別紙１

厚生労働科学研究委託費

革新的がん医療実用化研究事業

変異型 IDH を標的とした悪性脳腫瘍・肉腫・胆管がんに対する革新的治療法の開発

平成２６年度　委託業務成果報告書

業務主任者　北林　一生

平成２７（２０１５）年　３月

本報告書は、厚生労働省の革新的がん医療実用化研究事業による委託業務として、独立行政法人国立がん研究センターが実施した平成２６年度「変異型IDHを標的とした悪性脳腫瘍・肉腫・胆管がんに対する革新的治療法の開発」の成果を取りまとめたものです。

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別紙３

厚生労働科学研究委託費（革新的がん医療実用化研究事業）
委託業務成果報告（総括）

変異型 IDH を標的とした悪性脳腫瘍・肉腫・胆管がんに対する革新的治療法の開発

業務主任者　北林　一生　　国立がん研究センター研究所　造血器腫瘍研究分野

研究要旨　　IDH1 遺伝子及び IDH2 遺伝子は、脳腫瘍・急性骨髄性白血病・骨髄異形成症候群・胆管がん・軟骨肉腫・骨肉腫・血管免疫芽球性 T 細胞リンパ腫など様々ながんにおいて高頻度に変異が見られる。IDH 遺伝子変異を有する急性骨髄性白血病モデルマウスを開発し、変異型 IDH の発現ががんの維持に必須であることを証明した。変異型 IDH1 特異的阻害剤を開発し、IDH1 変異を有する急性骨髄性白血病モデルの白血病細胞を顕著に減少させることを明らかにした。新規の変異型 IDH1/2 に対する新規の特異的モノクローナル抗体（MsMab-1 抗体）を樹立した。MsMab-1 抗体は、複数の変異型 IDH1/2 に交差反応性を示し、この MsMab-1 抗体と以前に作製した HMab-1 抗体を組み合わせることにより、IDH1-R132H だけでなく、複数の変異型 IDH1/2 を高感度に検出することができ、診断に有用であることがわかった。

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Ａ．研究目的

本研究では、がん特異的な変異型イソクエン酸デヒドロゲナーゼ 1（IDH1）を発現する悪性脳腫瘍、胆道がん、軟骨肉腫などの稀少がんの治療法及び診断法を確立し、3 年以内に臨床研究に進めることを目的とする。変異型 IDH 遺伝子変異を有する患者由来の組織を免疫不全マウスに移植した異種移植片（PDX）のマウスモデルを作製し、これらのマウスモデルに独自に開発した変異型 IDH 阻害剤を投与することにより、変異型 IDH の標的妥当性を検証するとともに、変異型 IDH 阻害剤の治療効果を検証し、生体内での薬物動態を調べることにより、治療薬としての最適化を進める。さらに、変異型 IDH を有する患者の早期診断のため、変異型 IDH を特異的に認識する抗体を作製し、治療対象患者の診断法を確立する。

Ｂ．研究方法

①変異型 IDH 阻害剤の開発

脳腫瘍・軟骨肉腫・骨肉腫・胆管がんの患者由来組織片を NOG マウスの皮下又は各組織に移植することにより、各がんの患者由来組織片移植（PDX）モデルを作製する。

PDX モデルに変異型 IDH 阻害剤を投与し、腫瘍ボリュームの測定、染色像の観察、血漿中や腫瘍組織内の 2HG レベルの定量などを行い、変異型 IDH 阻害剤に対する感受性を調べる事により、脳腫瘍・軟骨肉腫・骨肉腫・胆管がん等における変異型 IDH の標的妥当性を検証すると共に、変異型 IDH 阻害剤の治療効果を確認する。

②診断法の確立

変異型 IDH のある患者の診断法を確立するため、変異型 IDH に特異的な抗体を作製し、ヒト病理切片に対して免疫組織染色を行い、有用性の確認を行う。

Ｃ．研究結果

IDH 遺伝子変異を有する急性骨髄性白血病モデルマウスを開発し、Cre-loxP システムを用いて変異型 IDH を除去すると急性骨髄性白血病の発症が抑制されることが示された。変異型 IDH を除去した白血病細胞を調べると、白血病幹細胞マーカーの発現が顕著に低下し、白血病誘導能が消失していることが明らかとなった。第一三共株式会社と共同で変異型 IDH1 に対する阻害剤を独自に開発した。急性骨髄性白血病モデルにおいて変異型 IDH1 阻害

剤が、変異型 IDH により産生される腫瘍細胞内及び血漿中の 2-ハイドロキシグルタレイトの量をほぼ完全に抑制し、白血病細胞を顕著に減少させることを証明した。物質特許及びこれらの抗腫瘍効果を含む主要特許を出願した。「IDH1 変異を有するモデル評価系の作出と新規薬剤感受性の試験」に関する研究計画を国立がん研究センターの倫理審査員会に提出し承認を得た。３例の変異型 IDH1 変異を有する脳腫瘍試料を免疫不全マウスの皮下に移植移植し、繰り返し移植可能な株を１株樹立した。

IDH1-R132G に反応し、野生型 IDH1 に反応しない複数のクローンを ELISA 法により選択した。次に、複数の変異型 IDH1/2 に対する ELISA を実施したところ、新規に樹立した MsMab-1 抗体（IgG_1, kappa）は、複数の変異型 IDH1/2 に交差反応性を示すことがわかった。ウェスタンブロット法により、IDH1-R132H、IDH1-R132S、IDH1-R132G、IDH2-R172M、IDH2-R172S、IDH2-R172G のリコンビナントタンパク質に対する反応性が見られた。さらに、免疫組織染色においても、MsMab-1 抗体は IDH1-R132H、IDH1-R132S 等の変異型 IDH1/2 を保有する glioma に対して高い反応性を示した。以上のことから、MsMab-1 抗体は、変異型 IDH1/2 陽性の glioma の診断マーカーとして有用であることがわかった。この MsMab-1 抗体と以前に作製した HMab-1 抗体（IDH1-R132H に対する特異的抗体）を組み合わせ、免疫組織染色を実施した結果、54 症例の gradeIII の glioma のうち、30 症例（55.6%）が IDH1/2 の変異陽性となった。さらに、IDH1/2 の変異陽性群と IDH1/2 の変異陰性群の glioma の予後の比較を行ったところ、IDH1/2 の変異陽性群の予後が有意に良いことがわかった。

D．考察

IDH 遺伝子変異を有する急性骨髄性白血病モデルを用いて、変異型 IDH の発現ががんの維持に必須であることを証明した。これらの結果は、変異型 IDH が治療標的として妥当であることを示している。さらに独自に開発した変異型 IDH1 阻害剤が白血病細胞を顕著に減少させることを明らかにした。この結果は、変異型 IDH1 阻害剤に抗腫瘍効果をあること示している。

変異型 IDH1/2 を検出する方法としては、Sanger 法によるダイレクト DNA シークエンス法、パイロシークエンス法、免疫組織染色法などが存在する。ダイレクト DNA シークエンス法が多くの施設で実施されており、比較的安価で感度も高い反面、試料に含まれる IDH の 20%以上が変異型でないと、IDH1/2 の変異を検出することができない。また、パイロシークエンス法は、すべての変異型 IDH1/2 を高感度に検出できるが、特殊な機器を必要とするため、汎用性がないのが現状である。一方、免疫組織染色法は、感度・特異度が高く、どの施設でも実施可能であり汎用性が高い反面、免疫組織染色に有用な特異的抗体が必要となる。そこで本研究では、変異型 IDH1/2 に対する特異的抗体の開発を実施した。我々はこれまで、glioma で見られる変異型 IDH1/2 の中で最も頻度の高い（約 90%）、IDH1-R132H に対する特異的抗体（HMab-1）を樹立した。HMab-1 抗体は、gradeIII の glioma の予後診断に有用である。しかしながら、HMab-1 抗体は他の変異型 IDH1/2 を認識することができないため、約 10%の変異型 IDH1/2 を見逃すことになる。従って本研究では、複数の変異型 IDH1/2 を同時に認識する抗体の樹立を目指した。その結果樹立した MsMab-1 抗体は、IDH1-R132H 以外の変異型も認識し、HMab-1 抗体単独よりも、gradeIII の glioma の予後を有意に診断することが可能となった。HMab-1 抗体と MsMab-1 抗体の有用性を比較した結果、より多くの変異型 IDH1/2 を検出するという観点からは、MsMab-1 抗体が有用である。一方、MsMab-1 抗体を用いた免疫組織染色においては、非特異反応も検出される割合が HMab-1 抗体よりも多く、施設間でのばらつきが多くなることが懸念される。HMab-1 抗体は IDH1-R132H 特異的であるが、非特異反応が少ないため、施設間でのばらつきのない安定した結果が得られる可能性が高い。今後、コンパニオン診断薬の開発という観点から、よりばらつきの少ない安定した検出系の開発を行う必要がある。

E．結論

変異型 IDH が治療標的として妥当であり、変異型 IDH1 阻害剤は治療薬として有望である。また、HMab-1/MsMab-1 抗体の組み合わせによる免疫組織染色において、変異型 IDH1/2 を高感度に検出することが可能となり、診断薬への応用が期待できる。

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H. 知的所有権の出願・登録状況

1. 特許取得

【発明の名称】変異型イソクエン酸デヒドロゲナーゼ１阻害剤としてのイソキサゾール誘導体

【発明者】斉藤昭一、伊藤雅夫、藤沢哲則、斉藤博直、清塚洋平、渡邉秀昭、松永大典、小川原陽子、

別紙３

厚生労働科学研究委託費（革新的がん医療実用化研究事業）
委託業務成果報告（業務項目）

変異型 IDH 阻害剤の開発

担当責任者　北林　一生　　国立がん研究センター研究所　造血器腫瘍研究分野

研究要旨　　IDH 遺伝子変異を有する急性骨髄性白血病モデルマウスを開発し、変異型 IDH の発現ががんの維持に必須であることを証明した。がん特異的な変異型イソクエン酸デヒドロゲナーゼ 1（IDH1）に対する阻害剤を独自に開発した。急性骨髄性白血病モデルにおいて変異型 IDH1 阻害剤が、変異型 IDH により産生される腫瘍細胞内及び血漿中の 2-ハイドロキシグルタレイトの量をほぼ完全に抑制し、白血病細胞を顕著に減少させることを証明した。これらの結果は、変異型 IDH1 阻害剤が抗腫瘍効果をあること示している。変異型 IDH1 変異を有する脳腫瘍試料を免疫不全マウスの皮下に移植移植し、繰り返し移植可能な株を樹立した。

Ａ．研究目的

本研究では、がん特異的な変異型イソクエン酸デヒドロゲナーゼ 1（IDH1）を発現する悪性脳腫瘍、胆道がん、軟骨肉腫などの稀少がんの治療法及び診断法を確立し、3 年以内に臨床研究に進めることを目的とする。変異型 IDH 遺伝子変異を有する患者由来の組織を免疫不全マウスに移植した異種移植片(PDX)のマウスモデルを作製し、これらのマウスモデルに独自に開発した変異型 IDH 阻害剤を投与することにより、変異型 IDH の標的妥当性を検証するとともに、変異型 IDH 阻害剤の治療効果を検証し、生体内での薬物動態を調べることにより、治療薬としての最適化を進める。さらに、変異型 IDH を有する患者の早期診断のため、変異型 IDH を特異的に認識する抗体を作製し、治療対象患者の診断法を確立する。

Ｂ．研究方法

脳腫瘍・軟骨肉腫・骨肉腫・胆管がんの患者由来組織片を NOG マウスの皮下又は各組織に移植することにより、各がんの患者由来組織片移植（PDX）モデルを作製する。PDX モデルに変異型 IDH 阻害剤を投与し、腫瘍ボリュームの測定、染色像の観察、血漿中や腫瘍組織内の 2HG レベルの定量などを行い、変異型 IDH 阻害剤に対する感受性を調べる事により、脳腫瘍・軟骨肉腫・骨肉腫・胆管がん等における変異型 IDH の標的妥当性を検証すると共に、変異型 IDH 阻害剤の治療効果を確認する。

Ｃ．研究結果

IDH 遺伝子変異を有する急性骨髄性白血病モデルマウスを開発し、Cre-loxP システムを用いて変異型 IDH を除去すると急性骨髄性白血病の発症が抑制されることが示された。変異型 IDH を除去した白血病細胞を調べると、白血病幹細胞マーカーの発現が顕著に低下し、白血病誘導能が消失していることが明らかとなった。第一三共株式会社と共同で変異型 IDH1 に対する阻害剤を独自に開発した。急性骨髄性白血病モデルにおいて変異型 IDH1 阻害剤が、変異型 IDH により産生される腫瘍細胞内及び血漿中の 2-ハイドロキシグルタレイトの量をほぼ完全に抑制し、白血病細胞を顕著に減少させることを証明した。物質特許及びこれらの抗腫瘍効果を含む主要特許を出願した。「IDH1 変異を有するモデル評価系の作出と新規薬剤感受性の試験」に関する研究計画を国立がん研究センターの倫理審査員会に提出し承認を得た。３例の変異型 IDH1 変異を有する脳腫瘍試料を免疫不全マウスの皮下に移植移植し、繰り返し移植可能な株を１株樹立した。

Ｄ．考察

IDH 遺伝子変異を有する急性骨髄性白血病モデルを用いて、変異型 IDH の発現ががんの維持に必須であることを証明した。これらの結果は、変異型 IDH が治療標的として妥当であることを示している。さらに独自に開発した変異型 IDH1 阻害剤が白血病細胞を顕著に減少させることを明らかにした。この結果は、変異型 IDH1 阻害剤に抗腫瘍効果をあること示している。

Ｅ．結論

変異型 IDH が治療標的として妥当であり、変異型 IDH1 阻害剤は治療薬として有望である。

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H．知的所有権の出願・登録状況

1. 特許取得

【発明の名称】変異型イソクエン酸デヒドロゲナーゼ１阻害剤としてのイソキサゾール誘導体

【発明者】斉藤昭一、伊藤雅夫、藤沢哲則、斉藤博直、清塚洋平、渡邉秀昭、松永大典、小川原陽子、北林一生

【出願日】2014/10/1

【出願番号】特願2014-203475

別紙３

厚生労働科学研究委託費（革新的がん医療実用化研究事業）
委託業務成果報告（業務項目）

変異型 IDH を有する腫瘍の診断法の確立

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研究要旨　　イソクエン酸デヒドロゲナーゼ 1/2 (IDH1/2)は、low grade glioma において高頻度に変異が生じ、α-ケトグルタル酸を oncometabolite の 2-ヒドロキシグルタル酸(2-HG)に変換する。一方、野生型 IDH1/2 と比べ、変異型 IDH1/2 を保持する glioma の予後は格段に良い。本研究では、新規の変異型 IDH1/2 に対する新規の特異的モノクローナル抗体を樹立し、glioma の臨床診断に応用することを目指した。まず、IDH1-R132G の合成ペプチドをマウスに免疫し、常法によりハイブリドーマの作製、ELISA 法による選択を行った。さらにウェスタンブロット法、免疫組織染色法にて、樹立した抗体の評価を行った結果、新たに樹立した MsMab-1 抗体は、複数の変異型 IDH1/2 に交差反応性を示すことがわかった。この MsMab-1 抗体と以前に作製した HMab-1 抗体を組み合わせることにより、IDH1-R132H だけでなく、複数の変異型 IDH1/2 を高感度に検出することができ、glioma の診断に有用であることがわかった。

A．研究目的

イソクエン酸デヒドロゲナーゼ 1/2 (IDH1/2)は、low grade glioma において高頻度に変異が生じ、α-ケトグルタル酸を oncometabolite の 2-ヒドロキシグルタル酸(2-HG)に変換する。一方、野生型 IDH1/2 と比べ、変異型 IDH1/2 を保持する glioma の予後は格段に良い。glioma 以外では、急性骨髄性白血病、軟骨系腫瘍、胆管がんなどにも変異型 IDH1/2 が発見されている。本研究では、変異型 IDH1/2 に対する新規の特異的モノクローナル抗体を樹立し、変異型 IDH1/2 を保有する腫瘍の臨床診断に応用することを目指した。

B．研究方法

IDH1-R132G の合成ペプチドをマウスに免疫し、常法によりハイブリドーマの作製、ELISA 法による選択を行った。まず、IDH1-R132G に反応し、野生型 IDH1 に反応しない複数のクローンを選択した。さらに、IDH1 の R132 を 19 種類のアミノ酸に変換したペプチド、IDH2 の R172 を 19 種類のアミノ酸に変換したペプチドを合成し、複数の変異型に反応性を示すクローンを選択した。また、ウェスタンブロット法、免疫組織染色法にて、樹立した抗体の評価を行った。最後に、我々が以前樹立した、IDH1-R132H に特異的な抗体である HMab-1 抗体と組み合わせ、glioma 患者の予後診断における有用性を評価した。

C．研究結果

IDH1-R132G に反応し、野生型 IDH1 に反応しない複数のクローンを ELISA 法により選択した。次に、複数の変異型 IDH1/2 に対する ELISA を実施したところ、新規に樹立した MsMab-1 抗体（IgG_1, kappa）は、複数の変異型 IDH1/2 に交差反応性を示すことがわかった。ウェスタンブロット法により、IDH1-R132H 、 IDH1-R132S 、 IDH1-R132G 、IDH2-R172M、IDH2-R172S、IDH2-R172G のリコンビナントタンパク質に対する反応性が見られた。さらに、免疫組織染色においても、MsMab-1 抗体は IDH1-R132H、IDH1-R132S 等の変異型 IDH1/2 を保有する glioma に対して高い反応性を示した。以上のことから、MsMab-1 抗体は、変異型 IDH1/2 陽性の glioma の診断マーカーとして有用であることがわかった。

この MsMab-1 抗体と以前に作製した HMab-1 抗体（IDH1-R132H に対する特異的抗体）を組み合わせ、免疫組織染色を実施した結果、54 症例の gradeIII の glioma のうち、30 症例（55.6%）が IDH1/2 の変異陽性となった。さらに、IDH1/2 の変異陽性群と IDH1/2 の変異陰性群の glioma の予後の比較を行ったところ、IDH1/2 の変異陽性群の予後

が有意に良いことがわかった。

D．考察

変異型 IDH1/2 を検出する方法としては、Sanger 法によるダイレクト DNA シークエンス法、パイロシークエンス法、免疫組織染色法などが存在する。ダイレクト DNA シークエンス法が多くの施設で実施されており、比較的安価で感度も高い反面、試料に含まれる IDH の 20%以上が変異型でないと、IDH1/2 の変異を検出することができない。また、パイロシークエンス法は、すべての変異型 IDH1/2 を高感度に検出できるが、特殊な機器を必要とするため、汎用性がないのが現状である。一方、免疫組織染色法は、感度・特異度が高く、どの施設でも実施可能であり汎用性が高い反面、免疫組織染色に有用な特異的抗体が必要となる。そこで本研究では、変異型 IDH1/2 に対する特異的抗体の開発を実施した。

我々はこれまで、glioma で見られる変異型 IDH1/2 の中で最も頻度の高い（約 90%）、IDH1-R132H に対する特異的抗体（HMab-1）を樹立した。HMab-1 抗体は、gradeIII の glioma の予後診断に有用である。しかしながら、HMab-1 抗体は他の変異型 IDH1/2 を認識することができないため、約 10%の変異型 IDH1/2 を見逃すことになる。従って本研究では、複数の変異型 IDH1/2 を同時に認識する抗体の樹立を目指した。その結果樹立した MsMab-1 抗体は、IDH1-R132H 以外の変異型も認識し、HMab-1 抗体単独よりも、gradeIII の glioma の予後を有意に診断することが可能となった。

HMab-1 抗体と MsMab-1 抗体の有用性を比較した結果、より多くの変異型 IDH1/2 を検出するという観点からは、MsMab-1 抗体が有用である。一方、MsMab-1 抗体を用いた免疫組織染色においては、非特異反応も検出される割合が HMab-1 抗体よりも多く、施設間でのばらつきが多くなることが懸念される。HMab-1 抗体は IDH1-R132H 特異的であるが、非特異反応が少ないため、施設間でのばらつきのない安定した結果が得られる可能性が高い。今後、コンパニオン診断薬の開発という観点から、よりばらつきの少ない安定した検出系の開発を行う必要がある。

E．結論

HMab-1/MsMab-1 抗体の組み合わせによる免疫組織染色において、変異型 IDH1/2 を高感度に検出することが可能となった。また、glioma の予後診断にも有用であることから、診断薬への応用が期待できる。

G．研究発表

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Liu X, Ogasawara S, Kaneko MK, Oki H, Hozumi Y, Goto K, Takagi M, Kato Y. A novel monoclonal antibody SMab-2 recognizes endogenous IDH2-R172S of chondrosarcoma Biochem Biophys Res Commun., in press

H．知的所有権の出願・登録状況

なし

様式第１９

学　会　等　発　表　実　績

委託業務題目「変異型IDHを標的とした悪性脳腫瘍・肉腫・胆管がんに対する革新的治療法の開発」
機関名　独立行政法人国立がん研究センター

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
Clonal evolution of stem cells and therapeutic strategy in acute myeloid leukemia（シンポジウム）	北林一生	第73回日本癌学会	2014年9月	国内
IDH mutant regulates metabolism and hypoxia pathway in AML（シンポジウム）	小川原陽子、北林一生	第76回日本血液学会	2014年11月	国内
変異型IDHの急性骨髄性白血病における機能（シンポジウム）	小川原陽子、北林一生	第87回日本生化学会	2014年10月	国内
がん幹細胞成立と維持における変異型IDHの役割と治療（シンポジウム）	北林一生	第87回日本生化学会	2014年10月	国内
Epigenetic regulation of stem cells in acute myeloid leukemia（シンポジウム）	北林一生	第37回日本分子生物学会	2014年11月	国内
神経膠腫におけるIDH1/2 変異に基づく治療戦略について（口頭）	成田善孝、大野誠、松下裕子、呂北康二、高橋雅道、小川隆弘、市村幸一	第15回日本分子脳外科学会	2014年9月	国内

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
IDH1/2 mutation detection in gliomas	Arita H, Narita Y, Yoshida A, Hashimoto N, Yoshimine T, Ichimura K.	Brain tumor pathology	2014	国外
Development of a robust and sensitive pyrosequencing assay for the detection of IDH1/2 mutations in gliomas	Arita H, Narita Y, Matsushita Y, Fukushima S, Yoshida A, Takami H, Miyakita Y, Ohno M, Shibui S, Ichimura K.	Brain tumor pathology	2014	国外
IDH2 and NPM1 mutations cooperate to activate Hoxa9/Meis1 and hypoxia pathways in acute myeloid leukemia.	Ogawara Y, Takuo Katsumoto T, Aikawa Y, Shima Y, Kagiyama Y, Soga T, Matsunaga H, Seki T, Araki K, Kitabayashi I	Cancer Res.	2015	国外
Bromodomain-PHD finger protein 1 is critical for leukemogenesis associated with MOZ-TIF2 fusion.	Shima H, Yamagata K, Aikawa Y, Shino M, Koseki H, Shimada H, Kitabayashi I.	Int. J. Hematology	2014	国外
The nuclear export signal (NES) within CALM is necessary for CALM-AF10-induced leukemia	Suzuki M, Yamagata K, Shino M, Aikawa Y, Akashi K, Watanabe T, Kitabayashi I	Cancer Sci.	2014	国外
MLL fusion proteins link transcriptional coactivators to previously active CpG-rich promoters	Okuda H, Kawaguchi M, Kanai A, Matsui H, Kawamura T, Inaba T, Kitabayashi I, Yokoyama A	Nucleic Acids Res.	2014	国外
The TIF1b-HP1 System Maintains Transcriptional Integrity of Hematopoietic Stem Cells	Miyagi S, Koide S, Saraya A, Wendt GR, Oshima M, Konuma T, Yamazaki S, Mochizuki-Kashio M, Nakajima-Takagi Y, Wang C, Chiba T, Kitabayashi I, Nakauchi H, Iwama A	Stem Cell Reports	2014	国外
Loss of NDRG2 expression activates PI3K-AKT signalling via PTEN phosphorylation in ATLL and other cancers.	Nakahata S, Ichikawa T, Maneesaay P, Saito Y, Nagai K, Tamura T, Manachai N, Yamakawa N, Hamasaki M, Kitabayashi I, Arai Y, Kanai Y, Taki T, Abe T, Kiyonari H	Nat Commun.	2014	国外
Hes1 suppresses acute myeloid leukemia development through FLT3 repression	Kato T, Sakata-Yanagimoto M, Nishikii H, Ueno M, Miyake Y, Yokoyama Y, Asabe Y, Kamada Y, Muto H, Obara N, Suzukawa K, Hasegawa Y, Kitabayashi I, Uchida K, Hirao	Leukemia	2015	国外
Detection of the G17V RHOA mutation in angioimmunoblastic T-cell lymphoma and related lymphomas using quantitative allele-specific PCR	Nakamoto-Matsubara R, Sakata-Yanagimoto M, Enami T, Yoshida K, Yanagimoto S, Shiozawa Y, Nanmoku T, Satomi K, Muto H, Obara N, Kato T, Kurita N, Yokoyama Y, Izutsu K, Ota Y, Sanada M, Shimizu S, Komeno T, Sato Y, Ito T, Kitabayashi I, Takeuchi K, Nakamura N, Ogawa S, Chiba S	PLoS One	2014	国外
Essential role of PU.1 in maintenance of MLL-associated leukemia stem cells	Aikawa Y, Yamagata K, Katsumoto T, Shima Y, Shino M, Stanley ER, Cleary ML, Akashi K, Tenen DG, Kitabayashi I	Cancer Sci.	2015	国外
Specific monoclonal antibodies against IDH1/2 mutations as diagnostic tools for gliomas	Kato Y.	Brain Tumor Pathol.	2015	国外

Diagnostic advantage of double immunohistochemistry using two mutation-specific anti-IDH antibodies (HMab-1 and MsMab-1) in gliomas	Takano S, Kato Y, Yamamoto T, Liu X, Ishikawa E, Kaneko MK, Ogasawara S, Matsuda M, Noguchi M, Matsumura A	Brain Tumor Pathol.	2014	国外
A novel monoclonal antibody SMab-2 recognizes endogenous IDH2-R172S of chondrosarcoma	Liu X, Ogasawara S, Kaneko MK, Oki H, Hozumi Y, Goto K, Takagi M, Kato Y	Biochem Biophys Res Commun.	2015	国外

（注１）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。

（注２）本様式はexcel形式にて作成し、甲が求める場合は別途電子データを納入すること。

Brain Tumor Pathol
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REVIEW ARTICLE

IDH1/2 mutation detection in gliomas

Hideyuki Arita · Yoshitaka Narita · Akihiko Yoshida · Naoya Hashimoto · Toshiki Yoshimine · Koichi Ichimura

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Abstract Somatic mutations of isocitrate dehydrogenase 1 and 2 (*IDH1/2*) are strongly associated with pathological subtypes, genetic profiles, and clinical features in gliomas. The *IDH1/2* status is currently regarded as one of the most important molecular markers in gliomas and should be assessed accurately and robustly. However, the methods used for *IDH1/2* testing are not fully standardized. The purpose of this paper is to review the clinical significance of *IDH1/2* mutations and the methods used for *IDH1/2* testing. The optimal method for *IDH1/2* testing varies depending on a number of factors, including the purpose, sample types, sample number, or laboratory equipment. It is therefore important to acknowledge the advantages and disadvantages of each method.

Keywords Glioma · *IDH1* · *IDH2*

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Introduction

Somatic mutations of isocitrate dehydrogenase 1 (*IDH1*, 2q34) were first identified in a small subset of glioblastomas [1, 2]. A large number of studies soon followed, reporting that *IDH1* mutations actually occur in the majority of lower grade gliomas [2–6]. Later, mutations of isocitrate dehydrogenase 2 (*IDH2*, 15q26) were also reported in a small number of gliomas [3, 4]. *IDH1/2* mutations are currently regarded as the earliest event in gliomagenesis and one of the most significant genetic alterations in glioma biology [4]. *IDH1/2* mutations also seem to be clinically significant based on their relationship with pathology, other genetic changes, and clinical presentation. A rapidly growing number of published studies constantly provide new information about *IDH1/2*, and *IDH1/2* status is almost routinely evaluated in laboratories. However, the methods for *IDH1/2* testing are not fully standardized. The purpose of this report is to review the clinical significance of *IDH1/2* mutation and the methods used for testing these mutations.

Genetic aspect of *IDH1/2* mutations

Mutational pattern of *IDH1/2* in gliomas (Fig. 1)

All reported *IDH1/2* mutations are missense mutations. They are almost always hemizygous and mutually exclusive to each other. IDH1 and IDH2 are homologous enzymes localized in the cytosol and mitochondria, respectively. IDH1/2 catalyze the conversion of isocitrate to α-ketoglutarate using $NADP^+$ as a cofactor. The *IDH1/2* mutations in gliomas result in the reduced ability to produce α-ketoglutarate and the acquisition of abnormal

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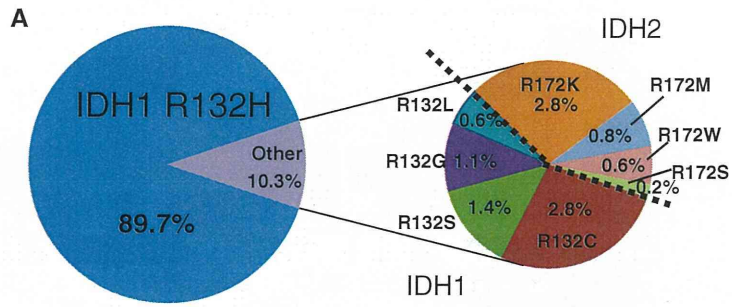

B

IDH1

Codon 132		
CGT		WT
CAT	c.394C>A	R132H
TGT	c.394C>T	R132C
GGT	c.394C>G	R132G
AGT	c.394C>A	R132S
CTT	c.395G>T	R132L
GTT	c.394C>G, c.395G>T	R132V

IDH2

Codon 172		
AGG		WT
AAG	c.515G>A	R172K
ATG	c.515G>T	R172M
TGG	c.514A>T	R172W
AGC	c.516G>C	R172S

Fig. 1 **a** Frequency of each mutation in *IDH1/2*. The ratio was calculated from the pooled data from eight independent studies, including the data on a total of 3,029 glioma cases [3, 6, 9–14]. The *left* circular chart represents the frequency of *IDH1* R132H mutation and non-R132H *IDH1/2* mutations. The *right chart* represents the frequency of each type of non-R132H *IDH1/2* mutation. **b** The nucleotide transitions in *IDH1/2*. All the mutations except R132V are point mutations

function to convert α-ketoglutarate into (R)-2-hydroxyglutarate ((R)-2HG) by using NADPH as a cofactor [7, 8]. (R)-2HG is considered to be a major oncometabolite, causing various biological effects on *IDH1/2* mutated tumors. *IDH1/2* mutations are therefore considered to be gain-of-function alterations [7].

The frequency of each mutation type varies according to the reports. The frequency of each mutation in a pooled data from 8 independent studies, describing the mutation types are shown in Fig. 1a [3, 6, 9–14]. The most common *IDH1/2* mutation in gliomas is c.395G>A transition in *IDH1*, which replaces the arginine with a histidine at codon 132 (R132H) (Fig. 1b). Other less common mutations also occur at codon 132 in *IDH1* or codon 172 in *IDH2*. The second most frequent mutations are R132C in *IDH1* and R172K in *IDH2* (2.8 % of all mutations, respectively). Other mutations include R132S, R132G, and R132L in *IDH1* and R172M, R172W, and R172S in *IDH2*. Extremely rare mutations include R132P, R132V, or those affecting R49, G97, and R100 in *IDH1* and R172T in *IDH2* ([15, 16] and references therein). The mutations affecting R140 in *IDH2* are among the most common mutations in myeloproliferative neoplasms; however, these are not observed in gliomas [8, 17].

Thus, the great majority of *IDH1/2* mutations involve codon 132 in *IDH1* or codon 172 in *IDH2*, and approximately 90 % of them are R132H in *IDH1*.

The relationship with other genetic alterations

IDH1/2 mutations show a distinct pattern with other genetic alteration characteristics in diffuse gliomas.

Genetic changes

IDH1/2 mutations are frequently observed in grade II–III gliomas, as described above, and are mostly associated with either *TP53* mutations or total 1p19q loss [4] (Fig. 2). *TP53* mutations are typically observed in grade II–III astrocytomas with *IDH1/2* mutations, and *ATRX* mutations are commonly observed in these populations [18]. Total 1p19q loss almost always coexists with *IDH1/2* mutations and *TERT* promoter mutations. This combination is typically observed in oligodendroglial tumors [9, 19]. *TP53* mutations and total 1p19q loss are mutually exclusive [4]. Similarly, *ATRX* and *TERT* promoter mutations also show mutually exclusive patterns [12, 19]. Thus, the combination of *TP53-ATRX* mutations or total 1p19q loss-*TERT* mutations is the hallmark of astrocytic or oligodendroglial tumors harboring *IDH1/2* mutations, respectively.

Epigenetic changes

Another important feature in *IDH1/2* mutated tumors is the epigenetic changes, which are some of the most

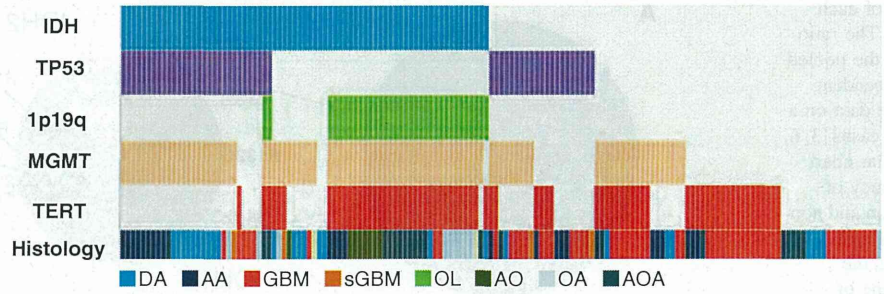

Fig. 2 Relationships among *IDH1/2* mutations, other genetic alterations, and histological subtypes. *Each column* represents individual tumors. *IDH1/2* mutations are strongly associated with *TP53* mutation or total 1p19q loss. Most *IDH1/2* mutated tumors show *MGMT* methylation. The data for the cases from National Cancer Center (Tokyo, Japan) are extracted and modified from the study by Arita et al. [9]. *DA* diffuse astrocytoma, *AA* anaplastic astrocytoma, *GBM* primary glioblastoma, *sGBM* secondary glioblastoma, *OL* oligodendroglioma, *AO* anaplastic astrocytoma, *OA* oligoastrocytoma, *AOA* anaplastic astrocytoma

fundamental alterations induced by these mutations. *IDH1/2* mutations have strongly been associated with the glioma-CpG island methylator phenotype (G-CIMP) and aberrant histone methylation [20, 21]. The mechanisms involved in the changes in DNA methylation and histone methylation status caused by *IDH1/2* mutations have been extensively discussed in several reviews [16, 22]. Thus, we only briefly summarized them in this review.

G-CIMP

G-CIMP was identified as a phenomenon in which DNA methylation in the CpG islands is increased genome-wide in a subset of gliomas [20]. It is well documented that G-CIMP is tightly associated with the presence of *IDH1/2* mutations and relative absence of typical copy-number alterations normally observed in glioblastomas, including *EGFR* amplification and chromosomal arm 10q loss. The tumors with G-CIMP present gene expression profiles of the proneural type [20]. DNA hypermethylation in these loci is considered to downregulate the expression of target genes, some of which may act as a tumor suppressor.

Aberrant (R)-2HG production in *IDH1/2* mutated tumors may inhibit α-ketoglutarate-dependent dioxygenase family, including ten–eleven-translocation (TET) [23]. The TET family catalyzes the conversion of 5-methylcytosine (5mC) to 5-hydroxymethycytosine (5hmC), which is a critical step in demethylating methylcytosine in the CpG dinucleotides, the main target of DNA methylation [24]. Inhibition of TET2 by (R)-2HG may possibly contribute to global DNA methylation in G-CIMP [21].

Histone modification

In *IDH1/2* mutated tumors, trimethylation at lysine residues of histone H3, including H3K9, H3K27, and H3K79 are increased [23]. Histone methylation is regulated by histone methyltransferase and demethylases and may affect gene transcription by altering chromatin structures. A Fe(II) and α-ketoglutarate-dependent subset of histone demethylase (e.g., lysine (K)-specific demethylase 6A (KDM6A)) is inhibited by (R)-2HG in *IDH1/2* mutated tumors, resulting in global alterations of histone demethylation and gene expression [23].

MGMT

CpG island methylation in O^6-methylguanine-DNA methyltransferase (*MGMT*) has also been associated with *IDH1/2* mutations [25, 26]. MGMT is a DNA-repair protein that removes alkyl adducts from O^6 position of guanine. Increased activity of MGMT reduces the chemosensitivity of alkylating agents, including temozolomide because O^6 position of guanine is the main target of DNA alkylation. *MGMT* methylation results in reduced MGMT expression and may lead to better response to temozolomide in glioblastoma [27]. *MGMT* methylation is invariably observed in *IDH1/2* mutated tumors [26]. On the other hand, about half of glioblastomas harbor *MGMT* methylation, regardless of the *IDH1/2* status (Fig. 2).

Clinical value

The relationship with pathology

In the pooled data from the eight reports (see above), *IDH1/2* mutations were observed frequently in grade II–III astrocytomas and oligodendrogliomas (53–83 %) as well as in secondary glioblastomas (54 %), but rarely in primary glioblastomas (6.3 %). *IDH1/2* mutations are highly specific to diffuse gliomas among CNS tumors. Other neuroepithelial tumors, including pilocytic astrocytoma, ependymoma, and ganglioglioma, rarely harbor *IDH1/2* mutations [15]. Non-neoplastic lesions mimicking gliomas never present these mutations [28]. The presence of *IDH1/*

2 mutations is a strong evidence of diffuse gliomas even in such cases (discussed further below).

Prognostic value

Several studies have suggested that patients with *IDH1/2* mutated tumor show longer survival than those with *IDH1/2* wild-type tumor, in most entities of gliomas [29].

The favorable prognosis of *IDH1/2* mutated glioblastomas was first reported in the pioneering study about *IDH1* [1], which was then followed by several other studies [3, 4, 14, 25, 30, 31]. *IDH1/2* mutations are generally regarded as a positive prognostic factor in glioblastomas. The overall survival in *IDH1/2* mutated cases is about twice longer than that of *IDH1/2* wild-type cases (24–31 vs. 9.9–15 months) [3, 25, 30]. Using a multivariate analysis, some studies have shown that *IDH1/2* status is an independent prognostic factor in glioblastomas [25], while others failed to reproduce this finding [31]. The small population of *IDH1/2* mutated glioblastomas might cause this controversy.

Several studies have reported that *IDH1/2* status is also a prognostic factor in grade III gliomas [14, 25, 32]. Some reports even demonstrated the positive prognostic value of *IDH1/2* mutations in each subtype: anaplastic astrocytomas [3] or anaplastic oligodendroglial tumors [33].

The prognostic value of *IDH1/2* status in grade II gliomas remains under debate. Some reports associated the presence of *IDH1/2* mutations with better prognosis [25, 34–37], while others did not [14, 38]. Sun et al. [39] investigated the prognostic value of *IDH1/2* status through a meta-analysis of ten previous studies and found that *IDH1/2* mutation was associated with longer survival in grade II gliomas. They pointed out several problems in the interpretation of currently available data, which include mixed cohorts of astrocytomas and oligodendroglial tumors, the close relationship with other prognostic factors, different methodology to evaluate *IDH1/2* status, and most importantly the lack of standard treatment in grade II gliomas. There are also other studies investigating the prognostic value in each subtype (i.e., astrocytomas or oligodendrogliomas); however, they remain inconclusive [14, 34, 35].

Overall, the independent value of *IDH1/2* as a biomarker remains somewhat controversial. This can be attributed to confounding factors or the study design as pointed out by Sun et al. [39] in their analysis of grade II gliomas. *IDH1/2* mutations are closely related to other prognostic/predictive factors, including patient age, *MGMT* status, or 1p19q copy number. Limited cohort size, different treatment, and the heterogeneity of tumor subtypes in each study may also result in conflicting results. Nonetheless, the *IDH1/2* status adds valuable information to the WHO grades in predicting the clinical course, and it should be considered as a stratification factor in clinical trials of gliomas [40].

Molecular classification and *IDH1/2*

One of the aims of the current WHO classification is to predict the clinical outcome of the patients harboring the tumor [41]. Nonetheless, the current diagnostic system poses the limitation that one type of tumor can include biologically and clinically different subsets of tumors. The molecular classification is expected to refine the current diagnostic system [42]. *IDH1/2* mutations present a strong association with the histological types and clinical outcome as described above. Therefore, these mutations are among the most promising markers.

The existence of *IDH1/2* mutations strongly supports the diagnosis of grade II–III gliomas. Intriguingly, Hartmann and colleagues reported that patients with *IDH1* wild-type anaplastic astrocytomas exhibited shorter survival than those with *IDH1*-mutated glioblastomas [43]. Underestimation of the tumor grades in histological diagnosis may lead to this observation along with the prognostic impact of the *IDH1* mutation itself. Malignant gliomas are histologically heterogeneous, and missampling can lead to undergrading [43]. Their findings argue for the significant value of *IDH1/2* mutation in the molecular classification combined with the current histological classification.

Combination of *IDH1/2* and other genetic status may aid in further predicting the subtypes of gliomas, because *IDH1/2* mutations show strong relationships with either the combination of *TP53-ATRX* mutations or total 1p19q loss-*TERT* mutation in astrocytic or oligodendroglial tumors, as described above.

The molecular diagnosis seems promising; however, it poses some significant limitations. The evaluation for molecular markers often needs DNA analysis, which requires expensive equipment and reduces the feasibility of the method in clinical use. Another issue is that standardization of the testing for each marker is needed. For example, *MGMT* methylation status is evaluated using various methods, including methylation-specific PCR (MSP) or pyrosequencing. However, these tests have not yet been standardized. Availability of molecular markers requires the accessibility of detection methods and their standardization.

IDH1/2 detection

IDH1/2 mutations undoubtedly divide diffuse gliomas into two groups, which have distinct biological and clinical features, as described above. *IDH1/2* mutations also have a

Table 1 The methods for *IDH1/2* detection

	Advantage	Limitation
Sanger sequencing	Gold standard	Modestly sensitive (>20 % of mutant allele is required)
	Detects all types of mutations	
Immunohistochemistry	Sensitive and robust	Detects only the mutation specific to the antibody used
	Available for FFPE samples without additional treatment	
Pyrosequencing	Sensitive	Needs special equipment
	Quantitative	The robustness depends on the assay design
Melting curve analysis	Rapid	Needs special equipment
	Detects all types of mutations	Modestly sensitive (improved by combining with COLD-PCR)
MRS	Noninvasive	Not validated and standardized for clinical use

MRS magnetic resonance spectroscopy, *FFPE* formalin-fixed paraffin-embedded, *COLD-PCR* co-amplification at lower denaturation temperature-polymerase chain reaction

significant impact on glioma diagnosis. Their highly specific distribution in grade II–III diffuse gliomas among CNS tumors also indicates that the presence of *IDH1/2* mutation is almost sufficient for the diagnosis of diffuse glioma, although the absence of these mutations does not exclude the diagnosis of gliomas [28]. Equivocal microscopic diagnosis can be derived from various factors, including small sample size (i.e., obtained by needle biopsy), sampling site (i.e., from infiltrative zone apart from the tumor core), or sample quality. The *IDH1/2* status may provide clinically important information in such cases. *IDH1/2* status therefore needs to be evaluated accurately.

IDH1/2 testing includes various methods targeting DNA sequence, mutant protein, or aberrant increase in (R)-2HG levels. However, *IDH1/2* testing should be easy to be incorporated into daily diagnostic practice. Sanger sequencing and immunohistochemistry (IHC) are conventionally applied for the assessment of *IDH1/2* status. Each assay has advantages and limitations (Table 1). Some of the currently available methods for *IDH1/2* testing are reviewed below.

Sanger sequencing

Sanger sequencing is the gold standard for detecting *IDH1/2* mutations, and most of the published data are based on this method [1–3]. This technique can detect all types of *IDH1/2* mutations and, if the mutation is detected, the result is reliable.

However, this technique requires sophisticated equipment and trained personnel [44]. Complicated procedures including DNA extraction, polymerase chain reaction (PCR), or purification of PCR products need to be optimized. The difference in the equipment or procedure may yield inconsistent results between laboratories [45].

Another caveat is that the results of this method largely depend on the tumor cell content of the samples. The source of non-neoplastic DNA includes adjacent normal brain, infiltrating lymphocytes, and microglia or endothelial cells, which may dilute mutant alleles and cause false-negative results [15]. At least 20 % of the mutant allele is required for detection by Sanger sequencing in our analysis, evaluating the sensitivity of *IDH1/2* detection [46] (Fig. 3a). Diagnosis for small samples obtained from tumor margin is challenging, but clinically important. The critical limitation of this method is its relatively low sensitivity, which might lead to missing out *IDH1/2* mutations.

Immunohistochemistry (IHC)

IHC is universally performed in clinical practice, and the IHC-based mutation detection is one of the most accessible technologies.

The specific antibodies for IDH1 R132H mutation, monoclonal antibody (mAb) H09 and Imab-1, are well characterized and commonly used [47, 48]. The cytoplasm of tumor cells with IDH1 R132H mutation is strongly stained, while tumor cells without this mutation are not stained (Fig. 3b). These antibodies are highly specific to the mutant protein, and residual brain tissues, including reactive glia, endothelial cells, or blood cells are not stained [47–49]. A weak diffuse background staining and a strong granular cytoplasmic staining of macrophages are also observed in IDH1 wild-type tumors. Meningiomas and schwannomas can show nonspecific positive-stained fibers. These patterns can be easily recognized and distinguished from the true-positive staining [49]. Based on these criteria, the specificity for IDH1 R132H mutation is considered to be nearly 100 % [50]. A cross-reactivity for R132L mutant has been reported [44]; however, this would not cause misdiagnosis.

This method is highly accurate. It can detect tumor cells with *IDH1/2* mutation in even tissues containing 6–9 % mutant allele [46, 51]. It has been claimed that the antibody can stain single cells reliably even in the infiltration zone

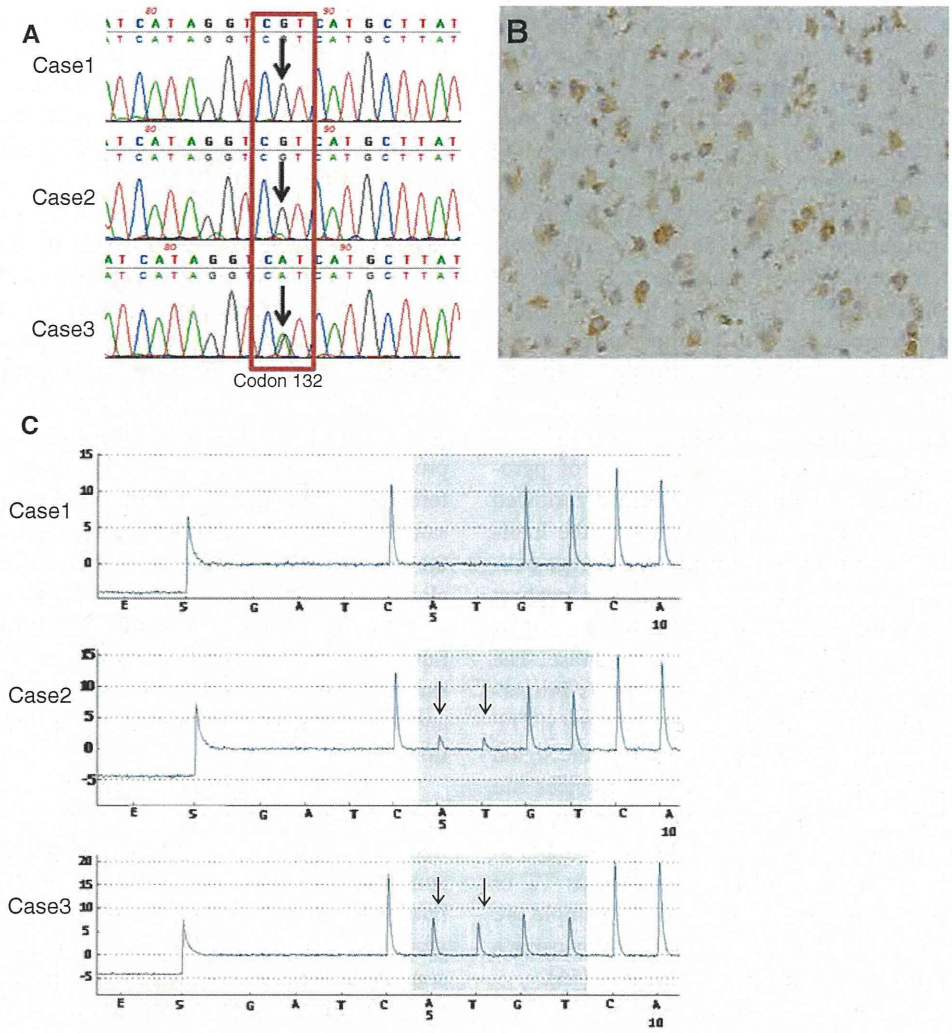

Fig. 3 **a** Sanger sequencing. Chromatograms of Sanger sequencing for *IDH1* in representative cases. The three cases are all anaplastic astrocytoma cases. *Case 1* presents a wild-type *IDH1,* while *cases 2* and *3* present the R132H mutant of *IDH1*. In case 3, a peak of adenine (*green*) indicates the c.395G>A transition. In case 2, a peak indicating the mutation is too low to be distinguished from other nonspecific peaks. **b** Immunohistochemistry. Immunohistochemical staining using anti-*IDH1* R132H mutant antibody. The cytoplasm of tumor cells with IDH R132H is strongly stained, while endothelial cells are not stained. (Original magnification ×200). **c** Pyosequencing. Pyrograms for *IDH1* in representative cases. The *arrows* indicate the specific peaks for the R132H mutants. The cases and analyzed samples are identical to those used for Sanger sequencing. The quantitative analysis of pyrosequencing reported that the frequency of R132H mutant alleles in each case was 0, 16, and 45 %, respectively. In case 2, the R132H mutation is apparent in pyrograms, although the result of Sanger sequencing is inconclusive

and that it enables differentiation of tumor cells from reactive glia [50]. The results of IHC using IDH1 R132H-specific antibodies are consistent across laboratories, even though different staining procedures were used [45].

The obvious limitation is that these antibodies cannot detect non-R132H mutations in IDH1/2 that correspond to approximately 10 % of all the IDH1/2 mutations [50]. Antibodies specific for other IDH1/2 mutations than R132H have also been developed and some of them are commercially available for IHC [52, 53]. A multispecific anti-mutated IDH1/2 antibody recognizing a subset of IDH1 and IDH2 mutations has also been reported [54]. The reactivity of this antibody varies depending on the methodology used, IHC, enzyme-linked immunosorbent assay (ELISA), and/or Western blotting. These approaches, especially the use of the multispecific anti-mutated IDH1/2 antibody, need to be further validated for clinical application.

Pyrosequencing

The principle of this method is a sequencing-by-synthesis analysis based on the real-time detection of nucleotide incorporation by DNA polymerase [55]. In practice, a DNA template amplified by PCR is hybridized to a primer for

pyrosequencing after purification, followed by pyrosequencing reactions using a pyrosequencer. Pyrosequencing reactions consist of four reactions: a DNA polymerase reaction, a sulfyrase reaction, a luciferase reaction, and nucleotide degradation by apyrase. Pyrosequencing reagents include the enzymes and the substrates for these reactions as well as adenosine 5′ phosphosulfate (APS) and luciferin (the four enzyme system) [56]. After adding the substrates and enzymes to the templates, each deoxynucleotide triphosphate (dNTP) is added to the samples stepwise, as programmed by the operator (dispensation order). If the injected dNTP is complementary to the template, the dNTP is incorporated by the DNA polymerase and the pyrophosphate is released. The concentration of ATP is then increased through the conversion of pyrophosphate and APS to ATP by the ATP sulfyrase, followed by the luciferase reaction. The light emitted by the luciferase reaction is quantitatively detected by a charge-coupled device (CCD) camera and represented as a peak at each nucleotide dispensation in the pyrogram. The unincorporated dNTP is rapidly degraded by the apyrase. The signal strength of the pyrosequencing reaction is proportional to the amount of pyrophosphate released and dNTP incorporated; hence the allele dosage. Therefore, the signal strength can be decreased when different sequences are mixed (i.e., heterozygous mutations) and also proportionally increased in a sequence containing homopolymers (a continuous stretch of the same nucleotide such as TT or CCC). The allele frequencies contained in the sample are automatically calculated from the signal strength by using a software developed for pyrosequencing analysis [56].

Several studies have applied pyrosequencing for *IDH1* testing [46, 57–59], some of which have validated the advantages of this technique over Sanger sequencing.

This method is highly sensitive. The minimum detectable frequency of the mutant allele is 5–7 % for pyrosequencing [57, 58], while Sanger sequencing sensitivity is at least 20 % for reliable detection (Fig. 3a, c) [15, 46]. Fragmented DNA template from the formalin-fixed paraffin-embedded (FFPE) specimen can be utilized for analysis [57, 59], as pyrosequencing allows the use of DNA templates under 100 bp [46].

Pyrosequencing requires expensive equipment and is available only in limited centers and laboratories. The robustness of pyrosequencing depends on the assay design, including the primers and the dispensation order [46].

Melting curve analysis

Melting curve analysis is a technique that allows the generation of a melting temperature profile of the double-stranded DNA. The melting temperature is unique to each nucleotide sequence, and even a single nucleotide substitution could alter the melting temperature. Thus, a missense mutation can be accurately detected by measuring the melting curve. Two different variants of this method were reported as an application for *IDH1/2* testing: fluorescent melting curve analysis (FMCA) and high-resolution DNA melting (HRM) [60–62].

In studies using FMCA, the PCR product of the target sequence is hybridized with a pair of fluorescent probes designed to complement the sequence, including mutation hotspot or its adjacent sequence [61, 62]. A real-time PCR system detects the fluorescent change derived by the denaturation of the probes during the gradual heating. In a mutant sequence, lower temperature is required for denaturation, unlike that in a wild-type sequence, because the probe imperfectly binds the mutant sequence. These differences appear in the patterns of the melting curve. A sample with only wild-type alleles shows a single peak in the melting curve, while a sample containing a mutant allele shows an additional peak at lower temperature [62]. This technique is also reported to be highly sensitive and rapid in detecting *IDH1/2* status even in FFPE tissue samples; the entire duration of this assay is about 80 min, and the minimum amount of mutated allele for the detection is 10 % [62].

A fluorescent dye intercalating double-stranded DNA is used in HRM. The amplified template with a saturating fluorescent dye is first denatured by heating, and then annealing is performed at the lower temperature. The fluorescence of the double-stranded DNA reduces by the gradual heating in a melting curve analysis. The samples containing mutant sequences show different melting curves because of the formation of heteroduplexed DNA after the denaturation phase; a lower melting temperature is observed [63]. The detection limit of this assay after conventional PCR amplification is similar to that of Sanger sequencing (25 %) [60].

These methods detect all types of mutations by detecting the melting temperature specific to each mutation [60, 62]. The disadvantage is an unsatisfactory sensitivity, but the sensitivity can be improved by combining co-amplification at lower denaturation temperature PCR (COLD-PCR) (reviewed below). COLD-PCR HRM and FMCA assays allowed the detection of 2 or 1 % mutant allele, respectively [60, 61]. Another disadvantage is that these methods require expensive equipment, which is usually used for another purpose in clinical settings [62].

Co-amplification at lower denaturation temperature PCR (COLD-PCR)

COLD-PCR is a method used to amplify a specific allele with mutation selectively. COLD-PCR itself is not an independent technique for DNA analysis, and this