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Establishment and quality evaluation of a glioma biobank in Beijing Tiantan Hospital

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Background: We established a glioma biobank at Beijing Tiantan Hospital in November, 2010.Specialized staffs have been trained to collect, store and manage the biobank in accordance with standard operating procedures.

Methods: One hundred samples were selected to evaluate the quality of glioma samples stored in the liquid nitrogen tank during different periods (from 2011 to 2015) by morphological examination, RNA integrity determination, DNA integrity determination, housekeeping gene expression and protein integrity determination.

Results: The majority of samples (95%) remain high RNA quality for further analysis with RIN≥6. All samples remain high DNA and protein quality without significant degradation.

Conclusion: Storage conditions of our biobank are suitable for long-term (at least 5 years) sample preservation with high molecular quality.

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Abstract :

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Introduction

Gliomas refer to a group of tumors that arise from the glial tissue of the central nervous system. Basically, they can occur anywhere in the central nervous system, both brain and spine. Gliomas are the most commonly occurring tumors of the central nervous system which make up about 27% of all brain tumors and 80% of all malignant primary brain tumors (Agnihotri et al.,2013; Wilson, Karajannis&Harter,2014; Aldape et al.,2015). The incidence rate of glioma is approximately 4.67-5.73 per 100000 persons (Ostrom et al.,2014). Glioblastoma (GBM) is the most malignant type of gliomas. The median survival time of patients with GBM is 12–15 months (Bryukhovetskiy et al.,2016).

Tumor biobanks aim to systematically collect, store, manage and utilize tumor tissues from surgical removal , blood samples and also the corresponding participant data for further use of scientific projects and more specifically, for clinical and translational research (Watson, Kay&Smith,2010; Kang et al.,2013). Relying on the neurosurgery discipline, a well-managed and normative glioma biobank was established in our hospital in November, 2010. Using morphological examination, RNA integrity determination, DNA integrity determination, housekeeping gene expression and protein integrity determination, the quality of the glioma tissues stored in the liquid nitrogen tank for 1-6 years (collected from 2011 to 2015) was evaluated in order to provide high quality samples for medical research.

Materials and methods

Laboratory equipment

The lab is equipped with -80℃ freezers, -150℃ freezers and liquid nitrogen tanks. Each freezer has a specialized temperature monitoring system to detect real-time temperature. When the temperature inside freezers is abnormal, alarm messages will be sent to biobank managers. Central air-conditioning system keeps the room temperature around 20℃.

Collection and storage of tumor tissues

Excised glioma tissues from patients were collected immediately by trained and experienced staffs as soon as tumor samples were removed from the body during neurosurgery. Consent to use resected tissues was obtained from all cancer patients prior to surgery. This study was approved by the Ethics Committee of Beijing Tiantan Hospital (KY2014-021-02).On the basis of pathological diagnosis, the remaining tissue samples were gathered. Blood and necrotic tissue on the surface of the samples were washed off with pre-cooling PBS. Then the tumor samples were cut into small pieces (about 0.5 cm x 0.5 cm, thickness < 0.5 cm) under aseptic conditions, repackaged in cryotubes and quickly frozen in liquid nitrogen tank in the operating room for temporary storage. All samples were frozen within 30 min after resection (Tonje et al.,2016). On the same day, they were transferred to liquid nitrogen tanks in the biobank for long-term preservation. Registration of samples were done at the same time. Clinical information of patients including name, gender, age, pathological diagnosis, clinical diagnosis and treatment were all recorded and stored in the database.

Morphology characteristics

One hundred samples were selected to conduct the following experiments from 2011 to 2015. For each year, twenty samples were selected randomly. Firstly, the tissue fragments were fixed in 10% formalin before thawed for 24 hours then embedded in paraffin. Next, five-micrometer tissue sections were cut, dewaxed and stained with Hematoxylin–Eosin (HE) in accordance with standard procedures (Iigen et al.,2014).The slides were observed by an experienced pathologist. Tumor samples which were comprised of ≥ 80% tumor nuclei and ≤ 20% necrosis (≤ 50% necrosis for GBM) were considered fit for other research.

RNA isolation

Total RNA was isolated using TRIZOL reagent (Invitrogen, Stockholm, Sweden). 1ml TRIZOL reagent was added into an RNAse-free tube. 100mg frozen tissue of each sample was then removed into the tube and homogenizated using RNAse-free pestles. The homogenizated

tissue was incubated in TRIZOL reagent for 10 minutes at room temperature. After those, 200ul chloroform was added into each tube, and the tubes were manually shaken for 15 seconds before placed at room temperature for 10 minutes. The tubes were centrifuged at 12,000rpm for 15 minutes at 4℃, after which the aqueous phase was transferred to a new RNAse-free tube. Afterwards, 500ul isopropyl alcohol was added into each tube and incubated at -20℃ for 20 minutes. The tubes were centrifuged at 12,000rpm for 10 minutes at 4℃. The supernatant was discarded, and 1ml 75% ethanol was added into each tube to wash RNA pellet. Finally, the tubes were centrifuged at 7500g for 10 minutes. The supernatant was discarded, and the RNA pellet was air-dried and subsequently dissolved into 30ul RNase-free water.

RNA yield and integrity determination

A Nanodrop spectrophotometer (Thermo Fisher Scientific) was used to determine concentrations (ng/ul) and purity of RNA samples. The A260/A280 ratio was measured to indicate RNA purity. The ratio of samples with high purity is 1.8~2.1 (Sanabria et al.,2014). An Agilent 2100 bioanalyzer in conjunction with the RNA 6000 Nano and the RNA 6000 Pico LabChip kits（Agilent Biotechnologies, Palo Alto, CA）was used to evaluate RNA integrity, from which RNA integrity number (RIN) was calculated. A scoring system between 1 and 10 were used in the RIN software, with 1 representing degraded RNA and 10 representing very high-quality, intact RNA (Griffin et al.,2012). In literature, samples were divided into four quality groups according to RIN: RIN<5, not reliable for demanding downstream analysis; 5≤RIN<6, suitable for quantitative reverse transcription-PCR (RT-qPCR); 6≤RIN<8, suitable for gene array analysis; and RIN>8, suitable for all downstream techniques (Kap et al.,2014). The assays were performed according to the manufacturer's instructions.

cDNA synthesis and real-time quantitative PCR (RT-qPCR) for housekeeping genes

Extracted RNA (1ug) was reverse-transcribed to first-strand cDNA using the PrimeScript RT reagent Kit With gDNA Eraser (TaKaRa). Afterwards, 2ul cDNA solution was used for a 40-cycle SYBR Green PCR assay with the SYBR Premix EX Taq reagent (TaKaRa). The same

sample was run three different times in the same experiment to remove any outliers. Primer sets were as follows: human ACTB: forward primer 5'-TTAGTTGCGTTACACCCTTTCTTG -3'; reverse primer 5'- GTCACCTTCACCGTTCCAGTTTT-3'; human GAPDH: forward primer 5'-CTATAAATTGAGCCCGCAGCC-3'; reverse primer 5'-GCGCCCAATACGACCAAATC-3', The Thermal Cycler DiceReal Time System (TaKaRa) was used for qRT-PCR under the following conditions: 95℃ for 30s, 95℃ for 5s，58℃ for 30 s，72℃ for 30 s.

DNA isolation

100mg frozen tissue of each sample was pulverized to isolate genome DNA. DNA isolation was accomplished using the EasyPure Genomic DNA kit (Transgen Biotech) according to the manufacturer's instructions. In the end, the DNA pallet was dissolved into 50ul ddwater(PH>8.0).

DNA yield and PCR for housekeeping genes

A Nanodrop spectrophotometer (Thermo Fisher Scientific) was used to determine concentrations (ng/ul) and the A260/A280 of DNA samples. After that, 50ng genomic DNA was used for a 40-cycle SYBR Green PCR assay with the SYBR Premix EX TaqⅡ reagent (TaKaRa). Each measurement was performed in triplicate to remove any outliers. Primer sets were as follows: human ACTB: forward primer 5'-AAGACCTGTACGCCAACACA-3'; reverse primer 5'-CTGGATGTGACAGCTCCCC-3'; the primer set of human GAPDH was the same as mentioned before.

β-Globin PCR amplification

Four different length fragments of the housekeeping gene β-globin were amplified to evaluate DNA quality. The maximum amplicon size positively correlates with DNA quality. PCR amplification was performed with 50 ng of tissue DNA using DNA Polymerase High Fidelity (TransTaq). The primers are shown in Table 1. PCR was performed using the following conditions: primerⅠ：initial denaturation at 94℃ for 5 min, followed by 35 cycles of denaturing at 94℃ for 30s, annealing at 56℃ for 30s, extension at 72℃ for 30s, and final extension at 72℃ for 5 min；primerⅡ：initial denaturation at 94℃ for 5 min, followed by 35 cycles of denaturing at 94℃ for 30s, annealing at 52℃ for 30s, extension at 72℃ for 30s, and final extension at 72℃

for 5 min ; primer Ⅲ : initial denaturation at 94℃ for 5 min, followed by 35 cycles of denaturing at 94℃ for 30s, annealing at 52℃ for 30s, extension at 72℃ for 1 min, and final extension at 72℃ for 5 min ; primer Ⅳ : initial denaturation at 94℃ for 5 min, followed by 35 cycles of denaturing at 94℃ for 30s, annealing at 54℃ for 30s, extension at 72℃ for 1min, and final extension at 72℃ for 5 min. PCR products were analyzed on agarose gels.

Protein isolation and western blot

100mg frozen tissue of each sample was pulverized to isolate tissue protein. First, the powdered tissue was incubated in 500ul RIPA (Applygen) containing 50x protease inhibitor (Applygen) on the ice for at least 15 minutes. Afterward, the solution was centrifuged at 12000rpm for 15 minutes at 4℃.Then, the supernatant was decanted to a new fresh tube, which was the final protein solution we needed. The protein concentration was measured by BCA assay (Applygen) according to the manufacturer's instructions. Electrophoresis was performed using 12% acrylamide gel, 20ug protein was run from each sample on the gel and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) .The membranes were blocked with 5% non-fatty milk in PBS containing 0.1% of Tween-20 for one hour and incubated with monoclonal anti-ACTB antibody (Tansgen) overnight. Then the membranes were washed thrice for 5 minutes before addition of HRP conjugated anti-mouse antibody for 1 hour. Membranes were then washed and treated with ECL Western Blotting Substrate (Fisher Scientific) according to manufacturer's instructions.

Statistical analysis

Statistical differences were analyzed using one-way ANOVA test. The criterion for significance was set at P value < 0.05.

Results

Composition of the biobank

The total number of cancer cases reached 3686 from 2010 to 2016. 3,686 patients were consented and tissue was collected from each patient. Fig.1 showed the constituent ratio of

glioma subtypes. The vast majority subtypes of the biobank were oligo-astrocytoma, astrocytoma and glioblastoma.

Morphology characteristics

According to the HE staining results, all tumor samples were comprised of ≥ 80% tumor nuclei and ≤ 20% necrosis (≤50% necrosis for GBM). Fig.2 shows the representative images from the 100 samples. No significant tissue decomposition was detected in all selected samples.

RNA integrity of different storage duration

The majority of RNA samples had a A260/A280 ratio ranging between 1.8 and 2.1 which indicate high purity (Fig.3). The RIN results of the selected 100 samples showed that 95% samples in the biobank were suitable for RT-qPCR and gene array analysis with RIN≥6 (Fig.4). Besides, no significant change in the RNA quality was found among different storage periods (from 2011 to 2015).

RT -qPCR for Housekeeping genes

RT -qPCR for housekeeping genes (GAPDH ， ACTB) was performed to verify the accuracy of RIN determination. Except for the 5% samples with RIN<6, of which the ct values were relatively higher, gene expression levels of all the other 95% samples remained stable under different storage periods (Table 2 and Fig.5). No significant difference was found.

DNA integrity of different storage durations

The A260/A280 and PCR for genomic housekeeping genes (GAPDH ， ACTB) were performed to evaluate DNA degradation level. DNA samples had a A260/A280 ratio ranging between 1.8 and 2.0 with the exception of two samples showed ratios of 2.05 and 2.07 (Fig.6), suggesting contamination during the phenol extraction. According to the PCR results, all the ct values were stable of different storage durations, no significant difference was found (Fig.7). DNA quality was assessed also by PCR amplification of ß-globin gene fragments. The integrity of DNA was scored according to the number of amplified bands of increasing size from 1 to 4. According to our results, 100% of extracted DNA samples were of good quality with at least

three amplified ß-globin bands of increasing size. No significant difference was found in DNA quality of different storage durations.

Western blot

Western blot of ACTB was performed to predict tissue protein quality. Western blot analyses revealed that all the samples were enriched for ACTB (Fig.8). No significant protein degradation was found.

Discussion

Tumor biobanks aim to collect and store sufficient number of tumor samples with high quality for basic cancer research .Clinical and basic researchers use these samples to carry out molecular biology, cell biology, genetics, transcriptomics, genomics and proteomics research in order to explore new standard of tumor classification, diagnosis, treatment and prognosis. Prolonged storage periods of tumor samples allows researchers to design studies to identify biomarkers of aggressiveness and responses to different drug treatments which increases their value (Hewitt,2011; Olivieri et al.,2014). Thus, establishment of tumor biobanks with high quality samples plays an important role in personal medicine and translational research.

Compared with other moleculers, the RNA molecule is less stable and more likely to degrade by RNases because its ribonucleotides contain a free hydroxyl group in the pentose ring (Zhang et al.,2016). However, in our study, we didn't find significant RNA degradation in samples preserved in the liquid nitrogen tank (-196℃) for 1-6 years (from 2011 to 2015). Except for few samples (5%) with RIN<6, the majority of them (95%) remain high quality for further analysis with RIN≥6. We verified the accuracy of RIN determination by RT-qPCR of housekeeping genes (GAPDH ， ACTB). The number of cycles required to reach a detectable threshold level for fluorescence is defined as ct. Ct is inversely correlated with the amount of template RNA (Riemer et al.,2012). Thus, RNA samples with high quality have a relatively lower ct value whereas RNA samples with degradation have a relatively higher ct value of housekeeping genes. Our results of RT-qPCR were in accordance with RIN determination. All the 95% samples with RIN≥6 have

stable ct values of housekeeping genes (ACTB: 14.32±0.47 ~ 14.70±0.65; GAPDH: 21.11±0.75 ~ 22.17±1.01). Our findings were consistent with those of Andreasson et al. (2013), who assessed RIN values in endocrine tissues stored at −80°C for approximately 30 years and found that long-term storage in −80°C did not adversely affect the quality of the RNA extracted from the tissues. In our study, we found that glioma tissues preserved in liquid nitrogen (-196℃) could well maintain RNA quality for at least 5 years. There are also other studies showed similar results such as Hebel et al.(2013)detected RIN values in blood samples stored at −80°C for 4–19 years and at −196°C for 11–19 years and found no adversely correlation between RNA quality and storage duration.DNA and protein are more stable than RNA. QPCR reactions of genomic housekeeping genes (GAPDH ，ACTB) showed no signs of DNA degradation as the ct values were stable (ACTB: 22.63±0.63 ~ 23.06±0.57; GAPDH: 32.77±0.63 ~ 33.32±1.50). β-Globin PCR amplification showed that 100% of extracted DNA samples were of good quality with at least three amplified ß-globin bands of increasing size. Since there is not a standard method to evaluate tissue protein quality, we just evaluated one housekeeping protein through western blot to roughly predict protein quality. Protein degradation was not found from ACTB western blot analysis.

According to our research, storage conditions of our biobank are suitable for long-term (at least 5 years) sample preservation with high molecular quality. However, there are also limitations of our research. Heterogeneity has been found in various human tumors and glioblastoma (GBM) is a highly heterogeneous tumor (Diaz-Cano,2012; Furnari et al.,2015). In our research, we only studied the tumor nuclei and necrosis proportion, but we did not study whether the pathological type of samples preserved in our biobank is consistent with the previous pathological diagnosis. About protein quality, we only studied one protein, but it is far from suggesting all proteins are of good quality. We only studied gliomas and are not sure whether the other tumors have similar quality results. Moreover, our biobank established only for a short period (presently up to 6 years), we should further verify the quality over time to find optimal storage periods.

Conclusion

Storage conditions of our biobank are suitable for long-term (at least 5 years) sample preservation with high molecular quality.

Acknowledgements

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Table 1(on next page)

Primers used for β-globin gene amplification by PCR

Amplicon	Forward primer	Reverse primer	Size (bp)
Ⅰ	GAAGAGCCAAGGACAGGTAC	CAACTTCATCCACGTTCACC	268
Ⅱ	GCTCACTCAGTGTGGCAAAG	GGTTGGCCAATCTACTCCCAGG	536
Ⅲ	ATTTTCCCACCCTTAGGCTG	TGGTAGCTGGATTGTAGCTG	989
Ⅳ	GGTTGGCCAATCTACTCCCAGG	TGGTAGCTGGATTGTAGCTG	1327

Table 2(on next page)

Gene expression levels under different storage durations

Year	gene	Ct values (Average±SD) of samples with		
		RIN < 5	5≤RIN<6	RIN≥6
2011	ACTB	16.17±0.14		14.50±0.41
	GAPDH	23.21±0.28		21.13±0.85
2012	ACTB	18.26±0.00		14.39±0.66
	GAPDH	26.10±0.00		21.69±1.00
2013	ACTB	15.79±0.00	15.11±0	14.40±0.58
	GAPDH	23.24±0.00	22.10±0	21.11±0.75
2014	ACTB			14.70±0.65
	GAPDH			22.17±1.01
2015	ACTB			14.32±0.47
	GAPDH			21.62±0.66

Figure 1

Constituent ratio of glioma subtypes

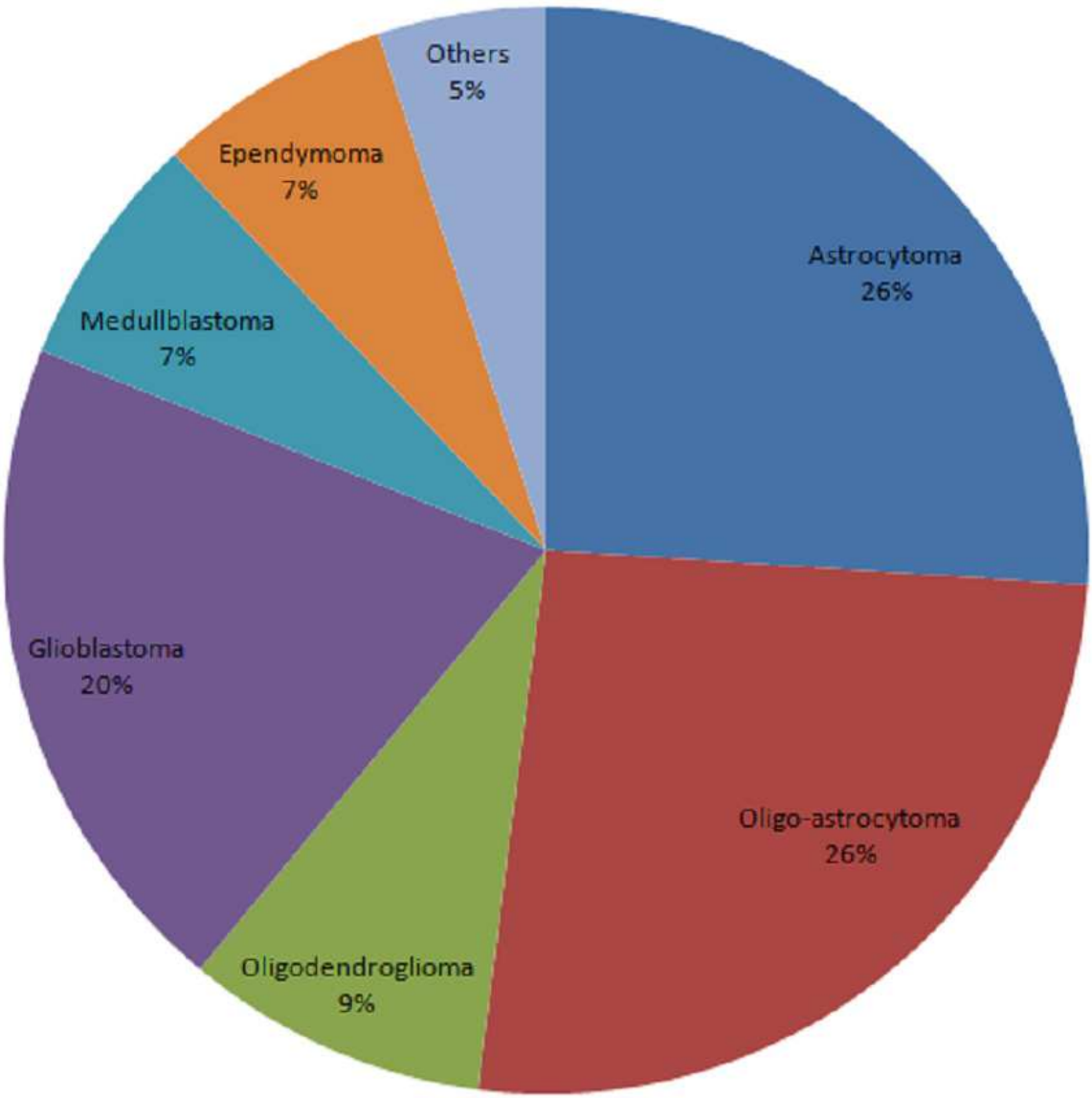

Figure 2

Morphology of glioma tissues preserved in liquid nitrogen of different storage periods (from 2011 to 2015)

(A) Morphology of glioma tissues preserved in liquid nitrogen in 2011. (B) Morphology of glioma tissues preserved in liquid nitrogen in 2012. (C) Morphology of glioma tissues preserved in liquid nitrogen in 2013. (D) Morphology of glioma tissues preserved in liquid nitrogen in 2014. (E) Morphology of glioma tissues preserved in liquid nitrogen in 2015.

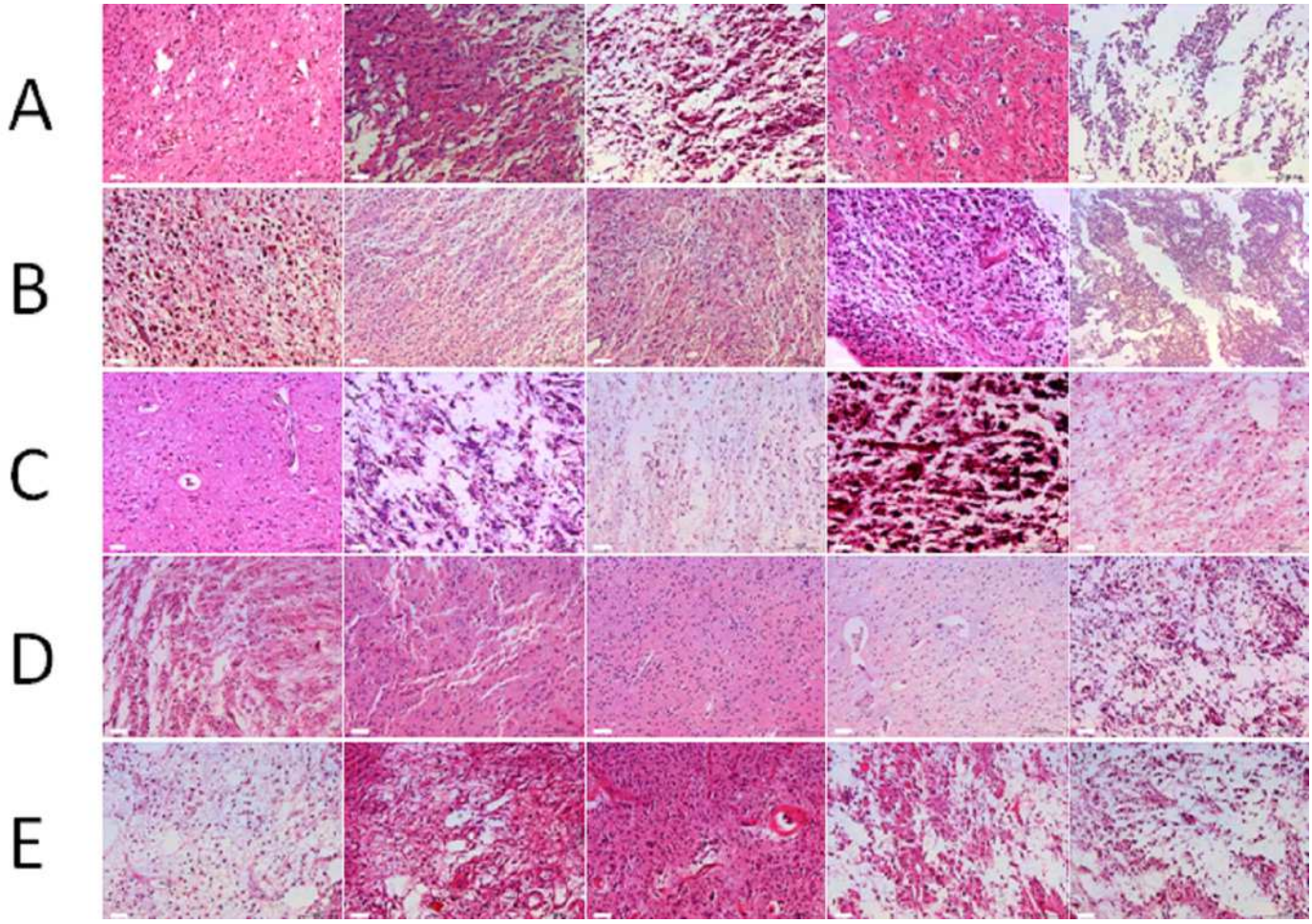

Figure 3

Ratio 260/280 of RNA samples

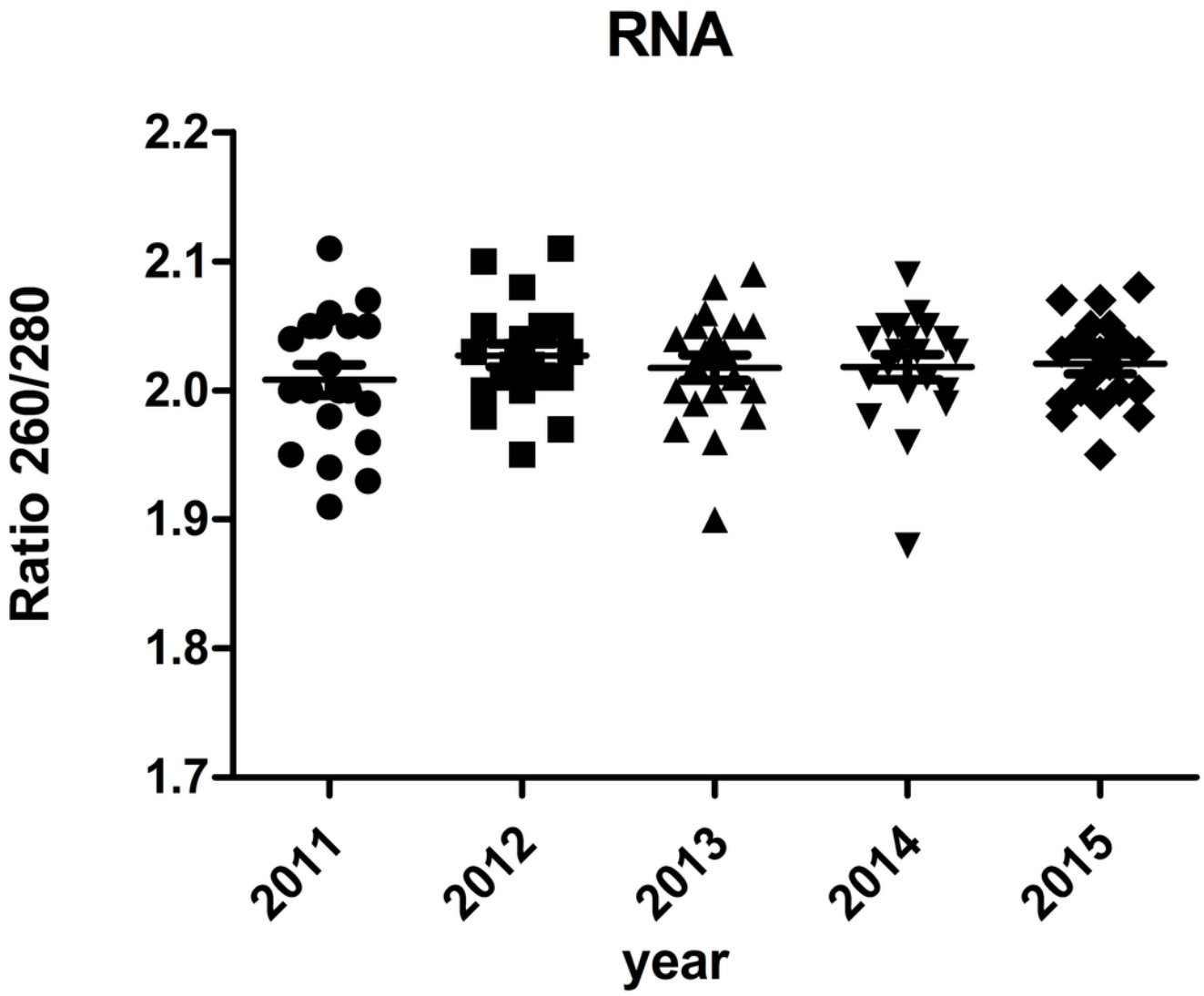

Figure 4

RNA integrity number of selected samples of different storage periods

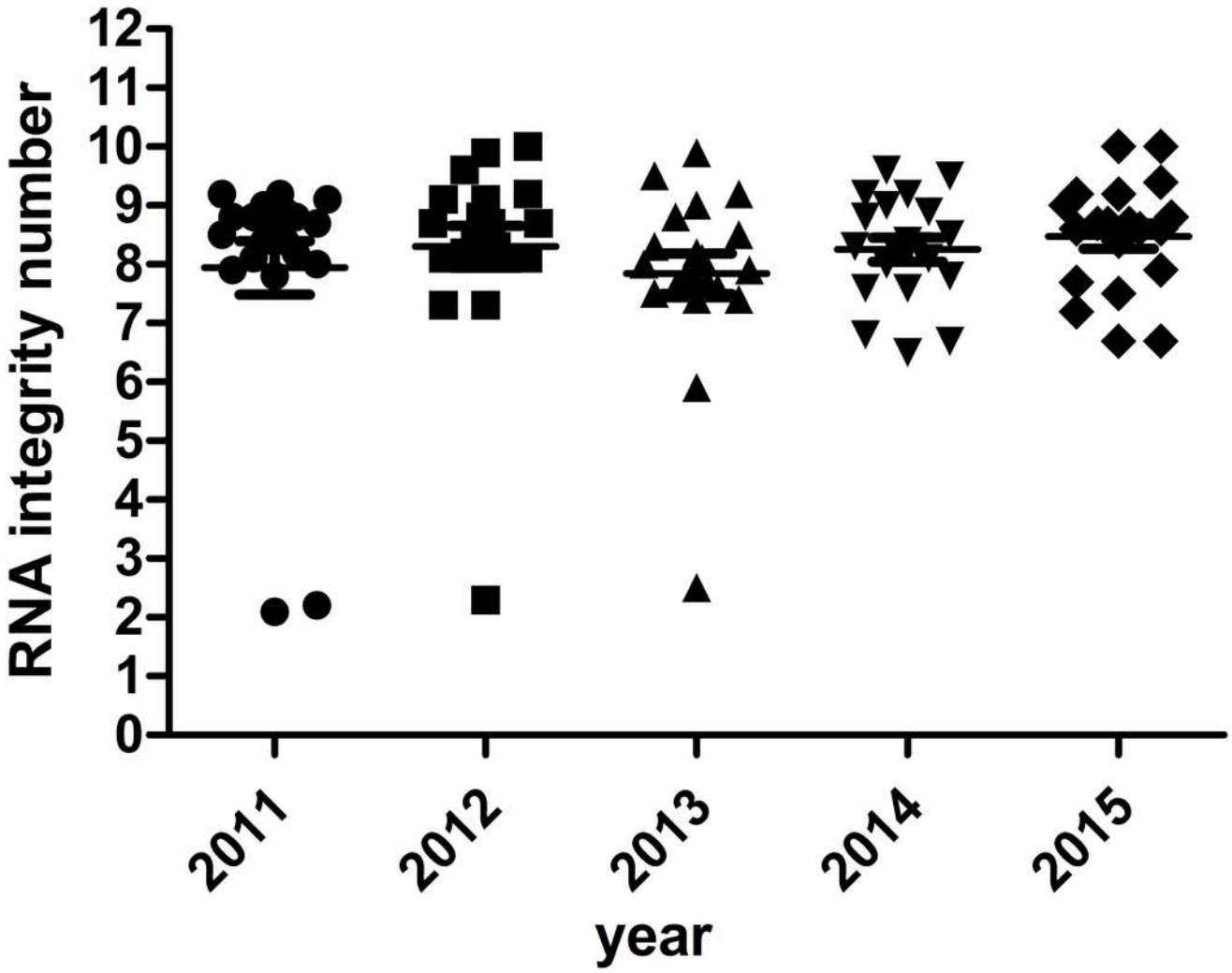

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Figure 5

Gene expression levels under different storage durations

(A) Gene expression levels of ACTB; (B) Gene expression levels of GAPDH.

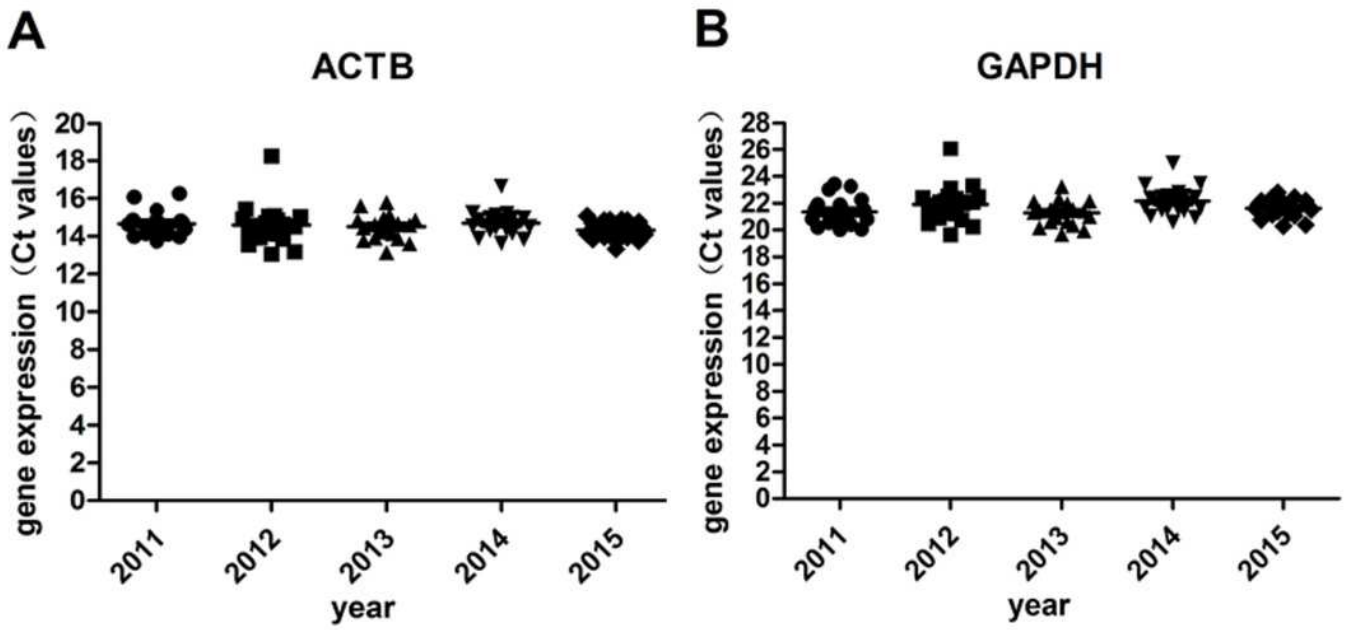

Figure 6

Ratio 260/280 of DNA samples

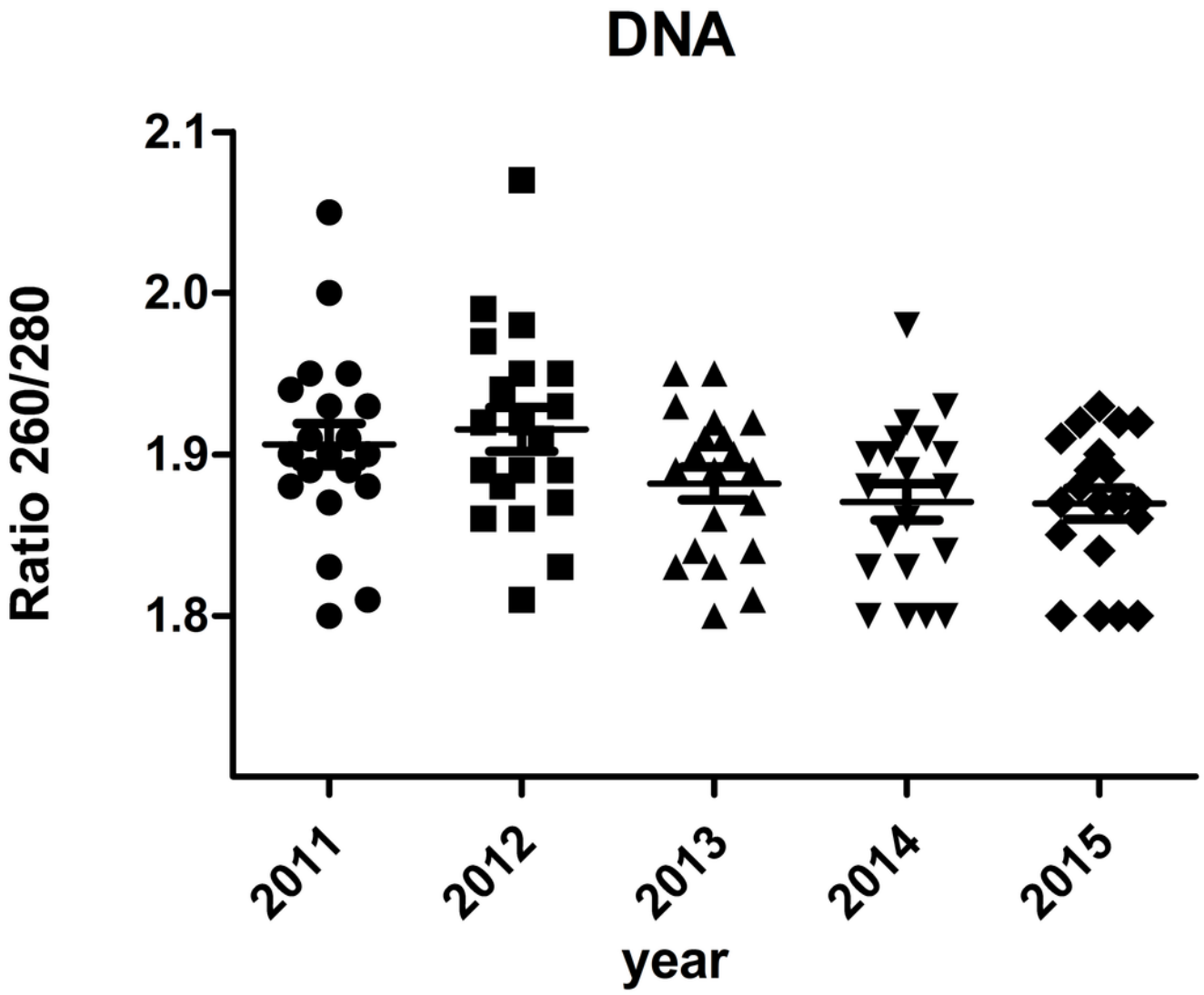

Figure 7

Ct values of genomic housekeeping genes under different storage durations

(A)Ct values of ACTB.(B)Ct values of GAPDH.

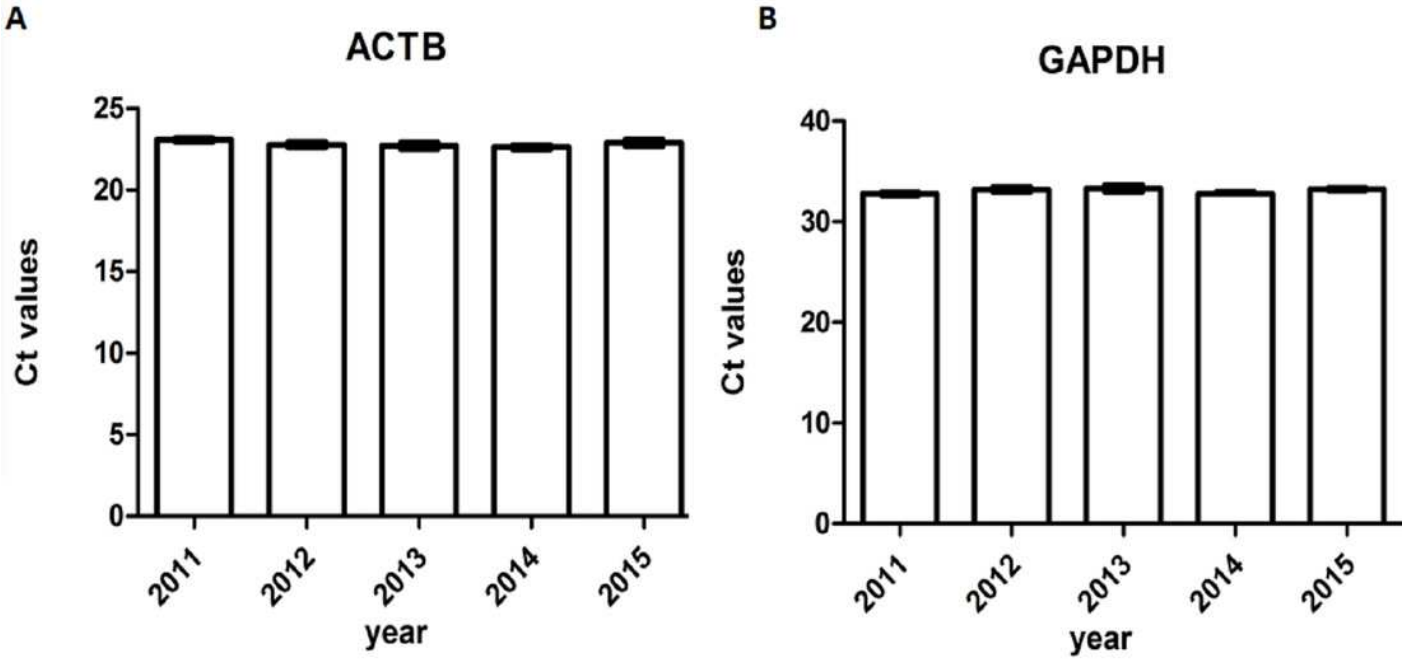

Figure 8

Western blot analysis of ACTB of selected samples under different storage periods

(A) Western blot analysis of ACTB of selected samples in 2011. (B) Western blot analysis of ACTB of selected samples in 2012. (C) Western blot analysis of ACTB of selected samples in 2013. (D) Western blot analysis of ACTB of selected samples in 2014. (E) Western blot analysis of ACTB of selected samples in 2015.

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