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MNS16A minisatellite genotypes in relation to risk of glioma and meningioma and to glioblastoma outcome

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ABSTRACT

The human telomerase reverse transcriptase (hTERT) gene is upregulated in a majority of malignant tumours. A variable tandem repeat, MNS16A, has been reported to be of functional significance for hTERT expression. Published data on the clinical relevance of MNS16A variants in brain tumours have been contradictory. The present population based study in the Nordic countries and the UK evaluated brain tumour risk and survival in relation to MNS16A minisatellite variants in 648 glioma cases, 473 meningioma cases and 1359 age, sex, and geographically matched controls. By PCR based genotyping all study subjects with fragments of 240 or 271 bp were judged as having short (S) alleles and subjects with 299 or 331 bp fragments as having long (L) alleles. Relative risk of glioma or meningioma was estimated with logistic regression adjusting for age, sex and country. Overall survival was analysed using Kaplan-Meier estimates and equality of survival distributions using the log-rank test, and by Cox proportional hazard ratios. The MNS16A genotype was not associated with risk of occurrence of glioma, glioblastoma (GBM) or meningioma. For GBM there were median survivals of 15.3, 11.0, and 10.7 months for the LL, LS, and SS genotypes, respectively; the hazard ratio for having the LS genotype compared with the LL was significantly increased HR 2.22 (1.41-3.49) and having the SS genotype versus the LL was non significantly increased HR 1.48 (0.80-2.77). When comparing the LL versus having one of the potentially functional variants LS and SS the HR was 2.05 (1.35-3.12). However functionality was not supported as there was no trend towards increasing HR with number of S alleles. Collected data from our and previous studies regarding both risk and survival for the MNS16A genotypes are contradictory and warrant further investigations.
INTRODUCTION

Telomere length maintenance is a requirement for infinite proliferative potential of cells and a majority of malignant tumours shows telomerase activity and thereby giving potential for an immortal phenotype. One critical factor determining the activity level of the telomerase complex is the reverse transcriptase component encoded by the \textit{hTERT} gene. Regulation of the \textit{hTERT} gene is complex and involves a large number of positively and negatively interacting factors. Wang et al \cite{1} suggested a novel \textit{hTERT} gene regulating mechanism based on the finding of an antisense transcript regulated by a variable tandem repeat polymorphism, MNS16A. This tandem repeat is located downstream of the \textit{hTERT} gene and was reported to have promoter activity in lung cancer cell lines \cite{1}. The functional significance of this antisense transcript activity in cells has not yet been proven. Wang et al \cite{2} also recently reported that the MNS16A genotype was associated with survival in glioblastoma in a US-based study. A subsequent study in France by Carpentier et al \cite{3}, however, reported no association of MNS16A genotype with survival, although it did report an association with risk of developing glioblastoma. We investigated the clinical impact of the MNS16A genotype in a large multinational series of patients with brain tumours and matched controls, estimating associations for risk and survival.

MATERIAL AND METHODS

\textit{Cases and controls}

Population based case-control studies of brain tumours were conducted in four countries as part of the international Interphone study, coordinated by the International Agency for Research on Cancer \cite{4}. The study population has been described before and blood samples were taken complementary to the study in the UK and the Nordic countries \cite{5}. Briefly, all studies followed the same core protocol. Cases and matched controls were recruited in
Denmark (nationwide), Finland (excluding Northern Lapland and Åland), Sweden (in the Stockholm, Lund, Gothenburg and Umeå areas) and the Thames region of southeast England. Cases aged 20-69 years in the Nordic countries, and 18-59 years in the UK, diagnosed from September 2000 to March 2004 (the exact dates within this period varied by centre) were comprehensively ascertained. Eligible cases were patients with glioma (comprising astrocytoma grade I-IV as well as oligodendroglioma and mixed oligoastrocytoma) or meningioma resident in the study areas without a prior diagnosis of a brain tumour. Histopathological diagnosis was available in 94% of the glioma cases and 89% of the meningioma cases. Controls in the Nordic countries were selected randomly from population registers, frequency matched to cases on sex, age in 5-year groups, and geographic region, whereas controls in the UK were randomly selected from general practitioner’s practice lists, within frequency matching. Potential cases and controls with a previous diagnosis of brain tumour were excluded. Blood samples were collected from consenting cases and controls. Eligible cases were invited by letter or personally to take part in the study. Controls were invited by letter. If no reply was received, a repeat letter was sent, or the potential participant was contacted by telephone. These procedures varied among countries; for example, in Finland, potential participants were contacted by telephone only if they did not respond to the second letter. In total, blood samples were collected from 45% of all interviewed cases and 48% of controls and were available from 728 glioma patients, 549 meningioma patients and 1610 controls (Table 1). In Denmark, Sweden and the UK, it was attempted to obtain blood samples from all subjects, with a success rate of 48% for glioma cases, 45% for meningioma cases and 49% for controls. In Finland, the investigators did not attempt to collect blood from all interviewed participants but, rather, blood samples were collected from three out of five centres, from a predetermined number of subjects (100 glioma, 100 meningioma, 100 controls). During the data collection period, all eligible Finnish participants were asked to
donate blood samples. The required number of samples was obtained by the end of the study period. For all study cases and controls with available DNA, the mean age and sex distribution did not differ from those where no DNA was available.

*Outcome data*

Outcome and treatment data of the patients were available and collected for the Swedish and Danish glioma patients. We restricted analyses of survival to patients with glioblastoma, the most common and aggressive form of glioma, to obtain a clinically homogeneous subgroup. Clinical data were extracted from medical records using a standardised registration form and date of death was identified from the Cause of Death Registry, where all Swedish and Danish inhabitants are registered after death. Median follow-up was 12.6 months (range 1-72 months). Cases were followed from date of diagnosis of glioblastoma to date of death or date of last follow up in the medical record for living patients, or October 31, 2006 whichever occurred first. At the end of follow up, 142 (92%) glioblastoma patients were deceased and 12 were alive. Surgery was categorised as biopsy only, partial resection or gross total resection. Information on chemotherapy and radiotherapy treatment was retrieved from medical records. Medical records were obtained for 96% of the Swedish and Danish glioblastoma cases. A pathological review was performed by two pathologists, the Swedish samples were reviewed by (TB) and the Danish samples were reviewed by (HB). The WHO 2000 classification [6] was used for the review. For samples where there were a discrepancy between the original report and the review pathologists, the slides were reviewed jointly by both HB and TB to obtain a consensus. 17 diagnoses were changed by pathology review; 12 to glioblastoma from another type of glioma and 5 from glioblastoma to anaplastic astrocytoma or oligodendroglioma.
Genotyping of MNS16A

DNA was extracted by standard procedures. 60 ng of DNA was amplified in the presence of 333 nM of each primer (5’-AGGATTCTGTATCTCTGAAGGGTG-3’, 5’-6FamTCTGCCTGAGGAAGGACGTATG-3’), 0.25 mM of each dNTPs, 1X GeneAmp PCR buffer II (Applied Biosystems), 0.6U AmpliTaq Gold (Applied Biosystems) and 2.5 mM MgCl$_2$ in 15 µl reaction volume. PCR was run on the GeneAmp 9700 instrument (Applied Biosystems). Cycling conditions were 95°C for 12 min, 10 cycles of 94 °C for 15 sec, 55 °C for 15 sec, 72 °C for 30 sec and 25 cycles of 89 °C for 15 sec, 55 °C for 15 sec, 72 °C for 30 sec followed by 72 °C for 10 min. The PCR products were diluted 1:10 with water and 0.7 µl of the diluted DNA was added to 10 µl of deionised formamide and 0.13 µl GeneScan-500 LIZ size standard (Applied Biosystems). The samples were denatured 95 °C for 5 min and quickly chilled on ice before analysis on the 3730xl DNA Analyzer (Applied Biosystems). Alleles were called with the GeneMapper version 3.0 software (Applied Biosystems). As a control for consistent genotyping, DNA from two breast carcinoma cell lines (T47D1 and BT20) was included on each analysed 96-well plate as well as a minimum of two negative controls (no DNA). We determined the MNS16A genotypes, as in the report by Wang et al [2], the MNS16A genotypes were determined by classifying the DNA amplicons of 240 or 271 base pairs as short (S) alleles and 299 or 331 base pairs as long (L) alleles. The MNS16A genotypes were defined as SS, LS, or LL.

Statistical analyses

We analysed the association of glioma and meningioma risk in relation to genotype, and the odds ratios were adjusted for age, sex and country by binary logistic regression.
Overall survival was analysed using Kaplan-Meier methods and statistical significance for equality of survival distributions evaluated using the log-rank test. In addition, the Cox proportional model was used to obtain hazard ratios.

RESULTS

Genotyping of MNS16A was successful in 648 (89%) glioma cases, 473 (86%) meningioma cases and 1359 (84%) controls. The frequency distribution of the MNS16A genotypes in glioma cases was: LL, 43.5%; LS, 42.8%; SS, 13.7%, in meningioma cases: LL, 44.8%; LS, 43.8%; SS, 11.4%, and in controls: LL, 47.8%; LS, 41.2%; SS, 11% (Table 2). There were no statistically significant differences in the distribution of the MNS16A genotypes by age, sex, or country. Moreover, there were no statistically significant differences between the different genotypes among glioma, glioblastoma or meningioma cases compared with controls (Table 2).

The median survival time for glioblastoma patients was 15.3 months for the LL genotype, 11.0 months for the LS genotype, and 10.7 months for the SS genotype. The hazard ratio for having the LS genotype compared with the LL was significantly increased HR 2.22 (1.41-3.49) and having the SS genotype versus the LL was non significantly increased HR 1.48 (0.80-2.77). When comparing the LL versus having one of the potentially functional variants LS and SS the HR was 2.05 (1.35-3.12). However functionality was not supported as there was no trend towards increasing HR with number of S alleles. When combining LS and SS, glioblastoma patients with the LL genotype had a significantly better survival than patients with the LS and SS genotypes (15.3 vs. 10.8 months; p=0.001) (Table 3 and 4). The hazard ratio for death was also significantly increased for having the LS or LS/SS genotype compared with the LL genotype, after adjusting in Cox proportional analyses for above or
below median age, sex, gross total resection or biopsy only, radiotherapy and chemotherapy (Table 4). Only including patients receiving radiotherapy did not change the result.

**DISCUSSION**

The present study did not show an association of risk of glioma, glioblastoma or meningioma with MNS16A genotype. Only one study previously reported on brain tumour risk in relation to MNS16A genotype, the French study by Carpentier et al [3], based on 205 glioblastoma cases and 305 controls. An odds ratio of 2.05 (95% CI: 1.22-3.44) was reported for subjects with SS compared with LL. The reason for this discrepancy between the current and the French study are unclear, but could include chance and the choice of control group. The French study had selected controls mainly from blood donors, who can be supposed to be healthier than the general population. If MNS16A is associated with increased risk for other cancer sites, this theoretically could have impact on the genotype distribution. Our control group was population based and matched geographically as well as for sex and age. The only exclusion criterion was a previous diagnosis of primary brain tumour.

We observed that the survival was significantly greater in subjects with the LL genotype compared to those with LS genotype but not compared with the SS genotype. This finding is in contradiction to that from the US study by Wang et al [2], which reported a significantly prolonged survival associated with the SS allele compared with the LS or LL alleles, based on 299 glioblastoma patients [2]. It is also discrepant with the French study by Carpentier et al [3], which did not find any survival differences for glioblastoma or anaplastic glioma patients depending on MNS16A genotypes. Thus, the three studies reported to date are contradictory and the discrepancies found are difficult to reconcile.
One reason for the opposing results could be the patient age distribution. A younger patient population could theoretically be enriched for secondary glioblastoma harbouring p53 mutations with more favourable prognosis which might interfere with the results [7]. In the US study the mean age of glioblastoma patients was 49.9 years, whereas the French study had a median age of 56.3 years. In our study the median age of glioblastoma patients was 55 years. Another difference is that the cases in our and the French study were population based whereas the US study was hospital based, which might have led to selection of the patients included. In the multivariate analyses gross total resection was an independent prognostic factor in the US study but not in ours nor in the French study. The definition of gross total resection is not standardised and only according to the local neurosurgeon’s estimation, which might be highly variable [8]. Chemotherapy and age were independent risk factors for prognosis in both studies [2][3].

Our study and the US study adjusted their results for age, type of surgery, and chemotherapy and both studies found significant, but opposing, results on the relation of MNS16A genotype to glioblastoma survival [2]. In the US study [2], only radiotherapy treated patients were included whereas in our study, 85 percent had received radiotherapy. Our results did not change, however, when including only radiotherapy treated patients (data not shown).

In their initial paper on MNS16A, Wang et al (1) identified the presence of an \( hTERT \) antisense RNA by in situ hybridization and suggested that the variable tandem repeat of MNS16A functioned as a repressor of a promoter with the ability to regulate the expression of this antisense RNA in lung cancer cell lines [1]. The short MNS16A allele was described to have less repressor activity, in theory leading to more antisense \( hTERT \) and less \( hTERT \) RNA expression (and thereby less telomerase activity). Based on these data it was argued that the
MNS16A polymorphism could have a functional relevance in e.g. tumour tissue. However, these are indirect evidence and formal proof that the MNS16A genotype can affect hTERT RNA expression has not been presented. In their subsequent paper on glioblastoma survival [2], Wang et al showed that the SS allele was associated with a better prognosis, which we and the French study [3] have not been able to confirm. The results from the three studies are inconsistent and it is at present not possible to assess the likelihood that MNS16A is a prognostic or risk factor in glioblastoma.

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