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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Biobanking of Fresh-frozen Human Colon Tissues: Impact of Tissue Ex-vivo Ischemia Times and Storage Periods on RNA Quality

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ABSTRACT

Background. Biobanking plays an important role in translational cancer research. The impact of tissue ex-vivo ischemia time and storage period on RNA integrity is not well documented.

Methods. Fresh-frozen colon tissues were collected in Taizhou Hospital of Zhejiang Province in China since 2004. Fifty-one colon cancer tissues with tumor cell content higher than 70 % and matched normal tissues during four storage periods (less than 15 months, 16–20 months, 21–25 months, and 26–40 months) were chosen to detect RNA quality. Fresh colon cancer tissues from 5 patients were cut into pieces and kept at room temperature or on ice for 0.5, 1, 2, and 4 h before snap freezing. RNA integrity was determined by microcapillary electrophoresis by the RNA integrity number (RIN) algorithm.

Results. Sixty-seven percent of normal colon tissues and 94 % of colon cancer specimens yielded RNA with a RIN of $\geq$7. Matched colon cancer and normal tissues showed significant difference in RNA quality. RNA remained stable in colon cancer tissues kept at room temperature and on ice for up to 4 h, and long-term storage of banked colon specimens did not negatively influence RNA quality (RNA with RIN of $\geq$7 banked less than 15 months, 83 %; 16–20 months, 78 %; 21–25 months, 77 %; 26–40 months, 90 %).

Conclusions. Frozen colon tissues yield high-quality RNA in approximately 80 % of specimens. Ex-vivo ischemia times and storage periods did not adversely affect RNA quality. This study showed that standard operation protocols and the maintenance of high-quality tissue repositories were the keys to translational medicine research.

Along with the emergence of new genomic technologies, particularly tissue-based microarrays, the need for high-quality cancer biospecimens is rapidly increasing. Researchers hope to obtain as much biological information as possible to identify the biological characteristics of cancer cells at a specific point in time. Clinicians hope to determine the therapy strategy by comparing the biological characteristics of cancer cells or tissues with the clinical information. Therefore, the availability and quality of biospecimens are critical to making progress in the fight against cancer.

In order to collect the tissue biospecimens for cancer research, many tumor tissue biobanks have been constructed in such countries as the United States, Canada, the United Kingdom, and France.[1,2] In 1987, the Cooperative Human Tissue Network was first established in the United States.[3] Recently, the National Cancer Institute developed a National Cancer Human Biobank (caHUB) to make high-quality tissues and data available to the cancer research community.[4,5] In China, cancer tissue bank construction began at the end of the 20th century. Several tissue biobanks were established at some large hospitals and cancer centers.[6–8]

Biospecimen quality is vital to tissue biobanks. It is dependent on standardized handling processes and on

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biospecimen research science that explores how collection, processing, storage, and transport procedures affects the biospecimen's molecular characteristics and ultimate usefulness in cancer research.[9,10] Although the importance of the quality of banked tissues has long been recognized, to date, few data exist on the quality of banked human tissues.[11–13]

The goal of the current study was to assess the quality of colon tissues held at the human tissue bank of Taizhou Hospital of Zhejiang Province. We investigated a panel of 51 fresh-frozen colon cancers and 51 matched normal colon tissues at various time points after surgery for RNA integrity.

MATERIALS AND METHODS

Human Colon Tissue Samples

Colon cancer tissue samples and matched normal colon tissues were collected from 101 patients who underwent surgery for colon cancer at Taizhou Hospital of Zhejiang Province from October 2007 to May 2010 and were stored in the Taizhou human tissue bank. The patients were not treated with chemotherapy or radiotherapy before their operation, and consent for the donation of specimens was obtained. The Tissue Bank Management Board and Ethics Committee of Taizhou Hospital of Zhejiang Province gave approval for the banking and use of the procured specimens. All patients undergoing colon resection for all histological diagnoses are eligible.

Tissue Banking Process

All patients who consent to donate their specimens are identified by the tissue bank assistant the day before operation. At the time of resection, the specimens are oriented by the attending surgeon and are transported immediately in prelabeled containers at room temperature to the pathology facility near the operation facility, and personnel immediately make a gross examination of the specimen. Tissues were collected in a range that would not affect diagnosis. The tumors were sectioned at the midline, and starting from the left side and advancing to the right, colon tissues $0.5 \times 0.5 \times 0.5$ cm^3 in size were collected. The mucosa layer of the adjacent normal colon tissues was also collected at the same time. The specimen then underwent routine histopathological processing. Immediately after removal, tumor and normal tissue aliquots were stored in labeled cryovials and snap-frozen in liquid nitrogen, then transferred to a low-temperature refrigerator (−80 °C). The clinical information and tissue ex-vivo ischemia times were recorded. Tissue ex-vivo ischemia time refers to the time from tissue excision to cryopreservation. The time of excision is defined as that of removal of the tissues from the operating table. The time of cryopreservation is defined as the time that the tissues was stored in a liquid nitrogen vapor freezer.

For this study, data were sorted into four groups according to storage periods (number of months between snap freezing and RNA extraction), as follows: less than 15 months, 16–20 months, 21–25 months, and 26–40 months. These data were graphed and analyzed by Microsoft Excel.

Histology

One small piece of tissues was used for hematoxylin and eosin (H&E) staining of each patient sample. All specimens were evaluated by a second H&E quality control of each aliquot because many cases had multiple aliquots to assure tumor cell content higher than 70 %. The tumor cell content was calculated on the basis of the ratio of the tumor area and the other area. The pieces from the frozen aliquots were fixed overnight in neutral buffered formalin and then transferred to 70 % ethanol before processing to a paraffin block. Five-micron-thick sections were microtomed, and the sections were placed on slides. The slides were then baked for 1 h at 60 °C in an oven and were stained with H&E. The histologic evaluation was completed by two experienced pathologists.

Tissue Ex-vivo Ischemia Times

For analyzing the impact of tissue ex-vivo ischemia times on RNA quality, fresh colon cancer tissues from five patients were obtained directly after surgical resection in the operation theater. The tissues were cut into nine cubes; one of the pieces was immediately put in a cryovial, frozen in liquid nitrogen, and transferred to a −80 °C environment. These samples served as references (time point 0 h). The remaining tissue cubes were transferred to eight different cryovials and then kept on ice or at room temperature. After 0.5, 1, 2, and 4 h, one of tissue pieces was removed from each condition, frozen in liquid nitrogen, and transferred to −80 °C until RNA extraction and RNA integrity detection.

In this study, tissue ex-vivo ischemia times of matched colon cancer and normal specimens stored routinely within the biobank were recorded and grouped as follows: ≤15 min, 16–30 min, and 31–60 min. These data were graphed and analyzed by Microsoft Excel.

RNA Quality

Fifty-one matched colon cancer and normal samples and others from the five patients we mentioned above were selected for RNA quality detection. Each specimen was

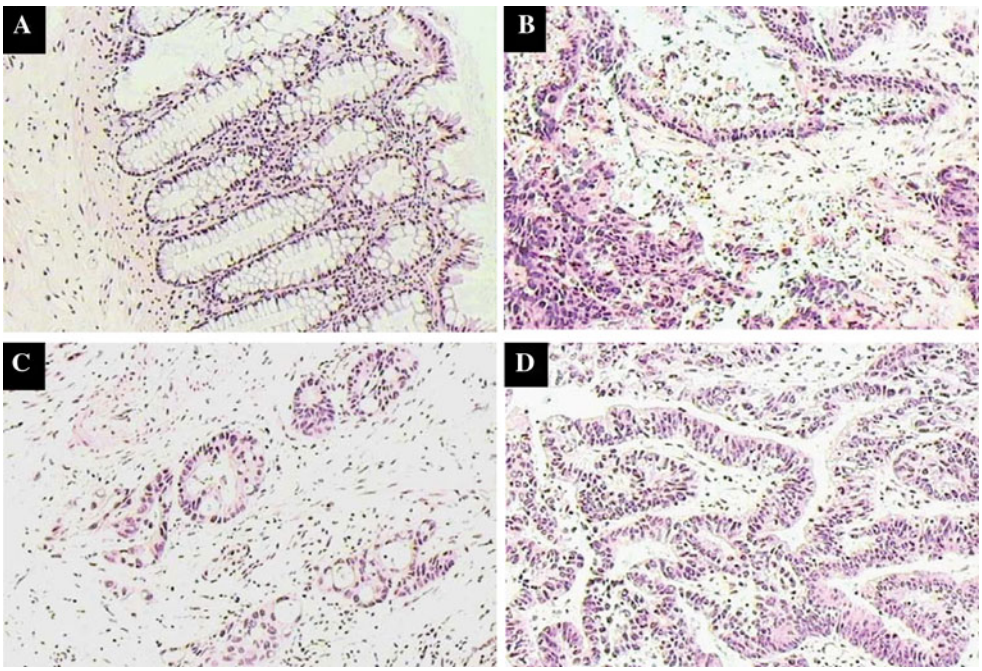

FIG. 1 Paraffin-embedded section of colon cancer samples. **a** Normal colon tissue. **b** Sample shows 80 % of sample is necrosis with about 20 % tumor. **c** Sample shows less than 20 % of sample is tumor. **d** Sample with more than 70 % of sample as tumor (×100)

homogenized in Trizol reagent (Invitrogen, Carlsbad, CA), and total RNA was extracted according to the manufacturers' instructions. The isolated RNA was analyzed on the Agilent 2100 bioanalyzer by Shanghai Biotechnology Corporation. To assess RNA integrity, RNA integrity numbers (RINs) are calculated by Agilent software. By means of this method, RNA integrity was determined by the entire electrophoretic trace of the RNA sample (total of 10 features), including the presence or absence of degradation products.

Statistical Analysis

Statistical analysis was performed by SPSS 13.0 (SPSS, Chicago, IL, USA). The differences of RNA concentration between colon cancer tissues and matched normal control were validated by the independent-sample *t* test. Pearson Chi-square tests were performed to compare the proportion of colon specimens with high-quality RNA (RIN of $\geq$7) banked during different tissue ex-vivo ischemia times and storage periods. A p value of <0.05 was considered to be statistically significant.

RESULTS

Histology

To keep the colon cancer tissue specimens histologically representative and to avoid areas of necrosis, aliquots of colon cancer tissues from 101 colon cancer patients in the human biobank of Taizhou Hospital of Zhejiang Province were sectioned and stained with H&E. The tumor cell content in 64 (63.4 %) of 101 was higher than 70 % (Fig. 1), and in these specimens, we randomly selected 51 colon cancer tissues and matched normal colon tissues for RNA quality evaluation.

RNA Quality of Tissue Samples Stored Routinely within the Biobank

A total of 102 colon tissue specimens including 51 colon cancer tissues and 51 matched normal colon tissues were detected for RNA quality. As the standard for RNA integrity, the RIN was calculated for all specimens.

A standard RIN of $\geq$7 was set as a cutoff for RNA quality sufficient for RNA microarray analyses and RNA expression arrays. The results indicated that overall, 80.4 % of all samples had a RIN of $\geq$7, with 94 % in colon cancer tissues (48 of 51) and 67 % in normal colon tissues (34 of 51), respectively. A significant difference was observed between colon cancer tissues and matched normal tissues ($p < 0.001$, Fig. 2a). The changes in RIN values for each matched cancer and normal sample are shown in Fig. 2c.

The RNA concentration was obviously higher in colon cancer tissues compared to the normal-tissue counterparts (0.39 ± 0.14 μg colon cancer vs. 0.24 ± 0.09 μg normal colon, $p < 0.001$, Fig. 2b).

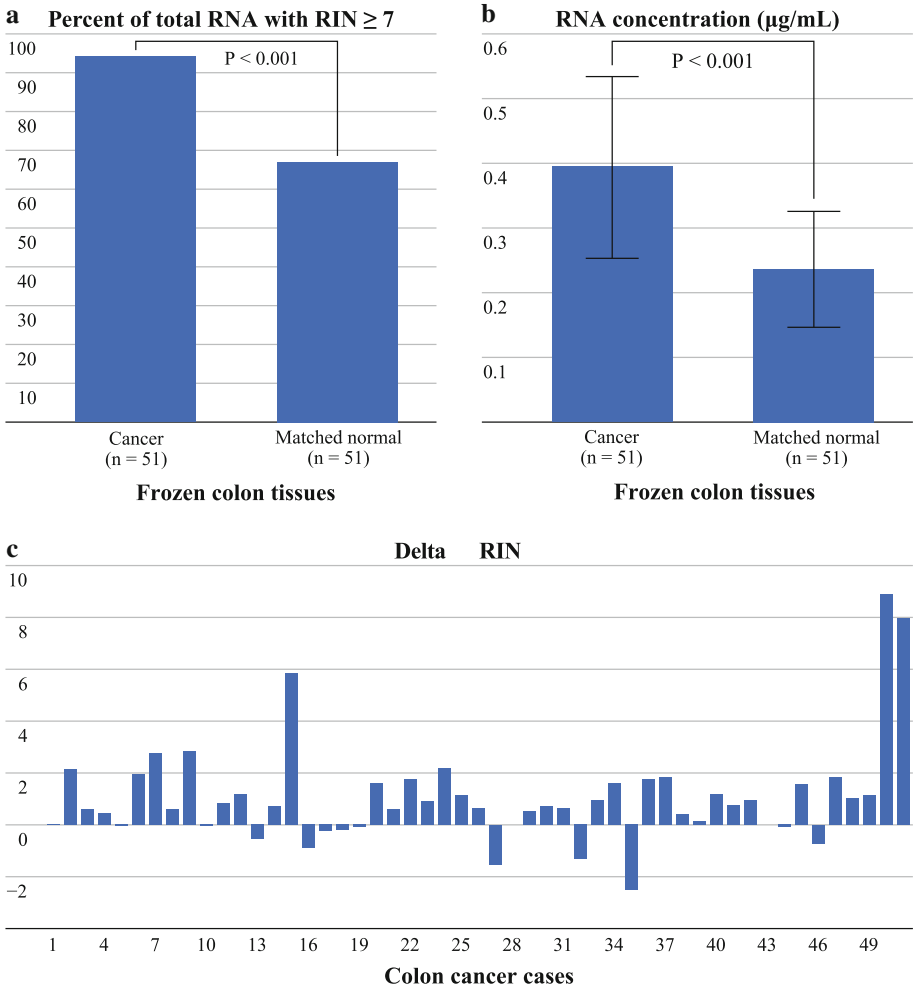

FIG. 2 RNA quality comparison between matched cancer and normal samples. **a** Significant difference was observed in the percentage of total RNA with a RIN of ≥7 between matched cancer and normal samples. **b** Significant difference was observed in RNA concentration between matched cancer and normal samples. **c** The change in RIN between RIN_{cancer} and RIN_{normal} for each matched sample

Effect of Tissue Ex-vivo Ischemia Times on RNA Integrity

To evaluate the influence of tissue ex-vivo ischemia times under controlled conditions on RNA integrity, fresh colon cancer tissues from five colon cancer patients were collected, separated, and placed on ice or held at room temperature at specific time points (0, 0.5, 1, 2, and 4 h) before snap freezing in liquid nitrogen. RNA was extracted, and the integrity was assessed on the basis of the electropherograms after microchip gel electrophoresis. The electropherograms of all samples showed distinct ribosomal peaks, indicating well-preserved RNA. No sign of degradation was detected during storage on ice or at room temperature, even after incubation for 4 h (Fig. 3a, b).

Furthermore, 39 fresh-frozen colon cancers and 39 matched normal colon tissues with their tissue ex-vivo ischemia times recorded and that were stored routinely within the biobank were grouped as follows: tissue ex-vivo ischemia times of ≤15 min, 16–30 min, and 31–60 min, and the RNA integrity detected. No obvious differences were observed among the different groups (Fig. 3c).

Relationship between Storage Periods and RNA Integrity

To assess the relationship between storage periods and RNA integrity, the storage periods of all 102 colon tissue samples were sorted into four groups: less than 15 months, 16–20 months, 21–25 months, and 26–40 months. The percentage of total RNA with RIN of ≥7 for colon specimens collected within the four time periods is shown in Fig. 4. The results indicated that the proportion of samples with high-quality RNA (RIN of ≥7) within the different storage periods did not statistically differ ($p < 0.05$, Fig. 4). The storage periods did not negatively affect RNA integrity.

DISCUSSION

Advances in genomics, proteomics, and biomarker studies have catalyzed changes in the field of cancer research. A

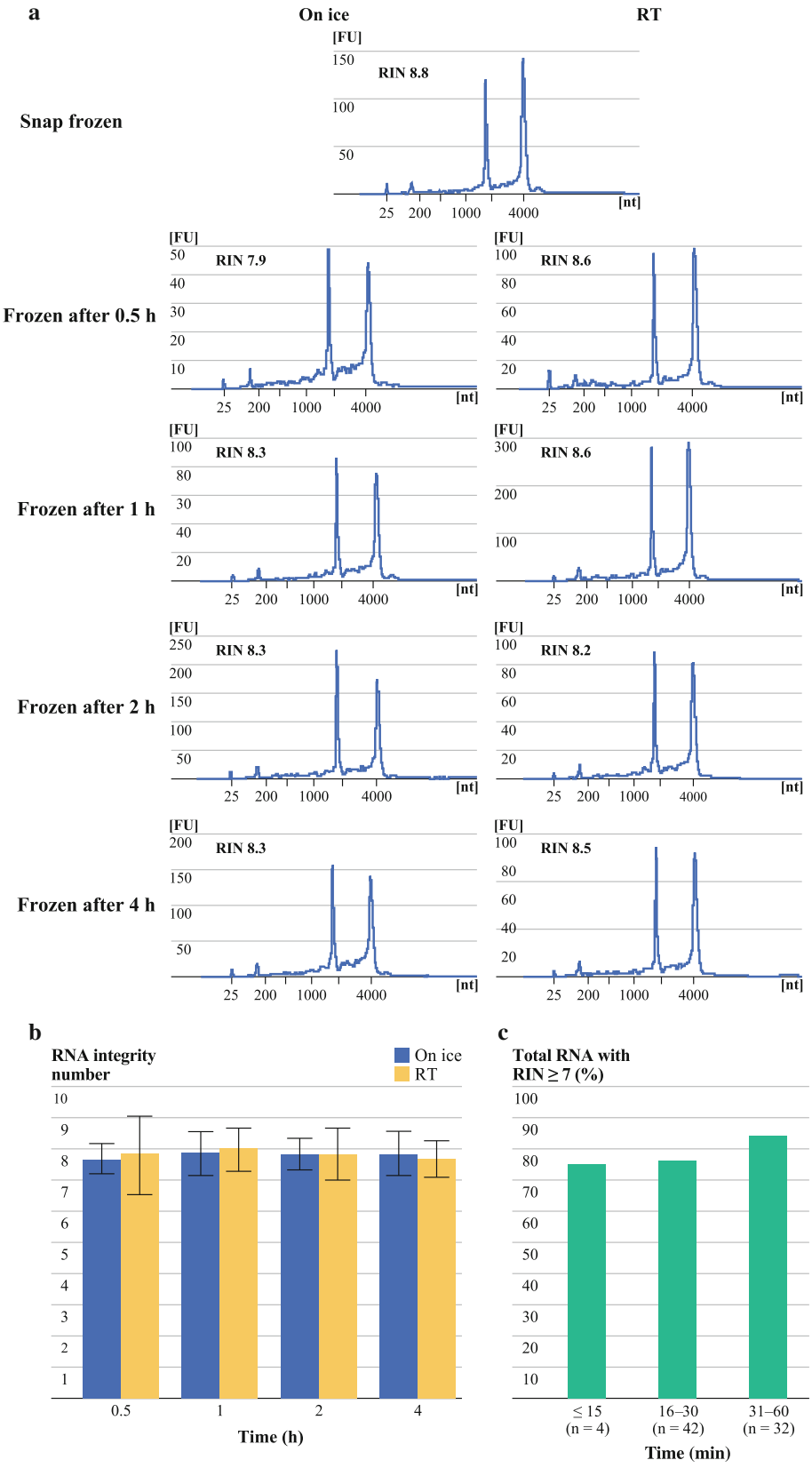

FIG. 3 Impact of various ex-vivo ischemia times on RNA integrity of colon tissues. Electropherograms obtained from purified RNA samples (**a**, showing an example from 1 study patient) reveal that RNA derived from snap frozen, kept on ice, or at room temperature ($t = 0.5$, 1, 2, 4 h) before snap freezing is all well preserved, with limited evidence of degradation. The mean RIN for all 5 patients for each time point and storage condition reflects these observations: a RIN of 10 indicates the highest quality, whereas a RIN of 0 represents the most degraded RNA. No statistical significance is achieved between all time points and sampling conditions. The 95 % confidence intervals indicated a relatively high level of confidence in the ability of the results to reflect the true mean (**b**). The percentage of total RNA with a RIN of ≥7 of 39 colon cancer and matched normal samples recorded at different ex-vivo ischemia times for ≤15 min, 16–30 min, and 31–60 min showed no statistical significance (**c**)

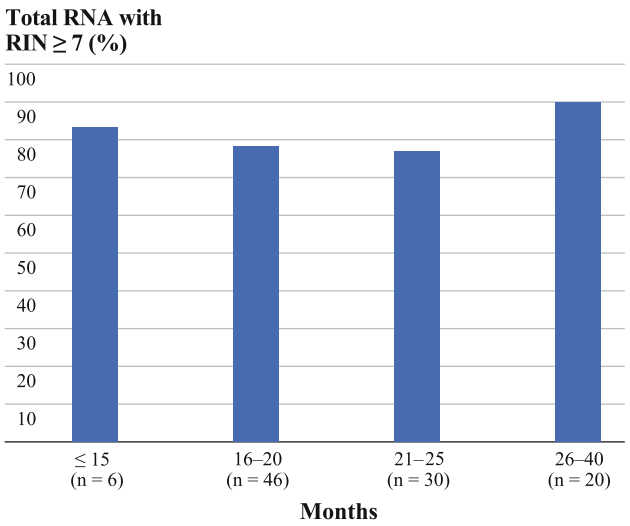

FIG. 4 Impact of storage periods on RNA integrity. The RNA was extracted and purified from 102 colon tissues frozen in the tissue biobank with Trizol and analyzed with an Agilent 2100 bioanalyzer. Percentage of tissue samples with a RIN of ≥7, including cancer samples and normal samples, is shown. The storage periods were less than 15 months, 16–20 months, 21–25 months, and 26–40 months, respectively

result of this transformation is the need for enough clinically annotated and well-characterized biospecimens to support the increasing needs of the cancer research community. Therefore, standard biobanks were established and developed, such as the National Cancer Human Biobank (caHUB) in the United States, TuBaFrost in Europe, and Shanghai Outdo Biotech Company Ltd. in China.[4,5,14,15]

For a biobank, the quality of the biospecimens is critical to cancer research. The quality of the collected specimens, clinical information, and storage must ensure that there is no loss of sample integrity. Despite ongoing research efforts by the Cooperative Human Tissue Network of the National Cancer Institute in the United States and other initiatives, few studies have examined the effects of preanalytic biospecimen collection variables on RNA quality.[16,17]

In Taizhou Hospital of Zhejiang Province, the tissue biobank was established in 2004. In this bank, frozen colon tissues from 398 colon cancer patients were collected and saved. Aliquots of colon cancer tissues from 101 patients were randomly selected for H&E staining, and the specimens with a tumor cell content higher than 70 % were used in the current study. This is to assure that the sample is histologically representative and to avoid areas of necrosis. It is identical to the research by Sandusky et al.[18] In that study, the specimens at least 65 % of the sample (aliquot) contained tumor that could be released to the researcher for genomic and proteomic analysis. Morente et al. also reported on the importance of histological representativeness for tissue quality in tissue banking.[19]

As we know, the integrity of the samples must be maintained during storage. In the past, the ratio of 18S to 28S has been used to evaluate RNA integrity. However, because the intensity of the rRNA band is affected by the condition of electrophoresis, the amount of loaded RNA, and the saturation level of ethidium bromide, subjective results are obtained by using a gross evaluation of the ratio of the 28S to the 18S bands on the agarose gel. Therefore, in recent years, RNA integrity has been assessed with a bioanalyzer to measure the RIN, which integrates 10 features from RNA electropherograms and which has been shown to be superior to the 18S-to-28S rRNA ratio.[20–23] The exact cutoff for the optimal RIN for microarray analysis has been shown to depend on the chosen platform and the investigators specifications for false inclusion and false exclusion. Rudloff et al. measured the RINs of human pancreas cancer tissues and chose a RIN cutoff of ≥7 to analyze the RNA quality.[24] Research has also demonstrated that human tissue with a RIN of >7 could be used for highly demanding gene array assays, those with a RIN between 4.1 and 6.9 could be used for qRT-PCR, and those with a RIN between 1 and 4 could be applied for PCR assays with short regions of amplification.[25] On the other hand, there are other characteristics of RNA that may be important for a particular assay and are not correlated with RIN. For instance, samples with a RIN of <7.0 are probably useful for gene array assays, particularly if they are compared to samples with similar RINs.[25]

In the current study, RNA quality was assessed by RIN, and a RIN of ≥7 was chosen as the cutoff value. Our research showed that about 80 % of banked colon tissue specimens under the specified protocol yield high-quality RNA. The results were higher than the 42 % of banked pancreas cancer biospecimens that yielded RNA with a RIN of ≥7 reported by Rudloff et al.[24] It might be because pancreas cancer samples are easier to degrade during the collection and storage. Furthermore, both the RNA concentration and the percentage of RNA with a RIN of ≥7 were obviously higher in colon cancer tissues compared to their normal tissue counterparts ($p < 0.001$). The reason may be the differences in tissue composition between cancer samples and normal samples. Cancer samples were rich in epithelial cancer cells because the tumor cell content was at least 70 % of the sample in our study. However, in the matched normal group with mucosa layer collected, the amount of the connective tissue was much richer than that in the cancer samples. Several studies have demonstrated that the connective tissue–rich specimens have a lower RIN. Fleige et al. analyzed RNA integrity in various bovine tissues and cell lines and found a lower RIN (mean 4.56) in extracts from connective tissue–rich organs like the jejunum than in RNA isolated from more cellular organs like the spleen (mean 7.28).[26] Bertilsson et al. also

reported that the extracts from samples with a high proportion of the prostate stroma showed lower RINs than samples containing more epithelium. [27]

Among the quality concerns of biospecimens, tissue ex-vivo ischemia time was considered as a primary quality assurance factor. Our research indicated that limited ex-vivo ischemia times and storage periods of fresh-frozen colon cancer tissues have no marked adverse effect on RNA quality. RNA integrity had no marked decline within 4 h after surgery. These results are consistent with previously published studies on this subject. In a study on the molecular quality of human tissues from the Cooperative Human Tissue Network, the RNA quality of various epithelial cancer specimens, including 67 colon tissues, was examined, and no obvious decline in RNA quality was found within the first 5 h after surgery.[28] Rudloff et al. reported that limited warm ex-vivo ischemia times and long-term storage of banked pancreas cancer biospecimens did not negatively influence RNA quality.[24] Micke et al. reported that RNA remained stable in colon tissues tested for up to 6–16 h under the following conditions: left at room temperature, kept on ice, kept in normal saline, kept in RNA later or snap frozen.[29] In breast cancer, Ohashi et al. found no loss of RNA integrity in normal breast tissue for up to 3 h after surgical removal.[17] However, on the other hand, recently presented research on the effect of tissue ex-vivo ischemia time of colorectal cancer tissues on the quality of RNA made different observations.[30] The study demonstrated that although the concentration of RNA was not affected by delayed freezing, the RNA integrity was decreased with increasing delayed freezing time. Spruessel et al. focused on the impact of tissue ex-vivo ischemia times on gene and protein expression profiles of healthy and malignant colon tissue and found that early changes in gene and protein expression profiles were already observed 5–8 min after colon resection. Fifteen minutes after surgery, 10–15 % of molecules, and after 30 min, 20 % of all detectable genes and proteins differed significantly from baseline values. Therefore, according to study's authors, the control of variables such as tissue ex-vivo ischemia time is needed to obtain reliable data in screening programs for molecular targets and diagnostic molecular patterns.[31]

In summary, this study evaluated the quality of banked colon tissues from a human tissue bank and the effect of tissue ex-vivo ischemia times and storage periods on the RNA integrity. Fresh-frozen colon tissues procured within a standardized research protocol yielded high-quality RNA in approximately 80 % of the cases. The quality of tissues in the biobank as assessed by RNA integrity was not adversely impacted by limited variations of tissue ex-vivo ischemia times or different storage periods. However, the presented data should be considered as an attempt to further standardize tissue biospecimen collection and banking, and it must be cautioned that quality control for RNA integrity of the tissues does not substitute for internal controls in the microarray experiments. Furthermore, one important limitation to this study is that the isolated RNA was not presented to gene expression analysis; this needs to be better understood and integrated into the research.

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