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Original research article

Comparison of DNA Sequencing and Multispecific Monoclonal Antibody (Msmab-1) based Immunohistochemical Detection of IDH1/2 Mutation in Acute Myeloid Leukemia Cases.

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Abstract:

Background: The *IDH1/2* mutation is an important epigenetic modifier involved in the pathogenesis of AML. It is associated with variable prognosis in AML cases. Lack of proper molecular diagnostic infrastructure and high cost limits the routine use of PCR with sequencing as routine diagnostic methodology. The aim of this study was to find the prevalence of IDH mutation in AML cases using both PCR with sequencing and Immunohistochemistry method.

Methods: We evaluated 60 patients registered at KGMU, Lucknow for diagnosis and treatment of AML. PCR followed by sequencing was done. IHC staining of the *IDH1/2* mutation was performed on all cases using bone biopsy or clot section (in cases of pediatric AML cases).

Results: Out of the total 60 patients of AML 4(6.7%) patients had IDH1R312 mutation and 5(8.3%) Patients had IDH2R172 mutation. IDH2 R140 mutation was not detected in any sample. On immunohistochemistry analysis 10 cases showed positive staining against anti IDH1/2 mutant (R132/R172) antibody, clone MsMab-1 with a sensitivity of 77.8% and specificity of 94.1%.

Conclusion: IHC could be an alternative method to direct Sanger sequencing for *IDH1/2* mutation detection in AML cases. However, the antibody used in the study is not effective for individual assessment of IDH1 and IDH2 mutation.

Keywords: Immunohistochemistry, Sanger sequencing, IDH1/2 Mutation, Acute Myeloid Leukemia.

Introduction

Hematological malignancies have been classified by the World Health Organization, 2001, into three broad categories of myeloid, lymphoid and blastic plasmacytoid dendritic cell neoplasms, depending on the clonal cell lineage affected. Myeloid neoplasms are further stratified according to maturity of cell lineage i.e. precursor neoplasm comprising of Acute myeloid leukemia and mature myeloid neoplasm comprising of Myeloproliferative neoplasm, Myeloproliferative/ Myelodysplastic neoplasm and Myelodysplastic neoplasm. Acute myeloid leukemia is further sub classified on the basis of cell morphology, immuno cytochemical characteristics and genetic abnormalities.

The new update of WHO classification on myeloid neoplasms illustrates that there is abundant impact of genetic markers on diagnosis and management of the disease. CEBPA, RUNX1, NPM1 have been included in the AML classification. Additional mutations that help in determining the clonal nature of disease, like ASXL1, EZH2, TET2, IDH1, IDH2, SRSF2 and SF3B1 have also been incorporated. BCR-ABL1 is well recognized new provisional entity with targetable genetic changes, which are now extensively used in treatment and follow up.

Epigenetic modifiers like TET2 and IDH1/2 have been vastly studied for their role in various malignancies[1s]. IDH1 (R132) and IDH2 (R140 & R 172) mutations are found in 19% and 34% of all hematological neoplasms, respectively [1]. Mutant IDH1 is found in 6-16% of de novo AML cases and is associated with poor outcome [2]. IDH2 mutations are found in 8-19% of de novo AML cases. It has been reported that IDH 2 (R140Q) mutation is more prevalent compared to IDH2 (R172K), accounting for 80 % and 20% of all IDH^{mt} cases respectively. [1,3,4,5,6]. Hypermethylated states due to these mutations lead to increased production of D2HG (an oncometabolite) that blocks hematopoietic stem cell/ progenitor differentiation, myeloid skewing and eventually leads to myeloid neoplasms. Ongoing researches so far have concluded that mutations in IDH1 and IDH2 (R172) confer a relatively poorer prognosis compared to IDH2 (R140) mutation which displays favorable or no impact on overall survival [7]. The detection of IDH1/2 mutations in AML has resulted in inclusion of various therapeutic approaches (hypomethylating agents, IDH mutant enzymes inhibitors, immunotherapy, BCL2 inhibition etc.) which aim at either restoring the normal IDH1/2 or at blocking the production of D2HG#. Few drugs like AG-120 and AG-221 are undergoing phased drug control trials [8].

The gold standard method for molecular diagnosis of the *IDH1R132/IDH2 R140, 172* mutation is direct Sanger sequencing, which is highly reliable and specific technique. However, in the present clinical setup in India there is a lack of availability of molecular diagnostic facilities and additional financial constraints on the patients' part restrict the use of molecular diagnostics as a routine investigation. On the other hand, Immunohistochemistry (IHC) is a routinely available and low-cost technique. Acknowledging the said fact, in the present study we plan to explore the effectiveness of Immunohistochemistry to be utilized as an alternative methodology for the assessment of IDH1/2 mutation in de novo AML.

MATERIAL & METHODS

Sixty cases of Acute Myeloid Leukemia registered in Leukemia Lymphoma lab, Department of Pathology, King Georg's Medical University, Lucknow, India, were included in this prospective study. Peripheral blood examination, Bone marrow aspiration, bone biopsy (or clot section in pediatric cases), flow cytometric evaluation, karyotyping and

FISH was routinely done on all the cases. The study was approved by the Institutional Ethical Committee board, King George's Medical University, Lucknow. Informed consent was obtained from all patients prior to sample collection.

Immunohistochemical detection of mutated IDH1/2 protein was done on 55 bone biopsies and 5 clot sections. Whole tissue sections (4 µm) were transferred to poly-L-lysine-coated adhesive slides and deparaffinised. Antigen retrieval was performed by autoclaving the slides in the Citrate buffer (Dako, Ph 6.0) for 20 minutes. Primary antibody MsMAb-1 was added at a concentration of 5µl/ml and incubated overnight. Secondary antibody (biotin conjugated anti-mouse IgG; Dako) was added and incubated for 20 minutes. Thereafter Horseradish peroxidase(HRP) was added to the sections and incubated for 30 minutes. Slides were then stained with diaminobenzidine (DAB, Dako) and incubated for 10 minutes. Counterstain with hematoxylin was done. No antibody was added in negative controls and sections of GBM were taken as positive control.

Slides were evaluated and cross confirmed by two pathologists who were blinded to the *IDH* mutational status. The expression of MsMab-1 was determined by semi quantitatively assessing the proportion of positively stained tumour cells. Cytoplasmic or nuclear staining in >10% tumor cells were considered as positive[9]. Non-specific staining RBC clusters and equivocally weak or focal cytoplasmic staining was considered as negative.

Genomic DNA was extracted from peripheral blood or bone marrow samples using Pure Link genomic DNA mini kit, Invitrogen. Sanger sequencing was performed as previously described. Exon 4 of the *IDH 1 and IDH 2* genes were amplified by polymerase chain reaction (PCR) using the primers pairs [IDH1F (5'- AGCTCTATATGCCATCACTGC-3'), IDH1R (5'- AACATGCAAAATCACATTATTGCC-3'), IDH2F- (5'- AATTTTAGGACCCCCGTCTG-3'), IDH2R (5'- CTGCAGAGACAAGAGGATGG-3')].For every PCR cycle 30 µl reaction consisted of 100 ng genomic DNA, 12.5 pmol of each oligonucleotide primer, 15µl of master mix and 12µl of DNAse free water .The amplification protocol for IDH1 comprised initial denaturation at 98°C for 30 seconds; 35 cycles of denaturation at 98°C for 10 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 30 seconds; followed by a final extension at 72°C for 5 minutes using platinum hot start PCR master mix(thermo fisher). For IDH2 initial denaturation at 98°C for 30 seconds; 35 cycles of denaturation at 98°C for 10 seconds, annealing at 61°C for 30 seconds, and extension at 72°C for 30 seconds; followed by a final extension at 72°C for 5 minutes. PCR products were electrophoresed on 2.5% (wt./vol) agarose gels and subsequently sequenced in both sense and antisense direction using the above-mentioned primers. DNA sequences and the *IDH* mutation were determined using a 3730XL DNA analyzer (thermo fisher Scientifics). The results were compared with wild type IDH1 and IDH2 cDNA (genbank accession number NG_023319.2 and NC_000015.10 respectively.

Statistical analyses were performed using SPSS version 19. Categorical variables are presented as numbers with percentages. All *P* values were two-sided and *P* values <0.05 were considered statistically significant.

Results:

Table 1: Clinical and Demographic Characteristics of AML Patients with Wild type and Mutant IDH.

Sl. No.	Characteristic	IDH wild		IDH1 mutated		IDH2 mutated		P value
		N	%	N	%	N	%	
1.	Age Group							
	1. <9	5	9.8	0	0	0	0	
	2. 10-19	8	15.7	0	0	1	20	0.909
	3. 20-50	31	60.8	3	75	3	60	
	4. >50	7	13.7	1	25	1	20	
2.	Sex							
	1. Male	33	64.7	2	50	2	40	0.491
	2. Female	18	35.3	2	50	3	60	
3.	Bleeding manifestation	15	29.4	1	25	2	40	
4.	Extra medullary disease	29	56.8	1	25	1	20	
5.	ECOG Score (%)							
	1. Fully active	0	0	0	0	0	0	
	2. Ambulatory+ restricted strenuous work	4	7.8	0	0	1	20	0.258
	3. Ambulatory+ self-care	6	11.8	1	25	2	40	
	4. Limited self-care	20	39.2	3	75	1	20	
	5. Completely disabled	21	41.2	0	0	1	20	
	6. dead	0	0	0	0	0	0	
9.	Prognostic group							
	1. Favorable	12	23.5	2	50	3	60	
	2. Intermediate	19	37.2	0	0	2	40	0.359
	3. unfavorable	20	39.2	2	50	0	0	
10.	Hemoglobin Median(range) (gm/dl)	6.5(2.7-13.0)		5.3(3.1-7.9)		8.2(7.1-9.1)		0.083
11.	Total Leukocyte Count Median(range) (X 10^6/cumm)	15.0(0.5-199)		15.5(3.2-40.0)		5.4(1.4-84.2)		0.479
12.	Platelet count Median(range)(x 10^9/L)	0.4(0.1-85)		1.95(0.5-3.5)		0.37(0.25-3.64)		0.073
13.	Anemia Grade							
	1. Grade 1 (10- lower limit of normal gm/dl)	7	13.7	0	0	0	0	0.000
	2. Grade 2 (8-10 gm/dl)	1	1.96	0	0	3	60	
	3. Grade 3 (6.5-7.9 gm/dl)	18	35.3	1	25	2	40	
	4. Grade 4 (<6.5 gm/dl)	25	49.1	3	75	0	0	
16	S.LDH Median(range) (IU/dl)	832.0 (150-7835.0)		798.5 (110.3-1008.0)		423.0 (301.0-967.0)		0.323
17.	Peripheral blood blast Median(range) (%)	34.0(0-98.0)		69.5(8.0-89.0)		46.0(0-83.0)		0.451
19.	Bone marrow blast Median(range) (%)	66.0 (22.0-98.0)		75.5 (62.0-90.0)		75.0 (41.0-90.0)		0.490
21.	dysplastic features (%)							
	1. Present	27	52.9	2	50	3	60	
	2. Absent	24	47.1	2	50	2	40	0.946

IDH mutation was detected 9(15%) out of 60 patients by DNA sequencing method. Four patients (6.7%) had IDH1R132 mutation (IDH1R132C-3, IDH1R132S-1), and five patients (8.3%) had IDH2R172 mutation (IDH2R172K-5). The cohort did not show any case with IDH2R140 mutation.

The demographic data of the entire cohort is present in Table 1.

Table 2 summarizes the results of Sanger sequencing and IHC staining for

the *IDH1/2* mutation in 60 bone marrow samples. The *IDH1/2* mutation was detected in 9 samples (15%) using direct sequencing (fig.1 and 2). However, there were 10 bone marrow samples (16.7), which were detected, positive by IHC with multispecific antibody for *IDH1/2* mutation (fig.3). Five samples displayed discrepant results compared to sequencing (three false positive and two false negative). The discordance rate between the two tests is 6.7%. The sensitivity and specificity of IHC for detecting the *IDH1/2* mutation in bone marrow samples were 77.8% and 94.1% respectively. The positive predictive value was 70% and the negative predictive value was 96%. Positive and negative likelihood ratio was 13.2 and 0.24 respectively. The accuracy if the test is 91.67%.

Table 2: Results of IHC and PCR for IDH1/2 detection in AML cases.

IHC	IDH1/2 mt present	IDH1/2 mt absent	TOTAL
Positive	7	3	10
Negative	2	48	50
TOTAL	9	51	60

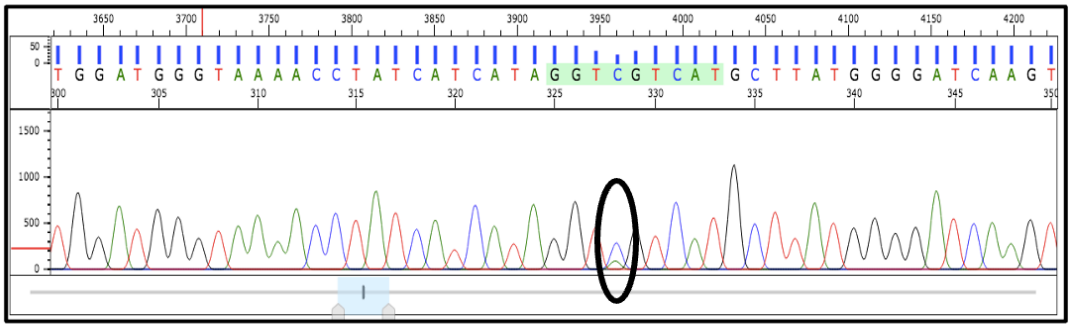

Figure 1: PCR sequence showing IDH1R132S mutation in forward sequence

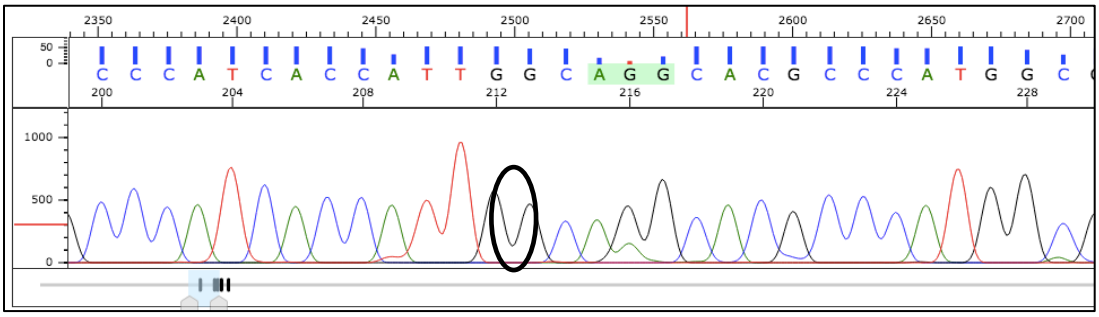

Figure 2: PCR sequence showing IDH2R172K mutation in forward sequence

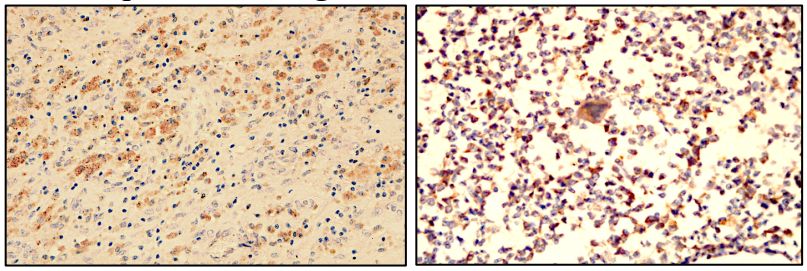

Figure 3: Left- Positive control IHC IDH1/2 in GBM case(10X). Right- Positive staining in cells with IDH1/2 mutation AML case bone biopsy(20X).

Discussion

Table 3: Comparison of immunohistochemistry with sequencing for IDH testing in various malignancies

Sl. No.	Study	Year	Tissue type	IDH	Sample size	Antibody used	Positive on IHC	Positive on PCR
1.	Takano [17]	2011	diffuse glioma	IDH1	49	Imab-1	12/49	10/49
2.	Loussouarn [18]	2012	Oligo	IDH1	90	DIA-H09	47/90	55/90
3.	Liu[19]	2013	Osteosarcoma	IDH1/2	12	MsMab	9/32	3/32
4.	Agarwal[20]	2013	Diffuse glioma	IDH1	50	DIA-H09	30/50	28/50
5.	Catteau [21]	2014	Diffuse glioma	IDH1	133	DIA-H09	61/133	66/133
6.	Kaneko[22]	2014	Giant cell tumour	IDH1/2	20	MsMab	20/20	16/20
7.	Dupuy[23]	2018	Angioimmunoblastic T-Cell Lymphoma	IDH2	42	ANTI IDH2(R172K)	13/42	12/42
8	Gondim[24]	2019	Glioma	IDH1	62	DIA-H09	12/62	15/62
9.	Bingqi[25]	2020	Intrahepatic cholangio Carcinoma	IDH1/2	130	MsMab	14/130	21/130

Acute myeloid leukemia involves the proliferation of myeloid precursors and has a prevalence of about 36.5% of all Indian pediatric/ adult leukemia patients [10]. WHO has laid down specific diagnostic criteria for its diagnosis along with its sub classification based on peripheral blood smear examination, bone marrow examination, Immunophenotyping, cytogenetic evaluation and analysis. As per WHO 2008 approximately 50% of all AML cases are cytogenetically normal (CN-AML). WHO 2008 stated NPM1, CEBPA and FLT3 gene mutations have some significance in the diagnosis/prognosis of CN-AML [11]. WHO 2016 update [12] has included the said mutations along with RUNX1 as separate entities in AML sub classification. Many new gene mutations have been mentioned along with their frequencies and prognostic significance in AML cases.

Since 2013, the cancer genome atlas project formulated functional complementation groups of genetic alterations in AML. In this grouping system, class 4 genetic alterations are related to DNA methylation – related genes. This class of epigenetic modifiers includes DNA hydroxymethylation (TET2, IDH1 and IDH2) and DNA methyltransferases (DNMT3A).

Epigenetic modifiers are a group of gene mutations causing epigenetic modifications that are known to regulate DNA and histone methylation and hence impair cell differentiation along different lineages, eventually leading to tumor development. DNMT3A, TET2, IDH1/2 are such epigenetic modifiers which are under study and their prognostic and therapeutic implications still under evaluation [13]. Mutations in factors regulating DNA methylation impairs cell differentiation along various lineages and leads to tumor development in coordination with other cancer genes and tumor causing factors. Mutations of the arginine residue in the active site (R132 in IDH1 and R140, R172 in IDH2) prevents the normal function of IDH enzyme and results in formation of a rare

oncometabolite 2 D hydroxyl glutarate(D2HG)5,6,7 [14,15,16]. D2HG competitively inhibits αKG dependent dioxygenases (TET DNA hydroxylases, Jumonji C domain containing histone demethylase and DNA methyltransferases etc.) leading to hypermethylation of CpG islands in promoter region. Hypermethylated states due to said mutations lead to blocked hematopoietic stem cell/ progenitor differentiation, myeloid skewing and eventually to myeloid neoplasms.

Ongoing researches have concluded so far that mutations in IDH1 and IDH2(R172) confers a relatively poorer prognosis compared to IDH2(R140) mutation which confers a favorable or no impact on overall survival. This makes it important to diagnose the IDH mutations in all AML cases, so that planned therapy, residual disease monitoring and prognosis could be provided to the patient. The discovery of IDH1/2 mutations have resulted in various therapeutic approaches (hypomethylating agents, IDH mutant enzymes inhibitors, immunotherapy, BCL2 inhibition etc.) aimed at either restoring the normal IDH1/2 or at blocking the D2HG production. Few drugs like AG-120 and AG-221 are undergoing phased drug control trials [8].

For the detection of IDH1/2 mutations, currently polymerase chain reaction with Sanger sequencing, Next Generation sequencing, and pyrosequencing are few diagnostic tests. Research has been done on the utility of evaluating oncometabolite D2HG levels to predict IDH1/2 mutation status. In the present clinical setup in India, there is a lack of availability of molecular diagnostic facilities and additional financial constraints on the patients' part restrict the use of these molecular diagnostics as routine investigation. Immunohistochemical detection of IDH1/2 mutation appears to be comparatively cost-effective and rapid substitute among all the available tests for screening of IDH mutation.

There are many studies and researches, which report comparison of accurate, rapid and cost effective diagnostic tests for the detection of various molecular aberrations (table 3). IDH mutations detection has been compared using IHC and PCR by Takano et al[17]. Since the relevance of IDH1R132 mutation is established in prognosis of gliomas, most of the comparative studies have been published on gliomas. Role of IDH mutation are being studied in the pathogenesis of various tumors, hence their comparative analysis between IHC and PCR have also been done in Osteosarcoma[19], Giant cell tumor[22], Angioimmunoblastic T cell lymphoma[23], intrahepatic cholangiocarcinoma[25]. Most of the studies have used mutation specific individual antibodies for IHC, however, since the availability of multispecific monoclonal antibodies against both IDH1R132 and IDH2R172, studies have utilized this antibody to simultaneously detect IDH1/2 mutations.

Although MsMab has been found to be useful in detection of IDH1R132 and IDH2R172 mutations, its multispecificity does not discriminates between IDH1 and IDH2 mutations. This antibody recognizes IDH1 with Arg 132 mutations(R132A/D/E/G/H/M/N/Q/SY) and IDH2 with Arg 172 mutations(R172A/C/D/E/G/L/Q/S/Y) [19,22]. Wild type IDH1/2 or other IDH1/2 mutations (IDHR132 C/F/I/K/L/P/R/T/V/W; IDH2-H175Y, R172 F/H/I/K/M/N/P/T/V/W) are not identified by this antibody. Moreover, Other IDH1/2 mutations at variable codons other than the one specified for by this MsMab may be missed.

We have used MsMab to assess its accuracy and cost effectiveness for screening of IDH mutation in AML cases. This antibody could not detect IDH2R140 mutations. We have still used this antibody as our study cohort had no reported IDH2R140 mutation case. To the best of our knowledge, this is the only study to compare IDH mutation detection in AML

cases using PCR with sequencing and IHC using MSMab-1.

Our study showed 15% of AML cases had IDH mutation (6.7% had IDH R132 and 8.3% had IDH2R172 mutation). This finding was similar to previous reports and suggested the tumorigenicity of cytogenetically normal AML by the aberrant of IDH1/2 coding sequences (Paschka et al., 2010[26]; Chotirat et al., 2012[27]; Ahmad et al., 2014[28]; Raveendran et al., 2015[29]). Immunohistochemistry showed a sensitivity and specificity of 77.8% and 94.1% respectively. IHC using MSmab-1 was not as sensitive as PCR with sequencing to detect IDH1/2 mutations. This variation could be because of heterogeneous expression of IDH1R132S and IDH2R172S protein in tumor cells. Moreover, there is a probability of degradation of the mutated proteins during bone biopsies processing in formic acid and formalin. IDH1R132C mutation reported in AML cases and the most common mutation in our study was also not detected by this antibody. Yet we had a discrepant result in one case where IDH1R132C mutation was detected positive by immunohistochemistry, which may be due to nonspecific binding of antibody.

To conclude, IHC could be an alternative approach to direct Sanger sequencing for detecting the *IDH1/2* mutation in bone marrow samples. However, Direct Sanger sequencing is the gold standard method for detecting the *IDH1/2* mutation and is highly specific. Since PCR and sequencing requires trained staff and high set up cost IHC can be better alternative approach in detecting the *IDH1/2* mutation. At the time of the commencement of study, due to non-availability of separate IDH1 and IDH2 specific antibodies, we have used common antibody to detect both the mutations. However, if different antibodies for IDH1 and IDH2 specific mutations were used, the results might have been concordant with the sequencing/PCR results.

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