RESEARCH ARTICLE

Identification of suitable endogenous controls for gene and miRNA expression studies in irradiated prostate cancer cells

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Abstract This study aimed to to evaluate the stability of commonly used endogenous control genes for messenger RNA (mRNA) ($N=16$) and miRNAs ($N=3$) expression studies in prostate cell lines following irradiation. The stability of endogenous control genes expression in irradiated (6 Gy) versus unirradiated controls was quantified using NormFinder and coefficient of variation analyses. HPRT1 and 18S were identified as most and least stable endogenous controls, respectively, for mRNA expression studies in irradiated prostate cells. SNORD48 and miR16 miRNA endogenous controls tested were associated with low coefficient of variations following irradiation (6 Gy). This study highlights that commonly used endogenous controls can be responsive to radiation and validation is required prior to gene/miRNAs expression studies.

Introduction

Gene expression levels differ greatly between individuals and contribute to individual differences in response to radiation [1]. Detecting differential regulation of genes is thus critical for a better understanding of radiation-induced molecular effects [2]. Quantitative real-time PCR (qRT-PCR) is a commonly used method to identify messenger RNA (mRNAs)

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and/or miRNAs differentially expressed between samples. qRT-PCR analysis requires normalisation of the target gene to an endogenous control gene to compensate for multiple factors related to the processes of RNA extraction, reverse transcription and PCR, known to introduce variation between samples. Constitutive genes that are required for the maintenance of basic cellular functions, such as glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) or β-actin (*ACTB*) are commonly used as reference genes in mRNA studies as their expression is assumed to be constant. However, a number of studies report differential expression of these genes in various tissues, as well as in response to environmental exposures [3–10]. Therefore, precise evaluation of potential genes for use as controls is required for each type of exposure and experimental model.

The identification of suitable endogenous controls in miRNA expression studies has not been as widely evaluated as for mRNA. Variability due to the RNA extraction method can be controlled using non-human (e.g., *C. elegans*) synthetic miRNA [11], but this may not minimise differences in template quality and efficacy of the reverse transcription reaction. Control for these differences requires the identification of a miRNA reference gene of the same length as the molecules of interest [12]. RNA, U6 small nuclear 2 (RNU6B), a member of the U6 small nuclear ribonucleoprotein family involved in the splicing of pre-mRNA, is one of the most commonly used reference gene [13]. Others include small nucleolar RNA, C/D box 44 (RNU44) [14], and small nucleolar RNA, C/D box 48 (RNU48) [15]. These small nucleolar RNAs (snoRNAs) are nonprotein-coding RNAs of approximately 70 nucleotides in length that are involved in site-specific modification of nucleotides in target RNAs [16] and 2′-O-ribose methylation in ribosome biogenesis [17]. SnoRNAs may be deregulated in cancer, and therefore their appropriateness as reference genes in the analysis of miRNA expression in tumour samples must be assessed. Candidates include the noncoding growth arrest

specific transcript 5 gene (*GAS5*) and U50. The down-regulation of *GAS5*, which encodes multiple snoRNAs, is associated with induction of growth arrest and apoptosis in breast and prostate cancer cell lines [18, 19], and is a negative prognostic factor in a number of solid tumours, such as gastric and colorectal cancer [20–22]. Another snoRNA, U50, is involved in the development of prostate and breast cancer [23, 24]. Its function remains unknown but could be associated with the regulation of cell proliferation [24]. For the assessment of circulating miRNAs in cancer patients, miR-1228 may represent a good candidate [25].

NormFinder is an algorithm-based analytical programme for the relative quantification of gene expression data that takes into account both amount of mRNA in the sample and the random variation caused by biological and experimental factors [26]. Quantification of the systematic error resulting from intra- and intergroup variations is achieved through the calculation of a stability measure, whose value increases with degree of unstability in expression levels across samples. The coefficient of variation (CtCV%) is another measure for the variation in the expression levels of miRNA/mRNAs among samples. CtCV% quantifies the dispersion of the data around the mean (standard deviation) as a percentage of the sample mean and is best suited to small datasets. In this analysis, stable expression is associated with a low CtCV% [27].

A few studies have validated the use of appropriate endogenous controls in prostate cancer [28, 29], but the suitability of commonly used endogenous controls in radiation studies has not been investigated. The aim of this study was to evaluate the stability of endogenous controls commonly used in mRNA and miRNA expression studies in irradiated prostate cells.

Material and methods

Cell culture

The prostate cell lines 22Rv1, LNCaP, DU145, PC3, PWR1E and RWPE1 were obtained from the American Type Culture Collection (Teddington, Middlesex, UK). 22Rv1, DU145, LnCaP and PC3 cells were grown in RPMI 1640 medium supplemented with 10 % foetal calf serum and 1 % streptomycin-penicillin (Lonza, Dublin, Ireland). PWR-1E and RWPE-1 were cultured in keratinocyte, serum-free medium with EGF (Lonza). A single 6-Gy dose fraction was delivered to 70 % confluent cells at a dose rate of 3.25 Gy/min using a RS225 Cell Irradiator (X-Strahl, Camberley, Surrey, UK). Samples were collected 4 h after irradiation.

RNA extraction and cDNA synthesis

TRI reagent (Sigma-Aldrich, Dublin, Ireland) was used to isolate total RNA, which was subsequently DNase treated (Ambion, Life Technologies, Dublin, Ireland). A NanoDrop 1000 spectrophotometer was used to measure the concentration and purity of the RNA in each sample. For mRNA, cDNA synthesis was carried out using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Dublin, Ireland) on 400 ng of RNA, following the manufacturer's protocol. For miRNA cDNA synthesis, the miCURY LNA(TM) universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark) was used according to the manufacturer's protocol on 20 ng of RNA.

Quantitative real-time PCR

All reagents, consumables and equipment for TaqMan polymerase chain reaction (PCR) were supplied from Applied Biosystems (Life Technologies, Dublin, Ireland), unless otherwise stated. The Human endogenous control array card (Part number: 4367563, Applied Biosystems) was analysed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's guidelines using 200 ng cDNA per sample. For the further evaluation of candidate mRNA genes, difference in the expression levels of genes between cell lines was measured by real-time TaqMan PCR on an ABI Prism 7500 HT Sequence Detection System (PerkinElmer, Foster City, CA). qRT-PCR for miRNA was performed using the mercury LNA Universal RT microRNA PCR kit (Exiqon) according to the manufacturer's instructions.

qRT-PCR NormFinder analysis

The freely available statistical programme NormFinder (Andersen et al. 2004) was used to rate the suitability of genes as endogenous controls. The NormFinder Excel plug-in allows for assessment of both inter- and intragroup variability and ranks the genes accordingly to give a measure of stability of which a lower value indicates increased stability in gene expression. This allows for the cycle threshold (Ct) variation within the irradiation/nonirradiation sample groups to be assessed for each gene as well as the variation between irradiation and nonirradiation groups. NormFinder compares inter- and intragroup variability and combines the two into a stability value. This adds the two sources of variation and represents a practical measure of systematic error that will be introduced when using the investigated gene.

qRT-PCR coefficient of variation analysis

The Ct coefficient of variation (CtCV%) compares the variation in one sample set to another and was used to

further test the performance of endogenous controls (Caradec et al. 2010). For each gene, CtCV% was calculated as the standard deviation divided by the mean Ct values of the two groups. It is expressed as a percentage and gives a measure of the spread of Ct values from the mean. A gene with a CtCV% <5 % was considered stable and a CtCV(%) <3 % very stable.

Statistical analysis

The difference in mean CT values between unirradiated and 6-Gy irradiated 22Rv1 and DU145 biological triplicates was examined using a Student's t test. Pearson R correlation analyses were used to identify least and most stable genes common to both CtCV(%) and NormFinder analyses and both cell lines tested. A p value less than 0.05 was considered significant.

Results

Endogenous control gene expression in irradiated and unirradiated prostate cells

This study evaluated the expression of commonly used endogenous controls for mRNA ($N=16$) and miRNA ($N=3$) studies in irradiated (6 Gy) and nonirradiated 22Rv1 and DU145 cells (Table 1). The summary statistics of the Ct values for both experimental groups are presented in Table 2. The standard deviation associated with the mean CT value across the three biological replicate tested was generally less than 1 for mRNA HKG (Mean=0.6, SD=0.45) and slightly higher for miRNAs HKG (Mean=1.6; SD=1.1). The mean Ct values for each gene were increased following irradiation. The difference was statistically significant for GAPDH ($p=0.04$), HPRT1 ($p=0.04$) and UBC ($p=0.049$) in DU145 cells and 18S ($p=0.04$), GAPDH ($p=0.03$), PGK1 ($p=0.02$), RPLPO ($p=0.02$) and YWHAZ ($p=0.02$). The variation of mean Ct values in irradiated (6 Gy) samples ranged from 11.28±0.88 in DU145 for 18S to 41.02±0.85 in 22Rv1 for RNU6B. The relationship between abundance (mean Ct value) and variability (SD) is represented in Fig. 1. The most abundant endogenous control 18S was observed to have one of the largest standard deviation across replicates in both cell lines and under both irradiated and nonirradiated conditions. All other genes tested were clustered around similar abundance and variability. RNU6B was consistently associated with large mean Ct values and low abundance.

Analysis of endogenous control gene stability following irradiation using NormFinder

NormFinder was used to evaluate the stability of the 16 mRNA endogenous control genes tested. The genes were first ranked based on their generated stability value under unirradiated conditions. The generated stability values were low

Table 1 Symbol, name and function of the commonly used endogenous control genes tested

Gene symbol	Gene name	Function
18S rRNA	18S ribosomal RNA	Ribosome subunit
ACTB	β-actin	Cytoskeletal structural protein
B2M	β-2-microglobuin	b-Chain of the MHC class I molecules
GADPH	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis
GUSB	β-glucuronidase	Lysosome exoglycosidase
HMBS	Hydroxymethylbilane synthase	Heme biosynthesis
HPRT	Hypoxanthine phosphoribosyltransferase 1	Purine generation through the purine salvage pathway
IPO8	Importin 8	Nuclear protein import
PGK1	Phosphoglycerate kinase 1	Glycolysis enzyme
POLR2A	Polymerase (RNA) II (DNA directed) polypeptide A	Synthesis of messenger RNA
PPIA	Cyclophilin A serine-threonine	Phosphatase inhibitor
RPLPO	Ribosomal protein, large	P0 ribosome subunit
TBP	TATA box-binding protein	Initiation of transcription by RNA polymerase II
TFRC	Transferrin receptor (p90, CD71)	Cellular iron uptake
UBC	Ubiquitin C	Ubiquitination
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5 monooxygenase activation protein, zeta polypeptide	Signal transduction
miR-16	–	Tumour suppressor, cell proliferation and apoptosis inhibition
SNORD48	Small nucleolar RNA, C/D box 48	Methylation of pre-ribosomal RNA
RNU6B	U6 small nuclear 2	Pre-mRNA splicing

Table 2 Mean cycle threshold (Ct) values and standard deviation (SD) of each endogenous gene tested across the three independent biological replicates of unirradiated and 6-Gy irradiated 22Rv1 and DU145 prostate cancer cell lines

	DU145				22Rv1			
	0 Gy		6 Gy		0 Gy		6 Gy	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
18S	11.26	0.88	12.83	1.18	11.94	0.20	14.12	1.25
ACTB	19.50	1.13	21.48	0.77	22.35	0.06	25.24	2.09
B2M	22.19	0.76	23.60	0.69	25.42	0.12	26.81	0.94
GAPDH	21.20	0.69	22.75	0.65	22.64	0.23	24.58	1.05
GUSB	25.95	0.74	27.24	0.56	27.15	0.07	29.35	2.22
HMBS	26.27	0.74	27.58	0.43	28.28	0.05	29.99	1.30
HPRT1	25.06	0.74	26.52	0.51	26.23	0.03	27.71	0.97
IPO8	26.91	0.88	28.50	0.56	27.87	0.12	28.95	1.22
PGK1	22.97	0.73	24.09	0.77	24.68	0.14	26.31	0.71
POLR2A	25.48	0.78	26.93	0.55	26.66	0.02	28.02	1.08
PPIA	20.63	0.84	22.06	0.59	21.57	0.15	23.28	1.18
RPLPO	20.70	0.65	21.85	0.47	21.73	0.02	22.56	0.41
TBP	31.13	0.85	32.24	1.01	28.55	0.31	30.13	0.95
TFRC	24.19	1.13	25.91	0.37	26.38	0.15	27.93	1.02
UBC	22.18	0.96	23.94	0.52	24.70	0.07	25.55	0.73
YWHAZ	25.98	0.71	27.04	0.50	26.51	0.19	27.98	0.69
miR16	18.55	0.12	19.65	0.77	22.7	2.84	21.7	2.21
SNORD48	18.55	2.20	16.93	0.29	21.05	1.64	20.97	0.25
RNU6B	33.9	2.25	40.53	2.99	42.1	2.98	41.02	0.85

$N=3$ biological replicates per sample

(DU145 0.01±0.009; 22Rv1 0.025±0.033) highlighting gene stability across biological triplicates (Table 3). The Ct data were next analysed to test the stability of the endogenous control genes following irradiation (6 Gy). The stability values generated were again relatively low across the panel of genes tested (DU145 0.011±0.014; 22Rv1 0.03±0.034). The effect

Fig. 1 Mean and standard deviation of Ct values of the tested endogenous controls in 22Rv1 and DU145 cells. Representation of the abundance (mean Ct value) and variability (standard deviation, SD) of mRNA ($N=16$) and miRNA ($N=3$) endogenous controls tested in **a** unirradiated DU145 cells, **b** 6-Gy irradiated DU145 cells, **c** unirradiated 22Rv1 and **d** 6-Gy irradiated 22Rv1 cells. Values are representative of three independent biological replicates
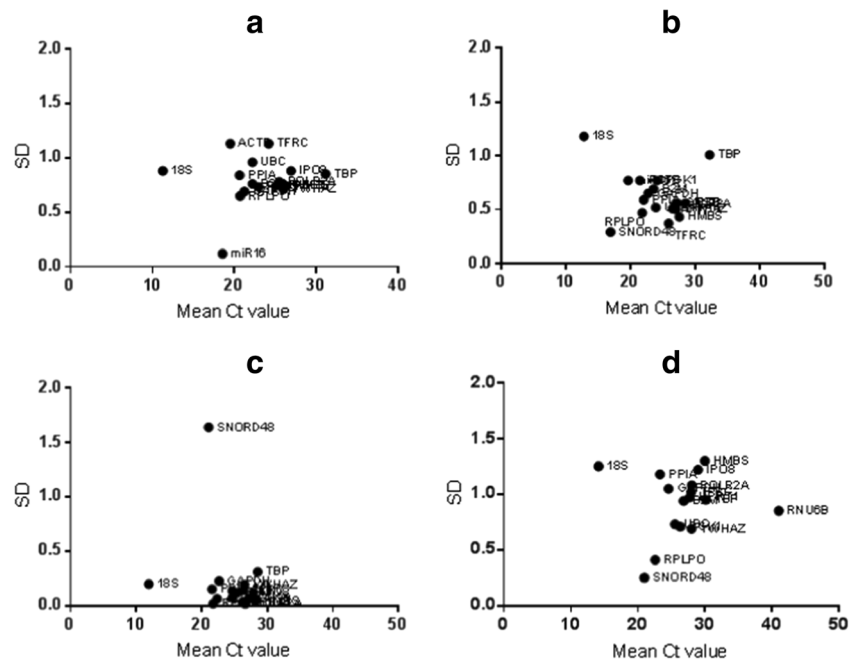
Table 3 Endogenous control genes ranked by their NormFinder stability value in unirradiated controls and in irradiated (6 Gy) compared to unirradiated controls

	DU145				22Rv1			
	Unirradiated control		Unirradiated control and IR			Unirradiated control		Unirradiated control and IR	
Rank	Gene	Stability value	Gene	Stability value	Rank	Gene	Stability value	Gene	Stability value
1	IPO8	0.002	IPO8	0.001	1	B2M	0.000	YWHAZ	0.002
2	PGK1	0.004	RPLPO	0.002	2	TFRC	0.000	TFRC	0.005
3	RPLPO	0.005	POLR2A	0.002	3	UBC	0.002	B2M	0.005
4	GAPDH	0.005	PPIA	0.004	4	YWHAZ	0.004	HPRT1	0.007
5	POLR2A	0.005	HPRT1	0.004	5	GAPDH	0.009	GAPDH	0.007
6	UBC	0.005	GUSB	0.006	6	HPRT1	0.015	PPIA	0.015
7	HPRT1	0.007	B2M	0.007	7	ACTB	0.016	IPO8	0.015
8	PPIA	0.007	GAPDH	0.008	8	POLR2A	0.016	UBC	0.023
9	GUSB	0.008	HMBS	0.008	9	RPLPO	0.019	PGK1	0.028
10	HMBS	0.008	UBC	0.009	10	PGK1	0.021	ACTB	0.030
11	B2M	0.011	PGK1	0.009	11	PPIA	0.022	GUSB	0.031
12	TFRC	0.011	TFRC	0.011	12	GUSB	0.022	HMBS	0.033
13	YWHAZ	0.012	YWHAZ	0.011	13	IPO8	0.022	RPLPO	0.039
14	TBP	0.013	ACTB	0.017	14	HMBS	0.028	TBP	0.048
15	ACTB	0.021	TBP	0.025	15	TBP	0.065	POLR2A	0.054
16	18S	0.041	18S	0.057	16	18S	0.135	18S	0.144
	Mean	0.010	Mean	0.011		Mean	0.025	Mean	0.03
	SD	0.009	SD	0.014		SD	0.033	SD	0.034

of irradiation on gene expression was nonetheless demonstrated by a shift in ranking of endogenous gene stability when the irradiated (6 Gy) and unirradiated conditions were analysed together compared to the unirradiated samples alone. GAPDH appeared the most stable across cell lines and in response to radiation exposure: in unirradiated controls, GAPDH ranked 4th (DU145) and 5th (22Rv1); and in irradiated cells, 8th (DU145) and 5th (22Rv1). There were, however, some disparities in rankings between the two cell lines. IPO8 performed the best

Fig. 2 Variability of the mRNA (*N*=16) and miRNA (*N*=3) endogenous control genes in response to 6-Gy irradiation in 22Rv1 and DU145. The mean and standard deviation of the combined unirradiated and irradiated Ct values is represented for **a** DU145 and **b** 22Rv1 cells. The CtCv% representing the spread of Ct values in unirradiated and 6-Gy irradiated samples is presented for each cell lines. **c** A CtCV(%) of 5 is commonly considered as a cut-off below which a gene is considered stable (*dashed line*)
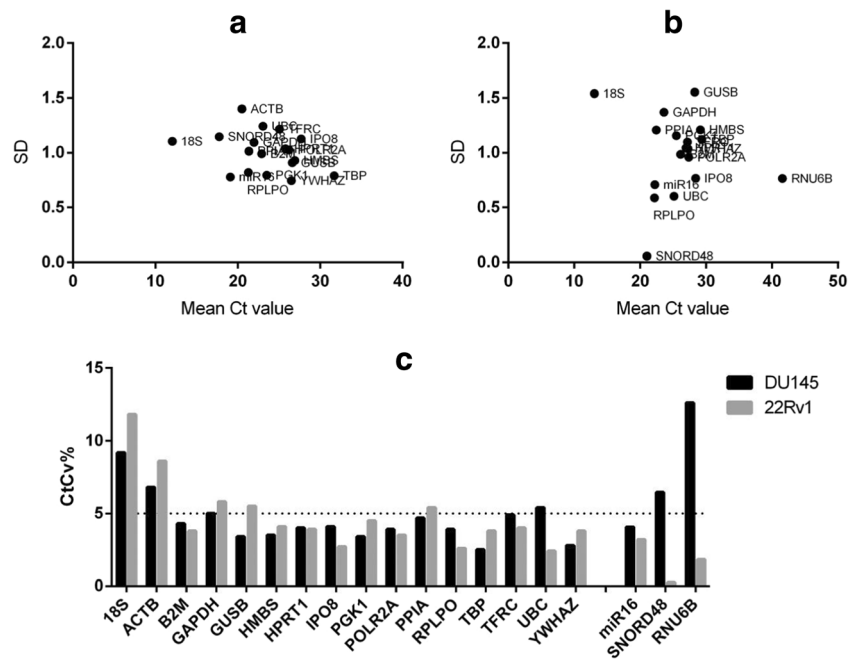
Table 4 Stability assessment of best and worst endogenous control genes following 6-Gy irradiation using coefficient of variation analysis in a panel of prostate cell lines

HRPT1					
	Mean Ct IR	Mean Ct C	Mean Ct IR and C	SD	CtCV%
22Rv1	27.70	26.23	26.97	1.04	3.9
DU145	26.52	25.05	25.79	1.03	4.0
PC3	28.39	29.67	29.03	0.64	2.20
LNCaP	26.29	28.07	27.18	0.89	3.27
PWR-1E	27.19	27.07	27.46	0.20	0.72
RWPE-1	26.17	26.03	26.1	0.07	0.26
18S					
	Mean Ct IR	Mean Ct C	Mean Ct IR and C	SD	CtCV%
22Rv1	14.12	11.94	13.03	1.54	11.8
DU145	12.83	11.26	12.05	1.10	9.2
PC3	16.69	17.23	16.96	0.38	2.25
LNCaP	13.45	14.96	14.2	1.06	7.51
PWR-1E	16.75	17.52	17.13	0.54	3.17
RWPE-1	13.56	14.11	13.83	0.38	2.81
miR-16					
	Mean Ct IR	Mean Ct C	Mean Ct IR and C	SD	CtCV%
22Rv1	21.7	22.7	22.2	0.7	3.19
DU145	19.65	18.55	19.1	0.77	4.07
PC3	18.89	17.92	18.40	0.68	3.72
LNCaP	21.11	19.60	20.40	0.99	4.89
PWR-1E	22.20	21.13	21.67	0.75	3.47
RWPE-1	20.91	19.16	20.03	1.23	6.17
SNORD48					
	Mean Ct IR	Mean Ct C	Mean Ct IR and C	SD	CtCV%
22Rv1	20.97	21.05	21.01	0.05	0.27
DU145	16.93	18.55	17.74	1.14	6.46
PC3	20.44	20.00	20.22	0.31	1.55
LNCaP	20.22	20.76	20.49	0.39	1.89
PWR-1E	22.33	22.16	22.19	0.05	0.22
RWPE-1	20.27	19.88	20.08	0.27	1.36

C control, *IR* 6-Gy irradiated

in DU145, with the lowest variability at 0.001. For 22Rv1, however, it was ranked 7th with a variability of 0.015. Conversely, YWHAZ was the best control gene in 22Rv1 cells (variability 0.002) but was ranked 13th for DU145 (variability 0.011). To further identify least and most stable gene common to both cell lines following irradiation, a correlation analysis of the stability values following irradiation generated in each cell lines was performed (Fig. 3a). 18S, TBP and ACTB consistently had the highest stability values. PPIA, UBC and PGK1 were the closest genes clustered around the correlation line, representing the most stable genes common to both cell lines. The number of miRNAs tested was not sufficient to allow NormFinder analysis.

Analysis of endogenous control gene stability following irradiation using CtCV% calculations

The mean and standard deviation of the combined Ct values of unirradiated and irradiated samples was calculated for each gene (Fig. 2a, b). Representation of abundance (mean Ct) against variability (standard deviation) indicates clustering of all genes around similar abundance and variability in DU145 cells. In 22Rv1 cells, 18S and RNU6B are clearly identified as outliers for high and low abundance, respectively, and RPLO and SNORD48 for low variability. The CtCV% of each of the candidate genes in response to radiation was next calculated (Fig. 2). A low CtCV% value (<5), representing a small spread of Ct values across samples is indicative of stable
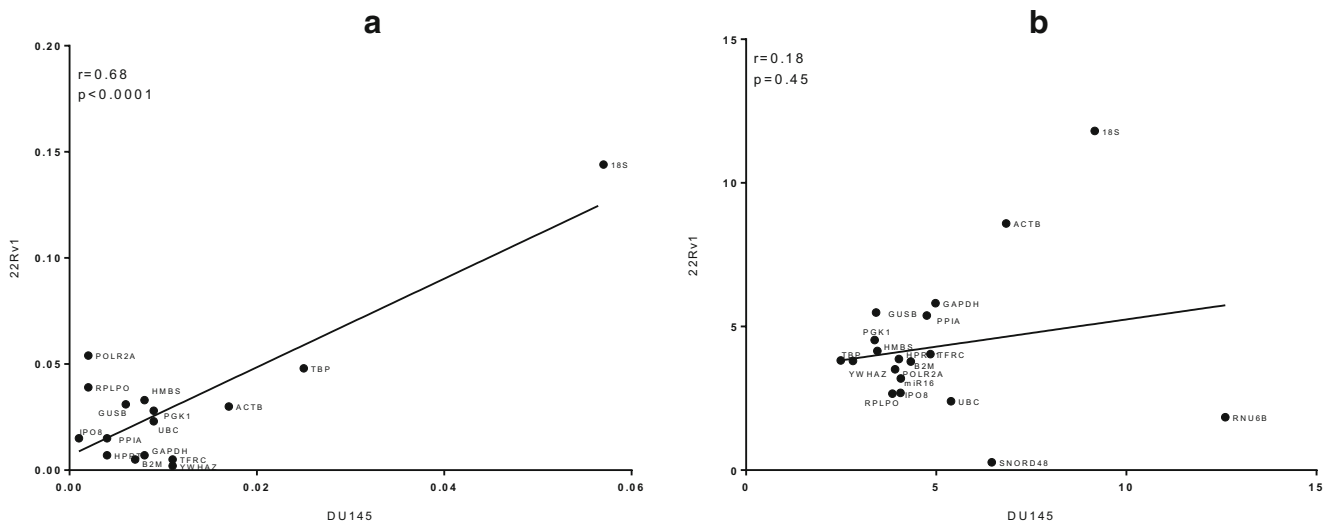
a

b

Fig. 3 Determination of the most and least stable genes common to both cell lines tested. Correlation analysis of **a** stability values and **b** CtCV(%) representing the expression stability for each gene following irradiation is presented for all genes tested. *r* represents the Pearson correlation coefficient; a *p*<0.05 was considered significant

expression. The CtCv% values were similar in both 22RV1 than DU145 cells in the majority of genes. All HKG tested were within the desirable <5 % CtCV% in both cell lines cells, except for 18S, ATBC, UBC, SNORD48 and RNU6B in DU145 cells and 18S, ACTB, GAPDH, GUSB and PPIA in 22Rv1 cells. The mRNA and miRNA endogenous controls were next independently ranked according to increasing CtCV% values in both cell lines (Table 4). The best ranking mRNA endogenous control gene in DU145 (IPO8) and 22Rv1 (B2M) cells did not rank as high in the other line (IPO8, 13th, 22Rv1; B2M, 7th, DU145). The worst ranking gene identified in both lines was 18S. The most stable miRNA endogenous control gene was miR-16 across both cell lines. To further identify the least and most stable gene common to both cell lines following irradiation, a correlation analysis of the CtCV(%) values following irradiation generated in each cell lines was performed (Fig. 3b). 18S, TBP and RNU6B consistently had the highest stability values. TBP, YWHAZ and HMBS were the closest genes clustered around the correlation line, representing the most stable genes common to both cell lines.

Comparison of NormFinder and CtCV% endogenous control gene stability analyses

Since CtCV(%) and NormFinder utilise different approaches for identifying stable genes, the observed differences in rankings between these two methodologies would be expected. A correlation analysis between CtCV% and stability values for each candidate HKG was conducted such that the most and least stable genes common to both methods and both cell lines could be identified. 18S, TBP and ACTB were consistently away from the correlation line in both cell lines, identifying

these genes as those commonly least stable (Fig. 4a, b). HPRT1, B2M and HMBS were clustered around the correlation line, representing the most stable genes common to both CtCV% and NormFinder in these lines (Fig. 4c, d).

Validation of most and least stable endogenous controls in additional prostate cell lines

HPRT1, 18S rRNA, miR-16 and SNORD48 were further investigated in two additional prostate cancer cell lines, PC3 and LNCaP, and in two normal prostate cell lines, PWR-1E and RWPE-1 (Fig. 5). NormFinder is unsuitable for the analysis of only two genes, so the CtCV% between the irradiation and control groups was calculated (Table 4). The results further validated HPRT1 as a stable endogenous control, expressing low variation (<5 CtCV%) in all cell lines, whereas 18S rRNA remained associated with large variations extending to a value of 7.51 in PWR-1E. miR-16 and SNORD 48 were also stable in these lines.

Discussion

MiRNA and gene expression can be easily and rapidly quantified by quantitative real-time PCR (qRT-PCR.). This sensitive method, widely used in cancer research, requires careful control of both the inherent biological variability in mRNA levels across tissues and between individuals and the reproducibility of RNA extraction yields and quality [8, 9]. Variability can be minimised through a normalisation process providing that accurate quantification has been achieved [6, 30]. Normalisation to one or several internal control genes, whose expression is assumed consistent across the physiological or
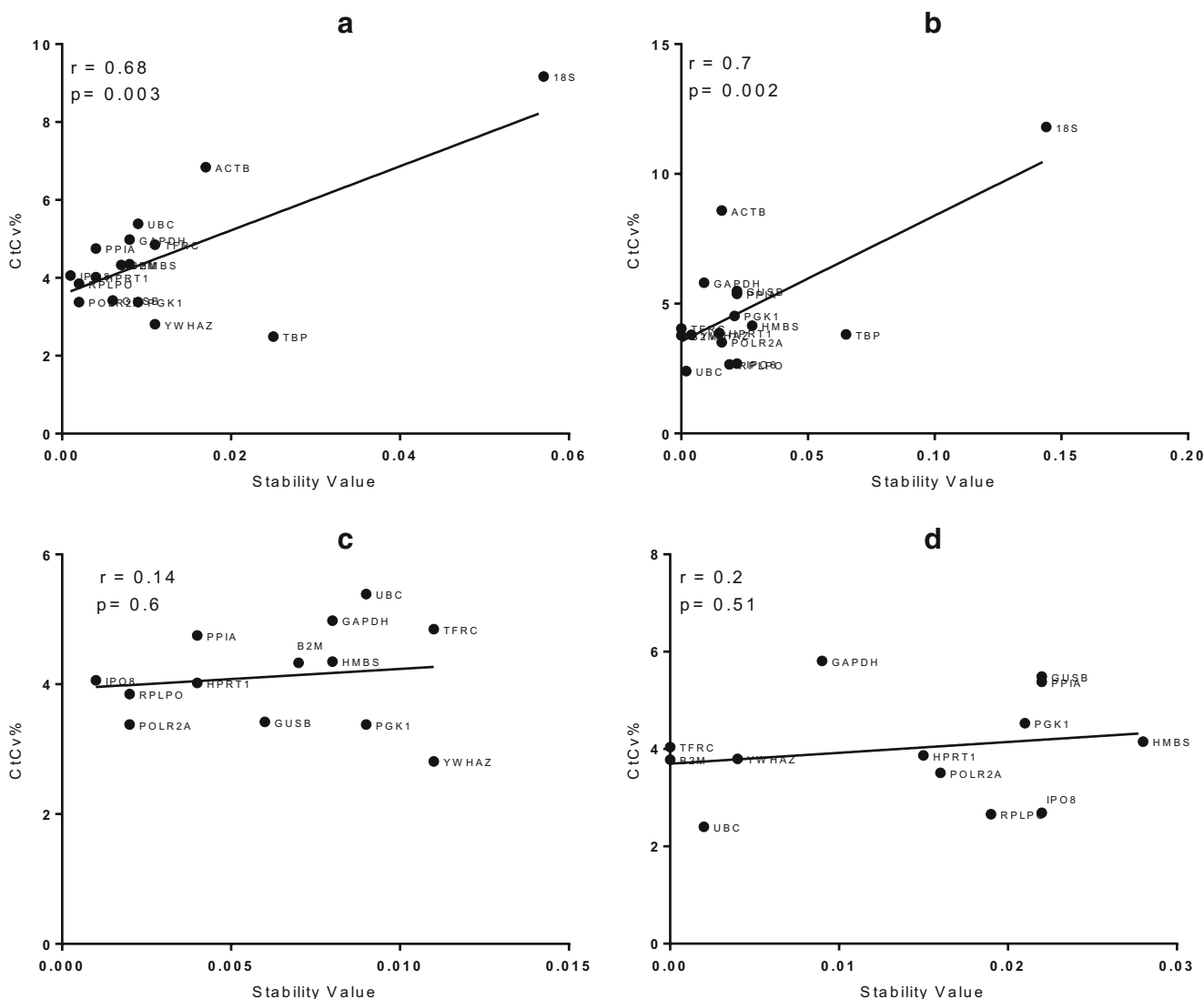
Fig. 4 Determination of the most and least stable genes common to both CtCV(%) and NormFinder and both cell lines tested. Correlation analysis of stability values and CtCV(%) representing the expression stability for each gene following irradiation is presented for all genes tested **a** DU145 and **b** 22Rv1 and following removal of the identified common least stable genes (ACTB, TBP, 18S) in **c** DU145 and **d** 22Rv1 cells. *r* represents the Pearson correlation coefficient; a *p*<0.05 was considered significant

experimental conditions tested, is a commonly used approach [26, 30, 31]. However, it remains associated with some fluctuation in the expression levels quantified for individual genes between cell lines and tissues [4, 32, 33] and endogenous control expression is not always sufficiently stable [34].

In this study, expression levels of a panel of endogenous control genes were measured in three biological replicates. The mean standard deviations for the Ct values across replicates were small for both mRNA and miRNA control genes tested highlighting reproducibility of our experimental conditions. Wide fluctuations in expression levels were observed between the six prostate cell lines and between the genes tested. Some variation in expression of endogenous controls in different cell lines and

tissues may reflect the role of the genes in the cell. For example, GAPDH may be higher in cells with higher energy demands [35]. Authors devise relevant conclusions regarding gene expression on the basis of the use in most cases of a unique or more rarely a specific set of endogenous controls. Nonetheless, the variations of that unique gene or set of genes have not been sufficiently studied, especially in irradiated samples.

In this study, stability was examined 4 h following exposure to a single 6-Gy radiation dose based on reports of maximum radio enhancibility of promoters following large radiation doses within a 12-h time frame (reviewed in [36]). Exposure to a single 6-Gy radiation dose increased the mean CT values of each gene in both cell lines, with a difference reaching statistical significance in several genes tested.

Fig. 5 Mean and standard deviation of Ct values of most and least stable endogenous controls following 6-Gy irradiation in a panel of prostate cell lines. The mean (abundance) and standard deviation (variability) of the combined unirradiated and irradiated Ct values in all cell lines tested is represented for **a** HRPT1, **b** 18S, **c** miR-16 and **d** SNORD48
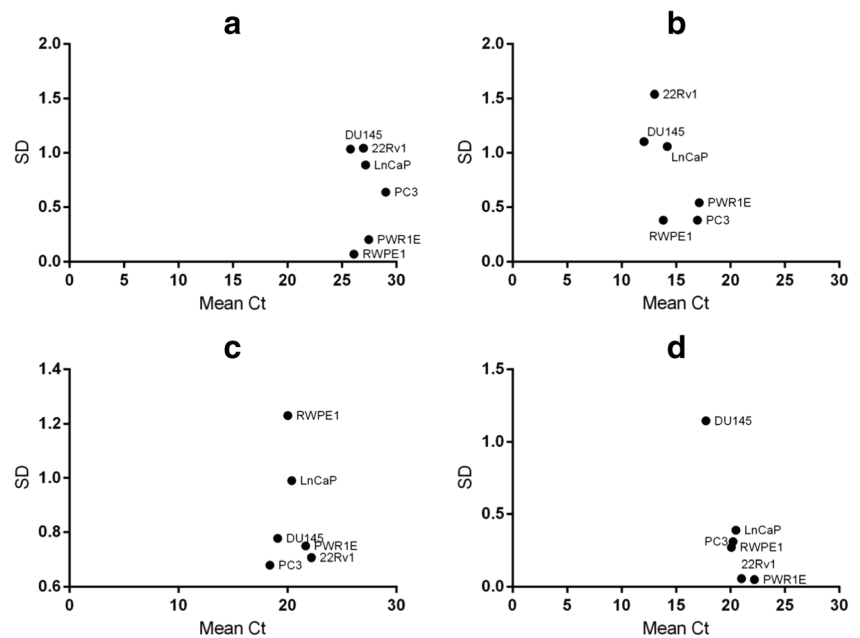
Mechanistic studies investigating the regulation of these genes by radiation would be important to examine this phenomenon. In our cell lines, all three miRNA endogenous controls tested performed well. However, the expression of the commonly used endogenous controls, such as ACTB and GAPDH varied under our experimental conditions. The validation of the stability of these genes using alternative radiation doses and time points is however warranted. Genes were ranked according to NormFinder and the coefficient of variation (CtCV%). The results showed that 18S rRNA was consistently ranked as the least stable endogenous control following irradiation in all cell lines tested. In contrast, HPRT1 had the lowest coefficient of variation values which suggest that it is the most stable housekeeping genes. The early amplification of 18S rRNA and its wide Ct range indicates that the gene exists in a large number of copies. During qRT-PCR, primers anneal more often to highly abundant transcripts, thus causing a greater variability of Ct values. Genes with lower mRNA expression, such as HPRT1, are amplified in later cycles with a narrower range of Ct values. The overall expression levels of the 16 endogenous controls analysed using NormFinder and the coefficient of variation (CtCV%) identified HPRT1 as one of the most stable genes in response to a 6-Gy single radiation exposure in both cell lines. This gene was previously recommended as a universal endogenous control gene for differential expression studies in cancer research [37]. Similarly, the stability of both SNORD48 and miR-16 following irradiation was high in all cell lines tested. SNORD48 was previously reported as a stable endogenous control for miRNA studies in prostate cancer tissues with a stability value of 2.05 using NormFinder [28].

Conclusion

This study assessed the effect of ionising radiation on the expression of commonly used endogenous controls in prostate cell lines. Our findings confirm the need to validate endogenous controls gene stability before use in qRT-PCR experiments. HPRT1 and SNORT48 were identified as stable endogenous control genes following irradiation in a panel of prostate cell lines and should be considered as the normalisation gene for future studies involving the analysis of gene expression patterns in irradiated prostate cells.

Conflicts of interest None

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