A NEW IDH1/2 PCR ASSAY FOR ONE-STEP DETECTION OF 12 IDH1 AND IDH2 MUTATIONS IN GLIOMA

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BACKGROUND

- Isocitrate dehydrogenase (IDH) mutational status is a strong diagnostic and prognostic marker in glioma which will probably be introduced in the next WHO classification system.
- In addition to the established 1p/19q codeletion and MGMT methylation, a series of new biomarkers such as IDH1/2, EGFR or BRAF mutations, and FGFR gene fusions, are increasingly documented to play a role as prognostic or predictive markers, and should progressively be introduced in the diagnostic and treatment decision algorithm for glioma.
- Current IDH mutations screening is performed with an IHC assay specific for IDH1 R132H, the most common mutation. Sequencing is recommended as a second-step test for IHC-negative or -equivocal cases. However, sequencing is not readily accessible in all centers, and its use generally leads to additional delay in providing a comprehensive IDH1/2 mutational status assessment. Moreover, IDH sequencing procedures can sometimes lead to inter-laboratory variability.
- Beyond IDH1 R132H, 11 other IDH mutations, 6 IDH1 and 5 IDH2, have been reported so far in literature, in large cohorts of gliomas.
- Recent data indicate that mutant specific IDH1-inhibitors impair growth of mutant-IDH1 gliomas in mice.
- A real-time PCR assay was designed to detect the 12 IDH1/2 mutations in one single step in FFPE samples, and identify the most frequent ones.

OBJECTIVES

1. Establish the analytical performance of the new IDH1/2 PCR assay
2. Validate the IDH1/2 PCR assay performance on FFPE glioma clinical samples by comparing PCR IDH mutational status to IHC and Sanger sequencing.

MATERIAL & METHODS

IDH1/2 one-step qPCR assay:

- PCR Clamping was used for the qualitative detection of IDH1 R132H and 11 additional IDH1/2 mutations. ARMS PCR technology was combined to selectively identify the most frequent IDH1 (R132H / R132C) and IDH2 (R172K) mutations (**Table 1**).

Evaluation of analytical sensitivity:

- Limit of Detection (LOD) (min. % mutant DNA detected in a WT background) was determined following CLSI/NCCLS EP17-A guidelines.
- 5 low positive samples (2-5-10-15 and 20%) obtained by mixing IDH mutant plasmid DNA with glioma IDH1/2 WT DNA were tested per mutation (n= 30 to 110 measurements per mutation and mutation percentage)

Validation of the IDH1/2 PCR assay:

- **Samples**
  - 171 FFPE glioma samples: 121 samples retrospectively collected in a reverse chronological order from 2 academic centers (C1=102; C2=19) and 50 additional commercial samples. No specific selection criteria beyond tumor characteristics assessed by local pathologists.
  - Samples selection: < 10 yrs; ≥ 50mm² tissue area with ≥ 40% tumoral cells.
  - DNA extracted from 10 µm FFPE sections (QIAamp DNA FFPE Tissue Kit, Qiagen)
  - qPCRs performed on 25 ng DNA acc. to *therascreen* IDH1/2 RGQ PCR Kit IFU (Qiagen) and Rotor-Gene Q 5plex HRM instrument (Qiagen)
- **Molecular biology methods**
  - IHC performed locally using the IDH1 R132H monoclonal antibody Clone H09 (Dianova)
  - Bidirectional Sequencing (centrally) using recommended primers for IDH1 codon 132 and IDH2 codon 172 [3] and newly designed primers for IDH1 codon 100.
  - Discordant PCR/Sanger Sequencing cases additionally tested by pyrosequencing +/- LNA-based sequencing (centrally).
- **Rare mutations testing**
  - 22 synthetic samples (30% and 40% Mutant DNA in WT DNA) for the 11 rare mutations, processed similarly to clinical samples

RESULTS

IDH1/2 ASSAY DESIGN

Gene / Codon	Mutation	Base change
IDH1 / R132	R132H *	395 G>A
	R132C *	394 C>T
	R132S	394 C>A
	R132G	394 C>G
	R132L	394 G>T
	R132V	394_395 CG>GT
IDH1 / R100	R100Q	299 G>A
IDH2 / R172	R172K *	515 G>A
	R172M	515 G>T
	R172W	514 A>T
	R172S	516 G>T
	R172G	514 A>G

Table 1 - IDH1/2 mutations detected and identified * with the IDH1/2 PCR Assay

- Detection of 12 mutations by PCR CLAMPING:
  - 6 within IDH1 codon R132
  - 5 within the homologous codon 172 of IDH2
  - one within IDH1 codon 100
- Identification of 3 major IDH1/2 mutations by ARMS:
  - IDH1 R132H
  - IDH1 R132C
  - IDH2 R172K

ANALYTICAL SENSITIVITY

- Analytical sensitivity ranged from 0,6 % to 15% according to mutations (mean = 3.3 %)
- LOD was < 5% for 11/12 mutations and ≤ 3% for 9 of them.
- The identification of the 3 major IDH1/2 mutations showed very high sensitivity with LOD of 0.78% (R132H), 1.19% (R132C) and 0.61 % (R172K) respectively

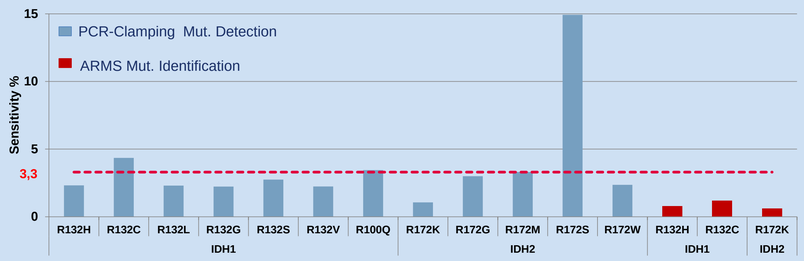

Fig 1 - IDH1/2 PCR test sensitivity

CLINICAL VALIDATION COHORT

- 147 samples met the selection criteria (103 academic and 44 commercial)
- The histological distribution reflected the observed subtypes in clinical routine in an academic center, with > 40% GBMs **(Fig 2)**
- IDH1/2 PCR technical success was 100% on samples collected in academic centers **(Fig 3)**

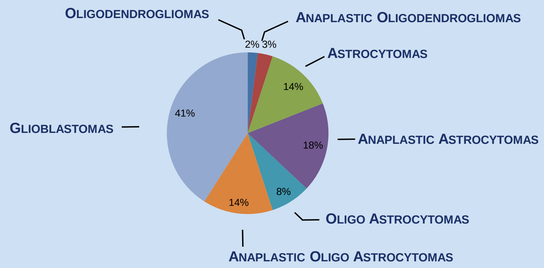

Fig 2 - Distribution of the samples by histology (n=147)

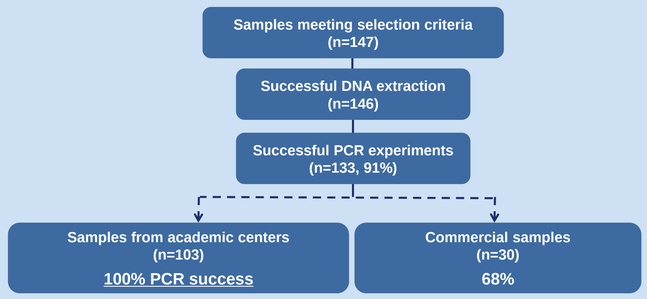

Fig 3 - IDH1/2 kit experimental flowchart: technical success rate

CLINICAL PERFORMANCE

1- Comparisons of PCR to IHC and Sanger sequencing

- Overall concordance between IHC and PCR for IDH1 R132H detection was 99% **(Tab 2)** - The only PCR/IHC discordant case was a sample of the commercial series
- Overall concordance between Sanger sequencing and PCR was 96%
  - PCR detected 5 additional mutated cases (2 IDH1 R132H, 1 IDH1 R132C, 1 IDH1 R132, 1 IDH2 172) compared to Sequencing **(Tab 3)**
- Positive agreement between PCR and IHC was 98.4 % [91.3;99.7] and was 100% between PCR and sequencing [94.6;100], meeting predefined target (PA ≥95%, lower CI limit ≥90%)
- Sequencing did not identify 3 IHC-positive cases (incl. the PCR-neg case, **Tab 2-4)**
- Out of the IHC-negative cases (n=72), PCR identified 12 rare mutations (17%, 10 IDH1, 2 IDH2), while only 9 (12 %) were detected by sequencing **(Tab 4)**

		IHC R132H POS	IHC R132H NEG	IHC TOTAL
IDH1/2 PCR	R132H	60	0	60
	NON-R132H	1	72	73
	TOTAL	61	72	133

Table 2 - IHC *vs* PCR for detection of IDH1/2 R132H mutation

		SEQUENCING IDH1/2 MUT+	SEQUENCING IDH1/2 MUT-	SEQUENCING TOTAL
IDH1/2 PCR	IDH1/2 MUT+	67	5	72
	IDH1/2 MUT-	0	61	61
	TOTAL	67	66	133

Table 3 - Sequencing *vs* PCR for IDH1/2 mutational status

	WT	IDH1 MUT R132H	IDH1 MUT R132C	IDH1 MUT R132 OTHER	IDH1 MUT R100	IDH2 MUT R172K	IDH2 MUT R172 OTHER	% Mutated Cases
IHC	72	61	0	0	0	0	0	46 %
SEQUENCING	66	58	2	6*	0	1	0	50 %
IDH1/2 PCR	61	60	3	7	0	1	1	54 %

* 3 R132S; 2 R132G; 1 R132L

Table 4 - Number and types of IDH 1/2 mutations detected by IHC, sequencing and PCR (n=133)

2- Analysis of discordant cases

CASE	IHC	IDH1/2 PCR	SANGER SEQ.	PYRO SEQ.	LNA-SEQ.	CONCL.	COMMENT
# 1 (COMMERCIAL)	POS	WT	WT	WT	WT	WT	IHC False-Pos
# 2	POS	R132H	WT	_	_	R132H	Low Mut Allele % (15%)*
# 3	POS	R132H	WT	_	_	R132H	Low Mut Allele % (10%)*
# 4 (COMMERCIAL)	NEG	R132C	WT	R132C	_	R132C	Low Mut Allele % (14%)*
# 5 (COMMERCIAL)	NEG	R132	WT	WT	WT	WT	PCR False-Pos**
# 6	NEG	R172	WT	WT	WT	WT	PCR False-Pos**

* Theoretical Mutant Allele % acc. to PCR LOD analysis - ** PCR test result close to LOD value

Table 5 - Analysis of the IHC/PCR and PCR/Sanger sequencing discordant cases

- Cases #1, #5, #6:
  Based on consistency of results obtained with the additional and highly sensitive techniques, the mutations detected by IHC (1 case) or PCR (2 cases) are likely false-positive
- Cases #2, #3, #4:
  Lack of mutation detection by Sanger Sequencing likely results from a low mutant allele content in the respective samples

3- Synthetic samples

- The IDH1/2 assay correctly detected the 11 IDH1/2 rare mutations at the two tested mutation frequencies (30% and 40%).

SUMMARY AND CONCLUSION

- The newly developed **IDH1/2 PCR assay** showed
  - **High technical success rate**
  - **High analytical sensitivity,** with an **LOD < 5%** for all but one (rare IDH2) mutations, below published references for sequencing techniques
- Positive concordance with IHC (R132H) and sequencing was high (98% & 100% resp.)
- Out of the 5 PCR/Sanger sequencing discordant cases (< 4%), 3 mutations detected by the PCR assay but not by Sanger Sequencing were confirmed by other sensitive techniques highlighting a higher sensitivity of the IDH1/2 PCR assay.
- The **IDH1/2 PCR assay** can reliably be performed on FFPE samples of up to 10 yrs of age, which should allow the assay to be used to retrospectively analyse clinical cohorts
- This new IDH1/2 PCR assay is able to detect the major IDH1R132H mutation and 11 rare IDH1/2 mutations in **one step.** This should facilitate the implementation of a comprehensive IDH1/2 testing protocol in routine clinical practice

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