Hypermethylation and Transcriptional Downregulation of the Carboxyl-Terminal Modulator Protein Gene in Glioblastomas

Christiane B. Knobbe, Julia Reifenberger, Britta Blaschke, Guido Reifenberger

The carboxyl-terminal modulator protein (CTMP) has been identified as a negative regulator of protein kinase B/Akt. Aberrant Akt signaling is frequently observed in glioblastomas, the most common and most malignant glial brain tumors. Because loss of CTMP function and/or expression may remove the inhibitory effects on Akt and promote tumorigenesis, we studied 93 primary glioblastomas and nine glioblastoma cell lines for CTMP deletion, mutation, promoter hypermethylation, and mRNA expression. None of the tumors or cell lines had CTMP-homozygous deletions or coding sequence mutations. However, CTMP mRNA expression was lower by at least 50% relative to non-neoplastic brain tissue in 37 (40%) glioblastomas and six (67%) glioma cell lines. Reduced CTMP mRNA levels were closely associated with hypermethylation of the CTMP promoter. Furthermore, treatment of CTMP-hypermethylated A172 glioma cells with the demethylating agent 5-aza-2'-deoxycytidine and the histone deacetylase inhibitor trichostatin A resulted in partial demethylation of the CTMP promoter and increased CTMP mRNA expression. Thus, epigenetic downregulation of CTMP transcription is a common aberration in glioblastomas. [J Natl Cancer Inst 2004;96:483–6]
with CTMP mRNA levels reduced by ≥50% and five tumors with CTMP mRNA levels of ≥60% relative to non-neoplastic brain tissue), the nine cell lines, and non-neoplastic brain tissue samples from four different individuals for methylation analysis by sequencing the CTMP 5′-CpG island after sodium bisulfite treatment of DNA revealed methylation of CpG sites in GB10D (arrows) but not in NB (shown is the reverse sequence from nucleotides 12 to 33). C) Assessment of CTMP methylation by PCR-based analysis of HpaII-digested (+) or undigested (−) DNA. Similar signals for HpaII-digested and undigested DNA indicate complete methylation of the HpaII site within each fragment (CTMP fragments 1, 3, or 5). No signal for HpaII-digested DNA indicates absent methylation, and a weak signal (e.g., fragment 5 in GB21D and GB110D) indicates partial methylation. D) Induction of CTMP mRNA expression in A172 but not T98G cells by 5-aza-2′-deoxycytidine (A172 = 1 μM for 72 hours; T98G = 0.5 μM for 48 hours) and trichostatin A (1 μM for 24 hours) treatment. Before treatment (−), A172 cells expressed very low levels of CTMP mRNA. After treatment (+), A172 cells expressed increased CTMP mRNA levels that are comparable with levels in NB. T98G cells expressed levels of CTMP mRNA comparable with levels in NB, independent of the treatment. bp = size of the respective PCR product in base pairs. E) Analysis of Akt expression and phosphorylation in tissue lysates from selected glioblastomas using western blot analysis with polyclonal antibodies specific for either total Akt or Akt phosphorylated at serine 473 (pAkt). The blots were additionally stained with a monoclonal antibody specific for β-actin (β-Act) to assess protein loading. Case numbers are given at the top of each lane. Signals for pAkt are markedly increased in all glioblastomas compared with signals in NB. kDa = molecular weight in kilodaltons.

Fig. 1. A) Reduced carboxyl-terminal modulator protein (CTMP) mRNA expression in glioblastoma GB10D shown by real-time reverse transcription-polymerase chain reaction (PCR). X-axis, cycle number; y-axis, relative amount of PCR product. Note identical curves for the housekeeping gene ADP-ribosylation factor 1 (ARF1) in GB10D and non-neoplastic brain tissue (NB), indicating equal expression (right panel). By contrast, the CTMP curve of GB10D is located to the right of the CTMP curve of NB, indicating lower CTMP expression in GB10D (left panel). The calculated CTMP mRNA level in GB10D was 20% of the level in NB. B) Sequence of the 5′-CpG island of CTMP after sodium bisulfite treatment of DNA revealed methylation of CpG sites in GB10D (arrows) but not in NB (shown is the reverse sequence from nucleotides 12 to 33). C) Assessment of CTMP methylation by PCR-based analysis of HpaII-digested (+) or undigested (−) DNA. Similar signals for HpaII-digested and undigested DNA indicate complete methylation of the HpaII site within each fragment (CTMP fragments 1, 3, or 5). No signal for HpaII-digested DNA indicates absent methylation, and a weak signal (e.g., fragment 5 in GB21D and GB110D) indicates partial methylation. D) Induction of CTMP mRNA expression in A172 but not T98G cells by 5-aza-2′-deoxycytidine (A172 = 1 μM for 72 hours; T98G = 0.5 μM for 48 hours) and trichostatin A (1 μM for 24 hours) treatment. Before treatment (−), A172 cells expressed very low levels of CTMP mRNA. After treatment (+), A172 cells expressed increased CTMP mRNA levels that are comparable with levels in NB. T98G cells expressed levels of CTMP mRNA comparable with levels in NB, independent of the treatment. bp = size of the respective PCR product in base pairs. E) Analysis of Akt expression and phosphorylation in tissue lysates from selected glioblastomas using western blot analysis with polyclonal antibodies specific for either total Akt or Akt phosphorylated at serine 473 (pAkt). The blots were additionally stained with a monoclonal antibody specific for β-actin (β-Act) to assess protein loading. Case numbers are given at the top of each lane. Signals for pAkt are markedly increased in all glioblastomas compared with signals in NB. kDa = molecular weight in kilodaltons.

To screen all 93 glioblastomas for CTMP hypermethylation, we digested tumor DNA with the methylation-sensitive restriction enzyme HpaII, and used PCR to amplify eight different HpaII site–containing fragments from the CTMP CpG island (Figs. 1, C, and 2, B). PCR conditions were optimized for each fragment by using DNA samples that had been analyzed by sequencing sodium bisulfite–modified DNA. HpaII restriction analysis revealed CTMP hypermethylation (i.e., methylation of at least four of eight HpaII site–containing fragments) in 29 of 37 (78%) glioblastomas with reduced CTMP mRNA levels and in two of 56 (4%) glioblas-
tomases with normal CTMP mRNA levels (Fig. 2, B). The relationship between CTMP hypermethylation and reduced mRNA expression was statistically significant ($P<.001$, two-sided Fisher’s exact test).

We next compared CTMP hypermethylation and/or reduced mRNA expression with previously published data (11) on PTEN and EGFR gene alterations in the 93 glioblastomas (Fig. 2, B). PTEN mutations were present in similar fractions of glioblastomas with and without reduced CTMP mRNA expression (27% versus 30%; $P = .599$, chi-square test) and with and without CTMP hypermethylation (32% versus 27%; $P = .749$, chi-square test). Similarly, five of six cell lines with CTMP hypermethylation (A172, U138MG, U178MG, U251MG, and U373MG) and two of three cell lines without CTMP hypermethylation (T98G and U118MG) carry PTEN mutations (12,13). Thus, we conclude that there is no association between PTEN inactivation and CTMP aberrations in glioblastomas. We also detected no relationship between CTMP hypermethylation and EGFR gene amplification (Fig. 2, B; $P = .505$, chi-square test).

To investigate the Akt activation status in primary glioblastomas, we performed western blot analyses of 15 selected tumors, including samples with CTMP hypermethylation (GB103D), PTEN mutations (GB47D and GB96D), EGFR amplification (GB98D, GB131D, GB139D, and GB140D), and alteration in none (GB60D, GB105D, GB147D, GB181D, and GS11D) or in any two or three of these genes (GB101D, GB191D, and GS3D). Relative to Akt protein levels in non-neoplastic brain tissue, all tumors showed increased levels of serine 473–phosphorylated Akt protein (Fig. 1, E). In agreement with previous studies (4,6), these findings indicate that Akt is activated in the majority of glioblastomas, including those tumors with alterations in expression or function of PTEN, CTMP, and/or EGFR.

We found that CTMP mRNA levels were reduced by 50% or more compared
with non-neoplastic brain tissue in 40% of primary glioblastomas and in 67% of glioma cell lines. Our findings suggest reduced CTMP expression as a novel molecular mechanism involved in the pathogenesis of glioblastomas. Reduced CTMP expression was closely associated with CTMP 5'-CpG island hypermethylation and could be restored by 5-aza-2'-deoxycytidine and trichostatin A treatment. These findings suggest a role for epigenetic DNA modification in the regulation of CTMP promoter activity. We found that CTMP gene promoter hypermethylation and reduced mRNA expression in glioblastomas are not associated with PTEN mutations and EGFR amplification. Whether our findings regarding CTMP expression and regulation are unique to glioblastomas or are also relevant to other tumors remains to be determined.

**REFERENCES**


**NOTES**

Supported by grants SFB 503/B7 and GRK 320 from the Deutsche Forschungsgemeinschaft, grants 10-1639-Re3 and 70-3088-Sal from the Deutsche Krebshilfe, and grant 9772182 from the Medical Faculty of Heinrich-Heine-University, Düsseldorf, Germany (to G. Reifenberger). J. Reifenberger is supported by the Lise Meitner Program of the Ministry of Science and Research of Northrhine-Westphalia.

All PCR primer sequences used in this study are available on request from G. Reifenberger.

Manuscript received August 14, 2003; revised December 24, 2003; accepted January 12, 2004.