Inhibition testing (Cq dilutions, spike or other) E Not performed by using dilutions of samples or universal inhibition assays. The amplification product for the reference gene assay obtained in each cDNA target has been considered.

Electrophoresis traces D Not performed

Processing procedure E Neuroblastoma tumors have been collected at the onset of disease. Each surgically resected tumor was processed by pathologists and immediately stored at –80°C.

Volume/mass of sample processed D

Processing/characterization E Neuroblastoma tumors have been collected at the onset of disease. All tumor samples were classified as Schwannian stroma-poor neuroblastoma according to the International Neuroblastoma Pathology Committee (Shimada H et al. Terminology and morphologic criteria of neuroblastic tumors: recommendations by the International Neuroblastoma Pathology Committee. Cancer. 1999;86:340-362) with at least 80% of neuroblasts.

Sample storage conditions and duration (especially for FFPE samples) E Samples were stored at –80°C

Neural-sciences extraction

Procedure and/or instrumentation E Total RNA was extracted by using the PerfectPure® RNA Tissue Kit (5Prime, Hamburg, Germany). Following manufacturer’s protocol. Homogenization of samples was performed by using the Tissue Lyser (QIAGEN, Germany) according to manufacturer’s instructions. Briefly, after adding Lysis solution to the cell pellet, the pellet was disintegrated from the tube by using a pipet tip. Sample was vortex vigorously to resuspend the pellet until there were no visible cell clumps in the lysate. Centrifugation steps were performed by using Centrifuge 5424 (Eppendorf AG, Hamburg, Germany). PRINCIPLE: Total RNA is purified by first adding cultured cells to a detergentsalt solution (a chaotrope guanidium isothiocyanate solution) to lyse the cells and eliminate endogenous Rnase activity. Lysis and homogenization disrupts the cell membranes releasing RNA into the lysis solution, and shears the genomic DNA, decreasing the viscosity of the lysate. Next, the lysates are applied to the purification column to bind the RNA and wash away proteins, DNA and other contaminants. Residual RNA is removed by on-column DNase treatment, and the DNA fragments and DNase are removed by subsequent washing steps. Finally, the purified RNA is eluted with DEPC-treated water.

Name of kit and details of any modifications E PerfectPure® RNA Tissue Kit (5Prime, Hamburg, Germany). We exactly followed manufacturer’s protocol. The only modification was in the Lysis step: the incubation time was extended for 30 minutes.

Source of additive/reagents used D 5Prime, Website (5Prime, Hamburg, Germany)

Details of DNase or RNase treatment E RNA was amplified and reverse transcribed by the WT-Ovation RNA Amplification System kit (NuGEN Technologies, San Carlos, CA) components

Contamination assessment (DNA or RNA) E RNA was amplified and reverse transcribed by using ultrasonic analysis (Agilent Technologies’ Bioanalyzer).

Instrument and method E Total RNA and small RNAs were quantified by RNA 6000 Nano Chip and GI-ME-N cells. We used VIC-labeled TaqMan Gene Expression assay (Applied Biosystems, Foster City, CA) in a total volume of 10 µl containing 10 ng of RNA, 2 µl Master Mix Probe (5Prime, Hamburg, Germany) and 25 primer/probe mix. Reactions were setup in realtime white tubes (Eppendorf, Hamburg, Germany) and they were run on the Mastercycler epGradient S system (Eppendorf). Cycling conditions were as follows: 35 cycles at 95°C for 15 seconds and at 60°C for 1 minute. The signal of the amplification plot was very late (Cq>40).

Probe sequence and modification E The amplified product was purified by according to manufacture’s instructions. Briefly, after adding Lysis solution to the cell pellet, the pellet was dislodged from the bottom of the tube by using a pipet tip. Sample was vortex vigorously to resuspend the pellet until there were no visible cell clumps in the lysate. Centrifugation steps were performed by using Centrifuge 5424 (Eppendorf AG, Hamburg, Germany). Additional 200 µl of DNase Wash Solution were added and the column was centrifuged at 13,000 g for 2 minutes. The column was then transfer to a new collection tube and we proceed with Wash 2 and elution as specified in the protocol.

Principle: Total RNA is purified by first adding cultured cells to a detergentsalt solution (a chaotrope guanidium isothiocyanate solution) to lyse the cells and eliminate endogenous Rnase activity. Lysis and homogenization disrupts the cell membranes releasing RNA into the lysis solution, and shears the genomic DNA, decreasing the viscosity of the lysate. Next, the lysates are applied to the purification column to bind the RNA and wash away proteins, DNA and other contaminants. Residual RNA is removed by on-column DNase treatment, and the DNA fragments and DNase are removed by subsequent washing steps. Finally, the purified RNA is eluted with DEPC-treated water.

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Complete reaction conditions E RNA was amplified and reverse transcribed by the WT-Ovation™ RNASynthesis Kit (NuGEN Technologies, San Carlos, CA) following manufacturer’s instructions and using the Mastercycler epGradient S system (Eppendorf). Cycling conditions were as follows: 35 cycles at 95°C for 15 seconds and at 60°C for 1 minute. The signal of the amplification plot was very late (Cq>40).

Amount of RNA and reaction volume E Amount of RNA: 50 ng; reaction volume: 42 µl

Prime reagents and dilution of (using QDR) and concentration E

Reverse transcriptase and concentration E WT-Ovation™ RNASynthesis Kit (NuGEN Technologies, San Carlos, CA) components

Temperature and time E Primer Annealing, 65°C for 5 minutes; Primers annealed: 4°C for 1 minute, 35°C for 10 minutes, 45°C for 10 minutes. Second step synthetic: 4°C for 1 minute, 35°C for 10 minutes, 50°C for 10 minutes, 70°C for 5 minutes. Post second step annealing: 4°C for 1 minute, 47°C for 10 minutes, 37°C for 15 minutes. 80°C for 20 minutes. SPATA™ amplification: 4°C for 1 minute, 47°C for 10 minutes, 37°C for 15 minutes. 80°C for 20 minutes.

Manufacture of reagents and catalogue numbers E

Cap with and without RT E All samples have been validated as RNA free by performing a no-reverse transcription control when first extracting RNA.

cDNA target concentrations E

Storage conditions of stock E

Sample manipulation and load of each assay E Not applicable since we performed monoplex qPCRs


Location of amplification D

Primary sources: Lymphatics, blood, and bone marrow of MDS patients with myelodysplastic syndrome and all the healthy controls. No reagents were used in this assay.
Amplification length

E 250 bp for each primer.

Library preparation

E primer mix: 150 nM / 10 µl reaction; 18S rRNA (pre-optimized by PrimerDesign Ltd, UK).

PCR validation

E melting curve analysis (ramping from 60°C to 95°C in 20 minutes) to ensure the presence of the specific amplicon.

Statistical methods for result significance

E Student’s t-test; Box-and-Whisker plots; Receiver Operating Characteristic (ROC) curves.

Number of replicates

E qPCR reactions were performed in duplicate.

NCQ determination

E runs performed as duplicate.

Outlier identification and disposition

E runs performed to minimize instrument and liquid handling variations were shown to be minimal (mean SD for non-amplified RNA: GI-ME-N=0.28, LAN-5=0.26).

Results of NTCs

E no false positives and unintended amplification products (e.g. primer dimers) by performing melting curve analysis after each T-UCR amplification.

Justification of number and choice of reference genes

E NCQ has been used as reference gene. This choice was based on:

1) Establishment of the optimal reference genes by the geNorm® Housekeeping Gene Selection Kit and software (v. 3.4) (PrimerDesign Ltd, UK).

2) Primer mix: 150 nM / 10 µl reaction.

3) Polymerase of specific detection of Eukaryotic 18S rRNA were supplied pre-optimized by Applied Biosystems, Foster City, CA. TaqMan® Gene Expression Assays were used as the reference standard.

Primer sequences

E identical primer/probe mix. Reactions were setup in 96-white-well plates (Eppendorf) by means of EpMotion 5070 Liquid Handling Workstation (Eppendorf, Hamburg, Germany). All reactions were performed in duplicate on the Mastercycler® epRealPlex S system (Eppendorf).

Location of each primer by exon or intron

E presence in the specific T-UCR assay.

Secondary structure analysis of amplicon

E noiseband method: the threshold is specified so that it is significantly (10 times the standard deviation) above the noise of the baseline. The baseline is automatically calculated for every sample individually.

Number and concordance of biological replicates

E data normalisation has been carried out against 18S rRNA as endogenous unregulated reference gene. For each cDNA, the duplicate T-UCR Cq values were averaged, and the normalized Cq was calculated by subtracting the mean Cq value for 18S rRNA from each T-UCR mean Cq value.

Pseudogenes, retrotransposons or other homologs?

E not applicable since we performed homo-geneous qPCR.

Rerun analysis

E none.

Abundance ratios of noiseband

E not applicable since we performed homo-geneous qPCR.

Statistical methods for result significance

E data normalisation has been carried out against 18S rRNA as endogenous unregulated reference gene. For each cDNA, the duplicate T-UCR Cq values were averaged, and the normalized Cq was calculated by subtracting the mean Cq value for 18S rRNA from each T-UCR mean Cq value.

NCQ validation number

E 18S rRNA assay among samples used in the present study is shown by the small difference of standard deviations for the Cq variance (≤0.12).

Dilution volume and amount of LC/DMMA

E reaction volume: 10 µl; Amount of LC/DMMA: 10 µg.

Additives (SYBR Green I, DMSO, etc.)

E SYBR Green I (5Prime, Hamburg, Germany).

Complete thermocycling parameters

E 95°C for 2 minutes, 40 cycles at 95°C for 15 seconds and at 60°C for 1 minutes, followed by a melting curve (ramping from 65°C to 95°C in 20 minutes) to ensure the presence of the specific amplicon.

Location and identity of any modifications

E T-UCR assays: no modifications (SBRY green detection chemistry). 18S rRNA: not available due to intellectual property of Applied Biosystems.

Complete thermocycling parameters

E 95°C for 2 minutes, 40 cycles at 95°C for 15 seconds and at 60°C for 1 minutes, followed by a melting curve (ramping from 65°C to 95°C in 20 minutes) to ensure the presence of the specific amplicon.

Buffer/kit identity and manufacturer

E 2.5x RealMaster Mix SYBR ROX for T-UCR assays / 2.5x RealMaster Mix Probe for 18S rRNA assay (5Prime, Hamburg, Germany).