Method Optimization for Extracting High-Quality RNA From the Human Pancreas Tissue[1,2] CrossMark

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Abstract

Nucleic acid sequencing is frequently used to determine the molecular basis of diseases. Therefore, proper storage of biological specimens is essential to inhibit nucleic acid degradation. RNA isolated from the human pancreas is generally of poor quality because of its high concentration of endogenous RNase. In this study, we optimized the method for extracting high quality RNA from paired tumor and normal pancreatic tissues obtained from eight pancreatic cancer patients post-surgery. RNA integrity number (RIN) was checked to evaluate the integrity of RNA, we tried to extract the RNA with an RIN value of 8 or higher that allows for the latest genetic analysis. The effect of several parameters, including the method used for tissue lysis, RNA*later* treatment, tissue weight at storage, and the time to storage after surgical resection, on the quantity and quality of RNA extracted was examined. Data showed that the highest quantity of RNA was isolated using a combination of manual and mechanical methods of tissue lysis. Additionally, sectioning the tissues into small pieces (<100 mg) and treating them with RNA*later* solution prior to storage increased RNA stability. Following these guidelines, high quality RNA was obtained from 100% (8/8) of tumor tissues and 75% (6/8) of normal tissues. High-quality RNA was still stable under repeated freezing and thawing. The application of these results during sample handling and storage in clinical settings will facilitate the genetic diagnosis of diseases and their subsequent treatment.

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Introduction

Genomic sequencing can determine the molecular basis of diseases in humans, which can be used to develop a plan comprising personalized medicine and therapeutic agents [1–3]. Several studies have recently reported the application of genetic approaches for taking medical decisions in various clinical fields [4–6]. However, to improve the accuracy and utilization of results obtained from using genetic approaches, it should be prioritized to secure a resource bank that can reflect various clinical environments and to obtain a high-quality genome from resource [7,8].

Ribonucleic acid (RNA) is highly susceptible to degradation by RNase, which is a ubiquitous enzyme. Sequencing of degraded RNA fragments yields less reproducible and unreliable data [9–11]. Therefore, it is critical to evaluate the quality of extracted RNA.

The Agilent 2100 BioAnalyzer is a microfluidic system introduced in 1999 capable of performing qualitative analysis of RNA in a high-
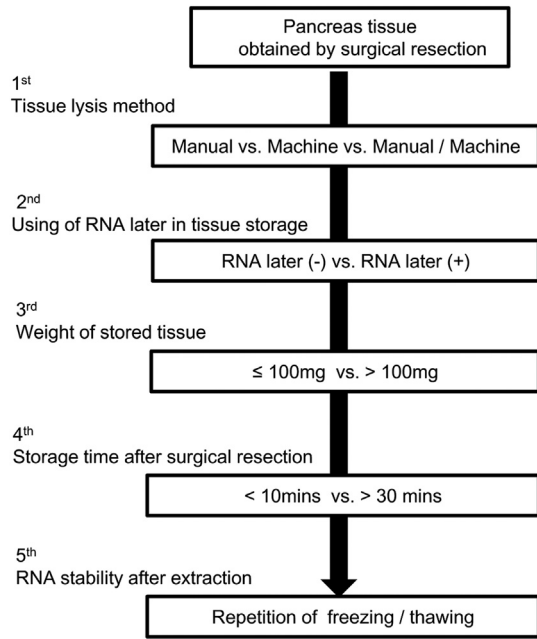
Figure 1. Schematic showing the study design used to optimize the method for RNA extraction from human pancreas.

throughput manner. The BioAnalyzer estimates the degree of degradation on a scale of 0–10 using a parameter called RNA integrity Number (RIN) [12]. The range of possible analyses of RNA depends on its RIN value; RNA with RIN ≤5 is not suitable for any analysis, 5–6 can be used for RT-qPCR, 6–8 can be used for microarray experiments, and >8 can be used with any latest technologies [13–16].

Human pancreatic tissue undergoes quick autolysis because of the abundance of endogenous RNase, DNase and proteases that are secreted during surgical dissection [17–19]. RNA extracted from these tissues exhibits varying degrees of degradation, as indicated by RIN values ranging from 2.5–8.7 (average 6.35) [20,21]. These RIN values of RNA isolated from pancreatic tumor tissue are substantially lower than those of RNA isolated from colon, breast and prostate tumors [22–25].

Here, we used normal and tumor tissues of the pancreas as targets for optimizing the method for extracting high quality RNA. We studied the effect of various parameters, including the method of tissue lysis, efficacy of storage solutions, amount of tissue stored and

time from tissue harvesting to storage on the stability of extracted RNA. Determining optimum limits for each of these parameters will enable efficient extraction of high quality RNA that can be analyzed using advanced techniques, thus allowing new diagnoses and therapeutic approaches.

Material and Methods

Study Design and Clinical Data

This study complied with the Declaration of Helsinki and was reviewed and approved by the Institutional Review Board (IRB) of Asan Medical Center (IRB No. 2015–0480). From June to December 2016, patients who underwent surgery for pancreatic cancer at Asan Medical Center were enrolled for this study. Written informed consent was acquired from all patients. Clinical information on the patients' age, sex, body mass index (BMI), diabetes mellitus (DM), preoperative chemotherapy, surgical procedure, pathologic diagnosis and findings of excised tissues was collected from their medical records.

Tissue Storage

Tumor and normal tissues were partially resected from surgical specimens and weighted, while taking appropriate measures to avoid RNase contamination. Depending on the amount, only a subset of the entire groups could be obtained in some cases. The paired group was defined as the case with the tissue stored both tubes with and without the RNA*later* (QIAGEN, MD, USA) as RNA stabilization reagent. If there was only a tissue stored in the RNA*later*, it was defined as an un-paired group. Depending on the weight, tissues were divided into two groups: <100 mg and >100 mg. The resected tissues were transferred to 1.5 ml cryotubes that were either empty or contained 700-μl of RNA*later* solution. Depending on the time elapsed between tissue harvesting and tissue storage, tissues were divided into two groups: <10 min and >30 min. During the time between harvesting and storing, tissues were stored in normal saline. Tissues in empty cryotubes were immediately stored in a deep freezer at –80 °C, whereas those in tubes containing RNA*later* were stored at 4 °C for 24 hours and later transferred to the deep freezer at –80 °C after carefully suctioning the RNA*later* solution.

Tissue Lysis and RNA Extraction

Tissues stored in cryotubes were cut into pieces weighing <40 mg and placed in 500-μl lysis buffer from the RNeasy Mini Kit (QIAGEN, MD, USA). To physically break the tissues, the tissue

Table 1. Clinicopathologic characteristics of enrolled patients

No.	Host Factor				Tumor factor				Tissue storage			
	Sex/ Age	BMI	DM	Preop CTx	OP	Tumor size (cm)	Histology	Diff	RNA later (–)	RNA*later* (+)		
										weight< 100 mg, time< 10mins	weight> 100 mg, time< 10mins	weight< 100 mg, time> 30mins
1	M/73	24.5	yes	no	DPS	3.8	PDAC	mod	X	O	O	O
2	M/54	23.8	yes	yes	DPS	3.2	PDAC	mod	X	O	X	X
3	M/75	22.4	no	no	DPS	4.3	PDAC	por	O	O	O	O
4	M/80	18.3	no	no	DPS	3.0	PDAC	mod	O	O	O	O
5	M/54	24.2	yes	no	DPS	2.3	ASC	n/a	O	O	O	O
6	F/59	21.1	no	no	DPS	1.8	PDAC	mod	O	O	O	O
7	M/55	23.6	yes	no	DPS	5.0	PDAC	mod	O	O	O	O
8	M/64	26.4	no	no	DPS	4.0	PDAC	mod	X	O	X	X

(Abbreviations: BMI; Body mass index, DM; Diabetes mellitus, Preop CTx; preoperative chemotherapy, OP; operation, Diff: differentiation, DPS; distal pancreatectomy and splenectomy, PDAC; pancreatic ductal adenocarcinoma, ASC; Adenosquamous carcinoma, mod; moderately, por; poorly.)
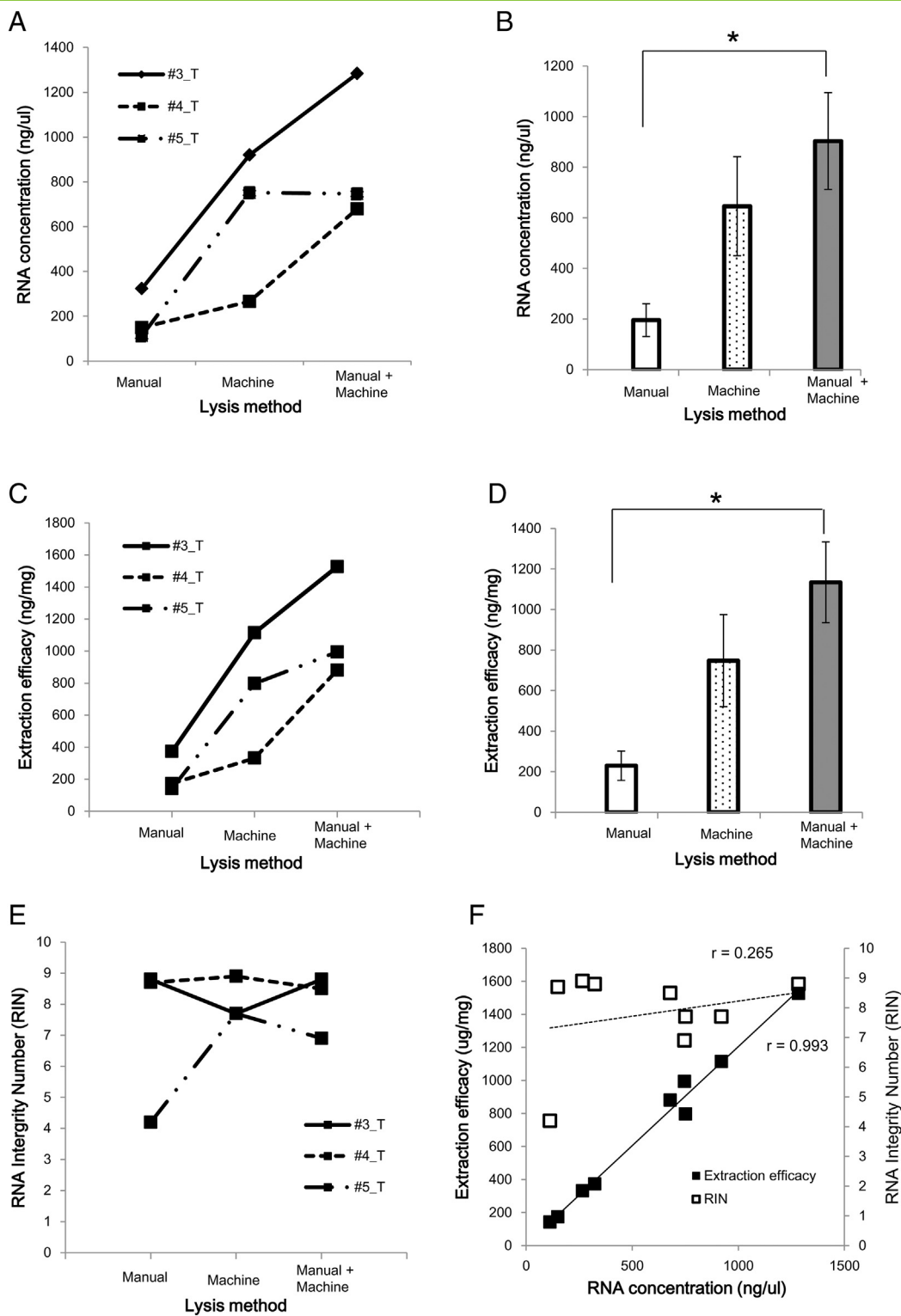
Figure 2. Effect of the tissue lysis method on RNA extraction efficacy and RNA integrity. A, Concentration of extracted RNA according to lysis methods in three tumor tissues (T; tumor tissue). B, Summary of RNA concentration extracted by lysis methods (*$P < .05$). C, Efficacy of RNA extraction according to lysis methods in three tumor tissues. D, Summary of extraction efficacy according to lysis methods (*$P < .05$). E, RNA integrity number (RIN) of extracted RNA according to lysis methods in three tumor tissues. F, Comparison of extraction efficacy and RIN based on the RNA concentration (r; Pearson correlation coefficient).

pieces were subjected to either manual disruption on ice using a disposable pestle (KIMBLE, NJ, USA), or mechanical disruption using the Tissue Ruptor II (QIAGEN, MD, USA) or a combination of the two. Tissue Ruptor II was used with a 1 min ON/15 s OFF cycle to avoid heat generation, and tube was moved to ice during the OFF phase. When using both methods, the Tissue Ruptor II was used after confirming that a large lump was lysed with a disposable pestle. For all samples, lysis was performed within 10 min.
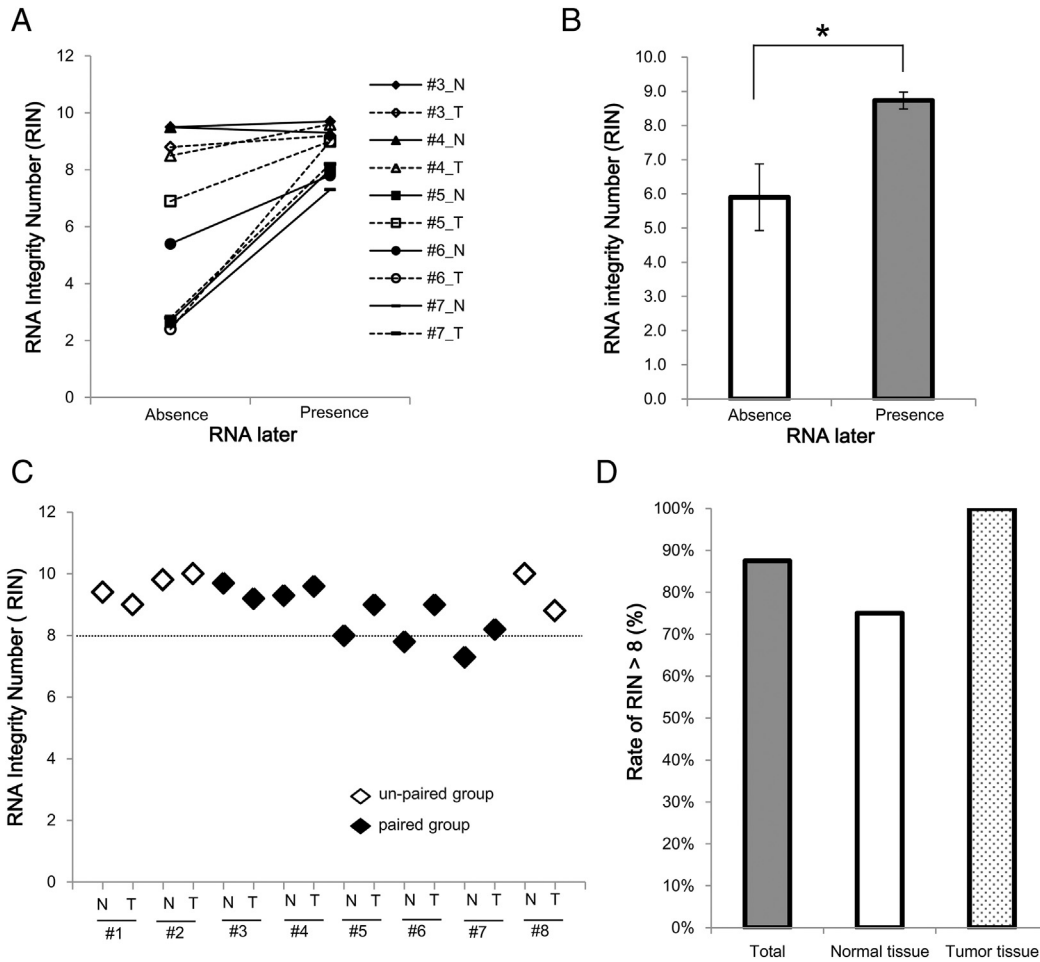
Figure 3. Effect of RNA*later* treatment of pancreas tissues on RNA integrity. A, RIN value of extracted RNA with or without RNA*later* treatment (N, normal tissue; T, tumor tissue). B, Summary of RIN values of extracted RNA with or without RNA*later* treatment (*$P < .05$). C, RIN values of all tissues using RNA*later* (dotted line; RIN = 8). D, Percentage of normal and tumor tissues with RIN >8.

Total RNA was extracted from the lysed tissue samples using RNeasy Mini Kit, according to the manufacturer's instructions, and eluted in 40-μl elution buffer. The concentration and purity of RNA were confirmed using NanoDrop-2000 Spectrophotometer (Thermo Fisher, MA, US), and the RNA was stored at −80 °C until needed for downstream experiments. RNA extraction efficacy was calculated by multiplying eluted volume and RNA concentration and dividing by weight of lysis tissue. To evaluate RNA stability, the extracted RNA was subjected to freezing at −80 °C followed by thawing at 37 °C for 1 hour. This freezing and thawing process was repeated several times.

RIN Estimation

The integrity of isolated RNAs was determined with the Agilent Bioanalyzer 2100 using an RNA 6000 Pico Lab Chip kit (Agilent Technologies, USA) [20]. The quality of each RNA sample was estimated on the basis of fragment size distribution indicated by two peaks corresponding to 18S and 28S ribosomal RNAs and a signal from small RNAs. The quality of RNA was assessed based on RIN values ranging from 1 to 10, with 1 being the most degraded and 10 being the most intact, using a RIN algorithm.

Statistical Analysis

Statistical analyses of RIN values were conducted using Student's *t* test and analysis of variance with SPSS for Windows version 21.0 (IBM Corp Armonk, NY, USA). Pearson correlation coefficient was used to analyze correlations between RNA quantity/quality and another variable. P-values less than 0.05 were considered statistically significant.

Result

Study Design and Clinicopathologic Characteristics of Enrolled Patients

In this study, we examined the effect of variables on the quantity and quality of RNA extracted from normal and tumor pancreatic tissues in five sequential steps (Figure 1). These variables included the method of tissue lysis, use of a commercial RNase-inactivating chemical called RNA*later* during tissue storage, weight of tissue during storage (≤ 100 mg vs. > 100 mg), storage time (< 10 min vs. > 30 min) and repeated freezing and thawing of RNA samples. Pancreatic tissues were obtained from patients diagnosed with pancreatic ductal adenocarcinoma (except one patient) who underwent distal pancreatectomy. The average size of pancreatic tumors was 3.4 cm (Table 1).
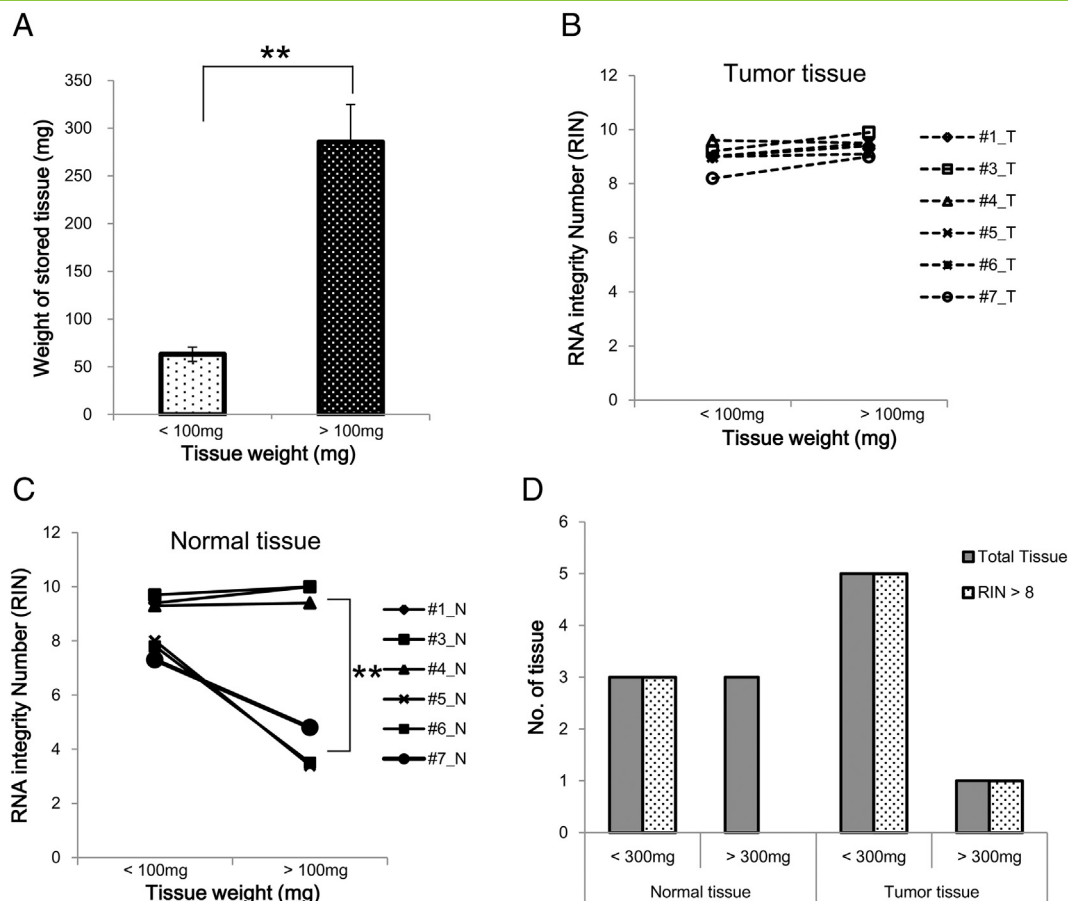
Figure 4. Effect of tissue weight during storage on RNA integrity. A. Comparison of tissue weights stored based on 100 mg of weight (**$P < .01$). B. RIN value of extracted RNA based on 100 mg of weight of tumor tissue. C. RIN value of extracted RNA based on 100 mg of weight of normal tissue (**$P < .01$). D. Based on 300 mg,), the number of normal and tumor tissues with RIN >8.

Effect of the Lysis Method on RNA Extraction Efficacy

Three tumor tissues were used to determine the effect of lysis method on the quantity and quality of the extracted RNA. In comparison to the manual method of tissue lysis (using disposable pestle), the combination of manual and mechanical lysis resulted in higher quantities of extracted RNA (Figure 2, A and B; $P = .025$). Moreover, a combination of manual and mechanical disruption significantly improved the extraction efficacy (Figure 2, C and D, Supplementary Table 1; $P = .013$). The RIN values of extracted RNA value was evaluated, but a certain pattern according to the method was not confirmed (Figure 2D). The RNA concentration obtained using each method showed a positive correlation with extraction efficacy ($r = 0.993$) but not quality ($r = 0.265$; Figure 2E, Supplementary Figure 1). Based on these data, a combination of manual and mechanical lysis was used for isolating RNA in all subsequent experiments

Effect of RNAlater Treatment on RNA Integrity

Extracted RNAs treated with and without RNA*later* solution showed an average RIN of 8.7 and 5.9, respectively (Figure 3, A and B, Supplementary Figure 2, A and B). Treatment of tissues with RNA*later* solution significantly reduced RNA degradation ($P = .012$). In eight pairs of cancerous and normal tissues, including three pairs of un-paired group, all eight tumors tissues showed RIN ≥8, whereas only six of eight normal tissues showed RIN ≥8 and the remaining two normal tissues showed RIN values of 7.3 and 7.8 (Figure 3, C and D).

Effect of Tissue Weight on RNA Integrity

RIN analysis was performed to determine the effect of the weight of tissue stored in RNA*later* on the RNA integrity. The average weight of the group weighing >100 mg was approximately 4.5-fold higher than that of the group weighing <100 mg (285.3 mg and 63.2 mg, respectively); this difference was statistically significant ($P < .001$) (Supplementary Table 2, Figure 4A). No significant differences were detected in RIN values between cancerous tissues weighing >100 mg and those weighing <100 mg (Supplementary Figure 3, A and B, Figure 4B). By contrast, among normal tissues, RNA was significantly degraded in tissues weighing >100 mg (Figure 4C, $P < .001$). In contrast to tumor tissues, none of the normal tissues weighting more than 300 mg showed RIN >8 (Figure 4D, Supplementary Figure 3C).

Effect of Time to Tissue Storage on RNA Integrity

Based on the time taken between harvesting the tissues from patients and storing them in RNA*later*, the tissues were divided into two groups, group 1 (<10 min) and group 2 (>30 min). We determined the effect of the time lapse between tissue harvesting and storage on RNA integrity by comparing the RIN values of RNA extracted from tissues in group 1 with those in group 2. No significant differences in RIN values were detected between group 1 and group 2 tissues regardless of whether the tissues were normal or cancerous (Figure 5A, Supplementary Figure 4). However, when comparing RNA extracted from tumor tissues with that extracted from normal
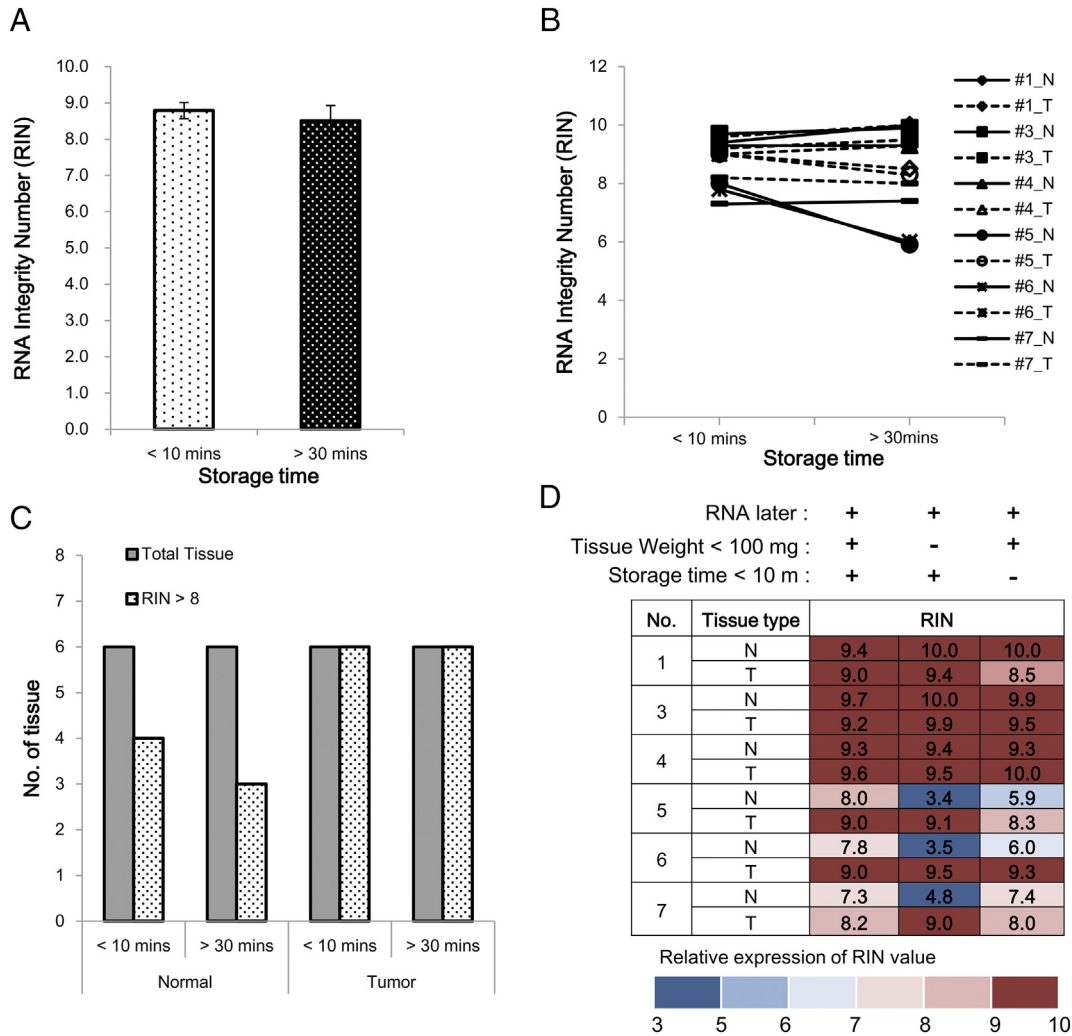
Figure 5. Effect of time to storage on RNA integrity. A, RIN values of extracted RNA according to the time to storage. B, RIN values of extracted RNA for each tissue according to the time to storage. C, The number of cases with RIN >8. D, RIN values and heat map of the extracted RNA according to each condition.

tissues, fewer normal tissues showed RIN ≥8 (Figure 5, B and C).). Overall, results of RNA*later* treatment, tissue weight (>100 mg) and time to storage (>30 min) showed a reduction in RNA stability, especially in normal tissues (Figure 5D).

Effect of Freeze–Thaw Cycles on RNA Integrity

After extracting high quality RNA, the effect of repeated freezing and thawing on RNA integrity was determined. Although the average RIN value decreased from 9.3 to 8.6 upon repeated freezing and thawing, this decrease was not statistically significant (Figure 6, A and B; Supplementary Figure 5). These data were consistent in both tumor and normal tissues.

Discussion

Rapid development of genetic analysis techniques has led to the identification of the molecular basis of various diseases and has redefined the fundamental understanding of disease pathogenesis [1,2]. For this approach, banking of quality-controlled human resources is essential; RNA integrity is one such quality indicator required for this banking [7,26,27]. Because RNA is highly susceptible to RNase-

mediated degradation, the degree of damage via RNase encountered in the tissue storage process can be used as an indicator for taking precautions during tissue storage [28,29].

There is a method to assess the quality of the extracted RNA [9]. RNA was electrophoresed on agarose gel and analyzed for specific patterns of ethidium bromide staining. Non-degraded RNA clearly showed two bands representing the 28S and 18S ribosomal RNAs, whereas degraded RNA showed diffused banding patterns with small-sized fragments. To automate the process of determining RNA quality, we used Agilent 2100 BioAnalyzer, which determines the quality of RNA using RIN values ranging from 1–10, where 1 indicates complete degradation and 10 indicates no degradation. RNAs with RIN ≥ 8 can be subjected to a range of downstream analyses using the latest techniques [13–16].

The average RIN values of the organ-specific RNAs in the tissue bank have been reported as 7.5 for colon cancer tissues [22], 7.87 and 8.9 for breast cancer tissues [23,24], 8.7 for prostate cancer tissues [25], and 6.35 for pancreatic tissues [20,21]. The relatively low RIN value of pancreas tissue compared with that of other tissues can be attributed to the peculiar features of the pancreas. The pancreas secretes digestive enzymes into the pancreatic duct and hormones into
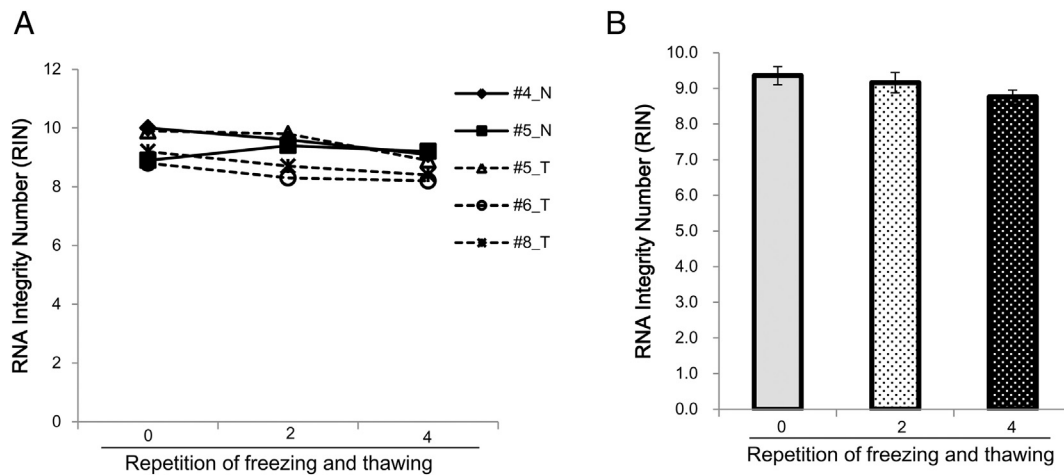
Figure 6. Effect of repeated freezing and thawing on RNA stability. A, Change in individual RIN values after freezing and thawing. B, Summary of changes in RIN values after freezing and thawing.

the blood stream to regulate glucose levels, and has an abundance of RNase [19]. Therefore, pancreatic RNAs are easy degraded during tissue harvesting, storage and tissue lysis.

In this study, we developed a procedure to isolate RNA from pancreatic tissue with a quality that is sufficient to enable genetic analysis. This involved optimizing various parameters in a phased approach and using an experimental setup that reflected a clinical setup after surgery. Results of this study revealed several practical clues for the extraction of high quality RNA. First, considering the unique characteristics of pancreatic cancers, proper using of manual and mechanical lysis was effective in obtaining sufficient amounts of RNA. Pancreatic cancer undergoes a pathological feature called desmoplasia, which is characterized by an increase in fibroblasts and the deposition of extracellular matrices [30,31]. Desmoplasia hardens the tumor tissue, making it partially resistant to lysis. Moreover, the amount of tissue available for research post-surgery is limited. Therefore, the tip of a disposable pestle is not sufficient for effective lysis of the tissue, as it rotates together with the tissue in the tube. To resolve this problem, we determined that breaking the hard tissue using direct manual pressure followed by mechanical lysis with tissue ruptor was most effective for extracting large quantities of RNA. Second, RNA degradation in pancreatic tissue was minimized by storing the tissue in RNA*later* solution. Although RNA*later* is a commercially available product, its use with pancreatic tissues containing high amounts of RNase has not yet been established. Recently, injecting RNA-*later* directly into the pancreatic parenchyma or pancreatic duct of mouse [17,32], those of pig has shown to increase RNA stability [33]. In this study, our results showed that RNA*later* dramatically improved RNA stability in human pancreatic tissues as well. Third, we demonstrated that high quality RNA isolated from tissues treated with RNA*later* was resistant to degradation by repetitive freeze–thaw cycles. Results showed that RNase activity was either completely absent or minimal in the tissue samples following RNA*later* treatment. Finally, we confirmed that normal tissues were more vulnerable to RNase degradation than tumor tissues, suggesting that clinicians should be extra careful while procuring and storing tissues.

Although experiments carried out in this study were designed considering various clinical settings to derive practical results, there

were some limitations to generalizing this method. First, this study focused only on tissues from pancreatic cancer. These methods should be validated in other organs and environments. However, because the extent of RNA degradation in pancreatic tissues is likely to be greater than that in other organs, we expect results of this study to be applicable to other organs. Second, although tissues for clinical research are stored in many ways, the most common being paraffin-embedded sections, all tissues used in this study were stored at –80 °C. Therefore, it is necessary to perform additional experiments using other storage methods to verify the results of this study.

Conclusions

In the study, we optimized the method to extract high quality RNA from human pancreas through a stepwise study in accordance with clinical situation. This approach is expected to be of practical assistance to clinicians, particularly those who procure tissues directly from pancreatic cancer patients. These results have the potential to improve the basic understanding of many diseases, especially pancreatic cancer, and consequently, to facilitate new diagnoses and therapeutic approaches.

Funding

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.04.004.

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