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# Evidence for association of mitochondrial DNA sequence amplification and nuclear localization in human low-grade gliomas

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**Abstract**

Gliomas are tumors which have been found to exhibit consistent genetic changes. Recent studies have shown mitochondrial DNA is also altered in these tumors, and include large deletions and gene amplification. Other studies of the mitochondrial genome in cancer have revealed a variety of different alterations, including the localization and insertion of mitochondrial DNA into the nucleus and nuclear genome in HeLa cells and diethylnitrosurea-induced hepatoma cells. Whether these changes are ontogenically early in the multistep pathway to the development of malignancy, or if this phenomenon occurs in human glial tumors is unknown. I sought to study these questions in a panel of unselected primary glial tumors of pathologically low grade. Fifteen tumors were assessed with a mitochondrial cDNA probe with homology to positions 1679–1948, and 2017–2057. All low-grade tumors revealed increases in copy number when compared to a normal brain control. Nuclear suspensions of these tumors were evaluated by fluorescent in situ hybridization (FISH), using the entire mitochondrial genome as a probe after labeling with rhodamine. All tumors showed evidence of mitochondrial sequence localization within the nuclei. A corresponding glioblastoma and two nonnal brain specimens were also evaluated which did not have amplification of the mitochondrial genome; FISH with the mitochondrial probe revealed minimal hybridization signal within the nuclei of these samples. Mitochondrial DNA nuclear localization can be found in primary low-grade brain neoplasms, and is correlated to increases in mitochondrial DNA.

*Keywords:* Mitochondria; Glioma; Gene amplification

## 1. Introduction

Gliomas comprise 60% of primary central nervous system tumors, and can be of low or high pathologic grade of malignancy. With the most malignant tumors, even with maximal multimodality therapy including surgery, radiation therapy, and chemotherapy, most patients will not survive 1 year (Grant et al., 1995). These neoplasms are thought to arise from sequential genetic events of nervous system precursors, resulting in cells which are unregulated with respect to growth and control (James et al., 1988). The variable recurrent aspects of this multistep progression include losses of genomic ma-

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terial as well as gene amplification (Venter and Thomas, 1991; Bello et al., 1994; Liang et al., 1994; Rubio et al., 1994).

Mitochondrial involvement in neoplasia has been hypothesized for many years (Warburg et al., 1926), although specific interest in the mitochondrial genome in cancer has been relatively recent. The mitochondrial genome consists of 16,569 base pairs arranged in a circular way, and is the only extranuclear source of genomic material in mammalian cells (Lewin, 1994). Alterations which have been noted in neoplastic disease include deletions, increased gene expression, abnormal physical structure, and gene amplifications (Baggetto, 1993). Recent work in gliomas has shown the mitochondrial genome is also altered, with increases in copy number of specific positions as well as recurrent deletions found, in both pathologically high- and low-grade tumors (Liang, submitted). Previous studies have revealed evidence for mitochondrial DNA sequence localization into the nucleus of HeLa cells (Shay and Werbin, 1992), as well as in rat hepatoma cells (Corral et al., 1989). These studies suggest mitochondrial sequences may act as transposable elements and thus may have importance in changing the nuclear genome (Shay and Werbin, 1992). Because these chimeric sequences are expressed, this has been proposed to be a possible pathogenetic mechanism in carcinogenesis (Gellissen and Michaelis, 1987; Shay and Werbin, 1992). It has been hypothesized that transfer of mitochondrial sequences from the mitochondria to the nucleus with subsequent integration may require a high copy number of the mitochondrial DNA (Gellissen and Michaelis, 1987). I evaluated the possibility of mitochondrial DNA localization into the nucleus of glioma cells by fluorescent in situ hybridization, in a panel of tumors which had mitochondrial DNA amplification. Further, I studied pathologically low-grade tumors since I wished to establish if this occurred as a ontologically early event.

## 2. Materials and methods

### *2.1. Tumors and brain samples*

Unselected tumor tissue was obtained at craniotomy which was not required for clinical analysis and would have otherwise been discarded. Tissues analyzed included 5 pilocytic astrocytomas, 3 gangliogliomas, 5 astrocytomas, and 2 neurocytomas, and were verified by a neuropathologist. In two tumors (P2, A3) normal brain tissue was available due to a temporal lobectomy that had been performed; these specimens were confirmed to be normal brain by a neuropathologist.

### *2.2. DNA extraction and hybridization analysis*

DNA preparation and analysis was performed as previously described (Liang et al., 1994; Liang et al., 1996). Frozen sections were prepared and evaluated microscopically for neoplastic change; adjacent non-neoplastic tissue was trimmed and not utilized for this study. Resultant tissue was then used for DNA extraction. Southern hybridization probes used included an extracted cDNA clone which has high homology to mitochondrial sequence (Genbank accession MDL U25123 with homology to mitochondrial sequence positions 1679–1948, and 2617–2057) and a control β-actin probe (purchased from Oncor, Gaithersburg MD). The mitochondrial sequence did not hybridize to any human chromosome when assessed by a somatic cell hybrid mapping panel of individual human chromosomes (Cornell Medical Institute, Cambden, NJ), although there was hybridization to whole cellular DNA. Gene amplification was defined as $\geq 5$ copies as determined by densitometric measurements when compared to a normal brain control.

### *2.3. Mitochondrial sequence detection*

Mitochondrial DNA probes for assessment of localization of sequences in the cell nuclei were obtained by PCR using primers which included virtually the entire mitochondrial genome (Tallini et al., 1994). PCR conditions were as described, with the exception of the replacement of 200 mM dTTP with 100 mM dTTP and 100 mM digoxigenin–12-dUTP (Boehringer Mannheim, Indianapolis, IN) as a fluorochrome marker. PCR reactions underwent Centricon-30 concentration (Beverly, MA), and the complete labeled mitochondrial genome probe was ethanol precipitated, and resuspended in sterile water.

### 2.4. FISH

Nuclear suspensions were prepared as per the manufacturer's protocol (Oncor, Gaithersburg, MD). For FISH, slides were denatured at 70°C in 70% formamide/2 × standard saline citrate (SSC) for 3 min, subsequently dehydrated through an alcohol series, and air dried. The slide was placed on a 42°C slide warmer for at least 1 min before hybridization was performed. Five hundred ng of the labeled mitochondrial DNA probe was combined with 10 mg of Cot-1 DNA, and subsequently lyophilized to near dryness. Ten μl of hybridization solution (50% formamide, 10% dextran sulfate, 1 × SSC) was added, and mixed briefly. This solution was placed in a 70°C water bath for 5 min, and subsequently into a 37°C water bath for at least 30 min. The probe was then placed on the slide, and a coverslip sealed in place with rubber cement. The slide was incubated in a moist chamber at 37°C overnight. Slides were then washed at 45°C twice in 50% formamide/2 × SSC for 15 min each, once in 2 × SSC, and once in 0.1 M sodium phosphate/0.1% NP-40 (PN) for 10 min each. Slides were then washed at room temperature in PN for 2 min, then incubated in a bath containing 5 mg/ml rhodamine conjugated anti-digoxigenin (BMB, Indianapolis IN) in PN, with 5% non-fat dry milk and 0.02% sodium azide for 30 min. The slides were washed twice in PN for 2 min each, and 15 ml antifade solution (10 mg/ml *p*-phenylamine dihydrochloride) containing 0.025 mg/ml 4,6-diamino-2-phenyl-indole (DAPI) was applied. Slides were viewed with a Leitz DM fluorescence microscope equipped with filters to allow viewing of rhodamine and DAPI signals.

## 3. Results

### 3.1. Amplification of mitochondrial DNA sequences in primary gliomas

To illustrate the presence of amplification of mitochondrial DNA in the glioma specimens, genomic DNA was digested with *Eco*RI, transferred to nylon membranes, and hybridized with a cDNA clone homologous to base positions 1679–1948 and 2017–2057. This cDNA has previously been reported to be amplified in a large percentage of human gliomas (Liang, submitted). Fig. 1 illustrates the results of the hybridization. The cDNA clone hybridizes to an 8-kb

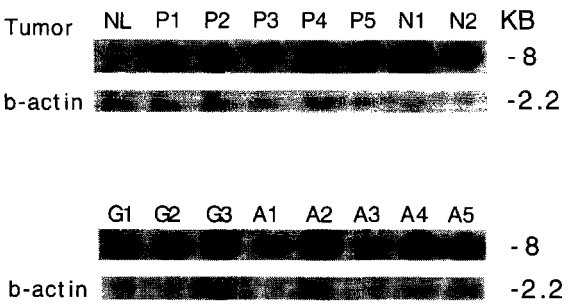


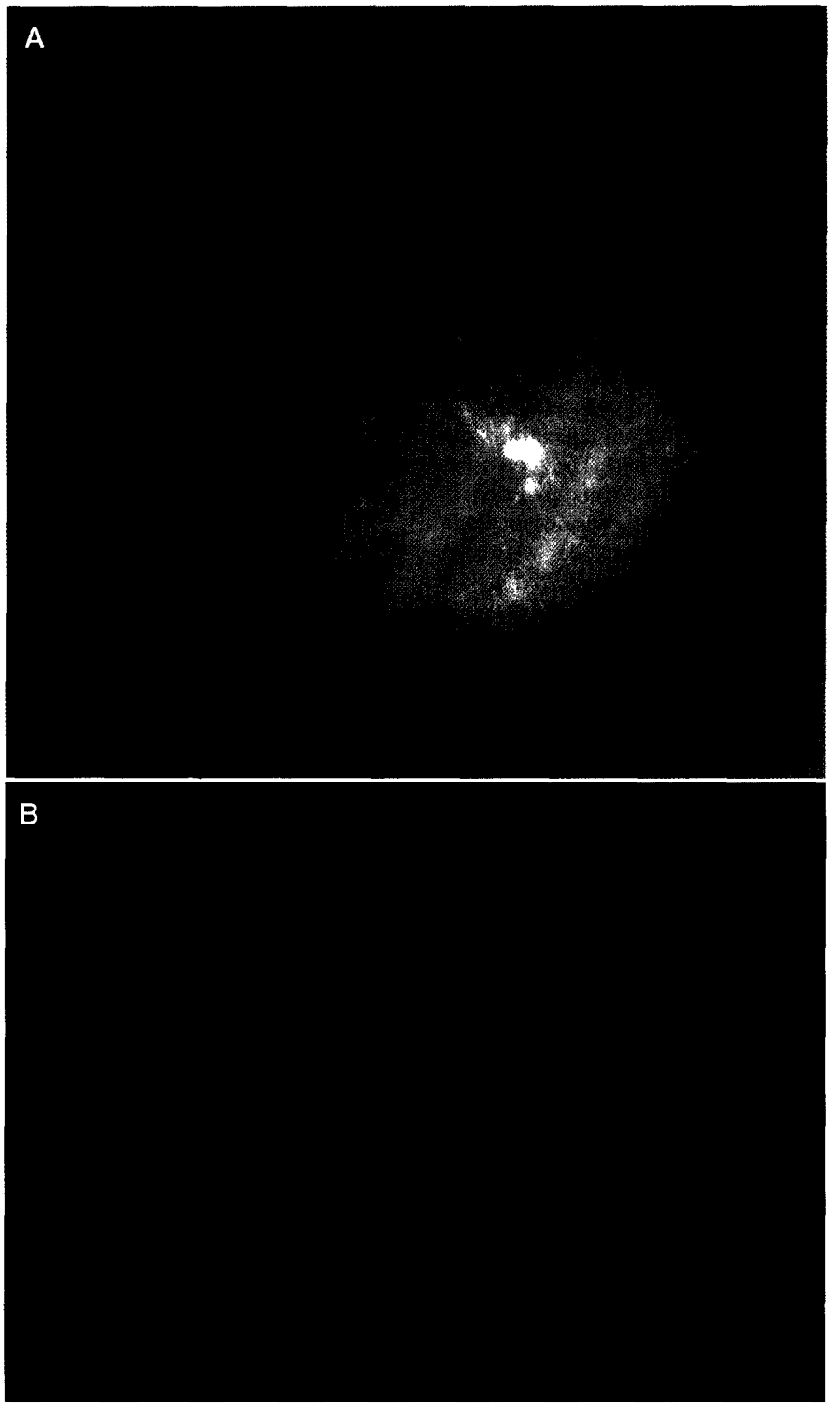

Fig. 1. Southern hybridization of cDNA clone with homology to mitochondrial positions 1679–1948, 2017–2057 (Genbank Accession Number MDL U25123). Increased hybridization is noted in all tumors when compared to a normal brain control (NL) and normalized to β-actin hybridization. P, pilocytic astrocytoma; G, ganglioglioma; A, astrocytoma; N, neurocytoma.

Table 1
Number of cells revealing mitochondrial DNA signal in pathologically low grade gliomas

| Tumor | Cells with mitochondrial DNA signal/total cells evaluated |
|---|---|
| P1 | 40/56 (71%) |
| P2 | 33/40 (83%) |
| P3 | 29/39 (74%) |
| P4 | 38/40 (95%) |
| P5 | 30/40 (75%) |
| G1 | 44/51 (86%) |
| G2 | 37/48 (77%) |
| G3 | 29/42 (69%) |
| A1 | 39/50 (78%) |
| A2 | 35/50 (70%) |
| A3 | 35/44 (80%) |
| A4 | 39/49 (80%) |
| A5 | 33/42 (79%) |
| N1 | 28/40 (70%) |
| N2 | 44/50 (88%) |
| GBM [a] | 2/51 (4%) |
| nl-P2 [a] | 3/50 (6%) |
| nl-A3 [a] | 4/32 (8%) |

P, pilocytic astrocytoma; G, ganglioglioma; A, astrocytoma; N, neurocytoma; GBM, glioblastoma; nl-P2, normal brain specimen of matched tumor P2; nl-A3, normal brain specimen of matched tumor A3.

[a] These samples did not reveal amplification of the mitochondrial cDNA probe.


A
B

*Eco*RI fragment of mitochondrial DNA. After normalization to a single copy control, increases in copy number were noted in every tumor, when compared to normal brain. The level of increased copy number detected was between 12- and 16-fold, with a median value of 13.

### 3.2. Insertion of mitochondrial DNA into amplified cell nuclei

Utilizing FISH of the entire mitochondrial genomic DNA, I attempted to identify if these sequences could be detected in the nucleus of the tumor cells. Table 1 summarizes the number of signals seen per cell in each tumor sample. Of the 15 samples evaluated, at least 69% of the of nuclei revealed mitochondrial sequence hybridization (69–95%; median 77%). In total, 533/681 (78%) nuclei showed a hybridization signal from the labeled mitochondrial DNA probe. In contrast, nuclear suspensions prepared from a glioblastoma (GBM) which did not reveal mitochondrial DNA amplification showed hybridization signal in only 2/51 (4%) of nuclei examined. Fig. 2A shows an example of the FISH of mitochondrial DNA to tumor P2 (a pilocytic astrocytoma). Prominent hybridization of the mitochondrial sequence is noted as manifest by the rhodamine (red/orange) signal within the nucleus of the cell. Fig. 2B shows the results of the hybridization of the labeled mitochondrial genomic probe to the nuclei of the glioblastoma which did not show mitochondrial sequence amplification; no hybridization signal is noted within the nucleus of this cell. Two other normal brain specimens were also evaluated, which were matched samples of tumors P2 and A3. Fig. 3 shows the Southern hybridization of the mitochondrial cDNA probe to the GBM and normal tissues; note increased hybridization of P2 and A3 compared to the matched normal brain controls; also note the lack of increased hybridization of sample GBM. When FISH of the entire mitochondrial genome was performed on nuclear suspensions of these normal brain specimens, < 8% of nuclei revealed hybridization signals.

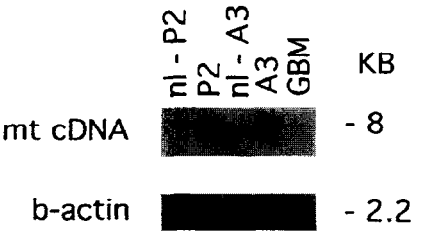



Fig. 3. Southern blot of matched normal brain control of tumors P2, A3, as well as the glioblastoma specimen, using the mitochondrial cDNA as probe. Note increases in hybridization of the tumor specimens P2 and A3, compared to the corresponding normal brain specimens and glioblastoma sample. Below is shown the β-actin loading control. nl-P2, normal brain matched DNA specimen; nl-A3, normal brain matched DNA specimen to tumor A3; GBM, glioblastoma.

## 4. Discussion

A major goal of this study was to determine if mitochondrial DNA sequences could be localized to glioma nuclei which revealed amplification of mitochondrial DNA sequences. All 15 brain neoplasms harbored amplification of mitochondrial sequences, and showed evidence of mitochondrial sequence localization to the nuclei of the tumor cells. If no amplification was present (e.g., the glioblastoma and matched normal brain specimens) only minimal mitochondrial sequence nuclear localization was noted. This provides evidence in glial tumors that mitochondrial DNA can be found in the nuclei, and is *associated* with amplification of the mitochondrial genome (although it is not known where the amplified mitochondrial sequences are specifically located). The other major goal was to determine if the localization of mitochondrial sequences could be detected in the nuclei of lower grade primary brain

Fig. 2. Results of fluorescent in situ hybridization (FISH) of the rhodamine-labeled mitochondrial genome probe to tumor nuclei. A: FISH to a nucleus of tumor P2 (pilocytic astrocytoma) which harbors amplification of mitochondrial sequence. Note rhodamine (red/orange) signal, indicating the presence of mitochondrial sequence located within the nucleus of the tumor cell. B: FISH to a glioblastoma nuclear sample which did not have mitochondrial sequence amplification. Only the DAPI counterstain on the nucleus is seen; no evidence of mitochondrial probe localization is noted.

tumors. Indeed, all the low-grade neoplasms revealed evidence of nuclear localization of mitochondrial sequence. Thus, these changes are found ontologically early in the multi-step progression to malignancy.

Localization of mitochondrial DNA sequences has been associated with insertion of these sequences within the nuclear genome (Shay and Werbin, 1992). In carcinogen-induced rat hepatoma models, higher copy number and abnormal organization has been shown of mitochondrial genes which encode the COI, COII, COIII and ND6 sequences, and are inserted into the rat nuclear genome (Corral et al., 1989). Furthermore, these changes could be temporally correlated with the development of tumors induced by exposure to diethylnitrosurea. HeLa cells have been noted to contain various parts of the mitochondrial genome inserted into the nucleus, including continuous but non-sequential positions 684–971, 6553–7302, and 10,606–11,159 (Shay and Werbin, 1992). This same study also found an insertion of mitochondrial DNA sequence into the *c-myc* oncogene, at exons 2 and 3, with the resultant hybrid transcribed. It is possible that a more detailed assessment of a larger amount of the genomic DNA surrounding genomic insertions may have revealed an open reading frame which was interrupted. Although it is unclear at present if these changes are functionally significant, our findings of amplification of the mitochondrial genome and localization of the genome to the nucleus in glioma cells adds impetus to, and suggests that, these phenomena may be a common event in carcinogenesis, and may play a role for inappropriate gene expression.

The finding of the mitochondrial DNA localization to within the nucleus of pathologically lower grade brain tumors is in agreement with studies performed in cirrhotic liver tissue, considered a premalignant lesion, surrounding hepatic tumors (Yamamoto et al., 1992), where both damaged and deleted mitochondrial DNA was found. The authors suggest abnormalities of mitochondrial DNA may reflect or indicate a precancerous lesion, and thus be an early event in carcinogenesis. Since gliomas are thought to arise from a multistep progression of events to malignancy, a similar phenomenon may be occurring in these tumors. It is interesting to note that the glioblastoma specimen, a high-grade glial tumor, did not have either amplification or mitochondrial sequence nuclear localization. This may be the result of differing pathways to malignancy, as described recently by Van Meyel et al. (1994). Further studies in both de novo and recurrent high-grade tumors will be interesting to test this hypothesis.

The mechanism of gene transfer and insertion of mitochondrial DNA into the nuclear genome is unknown. Shay et al. (1991) have proposed a comprehensive model for transfer of mitochondrial DNA to the nucleus. This involves incomplete degradation of mitochondria, resulting in the release of mitochondrial nucleic acids into the cytoplasm; this material is then free to travel to the nucleus. It has been proposed that the insertion of mitochondrial DNA is an ongoing process, which has occurred throughout evolution (Fukuda et al., 1985; Kamimura et al., 1989). Arguments have been made which suggest this is a stochastic process, (Gellissen and Michaelis, 1987; Shay and Werbin, 1992); because the human genome contains mostly non-coding sequence, in the normal situation, these insertions will not result in pathologic disease states. However, when insertions enter coding sequences, genomic alterations could result, as have been seen with other mobile element insertions (Van Arsdel et al., 1981; Wallace et al., 1991). The amplification of the mitochondrial genome could act as a larger source of mobile elements in the cytoplasm, thus driving additional amounts of the mitochondrial genome to the nucleus and increasing the probability of pathologically important genomic alterations. This should be a testable hypothesis since mitochondrial DNA can be depleted by exposure of cells to ethidium bromide (Desjardins et al., 1986).

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*Note added in proof:* After acceptance of the manuscript, two interesting articles have come to our attention in light of these results:

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