixation length and postmortem interval on the DNA quality of liver and spleen FFPE tissues in all autopsied cases. Q129/Q41 clearly decreased as formalin fixation was prolonged, and the threshold was exceeded only in three cases, with the maximum being 6 days (Fig. 2A). The DIN showed a similar trend, but spleen samples requiring longer fixation showed a divergence from the Q129/Q41 threshold value, with some cases exceeding the threshold (Fig. 2B). Neither Q129/Q41 nor DIN was associated with postmortem interval (Figs. 2C, D).

Evaluation of DNA extracted from formalin-fixed, paraffin-embedded tissues of all organs fixed within 6 days

DNA was extracted from FFPE tissues of all organs of three autopsied patients, fixed within 6 days (No. 1, 3, and 6: 2, 6, and 3 days, Table I). DNA yield was highest in the spleen, followed by the liver, kidney, bone marrow, and thymus, suggesting that organs with a higher cell density have a higher DNA yield (Fig. 3A). DNA purity (A260/280) was high (above 1.8) in all organs except the bone marrow (Fig. 3B). Q129/Q41 values exceeded the threshold in the clinical autopsy FFPE tissues of all organs, being comparable to those of surgically resected cancer FFPE tissues for positive controls, except for bone marrow (Fig. 3C). The results for DIN were similar to those for Q129/Q41 (Fig. 3D). Q305/Q41 values were low in all samples, except for cultured cells for positive controls (data not shown), suggesting that this ratio is an inappropriate indicator for assessing the quality of DNA extracted from clinical autopsy FFPE tissues. Therefore, regardless of organ type, good quality DNA is expected to be obtained for all clinical autopsy FFPE tissues if formalin fixation is performed within 6 days. Bone marrow requires decalcification steps, and formic acid was used in this

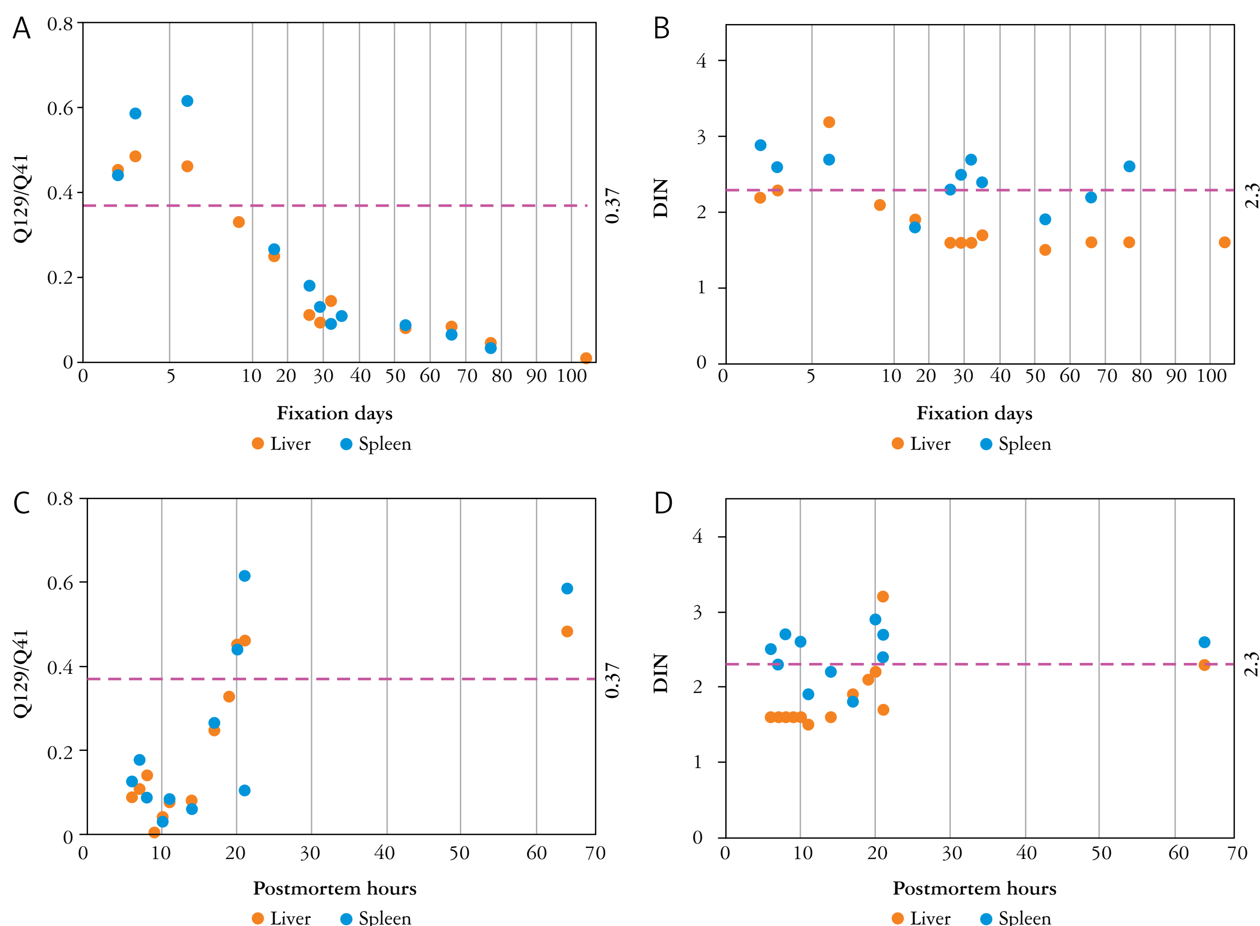

Fig. 2. Effects of formalin fixation duration and postmortem interval on DNA quality. The Q129/Q41 and DIN values of DNA extracted from liver and spleen FFPE tissues of all autopsy cases ($n = 15$) are plotted according to the number of days of formalin fixation (A, B) and postmortem hours (C, D)

DIN – DNA integrity number. The dashed lines indicate thresholds beyond which values were considered to indicate good DNA quality.

study; however, the use of acids for decalcification is known to cause nicking of nucleic acids [23]. Thus, the purity and quality of the DNA extracted from it would have been impaired.

Effects of proteinase K digestion prolongation and tissue thickness on DNA quality

Using liver FFPE tissues from three recent autopsy cases (No. 13–15, Table I), we examined the effects of prolonged proteinase K digestion and tissue section thickness on DNA of low quality. The numbers of days of formalin fixation for tissues obtained from these cases were 52, 33, and 20. Q129/Q41 tended to increase with an increase in proteinase K digestion time and thickness of sections; the matched-pair ANOVA showed significant differences among the 1 h/24 h/48 h conditions of proteinase K digestion ($p < 0.05$), and the matched-pair t-test showed that a tissue thickness of 10 μm was superior to that of 1 μm ($p < 0.05$). However, neither prolonged proteinase K digestion nor tissue section thickness had any effect on DIN. When these conditions were compared between the DNA extracted from 24 h fixed liver FFPE tissues and that from frozen liver tissues of the same patients (Fig. 4), DNA extracted from frozen livers showed superior quality in terms of Q129/Q41 ($p < 0.01$) and DIN ($p < 0.01$). DNA from 24 h fixed liver FFPE tissues did not show as good quality as that from frozen livers; however, it showed stable and good quality, which exceeded the thresholds for Q129/Q41 and DIN.

Optimal workflow for clinical autopsy tissue collection for molecular biology analyses

The workflow for collecting tissue specimens from clinical autopsies for possible future molecular biology analyses, as suggested by the results of this study, is shown in Figure 5. During the autopsy, in addition to the normal dissection procedure, organ fragments need to be routinely sampled from the removed organs. Candidate organ types for collection are those with gross abnormalities or, if no such organ types are present, the liver and spleen, which generally contain a large amount of tissue and DNA. Collected tissue fragments then undergo the formalin fixing and paraffin embedding processing workflow used for surgi-

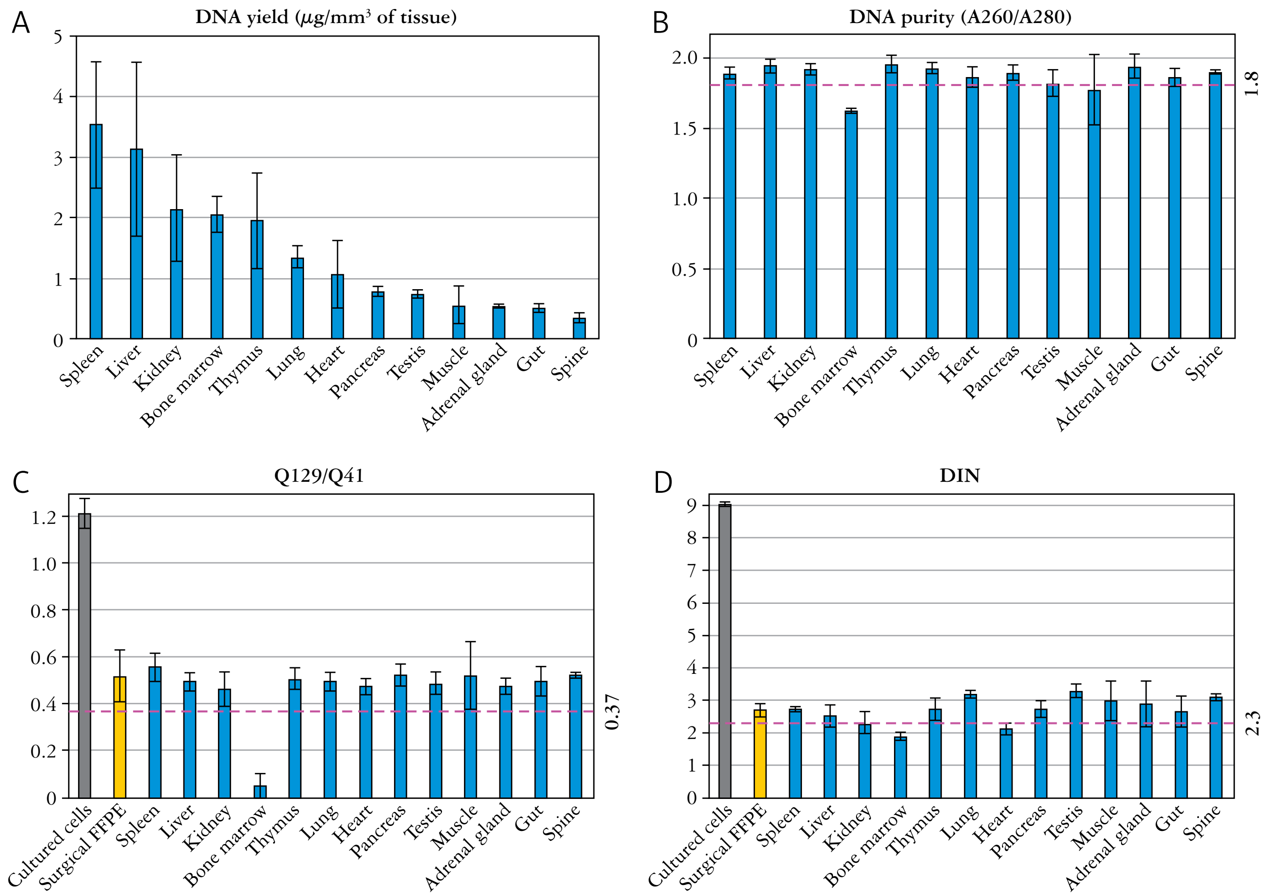

Fig. 3. DNA evaluation of clinical autopsy tissues fixed in formalin within 6 days of resection

DNA yield (A), DNA purity (B), Q129/Q41 (C), and DIN (D) values for clinical autopsy formalin-fixed, paraffin-embedded tissues in which formalin fixation was performed within 6 days of resection are shown by organ type (n = 3). DIN – DNA integrity number, FFPE – formalin-fixed, paraffin-embedded Data are presented as mean ±SE. Cultured cells and surgically resected lung cancer formalin-fixed, paraffin-embedded (FFPE) tissues (surgical FFPE) were used as positive control samples (n = 3 for each). Dashed lines indicate thresholds.

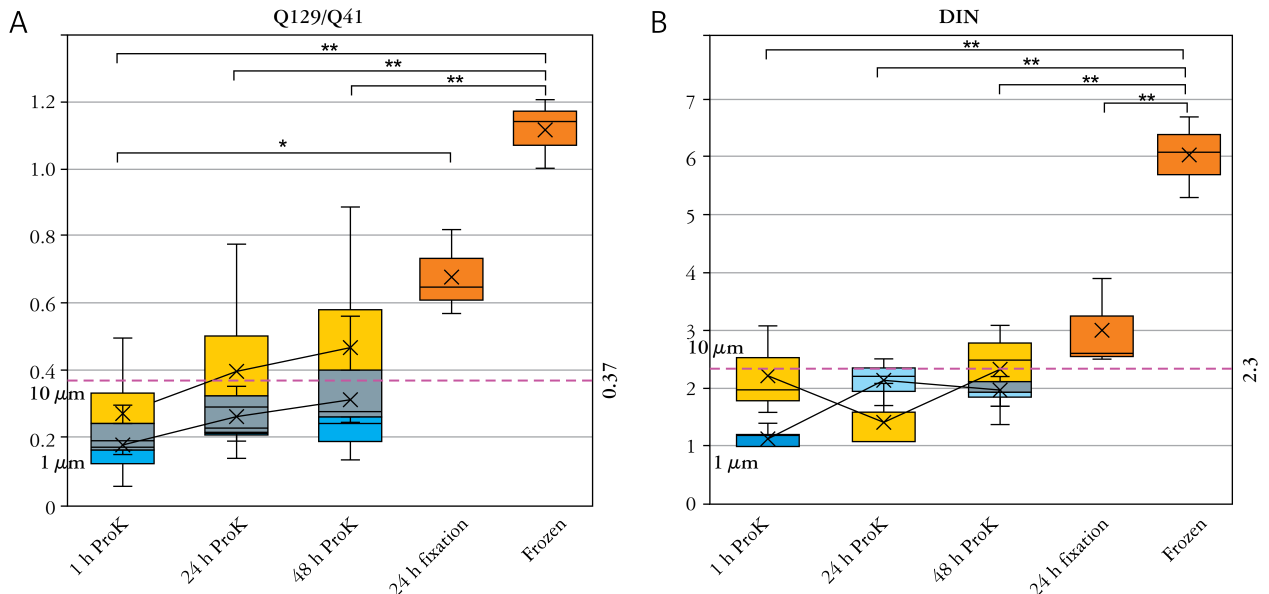

Fig. 4. Effects of prolonged proteinase K digestion and tissue thickness on DNA quality. Q129/Q41 (A) and DIN (B) values of clinical autopsy liver formalin-fixed, paraffin-embedded tissues in which DNA was extracted by proteinase K digestion for 1 h/24 h/48 h are presented

DIN – DNA integrity number
*10 μm thick and 1 μm thick sections were subjected to the same conditions (n = 3 for each). For comparison, liver formalin-fixed, paraffin-embedded tissues fixed in 10% neutral buffered formalin for 24 h (proteinase K digestion, 1 h; section thickness, 5 μm) and frozen liver tissues from the same patients were used. Data are presented as box plots and means, as determined by ANOVA with Tukey HSD test (*p < 0.05, **p < 0.01). Dashed lines indicate thresholds.*

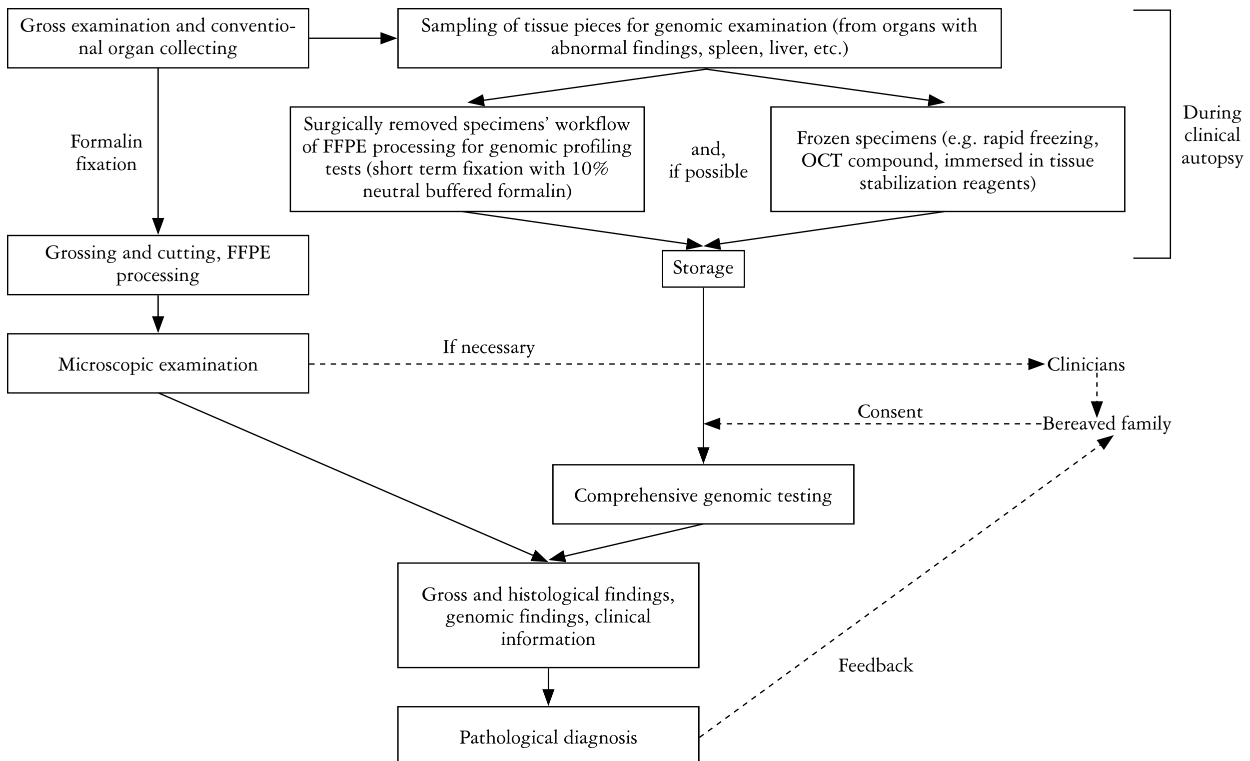

Fig. 5. Optimal workflow for clinical autopsy formalin-fixed, paraffin-embedded tissue collection for molecular biology analyses

FFPE – formalin-fixed, paraffin-embedded, OCT – optimal cutting temperature. In addition to the usual dissection procedures, tissues are routinely sampled for future genomic testing, transferred to a workflow of surgically resected specimens for cancer genomic profiling tests, and DNA-preserved formalin-fixed, paraffin-embedded tissues are routinely prepared. Further, if possible, frozen tissues are routinely prepared. Further, if possible, frozen tissues are also prepared for use in other molecular biology analyses.

cally resected specimens for cancer genomic profiling tests, wherein tissues are processed under ideal conditions for DNA preservation, such as fixation with 10% neutral buffered formalin between 6 and 48 h, decalcification with EDTA solution if necessary, and then stored as DNA-preserved FFPE tissues. Furthermore, if possible, tiny fragments of tissue in microtubes or tissue fragments embedded in optimal cutting temperature compound are frozen quickly and stored at –80°C. If immediate freezing is not possible, tissue fragments may be immersed in a tissue stabilization reagent (e.g., Allprotect Tissue Reagent, Qiagen, Inc.). Multiple tubes of frozen specimens should be prepared for possible use in DNA, RNA, and protein analyses. After gross and microscopic examinations, if necessary, comprehensive genomic testing is performed, with the bereaved family's consent obtained in cooperation with clinicians, and an integrated pathological diagnosis is made. Results are revealed to the bereaved family as per their wishes. If there are genetic variants to be disclosed, family members are referred for genetic counseling.

Discussion

Prolonged formalin fixation is notorious for causing DNA breaks and chemical modifications [24]. In this study, routinely prepared pediatric clinical autopsy FFPE tissues were judged to be of good quality only if they were fixed for up to 6 days. This duration for formalin fixation is the same as that reported by Lin *et al.*, who assessed the DNA quality of FFPE tissues from 12 cases of sudden cardiac death using NGS covering 95 cardiac genes [25]. They reported that 94–100% concordance was obtained for fixation within 2 to 6 days in comparison with non-fixed samples, but this concordance was lost after 8 days. Furthermore, a Japanese Society of Pathology guideline states that it is difficult to prepare NGS libraries if the fixation is done after 7 days [15]. Therefore, we believe that clinical autopsy tissues should be fixed in buffered formalin within 6 or 7 days to provide DNA of quality good enough to perform comprehensive genomic analyses. Our study showed that postmortem interval was not associated with DNA quality. In forensic medicine, many studies have reported the effects of postmortem interval on DNA quality [26, 27]; however, a postmortem interval up to 64 h, which was the maximum time in this study, would not affect DNA quality.

Prolonged proteinase K digestion and tissue section thickness mildly affected Q129/Q41 but not DIN. Frazer *et al.* previously reported that neither the ΔCq of the Illumina QC assay nor DIN was changed between 24 and 72 h of proteinase K digestion [28]. Viljoen *et al.* compared tissue thicknesses of 1 μm/3 μm/5 μm

and reported that the thinner the tissue section is, the higher is the DNA concentration and purity, and the lower is the DIN [20]. Our results overlap with these reports. However, 24 h fixed FFPE tissues in 10% neutral buffered formalin and frozen tissues showed more stable and good quality DNA; therefore, it was suggested that formalin fixing and embedding of tissues for 24 h and freezing tissues are effective approaches for obtaining DNA of good enough quality for molecular biology analyses.

Conclusions

The clinical autopsy FFPE tissue processing workflow is not standardized and varies among institutions. This study included autopsy specimens from a single children's hospital over the past year approximately; nevertheless, the number of days of formalin fixation varied widely, 2–104 days, and only 3 of 15 cases, for which fixation was performed within 6 days, provided DNA of good quality. This may be due to the small number of pathological staff in children's hospitals, which results in prioritization of diagnostic work on surgically resected tissues and delay in autopsy tissue-related work. Since it is practically difficult to perform fixation routinely within 6 days of resection in clinical autopsy cases, we propose separately collecting tissue fragments at autopsy and transferring them to the surgically resected tissue workflow for cancer genomic profiling tests, which is now widespread in children's hospitals, and, ideally, also freezing these tissues. Preparation of DNA-preserved FFPE tissues should be sufficient for whole genome/whole exome sequencing, whereas frozen specimens provide a better quality of DNA and can be used for future gene expression searches and functional analysis as needed. Workflows that reveal the most information from individual cases would benefit not only bereaved families but also the overall development of medicine.

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The authors declare no conflict of interest.

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