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Relationship between formalin reagent and success rate of targeted sequencing analysis using formalin fixed paraffin embedded tissues

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A B S T R A C T

Background: Tumor genetic alterations are determined to aid in selecting therapy and predicting prognosis. In routine clinical practice, targeted sequencing analysis is performed using formalin-fixed paraffin embedded (FFPE) tissues. However, successful genetic analysis remains challenging because FFPE DNA is fragmented during the sample preparation process.

Methods: Real-time PCR was performed to assess DNA quality and quantities. Targeted sequencing was performed using FFPE tissues fixed with different types of formalin.

Results: DNA was less fragmented from samples fixed in low formalin concentration (10% vs. 20%) and neutral buffered conditions (neutral buffered vs. non-neutral). DNA fragmentation increased over the fixation time. In a preliminary test study, we compared fixation using 10% neutral buffered formalin (n = 180) and 20% formalin (n = 26). The success rate of targeted analysis was higher using 10% neutral formalin (98.3%; 177/180) compared with 20% formalin (34.6%; 9/26). In a validation study with additional formalin-fixed paraffin embedded tissues fixed with 10% neutral buffered formalin (n = 860), we reproduced these results and achieved a high success rate for targeted sequencing analysis (98.4%; 846/860).

Conclusion: Our data show that 10% neutral buffered formalin is recommended for fixation of formalin-fixed paraffin embedded samples to achieve high success rate of targeted sequencing analysis.

1. Introduction

Formalin-fixed paraffin embedded (FFPE) tissues are routinely prepared and used for pathological diagnosis of various diseases. A large amount of archival FFPE tissues has been stored in the pathology department. FFPE tissues can be easily stored at room temperature for long periods of time and retrospectively analyzed [1]. Although formalin is a widely-used fixation reagent, it has a negative effect on the integrity of DNA [2] and causes DNA-DNA and/or DNA-protein cross-links, nucleotide transitions and DNA fragmentation [3–5]. These effects can interfere with subsequent sample analysis, such as next generation sequencing (NGS) analysis [6,7]. Although short read sequencing can be used to analyze fragmented DNA for tumor mutations to some extent, severely fragmented DNA at low concentrations is not applicable for preparation of a NGS library and analysis.

Because clinical specimens are valuable and finite, it is important to determine whether DNA quantity and quality are sufficient for NGS analysis at the pre-analytical phase to ensure high success of genetic analysis [8–10]. However, the precise formalin type and fixation conditions for FFPE sample preparation that ensure successful genetic analysis have not been clarified. In this study, we examined the effects of formalin concentration, neutral buffer condition and fixation times on DNA length and success rate of targeted sequencing analysis from FFPE samples.

2. Materials and methods

2.1. Samples and preparation

We assessed DNA fragmentation with different types of formalin reagents in a pilot study: 10% neutral buffered formalin, 10% formalin, 20% neutral buffered formalin and 20% formalin (Wako Pure Chemicals, Osaka, Japan). Resected human liver tissues were cut into 5 mm^3 sections with a trimming knife and soaked in each formalin reagent for various times (6 h, 12 h, 1 day, 3 days and 7 days). Fixed tissues were then embedded into paraffin and archival FFPE samples were produced. For preparing frozen tissues, samples were snap frozen in liquid nitrogen and stored at -80°C until DNA extraction.

For the preliminary test study, we used resected tissues or biopsies randomly fixed in either 10% neutral buffered formalin (n = 180) or

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Table 1
Success rate of targeted sequencing using FFPE DNA fixed with 10% neutral bufferd formalin and 20% formalin.

	10% neutral buffered formalin (n = 180)					20% formalin (n = 26)				
Tissue	Total (n)	Success (n)	Failure (n)	Success rate	Failure rate	Total (n)	Success (n)	Failure (n)	Success rate	Failure rate
Ope	138	136	2	98.6%	1.4%	15	6	9	40.0%	60.0%
Biopsy	42	41	1	97.6%	2.4%	11	3	8	27.3%	72.7%
Fixation (days)										
1	67	66	1	98.5%	1.5%	11	4	7	36.4%	63.6%
2	23	23	0	100%	0%	2	2	0	100%	0%
3	41	39	2	95.1%	4.9%	2	1	1	50.0%	50.0%
4	24	24	0	100%	0%	1	0	1	NA[a]	100%
5	11	11	0	100%	0%	8	1	7	12.5%	87.5%
6	8	8	0	100%	0%	2	1	1	50.0%	50.0%
7	6	6	0	100%	0%	0	0	0	NA[a]	NA[a]
Tissue										
Gastric	48	47	1	97.9%	2.1%	1	1	0	100%	0%
Lung	44	44	0	100%	0%	0	0	0	NA[a]	NA[a]
Liver	23	21	2	91.3%	8.7%	11	4	7	36.4%	63.6%
Lymphnode	25	25	0	100%	0%	2	0	2	0%	100%
Ovary	24	24	0	100%	0%	0	0	0	NA[a]	NA[a]
Breast	8	8	0	100%	0%	6	2	4	33.3%	66.7%
Skin	0	0	0	NA[a]	NA[a]	6	2	4	33.3%	66.7%
Peritoneum	4	4	0	100%	0%	0	0	0	NA[a]	NA[a]
Pancreas	2	2	0	100%	0%	0	0	0	NA[a]	NA[a]
Endometrium	1	1	0	100%	0%	0	0	0	NA[a]	NA[a]
Vagina	1	1	0	100%	0%	0	0	0	NA[a]	NA[a]
Overall	180	177	3	98.3%	1.7%	26	9	17	34.6%	65.4%

[a] NA; not applicable.

20% formalin (n = 26) for 1–7 days without selection bias. Tumor types of tissues were as follows: gastric cancers (n = 49), lung cancers (n = 44), hepatocellular carcinomas (n = 34), metastatic lymph node (n = 27), ovarian cancers (n = 24), breast cancers (n = 14), skin cancers (melanoma) (n = 6), peritoneum cancers (n = 4), pancreatic cancers (n = 2), endometrial cancer (n = 1) and vaginal cancer (n = 1). The distribution of these samples between the fixation condition groups is shown in Table 1.

For the validation study, we examined samples prepared in 10% neutral buffered formalin. After November 2013, all tissue samples were fixed with 10% neutral buffered formalin at our institute. A total of DNA samples from 860 FFPE sections were subjected to NGS analysis. Serial sections (10-μm thick) were prepared from FFPE samples and stored at room temperature overnight. The sections were then deparaffinized and stained with hematoxylin-eosin as follows: xylene for 5 min twice, 100% ethanol for 30 s, 95% ethanol for 30 s, 70% ethanol for 30 s, water for 30 s, Lillie-Mayer hematoxylin (Muto Pure Chemicals, Tokyo, Japan) for 30 s, water for 30 s, eosin Y (Merck, Darmstadt, Germany) for one dip, 70% ethanol for 30 s, 95% ethanol for 30 s, 100% ethanol for 30 s and xylene for 5 min twice.

Informed consent was obtained from all patients. This study was approved by the Institutional Review Board at Yamanashi Central Hospital.

2.2. Laser-capture microdissection and DNA extraction

Pathological review was performed by a pathologist (T.O.). To obtain the tumor or non-neoplastic area, we performed laser-capture microdissection using an Arcturus XT laser microdissection system (Thermo Fisher Scientific, Waltham, MA, USA) [11]. We confirmed that dissected tissues were attached on the CapSure Macro LCM Caps. Film was peeled from CapSure Macro LCM Caps with a pincette and transferred into 1.5 mL sterilized tubes. DNA extraction was performed with a QIAamp DNA FFPE kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with minor modifications. Buffer ATL (180 μL) and Proteinase K (20 μL) were added into film-containing 1.5 mL sterilized tubes. This sample was incubated at 56°C for 1 h and subsequently at 90°C for 1 h. Next, 200 μL Buffer AL and 200 μL ethanol were added and samples were mixed. The mixture was added into QIAamp MiniElute columns to bind DNA to membrane and washed with 500 μL Buffer AW1 and 500 μL Buffer AW2. FFPE DNA was eluted using 40–50 μL Buffer ATE. Frozen tissue was thawed and DNA was extracted using the QIAamp DNA FFPE kit (Qiagen) as described, without incubation step (90°C for 1 h).

2.3. FFPE DNA quality analysis

The integrity of FFPE DNA was assessed using the TaqMan RNase P Detection Reagents Kit (catalogue number 4316831) and the FFPE DNA QC Assay v2 (catalogue number 4324034) on a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific) according to the manufacturers' recommendations. The reagents included primer pairs amplifying a short- (87 bp) and a long- (268 bp) amplicon located on the RNase P locus. DNA quality analysis was performed as described previously [12]. Briefly, the PCR reaction mix included 1 μL TaqMan Assay, 10 μL TaqMan Fast Advanced Master Mix, 8 μL nuclease-free water and 1 μL FFPE DNA. The PCR reactions were transferred into a MicroAmp Fast Optical 96-well Reaction Plate and sealed plate with MicroAmp Optical Adhesive Film (Thermo Fisher Scientific). The plate was briefly mixed with 96-well plate mixer and centrifuged at 180 ×*g* for 3 min at 25°C. Human control genomic DNA (included with the TaqMan RNase P Detection Reagents Kit) was serially diluted four times for a five-point standard curve and the absolute DNA concentrations were determined. The following PCR protocol was used: 95°C for 20 s, followed by 45 cycles of 95°C for 1 s and 60°C for 20 s. Assessment of DNA fragmentation was estimated as the ratio of DNA (relative quantification, RQ) obtained for the long amplicon to the short amplicon. The RQ value is an indicator of the degradation level of genomic DNA; a higher RQ value indicates better quality genomic DNA.

2.4. Library quantity check and targeted sequencing

The Ion AmpliSeq Library kit v2.0 (Thermo Fisher Scientific) was used for amplicon-based NGS library construction. The commercially available panel or custom-made panels were used [13–16]. Multiplex PCR amplification was performed as follows: HiFi reagent, PCR primers and FFPE DNA were mixed, and nuclease-free water was added to bring the reaction to 20 μL. The PCR protocol was followed according to the manufacturer's instructions. Multiplex PCR products were treated with 2 μL FuPa reagents to partially digest primers and PCR was performed as follows: 50°C 10 min, 55°C 10 min and 60°C 20 min. Adaptor ligation was performed as follows: 22 μL of PCR products were mixed with 4 μL Switch solution, 1 μL nuclease-free water, 0.5 μL P1 adaptor, 0.5 μL Barcode Xpress and 2 μL DNA ligase. The adaptor-ligated library was purified with Agencourt AMPure XP reagents (Beckman Coulter, Brea, CA, USA). The purified library was quantified using an Ion Library quantitation kit (Thermo Fisher Scientific). Equimolar libraries were mixed and emersion PCR was carried out using the Ion OneTouch™ System or Ion Chef System (Thermo Fisher Scientific) according to the manufacturer's instructions. Targeted sequencing was performed on the Ion PGM system or Ion Proton system (Thermo Fisher Scientific) [17].

2.5. Data analysis

Sequence data were processed using standard Ion Torrent Suite Software running on the Torrent Server. Raw signal data were analyzed using Torrent Suite. The data processing pipeline involved signaling processing, base calling, quality score assignment, adapter trimming, PCR duplicate removal, read alignment to the human genome 19 reference (hg19), quality control of mapping quality, coverage analysis and variant calling.

2.6. Statistics

We calculated unbiased variance and used two sample t-test for equal variance and Welch t-test for unequal variance. R package (version 3.1.2) was used for statistical analysis.

3. Results

3.1. Type of formalin reagents and DNA fragmentation

We conducted a pilot study to examine the effects of various formalin reagents on DNA quality and quantities from FFPE samples. We selected four types of formalin reagents that are regularly used in routine clinical practice: 10% neutral buffered formalin, 10% formalin, 20% neutral buffered formalin and 20% formalin. Surgically resected liver tissues were fixed in one of the formalin reagents from 6 h to 7 days to generate FFPE samples. We also included frozen tissues for comparison. We then extracted DNA from the FFPE samples and assessed DNA length by quantitative-real time PCR. Compared with DNA extracted from frozen tissue, DNA from FFPE samples was degraded (Fig. 1A). We found that as the fixation time increased, the DNA samples became more fragmented. In addition, long DNA fragments could be extracted from FFPE tissues samples fixed from 6 h to 3 days; however, after fixation for 7 days, DNA was severely fragmented. The order of formalin reagents ranked in terms of increasing DNA fragment effects was 10% neutral buffered formalin, 10% formalin, 20% neutral buffered formalin and 20% formalin. As same condition, we also examined the long DNA quantity adjusted by the microdissection area. After fixation for 1 day, high amounts of long DNA fragments were found in the following order: 10% neutral buffered formalin, 20% neutral buffered formalin, 10% formalin, and 20% formalin (Fig. 1B). In samples fixed for over 3 days, the quantity of long DNA fragments was drastically decreased in the non-buffered formalin conditions. Together these results indicate that a low concentration of formalin that is neutrally buffered maintains DNA integrity and allows for preservation of DNA that is more suitable for further genetic analysis.

3.2. Preliminary test study

To estimate the DNA quality and quantity required for achieving successful NGS analysis, we compared FFPE tissue samples prepared under the worst condition (n = 26, 20% formalin) and the best condition (n = 180, 10% neutral buffered formalin) from our pilot analysis in a preliminary test study. Fixation time and storage periods were not significantly different between 10% neutral buffered formalin and 20% formalin fixation conditions (median fixed time 2.5 days vs. 2.5 days, $p = 0.39$; median storage period 10 vs. 13.5 months, $p = 0.22$) (Fig. 2A, B). Although the amounts of short DNA fragments were comparable from FFPE samples from both conditions ($p = 0.87$; Fig. 2C), long DNA fragments were more abundant using 10% neutral buffered formalin than 20% formalin ($p = 0.047$; Fig. 2D). Consistent with the data from pilot study, the DNA quality value (RQ) of samples from FFPE tissues fixed in 10% neutral buffered formalin was higher than samples from FFPE tissues fixed in 20% formalin (median RQ value 0.38 vs. 0.08, $p = 2.2 \times 10^{-16}$; Fig. 2E). Nested case-control study reproduced the results that RQ values were significantly higher in samples fixed with 10% neutral buffered formalin than with 20% formalin (n = 26, 20% formalin; n = 72, 10% neutral buffered formalin) (Supplemental Fig. 1).

To examine how the extracted DNA quality and quantity affect the success of subsequent NGS analysis, we compared DNA from tissues soaked in 20% formalin and 10% neutral buffered formalin. When we obtained sufficient mapped reads on target region and coverage depth, we considered NGS analysis was conducted successfully. As expected, the overall success rate using DNA extracted from 20% formalin was low (34.6%, 9/26 samples) compared with 10% neutral formalin (98.3%, 177/180 samples) (Table 1).

3.3. Formalin fixation time

We next examined the effect of fixation time on the success rate of NGS. The tissue samples used in the preliminary study had been prepared by fixing in the formalin reagents from 1–7 days (Table 1). We used these samples to conduct NGS. In samples prepared with 10% neutral buffered solution, the success rate was almost the same across samples fixed from 1–7 days (range: 95%–100%) (Table 1). However, in samples prepared with 20% formalin, the success rate was only 47% (7/15 samples) and 18% (2/11 samples) in samples with 1–3 day fixation and those with 4–7 day fixation, respectively; the RQ values for samples with 1–3 day fixation was 0.11 and 0.03 for samples with 4–7 day fixation (data not shown). These results supported our data showing that DNA was severely fragmented in these conditions after 3 days of fixation (Fig. 1) and demonstrated a failure of subsequent NGS analysis.

3.4. Biopsy and surgically resected tissues

Higher amounts of wet tissue can be obtained by surgical resection than biopsy. We next examined whether the amount of tissue could influence the NGS success rate. We obtained 153 surgically resected specimens and 53 biopsies from patients and fixed with formalin. Of these tissue samples, 180 samples were fixed with 10% neutral buffered formalin and 26 with 20% formalin (Table 1). The median microdissected area was 21.5 mm^2 (range: 0.3–73.2 mm^2) and 41.0 mm^2 (range: 2.8–250 mm^2) from biopsied and surgically resected tissues, respectively. The NGS success rate was 99% (136/138) from resected tissues and 98% (41/42) from biopsied tissues fixed using 10% neutral buffered formalin (Table 1), indicating that even a small amount of tissue can be used for successful targeted sequencing. Furthermore, tumor tissue collected from various organs (e.g. stomach, lung, liver, lymphnode, ovary, breast, peritoneum, pancreas, endometrium,

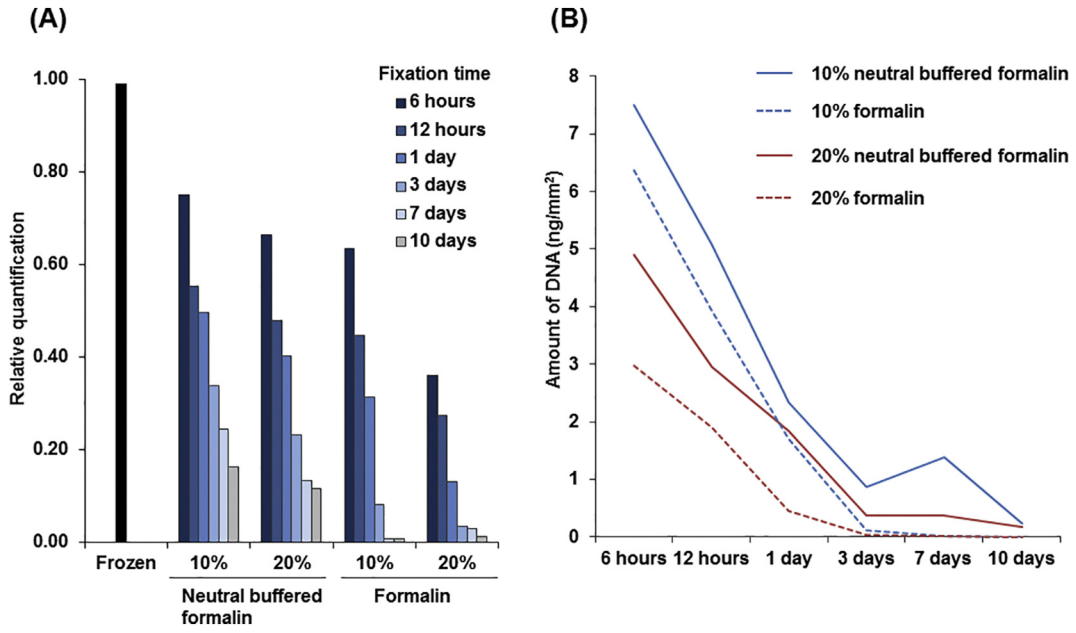

Fig. 1. DNA quality and quantities in FFPE samples prepared with various formalin reagents and fixation times.
A: FFPE DNA was obtained in samples prepared in the indicated formalin reagents for the indicated fixation times. DNA quality was evaluated by relative quantification values (see Methods). DNA extracted from frozen tissues was used as a high-quality control. B: Amount of long DNA was assessed in samples prepared in the indicated formalin reagents for the indicated fixation times as shown in (A).

vagina) underwent successful targeted sequencing, after they were fixed with 10% neutral buffered formalin. In contrast, we only observed a 40% NGS success rate (6/15) from resected tissues and 27% rate (3/11) from biopsied tissues fixed in 20% formalin. These results indicate that limited amounts of material fixed in 10% neutral buffered formalin is a key factor to achieve high success rate of NGS analysis.

3.5. Validation study

Our data suggested that a high NGS success rate could be achieved using FFPE samples fixed in 10% neutral buffered formalin. Based on these findings, all tissue samples have been fixed in 10% neutral buffered formalin at our institute since November 2013. To validate the findings from our previous data, we next performed NGS analysis using additional FFPE tissues fixed in 10% neutral buffered formalin (n = 860). These FFPE tissues were obtained surgically resected specimens or biopsies. Among the total 860 samples, NGS analysis was successfully performed in 846 samples (98.4%) (Fig. 3). Only five samples (0.6%) failed to achieve NGS library construction because of insufficient amount of DNA available. We further analyzed the quality of sequence data. In total, good quality data were not obtained in 9 samples due to the following reasons: sequencing reads were not sufficiently mapped onto the target region (4 samples); coverage depth was not sufficient (3 samples); and sequencing read size was short and insufficient on target rate and low coverage depth (2 samples) (Fig. 3). These data indicated that we can obtain sufficient nucleotide sequence data with a high frequency (846/860, 98.4%) if tissues are fixed with 10% neutral buffered formalin.

4. Discussion

FFPE tissues have been used for routine pathological diagnosis and

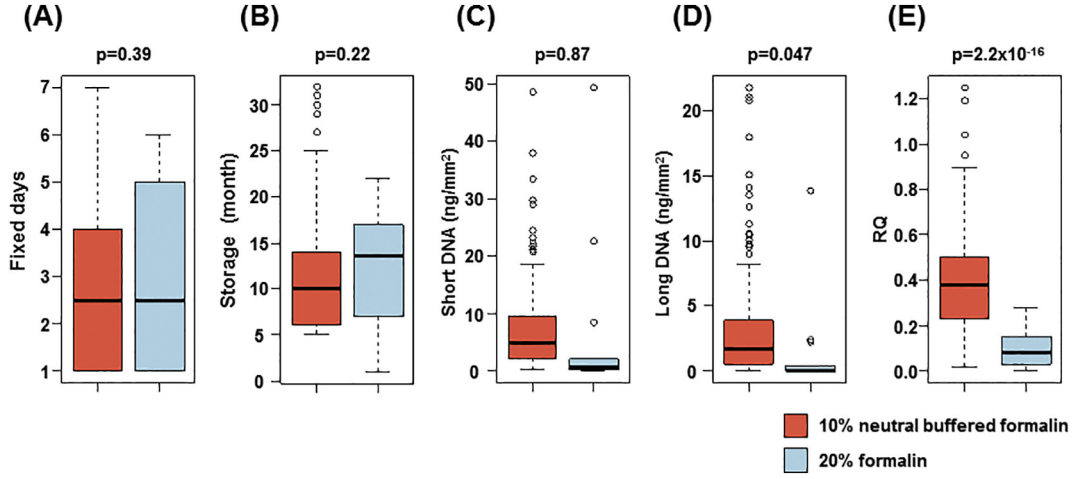

Fig. 2. Comparison of quality and quantities of FFPE DNA fixed with 10% neutral buffered formalin or 20% formalin.
DNA was extracted from FFPE tissues fixed with 10% neutral buffed formalin (n = 180) or 20% formalin (n = 26). Box plots show comparisons of A: the fixation days, B: storage period of FFPE blocks, C: amount of short DNA (ng/mm^2), D: amount of long DNA (ng/mm^2) and E: relative quantification (RQ) values.

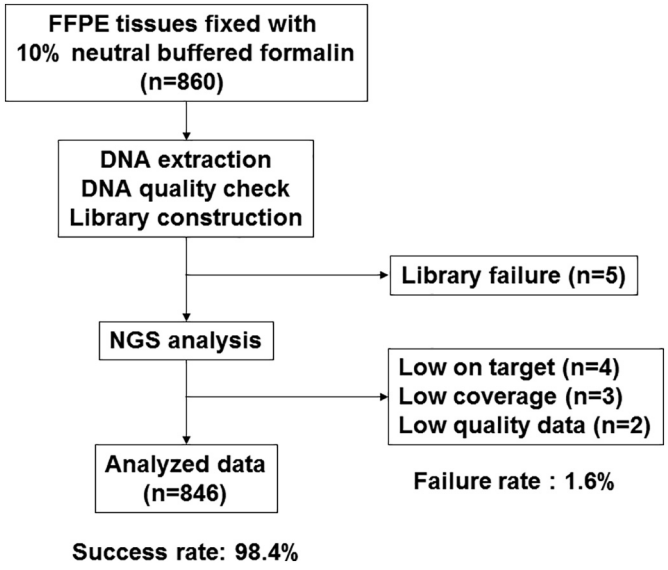

Fig. 3. Validation study of NGS success rate using DNA from FFPE fixed with 10% neutral buffered formalin.
FFPE samples were prepared in tissues fixed in 10% neutral buffered formalin (n = 860). The quality of FFPE DNA was checked and subjected to library preparation for targeted sequencing analysis. Library concentration was assessed by real-time PCR, and low library amount was considered as failure (n = 5). These samples were excluded from subsequent analysis. Targeted sequence was performed on a next generation sequencer. Data quality was assessed, and low quality data were obtained from 9 samples. Overall, we could analyze 98.4% of samples (846/860) using FFPE DNA from tissues fixed in 10% neutral buffered formalin.

immunohistochemical examination. Recently, NGS analysis has been frequently conducted on DNA extracted from FFPE tissues [18,19]. These genetic approaches have clarified new molecular subtypes across multiple diseases, including cancers, and changed the clinical practice by establishing precision medicine.

Large amounts of FFPE tissues have been stored in archives in multiple clinic, hospital and academic institutes worldwide. However, DNA extracted from FFPE tissues is often fragmented and shows cytosine to thymidine transitions and cross-linking modifications [20–23]. These changes are primarily dependent on the fixation time and concentration of formalin reagents and storage conditions [24,25]. Low quality FFPE DNA is not suitable for genetic analysis and can generate artifacts. However, only few studies have examined how DNA quality and subsequent success rate of NGS analysis are influenced by fixative reagent types and fixing time.

In this study, we examined the impact of formalin reagents on the extracted DNA quality and quantities and attempted to elucidate conditions that contribute to a high success rate of NGS analysis. To this end, we first examined levels of DNA fragmentation in samples fixed in several types of formalin reagents at different time points. Our data indicated that neutral buffered formalin and a low concentration of formalin (e.g. 10% neutral buffered formalin) generated less DNA fragmentation and enabled retrievable DNA. Previous report showed DNA degradation was determined by agarose gel electrophoresis and that high level of DNA degradation led to insufficient amount of DNA and failure of library preparation [7]. These results suggest that researchers should avoid using non-buffered formalin, because DNA was severely fragmented under these conditions and generated a low success rate in subsequent genetic analysis. The differences between 10% versus 20% and buffered and non-buffered conditions were remarkable (Table 1). Analysis of samples from our institute revealed a success rate of NGS analysis of 98.4% (846/860) using DNA extracted from FFPE tissues fixed with 10% neutral buffered formalin.

This high performance rate was achieved using multiple tumor types and specimen types (biopsy or resected tissues). Together these results demonstrate that 10% neutral buffered formalin is the best reagent to ensure successful NGS analysis.

High concentrations of formalin penetrate into tissues in a short time, and therefore 20% formalin is used in some institutes. A questionnaire survey was conducted by the Japan Pathological Society in 2011 in 324 institutes and revealed that only 44% of institutes used 10% neutral buffered formalin [26]. This suggests that 56% of the institutes in Japan may suffer a high failure rate of NGS analysis using FFPE DNA samples. Our institute has stored over 0.5 million FFPE blocks from 1980 to 2013. These “archive” samples accumulated for a long time, but almost of these archives maybe not suitable for targeted sequencing analysis unfortunately.

There are growing demands for sequence data obtained by NGS on DNA extracted from FFPE samples in routine clinical practice. To shorten the turn-around time and realize precision medicine, we have to make an effort to reduce the failure of genetic analysis as much as possible. For this purpose, quality control of DNA integrity at the pre-analytical phase is required. Our data demonstrated that selecting specific formalin reagents for preparation of FFPE samples is critical for achieving a high success rate of NGS analysis.

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Conflict of interests

All authors declare no competing interests.

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