Research Paper

Identification of Stably Expressed lncRNAs as Valid Endogenous Controls for Profiling of Human Glioma

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Abstract

Background: Recent research indicates that long non-coding RNAs (lncRNA) represent a new family of RNAs that is of fundamental importance for controlling transcription and translation. Thereby, there is increasing evidence that lncRNAs are also important in tumourigenesis. Thereby valid expression profiling using quantitative PCR requires suitable, stably expressed normalisers to achieve reliable and reproducible data. However, no systematic analysis of suitable references in lncRNA studies in human glioma has been performed yet.

Methods: In this study, we investigated 90 lncRNAs in 30 tissue specimen for the expression stability in human diffuse astrocytoma (WHO-Grade II), anaplastic astrocytoma (WHO-Grade III) and glioblastoma (WHO-Grade IV) both alone as well as in comparison with normal white matter. Our identification procedure included a rigorous bioinformatical selection process that resulted in the inclusion of only highly abundant, equally expressed lncRNAs for further analysis. Additionally, lncRNAs were classified according to their stability value using the NormFinder algorithm.

Results: We identified 24 appropriate normalisers suitable for studies in diffuse astrocytoma, 22 for studies in anaplastic astrocytoma and 12 for studies in glioblastoma. Comparing all three glioma entities 7 lncRNAs showed stable expression levels. Addition of normal brain tissue resulted in only 4 suitable lncRNAs.

Conclusions: Our findings indicate that 4 lncRNAs (HOXA6as, H19 upstream conserved 1 and 2, Zfhx2as and BC200) are suitable as normalisers in glioma and normal brain. These lncRNAs may thus be regarded as universal references being applicable for the accurate normalisation of lncRNA expression profiling in various glioma (WHO-Grades II-IV) alone and in combination with brain tissue. This enables to perform valid longitudinal studies, e.g. of glioma before and after malignisation to identify changes of lncRNA expressions probably driving malignant transformation.

Key words: long non-coding RNA, lncRNA, Glioma, References, qPCR, Profiling.

Introduction

Long non-coding RNA (lncRNA) has recently been observed not being just by-product in the process of transcription but being of significant importance in transcriptional and translational control as well as during splicing, cell cycle, apoptosis, pluripotency, reprogramming and imprinting [1-8]. This highlights a potential role of lncRNAs during tumour formation and progression. Nevertheless, only a minority of lncRNAs has so far been investigated and functionally annotated. [1, 9]. According to Ma et al. lncRNAs can be classified by *(1)* genomic location, *(2)* exerted effect on DNA sequences, *(3)* functional

mechanisms and *(4)* targeting mechanisms [10]. One of the best studied processes being associated with lncRNAs is the X-chromosome inactivation: A single X-inactivation center (Xic) of about 100-500 kbps in length [11, 12] is necessary and sufficient for X-chromosome inactivation [11, 13]. Thereby, Xic contains a well characterised lncRNA: Xist (X-inactive specific transcript). This is a 17-20 kbps long RNA that tags the X-chromosome during early X-chromosome inactivation [11, 12]. Subsequently, only the X-chromosome that expresses Xist is inactivated [11, 14-18]. It is obvious that disturbances in such powerful regulation mechanisms can propagate the development of numerous human diseases.

Especially in the context of tumourigenesis, distinct lncRNAs have been highlighted [19, 20]: HOTAIR (HOX antisense intergenic RNA) is a lncRNA being expressed in various cancers interacting with PRC2 (Polycomb repressive complex 2) [21]. Studies have shown that HOTAIR is associated with motility, invasion, and metastatic potential in tumours [22]. HOTTIP is another example of a lncRNA that is potentially associated with the differentiation status of cancer cells that may be regarded as a co-factor leading to genomic rearrangements in cancer [20, 23, 24]. In case of the antisense lncRNA ANRIL studies have shown that ANRIL is able to repress tumour suppressor loci and thus may promote cancer [25].

There is recent evidence, that lncRNAs may also play a crucial role in brain tumours [26-28]. Glioblastoma (GBM, WHO-Grade IV) represent not only the most frequent brain tumour entity of adults but also the most fatal one with a high risk of recurrence and a very dismal prognosis [29]. Thereby, primary and secondary GBMs can be distinguished: Whilst primary GBMs are tumours that originate *de novo*, secondary GBMs are tumours that derive from lower-grade gliomas, i.e. diffuse astrocytoma (DA, A II, WHO-Grade II) and anaplastic astrocytoma (AA, A III, WHO-Grade III) in a process of malignant transformation [29-31].

Especially by investigating changes in gliomas occurring during tumour progression, i.e. DA, AA, GBM, the selection of suitable references for expression profiling is a challenge. "Ideal" references are genes that do not show expression changes in all investigated tumour entities. They have to be identified and validated prior to quantitative data analysis for each tissue type under the desired experimental setup [32, 33]. As the stability and integrity of different RNA classes, e.g. mRNA, rRNA, miRNA, is highly variable [32-37] normalisers should ideally be composed of the same RNA class as the target RNAs to avoid computational errors by referring to different RNA classes.

So far, there are no lncRNA normalisers for quantitative PCR (qPCR) data in human gliomas available. Moreover, the importance to use a combination of at least two or more validated reverences for proper evaluation of data has not yet been considered [34-37]. This problem is now solved by us. In this study, we investigate 90 different lncRNAs in parallel on human diffuse astrocytoma, anaplastic astrocytoma, glioblastoma and normal white matter as "healthy", unaffected brain tissue. We determined stably expressed lncRNAs using the NormFinder algorithm for both each entity alone as well as in combination and in comparison with normal brain tissue. This way, we were able to identify stably expressed lncRNAs suitable as universal normalisers in studies investigating astrocytoma of different WHO-Grade, enabling longitudinally designed studies of tumours before and after malignant transformation.

Materials and Methods

Sample collection

For this study, we selected 30 different tissue specimen including five human diffuse astrocytoma (WHO-Grade II), five anaplastic astrocytoma (WHO-Grade III), and 15 glioblastoma (WHO-Grade IV) as well as 5 human occipital white matter specimens as "healthy" brain tissue.

Tumour samples were provided by the Brain Tumour Bank of the Center for Neuropathology. Written informed consent was obtained according to the guidelines of the local ethics committee. Surgical samples were fixed with 4% buffered formalin, paraffin embedded, and subjected to routine histological stains: H&E (Haematoxylin and Eosin), EvG (Elastic van Gieson), PAS (Periodic acid-Schiff), Gomori silver stain and immunohistochemistry using antibodies against GFAP (glial fibrillary acidic protein, monoclonal mouse antibody, clone 6F2, Dako), MAP2 (microtubule-associated protein 2, clone HM.2, Sigma) and Ki67 (monoclonal mouse antibody, clone MIB1, Dako). Tumour samples were classified according to the WHO (world health organisation) classification of tumours of the central nervous system, 4th edition, 2007 [29-31]. Determination of IDH1 (isocitrate dehydrogenase 1) mutations was performed using the pyrosequencing technique as described previously [38]. MGMT promoter methylation was determined as described previously [39]. LOH1p/19q (loss of heterozygosity of chromosome 1p and 19q) was detected as described previously [40]. Detailed information of patients can be found in **Table 1**.

Table 1. Tumour sample collection. Listed are all tumour samples used in this study including patient age at surgery, sex, tumour entity according to WHO-Grade, localisation and molecular genetic features of tumours. MGMT status: 0: not methylated; 1: methylated; 2: partially methylated; 9: not analysed due to low amount of tissue; IDH1-mutation: 0: wt; 1: R132H, 2: R132G; 3: R132C; 9: not analysed due to low amount of tissue; LOH1p/19q: 0: no; 1: 1p; 2: 19q; 3: 1p19q; 4: loss of some markers of 1p; 5: loss of some markers of 19q; 6: loss of some markers of 1p/19q; 9: not analysed due to low amount of tissue.

Sample	Age at surgery [y]	Sex	Tumor Entity	WHO-Grade	Localisation of tumor	MGMT status	IDH1-mutation	LOH1p/19q
AII_1	34	f	Diffuse astrocytoma	II	frontal left	1	3	0
AII_2	34	m	Diffuse astrocytoma	II	fronto-temporal right	1	1	2
AII_3	34	m	Diffuse astrocytoma	II	intraparenchymatous	0	9	0
AII_4	39	f	Diffuse astrocytoma	II	frontal right	2	2	0
AII_5	28	f	Diffuse astrocytoma	II	precentral right	1	9	2
AIII_1	21	f	Anaplastic astrocytoma	III	frontal right	1	1	0
AIII_2	50	f	Anaplastic astrocytoma	III	frontal right	0	0	0
AIII_3	25	m	Anaplastic astrocytoma	III	intraparenchymatous	1	2	0
AIII_4	40	m	Anaplastic astrocytoma	III	temporal left	1	1	0
AIII_5	39	m	Anaplastic astrocytoma	III	temporoparietal left	1	0	0
GBM_1	49	f	Glioblastoma	IV	frontal right	1	1	5
GBM_2	45	f	Glioblastoma	IV	frontal right	0	0	5
GBM_3	41	m	Glioblastoma	IV	frontal left	1	1	9
GBM_4	43	m	Glioblastoma	IV	temporal right	2	0	6
GBM_5	54	m	Glioblastoma	IV	intraparenchymatous	1	0	9
GBM_6	50	m	Glioblastoma	IV	temporoparietal left	0	0	2
GBM_7	44	m	Glioblastoma	IV	frontal left	1	1	9
GBM_8	46	m	Glioblastoma	IV	temporal right	0	0	9
GBM_9	61	m	Glioblastoma	IV	parietal left	0	0	0
GBM_10	55	f	Glioblastoma	IV	bifrontal	1	1	9
GBM_11	45	m	Glioblastoma	IV	frontal left	1	0	2
GBM_12	72	f	Glioblastoma	IV	temporal	1	0	0
GBM_13	73	m	Glioblastoma	IV	temporal left	1	0	5
GBM_14	67	m	Glioblastoma	IV	occipital right	0	0	0
GBM_15	34	f	Glioblastoma	IV	frontal left	1	0	2

Table 2. Control sample collection. Listed are all control samples used in this study including age of patients, sex, post-mortem intervals (PMI), investigated brain region and cause of death.

Sample	Age [y]	Sex	PMI [h]	Region	Cause of death
WM_1	63	m	18	Occipital white matter	Cardiac arrest
WM_2	75	m	27	Occipital white matter	Cardiac arrest
WM_3	77	f	20	Occipital white matter	Cardiac arrest
WM_4	53	m	22	Occipital white matter	Cardiac arrest
WM_5	55	f	14	Occipital white matter	Cardiac arrest

Human control tissue was provided by the Neurobiobank Munich (NBM). All investigated cases were collected and clinically as well as neuropathologically characterized according to the NBM standard protocols established by BrainNet Europe and BrainNet Germany. Patients did not show any neurological or psychiatric disorders. Written informed consent was obtained according to the guidelines of the local ethics committee. Detailed information of selected cases can be found in **Table 2**.

Extraction of RNA

Representative H&E stained slides of FFPE tumour samples were prepared and solid, viable tumour consisting of at least 90% tumour cells was microscopically identified. RNA extraction was performed on distinct, micro-dissected tumour regions.

In case of normal brain tissue, subcortical, occipital white matter was marked on H&E stained slides and isolation of distinct regions was performed similar to tumour samples. Extraction of RNA was performed using the RNeasy FFPE Kit (Qiagen) according to the manufacturer's protocols. Quantity and quality of RNA was determined using a NanoDrop system determining the 260/280 nm absorbance ratio. In all cases the ratio was between 1.90 and 2.20. In order to avoid amplification bias we did not use pre-amplification of RNA but proceeded directly with subsequent analysis.
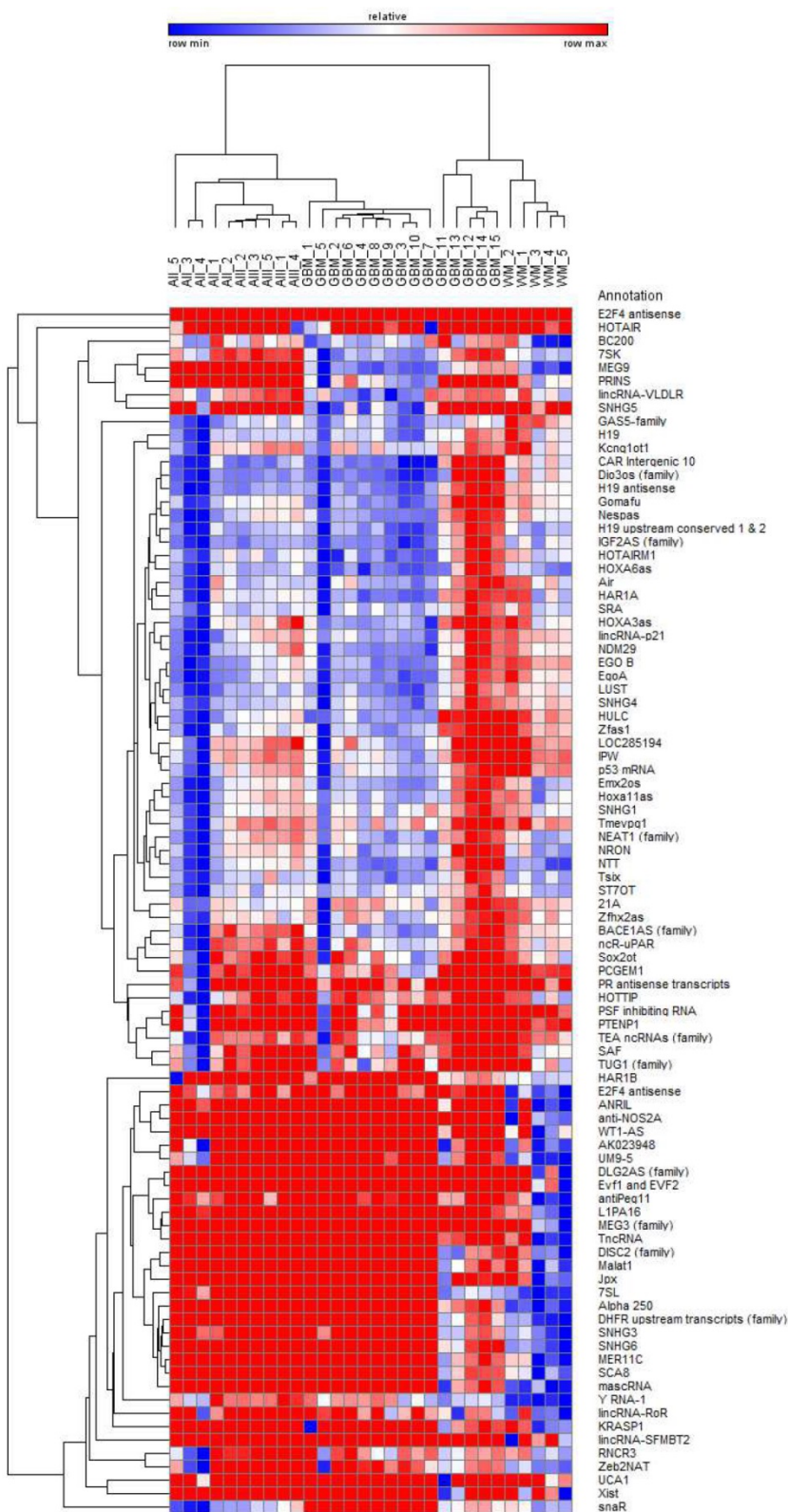
Figure 1. Bi-hierarchical clustering of all 90 investigated lncRNAs in 30 tissue specimen. We investigated 5 diffuse astrocytomas (A II, DA, WHO-Grade II), 5 anaplastic astrocytomas (A III, AA, WHO-Grade III), and 15 glioblastomas (GBM, WHO-Grade IV) as well as 5 occipital white matter tissues (WM) as healthy control. There is a clear clustering of tumour entities visible. Blue: low Ct-value; red: high Ct-value.

Polyadenylation reaction, reverse transcription and quantitative polymerase chain reaction (qPCR)

Polyadenylation and reverse transcription reactions were performed using the human LncProfiler qPCR Assay Kit (SBI). Equal amounts of RNA (750 ng) were used in the polyadenylation reaction. All procedures were performed according to the optimised manufacturer's protocols. Quantification of lncRNAs was performed using the validated pre-designed qPCR primer library of the human lncProfiler qPCR Assay Kit (SBI) allowing the parallel investigation of 90 annotated lncRNAs that are listed in the lncRNA database. Quantitative PCR was performed on a LightCycler 480 II device (Roche) using the SensiFAST SYBR No-ROX Kit (Bioline) and standard protocols.

Bioinformatical analysis

Clustering of expression data was performed using Gene-E software (http://www.broadinstitute.org/cancer/software/GENE-E/) and an un-supervised bi-hierarchical clustering algorithm based on pair-wise distance calculations. For the identification of stably expressed lncRNAs, cycle-threshold (Ct) values were used as basis for statistical analysis. To enhance reliability of data, only highly abundant lncRNAs (i.e. with Ct values < 30) were taken into consideration, lncRNAs with Ct values ≥ 30 were excluded due to low abundance and thus being not suitable as references. Furthermore, all lncRNAs that showed significant expression differences within different groups were excluded. For significance testing, GraphPad Prism software was used. We performed unpaired t-tests, p-values < 0.05 were considered as significant. Remaining lncRNAs were classified using the NormFinder algorithm taking both intra- and intergroup variations into consideration [41]. Only lncRNAs with stability values of < 0.1 were considered as appropriate.

Results

Each glioma entity shows different but distinct lncRNA profiles clearly separated from normal white matter

The analysis of 90 lncRNAs in 5 diffuse astrocytomas, 5 anaplastic astrocytomas, 15 glioblastomas and 5 normal white matter brain specimens show in an unsupervised bi-hierarchical clustering distinct lncRNA expression profiles (**Figure 1**): All diffuse astrocytomas build a distinct cluster that is flanked by anaplastic astrocytomas on one side. All anaplastic astrocytomas show another distinct cluster that is located between diffuse astrocytomas and glioblastomas. All glioblastomas cluster together and are flanked by anaplastic astrocytomas. Furthermore, all normal white matter specimens show a distinct cluster that is separated from all glioma clusters. The different clustering of lncRNA profiles emphasised that (1) lncRNA profiles of all tumour entities are individual representing different molecular pathways being activated in the tumours, (2) there is a step-wise change of lncRNA profiles that is represented by the clustering of diffuse astrocytomas being situated next to anaplastic astrocytomas that are situated next to glioblastomas and (3) separation of lncRNA profiles of different glioma entities mirrors the need for normalisers being equally expressed not only within one cluster but in all clusters to perform longitudinal studies on tumour specimen during tumour progression.

Identification of reference lncRNAs in diffuse astrocytoma, anaplastic astrocytoma and glioblastoma

The conscious analysis of lncRNA expression data was performed in a step-wise bioinformatical approach. To get reliable references, only highly abundant lncRNAs were taken into consideration, i.e. lncRNAs with Ct values of ≥ 30 were excluded due to low abundance indicating not being suitable as normalisers. All remaining lncRNAs were alaysed using the NormFinder algorithm [41] and ranked according to their stability values. Only lncRNAs with stability values of < 0.1 were considered as suitable normalisers.

We identified 24 lncRNAs suitable as normalisers in diffuse astrocytomas (**Table 3, Figure 2A**). The top five are Zfhx2as (stability value 0.008), H19 (stability value 0.010), EgoA (stability value 0.010), IGF2AS (stability value 0.010) and Hoxa11as (stability value 0.012). In case of anaplastic astrocytomas, we identified 22 suitable normalisers (**Table 3, Figure 2B**), the five most stable lncRNAs are SNHG4 (stability value 0.005), H19 upstream conserved 1 and 2 (stability value 0.006), Dio3os (stability value 0.010), Hoxa11as (stability value 0.011) and IGF2AS (stability value 0.013). In glioblastoma we identified 12 suitable lncRNAs (**Table 3, Figure 2C**), the top five are LUST (stability value 0.016), SNHG4 (stability value 0.019), EgoA (stability value 0.034), H19 upstream conserved 1 and 2 (stability value 0.035) and EGO B (stability value 0.036).

Table 3. Stably expressed lncRNAs in single glioma entities. We identified 24 suitable normalisers in diffuse astrocytoma (WHO-Grade II), 22 suitable normalisers in anaplastic astrocytoma (WHO-grade III) and 12 suitable normalisers in glioblastoma (WHO-Grade IV). All lncRNAs are arranged according to their stability values. Stability values are calculated using the NormFinder algorithm.

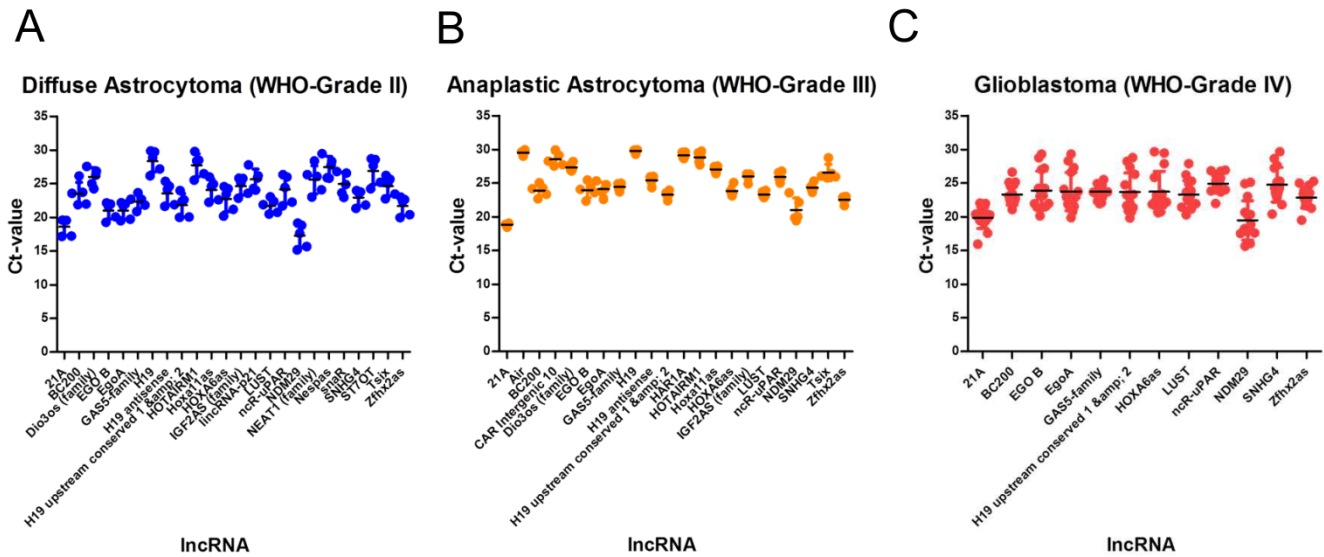| Diffuse Astrocytoma (WHO-Grade II)			Anaplastic Astrocytoma (WHO-Grade III)			Glioblastoma (WHO-Grade IV)		
Gene name	Stability value	Standard error	Gene name	Stability value	Standard error	Gene name	Stability value	Standard error
Zfhx2as	0.008	0.004	SNHG4	0.005	0.004	LUST	0.016	0.005
H19	0.010	0.004	H19 ups. cons. 1, 2	0.006	0.004	SNHG4	0.019	0.005
EgoA	0.010	0.004	Dio3os (family)	0.010	0.005	EgoA	0.034	0.007
IGF2AS (family)	0.010	0.004	Hoxa11as	0.011	0.005	H19 ups. cons. 1, 2	0.035	0.008
Hoxa11as	0.012	0.005	IGF2AS (family)	0.013	0.005	EGO B	0.036	0.008
SNHG4	0.013	0.005	HOXA6as	0.013	0.005	Zfhx2as	0.041	0.009
ST7OT	0.015	0.006	H19	0.013	0.006	ncR-uPAR	0.045	0.009
HOTAIRM1	0.015	0.006	HAR1A	0.013	0.006	21A	0.048	0.010
Nespas	0.016	0.006	H19 antisense	0.014	0.006	HOXA6as	0.051	0.010
21A	0.017	0.006	LUST	0.016	0.006	GAS5-family	0.065	0.013
H19 antisense	0.018	0.007	Air	0.017	0.007	NDM29	0.067	0.013
snaR	0.018	0.007	21A	0.019	0.007	BC200	0.086	0.017
Tsix	0.019	0.007	CAR Intergenic 10	0.020	0.008			
Dio3os (family)	0.019	0.007	ncR-uPAR	0.024	0.009			
EGO B	0.021	0.008	GAS5-family	0.025	0.009			
LUST	0.025	0.009	EgoA	0.027	0.010			
HOXA6as	0.025	0.009	HOTAIRM1	0.029	0.011			
lincRNA-p21	0.026	0.009	EGO B	0.030	0.011			
GAS5-family	0.027	0.010	BC200	0.034	0.012			
H19 ups. cons. 1, 2	0.029	0.010	Zfhx2as	0.037	0.013			
ncR-uPAR	0.029	0.010	Tsix	0.053	0.019			
NEAT1 (family)	0.030	0.011	NDM29	0.069	0.025			
BC200	0.042	0.015						
NDM29	0.045	0.016						

Figure 2. Identification of stable lncRNAs in diffuse astrocytoma in different glioma entities. In diffuse astrocytoma (WHO-Grade II) we identified 24 (A), in anaplastic astrocytoma (WHO-grade III) we identified 22 (B) and in glioblastoma (WHO-Grade IV) we identified 12 lncRNAs suitable as normalisers (C). Indicated are mean and SD.

Table 4. Stably expressed lncRNAs suitable for studies in different human glioma entities. We identified 7 lncRNAs that are suitable for studies in multiple glioma entities. All lncRNAs are arranged according to their stability values. Intragroup and intergroup variations indicated were calculated using the NormFinder algorithm.

Glioma		Intragroup variation			Intergroup variation		
Gene name	Stability value	A II	A III	GBM	A II	A III	GBM
Zfhx2as	0.014	0.000	0.001	0.001	0.000	-0.006	0.005
SNHG4	0.017	0.001	0.000	0.001	-0.013	0.001	0.013
HOXA6as	0.018	0.001	0.000	0.004	0.002	0.005	-0.007
H19 ups. cons. 1, 2	0.019	0.000	0.000	0.002	-0.017	0.004	0.013
ncR-uPAR	0.024	0.001	0.000	0.001	-0.004	0.023	-0.019
21A	0.025	0.000	0.000	0.002	0.005	-0.027	0.021
BC200	0.029	0.001	0.001	0.007	0.027	0.000	-0.027

Expression stability of lncRNA references suitable for comparison of gliomas and normal brain tissue

To investigate gliomas during malignant transformation it is important to perform longitudinally designed studies that include tumour specimen before and after malignant transformation. To identify lncRNA normalisers that are applicable for diffuse astrocytoma, anaplastic astrocytoma and glioblastoma, we applied an extended bioinformatical approach: (1) Exclusion of low abundant lncRNAs with Ct values of ≥ 30, (2) exclusion of all lncRNAs with stability values of ≥ 0.1 in NormFinder analysis and (3) exclusion of all lncRNAs with significantly different expression levels ($p < 0.05$) in significance testing in order to consider both intragroup and intergroup variations during selection process.

We identified 7 lncRNAs that fulfilled all criteria to be considered as stably expressed in all investi-gated tumour entities (**Table 4, Figure 3**). The five most stable lncRNAs are Zfhx2as (stability value 0.014), SNHG4 (stability value 0.017), HOXA6as (stability value 0.018), H19 upstream conserved 1 and 2 (stability value 0.019) and ncR-uPAR (stability value 0.024).

To identify lncRNAs being suitable as normalisers in different glioma entities and in normal brain tissue, we further enhanced our analysis by adding expression data of normal white matter. We applied the similar approach as in case of multiple glioma entities and found four stably expressed lncRNAs (**Table 5, Figure 4**): HOXA6as (stability value 0.019), H19 upstream conserved 1 and 2 (stability value 0.024), Zfhx2as (stability value 0.030) and BC200 (stability value 0.044). These lncRNAs may be regarded as universal normalisers being applicable in a broad range of studies focussing on human glioma research.
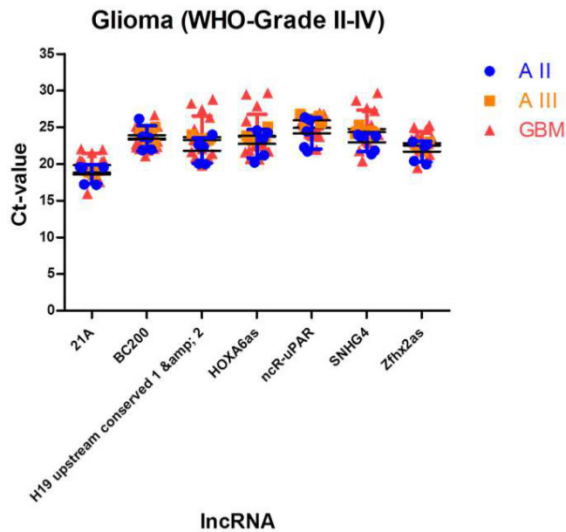
Figure 3. Stably expressed lncRNAs suitable for studies comparing different glioma entities. We identified 7 lncRNAs that are suitable as normalisers in studies investigating different glioma entities. Indicated are mean and SD.
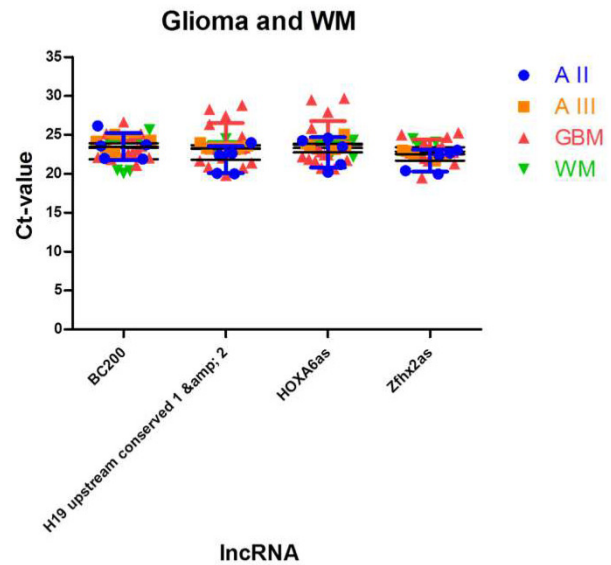

Figure 4. Identification of lncRNAs suitable as normalisers in studies investigating glioma and normal brain tissue. We identified 4 lncRNAs that show stable expression in different glioma entities and in normal white matter. These normalisers can be regarded as universal normalisers that are suitable as references in a broad range of lncRNA studies. Indicated are mean and SD.

Table 5. Stably expressed lncRNAs suitable for studies in human gliomas and normal brain tissue. We identified 4 lncRNAs that show stable expression levels in all three glioma entities and in normal occipital white matter. These lncRNAs may be regarded as universal normalisers applicable in a broad range of lncRNA studies. All lncRNAs are arranged according to their stability values. Intragroup and intergroup variations indicated were calculated using the NormFinder algorithm.

Glioma and white matter		Intragroup variation				Intergroup variation			
Gene name	Stability value	A II	A III	GBM	WM	A II	A III	GBM	WM
HOXA6as	0.019	0.001	0.000	0.003	0.001	-0.002	0.003	-0.004	0.002
H19 ups. cons. 1, 2	0.024	0.000	0.000	0.002	0.001	-0.025	-0.002	0.012	0.014
Zfhx2as	0.030	0.000	0.002	0.001	0.000	-0.015	-0.020	-0.003	0.038
BC200	0.044	0.001	0.001	0.007	0.005	0.042	0.018	-0.005	-0.055

Discussion

Recently it has been found that mRNA represents only a small fraction of RNA as the vast majority of DNA is transcribed into RNAs. In this context, lncRNAs are of emerging importance as there is increasing evidence that lncRNAs are of regulatory significance in numerous cellular processes [1, 4-7]. Since conscious quantifications of lncRNAs need adequate, reliable normalisation strategies, it is important to perform systematic evaluations of potential normalisers [42, 43].

The current study is the first systematic analysis of a wide range of lncRNAs in gliomas of different WHO-Grades (diffuse astrocytoma WHO-Grade II, anaplastic astrocytoma WHO-Grade III, glioblastoma WHO-Grade IV) as well as normal white matter using quantitative PCR (qPCR) to identify suitable normalisers allowing both longitudinal studies of gliomas before and after malignant transformation and studies comparing glioma and normal brain tissue. Investi-

gating 90 lncRNAs being annotated in the lncRNA database [9] we were able to detect suitable references for the different tumour entities both alone and in comparison with each other. Of the 90 lncRNAs we identified 24 suitable normalisers in diffuse astrocytomas, 22 in anaplastic astrocytomas and 12 in glioblastomas. Interestingly, we found that glioblastoma showed the lowest number of stably expressed lncRNAs. Applying a rigorous bioinformatical approach, the number of suitable normalisers was dramatically reduced by comparing expression levels across all three glioma entities: We identified 7 suitable normalisers for studies comparing different glioma entities (i.e. Zfhx2as, SNHG4, HOXA6as, H19 upstream conserved 1 and 2, ncR-uPAR, 21A and BC200). These normalisers are suitable for e.g. longitudinal studies of gliomas before and after malignant transformation. Interestingly, even highly ranked in single tumour entities many lncRNAs turned out to be unsuitable as normalisers across tumour subgroups as being differentially expressed in different entities and

thus not being suitable as proper intergroup normalisers. The addition of normal white matter resulted in only 4 suitable normalisers: HOXA6as, H19 upstream conserved 1 and 2, Zfhx2as and BC200. Thus, these 4 normalisers may be regarded as universal normalisers suitable for a broad range of lncRNA studies. SNHG4, ncR-uPAR and 21A showed to be differentially expressed in glioma and normal white matter being unsuitable as normalisers comparing normal tissue and glioma. As recent publications show that normalisation to a single reference gene is not sufficient, even if normalisers were identified in a conscious way, the optimal normalisation strategy should include at least a combination of two or more references [37, 44, 45]. Thus, we suggest a combination of all 4 universal normalisers in lncRNA studies. This strategy will further increase the reproducibility and validity of lncRNA studies in glioma and normal brain tissue.

In summary, we showed that the proper selection of normalisers is an absolute requirement for valid determination of lncRNA expression profiles using quantitative PCR. Only by selecting proper normalisers, valid and reproducible data can be generated. We identified a set of 4 normalisers applicable as references for accurate lncRNA studies in human diffuse astrocytoma (WHO-Grade II), anaplastic astrocytoma (WHO-grade III), glioblastoma (WHO-Grade IV) and normal white matter tissue. A combination of these 4 normalisers will result in unbiased results. Thus, the data presented in this study will further boost research on lncRNAs in human gliomas and allows even longitudinal studies of gliomas during malignant transformation and tumour progression.

Acknowledgement

Competing Interests

None declared.

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