Effects of *ex vivo* ischemia time and delayed processing on quality of specimens in tissue biobank

DAN GUO[1*], ANQI WANG[1*], TING XIE[1*], SUMEI ZHANG[1], DINGYAN CAO[1] and JIAN SUN[1,2]

[1]Clinical Biobank, Department of Medical Research Center; [2]Department of Pathology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, P.R. China

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Abstract. The RNA quality of tissue biobank is crucial for translational research; however, the effects of the *ex vivo* ischemia time on RNA integrity and expression of genes related to hypoxia, stress, apoptosis and autophagy remains elusive. A total of 18 carcinoma tissues were stored at room temperature for 15 min, 30 min, 1, 2, 4, 8 and 24 h. The integrity and purity of isolated RNA were analyzed. Furthermore, the gene expression of mTOR, hypoxia-inducible factor 1α, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit β isoform (PI3KCB), threonine kinase 1 (AKT1), NF-κB, protein kinase AMP-activated catalytic subunit α1 (AMPKα1), caspase 8 (CASP8), unc-51 like autophagy activating kinase 1 and Fas cell surface death receptor were analyzed using reverse transcription-quantitative PCR. The results demonstrated that RNA integrity numbers (RINs) remained stable in carcinoma tissues following *ex vivo* ischemia for 2 h at room temperature and that degradation began at 4 h ($P<0.001$). Additionally, the expression of PI3KCB, AKT1, AMPKα1 and CASP8 decreased at time points 8-24 h following *ex vivo* ischemia and delayed processing ($P<0.001$). In conclusion, >2 h of *ex vivo* ischemia and delayed processing induced RNA degradation and a decrease in RIN, and the gene expressions of PI3KCB, AKT1, AMPKα1 and CASP8 may be considered as markers to evaluate tissue quality at the gene expression level, providing a method for the standard processing and assessment of tissue specimen.

Correspondence to: Professor Jian Sun, Clinical Biobank, Department of Medical Research Center, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 1 Shuaifuyuan, Dongdan Road, Dongcheng, Beijing 100730, P.R. China
E-mail: sunjian0720@126.com

*Contributed equally

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Introduction

Tissue biobanks support the collection, analysis, storage and distribution of biospecimens for basic, translational and clinical research. With the advent of proteomics, transcriptomics and metabolomics, as well as the development of bioinformatics, molecular imaging and drug discovery, the demand for high-quality tissue biospecimens continues to increase (1,2), large-scale and high-flux research requirements for high quantity and quality biospecimens have allowed tissue biobanks to develop from undeveloped, small-scale storages to complex and multifaceted large-scale storages (3).

Quality assurance and quality control are critical for tissue biobanks to ensure that biospecimens distributed to researchers are comparable and produce accurate results; as a rough estimate, the number of stored tissue biospecimens was >300 million in the US around 2000, with this number increasing at the rate of 20 million/year (4). However, according to a survey taken by >700 cancer researchers, 47% of them were of the opinion that sample quality is difficult to control and 60% questioned the quality of samples (4).

There are various factors influencing the quality of biospecimens, and pre-analytical variables are crucial (5,6). Pre-analytical variables are divided into the pre-acquisition and post-acquisition phases. In the pre-acquisition phase, factors such as anesthesia (7), surgical trauma (8,9) and *in vivo* ischemia (10) might affect the cell integrity and gene expression profiles of the biospecimen, but they cannot be controlled very well because of the uncertainty of clinical treat requirements (11). Rather, factors such as *ex vivo* ischemia time, delayed processing time and processing temperature, which are crucial for bio-specimen quality (12), can be controlled and standardized effectively.

To the best of our knowledge, little research has been conducted of the impact of *ex vivo* ischemia time and delayed processing of biospecimens on hypoxic, stress, apoptotic and autophagic pathways. Therefore, the current study systematically assessed the impact of *ex vivo* ischemia time and delayed processing time on fresh tissue specimens in terms of the RNA quality and relative mRNA expression of genes involved in hypoxia, stress, apoptosis and autophagy to provide a theoretical basis for the standard processing and assessment of tissue biospecimens.

Materials and methods

Procurement of tissues. A total of 18 carcinoma tissue specimens were collected from patients who underwent surgery at Peking Union Medical College Hospital, Beijing, China from April 2017 to June 2017. The clinical characteristics of 18 patients are presented in Table I. The median age of the patients at diagnosis was 49.67±12.76 years (range, 29-79 years), of which 16 patients were female (88.9%) and 2 patients were male (11.1%). The collected tissues included specimens from 10 patients with thyroid cancer (55.5%), 3 with ovarian cancer (16.6%), 2 with breast cancer (11.1%), 1 with pancreatic cancer (5.6%), 1 with polymorphous adenocarcinoma (5.6%) and 1 with lung cancer (5.6%). These 18 specimens and 6 types of cancer were included due to their prevalence and commonality in the clinical and tissue biobank. All patients provided signed informed consent for the donation of the specimens. The current study was approved by the Ethics Committee of the Institutional Review Board of Peking Union Medical College Hospital.

Following gross examination, the tissues were resected and washed several times with PBS (pH 7.2) to remove cell debris and remaining blood. Tissues were then dissected into two parts, one part of the specimen (1 cm^3) was used for clinical pathology diagnosis, while the other part was divided to 7 smaller specimens (volume, 100 mm^3). One of the 7 small specimens was immediately snap-frozen in liquid nitrogen, thus the time from tissue resection to storage in liquid nitrogen was ~15 min, which was set as the first point of *ex vivo* ischemia time. The other 6 specimens were stored at room temperature for 15, 45, 105, 225, 465 and 1,425 min, and then were stored in liquid nitrogen, so the *ex vivo* ischemia time was 30 min, 1, 2, 4, 8 or 24 h, respectively. Following storage, the specimens were cryopreserved for the subsequent RNA extraction, gene expression analysis and morphological features assessment, which were reviewed by two experienced pathologists to confirm final diagnoses.

RNA extraction and integrity control. Total RNA was extracted from all tissue samples; ~30 mg of 30 mm^3 tissue specimen was homogenized using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) with a homogenizer (IKA Werke GmbH & Co. KG) for RNA extraction, according to the manufacturers' protocols. RNA concentration was quantified using NanoDrop™ One (Thermo Fisher Scientific, Inc.) by measuring the extinction coefficient at 260 nm. Additionally, the ratio of optical density (OD) 260/230 and OD 260/280, which indicates RNA purity, was tested using the NanoDrop One. An Agilent 2100 Bioanalyzer and an Agilent RNA 6000 Nano kit (Agilent Technologies, Inc.) were used to determine RNA quality, according to the manufacturers' protocol. RNA integrity numbers (RINs) were calculated using Agilent Technologies 2100 Bioanalyzer expert software (version B.02.0x; Agilent Technologies, Inc.), and were presented as a value (1-10) to indicate the degree of degradation.

Reverse transcription. Total RNA (1 μg) was diluted to a final volume of 25 μl. A reverse transcription master mix was prepared as follows to reverse transcribe RNA into cDNA: 5 μl 5X reaction buffer, 1 μl random primers (cat. no. C1181; Promega Corporation), 1.25 μl dNTPs (cat. no. U1330; Promega Corporation), 1 μl M-MLV reverse transcriptase (cat. no. M1701; Promega Corporation), 0.5 μl RNase inhibitors (Takara Bio, Inc.) and 6.25 μl nuclease-free water (cat. no. AM9937; Invitrogen; Thermo Fisher Scientific, Inc.). Following reverse transcription, all samples were diluted to a final volume of 100 μl.

Table I. Clinical characteristics of the patients (n=18).

Clinical characteristic	Number of patients, n (%)
Sex	
Male	2 (11.1)
Female	16 (88.9)
Age, years	
≤50	8 (44.4)
>50	10 (55.6)
Type of carcinoma	
Thyroid	10 (55.5)
Breast	3 (16.6)
Ovary	2 (11.1)
Pancreas	1 (5.6)
Polymorphous adenocarcinoma	1 (5.6)
Lung	1 (5.6)

Reverse transcription-quantitative PCR (RT-qPCR). All PCR procedures were set up using a QIAgility robotic system (Qiagen, Inc.). Mixes were prepared with 5 μl Fast SYBR1 Green Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.; cat. no. 4385612) and 2 μl primer mix (1.67 μM; Table II). Then, 2 μl cDNA (10 ng/μl) were added for a final volume of 10 μl/well. Gene expression quantification was performed via RT-qPCR using an Applied Biosystems® 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used: 95˚C for 20 sec; followed by 40 cycles of 95˚C for 3 sec and 60˚C for 30 sec. The expression of the housekeeping gene GAPDH was used for normalization and mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method (13,14). The primers used in the analysis of gene expression were associated with hypoxia, stress, apoptosis and autophagy: Caspase 8 (CASP8), Fas cell surface death receptor (FAS), hypoxia-inducible factor 1α (HIF1α), unc-51 like autophagy activating kinase 1 (ULK1), mTOR, NF-κB, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit β isoform (PI3KCB), AKT serine/threonine kinase 1 (AKT1), protein kinase AMP-activated catalytic subunit α1 (AMPKα1) and (Table II).

Histological assessment. The other part of all the fresh tissues specimen were used for the pathological clinical diagnosis. In addition, after the RNA isolation, the frozen thyroid tissues in liquid nitrogen with different *ex vivo* ischemia times were fixed in 10% neutral buffered formalin at room temperature for 24 h. Specimens were then embedded in paraffin. Sections (thickness, 5 μm) were microtomed and placed on slides. Slides were baked for 1 h at 60˚C in an oven and stained with

Table II. Primer sequences used for reverse transcription-quantitative PCR.

Gene	Primer sequences (5'→3')	Fragment size, bp
CASP8	F: GCCCCCATCTATGAGCTGAC	100
	R: TATCCCCCTGACAAGCCTGA	
FAS	F: GTGGACCCGCTCAGTACG	121
	R: GGACGATAATCTAGCAACAGACG	
HIF1α	F: TTGGCAACCTTGGATTGGATG	190
	R: AAATCTCCGTCCCTCAACCTC	
ULK1	F: GTCACACGCCACATAACAG	90
	R: TCTTCTAAGTCCAAGCACAGT	
mTOR	F: GCTTAGAGGACAGCGGGGAA	120
	R: AAGCATCTTGCCCTGAGGTT	
NF-κB	F: CCACAAGACAGAAGCTGAAG	149
	R: AGATACTATCTGTAAGTGAACC	
PI3KCB	F: TGGAACTTGGCACTGGAACT	114
	R: GCTGGGAAACAAGGGGAGAA	
AKT1	F: CTGGTCCTGTCTTCCTCATGTT	190
	R: AGGCAGCCCCTTTGACTTCT	
AMPKα1	F: TGAAAGAAAAGTGTGTGCTGT	64
	R: TGGGTGAGAAGATGAGGGAAAGA	
GAPDH	F: GAAGGTGAAGGTCGGAGTC	226
	R: GAAGATGGTGATGGGATTTC	

bp, base pair; CASP8, caspase 8; FAS, Fas cell surface death receptor; HIF1α, hypoxia-inducible factor 1α; ULK1, unc-51 like autophagy activating kinase 1; PI3KCB, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit β isoform; AKT1, threonine kinase 1; STAT3, signal transduced and activator of transcription.

hematoxylin and eosin, both at room temperature for 1 h with an autostainer XL (ST5010; Leica Microsystems GmbH). Histological evaluations were completed by two experienced pathologists on a light microscope (magnification, x100 or x200; Axio Scope.A1; Zeiss GmbH).

Statistical analysis. Statistical analysis was performed using SPSS (version 19.0; IBM Corp.) and Prism software (version 5; GraphPad Software, Inc.). One-way ANOVA followed by Tukey's post hoc test was used to analyse differences between multiple groups. Pearson's correlation coefficient was used for correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of ex-vivo ischemia time on RNA integrity. To evaluate the influence of tissue *ex vivo* ischemia time points on RNA integrity under controlled conditions, 18 fresh carcinoma tissues from different organs were collected, separated and stored at room temperature for various *ex-vivo* ischemia times prior to being snap-frozen in liquid nitrogen. The RNA quality was assessed according to integrity and purity. The RNA integrity data of tissues *ex vivo* ischemia at 15 min, 2, 8 and 24 h are presented in Fig. 1A. The distinct ribosomal peaks of 18S and 28S indicated good RNA integrity at 15 min and 2 h, but not at 8 and 24 h. The RINs of each group were 8.12±0.576, 7.78±0.546, 7.88±0.621, 7.67±0.523, 7.31±0.589, 7.15±0.844 and 6.63±0.688 for *ex vivo* ischemia time points 15, 30 min, 1, 2, 4, 8 and 24 h, respectively (Fig. 1B). SPSS analysis demonstrated that the RINs at 4, 8 and 24 h were significantly decreased compared with 15 min (P<0.001). Furthermore, compared with 30 min, RINs at 8 h (P<0.01) and 24 h (P<0.001) were significantly decreased. Similarly, compared with 1 h, the RINs at 4 h (P<0.05), 8 h (P<0.001) and 24 h (P<0.001) were decreased, and the RINs at 8 h (P<0.05) and 24 h (P<0.001) were significantly declined compared with 2 h. In addition, the RINs at 8 h were decreased compared with 4 h (P<0.01), and similarly, the RINs at 24 h were significantly decreased compared with 8 h (P<0.05). The results indicated that RNA remained intact following 2 h of *ex vivo* ischemia at room temperature and integrity began to gradually decline after 4 h.

The means of RNA OD260/280 and OD260/230 ratios of 7 time points were 2.05±0.1 and 1.78±0.43, respectively (n=126). The OD260/280 ratios of each group were 2.028±0.099, 2.101±0.144, 2.043±0.091, 2.051±0.085, 2.031±0.045, 2.011±0.076 and 2.085±0.128 for *ex vivo* ischemia time points 15, 30 min, 1, 2, 4, 8 and 24 h, respectively (Fig. 2). The OD260/230 ratios were 1.713±0.398, 1.960±0.330, 1.916±0.367, 1.675±0.561, 1.839±0.391, 1.550±0.500 and 1.799±0.481 for *ex vivo* ischemia time points 15, 30 min, 1, 2, 4, 8 and 24 h, respectively. The ratios of OD260/280 and OD260/230 of all groups indicated that the degradation of RNA integrity does not affect RNA purity reflected by absorbance under ultraviolet light.

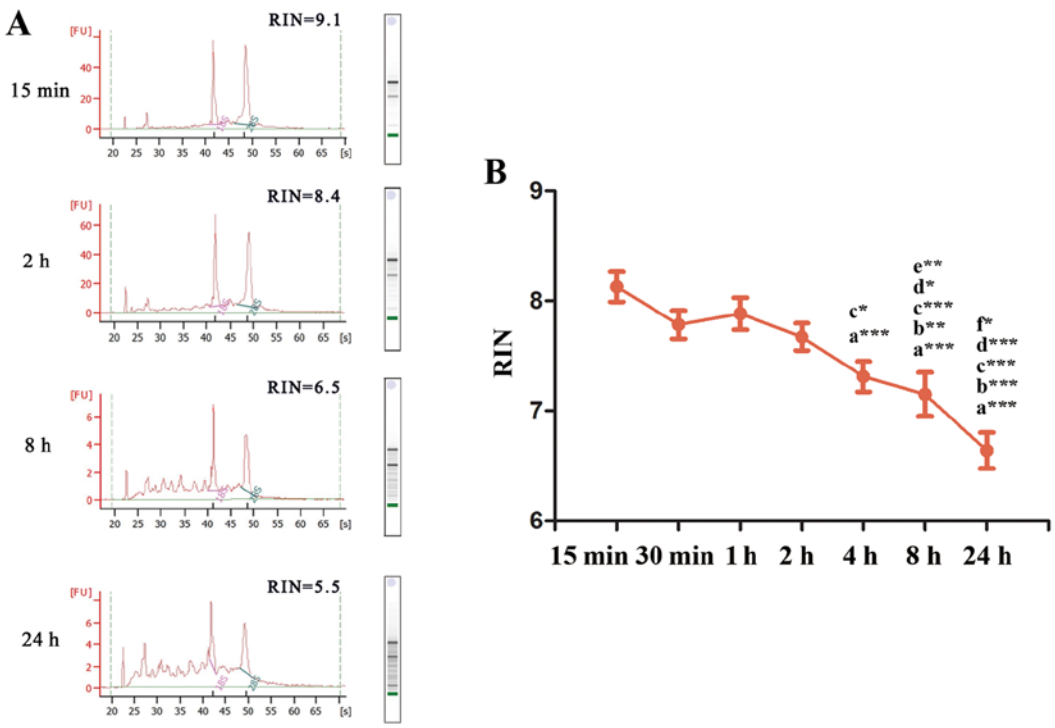

Figure 1. RNA integrity of tissues for various *ex vivo* ischemia time points. (A) mRNA RINs at 15 min, 2, 8 and 24 h of *ex vivo* ischemia. (B) Changes in RNA integrity at increasing *ex vivo* ischemia time points at room temperature. a, vs. 15 min; b, vs. 30 min; c, vs. 1 h; d, vs. 2 h; e, vs. 4 h; f, vs. 8 h. *P<0.05, **P<0.01, ***P<0.001. RINs, RNA integrity numbers.

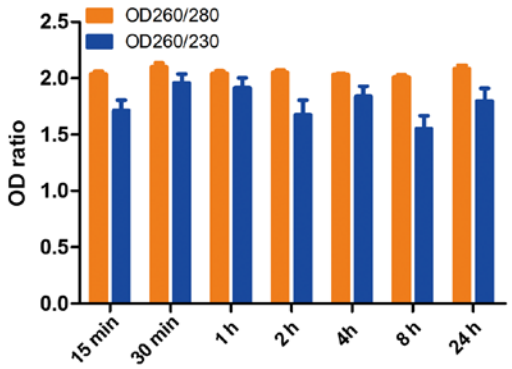

Figure 2. OD260/280 and OD260/230 of isolated RNA for various *ex vivo* ischemia time points. OD, optical density.

Effect of ex vivo ischemia time on gene expression. To explore the influence of *ex vivo* ischemia time points on cell functions involving hypoxia, stress, apoptosis and autophagy, the gene expression of mTOR, HIF1α, PI3KCB, AKT1, NF-κB, AMPKα1, CASP8, ULK1 and FAS were evaluated using RT-qPCR. The RINs of the 7 different *ex vivo* ischemia times indicated that RNA remained intact following 2 h of *ex vivo* ischemia at room temperature and integrity began to gradually decline after 4 h. Thus, the present study mainly focused on *ex vivo* ischemia times of 15 min, 2, 8 and 24 h, and more times will be explored in further study. The results demonstrated that AKT1 was significantly decreased at 8 h compared with 15 min (P<0.01) and that PI3KCB, AMPKα1 and CASP8 were significantly decreased at 24 h compared with 15 min (P<0.05; Fig. 3). However, the expression of mTOR, HIF1α, NF-κB, ULK1 and FAS were not significantly different between time points.

Further analysis indicated that there was no significant correlation between RIN and gene expression (Table III). In addition, the interclass Pearson correlation coefficients among the gene expression levels were strong, such as $r_{mTOR/PI3KCB}$=0.721, $r_{mTOR/AKT1}$=0.792, $r_{mTOR/NF-κB}$=0.640, $r_{mTOR/AMPKα1}$=0.630, $r_{PI3KCB/AKT1}$=0.741, $r_{PI3KCB/NF-κB}$=0.572, $r_{PI3KCB/AMPKα1}$=0.835, $r_{AKT1/NF-κB}$=0.646 and $r_{AKT1/AMPKα1}$=0.666, $r_{AKT1/CASP8}$=0.508, $r_{CASP8/FAS}$=0.762, $r_{CASP8/mTOR}$=0.607, $r_{HIF1α/ULK1}$=0.728 (P<0.001) (Table IV). In addition, HIF1α had slight positive correlation with mTOR, PI3KCB and AKT1 (r=0.281, r=0.293 and r=0.298, respectively; P<0.05), and FAS has correlation with NF-κB (r=0.281; P<0.05). In addition, there were no correlations observed between some gene expression (P>0.05), such as AKT1 vs. FAS, ULK1 vs. AKT1, AMPKα1 vs. FAS, ULK1 vs. mTOR, ULK1 vs. PI3KCB, ULK1 vs. NF-κB and FAS vs. PI3KCB. A scatter plot of gene expression, including the correlation between the expression of these genes is presented in Fig. 4; the scatterplot trend was consistent with the correlation coefficients of the gene expression presented in Table IV.

Histological morphology. To evaluate histomorphology at various *ex vivo* ischemia time points, frozen tissue specimens were fixed, sectioned and stained with H&E. The histological morphology demonstrated that follicular epithelial cells were arranged closely and maintained integrity at 15 min and 2 h in thyroid tissues (Fig. 5). However, the microstructure of the thyroid follicles was destroyed at 8 h and cells displayed looseness and follicle disintegration at 24 h. These results demonstrated that *ex vivo* ischemia and delayed processing induced morphological changes in thyroid carcinoma, which was accompanied by RNA degradation.

Table III. Correlation coefficients between RIN and gene expression.

	RIN	
Gene	Pearson correlation coefficient	Significance (bilateral)
mTOR	-0.065	0.590
PI3KCB	-0.051	0.668
AKT1	0.172	0.150
NF-κB	0.102	0.394
HIF1α	0.018	0.882
AMPKα1	-0.040	0.741
CASP8	0.133	0.267
FAS	0.066	0.582
ULK1	0.024	0.840

RIN, RNA integrity number; PI3KCB, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit β isoform; AKT1, threonine kinase 1; HIF1α, hypoxia-inducible factor 1α; AMPKα1, protein kinase AMP-activated catalytic subunit α1; CASP8, caspase 8; FAS, Fas cell surface death receptor; ULK1, unc-51 like autophagy activating kinase 1.

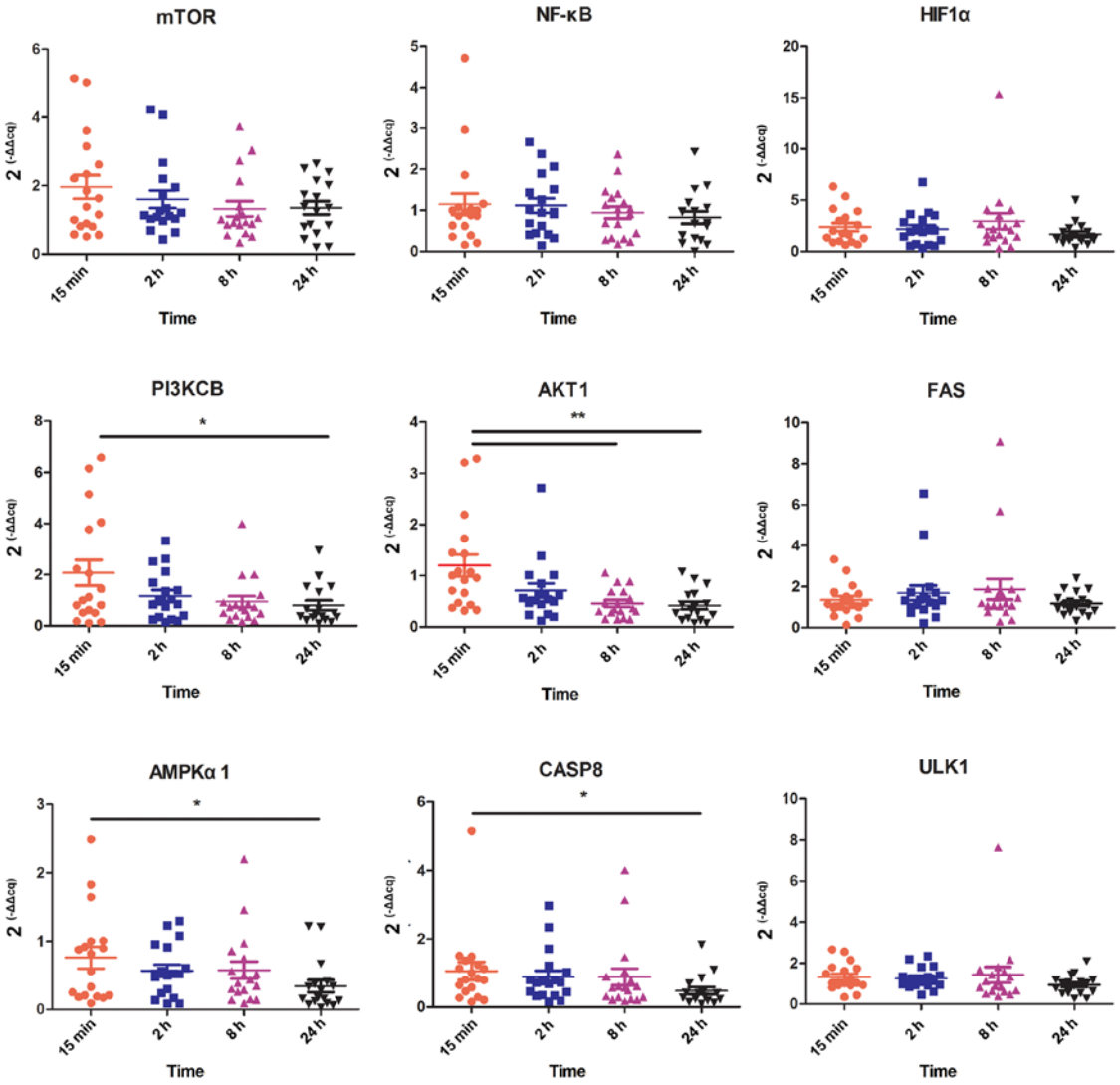

Figure 3. Gene expression of genes associated with hypoxic, stress, apoptotic and autophagic pathways for various *ex vivo* ischemia time points. HIF1α, hypoxia-inducible factor 1α; PI3KCB, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit β isoform; AKT1, threonine kinase 1; FAS, Fas cell surface death receptor; AMPKα1, protein kinase AMP-activated catalytic subunit α1; CASP8, caspase 8; ULK1, unc-51 like autophagy activating kinase 1. *P<0.05, **P<0.01.

Table IV. Pearson's correlation coefficients of gene expression.

	Gene expression								
Variable	mTOR	PI3KCB	AKT1	NF-κB	HIF1α	AMPKα1	CASP8	FAS	ULK1
mTOR									
r-value	1	0.721[c]	0.792[c]	0.640[c]	0.281a	0.630[c]	0.607[c]	0.409[c]	0.221
Significance (bilateral)	N/A	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.063
N	72	72	72	72	72	72	72	72	72
PI3KCB									
r-value	0.721[c]	1	0.741[c]	0.572[c]	0.293[a]	0.835[c]	0.372[b]	0.019	0.150
Significance (bilateral)	0.000	N/A	0.000	0.000	0.013	0.000	0.001	0.876	0.208
N	72	72	72	72	72	72	72	72	72
AKT1									
r-value	0.792[c]	0.741[c]	1	0.646[c]	0.298[a]	0.666[c]	0.508[c]	0.181	0.189
Significance (bilateral)	0.000	0.000	N/A	0.000	0.011	0.000	0.000	0.127	0.111
N	72	72	72	72	72	72	72	72	72
NF-κB									
r-value	0.640[c]	0.572[c]	0.646[c]	1	0.226	0.674[c]	0.658[c]	0.281[a]	0.021
Significance (bilateral)	0.000	0.000	0.000	N/A	0.056	0.000	0.000	0.017	0.861
N	72	72	72	72	72	72	72	72	72
HIF1α									
r-value	0.281[a]	0.293[a]	0.298[a]	0.226	1	0.433[c]	0.525[c]	0.482[c]	0.728[c]
Significance (bilateral)	0.017	0.013	0.011	0.056	N/A	0.000	0.000	0.000	0.000
N	72	72	72	72	72	72	72	72	72
AMPKα1									
r-value	0.630[c]	0.835[c]	0.666[c]	0.674[c]	0.433[c]	1	0.557[c]	0.137	0.342[c]
Significance (bilateral)	0.000	0.000	0.000	0.000	0.000	N/A	0.000	0.250	0.003
N	72	72	72	72	72	72	72	72	72
CASP8									
r-value	0.607[c]	0.372[c]	0.508[c]	0.658[c]	0.525[c]	0.557[c]	1	0.762[c]	0.467[c]
Significance (bilateral)	0.000	0.001	0.000	0.000	0.000	0.000	N/A	0.000	0.000
N	72	72	72	72	72	72	72	72	72
FAS									
r-value	0.409[c]	0.019	0.181	0.281[a]	0.482[c]	0.137	0.762[c]	1	0.501[c]
Significance (bilateral)	0.000	0.876	0.127	0.017	0.000	0.250	0.000	N/A	0.000
N	72	72	72	72	72	72	72	72	72
ULK1									
r-value	0.221	0.150	0.189	0.021	0.728[c]	0.342[b]	0.467[c]	0.501[c]	1
Significance (bilateral)	0.063	0.208	0.111	0.861	0.000	0.003	0.000	0.000	N/A
N	72	72	72	72	72	72	72	72	72

[a]P<0.05, [b]P<0.01, [c]P<0.001. PI3KCB, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit β isoform; AKT1, threonine kinase 1; HIF1α, hypoxia-inducible factor 1α; AMPKα1, protein kinase AMP-activated catalytic subunit α1; CASP8, caspase 8; FAS, Fas cell surface death receptor; ULK1, unc-51 like autophagy activating kinase 1; N/A, not applicable.

Discussion

RNA degradation is one of the major issues when storing tissues in biobanks, and previous studies have investigated the effect of tissue type, storage buffers and *ex vivo* ischemia times on RNA quality (15,16). Among these factors, *ex vivo* ischemia and delayed processing time are crucial pre-analysis challenges for RNA quality (6,12). The results of the current study demonstrated that RNA integrity decreased significantly following *ex vivo* ischemia for 4 h, indicating that RNA remained intact for 2 h at room temperature.

Previous research reported that the effects of *ex vivo* ischemia time on RNA integrity varied in different types of tumors. Bao *et al* (17) demonstrated that RNA remained stable both at room temperature and on ice for ≤4 h in colon cancer tissues. Additionally, retained ileum mucosa was revealed to exhibit high

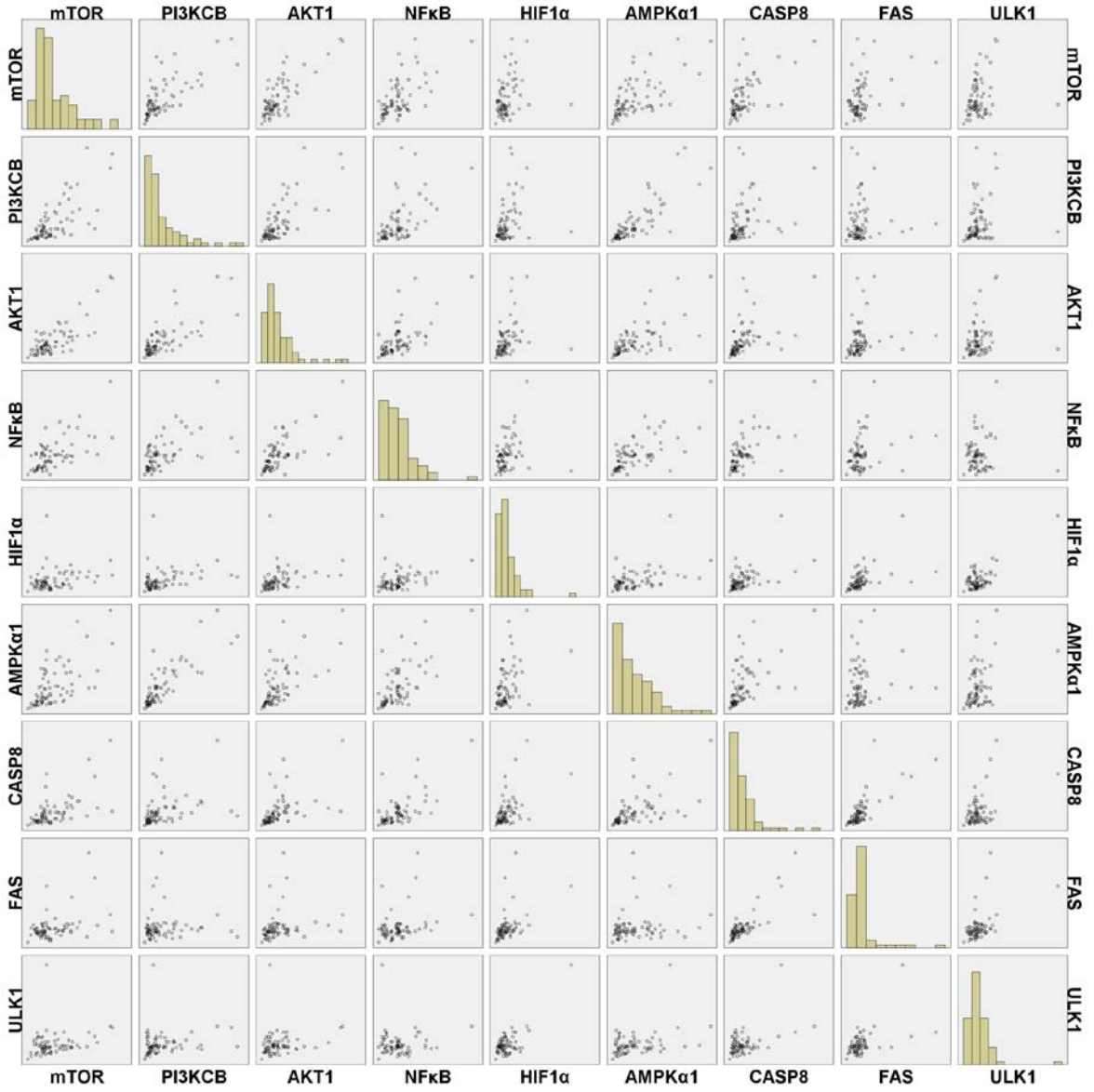

Figure 4. Scatter plots of the expression of genes associated with hypoxic, stress, apoptotic and autophagic pathways for various *ex vivo* ischemia time points. PI3KCB, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit β isoform; AKT1, threonine kinase 1; HIF1α, hypoxia-inducible factor 1α; AMPKα1, protein kinase AMP-activated catalytic subunit α1; CASP8, caspase 8; FAS, Fas cell surface death receptor; ULK1, unc-51 like autophagy activating kinase 1.

RNA integrity for 1.5 h at room temperature and 6 h at 4˚C (18). In kidney renal cell carcinoma tissues, RNA degradation was observed at room temperature for 1 h and degradation was not detected following storage on ice for 4 h (19,20). Similarly, RIN gradually decreased in tumor and adjacent normal tissues at 24˚C at ischemia time points 15, 30, 60 and 120 min in hepatocellular carcinoma tissues (21). Even though our study did not verify whether the RNA integrity from different tumor tissues (such as thyroid, ovary, breast, pancreas, jaw and lung) had different sensitivities to *ex vivo* ischemia time points, it could be verified by further studies with more carcinoma specimens.

In addition, a previous study demonstrated that the RIN of non-small cell lung cancer tissue was significantly lower (OR=0.08; P=0.01) in samples that were preserved for >3 h prior to cryopreservation, and prolonged *in vivo* (>2 h) and *ex vivo* ischemia (>10 h) times were associated with lower patient-derived xenograft engraftment rates (22). Furthermore, long term *ex vivo* ischemia of human brain tumor biopsy tissues affected ribosomal RNA integrity and increased RNA degradation (23). When snap freezing and multiple sampling of biopsies were combined, the percentage of specimens with degraded RNA was reduced by two-fold (23). However, another previous study on RNA integrity and gene expression in human myocardial tissue reported that there was no significant correlation between RIN and a post-mortem interval of 5-24 h at 4˚C (24). Furthermore, RNA integrity was not correlated with 1 h *ex vivo* ischemia time points in different types of tumors, including breast, colon, stomach, oesophageal, gall bladder, ovarian, kidney, liver, lung, pancreatic, small intestinal, spleen, common bile duct, retroperitoneum, testis and urinary bladder cancer (24,25).

Additionally, the duration of *ex vivo* ischemia affects the related gene expression profile (20). The results of the current study demonstrated that the gene expression of PI3KCB, AKT1,

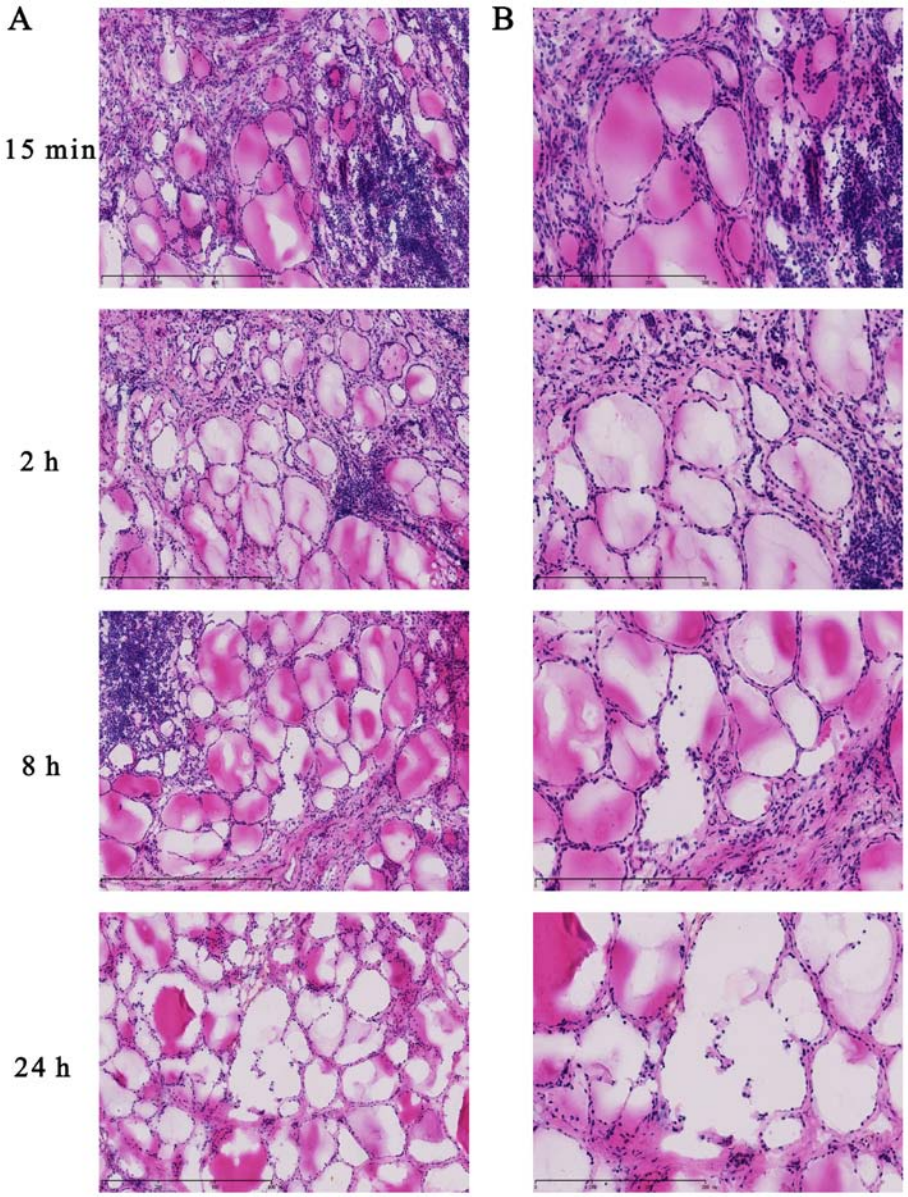

Figure 5. Haematoxylin and eosin-stained thyroid tissues at various *ex vivo* ischemia time points. Thyroid tissues at *ex vivo* ischemia time points of 15 min, 2, 8 and 24 h at (A) magnification x100 and (B) magnification x200.

AMPKα1 and CASP8 were significantly decreased following *ex vivo* ischemia at time points 8-24 h compared with 15 min. PI3KCB, AKT1, AMPKα1 and CASP8 serve important roles in the hypoxic, stress, apoptotic and autophagic signaling pathways (26,27). AMPKα1 is critical for hypoglycemia, hypoxia, ischemia and heat shock (28), and regulates growth, metabolism reprogramming, glucose and fatty acid metabolism and mitochondrial function (29). Oncogenes AKT1 and PI3KCB are major factors in the regulation of various cellular functions, including metabolism, growth, proliferation, survival, transcription and protein synthesis (30,31). Furthermore, AMPKα1 stimulates autophagy by inhibiting mTOR, whereas PI3KCB, Ras and AKT1 inhibit autophagy by activating mTOR (32). Additionally, CASP8 is induced by endoplasmic reticulum stress and proteotoxic stress (33), which participate in the apoptosis signal pathway (34).

An increased number of studies have demonstrated that when the blood supply is cut off, tissues undergo oxygen deprivation and mammalian cells undergo hypoxemia, autophagy and endoplasmic reticulum stress response (35). Then, hypoxemia, autophagy, apoptosis and endoplasmic reticulum stress occur and coordinate (36,37). Furthermore, a previous study has reported that exposure to *ex vivo* ischemia significantly altered gene expression of G-protein signaling 1 and eukaryotic translation elongation factor 1 α 1 in normal and colorectal cancer tissue samples (38).

Dr Carolyn Compton, a former Office of Biorepository and Biospecimen Research executive, proposed that when biological samples are excised, cut off from a blood supply and exposed to abrupt changes in temperature, cellular behavior becomes difficult to predict (4). Gene expression and protein phosphorylation fluctuate extensively and cellular self-destruct pathways may be activated (4). The American Society of Clinical Oncology, College of American Pathologists (2014), Biorepositories and Biospecimen Research Branch, National Cancer Institute (2016), Clinical Laboratory Standards

Institute (2011), Clinical Laboratory Standards Institute (2005), International Standards Organization/Technical Committee (2016) and European Committee for Standardization/Technical Committee (2015) demonstrated that *ex vivo* ischemia times in tissues should be as short as possible (optimally <20 min and not >1 h) for the analysis of biomarkers, molecular analysis, and RNA, DNA and protein isolation (6).

Although the results of the current study revealed that the expression of PI3KCB, AKT1, AMPKα1 and CASP8 decreased with time, there was no correlation between RIN and gene expression, indicating that the decrease in gene expression was not induced by RNA degradation. Instead, the decrease in gene expression was associated with hypoxia, stress, apoptosis and autophagy induced by *ex vivo* ischemia and delayed processing.

Furthermore, the results demonstrated positive correlations between the expression of mTOR, PI3KCB, AKT1, NF-κB and AMPKα1, indicating that their expression levels may be coordinated during *ex vivo* ischemia and delayed processing. Certain stress responsive transcription factors, including NF-κB, serve a major role in autophagic response (39) and orchestrate numerous transcriptional responses, including autophagy and execution of cell death (40). Wang *et al* (26) reported that endoplasmic reticulum stress and mitochondrial injury on myocardial ischemia and reperfusion upregulated phosphorylated PI3KCB and AKT1. Hypoxia and reperfusion can evoke autophagy and apoptosis via the AKT1 and mTOR signaling pathways in microglia cells and the expression of mTOR and AKT1 was not altered under hypoxic conditions for 6 h and reperfusion injury at 24; however, the levels of p-AKT1 and mTOR were altered (27). Thus, the results of the current study indicated that while mRNA expression was altered due to *ex vivo* ischemia and delayed processing, whether the expression of phosphorylated proteins, which is important in cell signal transduction, may be altered requires further study.

Furthermore, the results demonstrated that there were fewer positive correlations between HIF1α and mTOR, PI3KCB and AKT1. A prior study revealed that HIF1α promotes the expression of hundreds of genes involved in autonomous and non-autonomous cellular adaptations to hypoxia, which are upregulated at the protein level via mTOR or at the mRNA level via STAT3 and NF-κB signaling (41). Although the current study did not report ULK and/or FAS downregulation at the mRNA level or an association with the other factors, the activity of ULK is required for autophagy (42). ULK-mediated Beclin-1 phosphorylation *in vivo* is crucial for the function of Beclin-1 in autophagy (42).

Moreover, in addition to RNA degradation, the results of the present study showed that *ex vivo* ischemia and delayed processing induced morphological changes in thyroid carcinoma tissues, which is another factor to be evaluated following delayed processing.

In addition to the processing variables, biological and environmental factors, including donor sex, age (43), drug use (44) and diet, exercise and lifestyle habits, such as smoking, could affect the quality of biospecimens (45). Furthermore, various acquisition methods, such as collecting during the surgical operation (*in situ*) or after resection (*ex vivo*) (9), and surgical approaches, such as laparoscopic colectomy or open colectomy (8) could influence gene expression analysis.

There were certain limitations of the present study. Firstly, the current study was a preliminary study that could not verify whether RNA integrity from various tumor tissues had different sensitivities to *ex vivo* ischemia time due to the small sample size. Future studies should include larger sample sizes and more tumor specimens to comprehensively examine the effects of various *ex vivo* ischemia time points and delayed processing on RNA integrity and tissue quality in different carcinoma tissues. Secondly, tissue biobanks are the basic infrastructure that provide biospecimens to various institutions for basic, translational and clinical research, and their procedures for the collection, analysis, storage and distribution of biospecimens may affect the results of downstream studies. Furthermore, tissue banks should set up individual procedures according to the requirements by researchers to accommodate research institutions that require tissues for specific purposes. Therefore, a future study should investigate the commonality of more tissues in biobanks, such as breast, colon, stomach, oesophagus, gall bladder, ovary, kidney, liver, lung and pancreas, which could be used to provide research institutions with standardized processing procedures for specimens.

In conclusion, *ex vivo* ischemia time and delayed processing induced RNA degradation and altered gene expression in various carcinoma tissues. Fresh tissues should be processed within 2 h to avoid RNA degradation to acquire high quality biospecimens. Furthermore, the gene expression of PI3KCB, AKT1, AMPKα1 and CASP8 may be considered as markers to evaluate tissue quality at the gene expression level, providing a method for the standard processing and assessment of tissue specimens.

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Availability of data and materials

All data generated or analyzed during the current study are included in this published article.

Authors' contributions

AW, SZ and DC processed the tissue specimens, performed the RNA extraction and qPCR. DG and TX designed the study, contributed to the analysis of the data and wrote the manuscript. JS performed the histological analysis and interpreted the patient data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients provided signed informed consent for the donation of the specimens. The current study was approved by the

Ethics Committee of the Institutional Review Board of Peking Union Medical College Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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