Epigenetic Regulation of c-ROS Receptor Tyrosine Kinase Expression in Malignant Gliomas

Supplementary Information

Supplementary Methods

RT-PCR. The cycling conditions for the RT-PCR reactions of Figure 2 were: an initial denaturation step at 95°C for 5 min, followed by 38 cycles of 94°C for 45 sec, 52°C for 45 sec and 72°C for 50 sec and a final extension step at 72°C for 10 min. PCR cycling parameters for the RT-PCR reaction of Figure 4 are as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec and a final extension step at 72°C for 10 min. The PCR primers span exons 40 to 41of the c-ROS gene. All PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide. For QRT-PCR, $1/10^{th}$ of the RT reaction was amplified using Taqman® probes for human c-ROS (Hs00177228_m1) and human TBP (TATA-box binding protein) endogenous control on an ABI 7500 Real-Time PCR system (Applied Biosystem).

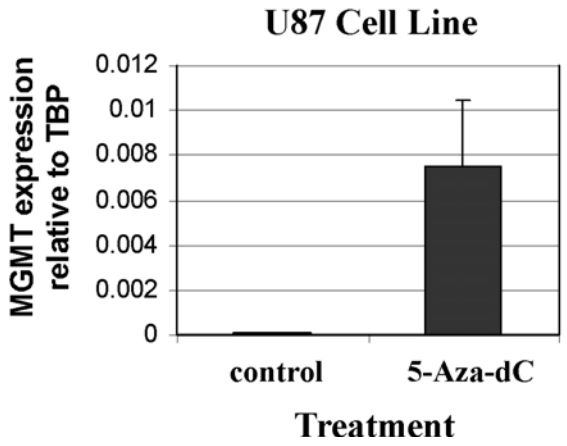
5-Aza-dC Treatment. The ROS-negative glioblastoma cell line U87 were cultured in medium and treated with 5 μmol/L of 5-azadC (Sigma) for 4 days (fresh drug was added every 24 hours). This treatment regimen has been demonstrated to result in demethylation of specific genes elsewhere (Mueller et.al., Downregulation of RUNX3 and TES by hypermethylation in glioblastoma. 2007. Oncogene:26, 583-93.).

Identification of c-ROS Transcription Start Site by Primer Extension. The primer extension experiments were performed using 2 μg of SW1088 and U87 poly(A)$^+$ mRNA that were mixed with 1-2 x10^5 cpm of ^{32}P end-labeled primers hROS-Primex3 or hROS-Primex8 (see Supplemental Table 1) as followed: ^{32}P end-labeled oligonucleotides and RNA were mixed, ethanol-precipitated, and resuspended in 30 μl of 50% formamide, 400 mM NaCl, 10 mM PIPES (pH 6.4), and 1 mM EDTA. Annealing was performed by incubation at 80 °C for 3 min followed by slow cooling to room temperature. The nucleic acids were precipitated with isopropanol and primer extension reactions were carried out in 20 μl of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 1 μg of actinomycin D, 125 μM of each deoxynucleoside triphosphate and 20 units of SuperScript^{TM} at 39 °C for 1 h. The reactions were stopped by the addition of EDTA to a final concentration of 25 mM, and the RNA was degraded with 5 μg of RNase A at 37 °C for 30 min. After phenol/chloroform extraction of the mixture and subsequent ethanol precipitation the primer extended products were analyzed on denaturing polyacrylamide sequencing gels. Size of extended products were measured and calculated using a DNA ladder that comigrated with the samples. The c-ROS transcription initiation site is indicated in Supplemental Figure 1.

Determining Promoter Activity of c-ROS by Luciferase Assays. The 5' region of the c-ROS gene (Figure S1) was assessed for promoter activity by performing luciferase reporter assay as follows: The PAC clone 92C8 (RPCI-1 library) that contains genomic DNA sequences corresponding to the 5' region of the human ROS gene was obtained from www.bacpac.chori.org. The 92C8 PAC was used as a template for amplification of several fragments of c-ROS 5' genomic sequences by PCR using the following primer pairs: hROSpromFowD/hROSpromRevA generated a fragment of 6858 bp (referred to as contruct -7kb relative to the ATG translation initiation codon), hROSpromFowH/hROSpromRevA generated a fragment of 3838 bp (referred to as contruct -4kb) and hROSpromFowH/hROSpromRevA generated a fragment of 1898 bp (referred to as contruct -2kb). The SV40 promoter was amplified from pcDNA3.1/myc-His vector (Invitrogen) by PCR and cloned into pGL3 plasmid. PCR fragments were purified (Qiagen Gel Extraction Kit, Qiagen, Valencia, CA, USA) and cloned in pCR-XL-TOPO (Invitrogen, Carlsbad, CA, USA) vector. After assessment of sequence integrity, the fragments were inserted in the pGL3 basic firefly luciferase vector (Promega, Madison, WI, USA).

Supplementary Figure S1.

Supplementary Figure S2.



Supplemental Figure Legends

Fig. S1. Nucleotide sequence of the 1.8 kb 5' region of the human c-ROS gene (- 2 kb construct Figure 2C). The transcriptional initiation site is indicated with a red arrow. CAAT and TATA box are underlined and italicized. Asterisks denote the 13 CpG dinucleotides constituting the CpG island. Arrows indicate oligonucleotide primers used in RT-PCR and primer extensions experiments.

Fig. S2. Assessment of the efficacy 5-Aza-dC treatment of U87 cells by QRT-PCR measurement of O6-methylguanine methyl transferase (MGMT) expression. U87 cells lack expression of MGMT, which is turned on by demethylation of its promoter sequences. Data values are mean +/- standard deviation, n=4.

Supplementary Table 1.

Table 1.Oligonucleotide sequence of c-ROS primers

Primer	Sequence
hROS1	5’-GCTTGATTTGGGTGCCTCTA-3’
hROS2	5’-GTTTGCTAAAGCATACGTG-3’
hROS3	5’-TTCCCTGCAGAGCTGAAAAGA-3’,
hROS4	5’-CCCACTGAAATGTTCCAGTTT-3’,
hROS5	5’-CCCTTGCTGAAATACAATTCG-3’,
hROS6	5’-CTAACCTTAGTCACTGGG-3’,
hROS7	5’-AATGGCAGATTTTTAGATGG-3’
hROS8/hROS-Primex8	5-‘TGCTTTAGCAAACAGTAGCAC-3’
hROS9	5’-GCTGTCTCGATAGACCTGAT-3’
hROS10	5’-ATGCCACTGCACTGTAGCCT-3’
hROS11	5’-CGCCTGTAATCCTAGCACTTT-3’
hROS20	5’-CTCACCTTGGTTGACCTT-3’
hROS80	5'-GAGTCCAAAGTCTCCAATCTTCAC-3'
hROS-Primex3	5’-GAGCTCTTTTCAGCTCTGCAGGGAATTGGG-3’
hROS-bisFow1a	5’-GTATGTGTTATTTATAGGGAGAATT-3’
hROS-bisRev2a	5’-CATTATACAAACTTCCTTCC-3’
hROSpromFowD	5’-AAAAAACTCGAGGAATTGCTCAGGCACTGTTGAATTGGGCAA-3’
hROSpromFowF	5’-AAAAAACTCGAGACTTCCCTGATACTGAAACCAGATAAAGAC-3’
hROSpromFowH	5’-AAAAAACTCGAGGAATTCTACTGCTGGGTATATGCCTAAAAG-3’
hROSpromRevA	5’-TTTTTTGGATCCGCTTCGGAATAAGACAGTAAATGTTCTTCAT-3’

Supplementary Table 2.

Table 2. Expected PCR fragment size

Primer Pair	Fragment Size (bp)
hROS7/6	262
hROS7/5	319
hROS7/4	358
hROS7/3	406
hROS7/2	445
hROS7/1	491
hROS8/9	256
hROS8/10	417
hROS8/11	634