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Prognostic Value of *IDH1* Mutations Identified with PCR-RFLP Assay in Glioblastoma Patients

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Abstract

Background and Objective: It has been shown that somatic missense mutations in codon 132 of the NADP+ dependent isocitrate dehydrogenase 1 (*IDH1*) gene occur frequently in primary brain tumors including highly malignant glioblastoma (GBM). The aim of this study was to evaluate a PCR-restriction fragment length polymorphism (RFLP)-based method for missense mutation detection and to estimate the prognostic value of the two most frequent *IDH1* codon 132 mutations, R132H and R132C, in patients with newly diagnosed GBM treated with radiation combined with temozolomide.

Methods: DNA was extracted from formalin-fixed, paraffin-embedded tissue. The PCR-RFLP method was adapted to *IDH1* codon 132 mutation screening. The mutation status was determined in a group of 58 patients.

Results: We found R132H mutations in 14% of patients. No R132C mutation was found in this study. Median follow-up for living patients was 31 (range 17–51) months. Median progression-free survival in the group of patients with *IDH1* mutation was 29 months compared with 10 months in the *IDH1* wild-type group ($p = 0.004$; hazard ratio [HR] 3.09, 95% CI 1.25, 4.78). Median overall survival in the group with *IDH1* mutation has not been reached, whereas in the group with wild-type *IDH1* it was 19.5 months ($p < 0.001$; HR 4.76, 95% CI 1.22, 6.30). Three-year overall survival was 60% in the group with *IDH1* mutation while in the wild-type *IDH1* group it dropped to 29%. *IDH1* mutations significantly correlated with younger age ($p = 0.02$).

Conclusions: Our results indicate that the *IDH1* R132H mutation is a powerful prognostic marker in GBM treated with chemoradiation. The PCR-RFLP method allows for a fast, inexpensive, and sensitive mutation screening.

Introduction

Glioblastoma (GBM), corresponding to WHO grade IV, is the most frequent primary brain tumor in adults. It represents 54% of all tumors originating from glial cells (Central Brain Tumor Registry of the United States, http://www.cbtrus.org), and is characterized by poor prognosis, with a median overall survival of 12 months.[1] Most GBMs (approximately 90%) develop rapidly *de novo* as a so-called primary GBM without histological evidence of transition from more differentiated astrocytic precursor lesions of lower grade stage. In contrast, secondary GBMs develop through progression from diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III). Primary and secondary GBMs, although difficult to discriminate in standard diagnostics, differ in terms of age of diagnosis (median 62 years for primary vs 45 years for secondary GBM), median survival (shorter for primary GBM), and genetic pathways.[2] In most Western countries, the current standard treatment of newly diagnosed GBM involves surgery and combined chemoradiotherapy with the

use of temozolomide followed by adjuvant temozolomide in 21-day cycles.[3]

Located in the chromosome region 2q33, isocitrate dehydrogenase 1 gene (*IDH1*) belongs to a group of five genes encoding human isocitrate dehydrogenases, enzymes which catalyze the reaction of transition of isocitrate to α-ketoglutarate. Four of the human isocitrate dehydrogenase enzymes are mitochondrial; only IDH1 is present mainly in cytoplasm and peroxisomes.[4] The physiological function of IDH1 remains unclear. Presumably, it plays a role in cytoplasmic nicotinamide adenine dinucleotide phosphate (NADPH) generation as well as fatty acid oxidation and degradation of phytanic acid in peroxisomes.

Previous studies showed that, in contrast to the majority of solid tumors, mutations in the *IDH1* gene occur frequently in brain cancers.[5-7] They are also evident in some patients with acute myeloid leukemia.[8] These mutations are limited to the small hot-spot region in exon 4, codon 132, which encodes arginine located in the active site of the enzyme. Substitution of this amino acid impairs interaction with isocitrate and dramatically reduces enzymatic activity of IDH1 against its natural substrate. Normally, IDH1 functions as a homodimer. When a mutant molecule binds to a wild-type protein it results in formation of inactive heterodimer. Therefore, mutations in codon 132 have dominant effects and their presence in at least one of the alleles leads to inactivation of the enzyme, increased levels of isocitrate, and decreased levels of α-ketoglutarate.[9] Recent findings demonstrated accumulation of isocitrate metabolite *R*-(−)-2-hexoglutarate in cells with an *IDH1* mutation.[10] At the cellular level, the consequences of these dramatic changes are not clear. Based on functional studies and the finding of increased levels of hypoxia inducible factor (HIF1α) in mutant cells, Zhao et al. proposed that *IDH1* may act in the hypoxic pathway as a tumor suppressor.[11] These results are inconsistent with clinical observations in patients with glioma. Despite the conclusion from functional studies, GBM patients harboring *IDH1* mutations have better outcomes than those with the wild-type gene.[12,13] In previous studies, patients carrying the mutant allele had a median overall survival (OS) of 31 months, compared with 15 months for patients with wild-type *IDH1*. *IDH1* mutations occur mainly in secondary GBMs (approximately 85% of these patients) and lower grade tumors. They are observed much less frequently in primary GBMs (5% of cases).[14-16] This means that *IDH1* mutation might be considered as the best known single genetic marker for diagnosis of secondary GBM.[17]

In previously published studies, *IDH1* mutations have been detected by genomic sequencing.[13-19] The fact that these mutations occur in one codon creates an opportunity for application of a simple test based on restriction digestion. In a study by Balss et al.,[19] 92.7% of all codon 132 mutations were the R132H substitution and the second most frequent mutation was R132C (3.6%). All other mutations accounted for only 3.7%.

In this study we developed a restriction fragment length polymorphism (RFLP) method suitable for detecting the most frequent *IDH1* mutations, R132H and R132C. We screened a group of patients with newly diagnosed GBM treated with standard radiotherapy with temozolomide and performed survival analysis of *IDH1* wild-type versus mutated patients.

Materials and Methods

Patients

This study involved 58 patients with newly diagnosed GBM, WHO grade IV, who were treated between 2002 and 2007. All patients had good performance status at the beginning of the treatment and underwent surgical tumor resection. Patients were treated with temozolomide according to the following scheme: 75 mg/m^2 of body surface area of temozolomide during radiotherapy (total fraction 60 Gy) and adjuvant temozolomide therapy for 5 days in 21-day cycles in a dose of 150–200 mg/m^2. In cases of tumor progression, second-line chemotherapy with lomustine (CCNU) was administered. In this group of patients the best known predictive molecular factor, *MGMT* promoter hypermethylation, was determined previously (manuscript in preparation) with the use of methylation-specific PCR.[20] Patients' characteristics are listed in table I.

The study protocol was approved by the Independent Ethics Committee of the Cancer Centre and Institute of Oncology, Warsaw, Poland.

DNA Samples Preparation

All DNA samples were obtained from formalin-fixed, paraffin-embedded (FFPE) tissue, using the Scherlock AX kit (A&A Biotechnology, Gdynia, Poland). The quality and integrity of DNA samples from formalin-fixed tissue were determined by multiplex PCR reaction designed for the BIOMED-2 study,[21] with a set of primers that amplify PCR products of various sizes (range 100–400 bp). All DNA samples included in the study allowed for efficient amplification of 200 bp PCR product.

Table I. Patient characteristics

Characteristic	Value
Patients (n)	58
Sex (n)	
female	24
male	34
Age (y)	
range	21–70
median	49
Surgery extension (n)	
total resection	41
subtotal resection	17
MGMT promoter hypermethylation status (n)	
positive	26
negative	32
Median progression-free survival (mo)	13
Median overall survival (mo)	22
Observation time for living patients (mo)	
range	17–51
median	31

Mutational Analysis by PCR-RFLP

To determine *IDH1* mutation status in nucleotide positions 395G>A (R132H) and 394C>T (R132C), we developed a new nested PCR-RFLP assay (figure 1). In the first step a 166 bp fragment of *IDH1* exon 4 was amplified. Twenty cycles of PCR reaction were performed in a total volume of 15 μL, consisting of 1x PCR buffer, 2 mmol/L $MgCl_2$, 250 μmol/L of each deoxynucleotide, 0.1 μmol/L of each PCR primer, 0.5 U of FastStart Taq DNA Polymerase (Roche, Mannheim, Germany), and 50 ng of DNA template. The PCR product was diluted ten times and reamplified in two parallel second-step reactions with two sets of primers designed for R132H and R132C mutation discrimination. One of the primers in each primer pair included a mismatch that created the restriction site for *Hsp*92II or *Psp*1405I enzymes for R132H and R132C mutation detection, respectively. The second primer in each pair included a mismatch introducing a control restriction site for the same enzyme that was used for mutation detection (i.e. *Hsp*92II or *Psp*1405I). Digestion of the control site in every PCR product excluded false negative results that might have occurred due to incomplete digestion by the specific enzyme used. The second PCR step was carried out for 30 cycles in a total volume of 15 μL, consisting of 1x PCR buffer, 3 mmol/L $MgCl_2$, 250 μmol/L of each deoxynucleotide, 0.15 μmol/L of each PCR primer, 0.5 U of FastStart Taq DNA Polymerase, and 1 μL of diluted first-step nested PCR product. All PCR reactions were performed in GeneAmp® system 2700 (Applied Biosystems) thermocycler. Primer sequences and PCR conditions are listed in table II. In this step, the size of the amplified products was 109 bp in the reaction to detect the R132H mutation, and 123 bp in the reaction to detect the R132C mutation. Incorporation of the restriction sites was confirmed by DNA sequencing of inner products of the selected DNA samples.

Half of the final volume of the inner PCR product was digested with the restriction enzyme *Hsp*92II (Promega) or *Psp*1406I (Fermentas) for R132H and R132C mutation detection, respectively. Digestion was performed for 4 hours in a total volume of 20 μL, containing 1x digestion buffer and 10 U of restriction enzyme. Digestion products were electrophoresed in 8% polyacrylamide gels (acrylamide/Bis 19 : 1) and visualized with ethidium bromide. Digestion of PCR products from wild-type template DNA occurred only in the control site. In the completely digested wild-type sample only one band on the gel was visible: 97 bp in R132H and 112 bp in R132C mutation test. In the mutated *IDH1* allele an additional restriction site was introduced in the PCR step and the mutation was confirmed by the presence of an additional 76 bp or 88 bp fragment that indicated R132H or R132C substitutions, respectively (figure 2).

All positive and negative RFLP results were confirmed by DNA sequencing of first-step PCR products with BigDye® Terminator v3.1 on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Statistical Analysis

The follow-up period was terminated in May 2009. OS was defined as the time from surgery to a patient's death or last follow-up contact before the moment of completion of data

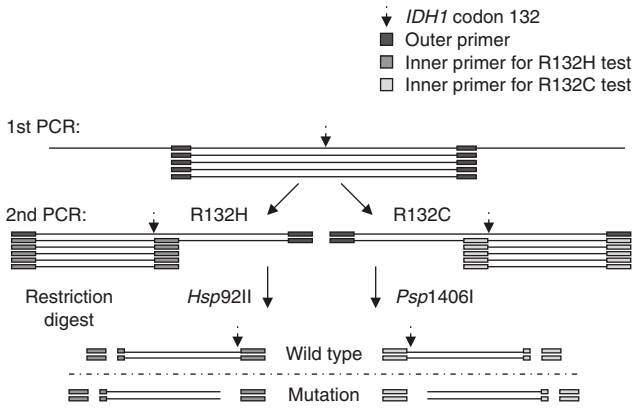

Fig. 1. PCR-RFLP procedure scheme.

Table II. Primer sequences and PCR conditions

	Primer sequence	PCR conditions
First-step PCR		
forward	5′ CGGTCTTCAGAGAAGCCATT 3′	95°C 3′ → 20x (95°C 30" → 60°C 30" → 72°C 30") → 72°C 7′
reverse	5′ CAAGTTGGAAATTTCTGGGC 3′	
Second-step PCR		
R132H forward	5′ TACGGTCATGAGAGAAGCCATTATC 3′	95°C 3′ → 30x (95°C 30" → 54°C 30" → 72°C 30") → 72°C 7′
R132H reverse	5′ GACTTACATGACCCCATAAGCATCA 3′	
R132C forward	5′ GGATGGGTAAAACCTATCATCAAACGT 3′	95°C 3′ → 30x (95°C 30" → 54°C 30" → 72°C 30") → 72°C 7′
R132C reverse	5′ TTAATTCAACGTTGGAAATTTCTGGGC 3′	

collection. Progression-free survival (PFS) was defined as the time from surgery to radiographic evidence of tumor progression or the date of recraniotomy. OS and PFS curves were estimated by the Kaplan-Meier method and compared by the use of two-sided log-rank test. The statistical relationship between various parameters was analyzed using the Fisher's exact test.

Results and Discussion

Using the RFLP-based approach we identified *IDH1* R132H mutations in 14% (8/58) of the patients. No R132C mutation was found in the studied group of GBM patients. The results of PCR-RFLP analysis were validated by genomic sequencing. All eight positive and 50 negative DNA samples underwent verification. We obtained consistent results confirming the usefulness of the PCR-RFLP approach, as well as its sensitivity and specificity compared with the DNA sequencing method. Sequencing analysis showed that all identified mutations were heterozygous.

Median PFS in the group of patients with mutated *IDH1* was 29 months compared with 9 months in the group with wild-type *IDH1* ($p = 0.004$; HR 3.09, 95% CI 1.25, 4.78) [figure 3]. Three-year OS was 60% in the group of patients with mutated *IDH1* and 29% in the group with wild-type *IDH1*. Median OS in the group with mutated *IDH1* has not been reached in the follow-up period. Median OS in the wild-type group was 19.5 months ($p < 0.001$; HR 4.76, 95% CI 1.22, 6.30) [figure 3]. Seven of eight patients with *IDH1* somatic mutations were younger than 49 years (median age of the group) and this difference is statistically significant ($p = 0.023$) at significance level of 0.05.

The frequency of *IDH1* mutations was also higher in the group of patients with *MGMT* promoter hypermethylation, although this difference is not statistically significant. In six of eight cases with mutated *IDH1*, the detailed histopathological examination provided evidence of secondary GBM. However, these patients did not have a history of prior diagnosis of a lower-grade glioma, which is one of the possible criteria of secondary GBM.

Combined chemoradiotherapy with temozolomide with adjuvant temozolomide has become a standard treatment of patients with newly diagnosed GBM. To our best knowledge, there are no reports on the mutation analysis of the *IDH1* gene and its role in clinical outcomes of patients treated with this regimen. In this study we performed analysis of *IDH1* in a demographically diverse group of GBM patients treated with this combined therapy.

IDH1 mutations are limited to the small single hot-spot in codon 132 of exon 4. From the diagnostic point of view this fact is very important because well defined single nucleotide genomic variations are easy to evaluate with the use of restriction enzymes.[22] In this study we proposed the PCR-RFLP-based approach for *IDH1* mutation detection. This technique allows for a fast and simple analysis, which can be performed in 1 day from DNA isolation to the final result.

The RFLP approach has several important advantages over other methods including genomic sequencing or real-time PCR instrument-based techniques such as high resolution melting

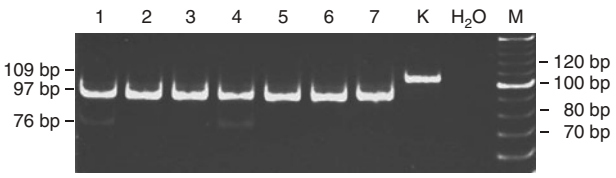

Fig. 2. An example of the PCR-RFLP result for *IDH1* R132H mutation detection, showing the polyacrylamide gel electrophoresis of the second-step PCR product (109 bp) after restriction digestion. Lanes 1-7 contain patients' samples, with samples 1 and 4 showing the *IDH1* R132H mutation. **H_2O** = no template control; **K** = undigested PCR control; **M** = 10 bp DNA ladder (Fermentas).

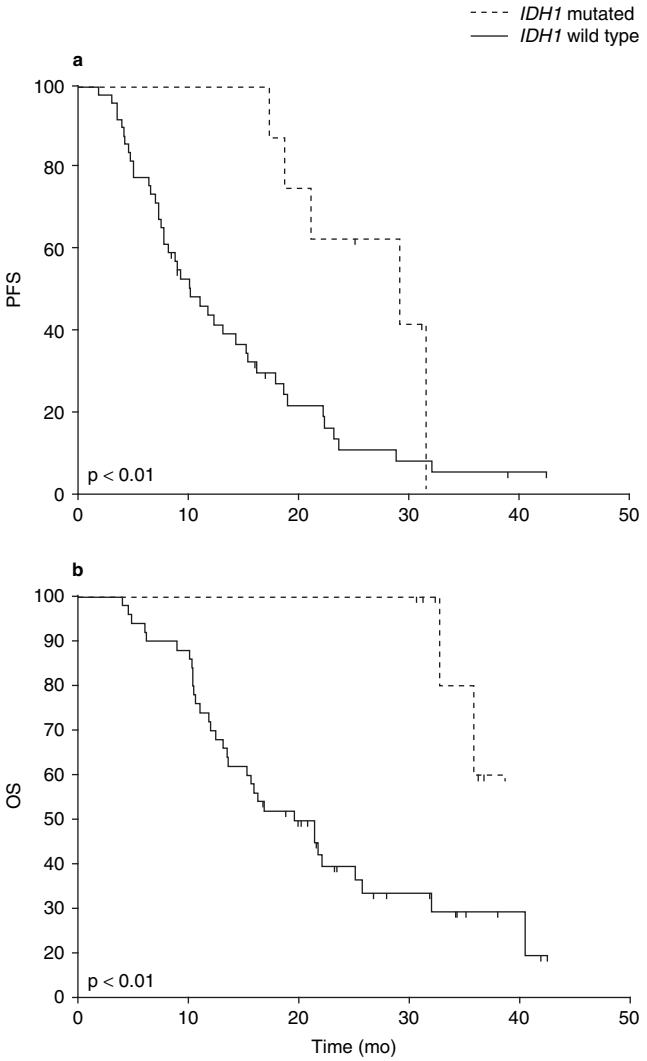

Fig. 3. (**a**) Progression-free survival (PFS) and (**b**) overall survival (OS) curves according to *IDH1* R132H mutation status.

point analysis or allelic discrimination. It is sensitive, cost effective, does not require any sophisticated equipment, and is at least as fast as other approaches. All of these features make this method very suitable for patient screening and diagnostics. In our experience, this approach was comparably sensitive and specific to genomic sequencing, the method that was previously shown to be useful for *IDH1* mutation detection in FFPE tissue.[18]

During the data collection period, a similar method was published by Meyer et al.[23] These authors proposed PCR-RFLP for five known *IDH1* mutations including the least frequent changes. Our approach differs in technical details. We designed PCR primers with the control restriction site. In our system, digestion of the PCR product results in formation of a specific control DNA fragment. The respective band on the gel indicates complete PCR product digestion. This internal control prevents eventual false-negative results caused by incomplete DNA restriction cleavage. We also designed shorter PCR products that may be more useful in analysis of low quality DNA, such as that isolated from FFPE tissue.

Recently, a monoclonal antibody that specifically recognizes the *IDH1* R132H mutant protein has been described.[24,25] This antibody was successfully used for mutation detection on the protein level either by Western blot assay or immunohistochemistry. Although Western blot analysis seems not to be useful in clinical diagnostics, a specific immunohistochemical staining may find application in a standard histopathological evaluation. This approach was previously shown to be at least as useful as DNA sequencing.[26] Although comparison of the PCR-RFLP assay needs further methodological study, based on reported data we can expect that it should show similar sensitivity.

In our opinion, PCR-based methods of mutation detection provide at least one significant advantage. The results are generated as simple binary data, such as presence or lack of an additional band on the gel in the PCR-RFLP. Consequently, these methods are not susceptible to errors resulting from subjective microscopic observation. On the other hand, the PCR-based methods are prone to false negative results resulting from contamination of analyzed tumor samples with normal cells: either normal glial cells, blood microvessels or infiltrating lymphocytes. In our study, in all cases of mutated samples, the bands on the gel corresponding to the mutant allele were less intense than the wild-type allele bands. In our interpretation this results from the fact that all diagnosed mutations were heterozygous and all tissue samples contained a combination of neoplastic and other cells. The problem of contamination of the tumor tissue with normal cells may be resolved with the use of micro- or macrodissection of the analyzed tissue sample. Such an approach has already been shown to improve detection of IDH1 mutations with the use of genomic sequencing.[18]

Previous reports indicate that *IDH1* mutations may be an important factor in the pathogenesis of GBM.[12,14] They also seem to be the best known genetic marker for secondary GBM tumors which, contrary to primary GBM, develop from better-differentiated astrocytic tumors (WHO grade II and III) and are diagnosed in younger patients. We observed the *IDH1* R132H mutation in 14% of patients, and significantly more frequently in younger patients. In most of these cases histopathological evaluation documented, at least focally, better-differentiated neoplastic astrocytes, indicating secondary GBM. We also observed the previously reported association

between *IDH1* mutations and *MGMT* promoter methylation.[16] However, in our study this relationship was not statistically significant, probably because of the small number of patients.

Similarly to the previous reports, our results showed that *IDH1* mutations are associated with better clinical outcome in terms of PFS and OS. It is not clear whether *IDH1* mutations are a prognostic factor *per se* or a predictive factor in the context of the specific therapy regimen. Our analysis includes only patients with GBM treated with the current standard approach, i.e. concomitant radiotherapy and temozolomide with adjuvant temozolomide. In most of the previously reported studies that indicate the role of *IDH1* mutation for patient survival the treatment regimen of patients was not specified. In this study we observed better prognosis for GBM patients compared with previously published results by Sanson et al.[16] and Yan et al.[14] This difference may result from younger age of the included patients, but also may suggest that the observed better clinical outcomes in our study is caused by the interaction of temozolomide treatment and *IDH1* function. Systematic prospective studies as well as more detailed retrospective analysis are needed to explain this relationship.

Conclusions

Our study showed usefulness of the PCR-RFLP technique for detection of point mutations in codon 132 of *IDH1* gene. This technique provides sensitivity and specificity similar to the genomic sequencing method. *IDH1* mutations occur more frequently in younger patients and correlate with better progression-free survival and overall survival in glioblastoma patients treated with current standard regimen of concomitant radiotherapy and temozolomide followed by adjuvant temozolomide. Therefore these point mutations appear to be important prognostic factors.

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