Abstract
The proto-oncogene tyrosine kinase c-ROS is an orphan receptor whose normal expression pattern is tightly spatiotemporally restricted during development. In glioma, c-ROS mRNA expression is frequently ectopically up-regulated. In this study, we determined by immunohistochemical means that c-ROS receptor protein is present in 25% of low-grade and 30% of malignant glioma tumor samples from tissue microarrays. We then explored the molecular basis for the up-regulation of c-ROS expression in these tumors. We identified and characterized the c-ROS gene promoter region and report that the ectopic expression of c-ROS in tumors is tied to hypomethylation of a CpG island in the c-ROS promoter. Bisulfite sequencing analysis in glioma tumor samples revealed that demethylation of the CpG island (−384 to −132 bp) correlated with c-ROS expression. Moreover, c-ROS expression could be activated by treatment of c-ROS–negative cells with the demethylating agent 5-aza-2’-deoxycytidine. These results establish a strong link between c-ROS promoter demethylation and gain of c-ROS expression and function in glioma. Our data suggest that epigenetic activation of c-ROS represents an important oncogenic mechanism for glioma initiation and progression and suggest that cautionary measures in the clinical use of 5-aza-dC for the treatment of glioma be taken into consideration.

Introduction

C-ROS is ectopically expressed in malignant gliomas (reviewed in ref. 1), one of the most devastating and lethal forms of human cancer. This suggests that over expression of c-ROS may serve as a tumorigenic event for these tumors and represents a potential new anticancer target for therapeutic development. Chromosomal rearrangements that result in c-ROS fusion kinases have been identified in sarcomas, glioblastoma multiforme (GBM), and non–small cell lung cancers (1). These studies established that a constitutively activated c-ROS kinase constitutes a potent oncogenic event, and therefore, understanding the mechanistic details of full-length c-ROS expression in tumors is imperative.

Gliomas are beleaguered with genetic aberrations including changes in the methylation pattern of CpG islands in promoter regions of important cancer genes (reviewed in ref. 2). In general, tumor-specific transcriptional silencing by promoter hypermethylation is often associated with tumor suppressor gene silencing. These observations spearheaded the design of clinical trials for cancer using the DNA methylation inhibitor decitabine (5-aza-dC; refs. 3, 4). Conversely, less is known regarding the role of hypomethylation in the initiation and development of tumors in general and, particularly, in gliomas. For example, a handful of cancer genes have been shown to be transcriptionally reactivated by DNA demethylation (5–15) and treatment of cancer cells with 5-aza-dC was shown to activate tumor-promoting genes and result in increased invasive and metastatic phenotypes (16, 17). These reports indicate that pharmacologic demethylation of important cancer genes lead to increased tumorigenicity and underscore the need for watchful monitoring of patients undergoing 5-aza-dC therapy.

In this study, we showed robust expression of c-ROS receptors in malignant astrocytomas using tissue microarrays. We also determined the architecture and strength of the c-ROS gene promoter and discovered that ectopic c-ROS expression observed in gliomas correlates with promoter hypomethylation. The results from this study show an epigenetic regulation of c-ROS expression in disease and suggest prudence in the use of 5-aza-dC for the treatment of gliomas.

Materials and Methods

Gliomas and cell culture. Human gliomas were kindly provided by Dr. Abhijit Guha (Labatt Brain Tumour Research Centre, Toronto, Canada) and were used in compliance with institutional policies. Cell lines were obtained from American Type Culture Collection. Cells were treated with 5 mmol/L 5-aza-dC (Sigma) for 96 h.

gDNA and RNA isolation. RNA and gDNA were extracted from tumors and cultured cells using the RNA/DNA isolation protocol from Qiagen. Poly(A)mRNA was isolated from cells using Oligotex Direct mRNA kit (Qiagen) according to the manufacturer’s protocol.

Reverse transcription-PCR and primer extension analysis. For reverse transcription (RT)- and QRT-PCR analysis of control and 5-aza-dC–treated cell lines, cDNAs were synthesized from DNase-treated total RNA using a SuperScript III RT-kit (Invitrogen) according to the manufacturer’s protocol. PCR cycling parameters and oligonucleotide sequences are listed in the Supplementary Data section. Primer extension experiments were performed using poly(A)mRNA mixed with 32P-end-labeled c-ROS oligonucleotides indicated in Supplementary Fig. S1.

CpG island identification and bisulfite modification of gDNA. c-ROS gDNA sequence was scanned using a CpG island search engine. For mapping of methylated cytosine residues, gDNA obtained from tumors and...
5-aza-dC–treated cellswas modified by NaHSO3 using the CpGenome-DNA Modification kit (Intergen) according to the manufacturer's protocol. The final products were amplified by PCR using primers designed to amplify the predicted bisulfite-modified sequences (Supplementary Data). After amplification, products were purified, cloned into TA-cloning vector (TOPO-TA; Invitrogen), and individual clones from each sample were sequenced.

**Tissue micro arrays.** Astrocytoma tissue micro arrays [US Biomax and ISU-ABXIS (Accumax)] were processed for immunohistochemistry as follows: after target retrieval, endogenous peroxidase activity was quenched in 0.3% H2O2, washed several time with PBS, preincubated in blocking solution [5% goat serum (Sigma) in PBS/0.3% Triton-X100] and incubated with antibody for c-ROS (ab5512, Abcam) followed by a biotinylated anti-rabbit secondary antibody. All antibodies were diluted in blocking solution. Immunobinding was detected using Vectastain ABC kit (Vector Laboratories) using 3,3′-diaminobenzidine (Vector Laboratories) as a substrate according to the manufacturer's protocol and counterstained with hematoxylin. Quantification of expression was tabulated by two independent individuals and averaged.

**Luciferase assays.** A PAC containing c-ROS gDNA sequence was used to amplify the indicated c-ROS fragments by PCR (Supplementary Data). Fragments were cloned in the pGL3 basic firefly luciferase vector (Promega). Cells were transfected (Fugene 6-Roche) with a 4:1 molar ratio to TK-renilla Luciferase reporter plasmid. Luciferase assays were performed 48 h later using Promega's Dual Glow Luciferase Assay system according to the manufacturer's instructions.

**Results and Discussion**

Previous detection of c-ROS expression in gliomas relied on measurement of mRNA levels (reviewed in ref. 1). To further these observations, we determined the extent of c-ROS receptor expression by immunohistochemistry on tissue microarrays. From a total of 231 astrocytic gliomas, we observed that 23% (11 of 47) of grade II, 17% (13 of 78) of grade III, and 29% (31 of 106) of GBMs expressed c-ROS, whereas the normal brain tissue did not (Fig. 1A and B). These results indicate that ectopic expression of the c-ROS receptor is observed in both low and high-grade gliomas.

To better understand the role of c-ROS in gliomagenesis, we sought to decipher the molecular mechanism(s) responsible for this expression. Using QRT-PCR, we screened 17 malignant astrocytoma cell lines for c-ROS expression (Fig. 1C). Forty-one percent (7 of 17) expressed c-ROS mRNA. Focusing on the cell line with the highest levels of c-ROS expression (SW1088), we aimed to determine the causality of this expression. Given how common gene amplifications in gliomas are, we performed a gene copy number assay on SW1088 gDNA using Taqman probes and did not detect amplification (data not shown). This is in accordance with studies that show no amplification at the c-ROS locus in GBMs (18, 19).
**Figure 2.** Characterization of upstream sequences of the c-ROS gene. A, identification of the transcriptional initiation site of c-ROS in SW1088 cells. RT-PCR reactions using primer pairs that successively extend upstream from the translational initiation codon of c-ROS. Primer sequences and PCR product sizes are listed in the Supplementary Data section. B, primer extension mapping of the transcription initiation site of c-ROS transcript. The indicated 32P-radiolabeled oligonucleotides were hybridized to mRNA from SW1088 cells (lanes 1), mouse liver (lanes 2), or yeast t-RNA (lanes 3) and extended products of 263 and 306 nt as determined by comigration with a sequencing ladder (data not shown). C, sequences upstream of c-ROS transcription start site have promoter activity. Schematic depiction of 5’ sequence deletion fragments fused to the firefly luciferase cDNA along with positive and negative control constructs (SV40 promoter and pGL3 empty vector). D, Luciferase activity of transiently transfected SW1088 cells for the constructs depicted in C. Columns, mean of triplicate; bars, SD.

**Figure 3.** Epigenetic modulation of c-ROS expression in glioblastoma cell lines. A, schematic representation of the position of the CpG island within the promoter region of the c-ROS gene. Circles, CpG dinucleotides. B, cell lines expressing c-ROS have a hypomethylated CpG island. Bisulfite treatment of gDNA isolated from GBM cells expressing (SW1088) and nonexpressing (U87) c-ROS reveal a correlation between methylation of the CpG island and expression. PCR products were cloned into TA-vector plasmids and individual clones were sequenced. ●, methylated; ○, unmethylated CpGs. C, treatment of U87 with 5-aza-dC increases the expression of c-ROS. Graphical representation of QRT-PCR reaction from total RNA isolated from the SW1088, U87, and 5-Aza-dC–treated U87. Expression is relative to TATA-box binding protein. Columns, mean (n = 4); bars, SD.
These results led us to hypothesize that this ectopic expression of c-ROS may be transcriptionally regulated. To address this, we characterized the c-ROS gene promoter region from SW1088 cells. We mapped the c-ROS transcription initiation site using RT-PCR, which indicated that an RNA message is initiated between nucleotides −856 and −694 relative to the translation initiation ATG (Fig. 2A). Using primer extension, we refined the transcription initiation site to 700 nucleotides upstream of the ATG codon (Fig. 2B). Taking the guanine residue as the +1 position, a "TATA"-like sequence and a "CAAT" motif are present at positions −22 and −55, respectively (Supplementary Fig. S1). Thus, the c-ROS promoter contains canonical elements for basic transcriptional activity.

Having established c-ROS transcript initiation architecture, we next determined the transcriptional strength of the upstream sequences by reporter gene assays. We created 5′ deletion constructs of the upstream region and subcloned them into a firefly luciferase reporter vector (Supplementary Data; Fig. 2C) and showed that in SW1088 cells, the −2 kb construct generated reporter activity 4-fold greater than that of control constructs. Interestingly, the −4 and −7 kb constructs did not display transcriptional activity much above background, perhaps suggesting the presence of DNA elements capable of masking the activity of the −2 kb sequences in this assay. From these results, we concluded that the sequences contained within the −2 kb construct and comprising the transcription initiation site have promoter activity in SW1088.

We further investigated the promoter sequence of the c-ROS gene by subjecting the −2 kb sequence to the CpG island search

![Figure 4](image_url)

**Figure 4.** Human glioma tumors expressing c-ROS have a hypomethylated CpG island promoter. A, RT-PCR reactions of RNA from human glioma tumors (T1–T28) were performed as described under Materials and Methods. The tumors are schwannomas (Schw), meningiomas (Men), anaplastic astrocytomas (A.Astr), glioblastomas (GBM), and gliosarcomas (GSM). RT reactions with (+) and without (−) enzyme. B, bisulfite sequencing was performed on gDNA isolated from tumors in A to determine the methylation status of the CpG island of c-ROS. PCR products were cloned into TA-vector plasmids and individual clones were sequenced. Tumors that showed expression of c-ROS by RT-PCR (A) are underlined and italicized. ●, methylated; ○, unmethylated CpGs.
engine and uncovered a 233-bp fragment between nucleotides −132 to −384 bp (relative to the transcription initiation site) containing 13 CpGs (Fig. 3A). This led us to hypothesize that c-ROS expression in gliomas may result from changes in promoter methylation. Therefore, we performed sodium bisulfite treatment of gDNA isolated from c-ROS expressing (SW1088) and non-expressing GBM cells (U87) and analyzed the methylation status of the CpG island. In U87 cells, the CpG island was found to be heavily methylated, whereas in the SW1088 cells, the island was hypomethylated (Fig. 3D) and that methylation status correlated with c-ROS expression (Fig. 3C). We then treated U87 cells with the methylation inhibitor 5-aza-dC and correlated c-ROS expression levels to promoter demethylation. Figure 3B to C show that demethylation of the CpG island through chemical means results in a substantial increase in c-ROS transcript levels. The efficiency of the 5-aza-dC treatment was controlled by measuring the expression of the MGMT gene, which is silenced in U87 cells by methylation (Supplementary Fig. S2). Our data indicate that the methylation status of the c-ROS promoter CpG island is associated with expression and, more importantly, that c-ROS expression can be acquired from a demethylation event.

We investigated this matter by screening 28 patients gliomas for c-ROS expression and concomitantly analyzed the methylation status of its CpG island as above (Fig. 4A). We found that 46% of the gliomas expressed c-ROS (Fig. 4A), and more importantly, this expression is associated with a hypomethylated c-ROS promoter (Fig. 4B). Altogether, our data show that c-ROS expression in gliomas result from demethylation events.

We established here that c-ROS expression in gliomas is dependent on the methylation of its promoter. Furthermore, sequencing of the exons of c-ROS from the expressing tumors failed to uncover mutations (data not shown). It is thus possible that c-ROS ectopic expression reflects an autocrine or paracrine loop–triggered oncogenic stimulus. The ligand for c-ROS remains elusive.

Our observations, its identification is more than ever a priority if we are to advanced our understanding of c-ROS's function during tumorigenesis.

Given the potency of receptor tyrosine kinase in the initiation and maintenance of cancer, clinical use of 5-aza-dC for the treatment of gliomas should be carefully considered. Currently, a handful of clinical trials using 5-aza-dC for cancers other than gliomas are under evaluation (3, 4). The scientific rationale is an effort at reactivating tumor suppressor gene functions that were lost due to epigenetic events. Our results, along with those of others (16, 17), suggest that the benefits of restoring tumor suppressor gene function may be mitigated by the activation of tumor-promoting events. As such, precautionary measures such as combination therapies with kinase inhibitors for example, should be considered.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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