Summary. Aims: Heat shock protein 27 (Hsp27) is induced by cell stress conditions. In the presence of oxidative stress it functions as an antioxidant. To study the putative expression patterns and clinical significance of Hsp27, we assessed the associations between Hsp27, \textit{IDH1} R132H mutation of Isocitrate dehydrogenase1 (\textit{IDH1}-R132H), Hypoxia-inducible factor subunit alpha (HIF-1 alpha), Carbonic anhydrase IX (CA IX), and patient prognosis in astrocytic gliomas.

Methods: Tissue micro-array samples of 295 grade II-IV astrocytomas were stained immunohistochemically for Hsp27, \textit{IDH1}-R132H, HIF-1 alpha, and CA IX. We tested their relationship with clinicopathological features and patient survival.

Results: There was a significant correlation between Hsp27 expression and increasing WHO grade (p<0.001). Hsp27 expression correlated significantly with \textit{IDH1} mutation when studied within the entire cohort (p<0.001) as well as separately in WHO grade II and III tumors (p=0.006 and 0.002, respectively). \textit{IDH1} mutation and HIF-1 alpha positive staining were detected simultaneously (p<0.001). In \textit{IDH1} mutated tumors, positive HIF-1 alpha staining correlated with CA IX expression (p=0.027), whereas no such correlation was found in \textit{IDH1} non-mutated tumors. \textit{IDH1} mutation was associated with a low cell proliferation index (p=0.001) and HIF-1 alpha with increasing proliferation (p=0.003). Hsp27 expression was associated with a shorter rate of patient survival in univariate survival analysis (p=0.001). In multivariate survival analysis, patient age, \textit{IDH1} mutation and HIF-1 alpha appeared as independent prognostic factors (p<0.000, <0.000 and 0.011 respectively).

Conclusions: Hsp27 expression is associated with increasing WHO grade and patient prognosis in astrocytic gliomas. The results suggest that \textit{IDH1} mutation may have an effect on the expression pathways of Hsp27 and CA IX.

Key words: Hsp27, Oxidative stress, Astrocytoma, \textit{IDH1}, Patient survival

Introduction

Diffusely infiltrating astrocytomas represent central nervous system (CNS) tumors which originate from astrocytic glial cells, or their precursors. They represent an important tumor entity accounting for 60% of all primary brain tumors (Louis et al., 2007). They can be assorted into grades II – IV according to WHO criteria. The most malignant type, glioblastoma (GBM), is the most prevalent form in adult patients. The devastating nature of GBM is highlighted by the 5-year survival rate of 10% using the latest therapeutic methods. Even the lower grade astrocytomas may relapse and proceed towards a more malignant grade. Primary GBMs most commonly occur as a new onset of disease, whereas secondary GBMs develop from lower grade astrocytomas. Being highly aggressive and necrotic tumors, astrocytic gliomas represent an ideal model for studying hypoxia and oxidative stress.
Heat shock protein 27 (Hsp27, also known as HSPB1) has a cytoprotective role within a normal cell. Acting as a chaperone in protein folding, it takes part in the proteasomal degradation of cytotoxic proteins (Parcellier et al., 2003). Hsp27 is also known to be able to prevent apoptosis in cells in unfavourable conditions that would normally undergo apoptosis (Con cannon et al., 2001). Although beneficial in normal tissues, the cytoprotective Hsp27 activity in cancer cells is usually disadvantageous, since it may facilitate a cancer cell's ability to function and survive (Calderwood et al., 2006). Recently, synthesis of Hsp27 has been shown to be induced by cell stress conditions, including hypoxia (Marotta et al., 2011) and oxidative stress (Yu et al., 2008). In the presence of oxidative stress, Hsp27 functions as an antioxidant. It lowers the levels of reactive oxygen species (ROS) by upholding intracellular glutathione in its reduced form and by lowering the levels of intracellular iron (Arrigo et al., 2005).

Isocitrate dehydrogenase 1 (IDH1) is an NADP+ dependent enzyme that catalyses oxidative decarboxylation of isocitrate to produce alpha-ketoglutarate (alpha-KG). In recent studies, IDH1 mutation occurs in 60-80% of grade II-III astrocytomas and in up to 88% of secondary GBMs, but less frequently in primary GBMs (Balss et al., 2008; Ohgaki and Kleihues, 2009; Yan et al., 2009; Mellai et al., 2011). The mutated IDH1 gene encodes a defective IDH1 enzyme, which, instead of producing alpha-KG, produces 2-hydroxyglutarate (2-HG) (Dang et al., 2009). Zhao et al. (2009) showed that in IDH1 mutated cells the expression of hypoxia-inducible factor subunit alpha (HIF-1 alpha) increases when alpha-KG concentration decreases. HIF-1 alpha is a transcription factor that facilitates tumor growth in hypoxic conditions. Whitlock et al. (2005) showed that expression of Hsp27 is upregulated by HIF-1 alpha. HIF-1 alpha also facilitates the transcription of carbonic anhydrase IX (CA IX) (Semenza, 2010).

CA IX is a member of the carbonic anhydrase enzymes (CAs). CAs catalyse the reversible reaction in which carbon dioxide is hydrated and gains a bicarbonate molecule and a hydrogen ion (Haapasalo et al., 2006). CA IX has an effect on the acid-base balance in tissue microenvironments, leading to the increased capability of tumour cells to survive and invade (Svastova and Pastorekova, 2013). CA IX expression has also been found to be associated with malignancy grades of diffusely infiltrating astrocytomas, and has a prognostic value in terms of patient survival in several brain tumors (Haapasalo et al., 2006; Nordfors et al., 2010).

The aim of the study was to assess the expression pattern of Hsp27 in the various grades of astrocytoma. Hsp27 expression has been associated with oxidative stress and hypoxia and thus we wanted to examine some of the key molecules involved in hypoxia pathways and associated regulatory mechanisms, particularly HIF-1 alpha, and CA IX. We also wanted to correlate IDH1 mutation status with Hsp27 expression, because the IDH1 mutation has been shown to be pathognomonic and one of the most powerful prognostic marker in gliomas (Boots-Sprenger et al., 2013). To our knowledge, this is the first study to assess the prognostic importance of Hsp27 in astrocytic gliomas.

Materials and methods

Study material

The study material consisted of 295 diffusely infiltrating astrocytomas (grade II: 43; grade III: 31; grade IV: 221). There were 264 patients with primary astrocytomas and 31 patients with recurrences only. The astrocytoma specimens were initially fixed in 4 % phosphate-buffered formaldehyde and then processed into paraffin blocks. On the basis of hematoxylin and eosin-stained slides, a neuropathologist (H.H.) evaluated the tumors according to WHO 2007 criteria (Louis et al., 2007). One histologically representative tumor region was selected from each specimen. From the selected regions, 1000 micrometer tissue cores were mounted into tissue microarray TMA blocks with a custom made instrument (Beecher Instruments, Silver Spring, MD, USA). Although the tissue microarray cores were evaluated as histologically representative, the relatively small 1000 micrometer cores may, in some cases, give an illustration of a rather regional expression of proteins.

Tumor samples were obtained from surgically operated patients at the Tampere University Hospital, Tampere, Finland, during the period 1983-2001. Tumors were removed using the highest level of safe resection. Data on radiotherapy and chemotherapy received was known in the case of 275 patients. Of these patients, 87 received only radiotherapy, compared to 4 that received only chemotherapy. 59 patients received both radio- and chemotherapy. None of the patients who were included in the study received Temozolomide treatment, due to the time period during which the study material was gathered. Patient survival was examined by a follow-up study of patients with a primary tumor. The follow-up time started after the primary resection of the astrocytoma. Patients were tracked until 2012 or until they died. The overall survival results were known for 247 patients. The mean patient age was 63 years (SD±14.6), the youngest patient was 20 years old, and the oldest 90 years old. The study protocol was approved by the Ethical Committee of Tampere University Hospital and the National Authority for Medicolegal Affairs of Finland.

Immunohistochemistry

5 micrometer thick sections were cut from multi-tissue paraffin blocks. Three similar contiguous sections were cut and stained immunohistochemically for Hsp27, IDH1, and HIF-1 alpha. CA IX immunohistochemistry
was performed as described previously (Haapasalo et al., 2006). Normal brain tissue was included in the TMA blocks as a control.

R132H point mutation specific mouse monoclonal antibody (Dianova GmbH, Hamburg, Germany) was used to detect IDH1-R132H specific gene mutations. Fully automated immunostaining was performed by a Bondmax immunostainer (Leica Biosystems Newcastle Ltd, Newcastle upon Tyne, United Kingdom). Bond Dewax Solution (catalogue No. AR9222) was used for deparaffinisation. For epitope retrieval, RTU Epitope Retrieval Solution 1, pH 5.9-6.1 (catalogue No. AR9961) was used for 30 min at 100°C. The slides were incubated for 30 minutes at room temperature with the IDH1-R132H point mutation specific antibody (dilution 1:50). The staining kit used was Bond Refine Detection kit. The slides were rinsed between steps with Bond Wash Solution (catalogue No. AR9590).

Hsp27 and HIF-1 alpha immunostaining was performed by the fully automated Ventana BenchMark LT Automated IHC Stainer (The BenchMark Series automated slide preparation system by Ventana Medical Systems, AZ, USA). Ventana EZ Prep solution (catalogue No 950-100) was used for deparaffinisation. For epitope retrieval, Tris -EDTA buffer with a pH value of 8.0 (catalogue No 950-124) was used at 95°C to 100°C for 30 minutes in Hsp27 immunostaining and for 90 minutes in HIF-1 alpha immunostaining. Slides were rinsed between steps with Ventana Tris-based Reaction buffer (catalogue No. 950-300). Slides were incubated at 37°C for 32 minutes with specific antibodies. Mouse monoclonal antibody specific for HIF-1 alpha (Abcam, Cambridge, UK) was used with a 1:50 dilution. Hsp27 specific antibody (Thermo Fisher Scientific, Fremont, CA, USA) was used with a 1:400 dilution. The staining kit used was the Ventana Ultraview DAB Detection Kit.

To demonstrate the simultaneous appearance of IDH1 mutation and CA IX expression, double immunohistochemical staining of selected representative slides was performed, using the Bondmax immunostainer. The same dilutions were utilised as the individual staining protocols. IDH1 staining was detected using the Bond Refine Red Detection kit (Cat# DS9390) and CAIX detected using the Bond Refine Detection kit.

Evaluation of the immunostaining

Staining for Hsp27 in the cytoplasm of tumor cells was measured and the staining intensity evaluated. Samples were considered either immuno-negative or immuno-positive (slight, moderate or strong positivity).

The IDH1 mutation status of each sample was evaluated as immuno-positive (mutated) or immuno-negative (non-mutated). The sample was considered immuno-positive if there was either a moderate or strong cytoplasmic signal, including nuclear positivity. The antibody used was R132H point mutation specific and the rare occurrence of other IDH1 mutations found in astrocytic gliomas cannot be excluded.

In the case of HIF-1 alpha, staining of nuclei was considered positive staining. Staining intensity was measured and samples were considered either immuno-negative or immuno-positive. The samples were considered immuno-positive if the staining was moderate or strong.

CA IX immunostaining and evaluation of the staining intensity have been previously reported (Haapasalo et al., 2006). The sample was considered CA IX immuno-positive if slight, moderate or strong staining was seen in tumor cells.

Statistical analysis

The statistical analyses were performed using IBM SPSS Statistics version 20.0. Significant associations were defined using the chi-square test and the Mann-Whitney U-test. Kaplan-Meier curves and the log-rank test were used in univariate survival analyses. Cox Regression analysis was used for multivariate survival analysis.

Results

Samples of normal brain tissue were immunohistochemically negative of all the examined molecules. Demographics details of the study material are presented in Table 1.

Hsp27

Hsp27 positive staining was seen in astrocytic tumor cells, endothelial cells and the extracellular matrix of tumor tissue. A clear difference in the staining of grade II-III tumors and GBMs was observed. In GBMs, the

<table>
<thead>
<tr>
<th>Table 1. Demographics of the study material</th>
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<tr>
<td><strong>Age</strong></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
</tr>
<tr>
<td><strong>Tumor grade</strong></td>
</tr>
<tr>
<td><strong>Resection</strong></td>
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<tr>
<td><strong>Radiotherapy</strong></td>
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<td><strong>Chemotherapy</strong></td>
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staining was often more intense than in lower grade tumors. Although great variation was also observed in the staining patterns within different grades, especially in GBMs, the staining varied considerably in different samples. The staining intensity often varied within the cells of the tumor as well, with both faintly and moderately stained cells often appearing side by side. In perinecrotic areas of GBMs, there were totally negative as well as strongly positive preinecrotic cells. Also, within non-ischemic areas, there was great variability in Hsp27 immunostaining. Fig. 1 demonstrates the staining patterns and different staining intensities of Hsp27 immunostaining. Cytoplasmic Hsp27 reactivity was seen in 128 of 295 astrocytomas (43.3%). Hsp27 expression correlated significantly with the increasing grade of astrocytoma (p<0.001, chi-square test). Percentages of Hsp27 positive samples in different grades are presented in Table 2. No significant correlation was found between Hsp27 and Ki-67 / MIB-1. Increasing Hsp27 expression predicted worse rates of patient survival when all grades were included within the univariate survival analysis (p=0.001, log-rank test). However, no significant associations were seen when patient outcome and Hsp27 expression were correlated separately in different grades.

### Table 2. Percentages of IDH1 mutation, HIF-1 alpha, Hsp27 and CA IX positive tumors in different WHO grades.

<table>
<thead>
<tr>
<th>Grade</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDH1</td>
<td>73.3%</td>
<td>69.4%</td>
<td>11.6%</td>
<td>25.0%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hsp27</td>
<td>16.3%</td>
<td>32.3%</td>
<td>50.2%</td>
<td>43.4%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CA IX</td>
<td>67.9%</td>
<td>79.2%</td>
<td>81.2%</td>
<td>36.4%</td>
<td>n.s.</td>
</tr>
<tr>
<td>HIF-1 alpha</td>
<td>10.5%</td>
<td>20.0%</td>
<td>43.5%</td>
<td>79.3%</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Fig. 1. A. Slightly Hsp27 positive grade II astrocytoma. B. Moderately Hsp27 positive grade III astrocytoma. C. Intensely positive Hsp27 immunostaining seen in the cytoplasm of a glioblastoma. D. Hsp27 immuno-negative glioblastoma. x 400
Similar results were observed for CA IX and HIF-1 alpha. The Kaplan-Meier curves for Hsp27 positive and negative cases are presented in Fig. 2.

**IDH1**

Intense and widely distributed IDH1 staining in cells was considered as truly positive staining. Immunopositive diffusely infiltrating tumor cells surrounded by normal cells were often detected at the border of the tumor and normal CNS tissue.

IDH1 immunostaining positivity was found to correlate with a lower grade of diffusely infiltrating astrocytoma (p<0.001, chi-square test). Percentages of IDH1 mutated tumors in different grades are presented in Table 2. Of the primary GBMs, 14 out of 196 (7.1%) had the IDH1 mutation, whereas in secondary GBMs (n=9), 4 were IDH1 mutated (44.4%) (p<0.001, chi-square test). The tumors with IDH1 mutation were less proliferative as measured by Ki-67/MIB-1, with median values of IDH1 mutated and non-mutated tumors 4.9 (mean 9.6±11.2) and 10.5 (mean 14.2±11.9) (p=0.001, Mann-Whitney test), respectively. In univariate survival analyses, IDH1 mutation positivity was found to be associated with longer patient survival (p<0.001, log-rank test). When the survival analyses were performed grade by grade, a significant difference in prognosis was found only in GBMs, in which IDH1 mutation was associated with better patient outcome (p<0.001).

**HIF-1 alpha**

HIF-1 alpha was strongly expressed in tumor cell nuclei in perinecrotic areas. However, HIF-1 alpha expression was also found in non-necrotic tumors. HIF-1 alpha immuno-positive staining and its pattern are demonstrated in Fig. 3.

HIF-1 alpha staining intensity was found to correlate with the increasing grade of diffusely infiltrating astrocytoma (p<0.001, chi-square test). Rates of positive staining in different grades are demonstrated in Table 2. HIF-1 alpha correlated with Ki-67/MIB-1 (p=0.003, Mann-Whitney U-test). Tumors not expressing HIF-1 alpha had a median Ki-67/MIB-1 index of 6.8 and mean of 11.5 (SD±11.7). In HIF-1 alpha positive tumors on the other hand, the median Ki-67/MIB-1 index was 13.2 and the mean 14.8 (SD±10.9). Univariate survival analysis showed a strong correlation between HIF-1 alpha expression and a patient’s worsened outcome (p=0.001, log-rank test).

**CA IX**

The immunostaining characteristics of the study material for CA IX were originally reported by Haapasalo et al. (2006). To simplify the survival analysis, CA IX intensities were divided into positive or negative staining, compared to the originally reported staining categories which were ‘no staining’, ‘slight’, ‘moderate’ and ‘strong’. In a similar way to the previous findings, CA IX positivity predicted poor patient outcome within the entire cohort (p=0.022, log-rank test).

**Correlations between Hsp27, IDH1, HIF-1 alpha, and CA IX**

To study regulatory mechanisms of carcinogenesis in diffusely infiltrating astrocytomas, HIF-1 alpha, Hsp27 and CA IX were investigated for their correlations with IDH1 mutation status and also for their correlations with...
each other. There was a significant correlation between IDH1 mutation and HIF-1 alpha (p=0.001, chi-square test). Positive staining for both the IDH1 mutation and HIF-1 alpha was detected in parallel specimens. Additionally, in the entire cohort, an association was found between Hsp27 positive staining and negative IDH1 mutation status (p<0.001, chi-square test). When the correlation was studied in different grades, IDH1 mutation was significantly associated. A similar situation was seen with Hsp27 expression in grades II and III (p=0.006 and 0.002, respectively, chi-square test). In GBMs, no significant correlation between IDH1 mutation and Hsp27 was found to exist. There was a correlation between IDH1 status and CA IX immunopositivity with 82% of IDH1 non-mutated tumors being CA IX positive (p=0.029, chi-square test).

In the entire cohort, a significant correlation was found between HIF-1 alpha and Hsp27 (p=0.020, chi-square test). Furthermore, HIF-1 alpha was found to correlate with CA IX (p=0.005, chi-square test). No significant association was seen between Hsp27 and CA IX (p=n.s., chi-square test).

When IDH1 mutated tumors were considered, HIF-1 alpha expression significantly correlated with CA IX positivity (p=0.027 chi-square test). Fig. 4A-C demonstrates the simultaneous appearance of IDH1-R132H point mutation and CA IX expression. Hsp27 did not correlate significantly with HIF-1 alpha expression in IDH1 mutated tumors (p=n.s., chi-square test).

**Multivariate survival analysis**

Multivariate survival analysis was performed to assess the significance of the studied parameters on patient prognosis. Altogether 166 primary grade II–IV astrocytom as with complete data for the multivariate analysis were included. The expression of Hsp27, HIF-1 alpha and CA IX, IDH1 mutation, WHO grade, whether chemotherapy had been received (yes or no), radiotherapy received (yes or no), and patient age was known in every case. The age categories were 20 to 57, 58 to 72 and 73 to 90 years old. Patient age, IDH1

| Table 3. The independent prognostic indicators of primary grade II-IV astrocytom as evaluated by Cox’s stepwise regression model (N=166). WHO grade (p=0.056), Hsp27 (p=0.141), CA IX (p=n.s.), radiation therapy (p=n.s.) and chemotherapy (p=n.s.) were left out of the model. |
|---|---|---|---|
| Significance | Hazard ratio* | 95% CI for Hazard ratio |
| | Upper | Lower |
| Patient age** | <0.000 | 2.299 | 1.880 | 2.840 |
| IDH1*** | <0.000 | 0.206 | 0.126 | 0.337 |
| HIF-1 alpha **** | 0.011 | 1.579 | 1.111 | 2.245 |

* Hazard ratio: <1: favourable prognosis, >1: unfavourable prognosis.
**Patient age cut off points 57 and 72 years. *** IDH1 non-mutated / mutated. **** HIF-1 alpha negative / positive
mutation and HIF-1 alpha appeared as independent prognostic factors. The results are presented in Table 3.

**Discussion**

Hsp27 has previously been associated with increasing tumor grade and poor differentiation in astrocytomas (Khalid et al., 1995; Hermisson et al., 2000; Assimakopoulou and Varakis, 2001; Shen et al., 2010). To our knowledge, our results show for the first time a significant association of Hsp27 with patient survival in astrocytic gliomas. Here we show that patients with Hsp27 immuno-positive tumors have a worse prognosis than those with Hsp27 immuno-negative tumors, which could be explained by the key factors in tumor aggressiveness – hypoxia and oxidative stress. Both factors increase Hsp27 gene expression (Yu et al., 2008; Marotta et al., 2011). This is supported by our results showing a significant correlation between Hsp27 and HIF-1 alpha expression and strong Hsp27 immuno-positive staining of hypoxic GBMs. Hsp27 positivity of tumors has also been connected with chemotherapy resistance (Calderwood and Ciocca, 2008). This probably relates to the cytoprotective role of Hsp27, which in the case of cancer, leads to an increase in unfavourable functions of malignant cells. The phenomenon may rationalise the survival study finding.

The association of the IDH1 mutation with Hsp27 or CA IX expression has not previously been assessed in gliomas. Our results show that one of the most important factors of glioma oncogenesis, IDH1 mutation status, is associated with the hypoxia-related molecules Hsp27, HIF-1 alpha, and CA IX. A statistically significant association between IDH1 mutation and Hsp27 expression was found in the entire cohort as well as in WHO grade II and III tumors. HIF-1 alpha was found to be associated with Hsp27 expression in the entire cohort, although it was not evident in IDH1 mutated tumors. Thus, it seems that there may be different Hsp27 expression pathways depending on the IDH1 mutation status of cancer cells.

HIF-1 alpha seems to mediate Hsp27 expression in some tumors, as shown by Whitlock et al. (2005). However, it seems that in IDH1 mutated tumors there may be other mechanisms activating Hsp27 overexpression. IDH1 mutation has been shown to alter the DNA’s histone methylation (Venneti and Thompson, 2013). Apparently, the changes caused via 2-HG overexpression can either increase or reduce expression of other molecules. Thus, the alternative histone methylation caused by IDH1 mutation may also have an effect on the expression of Hsp27.

In the entire cohort, HIF-1 alpha positive staining correlated significantly with higher CA IX expression, a situation which has been similarly described by others (Ivanov et al., 2001; Proescholdt et al., 2005; Said et al., 2007). In IDH1 mutated tumors, all samples with HIF-1 alpha positivity indicated the expression of CA IX. However, in IDH1 non-mutated astrocytomas no significant correlation was evident when expression of HIF-1 alpha was compared to CA IX. Thus, the present study suggests that CA IX signalling may be a part of both hypoxia-driven and hypoxia independent pathways, especially in lower grade diffusely infiltrating astrocytomas. Yet again, the epigenetic changes induced by IDH1 mutation may play a role in the expression pathways of CA IX.

HIF-1 alpha expression was also found to correlate with increasing cell proliferation. This is largely a feature of hypoxic GBMs, in which cell proliferation is high and HIF-1 alpha expression is suggested to be controlled by hypoxia, rather than IDH1 mutation. In our study, the majority of tumours were glioblastomas. This is most probably also the reason for the association found to exist between HIF-1 alpha expression and high cell proliferation in the analysis of the entire cohort.

In our large cohort of 295 diffusely infiltrating astrocytomas, we show that Hsp27 is a marker of poor patient prognosis. However, the prognostic value of Hsp27 is most probably due to other related features, such as HIF-1 alpha expression as well as IDH1 mutation, as suggested by the Cox multivariate analysis. The expression pathways of Hsp27 and CA IX seem to vary depending on whether the tumor cell has an IDH1 mutation or not. Furthermore, it seems that HIF-1 alpha could regulate the expression of CA IX in IDH1 mutated diffusely infiltrating astrocytomas, whereas other mechanisms activating CA IX expression could be more accurate in IDH1 non-mutated tumors. The findings support the hypothesis that, in addition to the conventional hypoxia inducible pathway, IDH1 mutation could be an essential factor in controlling the expression of Hsp27 and CA IX via a hypoxia-independent mechanism.

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