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Immunohistochemical analysis of 1844 human epithelial and hematopoietic tumors and sarcomas for IDH1R132H mutation

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Running title: Mutant IDH1-R132H protein in human tumors

Abstract

Aims: Mutations in the isocitrate dehydrogenase 1 gene have been recently identified to play a key role in diffuse astrocytoma and oligodendroglioma as well as in acute myeloid leukemia. In glioma, *IDH1R132H* is the most common mutation type, which is associated with younger patient age and a longer patient survival compared to wild type status. Sequencing analyses of carcinomas and lymphomas have detected *IDH1* mutations only in a small fraction of cases. In those studies, *IDH1R132H* was also the most frequent mutation. Aim of the present study was to analyze a comprehensive series of human tumors for *IDH1R132H* mutation. *Methods and results:* We stained a total of 1844 formalin-fixed paraffin-embedded tumors including carcinomas, sarcomas and hematopoietic tumors using a mutation specific antibody for *IDH1^{R132H}*. Our positive control series consisted of a collection of diffuse astrocytomas and oligodendrogliomas. No *IDH1R132H* mutation was found in our series. *Conclusions:* We conclude that *IDH1R132H* mutations occur almost exclusively in glioma and acute myeloid leukemia.

Introduction

Recently, various studies have shown that heterozygous mutations in the isocitrate dehydrogenase 1 (*IDH1*) gene, encoding a nicotinamide adenine dinucleotide phosphate (NADP⁺) dependent enzyme, occur frequently in astrocytic and oligodendroglial tumors of WHO grades II and III and in secondary glioblastoma¹. In a previously published series of 1010 gliomas 71 % of the tumors carried an *IDH1* mutation. Of these 93% were characterized by a base-pair exchange of guanine to adenine (G395A), leading to the substitution of the amino acid arginine by histidine (R132H)². In acute myeloid leukemia (AML), mutations of either *IDH1* or isocitrate dehydrogenase 2 (*IDH2*) were detected in up to 50% of the tumors³⁻⁶. In contrast to diffuse glioma, *IDH1R132C* mutations appear to be more frequent than *IDH1R132H* and very recent data suggest that *IDH2* mutations may represent the most frequent mutations in these tumors^{3, 5}. Sequencing analyses suggested that *IDH1* mutations are rare in other tumors⁷⁻⁸. The current clinical importance for knowledge on *IDH1* mutations in glioma is based on the observation in clinical trials and prospective studies: *IDH1* mutation is associated with a significant better prognosis⁹⁻¹⁰.

Biochemical studies revealed that mutant *IDH1* protein loses its ability to convert isocitrate to α -ketoglutarate¹¹⁻¹². However, mutant protein gains a novel function: it catalyzes the reduction of α -

ketoglutarate to 2-hydroxyglutarate (2HG) in a NADPH-consuming conversion¹³. Mutation of *IDH1* results in an approximately 100-fold increase of 2HG in glial tumors¹³. Interestingly, children with a defect in FAD-linked L-2-hydroxyglutarate-dehydrogenase and consecutively increased serum 2HG levels carry an increased risk for brain tumor development¹⁴⁻¹⁶. The potential cancer forming or promoting function of 2HG is under investigation. Recently, *IDH2* mutations have also been identified to produce 2HG⁵. Interestingly, novel observations point out that all mutations are localizing to the isocitrate binding site of the respective enzymes. *IDH1* and *IDH2* proteins exhibit an extraordinary high level of homology in the amino acid sequence corresponding to this binding site. Thus, increased 2HG levels may serve as a marker for different *IDH1* and *IDH2* mutations⁵⁻⁶.

Another potentially 2HG-independent mechanism of tumor promotion by *IDH1* mutations has been proposed: mutant *IDH1* results in a lack of α -ketoglutarate (α -KG) which is necessary for maintaining the activity of prolylhydroxylases. Active prolylhydroxylases in turn promote the degradation of HIF-1 α . *IDH1* mutation mediated reduction of prolylhydroxylases thus results in an increased HIF-1 α activity known for its tumor promoting effects¹⁷.

Therefore, analysis of tumor specimens for mutated *IDH1* protein, especially the most common variant *IDH1*^{R132H}, is important for both clinicians and researchers. The development of a mutation specific antibody suitable also for immunohistochemistry (IHC) on formalin-fixed paraffin-embedded (FFPE) tissue allows efficient screening of large tumor collections for the R132H mutation¹⁸⁻¹⁹. IHC allows diagnostic routine analysis and has the advantage of being rapidly performed and allows detection of *IDH1*^{R132H} protein in its morphological setting. In this study, we investigated 1844 tumors for presence of *IDH1*^{R132H} by IHC.

Material and Methods

Tissue specimens

FFPE tissues were provided by the Tissue Bank, National Center for Tumor Diseases Heidelberg (NCT) and the Department of General Pathology, Institute of Pathology Heidelberg, the Department of Pathology, University Hospital of Jena and the Institute of Neuropathology, University Hospital, Münster. The control series of glioma specimens¹⁸⁻¹⁹ was obtained from the Department of Neuropathology, Institute of Pathology, University Hospital Heidelberg. All tumors with exception of the 23 specimens of prostate carcinoma and in part the glioma control group were sampled on tissue micro arrays (TMA). Tumor entities and number of respective samples are given in table 1.

Immunohistochemistry

For immunohistochemistry we used the previously described antibody against mutant IDH1^{R132H} protein, clone H09 (Dianova, Hamburg, Germany)¹⁸⁻¹⁹. Sections cut to 3µm were incubated and processed with clone H09 on a Ventana BenchMark XT® immunostainer (Ventana Medical Systems, Tucson, AZ, USA). The Ventana staining procedure included pretreatment with cell conditioner 2 (pH 6) for 60min, followed by incubation H09 hybridoma supernatant at 37°C for 32min. Incubation was followed by Ventana standard signal amplification, UltraWash, counterstaining with one drop of hematoxylin for 4min and one drop of bluing reagent for 4min. For visualization, ultraView™ Universal DAB Detection Kit (Ventana Medical Systems) was used. Binding of antibody was scored as previously described¹⁹.

PCR amplification and direct sequencing

DNA was extracted from FFPE tissue of 7 cases with ambiguous binding pattern using standard protocols. A fragment spanning the catalytic domain of IDH1 including codon 132 was amplified using 60ng each of the primer *IDH1f* TGATGAGAAGAGGGTTGAGGA and the primer *IDH1r* ATCCCCATAAGCATGACGAC. Primer design was based on accession number NM_005896. For PCR, 20ng of DNA and 2x PCR Master Mix (Promega, Madison, WI, USA) were employed. PCR was performed in a total volume of 15µl, and included initial denaturation at 180°C for 30s, followed by 35 cycles with denaturation at 95°C for 30s, annealing at 56°C for 40s and extension at 72°C for 50s. Two microliters of the amplification product was submitted to sequencing using the BigDye®

Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Twenty-five cycles were performed employing 12ng of the sense primer *IDH1f* TGATGAGAAGAGGGTTGAGGA, with denaturation at 95°C for 30s, annealing at 56°C for 15s and extension at 60°C for 240s. Sequences were determined using the semiautomated sequencer (ABI® 3100 Genetic Analyzer, Applied Biosystems) and the Sequence Pilot version 3.1™ (JSI-Medisys, Kippenheim, Germany) software.

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Results

We analyzed 1844 tumors for mutant IDH1^{R132H} protein by IHC using the mouse monoclonal antibody H09. The group of epithelial tumors consisted of 23 prostate carcinomas, 326 colon carcinomas, 110 renal cell carcinomas (including clear-cell, papillary and chromophobe carcinomas), 143 non-small-cell lung carcinomas (including squamous cell and adenocarcinomas), 258 breast carcinomas (including invasive ductal and lobular subtypes), 4 thyroid carcinomas, 7 nasopharyngeal squamous cell carcinomas, 7 upper gastrointestinal carcinomas, 132 pancreatic carcinomas, 148 neuroendocrine gastrointestinal tumors and 98 carcinoid tumors. In the set of choroid plexus tumors, we analyzed 15 choroid plexus papillomas, 1 atypical choroid plexus papilloma and 3 choroid plexus carcinomas. Among 1275 epithelial tumors, 1268 were clearly negative and 7 yielded an ambiguous result. These 7 tumors were analyzed by direct sequencing which did not detect an *IDH1* mutation in codon 132. Thus, none of the 1275 epithelial tumors contained mutant IDH1^{R132H} protein. We further screened 364 different sarcomas, including 137 angiosarcomas, 35 leiomyosarcomas, 36 synovial sarcomas, 97 liposarcomas, 23 myxofibrosarcomas and 36 malignant fibrous histiocytomas. No *IDH1*R132H mutation was detected in sarcomas. The analysis of 179 lymphomas (including 34 follicular lymphomas, 41 diffuse large B cell lymphomas, 25 primary mediastinal B cell lymphomas, 17 mantle cell lymphomas, 16 marginal zone lymphomas and 46 Hodgkin's lymphomas) and 26 extramedullary plasmacytomas yielded also no *IDH1*R132H mutation. In the control series of 116 diffuse gliomas, the IDH1^{R132H} protein was found in 99/116 (85%) cases.

Discussion

Our series included 1275 epithelial tumors, 364 sarcomas, 205 hematopoietic tumors and a control series of gliomas. In none of the 1275 epithelial tumors IDH1^{R132H} protein was detected. The very close association of IDH1^{R132H} protein with *IDH1*R132H mutation has been demonstrated previously¹⁸⁻¹⁹. Our data set was controlled by the inclusion of 116 diffuse gliomas exhibiting the IDH1^{R132H} protein in 99/116 (85%) cases. Clone H09 is highly specific for tumors. No binding of H09 has been detected in reactive glial and non-glial tissues²⁰. Of note, because all *IDH1* mutations are heterozygous, wild type IDH1 protein is expressed in all: tumor tissues with mutation, tumor tissues without mutation, normal tissue and reactive cells. Thus, the *IDH1*R132H mutation appears to be absent or very rare in epithelial tumors. *IDH1* mutations in carcinoma have been analyzed in previous studies. Our series did not provide evidence for *IDH1*R132H mutations in colon, breast, kidney, lung, pancreatic, thyroid, upper gastrointestinal, nasopharyngeal and 3 choroid plexus carcinomas and in 15 plexus papillomas. In fact, six colon and 1 kidney carcinomas yielded ambiguous binding pattern, however, both, re-analysis on full tissue sections and DNA sequencing revealed wild type status in all seven instances. In the previous series, *IDH1*R132H mutations have been detected in a very low fraction of prostate, colon and thyroid carcinomas. In a series by Kang this alteration has been detected in 2/75 cases of prostate carcinoma⁸. Our series included only 23 prostate carcinoma specimens all of which were negative for IDH1R132H protein, therefore well in line with the published data. In a whole cancer genome sequencing approach, Sjöblom et al detected *IDH1*R132H mutation in 1/11 colon carcinoma²¹⁻²². The present series including 326 colon carcinomas did not exhibit a single case with IDH1R132H protein. Our findings are matched by absence of *IDH1* mutation in 128 and 97 colon carcinomas in the series by Bleeker et al. and Kang et al., respectively⁷⁻⁸. A very recent study detected 3 *IDH1* mutations in 38 thyroid cancers, however the mutations detected did not affect codon 132 of *IDH1*²³. We did not find either *IDH1*R132H mutation in the 4 thyroid carcinomas from our series. Data on *IDH1* status in sarcoma was not available so far. Our study contains 364 different sarcomas, including angiosarcoma, leiomyosarcoma, synovial sarcoma, liposarcoma, myxofibrosarcoma and malignant fibrous histiocytoma. IHC for IDH1^{R132H} protein did not reveal any mutation in sarcomas. All together, our data confirm on protein level the absence or extreme rarity of *IDH1*R132H mutations in epithelial and sarcomatous tumors. Acute myeloid leukemia (AML) so far is the only non CNS tumor described with a substantial number of *IDH1* mutations. We, therefore, extended our series to include additional hematopoietic neoplasms.

We did not detect IDH1^{R132H} protein in 179 lymphomas of different subtypes and in 26 plasmacytomas. We could demonstrate the ability of clone H09 to bind to hematopoietic lesions with *IDH1R132H* mutation in a preceding study demonstrating mutant protein in 3 of 71 patients with myelodysplastic syndrome²⁴. Thus our data complements a previous study by Kang et al. with a focus on various subtypes of leukemias in which 1 mutation among 186 leukemias has been reported⁸. It appears that *IDH1R132H* mutations are rare or absent in hematopoietic tumors apart from AML and precursor lesions thereof.

The availability of a mutation specific antibody for detection of mutant IDH1^{R132H} protein is of great value in the analysis of tumor entities carrying those alterations. It allows application of standard protocols for IHC and in comparison to DNA extraction followed by sequencing currently is more rapid and inexpensive. We performed our analyses on a semi-automated staining system (Ventana BenchMark XT® immunostainer, Ventana Medical Systems, Tucson, AZ, USA), however, antibody clone H09 has been successfully used with very different protocols (personal communication A. von Deimling). Another considerable advantage is the ability of IHC to detect small groups of or even single tumor cells, whereas DNA analysis may be compromised by presence of non tumorous cells contributing wild type DNA. TMA analysis with clone H09 is very rewarding because so far, all tumors examined appeared to carry the mutation in every tumor cell and all these cells expressed IDH1^{R132H} protein allowing efficient detection. However, *IDH1R132H* is predominantly seen in diffuse gliomas whereas it is virtually absent in carcinoma and sarcoma. While the diagnostic potential for clone H09 is evident for brain tumors, it appears to be irrelevant for most of the extracranial tumor entities. The value for such analysis in AML needs to be tested.

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Legends to table and figure

Table 1: Immunohistochemical results of IDH1^{R132H} specific antibody in epithelial, sarcomatous and hematopoietic tumors, glioma control group. *previously published

Figure 1: Top row shows negative staining with IDH1^{R132H} specific antibody in colon carcinoma (a,c), choroid plexus carcinoma (b), and breast carcinoma (d). Lower row shows diffuse glioma control group in tumors with *IDH1*R132H mutation (e,g,h) and *IDH1* wild type (f)

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Table 1

Entity	positiv	total
Prostate carcinoma	0	23
Colon carcinoma	0	326
Renal cell carcinoma	0	110
clear-cell	0	67
papillary	0	4
chromophobe	0	12
others	0	27
Non-small-cell lung carcinoma	0	143
Squamous cell carcinoma	0	59
Adenocarcinoma	0	62
Others	0	22
Breast carcinoma	0	258
invasive ductal	0	167
invasive lobular	0	43
others	0	48
Thyroid carcinoma	0	4
Nasopharyngeal squamous cell carcinoma	0	7
Upper gastrointestinal carcinoma	0	7
Pancreatic carcinoma	0	132
Neuroendocrine gastrointestinal tumors	0	148
Carcinoid	0	98
Choroid plexus papilloma	0	15
Atypical choroid plexus papilloma	0	1
Choroid plexus carcinoma	0	3
Angiosarcoma	0	137
Leiomyosarcoma	0	35
Synovial sarcoma	0	36
Myxoid liposarcoma	0	36
Dedifferentiated liposarcoma	0	48
Pleomorphic liposarcoma	0	13
Myxofibrosarcoma	0	23
Malignant fibrous histiocytoma	0	36
Extramedullary plasmacytoma	0	26
Follicular lymphoma	0	34
Diffuse large B cell lymphoma	0	41
Primary mediastinal B cell lymphoma	0	25
Mantle cell lymphoma	0	17
Marginal zone lymphoma	0	16
Hodgkin's lymphoma	0	46
Diffuse astrocytoma WHO grade II *	44	53
Anaplastic astrocytoma WHO grade III *	25	31
Oligodendroglioma WHO grade II *	16	16
Anaplastic oligodendroglioma WHO grade III *	14	16

Figure 1

