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Factors affecting *KRAS* mutation detection in colorectal cancer tissue

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ABSTRACT

Background: With the recent development of molecular tests for various biomarkers, it has become even more important to prepare adequate tissue samples. However, little is known about how the effect of cold ischemia time or formalin fixation time can affect *KRAS* mutation detection in colorectal cancer.

Methods: This study included the results of *KRAS* mutation tests for colorectal cancer in 401 specimens. We investigated clinicopathologic factors that may affect DNA quality of formalin-fixed, paraffin-embedded (FFPE) tissue including specimen type, cold ischemia time, and formalin fixation time and assessed the detection rate of the *KRAS* mutation in samples with varying DNA quality.

Results: Sample DNA quality for *KRAS* mutation test was better in biopsy specimens, which showed markedly shorter cold ischemia time and shorter formalin fixation time compared to resection specimens. A cold ischemia time of one hour or less was associated with better sample DNA quality. But the formalin fixation time was not a significant factor when it fell within the range performed in routine pathology diagnosis. When prolonged formalin fixation was tested, we confirmed that the specimen DNA quality gradually got worse from one month to three months.

Conclusions: The biopsy specimens showed better sample DNA quality for *KRAS* mutation test compared to resection specimens. In a routine diagnostic pathology setting, the cold ischemia time was an important factor affecting DNA quality and the formalin fixation had a wide time range for optimal DNA quality.

1. Introduction

As biomarkers are being more widely used in selecting treatment strategies, preparing adequate tissue samples for molecular tests has become more important. In the routine pathology diagnosis field, patient tissue samples are usually fixed in formalin and embedded in paraffin. During these processes, many factors affect the results of biomarker tests, including fixation delay (cold ischemia time), type of fixative used, and time in fixative. Delayed formalin fixation can change biomarker expression in breast biomarker assays or cause assays to be impossible to interpret [1], and formalin fixation time can affect the quality of extracted DNA from formalin-fixed paraffin-embedded (FFPE) tissue [2–4]. The effects of delayed fixation and prolonged fixation time on the expression of molecular markers have mainly been studied in breast cancer, with only one report published to date about tissue ischemia time and mRNA expression level in colorectal neoplasm [1,5–9].

The v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) is a proto-oncogene located on the short arm of chromosome 12 and is a well-known biomarker that predicts resistance to anti-EGFR monoclonal antibody therapy for various cancers [10,11]. The *KRAS* mutation is commonly tested for in pathology diagnosis and is detected in 30–50% of colorectal cancers [10,12–14]. Although various detection methods using FFPE have been developed for the *KRAS* mutation, there have been few studies focusing on the effect of FFPE sample quality on *KRAS* mutation detection [2,15–19].

In the present study, we evaluated the clinicopathological features that may affect the specimen DNA quality of FFPE tissue using colorectal cancer cases diagnosed by routine pathology, including cold ischemia and formalin fixation time. We additionally investigated *KRAS* mutation detection rate according to specimen DNA quality.

Abbreviations: Ct, cycle threshold; FFPE, formalin-fixed paraffin-embedded; H&E, hematoxylin and eosin; NBF, neutrally buffered formalin

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2. Materials and methods

2.1. Case selection

This retrospective study was approved by the Institutional Review Board of Kangbuk Samsung Hospital (Seoul, South Korea). We included 401 cases of *KRAS* mutation test using colorectal cancer tissue, which were all cases performed at Kangbuk Samsung Hospital between May 2013 and December 2015. Based on the review of electronic medical records, specimen type, tissue harvest site, histologic type, and results of the mutation tests were recorded, and the times of cold ischemia and formalin fixation of tissue specimens were calculated.

2.2. Specimen fixation and processing

The biopsy tissue was immediately placed in 10% neutrally buffered formalin (NBF) and transferred to the pathology department. The fresh resection specimens were transferred immediately to the pathology department and stored in a refrigerator before tissue preparation. After opening of the lumen, the colorectal resection specimens were fixed in 10% NBF. After fixation, the biopsy specimens were examined grossly and resection specimens were sectioned to 5 mm thickness during gross examination. The tissue processing was performed by an automated system (tissue process PELORIS II, Leica, Germany). The processed tissue samples were embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E), and prepared on glass slides for microscopic diagnosis. During microscopic diagnosis, the cancer areas were marked on the glass slide by pathologists and the tumor cellularity was recorded by percentage.

2.3. DNA extraction

The FFPE samples were sliced into 5-micrometer sections, and the areas marked as cancer were collected. Genomic DNA was extracted using an FFPE Purification Kit for DNA (Maxwell ®16 MDx, Promega, Madison, WI, USA). The concentration and purity of extracted DNA were examined using the NanoDrop ND-2000 UV/VIS spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). In addition, DNA quantity was also assessed using Quant-iT PicoGreen (Thermo Scientific, Waltham, MA).

2.4. Mutation assay

The *KRAS* mutation was evaluated with a PNAClamp™ *KRAS* Mutation Detection Kit (Panagene, Daejeon, Korea). Briefly, the 20 μl reaction volume containing 10 ng of extracted DNA, a primer and PNA probe set, and SYBR green PCR master mix were used for each test. Real-time PCR reactions were performed on a CFX96 PCR detection system (Bio-Rad, Philadelphia, PA, USA). The cycle threshold (Ct) and delta CT (ΔCT) values were automatically calculated with the sample tube without PNA mix to evaluate the acceptability of sample DNA quality. Based on the Ct value, the sample DNA quality was classified as optimal ($22 < Ct < 30$), acceptable ($30 \leq Ct < 34$) or invalid ($Ct \leq 22$ or $Ct \geq 34$). The presence of mutations was determined by ΔCT according to the manufacturer's guidelines.

2.5. Prolonged formalin fixation test

We performed additional experiments to assess the sample DNA quality of tissue fixed beyond the routine fixation time range, since gradual degradation of DNA by prolonged fixation in formalin has been reported [20]. To test the effect of over-fixation on DNA quality, we harvested additional tumor tissue from 5 colorectal cancer resection specimens following pathological diagnosis. Three 3 mm^3 sized pieces of cancer tissue were obtained per colorectal cancer specimen. The pieces were fixed in 10% NBF for 30, 60, or 90 days and were then processed and tested for the *KRAS* mutation using the methods described above.

Table 1
Baseline clinicopathologic features of 401 *KRAS* mutation tests.

Variables	Number of cases (%)
Specimen type	
Resection	327 (81.5)
Biopsy	74 (18.5)
Harvest site	
Right side colon	65 (16.2)
Left side colon	45 (11.2)
Rectosigmoid colon	291 (72.6)
Cold ischemia time (hours)	
≤ 1	119 (29.7)
1-3	167 (41.6)
> 3	115 (28.7)
Formalin fixation time (hours)	
≤ 24	228 (56.9)
24–48	80 (20.0)
48–72	29 (7.2)
72–96	54 (13.5)
> 96	10 (2.5)
DNA quality	
Optimal	361 (90.0)
Acceptable	40 (10.0)
Results of mutation test	
Wild type	253 (63.1)
Codon 12 mutation	119 (29.7)
Codon 13 mutation	29 (7.2)

3. Results

3.1. Clinicopathological data

The baseline clinicopathologic features of the 401 cases are shown in Table 1. Most of the cases (396 cases, 98.8%) were diagnosed as adenocarcinoma, two cases were diagnosed as mucinous carcinoma, and three cases were diagnosed as mixed adenoneuroendocrine carcinoma. The mean cold ischemia and formalin fixation times were 5.3 h (range: 0–29) and 35.2 h (range: 8–150), respectively. Among the 401 cases, 361 (90.0%) were in the optimal range for Ct values, and 40 cases (10%) had Ct values in the acceptable range.

3.2. Differences in sample DNA quality and processing times between specimen types

Because cold ischemia and formalin fixation time can depend on the size of the specimen, we analyzed the specimen types separately. All biopsy specimens were in the optimal DNA quality range ($P < 0.001$) and showed a significantly shorter cold ischemia ($P < 0.001$) and a relatively shorter formalin fixation time ($P = 0.030$) compared to resection specimens (Table 2).

Table 2
Differences of specimen DNA quality, processing times and tumor cell density according to specimen type.

	Resection	Biopsy	*P* value
Specimen DNA quality			< 0.001
Optimal (22 < Ct < 30)	287 (87.8)	74 (100.0)	
Acceptable (30 ≤ Ct < 34)	40 (12.2)	0 (0)	
Cold ischemia time (hour)	6.5 ± 6.3	0 ± 0	< 0.001
Formalin fixation time (hour)	36.6 ± 22.3	29.0 ± 26.5	0.030
Tumor cellularity (%)	66.20 ± 22.38	63.92 ± 25.15	0.243

Data are presented as n (%) or mean ± standard deviation.

Table 3
Sample DNA quality according to processing time distribution.

	All specimens			Resection only			Biopsy only
	Optimal (22 < Ct < 30)	Acceptable (30 ≤ Ct < 34)	*P* value	Optimal (22 < Ct < 30)	Acceptable (30 ≤ Ct < 34)	*P* value	Optimal (22 < Ct < 30)
Cold ischemia time (hours)			0.038			0.868	
≤ 1	114 (95.8)	5 (4.2)		40 (88.9)	5 (11.1)		74 (100.0)
1–3	145 (86.8)	22 (13.2)		145 (86.8)	22 (13.2)		0 (0)
> 3	102 (88.7)	13 (11.3)		102 (88.7)	13 (11.3)		0 (0)
Formalin fixation time (hours)			0.395			0.741	
≤ 24	202 (88.6)	26 (11.4)		168 (86.6)	26 (13.4)		194 (59.3)
24–48	72 (90.0)	8 (10.0)		51 (86.4)	8 (13.6)		59 (18.1)
48–72	28 (96.6)	1 (3.4)		22 (95.7)	1 (4.3)		23 (7.0)
72–96	50 (92.6)	4 (7.4)		38 (90.5)	4 (9.5)		42 (12.8)
> 96	9 (90.0)	1 (10.0)		8 (88.9)	1 (11.1)		9 (2.8)

Ct, cycle threshold.
Data are presented as n (%).

3.3. Factors affecting on specimen DNA quality

The cold ischemia and formalin fixation times for the specimens with optimal sample DNA quality ranged from 0 to 29 h and from 8 to 150 h, respectively. A cold ischemia time of less than 1 h for all specimens was associated with optimal DNA quality ($P = 0.038$) (Table 3). However, when resection specimens were analyzed separately, cold ischemia time was not associated with sample DNA quality ($P = 0.868$). The percentage of samples with optimal DNA quality tended to increase as the formalin fixation time increased to 72 h, after which, DNA quality again deteriorated, but this observation was not statistically significant. Other clinicopathologic features, such as treatment with chemotherapy before specimen harvest, tumor shape, T stage, histologic differentiation and tumor size, were not correlated with specimen DNA quality (Table 4).

3.4. Factors affecting detection rates of the KRAS mutation

The detection rate of the *KRAS* mutation was significantly associated with DNA quality, although there was no significant correlation between mutation detection and specimen type (Table 5). Notably, the mutation detection rate for the acceptable range group was less than half of that for the optimal range group ($P = 0.027$).

Table 4
Clinicopathologic factors affecting acceptability of resection specimens.

	Optimal (22 < Ct < 30)	Acceptable (30 ≤ Ct < 34)	*P* value
Neoadjuvant chemotherapy			> 0.999
Yes	259 (87.5)	37 (12.5)	
No	28 (90.3)	3 (9.7)	
Shape of tumor			0.385
Polypoid	45 (81.8)	10 (118.2)	
Ulcerofungating	119 (89.5)	14 (10.5)	
Ulceroinfiltrative	104 (87.4)	15 (12.6)	
Infiltrative	18 (9.7)	1 (5.3)	
T stage			0.292
1 or 2	55 (83.3)	11 (16.7.)	
3 or 4	221 (88.8)	28 (11.2)	
Differentiation			0.870
Well	14 (87.5)	2 (12.5)	
Moderate	256 (87.4)	37 (12.6)	
Poor	12 (92.3)	1 (7.7)	
Diameter of cancer (cm)	4.5 ± 2.2	4.3 ± 2.2	0.884
Tumor cellularity (%)	66.12 ± 22.84	62.63 ± 23.42	0.537

Data are presented as n (%) or mean ± standard deviation.
Ct, cycle threshold.

Table 5
Detection rate of *KRAS* mutation according to specimen type and sample DNA quality.

Variables	Wild type	Codon 12	Codon 13	*P* value
Sample DNA quality				0.027
Optimal (22 < Ct < 30)	220 (60.9)	113 (31.3)	28 (7.8)	
Acceptable (30 ≤ Ct < 34)	33 (82.5)	6 (15.0)	1 (2.5)	
Specimen type				0.287
Resection	201 (61.5)	100 (30.6)	26 (8.0)	
Biopsy	52 (70.3)	19 (25.7)	3 (4.1)	

Data are presented as n (%).

3.5. Prolonged formalin fixation affecting to DNA quality

We confirmed that specimen DNA quality was sufficient for the *KRAS* mutation test when tissue samples were processed in range of formalin fixation times routinely used in pathology diagnosis. However, in the prolonged fixation experiment, the mean Ct value began to deviate from the optimal range after a month of fixation. As fixation time increased, the mean Ct value increased gradually, and the mean Ct value of specimens at baseline and those fixed for 30 days were significantly different (Fig. 1). Despite degradation of specimen DNA quality over time, changes in microscopic features were not noticeable (Supplementary Fig. 1).

4. Discussion

In this retrospective study conducted to identify factors affecting results of the *KRAS* gene mutation test, we confirmed that the biopsy specimens showed better DNA quality comparing the resection specimens. The cold ischemia time was an important factor for optimal DNA quality, but the formalin fixation time was not a significant factor within the time range of routine pathology diagnosis. In additional experiments assessing prolonged formalin fixation, the specimen DNA quality began to deteriorate after one month.

Formalin is the most commonly used fixative in the laboratory as well as in routine pathology diagnosis due to its low cost and ease of use. Identifying the optimal formalin fixation time for molecular techniques is important because formalin fixation interferes with molecular experiments by cross-linking proteins and other molecules in tissues [3,7,21,22]. In this study, formalin fixation time was not a significant factor affecting DNA quality of colorectal cancer tissues for *KRAS* mutation testing in routine diagnostic pathology, and a wide range of formalin fixation times (from 8 to 150 h) resulted in optimal specimen DNA quality. However, during additional testing, the DNA quality of samples fixed for over one month became increasingly worse over time,

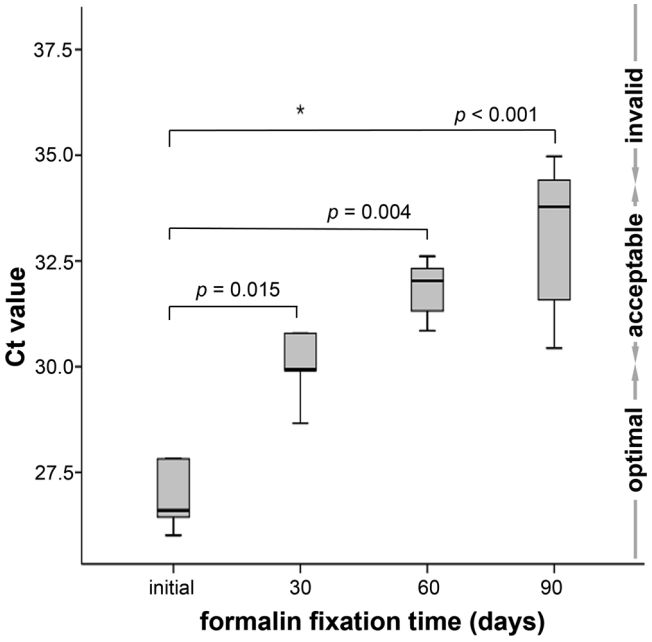

Fig. 1. Boxplot of cycle threshold (Ct) value according to formalin fixation time. The mean Ct value increases significantly as formalin fixation time increases. The mean Ct values at the time of the initial test and at 30 days, 60 days, and 90 days after the initial tests were 26.9, 31.1, 31.8, and 33.0, respectively. The mean Ct values at 30 days and after were all above the upper limit of the optimal range (optimal range: 22–30).

suggesting that specimens fixed for longer than one month should not be used for molecular tests. Castiglione et al. presented 24 h as the optimal formalin fixation time to preserve both tissue morphology and nucleic acids for molecular analysis of extracted COX-2 RNA [23]. However, the expression level of COX-2 was not significantly different between samples with 24 and 72 h of fixation time [23]. We similarly demonstrate the best DNA quality in samples with 48–72 hours of formalin fixation time, but further studies are needed to determine the optimal formalin fixation time to preserve the best quality DNA in colorectal resection specimens.

In this study, the biopsy specimens had better DNA quality than the resection specimens, which was presumably because the biopsy specimen had a short cold ischemia time. In Bray et al.'s study using human colorectal cancer tissue, changes in the RNA expression profile were detected after 15 min of tissue ischemic time and increased fourfold after 120 min [9]. One study using matched biopsies and excision specimens of breast cancer demonstrated that 3.4% of ER positive and 7.1% of PR positive biopsy specimens were matched to resection specimens that tested negative, suggesting that cold ischemia time and/or formalin fixation can change protein expression in immunohistochemical staining [21]. In a study using mouse liver, kidney and lung tissue, the authors demonstrated that tissue alkalosis resulting from cold ischemia could be a cause of gene expression change because increased cold ischemia time resulted in significantly increased pH [24]. Thus, the longer cold ischemia time of the resection specimen clearly affects the quality of DNA, RNA, and protein. However, there is controversy as to which sample should be used when biopsy and resection samples are present in the same tumor, since tumor heterogeneity also affects the results of the KRAS mutation test [25].

We note that there are some limitations in this study. First, we did not use matched tissue samples when comparing the results of biopsy and resection samples. Second, our results were derived from one mutation test for cancer in one organ. Therefore, these results should be validated with cancers of other organs at additional medical centers.

In conclusion, the specimen DNA quality for the *KRAS* mutation test was better in the biopsy samples than in the resection specimens, which was presumably due to shorter ischemia time. The formalin fixation time was not a significant factor for specimen DNA quality within the normal range of routine pathology diagnosis, but samples fixed in formalin for longer than one month showed significant quality degradation in a prolonged fixation experiment.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.02.018.

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