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Abstract

Glioblastoma multiforme (GBM) is the most frequent primary brain tumor in adults. Prognosis is poor. In a series of 214 GBM patients we observed an effect of the variant 5,10-methylenetetrahydrofolate reductase (MTHFR) c.677C>T on the overall survival. In patients younger than 60 years of age at diagnosis this effect was strongest (median overall survival in months ± standard error, genotype CC: 13±1; CT: 11±2; TT: 7±3; multivariate COX regression analysis: Wald=8.58; p=0.007). In addition, the MTHFR genotype significantly influenced the overall survival of patients with a postoperative Karnofsky score ≥ 70 (CC: 12±2; CT: 11±1; TT: 10±4; Wald=5.89; p=0.015). These data suggest the MTHFR c.677C>T variant as risk factor for survival in GBM patients.

Introduction

Methionine metabolism plays an essential role in DNA methylation (Figure 1). The enzyme 5-methyltetrahydrofolate-homocysteine S-methyltransferase (MTR) catalyses the remethylation of homocysteine to methionine. The latter is activated by ATP to S-adenosylmethionine (SAM), which provides the methyl groups used for DNA methylation. MTR depends on cobalamin (vitamin B12) and 5-methyltetrahydrofolate as cofactors. Most important transporter protein of cobalamin is transcobalamin 2 (Tc2), and 5-methyltetrahydrofolate is synthesized out of 5,10-methylenetetrahydrofolate by 5,10-methylenetetrahydrofolate reductase (MTHFR). Because 5,10-methylenetetrahydrofolate is also directed to synthesis of purines and thymidine, MTHFR activity regulates whether an one-carbon unit of tetrahydrofolate is utilized for methionine synthesis or for nucleic acid synthesis, and thus for DNA synthesis, stability and repair.[1]

Instability of genomic DNA and impaired DNA methylation are important for the development and progression of many tumors.[2] Therefore, functional genetic variants of methionine metabolism are attractive candidate factors influencing the development and the clinical course of human cancers. Indeed, the MTHFR missense variant c.677C>T (A222V) and the MTR missense variant c.2756A>G (D919G) have been correlated with the incidence of various malignancies.[3] Furthermore, functional synergisms have been suggested for the variants MTR c.2756A>G and Tc2 c.776C>G (P259R).[4] However, only a few studies investigated the role of the genetic variants of methionine metabolism as *prognostic* factor in patients with cancer. In the present study we analyzed the effects of four functional polymorphisms of the methionine metabolism on the clinical course of 214 GBM patients. Although our previous results did not identify the MTHFR c.677C>T variant as a risk factor for the incidence of GBM,[3] the present study suggests an influence of MTHFR c.677C>T on survival.

Material and Methods

Patients

For this study we analyzed 214 GBM patients (41% female, median age at diagnosis 62 years, range 23-80 years, mean age at diagnosis 58 years ± 12 years standard deviation) operated in the Dept. of Neurosurgery of the University Hospital Bonn, Germany, between 1994 and 2003. All histopathological diagnoses were made at the Department of Neuropathology/ German Brain Tumor Reference Center at the University of Bonn following the WHO criteria.[5] A gliosarcoma was diagnosed in 6%, and a giant cell GBM in 3% of cases. The study was approved by the Ethics Committee of the Medical Faculty of the University of Bonn. Surgical treatment consisted of a total resection in 52%, a subtotal/ partial resection in 43%, and a biopsy in 5% of cases. 82% of patients underwent standard fractionated postoperative radiotherapy, and 11% were administered additional adjuvant chemotherapy. Thus, adjuvant therapy after surgery was categorized as *none* (surgery only), *radiotherapy*, and *radiation and chemotherapy*. For 107 patients treatment at tumor relapse could not be ascertained. Hence therapy for recurrent tumors was not included in the statistical analysis. Median overall survival of all patients ± 1 standard deviation was 10±1 months (95% confidence interval: 9-11) with 7% censored.

Genotyping

The genotypes of four missense variants involved in methionine metabolism, MTHFR c.677C>T, MTHFR c.1298A>C, MTR c.2756A>G and Tc2 c.776C>G, were determined by PCR amplification of genomic DNA prepared from peripheral leukocytes with subsequent restriction analysis as published previously.[4]

Statistics

Log Rank test and Kaplan Meier curves were used to analyze the effect of the four variants on overall survival. Due to multiple testing, α was set to 0.0125. The effect of the MTHFR variant c.677C>T on overall survival was further investigated by COX regression with simultaneous analysis of MTHFR genotype, age, gender, postoperative Karnofsky index, extent of resection, and adjuvant therapy as covariables. In additional explorative analyses (univariate Log Rank tests and multivariate COX regression analysis with the co-variables listed above), the effect of the MTHFR c.677C>T variant was analyzed in subgroups of patients defined by adjuvant therapy, age (<60 years vs. ≥60 years, regarding the mean and median age at diagnosis in our population and regarding prior studies[6]; and postoperative Karnofsky index (<70 vs. ≥70).

Results

Log Rank testing demonstrated a significant influence of the MTHFR c.677C>T variant, but not of the other three genetic polymorphisms, on overall survival (median survival ± standard error in months): CC: 10±1; CT: 10±1; TT: 7±3; Log Rank=9.21; p=0.010 (Table 1). Multivariate COX regression including the MTHFR c.677C>T genotype and the clinical data revealed younger age (linear variable; Wald=7.10; p=0.008), higher Karnofsky score after surgery (linear variable; Wald=21.2; p<0.001) and adjuvant therapy (median survival in months ± standard error, none: 2±0.35; radiotherapy: 10±0.36; radiation and chemotherapy: 11±0.82; Wald=7.81; p=0.005) as important prognostic factors. The extent of resection had a statistically significant influence on survival only in the univariate Log Rank test (total resection: 10±0.69; subtotal/partial resection: 8±0.85; biopsy: 2±1.58; Log Rank=10.3; p=0.006), but not in the multivariate COX regression analysis (Wald=0.68; p=0.447). In the multivariate analysis, the effect of the MTHFR c.677C>T genotype on survival was not statistically significant, either (Wald=1.01; p=0.316).

Univariate Log Rank subgroup analysis revealed a highly significant association of the

MTHFR c.677C>T polymorphism with survival for patients younger than 60 years of age at diagnosis (37% of our study sample): CC: 13±1; CT: 11±2; TT: 7±3 (median survival in months ± standard error; Log Rank=9.94; p=0.007; Figure 3). Multivariate COX regression analysis showed that these data were robust, i.e. the effect of the MTHFR c.677C>T variant on survival in this patient subset was as strong as the effects of some of the clinical co-variables investigated simultaneously: MTHFR c.677C>T (Wald=8.58; p=0.003), extent of resection (Wald=2.10; p=0.148), Karnofsky score after surgery (Wald=12.3; p<0.001), adjuvant therapy (Wald=3.32; p=0.086). No significant effect was seen for the MTHFR c.677C>T polymorphism for patients >60 years (Wald=0.27; p=0.601).

Additionally, the MTHFR c.677C>T variant was found to significantly correlate with survival in the multivariate analysis among patients with a postoperative Karnofsky score ≥ 70 regardless of age (c.677CC: 12±2; CT: 11±1; TT: 10±4; Wald=5.89; p=0.015). No such effect was seen for patients with a postoperative Karnofsky score < 70 (c.677CC: 5±1; CT: 4±2; TT: 5±3; Wald=0.01; p=0.951). In none of the subgroups defined by adjuvant therapy the MTHFR c.677C>T polymorphism showed any significant correlation with survival.

Discussion

Overall prognosis for GBM patients is grim with a median survival of only 12-16 months even in selected series. However, certain subsets (young age at diagnosis, high extend of resection, high Karnofsky score after surgery) of patients can expect to survive for more than two years.[7] Small therapeutic advances will more likely translate into a clinically relevant survival benefit in patients with a relatively good prognosis. Hence the identification of prognostic survival factors may help to administer treatment more selectively, i.e. to spare those with an adverse prognosis a toxic therapy, and to allow for aggressive treatment in cases for which a meaningful prolongation of survival can be achieved.

Several *tumor* mutations have been correlated with the clinical course of GBM patients.[8] In addition, a few studies have indicated that *germline* genetic variants may have prognostic relevance as well, e.g. the ERCC1 c.8092C>A polymorphism, the deletion of glutathione S-transferase T1 (GSTT1),[9] and a polymorphism in the 5'-untranslated region of the epidermal growth factor gene (EGF g.61G>A).[10]

The major finding of the present study is an association of the c.677TT genotype of the germline variant MTHFR c.677C>T (A222V) with an adverse prognosis of GBM patients. Effects of the MTHFR c.677C>T polymorphism were in particular seen in patient subgroups with a comparatively good prognosis. In the subgroup of patients under 60 years the 6 months difference in median survival between patients homozygous for the MTHFR c.677CC genotype versus patients with c.677TT genotype suggests that the effect size contributed by the c.677C>T genotype is clinically relevant. Accordingly, the MTHFR c.677TT genotype was also associated with a poorer prognosis within the patient subgroup with a postoperative Karnofsky score ≥ 70, but not among patients with a worse performance status. The association of MTHFR c.677C>T with overall survival did not show any therapy specific effect. Given the limitations of any association study, our findings have to be reproduced in an independent dataset.

The T-allele of MTHFR c.677C>T, in particular when in homozygous state, leads to a clearly reduced enzyme activity.[11] Our findings may be explained by an effect on nucleic acid synthesis and thus on chromosomal stability.[1] In addition, a lack of MTHFR activity can cause a reduction of available SAM in several organs including the CNS, and SAM is the key methyl group donor for DNA methylation (Figure 1).[12] The T-allele of MTHFR c.677C>T has already been reported to be associated with global hypomethylation of genomic DNA.[13] Hypomethylation of the O(6)-methylguanine DNA methyltransferase (MGMT) promoter in GBM tumor cells has been associated with a shorter survival in GBM patients receiving

radiotherapy and treated with temozolomide,[14] and also a correlation between the T-allele of MTHFR c.677C>T variant and a decreased MGMT promoter hypermethylation has already been observed, however, in a series of uterine cervical cancers.[15] Thus, it is tempting to speculate that differential (tumor) DNA methylation due to MTHFR c.677C>T might constitute the molecular basis of the association between the MTHFR c.677C>T polymorphism and survival of GBM patients. Accordingly, Cadieux et al. most recently demonstrated that the MTHFR c.677C>T variant or a deletion encompassing the MTHFR gene locus on chromosome 1p36.3 was associated with global DNA hypomethylation in GBM tissue. Tumors with DNA hypomethylation or a MTHFR gene deletion exhibited an increased proliferation rate.[16] Our study provides the clinical data predicted by these observations: Reduced MTHFR activity due to presence of the T-allele of the c.677C>T variant is associated with a shorter overall survival. As limitation, we did not know the folate intake nor plasma folate levels of the GBM patients, which might have modified the effect of the MTHFR genotype on overall survival, as the effect of the c.677C>T variant on MTHFR activity is modified by the availability of folate.[11,17]

Our findings may have some therapeutic implications. Since the adverse biological effects of the T-allele of the MTHFR c.677C>T variant e.g. on DNA methylation may well become apparent only in the context of a low folate status,[17] folate supplementation or dietary strategies influencing methionine and further metabolites of methionine metabolism might be interesting candidate supportive therapies for GBM patients.

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Table 1: Genotypes and overall survival in months (all patients)

MTHFR c.677C>T	CC: 10±1	CT: 10±1	TT: 7±3	Log Rank=9.21; p=0.010
MTHFR c.1298A>C	AA: 10±1	AC: 9±1	CC: 14±4	Log Rank=0.60; p=0.742
MTR c.2756A>G	AA: 10±1	AG: 9±1	GG: 8±5	Log Rank=0.07; p=0.967
Tc2 c.776C>G	CC: 8±2	CG: 10±1	GG: 9±2	Log Rank=1.52; p=0.469

Kaplan Meier analysis of the genotypes on overall survival with median survival ± standard error in months and results of Log Rank test (two degrees of freedom). Due to multiple testing, threshold was defined with $\alpha=0.0125$. Censored for MTHFR c.677C>T, CC: n=9; CT: n=6; TT: n=0.

Table 2: MTHFR c.677C>T and overall survival in subgroups

	c.677CC	c.677CT	c.677TT	Log Rank	COX (Wald)
all patients	10±1	10±1	7±3	9.21; p=0.010	1.01; p=0.316
patients < 60y	13±1	11±2	7±3	9.94; p=0.007	8.58; p=0.003
patients ≥ 60y	9±8	8±6	6±5	2.56; p=0.278	0.27; p=0.601
KPI ≥ 70	12±2	11±1	10±4	5.23; p=0.051	5.89; p=0.015
KPI < 70	5±1	4±2	5±3	0.42; p=0.896	0.01; p=0.951

The influence of the MTHFR c.677C>T genotype on median overall survival ± standard error in months of all patients and of subgroups. Univariate analysis (Log Rank) and multivariate analysis with age (not included as covariable in the subgroups defined by age), gender, postoperative KPI (not included as covariable in the subgroups defined by KPI), extend of resection and adjuvant therapy as covariables. KPI = Karnofsky performance index/ Karnofsky score.

Figure legends

Figure 1. Methionine metabolism

In the human methionine metabolism, methionine becomes activated to S-adenosylmethionine (SAM) via methionine adenosyltransferase (MAT). SAM is an ubiquitious methyl group donor, e.g., for the synthesis of biogenic amines and DNA methylation. The degradation product of SAM is S-adenosylhomocysteine (SAH), which is hydrolyzed to homocysteine per homocysteine hydrolase (SAHH). Homocysteine can either be transsulfurated to cystathionine and cysteine via vitamin B6-dependent cystathionine beta-synthase (CBS) and cystathionine gamma lyase (CGL), or alternatively, homocysteine can be remethylated to methionine. This is done by 5-methyltetrahydrofolate-homocysteine S-methyltransferase (MTR, also called methionine synthase), which needs a derivate of vitamin B12 (methylcobalamin) as well as a derivate of folate (5-methyltetrahydrofolate; 5-MTHF) as cofactors. Vitamin B12 is transported by transcobalamin 2 (Tc2), and 5-methyltetrahydrofolate is synthesized by 5,10-methylenetetrahydrofolate reductase (MTHFR) from 5,10-methylenetetrahydrofolate (5,10-MTHF), which is synthesized from folate by dihydrofolate reductase (DHFR) in two subsequent steps.

Fig. 1. Human methionine metabolism