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IDH1 Mutations at Residue p.R132 (*IDH1*^{R132}) Occur Frequently in High-Grade Gliomas But Not in Other Solid Tumors

Fonnet E. Bleeker,[1,2] Simona Lamba,[2] Sieger Leenstra,[3,4] Dirk Troost,[5] Theo Hulsebos,[6] W. Peter Vandertop,[1,7] Milo Frattini,[8,9] Francesca Molinari,[9] Margaret Knowles,[10] Aniello Cerrato,[11] Monica Rodolfo,[8] Aldo Scarpa,[12] Lara Felicioni,[13] Fiamma Buttitta,[13] Sara Malatesta,[13] Antonio Marchetti,[13] and Alberto Bardelli[2,14]*

[1]*Neurosurgical Center Amsterdam, Location Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands*

[2]*Laboratory of Molecular Genetics, The OncoGenomics Center, Institute for Cancer Research and Treatment, University of Torino, Medical School, Candiolo, Italy*

[3]*Department of Neurosurgery, St. Elisabeth Ziekenhuis, Tilburg, The Netherlands*

[4]*Department of Neurosurgery, Erasmus Medical Center, Rotterdam, The Netherlands*

[5]*Departments of Neuropathology, The Netherlands*

[6]*Department of Neurogenetics, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands*

[7]*Neurosurgical Center Amsterdam, Location VU University Medical Center, Amsterdam, The Netherlands*

[8]*Department of Experimental Oncology, Istituto Nazionale Tumori; Milan, Italy*

[9]*Laboratory of Molecular Diagnostic Institute of Pathology, Locarno, Switzerland*

[10]*Section of Experimental Oncology, Leeds Institute for Molecular Medicine, Leeds, United Kingdom*

[11]*Institute of Endocrinology and Experimental Oncology, National Council of Research, Naples, Italy*

[12]*Department of Pathology, Section of Anatomic Pathology, University of Verona, Verona, Italy*

[13]*Clinical Research Center, Center of Excellence on Aging, University-Foundation, Chieti, Italy*

[14]*FIRC Institute of Molecular Oncology, Milan, Italy*

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ABSTRACT: Systematic sequence profiling of the Glioblastoma Multiforme (GBM) genome has recently led to the identification of somatic mutations in the isocitrate dehydrogenase 1 (*IDH1*) gene. Interestingly, only the evolutionarily conserved residue R132 located in the substrate binding site of *IDH1* was found mutated in GBM. At present, the occurrence and the relevance of p.R132 (*IDH1*^{R132}) variants in tumors other than GBMs is largely unknown. We searched for mutations at position R132 of the IDH1 gene in a panel of 672 tumor samples. These included high-grade glioma, gastrointestinal stromal tumors (GIST), melanoma, bladder, breast, colorectal, lung, ovarian, pancreas, prostate, and thyroid carcinoma specimens. In addition, we assessed a panel of 84 cell lines from different tumor lineages. Somatic mutations affecting the *IDH1*^{R132} residue were detected in 20% (23 of 113) high-grade glioma samples. In addition to the previously reported p.R132H and p.R132S alleles, we identified three novel somatic mutations (p.R132C, p.R132G, and p.R132L) affecting residue *IDH1*^{R132} in GBM. Strikingly, no *IDH1* mutations were detected in the other tumor types. These data indicate that cancer mutations affecting *IDH1*^{R132} are tissue-specific, and suggest that it plays a unique role in the development of high-grade gliomas.
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KEY WORDS: cancer; somatic mutation; IDH1; GBM; HGG

Introduction

The molecular profiling of tumor genomes is taking an enormous spurt these days. Genome-wide sequencing analyses have been performed in colorectal and breast cancer (Sjöblom et al., 2006; Wood et al., 2007), and most recently the same approach has been performed in pancreatic ductal adenocarcinoma (PDAC) (Jones et al., 2008) and glioblastoma multiforme (GBM) (Parsons et al., 2008). These mutational efforts have led to the identification of novel somatic mutations in genes that had not been previously linked to tumorigenesis. Of particular interest

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*Correspondence to: Alberto Bardelli, Laboratory of Molecular Genetics, Institute for Cancer Research and Treatment, University of Torino; Medical School, Str prov 142Km 3.95; Candiolo (TO), 10060, Italy. E-mail: a.bardelli@unito.it.

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is that the GBM mutational screen revealed 12% somatic mutations in the *IDH1* gene (MIM# 147700) (Parsons et al., 2008). The *IDH1* mutations were found predominantly in the group of secondary GBMs and younger patients. Furthermore, the patients with mutated *IDH1* had a significantly longer survival (Parsons et al., 2008). Other tumor type datasets analyzed for this gene have been relatively small thus far, and the reported mutation frequencies are generally low (0 of 11 breast, 1 of 11 colon) (Wood et al., 2007) (http://www.sanger.ac.uk/cosmic).

IDH1 encodes isocitrate dehydrogenase 1 (Geisbrecht and Gould, 1999), an enzyme that catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate (Koshland et al., 1985). This reaction leads to NADPH production, and is thought to play a role in the cellular control of oxidative damage (Lee et al., 2002). IDH1 is localized within the cytoplasm and peroxisomes (Geisbrecht and Gould, 1999). Two different mutations in *IDH1* have been described in GBM, both affecting the amino acid arginine at position 132 and leading to amino acid residue substitutions (p.R132H and p.R132S). R132 is evolutionarily highly conserved, and is localized in the substrate binding site of IDH1, where hydrophilic interactions between R132 and both the α- and β-carboxylate of isocitrate are formed (Xu et al., 2004). The $IDH1^{p.R132H\ and\ p.R132S}$ changes might affect these interactions and its enzymatic activity (Parsons et al., 2008). In this study we have investigated the mutational status of *IDH1* in 672 tumor samples and 84 cancer cell lines.

Materials and Methods

High-grade glioma (HGG; WHO grade III and grade IV) tumor samples (GBM, anaplastic astrocytoma, and anaplastic oligodendroglioma) and the matched normal DNA samples were obtained from the tumor bank maintained by the Departments of Neurosurgery and Neuropathology at the Academic Medical Center (Amsterdam, The Netherlands). DNA of melanoma, colorectal cancer, and gastrointestinal stromal tumors (GIST) samples was obtained from the Department of Experimental Oncology at the Istituto Nazionale Tumouri (Milan, Italy). DNA of PDAC xenografts was obtained from the Department of Pathology, Section of Anatomic Pathology at the University of Verona (Verona, Italy). DNA of breast, lung, ovarian, and thyroid (papillary carcinoma) cancer samples was obtained from the Clinical Research Center, Center of Excellence on Aging at the University-Foundation (Chieti, Italy). Additional DNA samples of thyroid carcinomas (medullary histotype), extracted from frozen tissues, were obtained from the Department of Cellular Biology and Molecular Pathology at the University of Naples (Naples, Italy). DNA of bladder cancer samples was obtained from the Section of Experimental Oncology at the Leeds Institute for Molecular Medicine (Leeds, UK). Tumor databases are listed in Table 1, and have been previously validated by showing that somatic mutations in common cancer genes could be detected at the expected frequencies.

In addition, a panel consisting of 84 cell lines from multiple tumor lineages was screened for $IDH1^{R132}$ mutations. Cell line details are shown in Supp. Table S1. The NCI-60 panel (the 60 human cancer cell lines of the National Cancer Institute) was obtained from ATCC (Middlesex, UK). In addition, 16 astrocytoma cell lines were included: the cell lines CCF-STTG1, Hs683, U87MG, U118MG, U251MG, U373MG, T98G (ATCC, Middlesex, UK), GAMG (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), SKMG-3 (a gift of Dr. C.Y. Thomas, University of Virginia Division of Hematology/Oncology, Charlottesville, VA), D384MG, SF763 (gifts of Dr. M.L. Lamfers, Department of Neurosurgery, Free University, Amsterdam, The Netherlands), SF126 (a gift of Dr. C. Van Bree,

Table 1. $IDH1^{R132}$ **mutations are specific for high grade gliomas**

Tumor type	Histotype	Number of samples analysed	Number of mutated samples	*P*-value (Fisher's exact test)
High grade glioma	Total	113	23	
	GBM primary	94	11	
	GBM secondary	15	11	
	AA	2	0	
	AO	2	1	
Bladder	Transitional Cell	34	0	0.002179
Breast	Total	127	0	8,03E-09
	Ductal	48		0.000292
	Lobular	45		0.000295
	Medullary	17		0.041578
	Mucinous	17		0.041578
Colorectal	Adenocarcinoma	128	0	7,26E-09
GIST		25	0	0.014124
Lung	Total	107	0	8,20E-09
	Adenocarcinoma	84		0.000001
	Carcinoid	7		0.343849
	Small Cell	16		0.073930
Melanoma		23	0	0.013501
Thyroid	Total	42	0	0.000576
	Medullary	21		0.023904
	Papillary	21		0.023904
Ovary	Adenocarcinoma	46		0.000284
Pancreas	Ductal Adenocarcinoma	23	0	0.013501
Prostate		4	0	0.584147

Tumor samples, tumor type, histotype, the number of samples analysed and the number of mutated samples are indicated. In addition, *P*-values of the Fisher's exact test, used to determine the tissue specificity for $IDH1^{R132}$ mutations in high grade gliomas, are listed. Abbreviations: AA; Anaplastic Astrocytoma, AO; Anaplastic Oligodendroglioma, GBM; Glioblastoma Multiforme, GIST; Gastrointestinal Stromal Tumors.

Table 2. Mutations affecting *IDH1*^{R132} identified in high grade gliomas

IDH1 mutation		Number of mutated samples	Histology of mutated samples	Previously described
Nucleotide	Amino Acid			
c.394C>T	p.R132C	3	GBM	yes
c.394C>G	p.R132G	1	GBM	no
c.394C>A	p.R132S	1	AO	yes
c.395G>T	p.R132L	1	GBM	no
c.395G>A	p.R132H	17	GBM	yes

The nucleotide and amino acid changes are listed alongside the number and histology of the mutated samples. In addition, we indicate whether the mutation has been described before in high grade gliomas. The nucleotide numbering uses the A of the ATG translation initiation start site as nucleotide +1, based on reference sequence NM__005896.2. All mutations are heterozygous. Abbreviations: AO; Anaplastic Oligodendroglioma, GBM; Glioblastoma Multiforme.

Table 3. Cancer cell lines in which the *IDH1*^{R132} mutations were analyzed

Tumor type	Number of cell lines analysed
Astrocytoma	20
Bladder	4
Breast	7
Cervix	1
Colon	9
HNSCC (tongue)	1
Kidney	6
Leukemia	5
Lung	9
Melanoma	8
Mesothelioma	1
Oesophageus	2
Ovary	8
Prostate	2
Thyroid	1

Cell lines are listed according to the tumor lineage from which they were originated. Abbreviation: HNSCC; Head and neck squamous cell carcinoma.

University of Amsterdam, Laboratory for Experimental Oncology and Radiation Biology, Amsterdam, The Netherlands), A58 and A60 (gifts of Dr. A. van Tilborg and Dr. P. De Witt Hamer, Department of Neurosurgery, Academic Medical Center, Amsterdam, The Netherlands), the xenograft cell line IGRG121 (a gift of Dr. B. Geoerger, Institut Gustave Roussy, Villejuif, France). Genomic DNA of the cell lines A1847, DU145, JAMA2, MCF7, ME180, MSTO-211H, NCI-H1299, NCI-H69, OE19, OE33, OVCA433, SCC9, SKCO1, and ZR-75-1 was provided by Dr. F. Di Nicolantonio (OncoGenomics Center, Institute for Cancer Research and Treatment, Italy). DNA from other cell lines was derived from our own laboratories.

Genomic DNA was isolated as previously described (Balakrishnan et al., 2007). PCR primers for the genomic region corresponding to *IDH1* (NM_005896.2) exon 4, which encodes codon R132, and the flanking intronic sequences, including splicing donor and acceptor regions were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers (forward 5′-AATGAGCTCTATATGCCATCACTG-3′, reverse 5′-TTCATACCTTGCTTAATGGGTGT-3′ and sequence 5′-GCCATCACTGCAGTTGTAGGTTA-3′) were synthesized by Invitrogen/Life Technologies, Inc. (Paisley, England). PCRs were performed in 96-well formats in 10 µl reaction volumes, containing 0.25 mmol/l deoxynucleotide triphosphates, 1 µmol/l each of the forward and reverse primers, 6% DMSO, 1 × PCR buffer, 1 ng/µl DNA, and 0.05 unit/µl Platinum Taq (Invitrogen/Life Technologies). A touchdown PCR program was used for PCR amplification (Peltier Thermocycler, PTC-200, MJ Research, Bio-Rad Laboratories, Inc., Italy).

PCR conditions were as follows: 94°C for 2 min; three cycles of 94°C for 15 sec, 64°C for 30 sec, 70°C for 30 sec; three cycles of 94°C for 15 sec, 61°C for 30 sec, 70°C for 30 sec; three cycles of 94°C for 15 sec, 58°C for 30 sec, 70°C for 30 sec; and 35 cycles of 94°C for 15 sec, 57°C for 30 sec, and 70°C for 30 sec, followed by 70°C for 5 min, and 12°C thereafter. PCR products were purified using AMPure (Agencourt Bioscience Corp., Beckman Coulter S.p.A, Milan, Italy). Cycle sequencing was carried out using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) with an initial denaturation at 97°C for 3 min, followed by 28 cycles of 97°C for 10 sec, 50°C for 20 sec, and 60°C for 2 min. Sequencing products were purified using CleanSeq (Agencourt Bioscience, Beckman Coulter) and analyzed on a 3730 DNA Analyzer, ABI capillary electrophoresis system (Applied Biosystems). Sequence traces were analyzed using the Mutation Surveyor software package (SoftGenetics, State College, PA).

A total of 756 PCR products, spanning 367 kb of tumor genomic DNA, were generated and subjected to direct sequencing. Changes previously described as SNPs were excluded from further analyses (http://www.ensembl.org/index.html). To ensure that the observed mutations were not PCR or sequencing artifacts, amplicons were independently reamplified and resequenced in the corresponding tumors. All verified tumor changes were resequenced in parallel with the matched normal DNA to distinguish between somatic mutations and SNPs not previously described. For samples in which mutations were found, matching between germ-line and tumor DNA was verified by direct sequencing of 26 single nucleotide polymorphism (SNP) at 24 loci (data not shown), to ensure that the observed changes are somatic mutations. Nucleotide and amino acid numbering uses the A of the ATG translation initiation start site (codon 1) as nucleotide +1, based on reference sequence NM_005896.2.

The Fisher's exact test (http://www.langsrud.com/fisher.htm) was used to determine the tissue specificity of *IDH1*^{R132} mutations in HGG. In this test, the absence of mutations of different tumor types was compared with the number of mutations found in HGG samples.

Results and Discussion

We determined the occurrence of *IDH1*^{R132} sequence variants in a panel of 672 tumor samples. These included 113 HGG samples (109 GBM, 2 anaplastic astrocytoma, and 2 anaplastic oligodendroglioma), 25 GIST, 23 melanoma, 34 bladder cancer, 127 breast cancer, 128 colorectal cancer, 107 lung cancer, 46 ovarian cancer, 4 prostate cancer, 42 thyroid cancer, and 23 PDAC specimens (Table 1). In addition, a panel consisting of 84 cell lines from multiple tumor lineages was screened for *IDH1*^{R132} mutations. Out of the 756 samples analyzed, 23 displayed heterozygous mutations at position R132 of the *IDH1* gene. Strikingly, mutations were only found in HGG (23 out of 113 samples corresponding to 20%; see Table 1). In agreement with previous results (Parsons et al., 2008), the most common change detected in our GBM tumor database is the *IDH1*$^{p.R132H}$ mutation. In addition to the reported p.R132H and p.R132S variants, we detected three novel heterozygous somatic mutations affecting

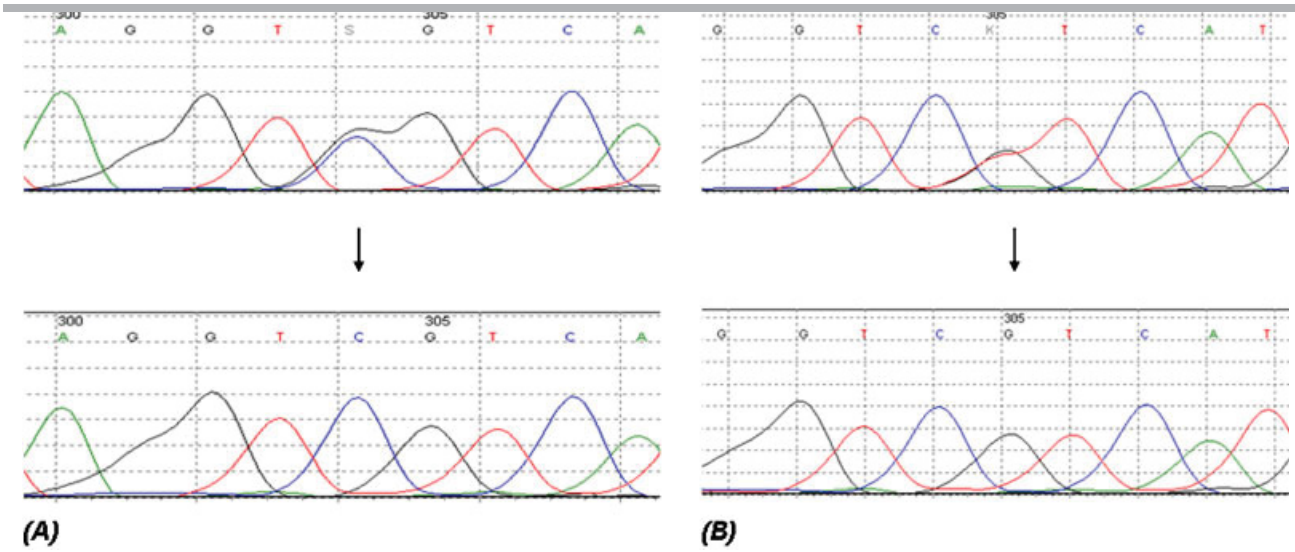

Figure 1. Examples of novel somatic *IDH1*^{R132} mutations identified in GBM. Top, chromatogram of the sequence of a tumor sample; bottom, chromatogram of the matched normal. Arrows, location of missense somatic mutations. Nucleotide and amino acid alterations are below the traces. The nucleotide numbering uses the A of the ATG translation initiation start site as nucleotide +1, based on reference sequence NM_005896.2. Numbers above the sequences are part of the software output. **A**: p.R132G mutation; **B**: p.R132L mutation.

residue R132 (p.R132C, p.R132G, and p.R132L) in GBM (Fig. 1, Table 2). As previously reported, most of the mutations were detected in glioblastomas. However, we also found a single mutated anaplastic oligodendroglioma. None of the other human solid tumor types displayed *IDH1*^{R132} variants, and this was in most cases statistically significant (see Table 1). Although one colorectal cancer sample has been previously described to have an *IDH1*$^{p.R132C}$ allele (Sjöblom et al., 2006), in our set of 128 colorectal cancer samples R132 variants were never detected.

Cancer cell lines represent unique tools for multiple aspects of biomedical research including the evaluation of the functional relevance of cancer alleles. We therefore searched for mutations at position R132 of *IDH1* in a panel of cancer cell lines, including 20 astrocytoma (Table 3 and Supp. Table S1). None of the cell lines displayed *IDH1*^{R132} variants. However, we found two previously unreported *IDH1* alleles in three cell lines: p.V71I was detected in the plasma cell myeloma line RPMI-8226, while p.G97D was found in the colorectal cancer cell lines DLD-1 and HCT-15. These two colorectal cancer cell lines are suggested to be genetically identical, and therefore may be derived from the same patient (Chen et al., 1995). As matched normal samples are not available for these tumor cell lines, we cannot assess whether the nature of these mutations is somatic. Neither the p.V71I nor the p.G97D variants have been reported previously as SNPs (http://www.ensembl.org/index.html). Considering that we did not find these alleles in any of the 672 tumor samples and 84 cell lines that we sequenced, we suspect that they are either very rare SNPs or novel *IDH1* somatic changes. Compared to the frequency (20%) that we found in HGG samples, the lack of *IDH1* mutations in our panel of 20 high-grade astrocytoma cell lines appears statistically significant (p-value = 0.024, Fisher's exact test). It is possible, however, that GBM cell lines are predominantly derived from primary GBM tumors, thus explaining our results.

GBM, WHO grade IV with predominant astrocytic differentiation, is the most common and most aggressive primary brain tumor (Louis et al., 2007). Most glioblastoma manifest rapidly de novo, without recognizable precursor lesions. These so-called primary GBMs typically present in middle age to elderly patients with a brief clinical history and show rapid progression and short survival time (Ohgaki and Kleihues, 2007; Scherer, 1940). In contrast, secondary GBMs are typically seen in younger patients, with a history of epilepsy caused by low-grade gliomas, which in years progress to GBM (Ohgaki and Kleihues, 2007; Scherer, 1940). Secondary GBMs are rare (5%) in comparison to primary GBM (Ohgaki et al., 2004), and can only be diagnosed with clinical (neuroimaging) or histological evidence of evolution from a less malignant astrocytoma (Ohgaki and Kleihues, 2007). Both subtypes are considered histopathologically indistinguishable. However, the classification in primary and secondary is nicely reflected by molecular mechanisms. Primary GBMs have a high rate of *EGFR* alterations, *MDM2* duplications, *PTEN* mutations, and homozygous *P16*^{INK4A} deletions, whereas *TP53* mutations are most prevalent in secondary GBMs (Ohgaki and Kleihues, 2007; Ohgaki et al., 2004). We observed the *IDH1* mutations predominantly in secondary GBM (11 of 94 vs. 11 of 15, p-value = 0.0000016, Fisher's exact test; see Table 1), in accordance with the results of Parsons and colleagues (2008). In addition to the *IDH1*$^{p.R132C/G/H/L}$ mutations in GBM, we identified an *IDH1*$^{p.R132S}$ mutation in an anaplastic oligodendroglioma sample. Interestingly, GBM patients with an *IDH1*^{R132} mutation have been reported to have a better survival (Parsons et al., 2008). No information on *IDH1* mutations in low-grade gliomas is available thus far; therefore, assessment of whether lower grade gliomas display *IDH1*^{R132} mutations and if they have a survival advantage are critical questions that should be addressed.

In conclusion, our data support the evidence that *IDH1* is a pivotal GBM cancer gene mutated predominantly in secondary glioblastomas. The identification of three novel mutations in *IDH1* affecting amino acid R132 may allow further structural and functional analysis of the function of this residue on the catalytic activity of isocitrate dehydrogenase 1. Our most relevant finding entails the unique and striking tissue-specific pattern of the *IDH1*^{R132} mutations in human solid cancer. The tissue specificity of cancer mutations has been observed in multiple cancer genes (e.g., *APC*, *AKT1*) (Bleeker et al., 2008a; Bleeker et al., 2008b). Why some genes are mutated in specific tumor types remains an

unsettled issue whose solution will be relevant for basic and clinical cancer research. As IDH1 is involved in a specific metabolic pathway, its mutations may potentially be exploited for therapeutic purposes. However, to therapeutically challenge the *IDH1* cancer variants it must first be assessed whether they functionally operate as oncogenes or tumor suppressor genes. The fact that we and others only found heterozygous mutations at one specific IDH1 residue involved in its catalytic activity, strongly suggests that these mutations could activate IDH1 in a pro-oncogenic (dominant) fashion in cancer cells. Studies revealing the functional role of the $IDH1^{R132}$ mutations are vital to confirm this hypothesis and to provide insights in the potential of mutated IDH1 as therapeutic target.

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