

Detection Of Mutations In The Isocitrate Dehydrogenase Genes (IDH1/IDH2) Using castPCR™ In Patients With AML And Their Clinical Impact In Mexico City

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Objective: Approximately 40–50% of patients with acute myeloid leukaemia (AML) have been reported to present with a normal karyotype and a variable disease-free period, most likely due to the molecular heterogeneity presented by these patients. A variety of mutations have been identified at the molecular level, such as those in the IDH1/2 gene, which causes a gain of function of the isocitrate dehydrogenase enzyme, generating high levels of the (R)-2-hydroxyglutarate oncometabolite, which competitively inhibits dioxygenase enzymes. Therefore, the objective of this study was to evaluate the incidence of IDH1/2 gene mutations in AML patients and their impact on survival.

Materials and methods: A total of 101 patients with a diagnosis of AML were included; mononuclear cells were obtained for DNA extraction and purification. Mutations were detected using TaqMan™ competitive allele-specific probes (castPCR™). Overall survival curves were plotted using IBM SPSS Statistics 23 software.

Results: The frequency of IDH gene mutations was 19.8%. For the IDH1 gene, 13.8% of the mutations identified included R132H, V178I, G105G and R132C. The frequency of mutations of the IDH2 gene was 5.9%; the variants included R172K and R140Q. The mean survival time in patients without IDH1 gene mutations was 173.15 days (120.20–226.10), while the mean survival time for patients with mutations was 54.95 days (9.7–100.18), $p = 0.001$.

Conclusion: The frequency of IDH1 and IDH2 gene mutations in the sample was similar to that reported in other studies. The analysis of these mutations in AML patients is of great importance as a prognostic factor due to their impact on survival and their use as potential therapeutic targets or as targets of inhibitors of IDH1 (Ivosidenib, Tibsovo) and IDH2 (Enasidenib, Idhifa).

Keywords: isocitrate dehydrogenase, competitive allele specific TaqMan, acute myeloid leukemia

Introduction

Acute myeloid leukaemia (AML) is one of the main haematological oncological neoplasias treated in reference centres; it is the most common type of acute leukaemia in adults, and its incidence increases with age.^{1–3} The incidence in Mexico is six cases per 100,000 inhabitants, with a mortality rate of 5.9/100,000 inhabitants. It has been reported that approximately 50% of AML patients present with normal karyotypes;

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however, at the molecular level, a variety of mutations have been identified, in which genes that regulate cell proliferation, differentiation and apoptosis are altered.^{4,5} These alterations impact the prognosis and survival of patients.^{6,7} It is important to highlight that the treatment of AML has consisted of a 7+3 protocol since 1973.^{8,9} However, the timely detection of gene alterations that cause AML will allow access to new drugs and therapeutic targets as inhibitors of the disease. The goal of kinase epigenetic modulators is to achieve greater overall survival.^{10,11} One type of mutation that is of great interest in AML is mutation of the IDH (isocitrate dehydrogenase) genes, which is present in > 80% of low-grade gliomas and secondary glioblastomas and in 20% of leukaemias.^{12–14} Three isoforms of the IDH enzyme have been identified: IDH1 is located in the cytoplasm, while IDH2 and IDH3 are located in the mitochondria.^{15,16} These genes have a high frequency of mutation in patients with AML and other tumour types.^{17–19} IDH mutations mainly affect the residues of the active site (e.g., IDH1^{R132}, IDH2^{R140} or IDH2^{R172}), resulting in a gain of function with increased activity of the neomorphic enzyme and therefore accumulation of D-2-hydroxyglutarate (2-HG).^{20–22} The oncometabolite 2-HG can competitively inhibit multiple α -KG-dependent dioxygenases, including key epigenetic regulators such as histone demethylases and TET proteins.^{23,24} Consequently, IDH mutations are associated with chromatin alterations, including global histone alterations and DNA methylation.^{25,26}

It is important to identify the presence of IDH1/2 mutations in a rapid manner so that patients can benefit from targeted therapies. Therefore, it is necessary to seek reliable methodological alternatives, such as castPCRTM, which is characterised by its high sensitivity and specificity, because it can detect minimal amounts of mutated DNA in a sample that contains large amounts of normal wild-type DNA.^{27,28} The castPCRTM technique uses specific oligonucleotides for the mutated allele that compete with an MGB-blocking oligonucleotide to suppress the normal allele.^{29,30} It can robustly detect mutant alleles at values as low as 0.1% in a wild-type background and has a > 99% concordance with other technologies, including technology based on digital PCR and Sanger sequencing.³¹

It is important to highlight that although Sanger sequencing is the “gold standard” for the identification of mutations due to its low rate of false positives and high specificity, it has a number of disadvantages, including low sensitivity, the time required to perform the assay,

requirement of high-quality tissue samples and the need for manual interpretation.³²

Unlike other methods, such as the amplification refractory mutation system (ARMS), for which the sensitivity is 1%, and Sanger sequencing, with 10–25% sensitivity castPCRTM has a sensitivity of 0.1%.³³ Moreover, next-generation sequencing (NGS) technology, which is used for the detection of multiple mutations, has the disadvantages of being time consuming and too costly to detect a specific genetic allele. In addition, the data produced by NGS are not suitable for routine clinical analysis.³⁴

Based on the above information, we evaluated the incidence of gene mutations (IDH1/2) by castPCRTM in AML patients and the clinical impact of these mutations on diagnosis, prognosis and survival.

Materials And Methods

Description Of The Study Population

A total of 101 patients with de novo AML treated at the General Hospital of Mexico with complete clinical records were included. Among the patients, 50.5% were female (n = 51), and the remaining patients were male (n = 50, 49.5%). The mean age was 46 years (15–92 years). The majority of patients were younger than 60 years (n = 68, 66.6%). The diagnosis of AML was based on both morphological findings and flow cytometry; the majority of patients had leukaemia with monocytoid characteristics (AML-M4, 60.4%), followed by those with differentiation (AML-M2, 30.7%). The mean haemoglobin level was 11 g/dl (4–91), with a mean leukocyte count of $150 \times 10^9/L$ ($0.2\text{--}4870 \times 10^9/L$) and a mean platelet count of $48 \times 10^9/L$ ($3\text{--}241 \times 10^9/L$). Regarding the main genetic alterations identified, 45.5% of patients had a normal karyotype, and only one patient with hyperdiploidy was detected. The other clinical and laboratory parameters are described in Table 1.

Approval for the present study was provided by the Ethics Committee of the Hospital General de Mexico “Dr. Eduardo Liceaga”. The informed written consents were collected from all enrolled patients and the entire study was performed based on the Declaration of Helsinki.

Type Of Treatment

The main part of the treatment was based on the 7 + 3 protocol; the intensity of the treatment was mainly based on the age and functional status of the patients. The

normal intensity 7 + 3 protocol (100 mg/m² cytarabine for 7 days plus 60 mg/m² daunorubicin for 3 days) was initiated in 78 patients (n = 77.2%), a total of 13 patients received a reduced dose of 7 + 3 (12.9%), and seven patients received cytarabine subcutaneously (6.9%).

Response To Treatment

After the initiation of induction therapy, the response to treatment was assessed according to the recovery of the blood count and the decrease in the number of blasts in bone marrow. Accordingly, patients were classified into different types of responses. A total of 36 patients (35.6%) met the criteria for complete remission, while 12 patients (11.9%) were considered to have a partial response.

Regarding refractory leukaemia, 21.8% (n = 22) of patients showed resistance to the first treatment protocol, while 30.7% (n = 31) died during the remission induction phase (26 cases of death due to aplasia and five cases of indeterminate death).

Separation Of Mononuclear Cells By A Ficoll-Hypaque Gradient

Peripheral blood samples were obtained from normal donors and AML patients. Mononuclear cells were separated using a Ficoll-Hypaque gradient (Lymphoprep, Nycomed Pharma AS, density 1.077 g/L), and isolated mononuclear cells were stored at -70°C.

Extraction And Purification Of DNA

Genomic DNA (gDNA) was extracted using DNAzol® (Thermo Fisher, Paisley, UK) according to the supplier's instructions. Isolated gDNA was analysed by 0.8% agarose gel electrophoresis to evaluate its quality. The amount of DNA was determined by the 260 nm/280 nm ratio using Genesys 10S UV-Vis (Thermo Scientific)

Subsequently, DNA purification was performed using a QIAamp DNA Mini Kit (Qiagen)

TaqMan Mutation Detection Assay (castPCR™)

The following TaqMan assays were designed for the detection of IDH1 and IDH2 gene mutations (GenBank Accession number, NM005896.3 and NM_002168.2, respectively): c.395 G>A p.R132H (Hs00000981_mu); c.394 C>T p.R132C (Hs00000982_mu); c.532 G>A p.

V178I; c.315 G>T p.G105G; c.515 G>A p.R172K; c.419 G>A p.R140Q. CastPCR™ analysis was performed using a real-time PCR System (Step OnePlus™ Real-Time PCR System, Applied Biosystems, USA, CA). The Master mix was prepared as recommended by the supplier. A total of 50 ng of gDNA per reaction and the probes described above were used. The cycling conditions were as follows: pre-PCR read 60°C for 30 s; holding stage 50°C for 2 min, 95°C for 10 min; cycling stage 95°C for 15 s, 60°C for 1 min for 40 cycles; and post-PCR 60°C for 30 s.

For each of the analysed IDH1/2 mutations, the limit of detection (LOD) of castPCR™ was determined by constructing dilution curves of samples from patients with and without IDH1/2 gene mutations. Each point was determined using different dilutions (1:1 to 1:50) of the mutated sample and a non-mutated sample (Figure 1).

Statistical Analysis

Multivariate analysis was performed based on the clinical parameters and the quantification of the mutations. Kaplan-Meier and log rank analyses were performed to assess survival in relation to each mutation; differences were considered significant when $p \leq 0.05$. SPSS software version 23 Statistical Package for Social Sciences, SPSS Inc, Chicago IL, USA) was used for the statistical analysis.

Results

Frequency And Clinical Association Of IDH Mutations In AML Patients

The frequency of IDH mutations in AML patients was 19.8% (20/101). In the particular case of the IDH1 gene, the frequency of mutations was 13.8% (14/101); the mutations identified included R132H, with a frequency of 3.9% (4/101), and V178I, with a frequency of 3.9% (4/101), while G105G (2/101) and R132C (2/101) exhibited a frequency of 1.9%. Two patients had two mutations, R132H and R132C, resulting in a frequency of 1.9% (2/101). The mutation frequency of the IDH2 gene was 5.9% (6/101); the variants included R172K, with a frequency of 3.9% (4/101), and R140Q, with a frequency of 1.9% (2/101) (Table 2).

Regarding the association between the karyotype and the presence of IDH gene mutations, 12% of

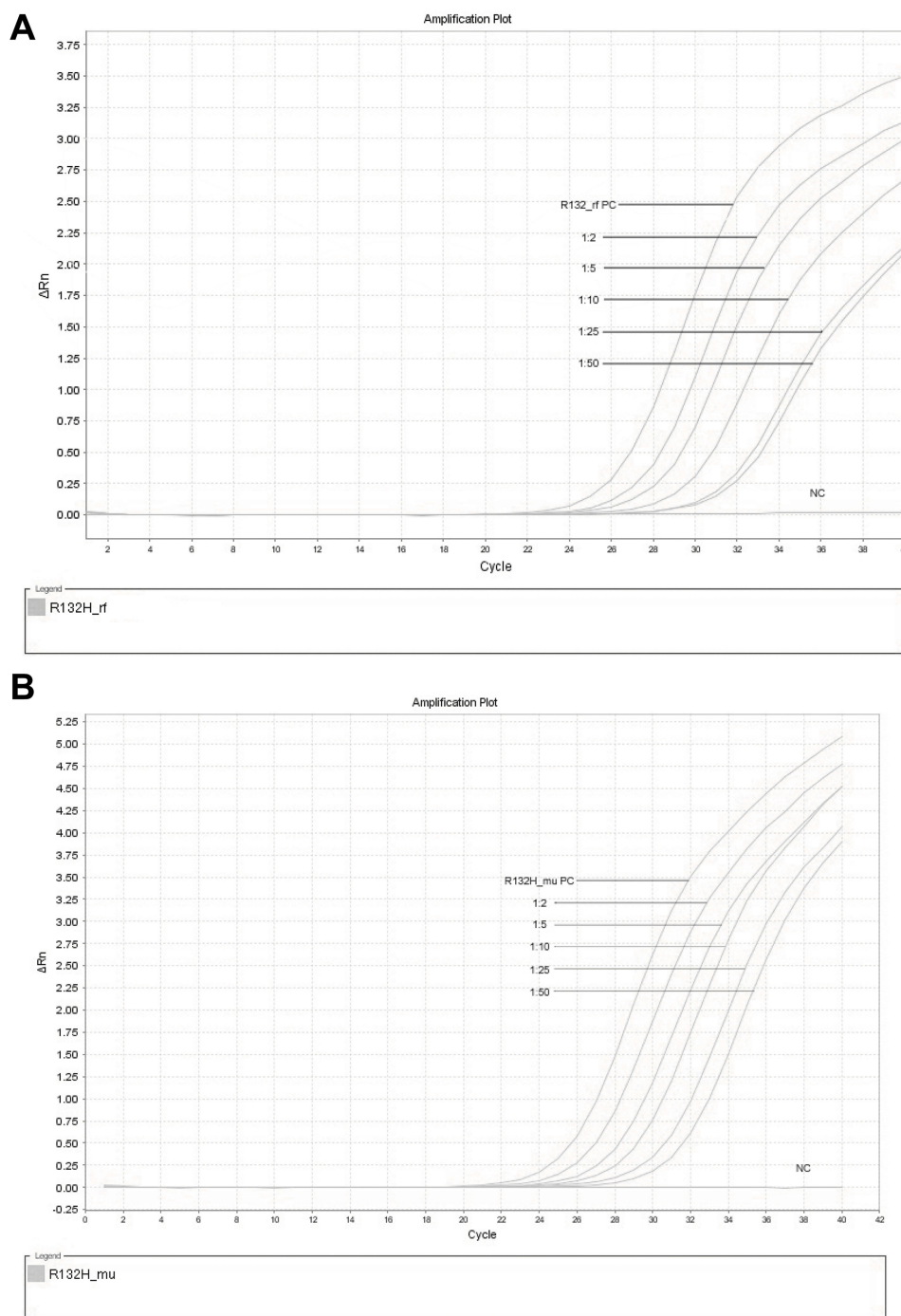


Figure 1 Evaluation of castPCR™ Limit of Detection (LOD). The LODs of castPCR™ are shown in Figure 1A and B. We found that the mutated alleles were detectable by castPCR™ up to a dilution of 1:50 for both cases: R132H_rf; CT = (25.04–31.14), R132H_mu; CT = (24.32–30.10).

patients did not present tumour growth. However, 21% of patients with a normal karyotype had mutations. The patient with an abnormal karyotype had no mutation in the IDH1 gene; in the case of IDH2, 1.8% of patients did not present tumour growth, and 12.19% of patients had a normal karyotype

Overall Survival Analysis In Patients With IDH1/2 Gene Mutations

The mean survival time of patients without IDH1 gene mutations was 173.15 days (120–226), while the mean survival time of patients with mutations was 54.95 days (9–100). Of the patients with mutations of this gene (n = 14),

Table 1 Clinical Characteristics Of The Population Analyzed (n=101)

Clinical features	
Age	
Mean \pm SD (range)	46 (15–92)
Median	47
Sex	
F	51 (50.5)
M	50 (49.5)
Laboratory data	
PB Blast count	
Mean \pm SD (range)	56 \pm 28(0–99)
Median	63
Mean WBC count, 10 ⁹ /L (range)	150 (0.2–4870)
Mean hemoglobin level, g/L (range)	11 (4–91)
Mean platelet count, 10 ⁹ /L (range)	48 (3–241)
Mean DHL (range)	684 (89–3921)
Biologic characteristics	
Immunophenotype (%)	
M1	2 (2.0)
M2	31 (30.7)
M4	61 (60.4)
M5	2 (2.0)
M6	3 (3.0)
M7	2 (2.0)
Cytogenetics	
Unsuccessful karyotype	54 (53.5)
Normal karyotype	46 (45.5)
Abnormal karyotype	1 (1.0)
Treatment scheme	
3+7	78 (77.2)
Mini 3+7	13 (12.9)
2+5	3 (3.0)
ARA C SC	7 (6.9)
Response	
Response complete	36 (35.6)
Remission partial	12 (11.9)
Refractory disease	22 (21.8)
Death in aplasia	26 (25.7)
Death by undetermined cause	5 (5.0)

85.7% died (12/14), log rank $p = 0.010$ (Figure 2). Among the six patients who presented mutations of the IDH2 gene, 66.7% died. The mean survival time in patients without mutations was 157.3 days (110–204), while in those with mutations, the mean survival time was 74.11 days (30–117). According to the log rank analysis ($p = 0.829$), there were no differences between the groups (Figure 3).

Table 2 Frequency Of Mutations Of The IDH Gene

Variants		n	%
IDH1	G105G	2	1.9
	R132H	4	3.9
	R132C	2	1.9
	V178I	4	3.9
	R132H, R132C	2	1.9
IDH2	R172K	4	3.9
	R140Q	2	1.9

Association Of IDH Mutations With The Prognosis

The impact of different clinical variables on a favourable response to remission induction (complete remission or partial response versus therapeutic failure due to refractoriness or death) as well as its impact on survival was analysed. Individually, clinical variables and age older than 60 years (OR 3.777, 1.45–9.85), intermediate-high risk (OR: 1.936, 0.83–4.51) and mutations in IDH, especially IDH2 (OR 7.974, 0.4362–145.78), were associated with an increased risk of therapeutic failure. Among these variables, patients who were diagnosed after 60 years of age ($p = 0.006$, 95% CI) and who required low-intensity treatment ($p = 0.045$, 95% CI) presented a significant risk for therapeutic failure. When evaluating the effect on medium-term survival, the IDH1 mutation (OR: 4.65, $p = 0.052$, 95% CI) and the presence of a leukocyte count above $30 \times 10^9/L$ at the time of diagnosis (OR: 3.33, $p = 0.008$, 95% CI) showed significant impacts, and unlike the response to induction therapy, both low-intensity treatment and age did not show an impact on survival. The different risk values of each variable are described in Table 3.

Discussion

In recent years, the biological roles of IDH enzymes in cancer have been described, including adaptation to hypoxia, histone demethylation and chromatin remodelling. However, the presence of mutations at the DNA level in these enzymes produces an increase in the production of the 2-HG oncometabolite, causing blockage of cell differentiation by inhibiting the activity of chromatin remodelling enzymes.³⁵ Due to the frequent occurrence of IDH1 gene mutations in solid tumours and mutations in the IDH2 gene in haematologic neoplasias,³⁶ we found that the frequency of mutations in the IDH gene in AML patients was 19.8%, which is consistent with values reported in other studies, and these mutations increased

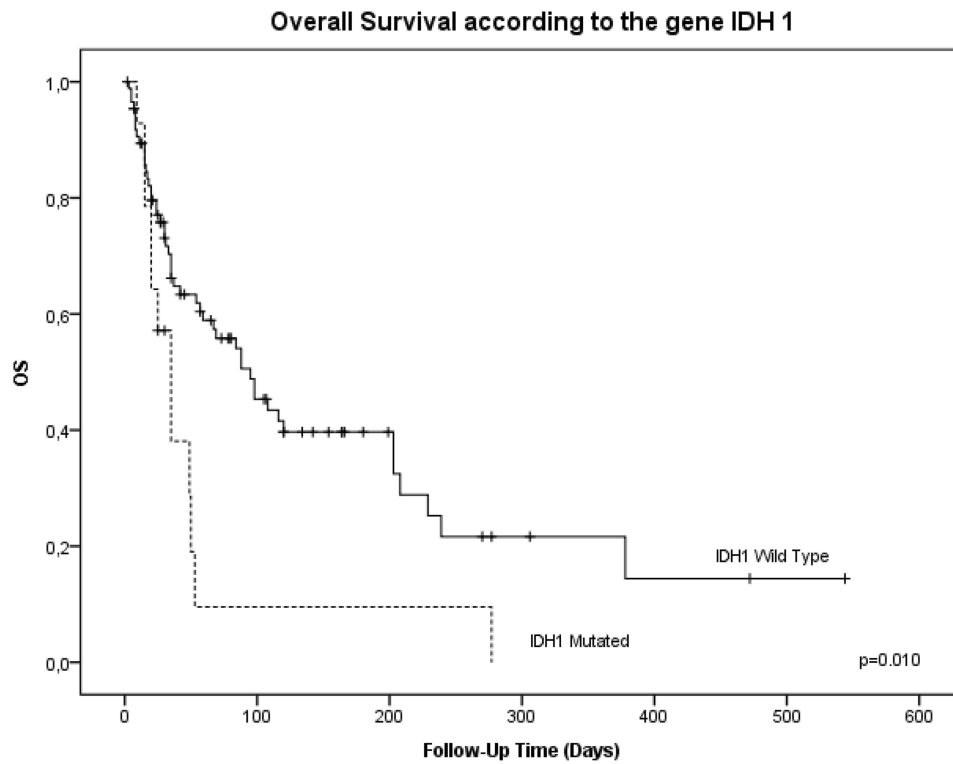


Figure 2 Global survival (OS) in patients with AML with mutated IDH1 gene. Of the patients with mutations in this gene, 85.7% died (12/14) (log rank $p = 0.010$).

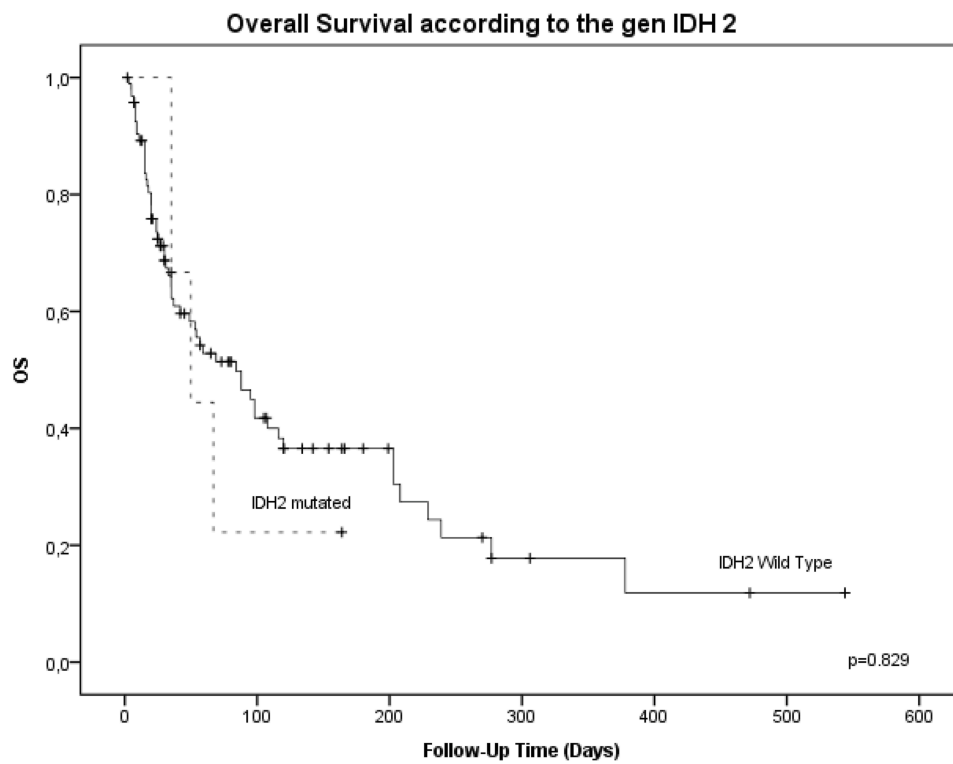


Figure 3 Global survival (OS) in patients with AML with mutated IDH2 gene. Of the patients with mutations in this gene, 66.7% died. There were no differences after the logrank test ($p = 0.829$, Figure 2).

Table 3 AML Patients Mortality And OR Model According To Mutations In IDH Gene

	Risk of Death Associated With Therapy			Risk Of Early Failure To Therapy		
	OR	(95% CI)	p	OR	(95% CI)	p
IDH1 mutated	4.65	0.98–22.05	0.052*	1.4545	0.42–5.02	0.593
IDH2 mutated	1.33	0.23–7.64	0.746	7.9748	0.44–145.79	0.161
Low intensity therapy	0.41	0.16–1.05	0.063	3.3043	1.03–10.63	0.045*
Intermediate-high risk	0.58	0.25–1.39	0.223	1.9368	0.83–4.52	0.126
Leukocytes >30 × 10 ³ /mcl	3.33	1.36–8.16	0.008*	3.1912	1.27–8.04	0.013*
Age > 60 years	0.71	0.31–1.61	0.818	3.7773	1.45–9.85	0.006*

Note: *Significance level $p < 0.05$.

Abbreviations: OR, odds ratio; CI, confidence interval.

at frequency of approximately 20% according to age.³⁷ Our study population showed mutations at younger ages, which is consistent with the diagnosis of this neoplasm in other populations. IDH1 gene mutations have been reported to be less common than IDH2 mutations in AML.³⁸ The IDH mutations identified in our AML patients included R132H, V178I, G105G and R132C, and R132H exhibited the highest frequency. In the case of the IDH2 gene, the mutations detected included R172K and R140Q. Other studies have reported that the R132C and R132H mutations present greater frequencies in the IDH1 gene.³⁹ In contrast to previous reports indicating a higher frequency of IDH2 mutations in AML, we found a higher frequency of IDH1 mutations due to the specificity and sensitivity of the castPCRTM technique, which had greater specificity and sensitivity than Sanger sequencing.

In the case of the G105G variant, a frequency of 10.6% has been reported. This is important because this variant can be a prognostic marker in patients with intermediate-risk FLT3-negative disease and can become a potential marker that benefits patients due to the development of new treatment alternatives.^{40,41} We found a frequency of 1.9% for this variant, which is much lower than that reported in the literature. In the case of the IDH2 gene, the mutations present included R140Q and R172H, the latter of which has been reported in adult patients.⁴² Only two AML patients showed two mutations in the IDH1 gene, R132H and R132C, and IDH1 and IDH2 mutations have been reported to be present in the same patient.^{43,44}

The advent of NGS technologies has accelerated the discovery of new genetic alterations in AML.^{45,46} Among the main mutations detected are those in the FLT3, NPM1, CEBPA, MLL, NRAS, KIT, WT1, RUNX1, TET2, IDH1/2, DNMT3A, ASXL1, PHF6, SRSF2 and PTPN11 genes,^{47–49} indicating that these genes may impact the prognosis of

AML patients.^{50,51} For example, mutations in FLT3 (37–46%) indicate unfavourable prognoses; in contrast, mutations in NPM1 (48–53%) and CEBPA (13–15%) indicate a better prognosis.⁵² In the case of mutations in DNMT3 and TET2, the prevalence rates of these mutation in the Mexican population are 2.7% and 11.8%, respectively, and they indicate an unfavourable prognosis.⁵⁰ The prognostic impact of IDH gene mutations in AML remains controversial. Some studies have associated these mutations with adverse reactions, while other studies have not been able to identify an association with the clinical response or survival.^{53,54} In the case of gliomas and glioblastomas, IDH1/2 mutations have been associated with increased survival; therefore, the use of IDH inhibitors is not necessary, unlike for AML.⁵⁵ A meta-analysis that included 8,121 AML patients showed that those with mutations in the IDH1 gene had a lower overall survival and a lower rate of complete remission than patients without mutations.⁵⁶ We found that AML patients with IDH1 mutations had decreased survival, which is consistent with previous reports. In the case of IDH2 gene mutations, no difference in survival was reported. These results agree with those reported for the IDH2 gene, in which the presence of a mutation has no effect on overall survival and complete remission.^{57,58} However, mutations have been associated with lower rates of complete remission and higher rates of relapse, which is similar to our results.

Finally, castPCRTM technology is considered a rapid and economical method for clinical practice that can be used as a diagnostic test for the detection of IDH1/2 gene mutations in AML.

Conclusion

The study of castPCRTM mutations allows rapid and timely diagnosis for the treatment of AML patients, and the importance of detecting other mutations, such as FLT3,

TET2 and DNMT3, has been demonstrated. Our results revealed the frequency of IDH mutations in our population of AML patients and their impact on overall survival and risk of therapy failure. The presence of IDH gene mutations allows stratification of patients by risk group. In Mexico, there are no studies that demonstrate the frequency of these mutations to assess specific therapies, such as the use Ofivosidenib (Tibsovo), an IDH1 inhibitor, and Enasidenib (Idhifa), an IDH2 inhibitor.

Acknowledgments

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Disclosure

The authors declare that they have no competing interests in this work.

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