The MDM2 gene is amplified and/or overexpressed in about 10% of glioblastomas and constitutes one of a number of ways the p53 pathway is disrupted in these tumours. MDM2 encodes a nuclear phosphoprotein that regulates several cell proteins by binding and/or ubiquitinating them, with p53 being a well-established partner. MDM2 has two promoters, P1 and P2, that give rise to transcripts with distinct 5' untranslated regions. Transcription from P2 is believed to be controlled by p53 and a single-nucleotide polymorphism (SNP309, T > G) in P2 is reported to be associated with increased risk for, and early development of, malignancies. The use of P1 and P2 has not been investigated in gliomas. We used RT–PCR to study P1- and P2-MDM2 transcript expression in astrocytic tumours, xenografts and cell lines with known MDM2, TP53 and p14ARF gene status. Both promoters were used in all genetic backgrounds, including the use of the P2 promoter in TP53 null cells, indicating a p53-independent induction of transcription. Transcripts from the P1 promoter formed a greater proportion of the total MDM2 transcripts in tumours with MDM2 amplification, despite these tumours having two wild-type TP53 alleles. Examination of SNP309 in glioblastoma patients showed a borderline association with survival but no apparent correlation with age at diagnosis nor with TP53 and p14ARF status of their tumours. Our findings also indicate that elevated MDM2 mRNA levels in tumours with MDM2 amplification are preferentially driven by the P1 promoter and that the P2 promoter is not only regulated by p53 but also by other transcription factor(s).

Keywords: MDM2; promoters; TP53; p14ARF; SNP309; gliomas

The evolutionarily conserved MDM2 gene (12q15) encodes a 491 amino-acid nuclear protein, whose activity and cellular localisation is believed to be controlled by post-translational modifications (Meek et al., 1997; Kubbutat et al., 1998). MDM2 interacts with many other proteins including Rb1, E2F1, and the ribosomal proteins L5, L11 and L23, indicating that MDM2 is involved in a complex circuit of interactions, affecting among others the cell cycle and apoptosis (Zhang and Zhang, 2005). The details of the control of these interactions have still to be determined.

MDM2 is amplified and/or overexpressed in a variety of human tumours of diverse tissue origins (Momand et al., 1998). Up to 10% of the most malignant astrocytic tumours, glioblastomas (WHO malignancy grade IV) (Louis et al., 2007) show MDM2 gene amplification with consequent mRNA overexpression. This is generally associated with primary (de novo) glioblastomas that have wild-type TP53 and p14ARF alleles (Reifenberger et al., 1993; Ichimura et al., 2000). Consequently, amplification and/or over-expression of MDM2 is believed to be an alternative mechanism for escaping p53-regulated control (Ichimura et al., 2000).

MDM2 gene transcription is regulated by two promoters, P1 and P2. The P1 promoter is located upstream of exon 1 and is active at basal constitutive levels in most cells (Mendrysa and Perry, 2000). Although motifs of the P1 promoter important for its activity have been defined, its control is still not understood (Chang et al., 2004; Phillips et al., 2006). The second promoter (P2) is located in intron 1, it has two p53-responsive elements (Zauberman et al., 1995) and p53 is believed to initiate MDM2 transcription from this promoter, thus forming an auto-regulatory feedback loop. Other p53-independent mechanisms have also been proposed (Qi et al., 1999; Ries et al., 2000; Phelps et al., 2003, 2005). In addition, a T > G polymorphism, referred to as SNP309 (rs2279744) in intron 1 of the human MDM2 gene, has been suggested to affect P2 activity by increasing the binding affinity of the Sp1 transcription factor (Bond et al., 2004). This is thought to result in higher MDM2 protein levels, which would potentially attenuate the p53 pathway and might facilitate cancer formation (Bond et al., 2004).

Transcripts derived from the P1 promoter (P1-MDM2) do not utilise exon 2 and have exon 1 as their 5' untranslated region (5' UTR), whereas P2-derived transcripts (P2-MDM2) have exon 2 (Barak et al., 1994). Bases 5–7 of exon 3 form the start codon and
are common to transcripts from both promoters. Translation of transcripts from the P1 promoter is eight-fold less efficient than translation of the P2 promoter transcripts (Landers et al, 1997), probably due to the presence of two short upstream open reading frames located in exon 1 (Jin et al, 2003).

The majority of studies have examined total MDM2 mRNA expression in normal and tumour tissues (Bueso-Ramos et al, 1995; Broll et al, 1999; Ko et al, 2000; Miyajima et al, 2001). The individual activity of the P1 and P2 promoters has been investigated in breast and oral cancer and head and neck squamous cell carcinoma (Rahman et al, 2000; Million et al, 2001; Okumura et al, 2002), but not in human gliomas. We have assessed how the gene status of MDM2, TP53 and p14ARF influences promoter usage in astrocytic gliomas (primary tumours, glioblastoma xenografts, glioblastoma cell lines). In addition, the SNP309 status was studied in glioblastoma patients and correlated to a number of genetic (i.e., TP53 and p14ARF) and clinical (i.e., survival and age at diagnosis) parameters.

**MATERIALS AND METHODS**

**Tumour tissue, xenografts and cell lines**

A total of 73 gliomas including 56 glioblastomas (prefixed as GB), 10 anaplastic astrocytomas (prefixed as AA) and 7 astrocytomas (prefixed as A) were used in the study. In addition, xenografts from three glioblastomas and three glioma cell lines were studied. The primary tumours were classified according to WHO classification (Louis et al, 2007). Each tumour piece analysed had a minimum tumour cell content of 75% but generally greater than 90%, as assessed by histology. The tumours have been included in previous studies using the same identification numbers (Reifenberger et al, 1993; Ichimura et al, 2000, 2008; Liu et al, 2005). Xenografts hold the same number as the tumour from which they were derived with the suffix X followed by passage number. Collection and handling of tumour tissues and xenografts were as described (Schmidt et al, 1999; Ichimura et al, 2000). The characteristics of the cell lines Tp365MG and Tp265MG have been reported elsewhere (Collins, 1983). CCF-STTG1 was purchased from ATCC (Middlesex, UK). Supplementary Table 1 lists specimen numbers and their diagnosis, and indicates in which part of the study each specimen was used. Table 1 lists the MDM2, TP53 and p14ARF gene status of the tissues, xenografts and cell lines used. The study was approved by the Ethical Committee of the Karolinska Hospital (No. 91:16) and the Cambridge Local Research Ethics Committee, Cambridge, UK (ref. LREC 03/115).

**TP53 analysis by multiplex PCR and MDM2 SNP309 genotyping**

DNA extraction from patients' peripheral blood and cell lines was as described previously (Ichimura et al, 1996). Multiplex PCR was performed by amplifying exon 2 (PC1046/PC1047) and exon 5 (PC929/PC931) of the TP53 gene together with exon 35 (PC2419/PC2420) of an internal control gene (DEPDC5). The latter is known to be genetically normal in the samples investigated (Segg et al, 2005). Products were carried out on 2% agarose gels and visualised by ethidium bromide staining. For sequences of the primers used, see Supplementary Table 2. The MDM2 SNP309 locus (rs2279744) was genotyped in the peripheral white blood cell DNA of 70 of the 73 astrocytic glioma patients in the series, using previously published primers and standard PCR conditions (Bond et al, 2004). The T to G variation at the 309th nucleotide of intron 1 of the MDM2 gene was sequenced using an ABI PRISM 3100-Avant Genetic Analyser (Applied Biosystems, Warrington, UK) and Accelrys Gene 2.0 (Accelrys, Cambridge, UK) sequencing analysis software.

**RT – PCR of TP53 and P1- and P2-DMDM2 transcripts**

Total RNA was extracted from tumour pieces and cell lines as described (Ichimura et al, 1996). The generation of cDNA and the PCR conditions for all the experiments have been previously reported (Ichimura et al, 1996, 2008; Liu et al, 2005). For sequences of the primers used, see Supplementary Table 2. Primer pairs PC180/50 and PC446/50 were used to amplify exons 4–9 and exons 7–9 of the TP53 cDNA, respectively. To amplify the P1- and P2-DMDM2 transcripts, forward primers PC3176 and PC3600 in the non-coding exons 1 or 2, respectively, were used with a common reverse primer PC3291 (exon 3) or PC3238 (exon 12) of the MDM2 cDNA. Standard cycling parameters (35 cycles) were used for the qualitative RT – PCR analysis on P1- and P2-DMDM2 transcript expression. Products were carried out on 2% agarose gels and visualised by ethidium bromide staining. The real-time analysis of MDM2 mRNA was performed using a LightCycler in an identical manner to that described previously for a similar analysis of other genes (Ichimura et al, 2008), but using primer pairs PC3176/PC3291 and PC4570/PC4573 (see Supplementary Table 2) for the quantitation of P1- and P2-DMDM2 transcript levels, respectively.

**Statistical analysis**

For the statistical analysis, the raw data obtained from the quantitative RT – PCR analysis were transformed into log2 values. To assess the effect of MDM2 gene status (amp or no amp) on P1- and P2-DMDM2 mRNA levels, a Mann – Whitney U test was performed using glioblastomas with MDM2 amplification, wt/wt TP53 and wt/wt p14ARF vs glioblastomas with no MDM2 amplification, wt/wt TP53 and wt/wt p14ARF. A Mann – Whitney U test was also used to compare the P1- and P2-DMDM2 mRNA expression within different tumour grades (GBs vs AAs and As) that have no aberrations on MDM2, TP53 and p14ARF genes. A two-way ANOVA was used to test the effect of TP53 and p14ARF gene status or their combination on P1 and P2 transcript levels. For the latter test, tumours were separated into two categories: (i) those with wt/wt allelic status and (ii) those with at least one defective allele (i.e., wt/mut, wt/–, mut/mut, mut/– and –/–). Survival curves were obtained using the Kaplan – Meier method and statistical differences were analysed using the log-rank test. A Mann – Whitney U test was used to compare the age at diagnosis for glioblastomas in relation to the SNP309 genotype. Differences were accepted as significant for P<0.05. All statistical analyses were performed using Minitab 13.0 and SPSS 15.0 software packages.

**RESULTS**

Transcription from the P2 promoter can be independent of p53 in astrocytic gliomas

To investigate whether expression using the P2 promoter can occur in the absence of p53, we studied P1- and P2-DMDM2 mRNA expression in a TP53-null glioma cell line (Tp265MG). Multiplex PCR and RT – PCR were first used respectively to confirm the TP53 homozygous deletion and lack of endogenous TP53 mRNA in Tp265MG (Figure 1A and B). To identify the presence of P1- and P2-derived MDM2 transcripts in Tp265MG, primer sets that amplify the two distinct 5’ UTRs of MDM2 mRNA were used. Tp265MG was shown to express exon 2-derived transcripts (Figure 1C), and quantitative PCR confirmed the presence of P2 transcripts albeit at a very low level (Table 1).

In a preliminary study, the promoter usage of the MDM2 gene was also investigated in a series of primary astrocytic tumours, comprising of 45 GBs, 5 AAs and 5 As (Supplementary Table 1) as well as in two additional glioblastoma cell lines (CCF-STTG1 and Tp365MG) and 3 glioblastoma xenografts (GB217X4, GB181X13 and GB166X1) with known MDM2, TP53 and p14ARF gene status.

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Table 1  Gene status of MDM2, TP53 and p14^{ARF}, P1- and P2-MDM2 transcript levels of each specimen and SNP309 genotype and age at diagnosis of the corresponding patient.

| Specimen no. | MDM2        | TP53        | p14^{ARF} | P1 | P2 | P1/P2 | SNP309  | Age at diagnosis |
|--------------|-------------|-------------|-----------|----|----|-------|---------|-----------------|
| A22          | No amp      | wt/wt       | wt/wt     | 0.84 | 1.01 | 0.832 | G/G     | NA              |
| A25          | No amp      | wt/wt       | wt/wt     | 0.68 | 2.02 | 0.337 | T/T     | NA              |
| A50          | No amp      | wt/wt       | wt/wt     | 0.40 | 0.63 | 0.635 | G/T     | NA              |
| A7           | No amp      | wt/wt       | wt/wt     | 0.07 | 0.49 | 0.143 | G/T     | NA              |
| A23          | No amp      | wt/wt       | wt/wt     | 0.58 | 0.63 | 0.921 | T/T     | NA              |
| A30          | No amp      | wt/wt       | wt/wt     | 0.30 | 0.24 | 1.250 | T/T     | NA              |
| AA104        | No amp      | wt/wt       | wt/wt     | 1.77 | 1.05 | 1.846 | G/T     | NA              |
| AA34         | No amp      | wt/wt       | wt/wt     | 2.27 | 1.02 | 2.225 | T/T     | NA              |
| AA59         | No amp      | wt/wt       | wt/wt     | 0.07 | 0.27 | 0.259 | T/T     | NA              |
| AA76         | No amp      | wt/wt       | wt/wt     | 0.80 | 3.42 | 0.234 | G/T     | NA              |
| AA107        | No amp      | wt/wt       | wt/wt     | 0.33 | 1.89 | 0.175 | G/T     | NA              |
| AA110        | No amp      | wt/wt       | wt/wt     | 6.44 | 1.07 | 0.458 | T/T     | NA              |
| AA15         | No amp      | wt/wt       | wt/wt     | 0.36 | 0.73 | 0.493 | T/T     | NA              |
| AA50         | No amp      | wt/wt       | wt/wt     | 0.36 | 0.44 | 0.818 | T/T     | NA              |
| AA49         | No amp      | wt/wt       | wt/wt     | 0.11 | 0.230 | 0.478 | G/G     | NA              |
| AA90         | No amp      | wt/wt       | wt/wt     | 25.72 | 2.23 | 11.67 | G/G     | NA              |
| GB180        | Amp         | wt/wt       | wt/wt     | 0.97 | 0.69 | 1.437 | T/T     | NA              |
| GB246        | Amp         | wt/wt       | wt/wt     | 18.28 | 9.4 | 1.945 | G/T     | NA              |
| GB35         | Amp         | wt/wt       | wt/wt     | 41.60 | 9.52 | 4.370 | G/T     | NA              |
| GB37         | Amp         | wt/wt       | wt/wt     | 50.68 | 13.86 | 3.567 | T/T     | NA              |
| GB90         | Amp         | wt/wt       | wt/wt     | 0.42 | 2.04 | 0.206 | G/G     | NA              |
| GB140        | Amp         | wt/wt       | wt/wt     | 9.91 | 5.66 | 1.751 | G/T     | NA              |
| GB223        | Amp         | wt/wt       | wt/wt     | 129.97 | 12.06 | 10.777 | T/T     | NA              |
| GB81         | Amp         | wt/wt       | wt/wt     | 23.76 | 12.95 | 1.835 | T/T     | NA              |
| GB327        | Amp         | wt/wt       | wt/wt     | 0.83 | 2.64 | 0.314 | T/T     | NA              |
| GB30         | No amp      | wt/wt       | wt/wt     | 0.42 | 2.04 | 0.206 | G/G     | NA              |
| GB189        | No amp      | wt/wt       | wt/wt     | 0.77 | 0.69 | 1.116 | T/T     | NA              |
| GB49         | No amp      | wt/wt       | wt/wt     | 0.74 | 1.84 | 0.407 | G/T     | NA              |
| GB247        | No amp      | wt/wt       | wt/wt     | 0.64 | 1.85 | 0.346 | G/T     | NA              |
| GB250        | No amp      | wt/wt       | wt/wt     | 0.83 | 2.64 | 0.314 | T/T     | NA              |
| GB30         | No amp      | wt/wt       | wt/wt     | 2.09 | 1.95 | 1.072 | T/T     | NA              |
| GB34         | No amp      | wt/wt       | wt/wt     | 0.45 | 1.77 | 0.254 | T/T     | NA              |
| GB52         | No amp      | wt/wt       | wt/wt     | 0.14 | 1.65 | 0.865 | T/T     | NA              |
| GB56         | No amp      | wt/wt       | wt/wt     | 1.76 | 3.08 | 0.571 | G/T     | NA              |
| GB57         | No amp      | wt/wt       | wt/wt     | 1.16 | 2.13 | 0.545 | G/T     | NA              |
| GB63         | No amp      | wt/wt       | wt/wt     | 0.75 | 1.83 | 0.410 | G/G     | NA              |
| GB8          | No amp      | wt/wt       | wt/wt     | 0.67 | 1.76 | 0.381 | G/T     | NA              |
| GB84         | No amp      | wt/wt       | wt/wt     | 0.57 | 2.77 | 0.206 | G/T     | NA              |
| GB94         | No amp      | wt/wt       | wt/wt     | 0.91 | 4.78 | 0.190 | G/T     | NA              |
| GB41         | No amp      | wt/wt       | wt/wt     | 0.28 | 0.75 | 0.373 | G/T     | NA              |
| GB9         | No amp      | wt/wt       | wt/wt     | 1.19 | 2.47 | 0.493 | T/T     | NA              |
| GB1         | No amp      | wt/wt       | wt/wt     | 0.31 | 0.71 | 0.437 | T/T     | NA              |
| GB51         | No amp      | wt/wt       | wt/wt     | 0.07 | 0.36 | 0.194 | T/T     | NA              |
| GB46         | No amp      | wt/wt       | wt/wt     | 1.14 | 5.03 | 0.227 | T/T     | NA              |
| GB59         | No amp      | wt/wt       | wt/wt     | 0.10 | 0.48 | 0.208 | G/T     | NA              |
| GB221        | No amp      | wt/wt       | wt/wt     | 0.57 | 1.43 | 0.393 | G/T     | NA              |
| GB27         | No amp      | mut/mut     | wt/wt     | 1.61 | 0.47 | 3.426 | T/T     | NA              |
| GB164        | No amp      | mut/mut     | wt/wt     | 0.29 | 1.96 | 0.148 | G/T     | NA              |
| GB61         | No amp      | mut/mut     | wt/wt     | 3.00 | 1.45 | 2.069 | T/T     | NA              |
| GB103        | No amp      | mut/mut     | wt/wt     | 4.84 | 1.32 | 3.667 | G/T     | NA              |
| GB131        | No amp      | mut/mut     | wt/wt     | 1.37 | 3.63 | 0.377 | T/T     | NA              |
| GB29         | No amp      | mut/mut     | wt/wt     | 0.09 | 0.93 | 0.393 | T/T     | NA              |
| GB132        | No amp      | mut/mut     | wt/wt     | 1.51 | 2.5 | 0.604 | G/T     | NA              |
| GB17         | No amp      | mut/mut     | wt/wt     | 0.86 | 0.61 | 1.410 | G/T     | NA              |
| GB138        | No amp      | mut/mut     | wt/wt     | 0.22 | 0.36 | 0.611 | G/G     | NA              |
| GB22         | No amp      | mut/mut     | wt/wt     | 0.31 | 0.71 | 0.437 | T/T     | NA              |
| GB5          | No amp      | mut/mut     | wt/wt     | 1.07 | 1.29 | 0.829 | T/T     | NA              |
| GB4         | No amp      | mut/mut     | wt/wt     | NA   | NA   | NA    | NA      | NA              |
| GB166        | No amp      | mut/mut     | wt/wt     | 0.31 | 0.5 | 0.620 | T/T     | NA              |

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Table 1 (Continued)

| Specimen no. | MDM2 | TP53 | p14ARF | P1 | P2 | P1/P2 | SNP309 | Age at diagnosis |
|--------------|------|------|--------|----|----|-------|--------|-----------------|
| GB16         | No amp | mut/– | –/–   | 0.14 | 0.12 | 1.167 | G/T    | 73              |
| GB33         | No amp | mut/– | –/–   | 0.52 | 0.41 | 1.268 | G/G    | 66              |
| GB55         | No amp | mut/– | –/–   | 1.42 | 2.01 | 0.706 | T/T    | 74              |
| GB217X4b     | Amp   | w/t/w/t | w/t/wt | 28.8 | 25.8 | 11.162 | NA     | NA              |
| GB181X1b*    | No amp | w/t/– | –/–   | 0.03 | 0.44 | 0.068 | NA     | NA              |
| GB166X1b*    | No amp | mut/– | –/–   | 0.01 | 0.01 | 1.268 | G/G    | NA              |
| CCF-STTG1*   | Amp   | w/t/wt | w/t/– | NA  | NA  | NA    | NA     | NA              |
| Tp365MGc     | No amp | –/–   | –/–   | 0.14 | 0.01 | 41    | G/G    | NA              |
| Tp365MGc     | No amp | –/–   | –/–   | 0.14 | 0.01 | 41    | G/G    | NA              |

A = astrocytoma; AA = anaplastic astrocytoma; GB = glioblastoma; no amp = no amplification; amp = amplification (≥ 5 copies); w/t/wt = two wild-type copies; –/– = homozgyous deletion (see text); w/t = loss of one allele; wt/mut = retention of one wild-type allele and one mutated allele; mut/mut = both alleles with unique mutations; mut/– = loss of one allele and retained alleles mutated; NA = not applicable. aNormalised target/reference (MDM2/18S) ratio. bGlioblastoma xenografts hold the same numbers as the tumour from which they were derived with the suffix X followed by passage number. cGlioblastoma cell lines.

Variation in the usage of the P1 and P2 promoters in astrocytic gliomas

Tumours with MDM2 amplification were grouped and compared to those with no MDM2 amplification. This comparison showed a statistically significant increase in mRNA levels from both the P1 promoter (P = 0.004, Mann–Whitney U test, Figure 2A) and the P2 promoter (P = 0.004, Mann–Whitney U test, Figure 2B) in the MDM2-amplified group. This indicates that both promoters are utilised in cases with amplification of the MDM2 gene. We next compared changes in the P1/P2 ratio in the same samples (Table 1). There was a significant difference in the P1/P2 ratio between tumours with MDM2 amplification as compared to those without amplification (P = 0.01, Mann–Whitney U test, Figure 2C), indicating that the elevated MDM2 mRNA expression seen in the MDM2-amplified astrocytic tumours is not equally driven by both promoters. MDM2 amplification had a higher impact on P1 than P2 expression, as indicated from the comparison of the medians of (a) P1 expression in the amplified cases (median = 22) vs P1 in the non-amplified cases (median = 0.74) and (b) P2 expression in the amplified cases (median = 9.46) vs P2 in the non-amplified cases (median = 1.85).

The effect of TP53 and p14ARF gene status was then investigated to evaluate whether any TP53 mutation (wt/mut, mut/mut or mut/–), p14ARF hemi- and nullizygosity (wt/– or –/–) or a combination of the two have an effect on P1 and/or P2 transcript levels. A two-way ANOVA indicated that any TP53 mutation was significantly associated with lower P2-DM2 mRNA levels (P = 0.014, two-way ANOVA, Figure 2D) and did not affect the activity of the P1 promoter (P = 0.575, two-way ANOVA). This was also observed when changes in the P1/P2 ratio were examined with a correlation seen between TP53 gene status and the P1/P2 ratio (P = 0.039, two-way ANOVA, Figure 2E). Neither the p14ARF gene status alone nor the combination of TP53 and p14ARF aberrations had any significant correlation with P1 or P2 transcript expression, nor with P1/P2 ratio (P > 0.05, two-way ANOVA).

Finally, we tested whether there is a difference in the P1-, P2- or P1/P2 ratio of MDM2 mRNA among high-grade (GBs) vs lower-grade gliomas (AAs and As) with no MDM2 gene amplification and wild-type TP53 and p14ARF genes. No statistical difference was observed (P > 0.05, Mann–Whitney U test), suggesting that tumour grade does not affect MDM2 mRNA expression in a TP53 and p14ARF wild-type background.

The P2 promoter polymorphism (SNP309) does not appear to be significant in astrocytic gliomas

To document the incidence of the SNP309 (rs2279744) polymorphism in astrocytic gliomas, DNA from peripheral white blood cells of 70 primary cases was studied (Supplementary Table 1). In addition to these, a glioblastoma cell line (Tp265MG) and a glioblastoma xenograft (GB181X13) were also investigated.

Table 1 summarises the genotypes obtained. Of the 70 patients genotyped for the SNP309, 34 (48.5%) were T/T, 27 (38.5%) were G/T and 9 (12.8%) were G/G. These frequencies are very similar to those seen in healthy Caucasian volunteers (48% T/T, 40% T/G and 12% G/G) (Bond et al, 2004). Notably, Tp35-null specimens, Tp265MG (see above) and AA90 (confirmed by array-CGH, manuscript in preparation) were homozygous for the variant G allele.

To examine the effect of SNP309 on MDM2 expression, we compared the levels of P2-DM2 mRNA in glioblastomas without MDM2 gene amplification in relation to SNP309 genotype (Figure 2F). The results showed that glioblastomas homozygous for G/G did not have significantly higher levels of P2-DM2 mRNA expression when compared to glioblastomas homozygous for T/T at this locus. Overall, the expression levels between the different SNP309 genotypes (T/T, G/T and G/G) did not appear to be substantially affected by the polymorphism at this locus. As shown by others (Bond et al, 2004; Swinney et al, 2005) the age of cancer formation in patients with SNP309 G/G can vary greatly from those individuals with a T/T genotype. We compared the age distribution at the time of diagnosis for 54 glioblastoma patients who had homozygous T/T genotype with that of patients who had either heterozygous (G/T) or homozygous (G/G) variant
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The usage of the two promoters of the MDM2 gene was analysed here for the first time in astrocytic tumours, glioblastoma xenografts and glioblastoma cell lines with known MDM2, TP53 and p14ARF gene status. By documenting P1- and P2-MDM2-induced transcripts in a glioblastoma cell line (Tp265MG) with no MDM2 gene amplification and homozygously deleted TP53 and p14ARF, it was demonstrated that exon 2-derived transcripts, that is, transcripts initiated from the supposedly p53-responsive promoter (P2), were detectable even in the complete absence of all protein products of the TP53 gene. Similar findings were made in an anaplastic astrocytoma case (AA90) with no MDM2 gene amplification, homozygously deleted TP53 and two wild-type copies of p14ARF (Table 1). In addition, GB103 retains only one allele of TP53 with the R175H mutation (Backlund et al, 2003) and the tumour also expresses MDM2 transcripts from the P2 promoter. The R175H mutation would affect all known splice variants of TP53 (Bourdon et al, 2005) and has been shown to be transactivation defective (Soussi and Wiman, 2007).

P1- and P2-induced transcripts were expressed from other astrocytic gliomas of the series, which also lacked wild-type TP53. There were seven glioblastoma cases (GB164, GB61, GB131, GB29, GB181X13) that also lacked wild-type TP53.

**DISCUSSION**

The usage of the two promoters of the MDM2 gene was analysed here for the first time in astrocytic tumours, glioblastoma xenografts and glioblastoma cell lines with known MDM2, TP53 and p14ARF gene status. By documenting P1- and P2-MDM2-induced transcripts in a glioblastoma cell line (Tp265MG) with no MDM2 gene amplification and homozygously deleted TP53 and p14ARF, it was demonstrated that exon 2-derived transcripts, that is, transcripts initiated from the supposedly p53-responsive promoter (P2), were detectable even in the complete absence of all protein products of the TP53 gene. Similar findings were made in an anaplastic astrocytoma case (AA90) with no MDM2 gene amplification, homozygously deleted TP53 and two wild-type copies of p14ARF (Table 1). In addition, GB103 retains only one allele of TP53 with the R175H mutation (Backlund et al, 2003) and the tumour also expresses MDM2 transcripts from the P2 promoter. The R175H mutation would affect all known splice variants of TP53 (Bourdon et al, 2005) and has been shown to be transactivation defective (Soussi and Wiman, 2007).

P1- and P2-induced transcripts were expressed from other astrocytic gliomas of the series, which also lacked wild-type TP53. There were seven glioblastoma cases (GB164, GB61, GB131, GB29, GB181X13) that also lacked wild-type TP53.

**Figure 1** Agarose gels electrophoresis of PCR products confirming the homozygous deletion of TP53 in Tp265MG and showing that expression from the P2 promoter is independent of TP53 status. (A) Multiplex PCR using genomic DNA as template and independent pairs of primers (Supplementary Table 2) for exons 2 and 5 of TP53, and an unrelated retained locus (DEPDC5), confirming the homozygous deletion of TP53 in Tp265MG. (B) RT–PCR analysis of TP53 transcripts encompassing exons 4–9 and exons 7–9 of the TP53 cDNA showing that no TP53 mRNA was detectable in Tp265MG. cDNA from the GB181X13 xenograft (TP53wt/wt) was used as a control template. (C) RT–PCR analysis of MDM2 transcripts using a forward primer located in either exon 1 (PC3176; 5′ P1 promoter) or exon 2 (PC3600; 5′ P2 promoter) with a common reverse primer (PC3238; sequence equivalent to 3′ UTR) and cDNA as template to amplify P1- and P2-MDM2 transcripts in Tp265MG. cDNA from GB181X13 (TP53mut/mut, expected to express MDM2 mRNA from both promoters) was used as a control. Note that MDM2 transcripts are expressed from both P1 and P2 promoters in Tp265MG glioma cell line and in GB181X13.
GB166, GB16 and GB33) that showed loss of one TP53 allele and mutation of the DNA-binding domain in the retained allele (Ichimura et al, 2000; Backlund et al, 2003). Such mutations affect all the proteins encoded by TP53 transcript variants (Bourdon et al, 2005). While there is no information about the impact on function of the specific mutations affecting each case, these seven tumours can only generate mutant p53 tetramers, which according to the model proposed by Chan et al (2004) will be inactive.

Finally, GB132 retained only one mutated TP53 copy (R342X), affecting the sequence coding for the p53 oligomerisation domain, and this would prevent the formation of full-length p53 tetramers and the oligomerisation of the six other isoforms known to utilise this region of code (i.e., p53β, p53γ, Δ40p53β, Δ40p53γ, Δ133p53β and Δ133p53γ), but would not impact on two p53 isoforms (i.e., Δ40p53 and Δ133p53) (Ichimura et al, 2000; Backlund et al, 2003; Bourdon et al, 2005).

While it can be argued that the P2-derived MDM2 transcripts came from normal cells present in the tumour tissue analysed, both the Tp265MG cell line and the GB166X1 xenograft contain no normal human cells, yet both expressed the P2 transcripts (primers used for analysis of the xenografts were human-sequence specific) and this would argue for at least some of the P2 transcripts coming from the tumour cells themselves. Overall, our findings provide evidence that (i) wild-type p53 is not necessary to induce MDM2 transcription through the p53-responsive promoter (P2) and that (ii) other transcriptional factor(s) can regulate MDM2 mRNA expression through P2 in astrocytic gliomas that lack endogenous p53 protein expression.

**Figure 2** Box plot diagrams showing the distribution of the MDM2 mRNA levels (i.e., P1, P2 or P1/P2) obtained for glioblastomas in relation to their MDM2 gene status (amplification or no amplification), TP53 gene status (wild-type or mutation) and SNP309 genotype (T/T, G/T or G/G). The upper and lower limits of the boxes and the line across the boxes indicate the 75th and 25th percentiles and the median, respectively. The upper and lower horizontal bars indicate the 95th and 5th percentiles, respectively. Outliers are illustrated as circles. P-values are indicated at the top of the plots and were determined using the Mann–Whitney U test or the two-way ANOVA (see ‘Results’ section for details). (A and B) Log2 P1- and Log2 P2-MDM2 mRNA expression in glioblastomas with and without MDM2 gene amplification. (C) P1/P2 MDM2 mRNA ratio in glioblastomas with and without MDM2 gene amplification. (D) Log2 P2-MDM2 mRNA expression in glioblastomas with wild-type or mutated TP53. (E) P1/P2 MDM2 mRNA ratio in glioblastomas with wild-type or mutated TP53. (F) P2-MDM2 mRNA expression levels in glioblastomas with no MDM2 amplification in relation to their T/T, G/T or G/G genotypes for the SNP309 locus. Expression levels were measured as described in the ‘Materials and Methods’ section. There was no statistically significant difference in the expression levels between the T/T, G/T or G/G genotypes.
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Studies have shown that different p73 isoforms can transactivate the P2-MDM2 promoter in p53-null cells (Zhu et al, 1998; Alarcon-Vargas et al, 2000; Wang et al, 2001), although this is debated (Yu et al, 1999). The TP73 gene is located at 1p36, a region reported to show deletions in astrocytic gliomas (Barbashina et al, 2005). However, this series of cases has been studied in detail as to their copy-number status at 1p36 and this has shown that the majority of the tumours have two copies of TP73 (Ichimura et al, 2008). The 13 cases that had only one copy of TP73 (A50, A23, A30, AA34, AA49, GB96, GB250, GB3, GB52, GB56, GB63, GB84 and GB41) retained two wild-type copies of TP73 (Ichimura et al, 2008). Thus, the p53-independent expression of P2-MDM2 could be explained by p73 in astrocytic gliomas. Less is known about the TP63 gene, but the p63α isoform has also been shown to weakly activate the MDM2 promoter in H1299, a p53-null non-small cell lung carcinoma cell line (Dohn et al, 2001).

Other functional, p53-independent elements have been reported in the P2 promoter. These include two thyroid hormone response elements (Qi et al, 1999) and an AP1-ETS motif together with a non-conserved upstream repeat sequence (nmGGGGC) in H1299 (Phelps et al, 2003). At present, there are no data to indicate the relevance of p53-independent cis-acting elements for P2-MDM2 promoter activity in gliomas with no wild-type TP53.

In an attempt to provide further insights into the contribution of P1 and P2 promoters on the regulation of MDM2 mRNA expression in astrocytic gliomas, quantitative RT–PCR analysis was used. Amplification of the MDM2 gene in glioblastomas with wild-type TP53 and p14ARF alleles was clearly reflected at the transcriptional level. Although expression from both promoters

Figure 3 SNP309 in relation to glioma tumourigenesis. (A) Survival curves of patients with glioblastoma, comparing those with T/T to those with G/G or G/T for MDM2 SNP309. A borderline association in survival based on the presence or absence of the G allele was observed. (B) MDM2 SNP309 genotype in glioblastomas and TP53 status (i.e., wild-type or mutation) in relation to survival. (i) Survival curves of glioblastoma patients with the common SNP309 genotype (T/T) by TP53 gene status. (ii) Survival curves of glioblastoma patients with the variant SNP309 genotype (G/T or G/G) by TP53 gene status. (C) MDM2 SNP309 genotype of glioblastomas and p14ARF gene status (i.e., wild-type or hemi/nullizygosity) in relation to survival. (i) Survival curves of glioblastoma patients with the common SNP309 genotype (T/T) by p14ARF gene status. (ii) Survival curves of glioblastoma patients with the variant SNP309 genotype (G/T or G/G) by p14ARF gene status. The crosses indicate censored patients. Note that no significant difference was observed in survival in conjunction with TP53 or p14ARF gene status.
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was increased in cases with MDM2 amplification, the amplification event had a higher impact on the P1 promoter. Assuming that the MDM2/p53 autoregulatory negative feedback loop is functional at the protein level in astrocytic tumours with amplified copies of the MDM2 gene, one would expect the P1 promoter to give rise to high MDM2 mRNA and protein levels in these neoplasms, as all MDM2 gene coded proteins with a p53-binding domain would inhibit the transcriptional activation of the P2 promoter by p53, reducing MDM2 mRNA expression from this promoter. This would result in higher MDM2 mRNA levels from the P1 promoter, as was observed.

Importantly, there also appeared to be a clear correlation between P2-MDM2 transcript levels and TP53 gene status (Figure 2D). The statistical analyses of these data are in agreement with two independent studies in oral squamous cell carcinomas and in cell lines established from head and neck cancers or sarcomas, where P2-MDM2 transcript levels correlated with TP53 gene status (Ralhan et al, 2000; Millon et al, 2001). In contrast, very little correlation was observed between P1/P2 values and p53 mutational status in human breast cancer specimens (Okumura et al, 2002).

Overall, it would seem that complete loss of wild-type p53 is not sufficient to inhibit the P2 promoter in astrocytic gliomas. Thus, P2 transcripts cannot be used to predict the presence of transcriptionally active p53 in astrocytic gliomas as has been suggested in other tumours by some authors (Bull et al, 1998; Millon et al, 2001).

In an attempt to further understand control of the P2 promoter in these tumours, we examined the SNP309 (rs2279744). This polymorphism in the P2 promoter has been found to affect the binding of the Sp1 transcription factor to the promoter sequence and the G allele has been associated with increased binding of Sp1 and expression of MDM2 transcripts and protein. The G allele has also been associated with accelerated tumour formation in both hereditary and sporadic cancers in humans (Bond et al, 2004).

Sequencing of the SNP309 region of the P2 promoter in the peripheral blood white cell DNA of 70 astrocytic glioma patients showed that 48% of the patients were homozygous for T/T, 38% heterozygous for T/G and 12% homozygous for G/G. These frequencies were similar to the small cohort of healthy Caucasian volunteers studied by Bond et al (2004). Interestingly, both Tp265 MG and AA90 with homozygous deletion of TP53 were homozygous for the G allele. The T to G substitution, which increases the binding affinity of the Sp1 transcriptional activator, might facilitate MDM2 mRNA expression from the P2 promoter in these samples. However, the P2-MDM2 expression observed in astrocytic gliomas with no wild-type TP53 cannot be solely explained by the involvement of the Sp1 trans-acting factor, as 5 of 9 (55.5%) of these cases were found to be homozygous for the T allele (Table 1).

The G/G and G/T SNP309 in patients with glioblastoma showed a borderline association with poorer survival but no correlation with age at diagnosis or with the TP53 and p14ARF status of their tumours. Our data are in agreement with three independent studies, where SNP309 was shown not to have a significant involvement in glioma tumourigenesis (El Hallani et al, 2007; Tsuiki et al, 2007; Idbaih et al, 2008). Null results have also been reported in other types of cancer in relation to this polymorphism (Campbell et al, 2006; Petenkaya et al, 2006; Fine et al, 2006; Talseth et al, 2007).

Finally, to examine the effect of the SNP309 polymorphism on MDM2 expression, the MDM2 mRNA transcriptional levels obtained by quantitative RT–PCR analysis were compared with SNP309 genetic status. In contrast to previous reports (Bond et al, 2004; Hong et al, 2005; Sanchez-Carbayo et al, 2007), the data did not show that G/G patients have a significantly higher P2-MDM2 mRNA expression levels as compared with the levels seen in astrocytic glioma patients with the T/T genotype. Overall, larger, prospective studies are needed to verify whether there is a clear involvement of this or other MDM2 polymorphism(s) in glioma tumourigenesis.

In summary, we report that both the P1 and P2 promoters are used in all genetic backgrounds, including the use of the P2 promoter in TP53-null cells, indicating a p53-independent induction of transcription from P2. In glioblastomas with amplification of the MDM2 gene, transcripts from the P1 promoter dominate despite all such cases having two wild-type TP53 alleles. We also found no clear correlation between the SNP309 (rs2279744) locus in MDM2 and age of presentation or survival in glioblastoma patients.

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