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TaqMan PCR assay in the control of RNA normalization in human post-mortem brain tissue

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Abstract

The brain tissue obtained after death is subjected to several circumstances that can affect RNA integrity. The present study has been directed to reveal possible pitfalls and to control RNA normalization in post-mortem samples in order to recognize the limitations and minimize errors when using TaqMan PCR technology. This has been carried out in samples of the frontal cortex in a series of control and diseased cases covering Parkinson's disease, dementia with Lewy bodies pure form and common form, and Alzheimer's disease. Special attention has been paid to the value of the agonal state, post-mortem delay and pH of the nervous tissue as approximate predictors of the quality of RNA, as well as to the use of the Bioanalyzer to confirm RNA preservation. In addition, since possible disease-modified mRNAs have to be normalized with ideal unaltered RNAs, TaqMan® human endogenous control plates have been used to determine the endogenous control most appropriate for the study. β-glucuronidase (GUS) and β-actin were good endogenous controls because their expression levels showed a small variation across a representative number of control and pathological cases. RNA stability was also analysed in a paradigm mimicking cumulative delay in tissue processing. GUS mRNA levels were not modified although β-actin mRNA levels showed degradation at 22 h. Finally, the control of RNA degradation for the normalization of genes of interest was also tested. mRNA expression levels for superoxide dismutase 1 (SOD1) and metalloproteinase domain 22 (ADAM22) were examined at several artificial post-mortem times, and their expression levels compared with those for putative controls β-actin and GUS. In our paradigm, the expressions of SOD1 and ADAM22 were apparently not modified when normalized with β-actin. Yet their expression levels were reduced with post-mortem delay when values were normalized with GUS. Taken together, these observations point to practical consequences in TaqMan PCR studies. Short post-mortem delays and acceptable pH of the brain are not sufficient to rule out RNA degradation. The selection of adequate endogenous controls is pivotal in the study. β-actin and GUS are found to be good endogenous controls in these pathologies, although GUS but not β-actin expression levels are preserved in samples with long post-mortem delay.

Keywords: TaqMan; Post-mortem brain; RNA

1. Introduction

Gene expression analysis is an important aspect of the study of neurodegenerative diseases as Parkinson's disease (PD), dementia with Lewy bodies (DLB) and Alzheimer's disease (AD). The determination of genes abnormally regulated will permit a better understanding of the causes and progression of these diseases, as well as the pointing to new diagnostic, prognostic and therapeutic targets. The human material necessary for study is usually the brain obtained after death, as a generous donation for research. Donor programs and brain banks have proven to be excellent initiatives serving this purpose. No significant modifications at the mRNA levels of key pathogenic proteins as tau and amyloid-β protein precursor (APP) (Oyama et al., 1993), and α-synuclein (Tan et al., 2005) have been seen in post-mortem brain. However, the brain tissue obtained after death is subject to several determinants that can affect RNA and protein integrity. Although some studies have suggested stability of human post-mortem brain RNA (Leonard et al., 1993; Cummings et al., 2001; Yasojima et al., 2001), many others have shown that RNA integrity is largely dependent on several

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factors, including the agonal state, hypoxia, and the post-mortem delay between death and tissue processing, among others (Yates et al., 1990; Ross et al., 1992; Harrison et al., 1995; Preece and Cairns, 2003; Li et al., 2004; Tomita et al., 2004). Based on these findings, it is commonly accepted that prolonged agonal state, hypoxia, low cerebrospinal fluid or brain tissue pH (as an indicator of acidosis), and long post-mortem delays have to be considered in the study of RNAs and proteins in post-mortem material. All these factors are of major concern in brain banks as facilities that provide human brain samples for further research. Yet it is difficult to predict adequate or inappropriate conditions for RNA preservation solely on the basis of these parameters.

In the present work, we have explored ways to improve reliability in the analysis of RNA in post-mortem brain tissue by using TaqMan PCR technology, which has emerged as a rapid and reliable method for semi-quantitative analysis of gene expression in many tissues, including the nervous system (Horii et al., 2002).

2. Experimental procedures

2.1. Brain samples

Frozen samples of the cerebral cortex frontal lobe (area 8, according to Brodmann) were collected from three patients with Parkinson's disease (PD), three with DLB pure form (DLBp), five with DLB common form (DLBc), seven with Alzheimer's disease (AD) stages I/II-A/B and nine with AD stages V/VI-C of Braak and Braak, and six age-matched controls, following informed consent of the patients or their relatives and approval by the local ethics committee. Also the study conforms with The Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (18 July 1964). No cases showed evidence of pyrexia, seizures, coma, hypoxia or long agonal state. These aspects were explored in detail by direct interviews with the persons who took care of the patients during the hours previous to decease. Both genders were represented equally; the age range was between 55 and 93 years (mean age 84 years); the average time from death to tissue processing (post-mortem delay) was between 2 and 13 h. Half of the brain was immediately cut in coronal sections, 1 cm thick, frozen on dry ice and stored at −80 °C until use. Brain pH was measured after homogenizing tissue samples in distilled water (100 mg/ml) and taking duplicate measurements of the supernatant with a calibrated pH meter. pH varied from 6.11 to

Table 1
Summary of the main clinical and neuropathological findings in the present series

Case	Disease	Gender	Age	Post-mortem (h)	Braak stages, βA-amyloid–NFT	pH	A_{260}/A_{280}
1	Control	F	73	5	0-0	6.93	1.79
2	Control	M	75	6	0-0	6.58	1.74
3	Control	F	73	7	0-0	6.71	1.65
4	Control	M	79	7	0-0	6.11	1.83
5	Control	F	73	10	0-0	6.58	1.7
6	Control	M	55	13	0-0	6.54	1.80
7	PD	F	70	4	0-0	6.26	1.79
8	PD	M	81	5	A-I	6.71	1.78
9	PD	M	88	10	0-0	6.42	1.86
10	DLBp	M	77	5	0-0	6.33	1.72
11	DLBp	M	68	12	B-0	6.46	1.74
12	DLBp	M	82	8	0-0	6.69	1.81
13	DLBc	M	71	5	C-V	6.59	1.71
14	DLBc	M	91	5	C-V	6.38	1.71
15	DLBc	M	78	6	C-V	6.85	1.71
16	DLBc	M	78	7	C-V	6.65	1.72
17	DLBc	F	72	7	C-V	6.22	1.82
18	ADI	M	75	4	B-I	6.65	1.91
19	ADI	F	73	4	A-II	6.55	1.75
20	ADI	F	79	5	A-I	6.5	1.87
21	ADI	M	59	7	A-II	6.44	1.75
22	ADI	M	69	10	B-II	6.9	2
23	ADI	F	84	5	A-II	6.4	1.81
24	ADI	M	78	7	B-II	6.68	1.65
25	ADV	M	75	2	C-VI	6.62	1.64
26	ADV	M	69	3	C-VI	7.02	1.72
27	ADV	F	86	10	C-V	6.54	1.94
28	ADV	M	69	6	C-V	6.13	1.7
29	ADV	M	93	7	C-V	6.54	2
30	ADV	F	84	2	C-V	6.5	2.1
31	ADV	F	78	3	C-VI	6.41	1.76
32	ADV	F	81	3	C-VI	6.3	1.71
33	ADV	M	77	4	C-VI	6.43	1.74

Parkinson's disease (PD), dementia with Lewy bodies pure form (DLBp) and common form (DLBc), Alzheimer's disease stages I/II-A-B (ADI) and V/VI-C (ADV) of Braak and Braak, and controls. M, male; F, female; βA-amyloid, senile plaques; NFT, neurofibrillary tangle; A_{260}/A_{280}, absorbance at 260 and 280 nm.

7.02 in control and diseased brains. For morphological examinations, the brains were fixed by immersion in 10% buffered formalin for 2 or 3 weeks. The neuropathological study was carried out on de-waxed 4 μm-thick paraffin sections of the frontal (area 8, according to Brodmann), primary motor, primary sensory, parietal, temporal superior, temporal inferior, anterior cingulated, anterior insular, and primary and associative visual cortices; entorhinal cortex and hippocampus; caudate, putamen and pallidum; medial and posterior thalamus; subthalamus; Meynert nucleus; amygdala; midbrain (two levels), pons and medulla oblongata; and cerebellar cortex and dentate nucleus. The sections were stained with haematoxylin and eosin, Klüver Barrera, and processed for immunohistochemistry with the streptavidin LSAB method (Dako, Dakopats, Barcelona, Spain) to glial fibrillary acidic protein, CD68 and *Licopericum esculentum* lectin for microglia, βA-amyloid, pan-*tau*, phosphorylation-specific *tau* Thr181, Ser202, Ser214, Ser262, Ser396 and Ser422, and αB-crystallin, α-synuclein and ubiquitin.

Neuropathological criteria for PD have been detailed elsewhere (Jellinger and Mizuno, 2003). Staging of AD was carried out following the criteria proposed by Braak and Braak (1999). Stages of βA-amyloid deposition (senile plaques) refer to initial deposits in the basal neocortex (stage A), deposits extended to the association areas of the neocortex (stage B) and heavy deposition throughout the entire cortex (stage C). Stages of neurofibrillary pathology correspond to transentorhinal (I–II), limbic (III–IV) and neocortical (V and VI). Characterization of DLB was made according to consensus guidelines of the consortium on DLB international workshop (McKeith et al., 1996,2000). Associated AD stages were further established depending on the βA-amyloid deposition burden and neurofibrillary pathology, following the nomenclature of AD. A summary of the main neuropathological changes, post-mortem delay and pH of the brain tissue in every case is shown in Table 1.

In addition, eight samples (two controls, three PD, one AD and two DLBp) with similar clinical parameters, brain pH and post-mortem delay but showing RNA degradation (Table 2), were used in certain paradigms.

Finally, samples of the frontal cortex from one control individual were obtained at 3 h post-mortem and immediately frozen (time 0), or stored at 4 °C for 3, 6 or 22 h, and then frozen to mimic cumulative post-mortem delay in tissue processing.

2.2. mRNA isolation

mRNA isolation was carried out in two steps. Total RNA was isolated using TRizol Reagent (Life Technologies, Barcelona, Spain) followed by the RNeasy Midi Kit (Qiagen, Barcelona, Spain). Frozen human brain samples (area 8, according to Brodmann) were directly homogenized in 1 ml of TRizol Reagent per 100 mg tissue and left for 5 min at room temperature. Next, 200 μl of chloroform were added and mixed vigorously. After 3 min at room temperature, the samples were centrifuged (12,000 × *g*, 15 min, 4 °C). The resulting supernatants were mixed with 0.5 ml of isopropanol, left at room temperature for 10 min and then centrifuged (12,000 × *g*, 10 min, 4 °C). After this step, the pellets were washed with 1 ml of ethanol 70% and centrifuged at 7500 × *g* for 5 min at 4 °C. Then the pellets were dried at room temperature for 10 min and re-suspended in 100 μl of RNase-free water and incubated in a water bath at 55–60 °C for 10 min.

Purified total RNA was mixed with 350 μl of RTL buffer (containing β-mercaptoethanol) provided with the RNeasy Midi Kit and 250 μl of ethanol 96%. The resulting solution was poured into an RNeasy column and centrifuged at 8000 × *g* for 15 s. Next, 500 μl of RPE buffer (provided with the RNeasy Midi Kit) was also poured into the column and centrifuged at 8000 × *g* for 15 s. This step was done twice followed by a centrifugation at 8000 × *g* for 2 min to completely elute the RPE buffer. Finally, the RNA was eluted by adding 30–40 μl of RNase-free water and centrifuging the column at 8000 × *g* for 1 min. The columns used exclude tRNA, 5S and 5.8S ribosomal RNAs.

The concentration of each sample was obtained from A_{260} measurements. RNA integrity was tested by gel electrophoresis, and confirmed by using the Agilent 2100 BioAnalyzer (Agilent). It is an instrument based on capillary electrophoresis that shows a progressive decrease of the 18S and 28S peaks with RNA degradation; this is associated with the appearance of new peaks in a molecular weight range between the 18S peak and small RNAs (Panaro et al., 2000; Miller et al., 2004). Only samples with RNA profiles showing preservation of the 18S and 28S peaks are suitable for RNA studies; the lower the two-peak profile, the lower the quality of the RNA.

2.3. cDNA synthesis

For each 100 μl reverse transcription reaction, 2 μg human RNA was mixed with 2.5 μM random hexamers, 1× TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM each dATP, dTTP, dCTP and dGTP, 0.4 U/μl RNase inhibitor and 1.25 U/μl MultiScribe Reverse Transcriptase (Applied Biosystems). Reactions were carried out at 25 °C for 10 min to maximize primer–RNA template binding, followed by 30 min at 48 °C, and then by incubation for 5 min at 95 °C to de-activate reverse transcriptase. Parallel reactions for each RNA sample were run in the absence of MultiScribe Reverse Transcriptase to assess the degree of contaminating genomic DNA.

2.4. TaqMan probes and TaqMan endogenous control plate

Human β-glucuronidase (GUS) (Hs99999908_m1, TaqMan probe 5′-GACTGAACAGTCACCGACGAGAGTG-3′), human β-actin (Hs99999903_m1, TaqMan probe 5′-TCGCCTTTGCCGATCCGCCGCCCGT-3′), human superoxide dismutase 1 (SOD1) (Hs00533490_m1, TaqMan probe 5′-CATCAATTTCGAGCAGAAGGAAAGT-3′) and human disintegrin and metalloproteinase domain 22 (ADAM22) (Hs00244640_m1, TaqMan probe 5′-ACGGACTTCATGGGATGTTCTATGA-3′) (Applied Biosystems) were examined in the present study. The TaqMan assay for GUS is located between 11 and 12 exon boundary, at position 1816 of NM_000181.1 transcript sequence. The predicted amplicon size is about 81 base pairs. The TaqMan assay for β-actin is located in the 5′ UTR region at position 36 of NM_001101.2 transcript generating an amplicon of 171 base pairs. The assay for SOD1 generates an amplicon of 60 base pairs from the NM_000454.4 transcript and it is located at position 216. The assay for ADAM22 is located at position 556 of NM_016351.3 transcript between 5 and 6 exon boundary generating an amplicon size of 75 base pairs.

To determine the endogenous control most appropriate for the study, we used two lots of a commercially available TaqMan® human endogenous control plate (Applied Biosystems). This plate simultaneously evaluates 11

Table 2
Summary of the cases with degraded RNA

Case	Disease	Gender	Age	Post-mortem (h)	Braak stages, βA-amyloid–NFT	pH	A_{260}/A_{280}
1	Control	F	80	3.30	0-0	6.55	1.83
2	Control	M	52	4	0-0	6.6	1.74
3	PD	F	84	4	0-0	6.75	1.84
4	PD	M	66	5	0-0	6.53	1.90
5	PD	M	76	11	0-0	6.5	1.75
6	DLBp	M	75	12	0-0	6.2	1.85
7	DLBp	M	82	16	0-0	6.73	1.81
8	ADV	M	70	10	C-V	6.58	1.82

Parkinson's disease (PD), dementia with Lewy bodies pure form (DLBp), Alzheimer's disease stage V/VI-C (ADV) of Braak and Braak, and controls. M, male; F, female; βA-amyloid, senile plaques; NFT, neurofibrillary tangle; A_{260}/A_{280}, absorbance at 260 and 280 nm. The RNA profile obtained with Agilent 2100 BioAnalyzer is shown in Fig. 1B.

candidate controls that cover a broad range of biological functions. We followed the protocol suggested by the supplier, performing reverse transcription reaction for all amplicons except 18S ribosomal RNA (rRNA). With the exception of 18S rRNA, all assays present on the TaqMan® human endogenous control plate are cDNA-specific. This is the reason why the protocol provided by the supplier recommends a specific RT reaction for 18S rRNA. The remaining 10 candidate controls are the following: huPO, human acidic ribosomal protein; β-actin; CYC, cyclophylin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerokinase; β2m, β_2-microglobulin; GUS, β-glucuronidase; HPRT, hypoxanthine phosphoribosyltransferase; TBP, transcription factor IID TATA binding protein; TfR, transferrin receptor. This plate also includes an internal positive control (IPC), which consists of a synthetic amplicon, primer and probe designed to detect the presence of PCR inhibitors in the test samples. The results of the endogenous control plate were calculated in relation to the control samples as ΔCt values ($Ct_{control} - Ct_{non\text{-}control}$).

2.5. TaqMan PCR

TaqMan PCR assays for SOD1 and ADAM22 were performed in duplicate on cDNA samples in 96-well optical plates using an ABI Prism 7700 Sequence Detection system (Applied Biosystems). The plates were capped using optical caps (Applied Biosystems). The ABI Prism 7700 measures the fluorescent accumulation of the PCR product by continuously monitoring cycle threshold (Ct), which is an arbitrary value assigned manually to a level somewhere above the baseline but in the exponential phase of PCR where there are no rate-limiting components. The Ct value sets the point at which the sample amplification plot crosses the threshold. The Ct values correlate with the initial amount of specific template.

For each 20 μl TaqMan reaction, 9 μl cDNA (diluted 1/50, which corresponds approximately to the cDNA from 4 ng of RNA) was mixed with 1 μl 20× TaqMan® Gene Expression Assays and 10 μl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems). Parallel assays for each sample were carried out using primers and probes with β-actin and GUS for normalization. The reactions were carried out using the following parameters: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Standard curves were prepared for SOD1, ADAM22, β-actin and GUS using serial dilutions of control human brain RNA. Finally, all TaqMan PCR data were captured using the Sequence Detector Software (SDS version 1.9, Applied Biosystems).

2.6. Data analysis

ANOVA with post-hoc Fisher's least significant difference (LSD) test was performed with Statgraphics Plus v5 software.

In Fig. 2A, the medians and percentiles of Ct values for each endogenous control are shown. In these experiments, 14 human frontal cortex samples were used because each commercial plate only allows the comparison of four samples (for more details see Section 3). In Fig. 2B, the average Ct value of control samples for GUS and β-actin was calculated for use as the calibrator value. Then, ΔCt values ($Ct_{calibrator} - Ct_{pathology}$) were determined for β-actin and GUS in all disease cases shown in Table 1. Then the average ΔCt and standard deviation values were calculated for PD, DLB and AD. In Fig. 3A, the calibrator value was the Ct value of the sample with a post-mortem time of 0 h. In Fig. 3B, the calibrator value corresponds to the average Ct value of the samples with non-degraded RNA.

3. Results

3.1. Agarose gel electrophoresis versus Bioanalyzer technology

To test the integrity of mRNA samples two methods are generally used: agarose gel electrophoresis and electropherograms. Preserved RNA is seen as two bands on agarose gels and as two peaks, corresponding to 28S and 18S ribosomal RNA, using the Agilent RNA LabChip and 2100 BioAnalyzer technology (Fig. 1A, upper panel: RNA1). However, the inspection of 28S and 18S ribosomal RNA bands using agarose gel electrophoresis may show apparently non-degraded RNA, whereas electropherograms from the same material obtained using the Bioanalyzer may evidence a shift in the RNA size distribution toward smaller fragments, indicating their poor quality (Fig. 1A, lower panel: RNA2).

Eighteen of the 26 cases initially selected were those with preserved RNA as revealed in the electropherograms (Fig. 1B, upper panel, cases 1 and 18 shown in Table 1 are representative of acceptable RNA preservation). The other eight cases with similar agonal state, post-mortem delay and brain pH (between 6.2 and 6.75, Table 2) showed impaired RNA preservation as revealed by a decrease of the main peaks and the presence of multiple smaller fragments (Fig. 1B, lower panel). Note that marked RNA degradation is found in two controls and two PD cases with relatively short post-mortem delays. We found no correlation among age, post-mortem delay, pH of the brain and the results of conventional RNA analysis.

The criterion followed to distinguish between good and poor quality mRNA was based on the RNA integrity number (RIN), which is provided by the Agilent RNA LabChip and 2100 BioAnalyzer technology. The RIN is a software algorithm that assesses the RNA quality by the entire electrophoretic trace of the RNA sample including the presence or absence of degradation products. The cases selected in the present study were those with a RIN number higher than 7.5 or between the range $7.5 > RIN > 6$. The cases considered with poor quality RNA presented a $RIN < 6$.

3.2. Purification of initial mRNA

A critical measure in comparing different cDNAs amplified in the PCR reaction is the purification grade of the initial mRNA due to differences in template amounts among samples in the RT reaction. The mRNAs of cases selected here showed optimal absorbance at 260 and 280 nm; the A_{260}/A_{280} ratio had an average standard deviation of 1.79 ± 0.11 (Table 1).

3.3. Endogenous controls

The commercial TaqMan endogenous control plate was used to determine the endogenous control gene with the least variability among the pathologies analysed. The plate contains several supposed housekeeping genes, including huPO, β-actin, CYC, GAPDH, PGK, β2m, GUS, HPRT, TBP and TfR. The median Ct value of a group of samples corresponding to three controls, and two PD, one DLBp, two DLBc, three ADI and three ADV samples, is shown in Fig. 2. The 25th and 75th Ct percentile values and ranges for each housekeeping gene are also shown in the same figure. Based on these data, GUS and β-actin appear to be the endogenous controls with the least variability (Fig. 2A).

In order to verify these results, TaqMan PCR was carried out for the selected endogenous controls with a larger group of

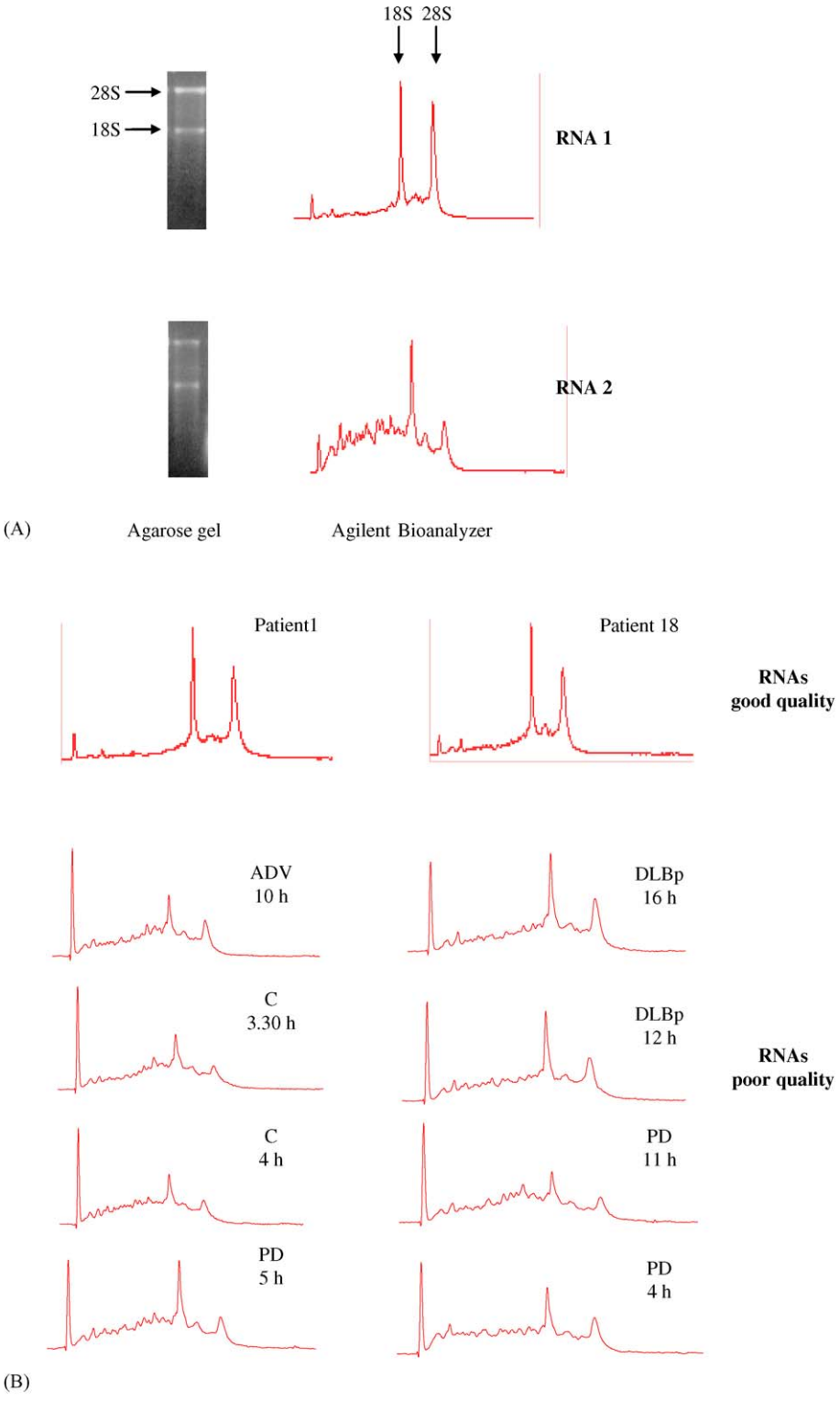

Fig. 1. (A) RNAs purified from two human frozen frontal cortex samples (RNA1 and RNA2) are analysed using formaldehyde–agarose gel electrophoresis, showing an apparently good quality. However, RNA2 shows significant degradation when it is analysed using an Agilent 2100 BioAnalyzer. One microgram of each RNA was used for formaldehyde–agarose gel analysis and 25 ng for the Agilent 2100 BioAnalyzer. (B) Examples of acceptable mRNAs (upper panel, corresponding to cases 1 and 18 in Table 1) and degraded RNA (lower panel, Table 2). Note the short post-mortem delay in many of the latter cases. All the degraded RNAs presented a RIN number lower than 6. ADV, Alzheimer's disease stage V of Braak and Braak; C, control; PD, Parkinson's disease; DLBp, dementia with Lewy bodies pure form.

control and pathological samples. The ΔCt average values for GUS and β-actin were calculated for all PD, DLBp, DLBc, ADI and ADV cases (Fig. 2B). Although results were more homogeneous for GUS than for β-actin in PD, DLBp and DLBc, no significant differences were observed between control and diseased brains ($p < 0.05$, ANOVA with post-hoc LSD test). ΔCt values for GUS and β-actin were very similar between control and AD cases (Fig. 2B).

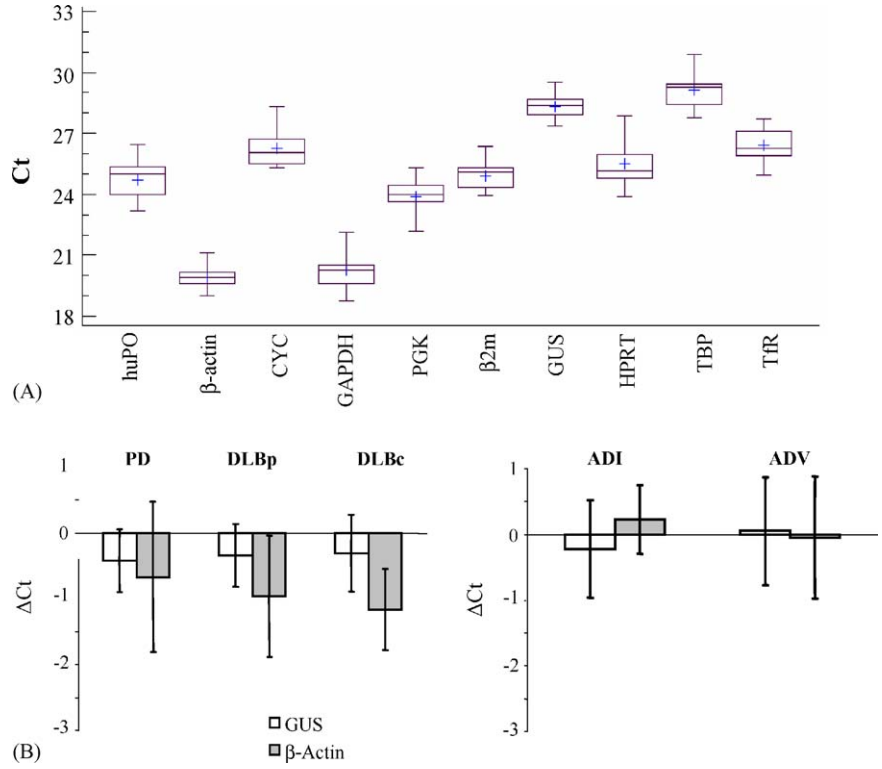

Fig. 2. (A) Expression levels of 10 ''housekeeping'' genes (Ct value) contained in the TaqMan® endogenous control plate (medians, lines; 25th–75th percentile, white boxes; ranges, whiskers) in 14 human frontal cortex samples (three controls, two PD, one DLBp, two DLBc, three AD stage IA of Braak and Braak, and three AD stage VC of Braak and Braak) selected at random from cases of Table 1. huPO, human acidic ribosomal protein; β-actin; CYC, cyclophylin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerokinase; β2m, β_2-microglobulin; GUS, β-glucuronidase; HPRT, hypoxanthine phosphoribosyltransferase; TBP, transcription factor IID TATA binding protein; TfR, transferrin receptor. (B) Average ΔCt values ($Ct_{control} - Ct_{pathology}$) calculated for β-actin and GUS in all disease cases shown in Table 1: PD ($n = 3$), DLBp ($n = 3$), DLBc ($n = 5$), AD stage I ($n = 7$) and AD stage VC ($n = 9$). Each ΔCt value was calculated as described in Section 2, taking the average of control Ct values as the baseline.

3.4. Effect of RNA degradation on RNA normalization

In order to document variations in the expression of ''control'' genes related with post-mortem delay in the greatest detail possible, samples of the frontal cortex from one control individual obtained at 3 h post-mortem and immediately frozen, or stored at 4 °C for 3, 6 or 22 h, and then frozen to mimic variable post-mortem delay in tissue processing, were used to analyse the ΔCt value for β-actin and GUS. Ideal short post-mortem times (3 and 6 h) had no effect on β-actin and GUS mRNA expression (Fig. 3A). However, a negative ΔCt value was detected for β-actin at 22 h, whereas the ΔCt value for GUS was very close to zero at the same time.

The same determination was carried out by comparing a pool of samples with degraded RNA ($n = 8$, corresponding to samples represented in Fig. 1B, lower panel, and in Table 2) to a pool of samples with non-degraded RNA ($n = 6$, corresponding to cases of Table 1 selected at random). The median ΔCt value for β-actin was negative, indicating that β-actin mRNA is more affected by degradation than GUS (Fig. 3B, $p < 0.05$, ANOVA with post-hoc LSD test).

Selection of the appropriate control at a given time (post-mortem delay) is important, as illustrated in the following paradigm. mRNA expression levels of two genes randomly selected, superoxide dismutase 1 (SOD1) and metalloprotei-

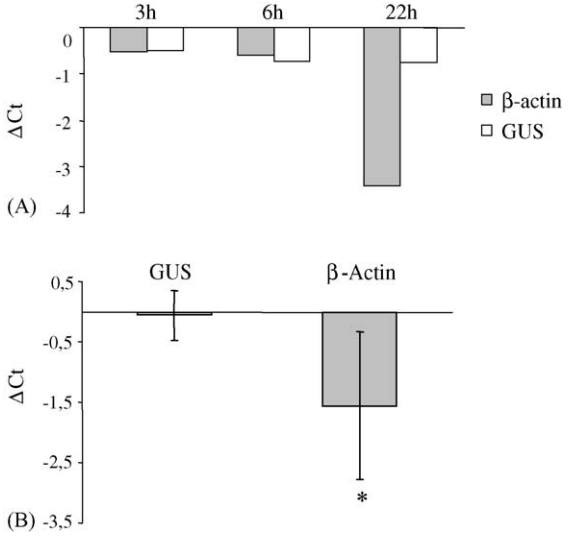

Fig. 3. (A) ΔCt ($Ct_{0\ h} - Ct_{time}$) value for β-actin and GUS in the frontal cortex of the same brain in which samples were left at room temperature for 3, 6 or 22 h and then frozen in liquid nitrogen (initial post-mortem delay, 3 h). RNA degradation was observed at 22 h (Buesa et al., 2004). (B) ΔCt values ($Ct_{non\text{-}degraded} - Ct_{degraded}$) for β-actin and GUS in an RNA-degraded pool obtained from eight human frozen frontal cortex samples (represented in Fig. 1B lower panel) compared with six non-degraded RNA samples (chosen at random from cases summarized in Table 1). ANOVA with post-hoc LSD test, $^{*}p < 0.05$.

nase domain 22 (ADAM22), were examined at several artificial post-mortem times, and their expression levels were compared with those for putative controls β-actin and GUS (Fig. 4A). Variations in the Ct values were observed for β-actin, SOD1 and ADAM22 at 22 h of artificial post-mortem delay, with no modifications in the Ct values for GUS at the same time-point. In Fig. 4B, the amount of target (SOD1 and ADAM22) and endogenous reference (GUS and β-actin) was determined for each experimental sample from the appropriate standard curve, which was plotted showing the cycle threshold, Ct (y), versus log ng total control RNA (x). The amount of each target was divided by the endogenous reference amount to obtain a normalized target value (arbitrary units), which permits the determination of the relative mRNA levels of SOD1 and

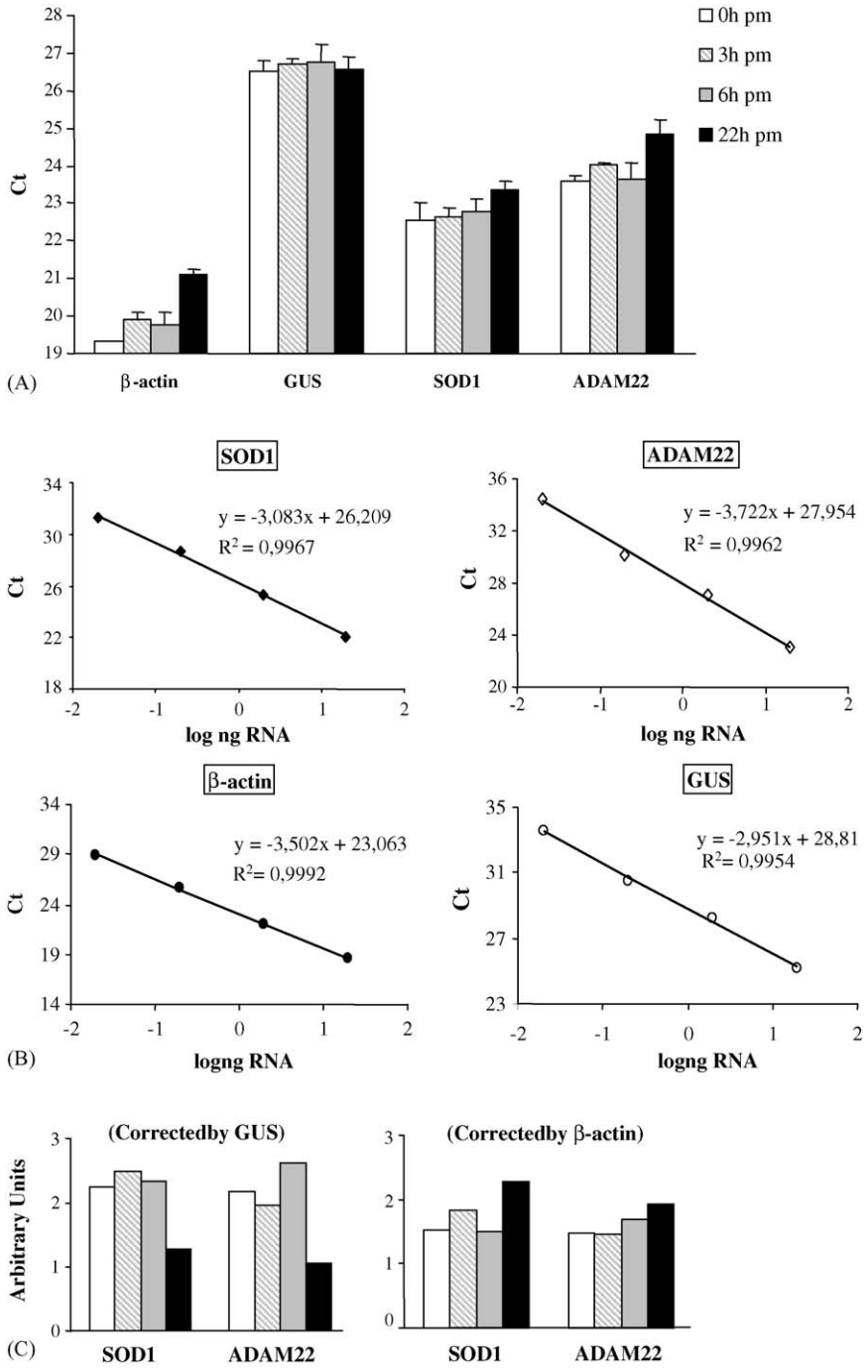

Fig. 4. (A) Ct values for β-actin, GUS, superoxide dismutase 1 (SOD1) and metalloproteinase domain 22 (ADAM22) in the frontal cortex of the same brain in which samples were immediately frozen at autopsy (initial post-mortem delay, 3:0 h) and left at 4 °C for 3, 6 or 22 h and then frozen in liquid nitrogen (3, 6 and 22 h). RNA degradation affects β-actin, SOD1 and ADAM22 mRNA expression levels, increasing their Ct value at 22 h. (B) SOD1 and ADAM22 mRNA levels normalized (arbitrary units) with GUS and β-actin in the same mentioned samples. (C) Representative standard curves for SOD1, ADAM22, β-actin and GUS constructed from several concentrations of control human brain RNA. CT values (y-axis) vs. log of several RNA concentrations of control samples (x-axis) show a reverse linear correlation.

ADAM22 in control and pathological samples. The standard curves were constructed from several concentrations of control human brain RNA (Fig. 4B).

The effect of RNA degradation (22 h) can be detected if SOD1 and ADAM22 mRNA expression levels are normalized with GUS, but they are abolished if normalization is carried out with β-actin (Fig. 4C).

4. Discussion

In a previous study, we reviewed several points affecting DNA array technology and also procedures to minimize pitfalls inherent to a degrading milieu in the context of brain banks and the study of degenerative diseases of the nervous system (Buesa et al., 2004). The present findings have shown that current parameters used as putative predictors of sample preservation in brain banks are not sufficient to eliminate RNA degradation in individual cases. All the cases initially included in this study had no prolonged agonal state, pyrexia, hypoxia, coma or seizures; the post-mortem delay was between 2 and 16 h; and the pH of the cerebral tissue was between 6 and 7. Yet several control and diseased cases disclosed very degraded RNA in spite of a post-mortem delay as short as 3 h. Since the reasons for these individual variations are not known, examination of RNA preservation must be carried out in every individual subject of RNA expression studies. Although agarose gel electrophoresis has generally been utilized in the past to reveal RNA smears in RNA isolated samples, this method is not sensitive enough when compared with modern technologies as the Agilent 2100 BioAnalyzer. The purification grade of RNAs is further analysed by examining the ratio of absorbance at 260 and 280 nm. Values close to 1.80 are indicative of acceptable RNA purification.

Possible modifications in the expression levels of a target gene have to be examined following normalization (correction) of its expression level with the expression levels of endogenous control genes. Commonly used endogenous controls belong to the group of so-called ‘‘housekeeping’’ genes which ideally are constitutively expressed by all cell types and which are not affected by disease. For example, there is a certain concern regarding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression as a suitable endogenous control in the study of degenerative diseases of the nervous system because GAPDH protein is implicated in apoptosis and neurodegeneration (Tatton et al., 2000). In the same line, mRNA expression levels of β-actin and other genes regulating the expression of cytoskeletal proteins α- and β-tubulins might be considered sub-optimal controls, as β-actin and α- and β-tubulins might be down-regulated in degenerative diseases of the nervous system. Yet, although the expression of β-actin is more variable than the expression of cyclophylin and GAPDH mRNAs in several tissues, mRNAs of these three genes show little fluctuation among several CNS tissues and disease models (Medhurst et al., 2000). In our study, the use of a commercial plate with 11 endogenous controls has proved a good tool for initial screening of the most appropriate endogenous controls. We determined the ΔCt values ($Ct_{control} - Ct_{pathology}$) in PD, DLBp, DLBc and AD in relation to control samples. Since the control Ct serves as a baseline for the assay, pathological samples with positive and negative ΔCt values have higher and lower initial template concentrations than the control samples, respectively. Ideally, the best control is expressed at a constant level in all samples. Because the ΔCt indicates the level of gene expression relative to the calibrator, the ΔCt values of a good control do not vary very much from zero. GUS and β-actin proved to be good endogenous controls because their expression levels showed a small variation across a representative number of controls, and PD, DLBp, DLBc and AD cases.

Normalization to total RNA concentration was suggested in a recent report when comparing tissue biopsies obtained from different individuals (Tricarico et al., 2002). However, the present findings have shown a more complex scenario in post-mortem brains subjected to several determining factors, some of which are unpredictable. The individual study of each sample for RNA degradation is essential in control and diseased cases. Finally, normalization with a particular control must be monitored. Yet ideal controls might also be degraded by several factors, including post-mortem delay. For this reason, RNA stability was examined in a paradigm mimicking cumulative delay in tissue processing. GUS mRNA levels were not modified although β-actin showed degradation at 22 h. These results were confirmed when a pool of samples with degraded RNA was compared with a pool of samples with non-degraded RNA. These findings give further support to the idea that individual genes should be tested for possible degradation with post-mortem delay, and that endogenous controls, such as GUS and β-actin, are differentially degraded with the time of post-mortem. Control of this factor is also crucial in the interpretation of the crude results of target mRNAs, the expression of which is suspected of being altered by disease. In our paradigm, the expressions of two candidate genes, SOD1 and ADAM22, were apparently not modified when normalized with β-actin. Yet their expression levels were reduced with post-mortem delay when values were normalized with GUS. Similar situations may occur in current practice when the mRNAs of controls and genes of interest are not tested individually for their integrity, including the possible decay post-mortem.

Based on these findings, it may also be inferred that a false down-regulation of gene expression will be obtained if RNA degradation is higher in pathological than in control samples, whereas a false up-regulation will occur with high RNA degradation in control samples. Therefore, minimizing RNA degradation in post-mortem brain is very important to improve the efficiency and reliability of TaqMan PCR in the study of human neurodegenerative diseases and in the search of new theurapeutical gene targets.

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