

## SHORT REPORT

# Comparison between mitochondrial DNA sequences in low grade astrocytomas and corresponding blood samples

E Kirches, G Krause, S Weis, C Mawrin, K Dietzmann

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**Background/Aims:** To identify somatic mutations in the mitochondrial DNA of glioblastomas, in a previous study the displacement loops of 17 glioblastomas and corresponding blood samples were sequenced and instabilities in repeats or transitions were detected in seven tumours. This study was extended by sequencing 10 DNA samples of diffuse astrocytomas (World Health Organisation grade II) and corresponding blood samples.

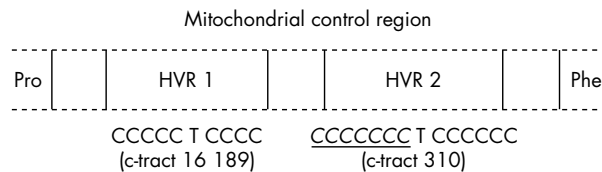
**Methods:** The 10 DNA samples of diffuse astrocytomas and corresponding blood samples were amplified and sequenced using fluorescent nucleotides.

**Results:** No sequence differences were detected, with the exception of a quantitative shift between two genotypes heteroplasmic within the hypervariable region 2, which can be interpreted as mitotic drift. In the glioblastoma series, any particular somatic mutation was usually found in only one tumour. The only frequent alteration was coupled to a mitochondrial germline polymorphism under-represented in the low grade astrocytoma group. Moreover, a single mutation in two patients with secondary glioblastomas had already been detected in diffuse astrocytomas of these individuals.

**Conclusions:** A lower percentage of mitochondrial DNA mutations in low grade tumours cannot be deduced from these data.

During the past years, an increasing number of reports have described structural rearrangements or point mutations in the mitochondrial DNA (mtDNA) of various cancers.<sup>1–5</sup> Recently, we detected a high frequency of sequence differences in the mitochondrial displacement loop (D loop; fig 1) in patients with glioblastoma (n = 17) between the tumour and the corresponding blood sample by direct sequencing.<sup>6</sup> The observed alterations consisted of instabilities in two polycytosine tracts in the hypervariable regions HVR1 and HVR2—a CA repeat instability and several base transitions. Transitions occurred at high frequency in one of the glioblastomas: five sites in HVR1 and four sites in HVR2. In addition, we detected a low frequency of instability in the HVR2 polycytosine tract with a polymerase chain reaction (PCR) approach in a large series of glioblastomas by comparing the tumour with the adjacent tumour free brain tissue. In two of these patients, who underwent malignant progression, material from a low grade tumour had been available (World Health Organisation (WHO) grade II), which showed the aberrant genotypes.

“Recently, we detected a high frequency of sequence differences in the mitochondrial displacement loop in patients with glioblastoma between the tumour and the corresponding blood sample by direct sequencing”



**Figure 1** Diagram showing the mitochondrial control region between the proline (Pro) and phenylalanine (Phe) tRNAs encoded by the mitochondrial genome. The numbers refer to the positions of the thymine residues within the two incomplete c-tracts according to the originally published sequence.<sup>7</sup> HVR, hypervariable region.

The transitions found by direct sequencing of the glioblastomas were homoplasmic and the new genotypes in the homopolymeric regions at least occurred at high levels of heteroplasmy. Therefore, the new genotypes must have been strongly accumulated in the tumour tissue. This is in accordance with the fact that many homoplasmic somatic mtDNA mutations are found in other tumours and also agrees with the observations made by Polyak and colleagues<sup>1</sup> in cell cultures. It was suggested that some somatic alterations might be detectable in low grade tumours, perhaps with a lower amount of mutated DNA. Therefore, in our present study we compared the complete D loop of 10 WHO grade II astrocytomas with corresponding blood samples by direct sequencing.

## MATERIALS AND METHODS

### Materials

DNA from 10 diffuse astrocytomas (WHO grade II) and corresponding blood was prepared by standard phenol/chloroform extraction<sup>8</sup> and was used for direct sequencing of the D loop.

### Sequencing reactions

A 1.3 kb mtDNA region including the complete D loop was amplified with the primers 5'-ATACACCAGTCTGTAAACC-3' (forward) and 5'-AGAAAGGCTAGGACCAAACC-3' (reverse), separated on 0.7% agarose gels, and the D loop was sequenced using fluorescent nucleotides (dyanamic ET cycle sequencing premix kit; Amersham, Piscataway, New Jersey, USA) in overlapping fragments less than 400 bp on a 373A sequencer (Applied Biosystems, Foster City, California, USA).

## RESULTS

In none of the 10 low grade astrocytomas were transitions, transversions, or other point mutations outside the two homopolymeric regions detected in the complete D loop. In

**Abbreviations:** D loop, displacement loop; HVR, hypervariable region; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; WHO, World Health Organisation

addition, no deletions or insertions were seen. A frequent mitochondrial germline polymorphism, the transition T16189C, was present in the blood (and tumour) of four individuals included in our previous glioblastoma study. It was coupled to the occurrence of new length variants in the corresponding HVR1 polycytosine tract in the tumour in all four cases.<sup>6</sup> In the low grade astrocytoma group, this polymorphism occurred in only one individual, but was not associated with a detectable polycytosine tract instability in the tumour in this case.

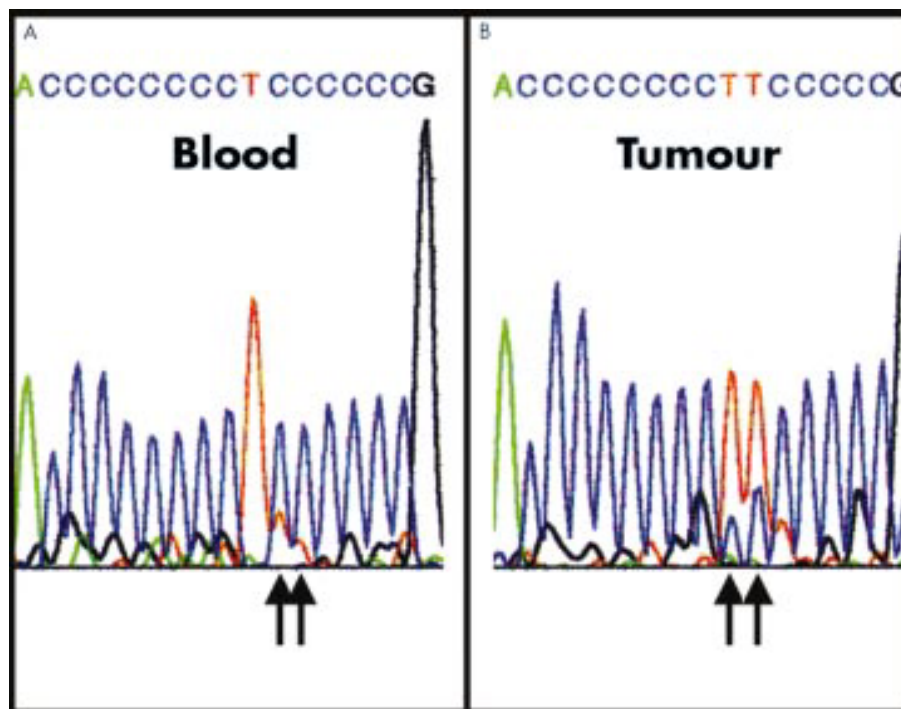
The only sequence difference between tumour tissue and blood detected in the low grade astrocytomas was a quantitative shift between two heteroplasmic genotypes in the incomplete HVR2 polycytosine tract. In the first half of this tract (underlined in fig 1), one or more cytosine residues may be inserted or deleted between nucleotide positions 303 and 309.<sup>7</sup> This position is a known polymorphic site and genotypes with seven or eight cytosine residues in the first half of this tract are frequently found in blood samples. In one patient, heteroplasmy occurred in the blood. The most abundant mtDNA contained eight cytosine residues, but low amounts of additional mtDNAs with nine and 10 cytosine residues were detected. This can be deduced from the small additional thymine peaks (arrows in fig 2; blood sequence), which represent the thymine residue 310 of the longer molecules shifted by one or two bases compared with the most abundant type in the sequencing reaction. In the corresponding tumour tissue, the proportion of molecules with nine cytosines was increased compared with the molecules with eight cytosines, as seen from the occurrence of a cytosine peak beneath the first thymine, from the relative increase of the second thymine peak, and from the decrease of the corresponding cytosine peak (arrows in fig 2; tumour sequence). This change cannot result from a newly generated mutation in the tumour, but can be explained by a genetic drift among mtDNAs, altering the proportions of existing heteroplasmic genotypes in this individual.

## DISCUSSION

Probably merely by chance, only one individual in the low grade astrocytoma group carried the T16189C germline polymorphism in blood compared with four individuals in the glioblastoma group. The elimination of the thymine generates a longer HVR1 polycytosine tract (fig 1), which is generally believed to exhibit higher instability. If a coupling between germline polymorphism and instability in the tumour tissue is suggested, only individuals with the T16189C transition can be taken into account ( $n = 5$ ) and the difference of polycytosine tract instability between both tumour groups is not important.

In addition, all other sequence differences between glioblastomas and corresponding blood samples found in our previous study<sup>6</sup> occurred in only one of the 17 individuals. Among the individuals of this glioblastoma series without the T16189C germline transition ( $n = 13$ ), only three carried somatic mutations in the tumour compared with none of nine in the low grade astrocytoma group. Therefore, the frequency of cases with other mutations was too low to deduce a difference between the tumour grades. In our previous study,<sup>6</sup> using a PCR approach, we found an aberrant genotype in at least two paraffin wax embedded astrocytomas of WHO grade II, which was absent in the adjacent brain tissue. The combination of both studies confirms the occurrence of mtDNA mutations in astrocytic tumours, but there is no convincing evidence to indicate differences between grades. Whether somatic mutations in low grade tumours often remain at low levels of heteroplasmy undetectable by direct sequencing remains unanswered. A positive selection of certain heteroplasmic mtDNAs that has no predictable influence on oxidative phosphorylation has been shown in the mouse<sup>9</sup> and might also occur in tumours.<sup>1</sup>

The quantitative shift between two heteroplasmic genotypes differing in the HVR2 polycytosine tract (fig 2) illustrates a general problem, inherent to all studies screening for somatic mtDNA mutations in tumours. The observed



**Figure 2** Sequence difference of the hypervariable region 2 c-tract between blood and tumour in a patient with an astrocytoma of WHO grade II, which can be misinterpreted as a somatic C to T transition in nucleotide 311 of the tumour mitochondrial DNA (mtDNA). However, the presence of thymine and cytosine peaks in the labelled positions (arrows) indicates heteroplasmy of various length variants of the c-tract. This heteroplasmy is known to occur in the first half of the incomplete c-tract (underlined in fig 1). The difference between blood and tumour might rely upon mitotic drift of heteroplasmic mtDNA genotypes and does not necessarily represent a mutation in the neoplastic cells.

alteration cannot be interpreted as a mutation in the tumour, because low amounts of the aberrant mtDNAs were present in blood. However, it cannot be ruled out that a low amount of maternally inherited heteroplasmy exists in an individual, which is beyond the detection limit of the screening method used. Recently, we clearly demonstrated the loss of one of two heteroplasmic brain mtDNAs in the corresponding tumour in a single patient with glioblastoma.<sup>10</sup> If a loss of certain genotypes in tumours of heteroplasmic individuals can occur—for example, by mitotic drift—there is no reason to assume that the same mechanism cannot lead to an accumulation of minor mtDNAs in brain to detectable amounts in the tumour.

“To test the hypothesis of whether other mitochondrial DNA mutations might occur in low grade astrocytomas, a higher number of cases must be examined”

In contrast to studies of other tumour entities, it is usually not possible to obtain high quality DNA from fresh surgical samples of adjacent tumour free tissue in patients with glioma. Therefore, the complete tumour D loop sequences could not be compared with adjacent brain tissue. This comparison is only possible in cases that allow the microdissection of completely tumour free areas from paraffin wax slices. In these cases, the detection of low amounts of mutant mtDNA is strongly hampered by the higher background in the sequencing reactions. In our study, blood samples were taken as a reference, although this includes a higher risk of undetected heteroplasmy in the brain, which might be misinterpreted to be somatic mutations in the tumour. Because no new sequence variants were seen in the 10 diffuse astrocytomas, this problem of a hidden heteroplasmy in the brain did not affect the results. In addition, new sequence variants of the HVR2 polycytosine tract in comparison with adjacent brain had been seen in a small fraction of glioblastomas.<sup>6</sup> They were also detected in two diffuse astrocytomas,<sup>6</sup> which later progressed into secondary glioblastomas, and there was no evidence that these patients had a different clinicopathological profile to the 10 negative cases in our study. To test the hypothesis of whether other mtDNA mutations might occur in low grade astrocytomas, a higher number of cases must be examined. Although it is difficult to prove that an aberrant sequence variant really occurred for the first time in the neoplastic cells, minor heteroplasmic genotypes can be accumulated in tumours to reach homoplasmy. It is debateable whether mitotic drift alone is a sufficient explanation or whether selective pressure is required.<sup>1</sup>

Probably most of the non-coding sequence alterations described in the literature—for example, in the D loop—might be randomly accumulated in the tumour tissue and play no role in tumorigenesis. Random mitotic drift might lead to such accumulation, irrespective of the origin of the sequence variant (somatic mutation or brain heteroplasmy). Although tRNA and missense mutations have a higher potential to be functionally relevant in tumorigenesis, even some mutations with no obvious functional impact on the respiratory chain can be non-randomly selected in normal tissues<sup>9</sup> and in tumours.<sup>1</sup> They must transfer some functional advantage to the cell, either directly and independently from the nuclear background,<sup>9</sup> or indirectly by improving the cooperation

### Take home messages

- No sequence differences were detected, except for a quantitative shift between two genotypes heteroplasmic within the hypervariable region 2, which can be interpreted as mitotic drift
- These data do not provide evidence for a lower percentage of mitochondrial DNA mutations in low grade tumours

between the nuclear and mitochondrially encoded components. The screening of 43% of the coding region in a glioblastoma with 11 mutations in the D loop revealed an additional eight mutations in the coding region, two of which were missense mutations.<sup>6</sup> This shows that in some cases a high number of mutations may be scattered over the entire mitochondrial genome. However, the pathogenetic relevance of most tRNA or missense mutations cannot be deduced from the DNA sequence and functional studies of the role of mtDNA in tumorigenesis are required in the future.

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