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

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Abstract :

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%) had high RNA quality for further analysis with RIN≥6. Quality of DNA and protein of all samples remained stable without significant degradation.

28   **Conclusion:** Storage conditions of our biobank are suitable for long-term (at least 5 years)

29   sample preservation with high molecular quality.

Introduction

31   Gliomas refer to a group of tumors that arise from the glial tissue of the central nervous

32   system. Basically, they can occur anywhere in the central nervous system, both brain and spine.

33   Gliomas are the most commonly occurring tumors of the central nervous system which make up

34   about 27% of all brain tumors and 80% of all malignant primary brain tumors (Agnihotri et

35   al.,2013; Wilson, Karajannis&Harter,2014; Aldape et al.,2015). The incidence rate of glioma is

36   approximately 4.67-5.73 per 100000 persons (Ostrom et al.,2014). Glioblastoma (GBM) is the

37   most malignant type of gliomas. The median survival time of patients with GBM is 12–15

38   months (Bryukhovetskiy et al.,2016).

39   Tumor biobanks aim to systematically collect, store, manage and utilize tumor tissues from

40   surgical removal , blood samples and also the corresponding participant data for further use of

41   scientific projects and more specifically, for clinical and translational research (Watson,

42   Kay&Smith,2010; Kang et al.,2013). Relying on the neurosurgery discipline, a well-managed and

43   normative glioma biobank was established in our hospital in November, 2010. Using

44   morphological examination, RNA integrity determination, DNA integrity determination,

45   housekeeping gene expression and protein integrity determination, the quality of the glioma

46   tissues stored in the liquid nitrogen tank for 1-6 years (collected from 2011 to 2015) was

47   evaluated in order to provide high quality samples for medical research.

Materials and methods

49   *Laboratory equipment*

50   The lab is equipped with -80℃ freezers, -150℃ freezers and liquid nitrogen tanks. Each freezer

51   has a specialized temperature monitoring system to detect real-time temperature. When the

52   temperature inside freezers is abnormal, alarm messages will be sent to biobank managers.

53   Central air-conditioning system keeps the room temperature around 20℃.

54   *Collection and storage of tumor tissues*

55   Excised glioma tissues from patients were collected immediately by trained and experienced

56   staffs as soon as tumor samples were removed from the body during neurosurgery. Consent to use

57   resected tissues was obtained from all cancer patients prior to surgery. This study was approved

58   by the Ethics Committee of Beijing Tiantan Hospital (KY2014-021-02).On the basis of

59   pathological diagnosis, the remaining tissue samples were gathered. Blood and necrotic tissue on

60   the surface of the samples were washed off with pre-cooling PBS. Then the tumor samples were

61   cut into small pieces (about 0.5 cm x 0.5 cm, thickness < 0.5 cm) under aseptic conditions,

62   repackaged in cryotubes and quickly frozen in liquid nitrogen tank in the operating room for

63   temporary storage. All samples were frozen within 30 min after resection (Tonje et al.,2016). On

64   the same day, they were transferred to liquid nitrogen tanks in the biobank for long-term

65   preservation. Registration of samples were done at the same time. Clinical information of patients

66   including name, gender, age, pathological diagnosis, clinical diagnosis and treatment were all

67   recorded and stored in the database.

68   *Morphology characteristics*

69   One hundred samples were selected to conduct the following experiments from 2011 to 2015.

70   For each year, twenty samples were selected randomly. Firstly, the tissue fragments were fixed

71   in 10% formalin before thawed for 24 hours then embedded in paraffin. Next, five-micrometer

72   tissue sections were cut, dewaxed and stained with Hematoxylin–Eosin (HE) in accordance with

73   standard procedures (Iigen et al.,2014).The slides were observed by an experienced pathologist.

74   Tumor samples which were comprised of ≥ 80% tumor nuclei and ≤ 20% necrosis (≤ 50%

75   necrosis for GBM) were considered fit for other research.

76   *RNA isolation*

77   Total RNA was isolated using TRIZOL reagent (Invitrogen, Stockholm, Sweden). 1ml

78   TRIZOL reagent was added into an RNAse-free tube. 100mg frozen tissue of each sample was

79   then removed into the tube and homogenized using RNAse-free pestles. The homogenized

80   tissue was incubated in TRIZOL reagent for 10 minutes at room temperature. After those, 200ul

81   chloroform was added into each tube, and the tubes were manually shaken for 15 seconds before

82   placed at room temperature for 10 minutes. The tubes were centrifuged at 12,000rpm for 15

83   minutes at 4℃, after which the aqueous phase was transferred to a new RNAse-free tube.

84   Afterwards, 500ul isopropyl alcohol was added into each tube and incubated at -20℃ for 20

85   minutes. The tubes were centrifuged at 12,000rpm for 10 minutes at 4℃. The supernatant was

86   discarded, and 1ml 75% ethanol was added into each tube to wash RNA pellet. Finally, the tubes

87   were centrifuged at 7500g for 10 minutes. The supernatant was discarded, and the RNA pellet

88   was air-dried and subsequently dissolved into 30ul RNase-free water.

89   ***RNA yield and integrity determination***

90   A Nanodrop spectrophotometer (Thermo Fisher Scientific) was used to determine

91   concentrations (ng/ul) and purity of RNA samples. The A260/A280 ratio was measured to

92   indicate RNA purity. The ratio of samples with high purity is 1.8~2.1 (Sanabria et al.,2014). An

93   Agilent 2100 bioanalyzer in conjunction with the RNA 6000 Nano and the RNA 6000 Pico

94   LabChip kits(Agilent Biotechnologies, Palo Alto, CA)was used to evaluate RNA integrity,

95   from which RNA integrity number (RIN) was calculated. A scoring system between 1 and 10

96   were used in the RIN software, with 1 representing degraded RNA and 10 representing very high-

97   quality, intact RNA (Griffin et al.,2012). In literature, samples were divided into four quality

98   groups according to RIN: RIN<5, not reliable for demanding downstream analysis; $5 \leq RIN < 6$,

99   suitable for quantitative reverse transcription-PCR (RT-qPCR); $6 \leq RIN < 8$, suitable for gene array

100 analysis; and RIN>8, suitable for all downstream techniques (Kap et al.,2014). The assays were

101 performed according to the manufacturer's instructions.

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

103 Extracted RNA (1ug) was reverse-transcribed to first-strand cDNA using the PrimeScript RT

104 reagent Kit With gDNA Eraser (TaKaRa). Afterwards, 2ul cDNA solution was used for a 40-

105 cycle SYBR Green PCR assay with the SYBR Premix EX Taq reagent (TaKaRa). The same

106   sample was run three different times in the same experiment to remove any outliers. Primer sets

107   were as follows: human ACTB: forward primer 5'-TTAGTTGCGTTACACCCTTTCTTG -3';

108   reverse primer 5'- GTCACCTTCACCGTTCCAGTTTT-3'; human GAPDH: forward primer 5'-

109   CTATAAATTGAGCCCGCAGCC-3'; reverse primer 5'-GCGCCCAATACGACCAAATC-3',

110   The Thermal Cycler DiceReal Time System (TaKaRa) was used for qRT-PCR under the

111   following conditions: 95℃ for 30s, 95℃ for 5s﹐58℃ for 30 s﹐72℃ for 30 s.

112   ***DNA isolation***

113   100mg frozen tissue of each sample was pulverized to isolate genome DNA. DNA isolation

114   was accomplished using the EasyPure Genomic DNA kit (Transgen Biotech) according to the

115   manufacturer's instructions. In the end, the DNA pallet was dissolved into 50ul ddwater(PH>8.0).

116   ***DNA yield and PCR for housekeeping genes***

117   A Nanodrop spectrophotometer (Thermo Fisher Scientific) was used to determine concentrations

118   (ng/ul) and the A260/A280 of DNA samples. After that, 50ng genomic DNA was used for a 40-

119   cycle SYBR Green PCR assay with the SYBR Premix EX TaqⅡ reagent (TaKaRa). Each

120   measurement was performed in triplicate to remove any outliers. Primer sets were as follows:

121   human ACTB: forward primer 5'-AAGACCTGTACGCCAACACA-3'; reverse primer 5'-

122   CTGGATGTGACAGCTCCCC-3'; the primer set of human GAPDH was the same as mentioned

123   before.

124   ***β-Globin PCR amplification***

125   Four different length fragments of the housekeeping gene β-globin were amplified to evaluate

126   DNA quality. The maximum amplicon size positively correlates with DNA quality. PCR

127   amplification was performed with 50 ng of tissue DNA using DNA Polymerase High Fidelity

128   (TransTaq). The primers are shown in Table 1. PCR was performed using the following

129   conditions: primerⅠ﹕initial denaturation at 94℃ for 5 min, followed by 35 cycles of denaturing

130   at 94℃ for 30s, annealing at 56℃ for 30s, extension at 72℃ for 30s, and final extension at 72℃

131   for 5 min﹔primerⅡ﹕initial denaturation at 94℃ for 5 min, followed by 35 cycles of denaturing

132   at 94℃ for 30s, annealing at 52℃ for 30s, extension at 72℃ for 30s, and final extension at 72℃

133   for 5 min ; primer Ⅲ : initial denaturation at 94℃ for 5 min, followed by 35 cycles of denaturing

134   at 94℃ for 30s, annealing at 52℃ for 30s, extension at 72℃ for 1 min, and final extension at

135   72℃ for 5 min ; primer Ⅳ : initial denaturation at 94℃ for 5 min, followed by 35 cycles of

136   denaturing at 94℃ for 30s, annealing at 54℃ for 30s, extension at 72℃ for 1min, and final

137   extension at 72℃ for 5 min. PCR products were analyzed on agarose gels.

138   ***Protein isolation and western blot***

139   100mg frozen tissue of each sample was pulverized to isolate tissue protein. First, the

140   powdered tissue was incubated in 500ul RIPA (Applygen) containing 50x protease inhibitor

141   (Applygen) on the ice for at least 15 minutes. Afterward, the solution was centrifuged at

142   12000rpm for 15 minutes at 4℃.Then, the supernatant was decanted to a new fresh tube, which

143   was the final protein solution we needed. The protein concentration was measured by BCA assay

144   (Applygen) according to the manufacturer's instructions. Electrophoresis was performed using

145   12% acrylamide gel, 20ug protein was run from each sample on the gel and proteins were

146   transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) .The membranes were

147   blocked with 5% non-fatty milk in PBS containing 0.1% of Tween-20 for one hour and incubated

148   with monoclonal anti-ACTB antibody (Tansgen) overnight. Then the membranes were washed

149   thrice for 5 minutes before addition of HRP conjugated anti-mouse antibody for 1 hour.

150   Membranes were then washed and treated with ECL Western Blotting Substrate (Fisher

151   Scientific) according to manufacturer's instructions.

152   ***Statistical analysis***

153   Statistical differences were analyzed using one-way ANOVA test. The criterion for significance

154   was set at *P* value < 0.05.

155   **Results**

156   ***Composition of the biobank***

157   The total number of cancer cases reached 3686 from 2010 to 2016. 3,686 patients were

158   consented and tissue was collected from each patient. Fig.1 showed the constituent ratio of

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

160 and glioblastoma.

161 *Morphology characteristics*

162 According to the HE staining results, all tumor samples were comprised of ≥ 80% tumor

163 nuclei and ≤ 20% necrosis (≤50% necrosis for GBM). Fig.2 shows the representative images

164 from the 100 samples. No significant tissue decomposition was detected in all selected samples.

165 *RNA integrity of different storage duration*

166 The majority of RNA samples had a A260/A280 ratio ranging between 1.8 and 2.1 which

167 indicate high purity (Fig.3). The RIN results of the selected 100 samples showed that 95%

168 samples in the biobank were suitable for RT-qPCR and gene array analysis with RIN≥6 (Fig.4).

169 Besides, no significant change in the RNA quality was found among different storage periods

170 (from 2011 to 2015).

171 *RT -qPCR for Housekeeping genes*

172 RT -qPCR for housekeeping genes (GAPDH ,ACTB) was performed to verify the accuracy of

173 RIN determination. Except for the 5% samples with RIN<6, of which the ct values were

174 relatively higher, gene expression levels of all the other 95% samples remained stable under

175 different storage periods (Table 2 and Fig.5). No significant difference was found.

176 *DNA integrity of different storage durations*

177 The A260/A280 and PCR for genomic housekeeping genes (GAPDH ,ACTB) were

178 performed to evaluate DNA degradation level. DNA samples had a A260/A280 ratio ranging

179 between 1.8 and 2.0 with the exception of two samples showed ratios of 2.05 and 2.07 (Fig.6),

180 suggesting contamination during the phenol extraction. According to the PCR results, all the ct

181 values were stable of different storage durations, no significant difference was found (Fig.7).

182 DNA quality was assessed also by PCR amplification of ß-globin gene fragments. The integrity

183 of DNA was scored according to the number of amplified bands of increasing size from 1 to 4.

184 According to our results, 100% of extracted DNA samples were of good quality with at least

185   three amplified ß-globin bands of increasing size. No significant difference was found in DNA

186   quality of different storage durations.

187   ***Western blot***

188   Western blot of ACTB was performed to predict tissue protein quality. Western blot analyses

189   revealed that all the samples were enriched for ACTB (Fig.8). No significant protein degradation

190   was found.

191   **Discussion**

192   Tumor biobanks aim to collect and store sufficient number of tumor samples with high quality

193   for basic cancer research .Clinical and basic researchers use these samples to carry out molecular

194   biology, cell biology, genetics, transcriptomics, genomics and proteomics research in order to

195   explore new standard of tumor classification, diagnosis, treatment and prognosis. Prolonged

196   storage periods of tumor samples allows researchers to design studies to identify biomarkers of

197   aggressiveness and responses to different drug treatments which increases their value

198   (Hewitt,2011; Olivieri et al.,2014). Thus, establishment of tumor biobanks with high quality

199   samples plays an important role in personal medicine and translational research.

200   Compared with other moleculers, the RNA molecule is less stable and more likely to degrade

201   by RNases because its ribonucleotides contain a free hydroxyl group in the pentose ring (Zhang

202   et al.,2016). However, in our study, we didn't find significant RNA degradation in samples

203   preserved in the liquid nitrogen tank (-196℃) for 1-6 years (from 2011 to 2015). Except for few

204   samples (5%) with RIN<6, the majority of them (95%) remain high quality for further analysis

205   with RIN≥6. We verified the accuracy of RIN determination by RT-qPCR of housekeeping genes

206   (GAPDH ﹑ACTB). The number of cycles required to reach a detectable threshold level for

207   fluorescence is defined as ct. Ct is inversely correlated with the amount of template RNA (Riemer

208   et al.,2012). Thus, RNA samples with high quality have a relatively lower ct value whereas RNA

209   samples with degradation have a relatively higher ct value of housekeeping genes. Our results of

210   RT-qPCR were in accordance with RIN determination. All the 95% samples with RIN≥6 have

211   stable ct values of housekeeping genes (ACTB: 14.32±0.47 ~ 14.70±0.65; GAPDH: 21.11±0.75

212   ~ 22.17±1.01). Our findings were consistent with those of Andreasson et al. (2013), who

213   assessed RIN values in endocrine tissues stored at −80°C for approximately 30 years and found

214   that long-term storage in −80°C did not adversely affect the quality of the RNA extracted from

215   the tissues. In our study, we found that glioma tissues preserved in liquid nitrogen (-196℃) could

216   well maintain RNA quality for at least 5 years. There are also other studies showed similar results

217   such as Hebel et al.(2013)detected RIN values in blood samples stored at −80°C for 4–19 years

218   and at −196°C for 11–19 years and found no adversely correlation between RNA quality and

219   storage duration.DNA and protein are more stable than RNA. QPCR reactions of genomic

220   housekeeping genes (GAPDH ,ACTB) showed no signs of DNA degradation as the ct values

221   were stable (ACTB: 22.63±0.63 ~ 23.06±0.57; GAPDH: 32.77±0.63 ~ 33.32±1.50). β-Globin

222   PCR amplification showed that 100% of extracted DNA samples were of good quality with at

223   least three amplified ß-globin bands of increasing size. Since there is not a standard method to

224   evaluate tissue protein quality, we just evaluated one housekeeping protein through western blot

225   to roughly predict protein quality. Protein degradation was not found from ACTB western blot

226   analysis.

227   According to our research, storage conditions of our biobank are suitable for long-term (at least

228   5 years) sample preservation with high molecular quality. However, there are also limitations of

229   our research. Heterogeneity has been found in various human tumors and glioblastoma (GBM) is

230   a highly heterogeneous tumor (Diaz-Cano,2012; Furnari et al.,2015). In our research, we only

231   studied the tumor nuclei and necrosis proportion, but we did not study whether the pathological

232   type of samples preserved in our biobank is consistent with the previous pathological diagnosis.

233   About protein quality, we only studied one protein, but it is far from suggesting all proteins are of

234   good quality. We only studied gliomas and are not sure whether the other tumors have similar

235   quality results. Moreover, our biobank established only for a short period (presently up to 6

236   years), we should further verify the quality over time to find optimal storage periods.

237   **Conclusion**

238   Storage conditions of our biobank are suitable for long-term (at least 5 years) sample

239   preservation with high molecular quality.

240   **Acknowledgements**

241   The authors kindly thank staffs of the Neurosurgical department of Beijing Tiantan Hospital in

242   helping with the collection of tissue samples.

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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

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
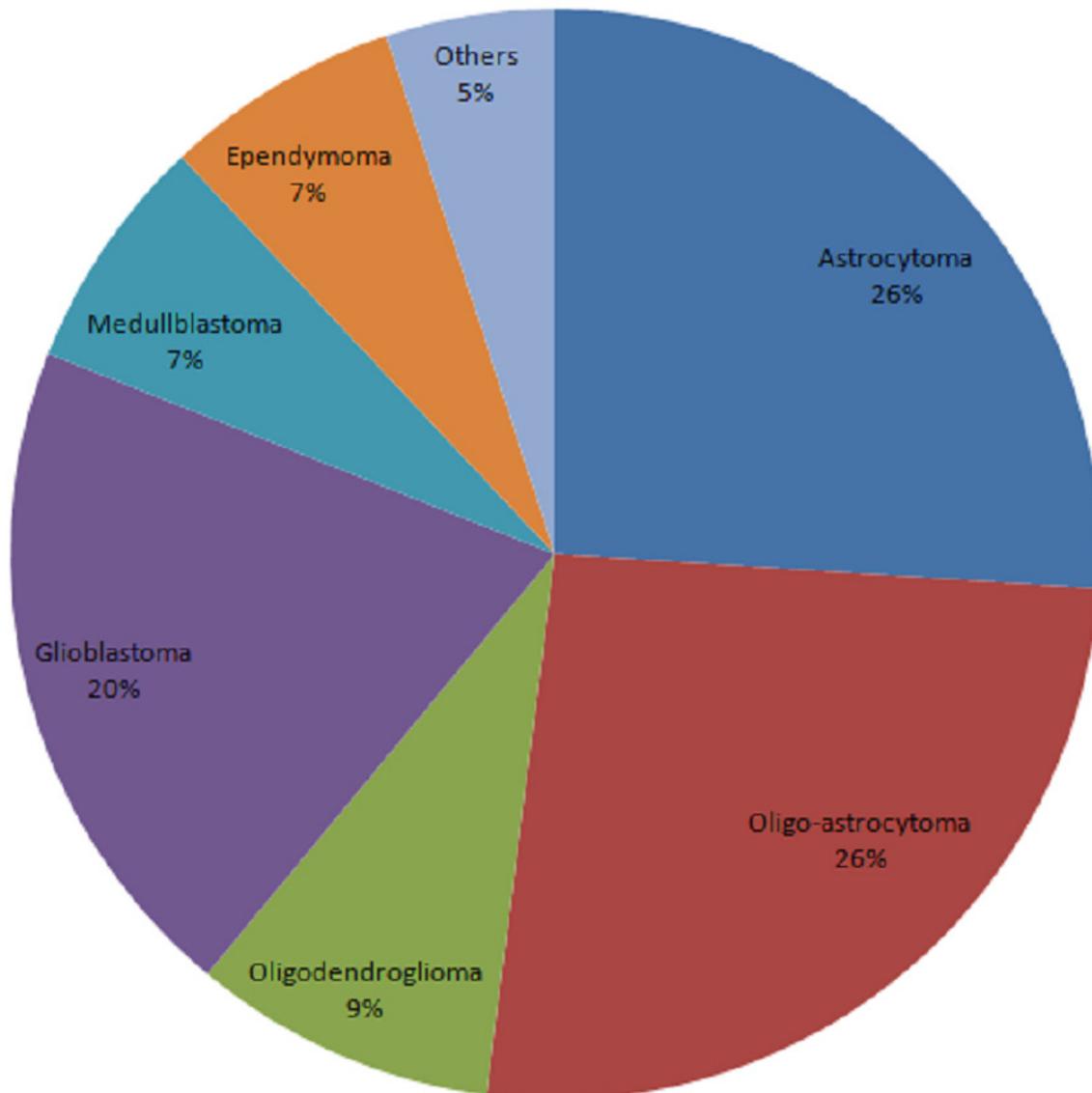
Figure 2

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.
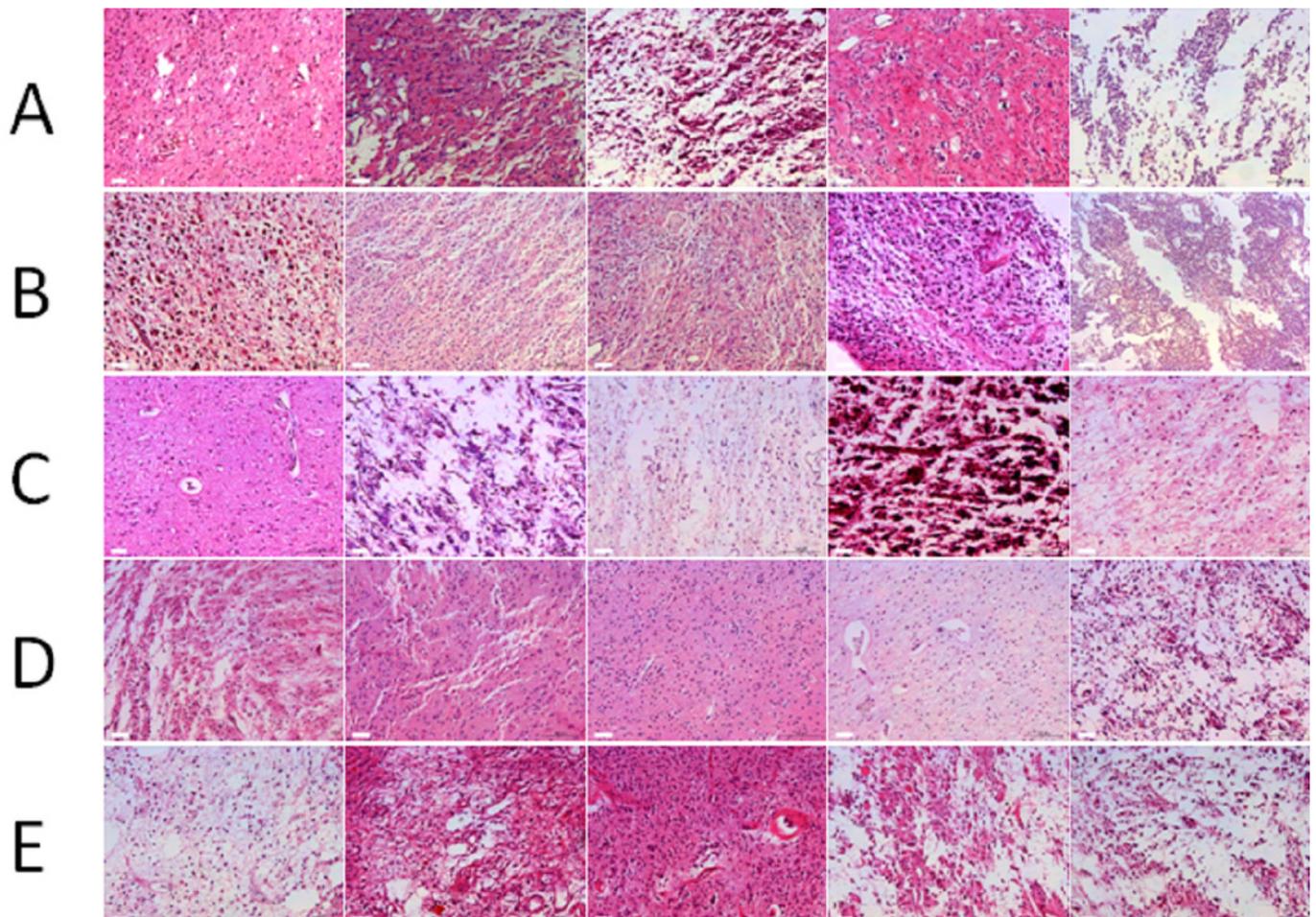
Figure 3

Ratio 260/280 of RNA samples
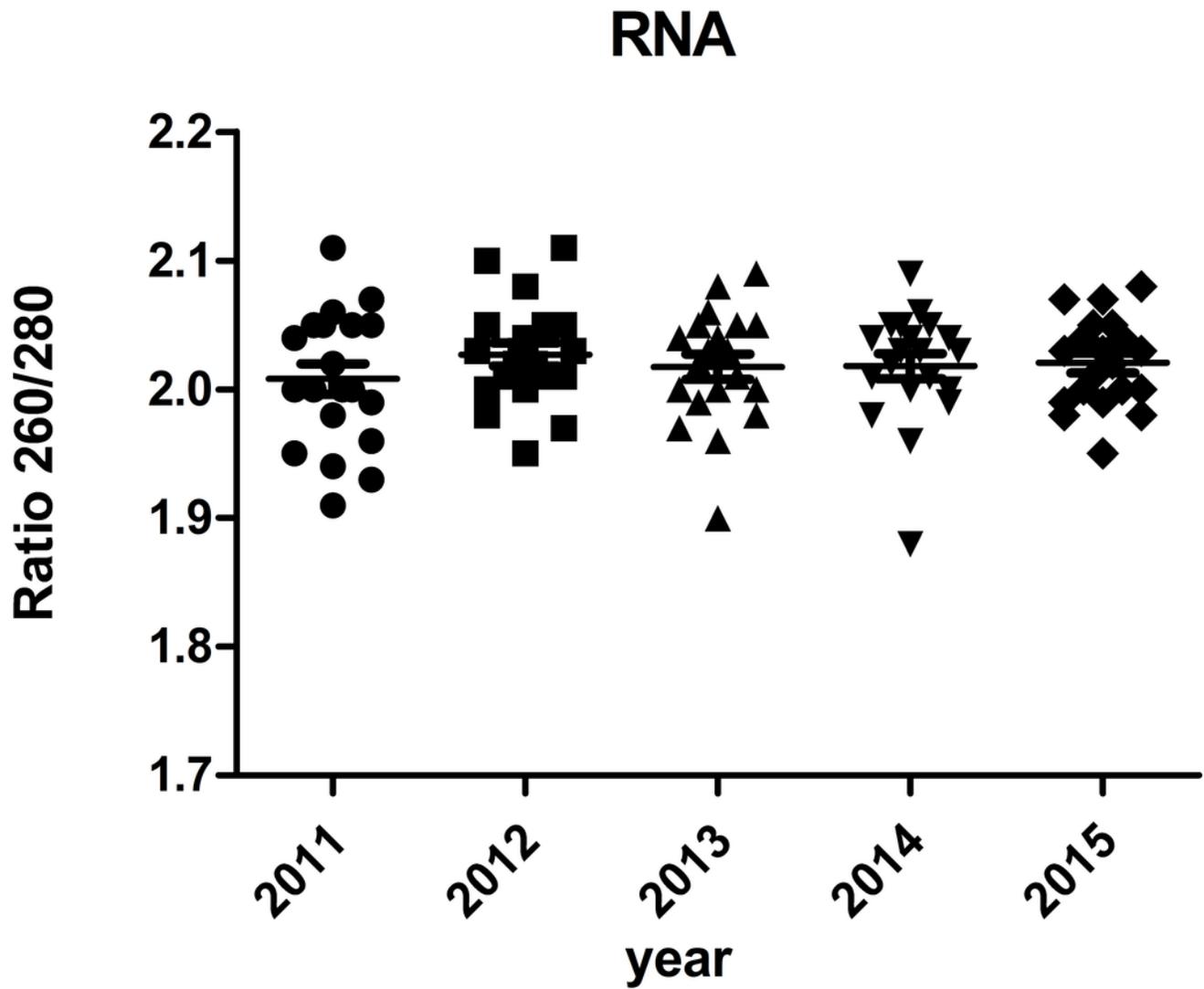
Figure 4

RNA integrity number of selected samples of different storage periods
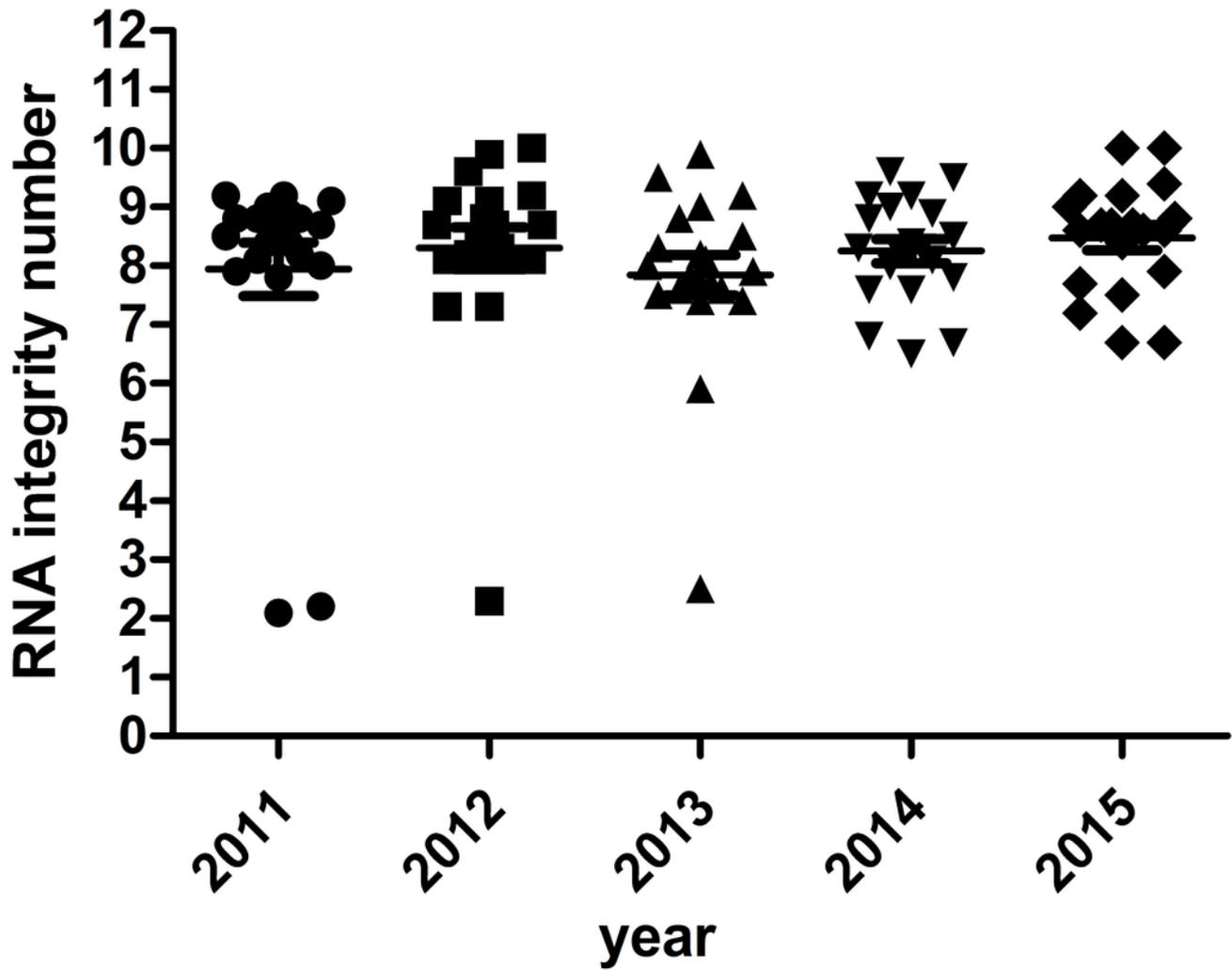
Figure 5

Gene expression levels under different storage durations

(A) Gene expression levels of ACTB; (B) Gene expression levels of GAPDH.
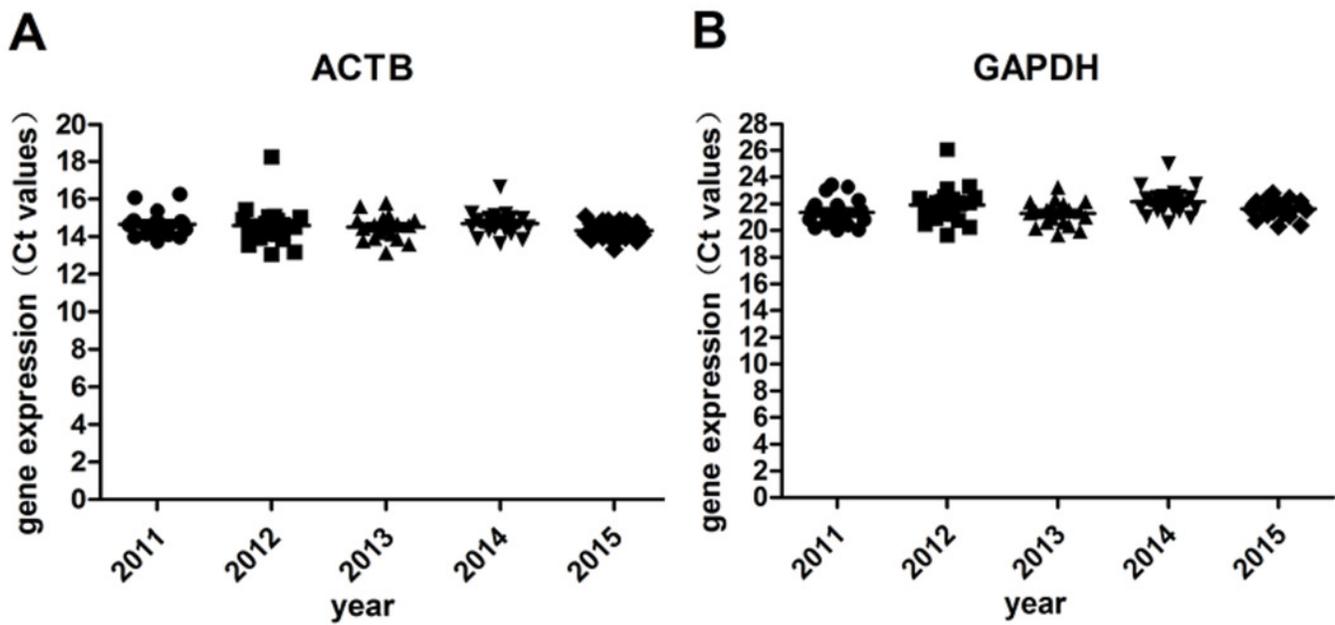
Figure 6

Ratio 260/280 of DNA samples
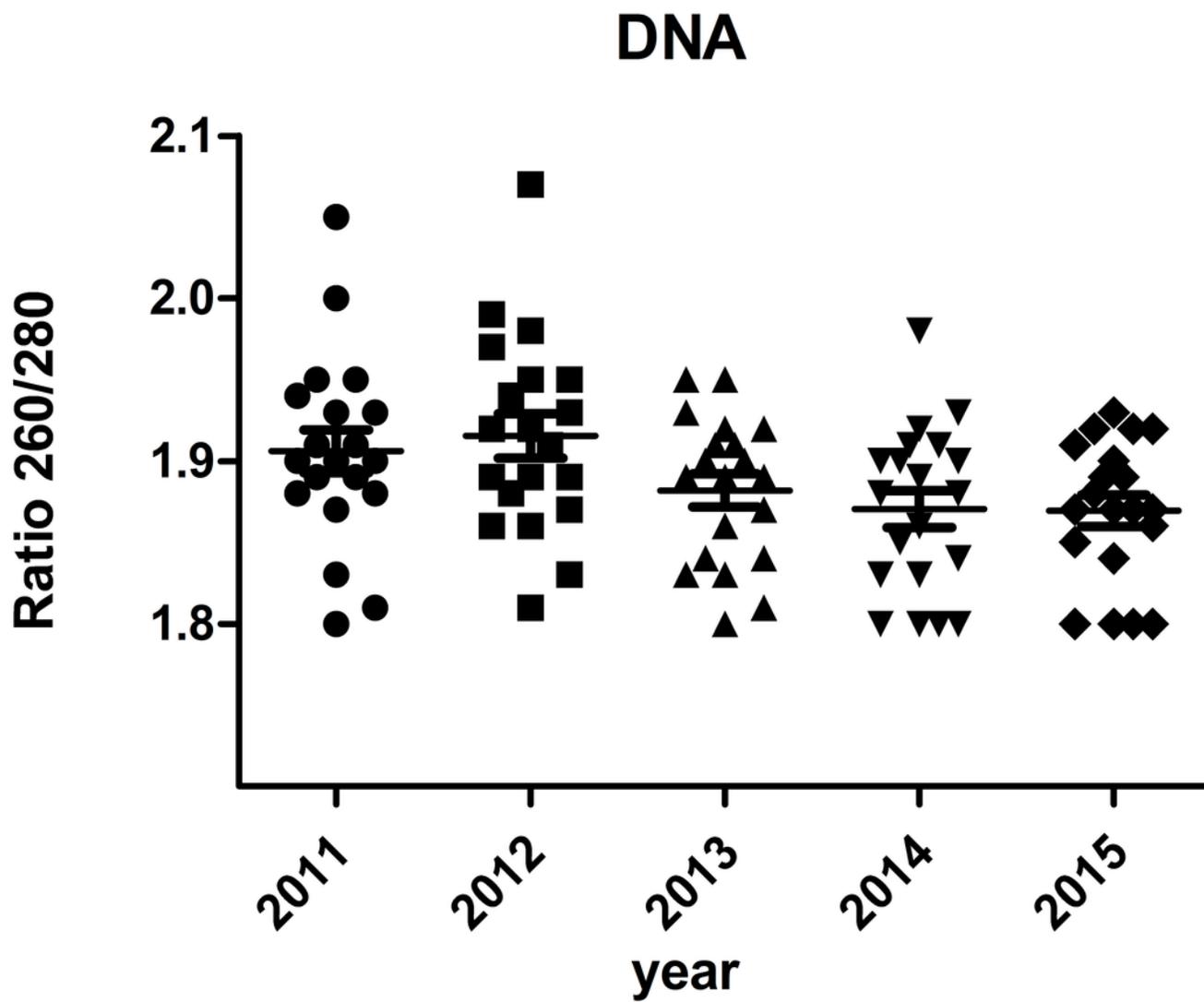
Figure 7

Ct values of genomic housekeeping genes under different storage durations

(A)Ct values of ACTB.(B)Ct values of GAPDH.
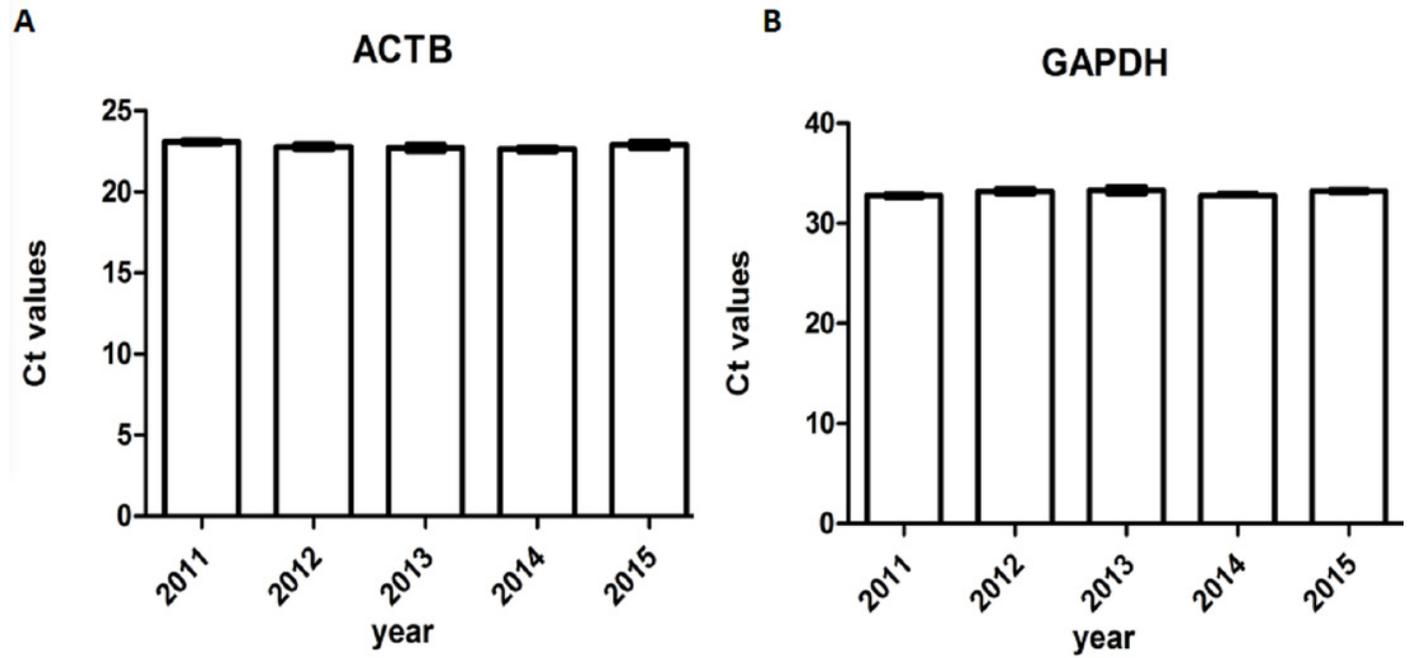
Figure 8

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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